

Epstein-Barr virus genes and nasopharyngeal cancer

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ABSTRACT

Epstein-Barr virus (EBV) is a gammaherpesvirus and is widespread in all areas of the world, infecting over 95% of the adult population. EBV primarily infects and replicates in the stratified squamous epithelium of the oropharynx during acute infection. Besides, its well-known tropism for B cells, the targets of EBV infection may also include epithelial cells, T cells, and cells of the macrocytic, granulocytic, and natural killer lineages. Although most humans coexist with the virus without serious sequelae, a small proportion will develop tumors. Almost every undifferentiated nasopharyngeal carcinoma (NPC) is EBV positive, despite geographical origin. EBV-derived IL-10 which is considered to play a role in the establishment of latent infection by suppression of the host immune system, may contribute to the growth of the tumor and to immune evasion. Latent membrane protein-1 (LMP-1) has transforming ability and support to the concept that EBV is involved in the pathogenesis of NPC. The association of NPC with EBV has been firmly established however the evidence indicating a role for the virus in the pathogenesis is still unknown and controversial. The main question is how the EBV-infected cells can escape from the immune response. [Turk J Cancer 2006;36(3):97-107].

KEY WORDS:

Epstein-Barr virus, nasopharyngeal cancer

INTRODUCTION

Epstein-Barr Virus (EBV) is a gammaherpesvirus discovered in 1964 by Epstein and Barr. It's widespread in all areas of the world, infecting over 95% of the adult population worldwide (1,2). Being a life-long persistent infection virus, transmitted by salivary contact, its primary infection occurs during childhood with latent infection of B lymphocytes (3-5).

EBV is an enveloped virus with double-stranded DNA genome that encodes more than 85 genes (6). The viral genome consists of a series of 0.5-kb terminal direct repeats and internal repeat sequences that have most of the coding capacity (7). Two subtypes of EBV are known to infect human beings: EBV-1 and EBV-2. The gene organization that code for the EBV nuclear antigen (EBNA-2, EBNA-3a, b, c) differs in the two types (8). EBV-2 transforms B cells less efficiently than EBV-1 in vitro, and the viability of EBV-2 lymphoblastoid cell lines is less than that of EBV-1 lines (9). Such differences may relate to divergence in the EBNA-2 sequences (10).

During acute infection, EBV primarily infects and replicates in the stratified squamous epithelium of the oropharynx (11). This is followed by a latent infection of the B lymphocytes in which generally the virus persists in circulating memory B cells (12,13). EBV was the first human virus to be directly implicated in carcinogenesis.

Although most human beings live with the virus without serious sequelae, a small proportion will develop tumors. Susceptibility to EBV-related tumors differs among human beings as demonstrated by geographical and immunological variations in the prevalence of these cancers. Tumors of lymphoid and epithelial origin are strongly associated with EBV, including Burkitt's lymphoma, Hodgkin's lymphoma, non-Hodgkin's disease, nasopharyngeal carcinoma (NPC), gastric and breast tumors as well as leiomyosarcomas (14-32).

The presence of EBV in NPC was firmly established as early as 1973 (33). Until today, the evidence of the role of this virus in the pathogenesis of NPC is still circumstantial and controversial (34). Due to its association with NPC, EBV has been classified as a group I carcinogen by the International Agency for Research on Cancer (IARC) (34). In most parts of the world, nasopharyngeal carcinomas occur rarely, with an annual incidence rate less than 1 per 100,000 (35). This cancer is found in an endemic form with an increasing incidence (10-30 fold higher) in the southern parts of China, Southeast Asia, and the Mediterranean region. The etiology of NPC is multifactorial and includes virological, genetic, environmental factors (36-38).

