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REVIEW

Therapeutic implications of Epstein–Barr virus infection for the treatment of nasopharyngeal carcinoma

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Abstract: Nasopharyngeal carcinoma (NPC) is highly endemic in certain regions including the People's Republic of China and Southeast Asia. Its etiology is unique and multifactorial, involving genetic background, epigenetic, and environment factors, including Epstein–Barr virus (EBV) infection. The presence of EBV in all tumor cells, aberrant pattern of antibodies against EBV antigens in patient sera, and elevated viral DNA in patient circulation as well as nasopharyngeal site underline the role of EBV during NPC development. In NPC tumors, EBV expresses latency type II, where three EBV-encoded proteins, Epstein–Barr nuclear antigen 1, latent membrane protein 1 and 2 (LMP1, 2), are expressed along with BamH1-A rightward reading frame 1, Epstein–Barr virus-encoded small nuclear RNAs, and BamH1-A rightward transcripts. Among all encoded proteins, LMP1 plays a central role in the propagation of NPC. Standard treatment of NPC consists of radiotherapy with or without chemotherapy for early stage, concurrent chemoradiotherapy in locally advanced tumors, and palliative systemic chemotherapy in metastatic disease. However, this standard care has limitations, allowing recurrences and disease progression in a certain proportion of cases. Although the pathophysiological link and molecular process of EBV-induced oncogenesis are not fully understood, therapeutic approaches targeting the virus may increase the cure rate and add clinical benefit. The promising results of early phase clinical trials on EBV-specific immunotherapy, epigenetic therapy, and treatment with viral lytic induction offer new options for treating NPC.

Keywords: immunotherapy, epigenetic therapy, viral lytic induction therapy

Introduction

Nasopharyngeal carcinoma (NPC) is a malignancy that originates from the epithelial cells extending over the nasopharyngeal surface.[1] The World Health Organization (WHO) has categorized NPC into three histopathological types including keratinizing squamous cell carcinoma (WHO type I), with varying degrees of differentiation; nonkeratinizing squamous cell carcinoma (WHO type II), retaining epithelial cell shape and growth pattern; and undifferentiated carcinoma (WHO type III), which does not produce keratin and lacks a distinctive growth pattern. For prognostic significance, WHO types II and III are considered together.[2,3]

Epidemiology studies on NPC show an uncommon geographical incidence. Even though it occurs sporadically in most countries throughout the world, NPC is prevalent in Southeast Asian countries and in native populations of the Arctic region, Northern Africa, and the Middle East. Furthermore, NPC is endemic in southern China, with annual incidence exceeding 20/100,000 population[4–6] as well as in Sarawak, Malaysia, representing a regional hot spot with annual incidence of 30/100,000 population.[7]

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The etiology of NPC involves genetic background and environmental factors. Chinese ethnicity is considered a significant susceptibility factor for NPC because communities living in, and migrated from, the southern part of the People's Republic of China are known to have the highest NPC incidence in the world, which is retained by Chinese offspring settling in other countries.[8,9] NPC family aggregation also indicates a strong genetic influence, with excess risk among individuals with a first-degree relative with NPC being four to ten-fold.[10–13] Familial NPC has been linked to genetic predisposition such as human leukocyte antigen (HLA) genotypes and susceptibility loci on chromosome 9, 4, and 3.[14–16] However, recent reports of genome-wide association studies indicated wider complexity of genetic factors.[17,18] It is suggested that genetic factors and environmental exposures play a combined role in triggering NPC. Environmental determinants for NPC risk include food, tobacco smoke, alcohol consumption, occupational dust, inhalant, and Epstein–Barr virus (EBV) infection. The Cantonese-style salted fish containing nitrosamines,[19–21] and preserved food containing butyrates,[22,23] heavy smoking,[24] and exposure to phorbol esters[25] are carcinogenic items that have been consistently linked to NPC.

Among environmental factors, EBV infection has attracted the greatest attention, and its association to NPC is highly documented. Interestingly, EBV reactivation is triggered by the (co)carcinogenic agents mentioned above, suggesting a synergistic effect.[25] The presence of viral DNA, RNA, and protein in all tumor cells, viral reactivation, and the aberrant antibody profiles against EBV antigens in patient sera highlight the role of EBV in NPC development.[26,27] Moreover, the existence of EBV in all NPC tumor cells provides opportunities for the development of diagnostic and therapeutic approaches.

EBV infection and NPC pathogenesis

General features of EBV infection

EBV is a gamma herpes virus that infects most adults in the world.[28] Humans are the only natural host for EBV, which transmits via salivary contact.[29] Primary infection generally takes place in early childhood and causes no or only mild nonspecific symptoms. Infection during adolescence or adulthood may result in infectious mononucleosis from which most recover without any sequelae.[30] More recently, EBV is implicated as the causal factor in several chronic and autoimmune disorders as well as cancer.[31–33] In 1997, WHO officially declared EBV a class I human carcinogenic agent for its causal role in the pathogenesis of multiple distinct lymphomas and carcinomas.[34]

Upon infection, EBV enters a latent state in "immortalized" circulating B-lymphocytes in the peripheral blood, causing the infected individual to be an asymptomatic carrier for life.[35–38] The basis for B-cell immortalization and latent persistence is formed by innate functions of a small number of viral gene products, which also drive the malignant phenotype of EBV-associated malignancies.[27] Epithelial coinfection generally occurs parallel to B-cell infection, leading to persistent virus secretion in saliva.[36,38] Latent EBV has no serious consequences in the vast majority of healthy individuals, as long as the immune system remains unaffected. However, in particular conditions, the virus or its infected host cell may be activated and subsequently plays a role in the pathogenesis of a wide spectrum of EBV-related disorders.[31] The EBV-associated malignancies include epithelial tumors such as nasopharyngeal and gastric carcinomas, mesenchymal tumors such as follicular dendritic cell tumor/sarcoma, EBV-driven lymphoid malignancies including Burkitt's lymphoma, acquired immunodeficiency syndrome (AIDS)-associated and immunodeficiency-associated lymphoproliferative disorders and lymphoma, extranodal natural killer (NK) cell/T-cell lymphoma, Hodgkin's lymphoma, and B-cell lymphoma in elderly persons.[27,31,39]