EPSTEIN-BARR VIRUS

EBV alters B-lymphocyte growth, resulting in permanent growth transformation by regulated expression of multiple viral genes (1). These genes include three integral membrane proteins, latent membrane proteins 1, 2A, and 2B (LMP), six EBV nuclear antigens (EBNA1, 2, 3A, 3B, 3C, and EBNA-LP), and two small, non-coding nuclear RNAs (EBERs) (6). The gene related products interact with or present homology to various antiapoptotic molecules, cytokines, and signal transducers, promoting EBV infection, immortalization, and transformation.

EBV nuclear antigens (EBNA)

EBNA-1 is a sequence-specific DNA binding phosphoprotein that is required for the replication and maintenance of the EBV genome (39). It also has a central role in maintaining latent EBV infection. The EBNA-1 coding sequence lies in the BKRF1 open reading frame (40,41).

EBNA-2 is a transcriptional coactivator that coordinates viral gene expression and also transactivates many cell genes while playing a critical role in cell immortalization (41,42). EBNA-2 and LP are the first latent proteins detected after EBV infection (11). EBNA-2 primarily serves to up-regulate the expression of viral and cellular genes such as CD23 (a surface marker of activated B-cells), c-myc (a cellular proto-oncogene), and viral EBNA-C promoter (43,45).

EBNA-LP, also known as EBNA-5, is one of the first viral proteins produced during EBV infection of B cells (40,41). EBNA-LP interacts with EBNA-2 to drive resting B lymphocytes into the G1 phase of the cell cycle by binding and inactivating cellular p53 and retinoblastoma protein tumor suppressor gene products (46,47). EBNA-3A, EBNA-3B, and EBNA-3C are transcriptional regulators (11). The EBNA-2 and -3 proteins are the major targets of cytotoxic T-lymphocytes that eliminate latently infected, growth-transformed B-cells (48).

Latent membrane protein (LMP-1)

LMP-1 is an integral membrane protein with six hydrophobic membrane-spanning segments and a COOH-terminal cytoplasmic tail, which contains the effector (49). LMP-1 changes the lymphoid cells by expression of B-cell activation antigens, adhesion molecules, transferrin receptor and sensitivity to TGF-beta (6). It inhibits apoptosis by elevating levels of Bcl-2 (50).

LMP-1 mimics the cellular growth signal that normally results from the binding of CD40 ligand by associating with the same tumor necrosis factor receptor-associated factors (TRAFs) (50-54). The COOH-terminal domain of LMP-1 interacts with TRAF-1 and TRAF-2 and with tumor necrosis factor receptor-associated death domain protein (TRADD) (52-56). Nuclear factor- NF- κ B, c-Jun NH₂-terminal kinase, p38 mitogen-activated protein kinase, and Janus kinase/signal transducers and activators of transcription are implicated in the function of LMP-1 (57). The consequences of NF- κ B activation are upregulation of antiapoptotic gene products Bcl-2, Bfl-1, A20, and cIAPs; proinflammatory cytokines such as IL6 and IL8; cell-surface antigens such as CD40, CD54, and CD95; and angiogenesis factors such as COX2 and VEGF (57).

MAPK family includes the ERK, p38, and JNK kinase (57,58). They play important roles in cellular responses to growth factor stimulation and stress signals. The JAK/STAT pathway is commonly activated by growth factors and cytokines and is involved in regulation of gene transcription and diverse cellular functions. PI3K is also activated by a wide variety of growth factors and cytokines. The ability of LMP-1 to activate PI3K/c-Akt may provide a fail-safe mechanism, protecting cells from LMP-1-mediated cytotoxicity by counteracting the proapoptotic activity of JNK (57-60).

LMP-1 expression is detectable in 50% to 65% of tissue samples from patients with NPC using western blotting or immunohistochemistry (61). This results support the evidence that EBV is involved in the pathogenesis of NPC. LMP-1 positive tumors are reported to more frequently extend to the outside of the nasopharynx and to have a lower tendency to recur compared to LMP-1 negative tumors (62).