Characteristics of EBV infection in NPC development

Following an initial infection, saliva which contains EBV virions is sampled by the tonsil, where infection occurs at the crypt of the tonsil.[36] EBV passes over the epithelial barrier to reach submucosal naïve B-cells residing in the mantle zone. Thus, the incoming virus infects epithelial cells or infiltrating B-lymphocytes in the lymphoepithelium of the naso- or oropharyngeal mucosa. At those places, it establishes a primary focus of latent infection (transformation) and lytic replication. Virus released from EBV-infected epithelial cells or B-cells with lytic infection can be transmitted from host to host via saliva or by infecting other mucosal cells.[40] The virus that enters resting B-lymphocytes will spread throughout the lymphoid tissue and express several latency programs to ensure genome maintenance and persistence.[38] Upon B-cell infection, EBV drives the B-cell into immortalization and cellular proliferation in an efficient stepwise process while virus replication is suppressed by methylation, a situation mirrored in most EBV-positive lymphoblastoid cell lines in vitro.[27] This latency is called latency type III, where EBV expresses the full spectrum of eleven latent gene products.

These include Epstein–Barr nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C, and EBNA-LP), three latent membrane proteins (latent membrane protein 1/LMP1, LMP2A, and LMP2B), two Epstein–Barr virus-encoded small nuclear RNAs (EBERs), and microRNAs (miRNAs) mapped to the BHRF1 and BART regions of the EBV genome, the latter recently found to comprise over 40 independent miRNA species.[29] In vivo, virus-specific cytotoxic T-cells (CTLs) will eliminate these proliferating EBV-infected cells.[41] However, a few cells may escape the immune response and generate resting memory B-cells, where viral antigen expression is mostly suppressed by methylation. This latency is typically called latency 0 (true latency), with only noncoding EBERs and BARTs being expressed, or latency I, when EBNA1 is coexpressed to secure genome maintenance in dividing cells.[42] EBV-infected memory B-cells preferentially home to the nasal–oral mucosal lymphoid tissues of the Waldeyer ring, where they can differentiate into plasma cells and produce EBV progeny.[43] Otherwise, EBV-infected memory B-cells persist in the peripheral blood and indefinitely serve as viral reservoir and can switch to an activated state of latency that ensures immune evasion and mimic a germinal center reaction through well-regulated coexpression of EBNA1, LMP1, and LMP2, which is called the default program or latency II.[44–46] When triggered by antigen, EBV-infected B-cells become plasma cells that support viral replication close to the mucosal epithelium and provide a source of infectious virions for other B-cells or local epithelial cells.[43]

EBV infection has been shown to be an early event in the development of NPC. The undifferentiated form, WHO type III NPC, shows the most consistent worldwide link with EBV.[47,48] This type of tumor is characterized by the presence of carcinoma cells and a prominent lymphocytic infiltrate. Interaction between tumor cells and lymphocytes seems to be decisive for the continued growth of the malignant component.[27] Unlike many other cancers, namely cervical or breast cancer, early premalignant lesion of NPC such as dysplasia and carcinoma in situ (CIS) is uncommon. Rarity of lesions without associated carcinoma (3%) and a rapid development of invasive carcinoma strongly implies a swift growth sequence of the initiated cell from dysplasia to CIS and invasive malignancy. This contrasts with human papillomavirus-associated cancers in which CIS may remain for years.[48,49] Despite infrequent premalignant lesions, high grade dysplasia and isolated CIS show the presence of EBV. All cases of CIS expressed LMP1 and EBERs, a hallmark of latent EBV infection, and contained clonal EBV genomes,[48,49] suggesting that the preinvasive tissue represents the outgrowth of a single EBV-infected progenitor cell.[50] Conversely, normal nasopharyngeal epithelium or low-grade dysplasia lesions do not show EBV infection.[51,52]

The pathophysiological link and molecular mechanisms of EBV-mediated carcinogenesis in NPC are not fully understood. Consistent expression of specific viral genes in every cell of NPC and in premalignant lesions underline the important role of EBV in disease development.[47] Gene expression is mainly restricted to the *EBNA1*, *LMP1*, *LMP2*, plus *BARF1* and the noncoding EBERs and BARTs, classified as type II latency.[27] Functional aspects of these genes have been recently reviewed in detail.[53–59] Among the latent genes expressed in NPC, LMP1 is considered the primary viral oncoprotein. The expression of LMP1 is important for EBV to facilitate tumor cell growth and survival advantages, and thus keep the malignant phenotype.[27,60,61] LMP1 has pivotal effects on cellular gene expression of multiple genes including the promotion of cell growth, antiapoptotic functions, and enhancement in cell motility.[61,62] LMP1 plays a role in the invasive and metastatic property of NPC by inducing matrix metalloproteinase 9, upregulating the expression of mucin 1, and downregulating cell–cell adhesion.[63–65] It also associates with exosomes, secreted endosome-derived vesicles that carry proteins, mRNAs, and miRNAs to adjacent or distant cells to modulate immune function, angiogenesis, cell proliferation, tumor invasion, and intercellular communication.[66–68] By this association, LMP1 may regulate its transforming capacity and modulate the cellular microenvironment to escape immune recognition.[69–72] In epithelial cells, LMP1 activates transcription of the epidermal growth factor receptor (EGFR), which is also detected at high levels in NPC.[73] However, its expression in NPC tissues is highly variable in frequency (20%–90% of cases) and level[48,68,74,75] in contrast to other EBV-related malignancies such as Hodgkin lymphoma and nasal NK/T-cell lymphoma, which mostly express clearly detectable levels of LMP1.[76,77]

There is abundant expression of BARTs and BARF1 mRNA in NPC samples, suggesting their crucial role in the pathogenesis.[56,78,79] Multiple EBV-encoded miRNAs have been detected in NPC, encoded in the BART region.[80] The virus actively employs its miRNAs to manipulate various viral and cellular functions. Some miRNAs may inhibit viral replication by targeting viral DNA polymerase for degradation.[81] Cluster one BART miRNAs can downregulate the expression of the viral LMP1.[82] Other miRNAs associate with viral replication, modulate host cell homeostasis and immune responses, and promote host cell survival.[59,83,84]

EBV reactivation from latency requires expression of viral immediate early transactivators, Zta and Rta, which drive further lytic gene expression including thymidine kinase, protein kinase, and the EBV-encoded DNA polymerase. These gene products are essential to create new viral genomes.[62,85,86] As indicated above, EBV may sporadically reactivate to a lytic state in epithelial cells, where newly expressed viral proteins become an antigenic stimulus that induce characteristic immune responses.[50,87,88] High titers of immunoglobulin A (IgA) against EBV replicative antigens suggest an increased viral lytic replication at early stages and are shown to precede the development of NPC clinical presentation.[89,90] Elevated IgA titers may also reflect enhanced epithelial infection.[91] Epithelial cells carrying EBV may be more susceptible to DNA damage[92,93] and allow for genetic changes induced by other environmental carcinogens.[94] Further, genetic changes may contribute to, and facilitate, latent infection or interact with EBV transforming proteins during the tumor propagation.[47]

Immune responses against EBV and viral immune evasion in NPC

Normally, virus infection elicits host innate and adaptive immune responses; the latter involving diverse antibody (humoral) and T-cell-based (cellular) reactions to multiple EBV antigens.[41,95] To manipulate immune recognition and survive destruction by the immune system, EBV has evolved variable strategies of immune evasion.[96,97] EBV is in fact a highly immunogenic virus, as shown by the strong response generated in infectious mononucleosis at the time of primary contact.[98–100] During lytic replication, the virus downmodulates HLA I and HLA II to escape CD4 and CD8 T-cell recognition. It also interferes with the effector T-cell action through the viral interleukin (IL)-10 homologue encoded by the BCRF1 gene. During latency in memory B-cells, EBV markedly reduces the expression of the most immunogenic latent proteins, such as members of the EBNA3 family. It restricts gene expression to only LMP-2 and/or EBNA1.[27] The EBNA1 protein is crucial for the maintenance of the EBV episome in the dividing cells through sequence-specific binding at origin of plasmid replication and the chromosome.[101] The presence of a Gly-Ala repeat domain in its sequence allows EBNA1 to escape the recognition of CD8 T-cells by preventing it from proteasomal degradation and presentation to major histocompatibility complex (MHC) class I.[102–104] The detection of BARF1- and LMP1-containing exosomes in the circulation of NPC cases indicates that EBV continuously intervenes with the immune system during malignant development. Secreted exosomes have been observed to associate with LMP1 to silence local T-cells or cell regulatory molecules such as galectin-9.[69,70,105,106] In addition, exosomes carry viral miRNAs that can modulate host immune cells.[68] The secreted BARF1 protein has immunomodulating potential via colony stimulating factor-1 binding, leading to altered behavior of tumor-associated macrophages.[57,107]

The vast majority of NPC patients demonstrate aberrant immunity against EBV. EBV-specific antibody responses in NPC are more robust and more variable than those in healthy EBV carriers, and therefore have diagnostic significance.[88] Arising from the nasopharyngeal mucosal epithelium, NPC is marked by infiltrating lymphocytes that secrete various immunomodulatory cytokines such as transforming growth factor β, IL-5, IL-6, and IL-10, which influence antibody class switching and lead to the generation of IgA.[108,109] Clinical onset of NPC is generally associated with high titers of IgA antibodies, especially against tumor-derived latent EBNA1 and EBV lytic cycle antigens, suggesting that lytic virus reactivation accompanies the process of malignant growth.[88,110] Elevated IgA-viral capsid antigen/EBNA1 antibody titer may be detectable long before NPC clinical appearance and thus has clinical relevance for NPC diagnostic and screening.[89,111–113] In contrast to EBNA1 and viral lytic antigens that evoke a strong humoral immune response during tumor propagation, tumor-associated membrane proteins such as LMP1, LMP2, and BARF1 barely induce a significant antibody response.[114–117]

Quite the opposite of humoral response, the EBV-specific cellular responses are normal or suppressed compared to those in healthy EBV carriers. In fact, during lytic viral replication, the expression of all viral genes, including lytic proteins and over 80 viral gene products, elicit many T-cell antigens that give rise to a significant response of CD4 and CD8 T-lymphocytes.[41] Despite abundant cellular response to latent and lytic gene products, EBV can successfully replicate. Multiple viral gene products interfere with the antigen processing machinery and the MHC molecule expression in infected cells. These include the expression of BNLF2a, which prevents peptide-loading of MHC class I molecules through inhibition of the transporter associated with antigen processing, leading to reduced presentation of viral antigens.[118] Another viral lytic protein, BGLF5, blocks the synthesis of new MHC class I molecules and modulates the expression of MHC class II molecules.[119] Viral BILF1 downregulates MHC class I molecules that present at the cell surface.[120] Viral IL-10 homologue encoded by the BCRF1 gene interferes with the effector T-cell action.[41,121]

All together, EBV lytic proteins effectively interfere with CD8 and CD4 T-cell surveillance, allowing EBV to continue generating viral progeny.[97] A reduced T-cell response occurs at the level of tumor-infiltrating lymphocytes, suggesting a local tumor-induced immune evasion despite host immune competence.[122] Specifically, NPC tumor containing EBV uses resistance to apoptosis and local T-cell silencing as its strategy to evade T-cell surveillance and responses.[69–71,122–125]