The activating cascades associated with LMP-1 lead to the enhanced expression of B-cell adhesion molecules (LFA1, CD54, and CD58), enhanced expression of B-cell activation markers (CD23, CD39, CD40, CD44, and HLA class II), and morphological changes such as cellular clumping (63,64). In epithelial cells, LMP-1 specifically inhibits p53-mediated apoptosis but not p53-induced cell cycle arrest (65,66). This protection from p53-mediated apoptosis may be responsible for the lack of p53 mutations in EBV associated cancers that express LMP-1 such as nasopharyngeal carcinoma (67).

The LMP-2 proteins are encoded by mRNAs (68). Between the two forms of LMP-2 (LMP-2A and 2B) only LMP-2A has a 119 amino acid N-terminal cytoplasmic domain. This domain contains nine tyrosine residues with two of the tyrosines forming an immunoreceptor-tyrosine-based activation motif (ITAM) (69).

The EBV encoded noncoding RNAs-EBERs

The most abundant RNAs in EBV infected cells are small nuclear EBER RNAs (70). The EBERs are expressed in many of the malignancies linked to EBV and most likely contribute in some way to the maintenance of latency in vivo.

Complementary strand transcripts or Bam A rightward transcripts

Complementary strand transcripts are transcribed from a region mapping to the Bam H1A fragment of the viral genome (71). These transcripts are present in many types of EBV infections but are high in nasopharyngeal cancers.

EBV proteins that show sequence and functional homology to diverse human proteins

BCRF1 and IL-10

EBV- BCRF1 protein shows 84% sequence homology to human IL-10 (37,72). IL-10 inhibits activation and effector function of T cells, monocytes, and macrophages. IL-10 is also a known growth and activation factor for B cells (73,74). EBV-derived IL-10 is thought to play a role in the establishment of latent infection by suppression of the host immune system (75,76).

BDLF2 and cyclin B1

Human cyclin B1 regulates the G₂-M transition in the cell division cycle by activating particular cyclin-dependent protein kinases. It has been suggested that it is a late gene expressed during the lytic cycle (37).

BHRF1 and BCL-2

BHRF1 shows partial (25%) sequence homology to the human BCL-2 proto-oncogene, since both protect human B lymphocytes from apoptosis (77). BHRF2 products can also interfere with epithelial cellular differentiation (78). BHRF1 may increase cell survival, by letting oncogenic mutations to aggregate (79).

BARF-1 and intracellular adhesion molecule 1

BARF-1 exists in immune suppression by either being an antagonist to colony-stimulating factor 1 receptor or by occupying intracellular adhesion molecule 1 receptors on T lymphocytes without leading to the proper stimuli necessary for T-cell activation (80).

EBV LIFE CYCLE

EBV can infect B-cells with virus attachment to the cell surface protein CR2 (CD21) through the viral glyco-

protein gp350/220 (81). EBV can also infect epithelial cells that lack CD21 via the viral gH glycoprotein (82). Virus produced by epithelial cells then goes to infect B-lymphocytes where it can establish long-term latency (81,82). Cells infected with EBV avoid apoptosis in this environment by expressing EBV latent membrane proteins (LMP) 1 and 2a. These molecules together could provide the necessary survival signals because LMP-1 is a CD40 homologue and LMP-2a mimics BCR engagement (52,53,83,84). This mechanism reduces the loss of EBV-infected B cells by supporting their progression into the long-lived memory B cell population.

After primary infection and the establishment of latency, EBV gene expression is restricted, possibly to only LMP-2a, a protein that maintains latency by providing the survival signals, and inhibiting B cell activation and lytic cycle entry (85,86). This type of latency has been designated type 0.

EBV in order to infect other susceptible individuals must enter the lytic cycle again. When reactivation occurs, several lytic viral proteins are expressed which actively inhibit immune mechanisms. These include an interleukin 10 homologue that inhibits the costimulatory and antigen-presenting functions of monocytes/macrophages, and several proteins that impair the release of cytokines, particularly interferon (a and b) (87-89). In addition, bcl-2 homologue prolongs cell survival by inhibiting apoptosis (79). However, in normal human beings due to the balance between the host and the virus, the virus persists and replicates without endangering the host.