Epigenetic mechanisms in NPC

Epigenetics describes the molecular processes that controls gene transcription, independent of DNA sequence. Mechanisms involved in epigenetic regulation include DNA methylation, histone deacetylation, and RNA interference. Among these, DNA methylation is the major modification most widely studied in NPC. DNA methylation refers to a post replication modification in which a methyl group is covalently added to the 5-carbon of cytosine bases that are located in cytosine-guanosine dinucleotides (generally named CpGs).[126] The contributions of CpG methylation in NPC pathogenesis include the silencing of EBV immunodominant antigens and various tumor suppressor genes (TSGs).[127,128]

Epigenetic regulation of EBV gene expression

EBV uses the host DNA methylation mechanism for viral persistence, either in normal or neoplastic tissue. By epigenetic modulation, EBV controls its own promoters and restricts expression of latent, episomal genomes. Tight latency is characterized by absence of virus production and only a limited set of viral promoters being expressed, allowing EBV to contribute to NPC development. Gene expression in latency is regulated mostly through various modes of promoter utilization to control the expression of viral genes associated with growth and transformation. DNA methylation suppresses the expression of Wp and Cp, which are the initial promoters of transcripts encoding nuclear antigens EBNA1–6 at early stages of B-cell infection and transformation.[129] Epigenetic mechanisms also silence the promoters of LMP1, LMP2A, and LMP2B and suppress expression of transmembrane proteins. However, DNA methylation does not control Qp, a major latent promoter for EBNA1 transcripts. By using Qp, EBV expresses the indispensable viral protein EBNA1 when all other EBV proteins are switched off, including the immunodominant antigens (EBNA2, 3A, 3B, 3C).[130] A fourth promoter driving EBNA1 expression is Fp, an early lytic promoter activated when EBV switches to the lytic cycle. Fp is nearly silenced in latency I and II tumors, including NPC.[127] Interestingly, the promoter for viral BARF1, a viral gene universally expressed in NPC and considered to contribute to tumor cell growth and immune evasion, is highly methylated and appears to be regulated in latency by deltaNp63, a transcription factor essential for maintenance of the undifferentiated state in NPC tumor cells (Hoebe dissertation, unpublished data, VU University, Amsterdam, 2014).[57]

The EBV lytic cycle is initiated by expression of immediate early genes, Zta and Rta, that are driven from the early gene promoters, Zp and Rp. The tight regulation of Zp and Rp is equally important for viral persistence as for Wp and Cp. Zp and Rp are hypermethylated in nearly all latency I and II EBV Burkitt's lymphoma cell lines. In EBV-associated tumors, including NPC, Zta and Rta transcripts are barely detectable because Zp and Rp promoters are found to be heavily methylated.[131]

Epigenetics of tumor suppressor genes

NPC is marked by the number of genes targeted for silencing by promoter methylation. Well-established TSGs such as p53 and Rb, which are altered in many tumors, are rarely mutated in NPC. Studies on activated oncogenes or inactivated TSGs did not demonstrate specific translocations, p53 gene mutations, or Rb gene alterations, nor activating Ras mutations.[132–134] EBV gene products have alternative functions that directly or indirectly affect these pathways. However, as indicated above, the intrinsic properties of EBNA1 may permit additional genetic changes to develop during malignant progression and contribute to tumor growth and metastasis. In contrast to genetic changes, epigenetic abnormalities of various TSGs are commonly detected in NPC. Aberrant epigenetic mechanisms disrupt multiple normal cellular regulatory and signaling pathways through DNA methylation of promoter CpG islands and/or histone modifications. These activities can provide cell growth and survival advantage and may be involved in the initiation and progression of NPC pathogenesis.[128] The Ras-association family 1 gene (RASSF1A), which has a role in Ras signaling, cell cycle arrest, apoptosis, and DNA repair, together with p16, a cell cycle regulation gene, were two of the first TSGs observed to be hypermethylated in NPC.[135,136] Polymerase chain reaction screening in NPC samples detected frequent loss of heterozygosity, indicating specific loss of DNA sequences involving the p16 cyclin dependent kinase inhibitor at 9p21 and the RASSF1A gene at 3p21. In vitro studies demonstrated that reintroduction of the RASSF1A gene into an NPC cell line inhibited cell growth. This suggests that

inactivation of RASSF1A can be an important contributor to NPC development, as it does in many cancer types.[135,136] More extensive studies demonstrated that high levels of CpG methylation spread throughout the cellular genome in EBV-associated NPC, and many TSGs were aberrantly methylated in their 5′ CpG islands. Multistep oncogenesis may involve TSGs that function in apoptosis, cell cycle and mitotic checkpoint regulation, intracellular adhesion, DNA damage repair, cytoskeleton organization, Wnt-signaling pathway, tumor invasion, and metastasis.[128,137,138] Furthermore, the frequent hypermethylation of multiple TSGs has potential value for diagnostic purposes and early detection in NPC.[139–141]

Besides silencing the viral gene promoters, EBV also modifies the host genome methylation pattern. Such altered methylation profiles in key cancer-related genes may contribute to pivotal mechanisms during NPC pathogenesis.[142] EBV-encoded LMP1 upregulates DNA methyltransferases (DNMTs) via the JNK/AP1-signaling pathway, inducing aberrant promoter methylation and reduced expression of certain cellular genes.[138,143,144] Treatment with demethylating drugs in LMP1 expressing epithelial cells has been observed to reupregulate the expression of the corresponding gene.[145] Elevated expression of DNMTs and other epigenetic modifiers, polycomb repressive complexes, are found in various tumors including NPC. In addition to DNMTs, activated polycomb repressive complexes could also modulate multiple cellular signaling pathways through EBV-encoded proteins.[146,147] All together, this suggests that LMP1 can regulate both maintenance and de novo methylation. Using epigenetic strategies, EBV alters host gene expression to facilitate its existence.

Therapeutic implications

Standard of NPC treatment

NPC is highly radiosensitive and therefore radiotherapy remains the standard treatment for all stages of nondisseminated disease.[148] Cases of stage I are treated by radiotherapy alone while stage III, IVA, and IVB disease, according to the American Joint Committee on Cancer 2010, are treated by radiotherapy with concurrent chemotherapy.[149,150] For stage II, the combination of chemotherapy and radiotherapy is recommended to prevent distant failures, although randomized-controlled evidence is lacking.[151] The standard care for stage I disease can achieve 5-year local control and a survival rate of 90%.[152] In nonmetastatic diseases, the standard management can achieve a 3-year disease-free survival and an overall survival of 82%–89%.[153,154] However, disease control is often associated with radiation- or chemotherapy-related toxicities, resulting in a decreased quality of life.[155] Furthermore, about 10% of cases experience recurrence, either local or regional. In cases of local recurrence, the best management remains to be determined. Treatment options include brachytherapy,[156] photodynamic therapy,[157] stereotactic radiosurgery,[158] and nasopharyngectomy.[159] For regional recurrence, the optimal treatment method is a neck dissection.[160] Survival after recurrences is variable, depending on previous strategy, duration of the disease-free interval, and retreatment approach.[161,162] Cases with metastatic NPC (stage IVC) can be treated with palliative therapy only. When patients are chemonaïve, platinum-based regimens give the best results.[163] A recent study showed the benefit of a combination of chemotherapy and radiation for locoregional disease in cases of distant metastases at diagnoses.[164] However, when the above mentioned strategies have failed, limited options are available. The best response rates are found with gemcitabine, capecitabine, or docetaxel, which result in median survival of 9.5–15 months.[165] Additional to the poor outcome, combination chemotherapy in metastatic NPC may relate to an unavoidable increased toxicity.[166] Overall, these limitations urge the development of additional strategies to overcome the primary challenges in NPC treatment, which include reducing toxicity, maintaining rates of good local control, and decreasing rates of distant metastasis in locoregional disease.[155]

New targeted therapies

As tumor biology is highly explored, the role of targeted therapy brings hope for tailored treatment for all types of cancer, including NPC. The advances in molecular targeted therapy and personalized medicine have provided grounds for more specific treatment in NPC and have become the focus of recent research and development. More focused therapy targeting disease etiology may increase cure rates since standard modalities using radiation with or without chemotherapy cannot achieve it. Results from studies combining targeted therapy in NPC with current treatments have shown some clinical benefit and require further trials to determine their advantages. These treatments include drugs targeting EGFR,[167,168] vascular endothelial growth factor (VEGF),[169] inhibitor of mammalian target of rapamycin,[170] and tumor hypoxia.[171]

Overexpression of the EGFR has been detected in a high proportion of NPC patient tumors.[172] The EBV oncoprotein LMP1 is known to activate transcription of the gene,[73] underlining the importance of EGFR signaling in NPC pathogenesis. A chimeric anti-EGFR immunoglobulin

G1 monoclonal antibody, cetuximab, has been developed and tested in a Phase II study in combination with carboplatin. In 60 recruited NPC cases with recurrent or metastatic disease, this trial demonstrated some clinical responses (11.7% of partial response and 48.3% of stable disease). Toxicities of grade 3–4 leukopenia and thrombocytopenia occurred in only 5% and 10% cases, respectively.[173] Cetuximab was also tested in a Phase II trial in combination with cisplatin and intensity-modulated radiotherapy involving 30 patients with stage III/IV NPC. Although achieving 86.5% of 2-year progression-free survival, this protocol was associated with a high incidence of grade 3–4 mucositis and 20% grade 2 radiotherapy-related dermatitis.[174] Two Phase II clinical studies have used another EGFR inhibitor, gefitinib, in patients with recurrent or metastatic NPC but failed to demonstrate any clinical response.[167,168] Angiogenesis is another promising treatment target, and the expression of VEGF has been observed to significantly associate with angiogenesis and metastases in NPC.[175] Bevacizumab, a chimeric monoclonal antibody targeting VEGF, has been tested in a Phase II multinational trial when added to standard chemoradiation treatment in 46 NPC patients with locally advanced disease. This study proved the protocol to be safe, as only grade 1–2 hemorrhagic events occurred in nine patients. Clinical responses included 90.8% of 2-year distant metastasis-free interval, 74.7% of 2-year progression-free survival, and 90.9% of 2-year overall survival.[169] A tyrosine kinase inhibitor targeting VEGF receptor, sunitinib, has also been tested in a Phase II study in 13 metastatic NPC patients. However, this study prematurely stopped because of severe hemorrhagic events affecting nine (69%) patients, even though five patients showed tumor shrinkage, indicating a good clinical response.[176] Another small molecule targeting VEGF, sorafenib, has reached Phase II clinical trial, showing its modest efficacy in recurrent or metastatic NPC cases.[177] Everolimus, a drug affecting mammalian target of rapamycin, has been tested on NPC cell lines, such as HK1, HONE-1, CNE-1, CNE-2, and C666-1, and was observed to have potential therapeutic effect for NPC.[170] Moreover, drugs targeting tumor hypoxia have been developed and tested in a clinical trial showing their capability as a promising strategy for NPC treatment.[171]

EBV targeting therapies

The presence of EBV in nearly all NPC cells emphasizes its potential to be an effective target in treatment of NPC. Although the natural role of EBV in NPC pathogenesis is not yet fully understood, this association serves as a target of exploitation in a therapeutic capacity.[178] Besides the options of targeted therapy mentioned previously, EBV viral antigen expression has attracted many studies for NPC treatment development. These include immunotherapy targeting EBV (EBV-specific T-cell infusions, EBV-based therapeutic vaccinations), EBV-targeted antibody-based therapies, epigenetic approaches, and viral lytic induction treatment.[178–180]

Immunotherapy targeting EBV

It has been observed that EBV-carrying NPC cells are capable of immunologic processing of internal antigens for CTL recognition and stimulating CTL CD8 elimination.[181] Functional CTLs are considered to be competent to attack tumor cells leading to tumor shrinkage.[182] However, it requires the tumor cells to express MHC class I and be low in apoptosis resistance function that might counteract the granzyme-B attack by CTLs. Previous studies have indicated significant MHC class I heterogeneity among NPC cases as well as expression of Bcl-2 and XIAP, which correlated with poor prognosis.[122–124,183–185] In addition, the immunosuppressive microenvironment of NPC cells in vivo may silence infiltrating activated T-cells, thereby diminishing the intended therapeutic effect.[121,122] Despite these observations, several immunotherapy techniques have been developed for EBV-associated malignancies. Two different approaches have been tested to treat NPC, namely adoptive immunotherapy, in which immune cells are passively transferred to patients, and active immunotherapy, in which an immunogen is administered to stimulate a response from the patient's immune system.[179,186]

The majority of adoptive immunotherapy studies in NPC patients have applied autologous CTL treatment. The infusion of autologous EBV CTLs expanded ex vivo by repeated stimulation with lymphoblastoid cell lines was first carried out in patients with advanced disease, leading to increased CTL levels and a reduced plasma EBV DNA level. Even though it failed to prove the existence of a clinical benefit, this trial has demonstrated the feasibility of CTL transfer in NPC patients.[187] A further trial enrolled ten endstage NPC patients who experienced progression after conventional chemoradiation therapy for intravenous autologus EBV-specific CTLs. All cases showed generation of EBV-specific CTLs that were able to specifically kill in vitro autologous EBV-infected cells. Clinically, this study protocol resulted in disease control in six patients and a progression in four patients.[188] Another similar clinical trial included ten NPC patients and demonstrated significant antitumor results. Four patients were in complete remission,

whereas the other six had a recurrent or metastatic disease. A mild swelling at the tumor site was the only toxicity effect caused by the study protocol.[189] Overall, these early trials showed promising results, including safety and tolerability of the use of EBV-specific CTLs in patients with advanced NPC cases.

In order to optimize the cell therapy approach, a study increased the dose of EBV-specific CTLs and administered it after nonmyeloablative, lymphodepleting chemotherapy. All eleven advanced NPC cases recruited in this trial tolerated the protocol well. Six patients demonstrated a stable disease that lasted for more than 4 months. Although this study confirmed its previous series on the safety of CTL treatment in advanced NPC,[188] administration of lymphodepleting chemotherapy did not add any improved tumor control to the previous results.[190]

Improvement of CTL generation that is more antigen-specific is very crucial to increase treatment efficacy. The above mentioned studies have been applied using lymphoblastoid cell lines to effectively generate CTLs in posttransplant lymphoproliferative disorder (PTLD) cases.[191] This modality evokes CTL responses targeting the immunodominant EBV antigens, EBNA3–6. Unlike PTLD, NPC cells express latency type II that includes LMP1, LMP2, and EBNA1, which have poor immunogenicity. To enhance the specificity of CTLs and antitumor response, the immunotherapeutic approach in NPC should only target latency type II antigens. For this reason, an adenovirus-based adoptive immunotherapy has been developed that encodes EBNA1 fused to multiple CD8 T-cell epitopes from LMP1 and LMP2. Clinically, it has been assessed in a Phase I study involving NPC cases with recurrent and metastatic disease. Out of 24 cases, T-cells were successfully expanded from 16 patients. Fourteen patients experienced mild toxicities such as grade 1 flu-like symptoms and malaise. Disease control was achieved with a mean time to progression of 136 days. The study protocol successfully demonstrated an increased overall survival from 220 to 523 days when compared with a patient cohort that did not receive any T-cell therapy. This adoptive immunotherapy indicates that CTL infusions with a polyepitope approach has the potential to prevent recurrent or metastatic disease after primary treatment.[180,192] More recently, the safety and tolerability of LMP-specific autologous CTLs were further proven by another study in the case of a recurrent NPC patient with multiple pulmonary metastases. This strategy demonstrated a remarkable effect on metastatic sites, where the majority of pulmonary lesions disappeared although the tumor at the primary site did not decrease.[193]

A Phase II clinical trial for the first time evaluated CTL infusion therapy as a first-line treatment in locally recurrent or metastatic NPC. The trial recruited only Asian patients to focus on the most prevalent population with the malignancy. The study protocol included up to six sequential infusions containing LMP2-specific T-cell following four cycles of chemotherapy. Of 35 patients receiving EBV-CTL, the study provided a 71.4% response rate with a 2- and 3-year overall survival of 62.9% and 37.1%, respectively. In addition to demonstrating improved survival outcome in advanced NPC patients, this study has set the groundwork for a future Phase III clinical trial of standard chemotherapy with and without EBV-CTL therapy.[194]

In active immunotherapy, an EBV-specific vaccine was developed, aiming to enhance the immune response in patients with EBV-related malignancy. For this type of EBV-targeted treatment, two strategies have been established: dendritic cell (DC) vaccination and peptide vaccination. DCs are professional antigen-presenting cells that function to activate naïve CD4 and CD8 T-cells. This approach has been developed by culturing autologous monocyte-derived DCs from patients with advanced NPC and pulsed with LMP2-peptide. A clinical trial has applied this vaccination to induce epitope-specific CD8 T-cell responses in 16 local recurrent and metastatic NPC patients. After vaccination, all patients elicited substantial immune response, generated as epitope-specific CTLs in their peripheral blood although the study only showed a few clinical responses (two patients showed partial tumor reduction and 14 patients developed disease progression). Moreover, this study protocol was well tolerated and caused no significant side effects.[195] Recently, a Phase II study reported clinical and immunologic effects of a DC vaccine transduced by an adenovirus truncated LMP1 and full length LMP2 in 16 cases of metastatic NPC. Although showing safety in administration, the current vaccine induced only modest efficacy, with only three subjects demonstrating clinical response. Immunologically, delayed-type hypersensitivity responses were shown in nine patients but with no increase in the frequency of peripheral LMP1/2 specific T-cells. Further modifications to the DC and combination with other cellular immunotherapies may be needed to improve the vaccine's effectiveness.[196] In another trial, an autologous DC vaccination was assessed as an adjunct strategy after radiotherapy in 38 patients with stage II/III NPC. The autologous DCs were pulsed with HLA-A2 restricted LMP2 peptides. Following treatment, delayed-type hypersensitivity responses were elicited in nine patients who also showed significant decrease of serum EBV DNA level. Serum levels of IL-2 and interferon gamma as well as the percentage of NK

and CD4 T-cells significantly enhanced. Patients also tolerated this regimen well, without any significant toxicity.[197]

Viral vector loading with EBV peptides has been under experiment. A vaccine approach was developed to incorporate scrambled DNA sequences of EBNA1, LMP1, and LMP2 and insert them into an adenoviral vector.[198] The construct of this EBV antigen-based NPC vaccine has been described, and the formulation potentially stimulates CD4 and CD8 T-cells against EBNA1 and LMPs. However, clinical trials on this technology are not yet available.[199] A different vaccine approach was developed by another group with a modified vaccinia Ankara (MVA) recombination vector expressing NPC-associated viral antigens. The vaccine virus, MVA-EL, was constructed using sequences cloned from a typical Chinese EBV strain and encodes functionally inactive fusion protein containing the C-terminal half of EBNA1 and full length LMP2.[200] Further, a Phase I clinical trial tested this strategy in patients who were in remission after standard therapy of NPC, aiming to determine its tolerability and its capacity to induce an EBNA1 and/or LMP2 CTL response. Three intradermal MVA-EL vaccinations were administered every 3 weeks using five escalating dose levels. Of 18 patients recruited, 15 cases showed increasing T-cell responses to one or both vaccine antigens. This trial proved that the MVA-EL vaccine is well tolerated. It also determined the highest and most consistently immunogenic dose to be chosen for further Phase II trial to determine its clinical efficacy.[201]

Antibody targeting options

Despite some clinical efficacy shown by antibody-based targeted therapy as mentioned under section of new targeted therapies, the utilization of such a management option in developing countries was hampered by high cost. Alternatively, antibodies targeting the LMP1 and LMP2 outer membrane loops may serve as therapeutic targets, as they can mediate cell killing complement activation.[202] Such antibodies may not be limited by local immunosupression in the NPC tumor environment and may mediate tumor killing by drug conjugation. Furthermore, they can be generated by antibody phage libraries (Middeldorp patent China CN1526072; US7811581).[203] A previous study successfully developed a novel human antibody fragment, antigen-binding against the LMP1 extracellular domain, which was subsequently conjugated with mitomycin C, thus forming an immunoconjugate. This biotherapy showed an effect on proliferation and apoptosis in NPC cell lines HNE2/LMP1 and the inhibition of growth rate of NPC xenografts in nude mice, proving its potential as a therapeutic agent in the treatment of LMP1-expressing NPC.[204] The resulting antibodies may be more specific than those targeting EGFR. Another study showed that immunization against short external loops of viral LMPs could be another low-cost option for antibody-based therapy development.[202,205] These extracellular loops are normally barely immunogenic but can be linked to approved human immunogens, such as tetanus toxoid or keyhole limpet hemocyanin, to improve immunogenicity and provide an economically affordable alternative to prior therapeutic vaccine approaches.[126] However, this option requires more research, and clinical trials must be conducted to demonstrate its clinical efficacy.

Drugs targeting epigenetic pathway

CpG methylation can be reversed with pharmacological demethylation using epigenetic agents,[206,207] providing the opportunity to explore epigenetic treatment as a novel therapeutic approach or as a combinational intervention with other modalities. Reactivating methylated and silenced TSGs would be expected to restore normal cell growth control, promote apoptosis in tumor cells, or evoke immune response. Demethylation would also reactivate the expression of EBV early and lytic genes in latently-infected NPC cells, so that highly immunogenic EBV antigens would be recognized by the immune system, leading to tumor killing. Drugs targeting epigenetic mechanisms include DNA methyltransferase inhibitors (nucleoside analogues such as 5-aza-2′-deoxycytidine/decitabine/DAC, 5-azacytidine, and zebularine)[208] and various histone deacetylase (HDAC) inhibitors.[206,207] These agents have been tested before in various type of cancers such as colon, head, neck, renal, and lung cancers, which resulted in only partial response in some patients.[209,210] A clinical trial of azacitidine was carried out in patients with NPC and EBV-positive AIDS-associated Burkitt's lymphoma. Comparison on pre- and post-treatment tumor biopsies showed significant demethylation of the latent and early lytic EBV promoters (Cp, Wp, LMP1p [ED-L1], Zp, Rp), with the reactivation of viral antigen expression (Zta),[211] signifying the potential of epigenetic therapy for NPC. Moreover, demethylating agents are currently used in combination with drugs inducing EBV lytic phase and nucleoside analogues.

Drugs targeting the viral lytic phase

The presence of EBV in the NPC cells may facilitate therapeutic killing of virus-carrying tumor cells. In latency EBV proteins cannot be recognized by host immune system because methylation of viral promoters suppresses viral imunogenic proteins. In lytic cycle many viral antigens are

exposed to immune system so in this state strong host immune response can be generated against EBV. In lytic replication, EBV-positive tumor cells commonly have intact antigen-presenting capacity to present viral epitopes in the context of MHC class I and/or MHC class II, giving rise to immune recognition and subsequent CTL killing.[41] Most NPC patients are also observed to generate functional CTLs with specificity against EBV proteins.[181,212] Substances that effectively activate the lytic cycle of EBV include chemotherapeutic agents affecting DNA synthesis and drugs affecting host DNA methylation and histone deacetylation.[213–217] EBV can be eliminated during lytic replication in vitro by nucleoside analogues such as acyclovir and ganciclovir.[218,219] However, these drugs must first be phosphorylated before incorporation by viral or cellular DNA polymerase into DNA. Cells containing latent EBV infection cannot efficiently phosphorylate either acyclovir or ganciclovir. In contrast, cells infected with the lytic form of viral infection express two virally encoded kinases (EBV thymidine kinase and the BGLF4 gene product, protein kinase), and thus allow phosphorylation or activation of both antiviral drugs in these cells. Simultaneous to viral lytic replication induced by stimulating agents, expression of EBV kinases increases susceptibility of the EBV-infected cells to antiviral treatment. Therefore, the combination of agents inducing viral replication and antiviral nucleoside analogues merits further evaluation as an alternative strategy to selectively eliminate EBV-carrying cells.[220,221] Moreover, lytic induction will also reexpress host TSGs, leading to the promotion of apoptosis in the tumor cells. The schematic concept of EBV lytic induction therapy is displayed in Figure 1.

The treatment concept of EBV lytic induction was first applied to a patient suffering from EBV-positive lymphoma using a HDAC inhibitor, arginine butyrate, in combination with antiviral.[222] A subsequent study used valproic acid instead of arginine butyrate for activating viral promoters.[223] A combination of chemotherapy, 5-fluorouracil, with a HDAC inhibitor was then used to increase the effectiveness of lytic induction. The combination was administered to an endstage NPC patient, while simultaneously adding valganciclovir. This study protocol revealed an increase of viral DNA in the circulation, indicating shedding of apoptotic fragments from the tumor which did not occur before therapy.[87] More recently, a novel combination therapy was developed and validated in a naturally EBV-infected NPC and in EBV-positive gastric cancer cell lines, showing strong synergistic effect. The drugs used consisted of chemotherapy gemcitabine, valproic acid, and valganciclovir. Application of this combination was carried out as a new treatment option in three NPC cases for which no curable treatment modalities were available. All patients showed increased levels of viral DNA in the blood. Regarding clinical parameters, patients were in stable condition, developed only transient and moderate side effects, and experienced improvement in quality of life during and after

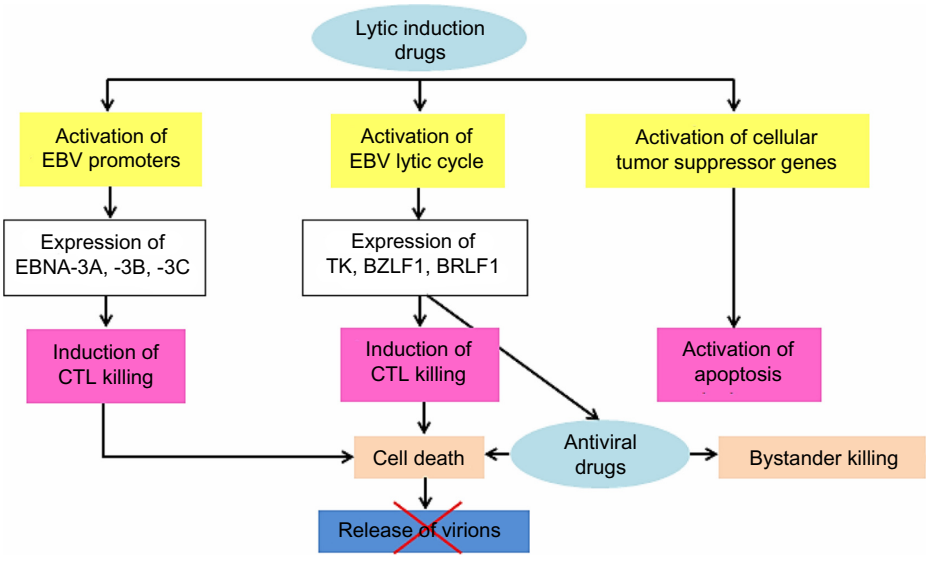

Figure 1 Schematic concept of treatment in NPC targeting EBV using a combination of lytic inducing regimens and antiviral drugs.
Notes: Lytic inducing drugs cause an effect in three mechanisms. They activate EBV promoters that lead to transcription of immunodominant latent and lytic gene products. Expression of the highly immunogenic lytic viral proteins such as EBNA3s will evoke the immune system and subsequent CTL elimination, thus inhibiting release of new virions. Antiviral treatment that is administered at an early lytic stage is converted into a cytotoxic drug by viral kinases and induce susceptibility of EBV-carrying tumor cells to CTL killing. Reexpression of host tumor suppressor genes may promote apoptosis of the EBV-infected cells. The simultaneous processes will result in tumor debulking.
Abbreviations: CTL, cytotoxic T-cells; EBV, Epstein–Barr virus; NPC, nasopharyngeal carcinoma; TK, thymidine kinase.

treatment.[224] Based on the results in this small population, a clinical trial with a larger sample size is currently underway in our center in collaboration with our Dutch colleagues.

Conclusion

NPC is highly prevalent in certain regions including southern China and Southeast Asia. EBV infection is associated with the vast majority of cases shown by the presence of viral transcripts and protein antigens in tumor cells. Given the premise of this tight relationship, EBV serves as a target for therapeutic implications. The fact that patients may relapse after primary treatment using radiotherapy, a combination of chemoradiation, or systemic therapy urges the development of personalized medicine that provides better disease control and prevents recurrence or metastases. Novel therapies targeting EBV have currently become a center of interest in research and development of NPC treatment. Currently, immune-based strategies represent options with the most clinical benefit. Such treatment offers promising application and success in patients with EBV-associated NPC and may augment clinical response to achieve disease control and reduce risk of recurrence, especially in cases with limited response after conventional therapy. Further Phase III trials are needed to assess the clinical efficacy of these strategies. Beside immune-based treatment, viral lytic induction therapy also shows potential as a treatment strategy in patients with NPC and currently has reached early phase clinical trials.

Disclosure

The authors report no conflicts of interest in this work.

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