EBV latency forms are characterized with different promoter usage and reflect different types of virus–cell interactions and virus needs to live in the human host. EBV can cause acute infectious mononucleosis, which is a self-limited disease due to a complex and effective T-cell immune response directed at EBV antigens (41). However, by not eradicating the virus, this response results in the establishment of EBV latency in a small number of B-lymphocytes where the virus exists without clinical symptoms.

Even though EBV commonly assumed to attack B cells and epithelial cells of the oropharynx, more recently, it has

been observed that EBV can also infect and replicate in human monocytes and macrophages (90,91). In early stages of viral infection, monocytes and macrophages are rapidly mobilized in tissues and impose a significant influence on almost all aspects of immunological and inflammatory responses (92). Moreover, monocyte/macrophages play an essential role in the induction and regulation of specific antiviral T cell responses through binding of antigenic-major histocompatibility complexes (MHC) to specific T cell receptor (TCR) complex and release of immunomodulatory cytokines (92,93). Among the different accessory molecules expressed on macrophages, the members of the B7 family CD80 and CD86, and intracellular adhesion molecule-1 (ICAM-1), were found to play important part in T cell activation by interacting with their counter receptors CD28/CTLA-4 and leukocyte function-associated antigen-1 (LFA-1), respectively (94,95). EBV has evolved extensive strategies to avoid detection by the host immune system and to persist chronically within the host (96). For instance, vIL-10 may facilitate the establishment of latent infection by inhibiting or partially reducing the host's immunity, especially T cell responses (93,97).

EBV-positive malignancies are associated with the other three latent forms of infection (98). B-cells infection with EBV is linked with cell immortalization and the establishment of viral latency that is characterized by a defined pattern of EBV gene expression, referred to as latency III. Nine EBV proteins -EBNA-1, 2, 3A, B, C,-LP, LMP-1, 2A, 2B- are expressed in latency III. Burkitt's lymphoma tissues infected with EBV express only a very restricted number of viral proteins -EBNA-1, LMP-2A- a virus latency pattern referred as latency I. Whereas Nasopharyngeal carcinomas (NPCs) display a different pattern of latency, with expression of EBNA-1, LMP-1, LMP-2, EBER a viral latency pattern referred as latency II.

EBV IN PATHOGENESIS of NPC

Malignancies such as Burkitt's lymphoma, nasopharyngeal carcinoma, and Hodgkin's disease can emerge from a clone of EBV-infected cells after several years of infection. Being clonal, EBV clearly sets the stage for progression to tumor. As EBV genomes are monoclonal in nature, it is

presumed that EBV infection in NPC occurred prior to the expansion of the malignant clone (99). Also, specific failure of immune recognition; stimulation of B-cell proliferation by other infections; and/or appearance of secondary genetic aberrations or mutations can be additional factors for carcinogenesis.

In undifferentiated nasopharyngeal carcinoma, EBV infects the epithelial cells of the posterior nasopharynx in Rosenmuller's fossa in Waldeyer's ring (100). There have been two models to explain infection of these cells by EBV. Although an EBV-compatible receptor on epithelial cells has not been found, a surface protein is antigenically related to the B cell. CD21 receptor has been described (101). Alternatively, it has been suggested that EBV may gain entry into nasopharyngeal cells through IgA-mediated endocytosis (42,102).

EBV has also been detected in in situ nasopharyngeal carcinoma, a precursor of undifferentiated nasopharyngeal carcinoma (42,103). These findings suggest that EBV infection occurs before neoplasia and is necessary for the progression of the malignant phenotype. EBV-1 and EBV-2 have both been implicated in nasopharyngeal carcinoma. EBV undergoes latency II expression in undifferentiated nasopharyngeal carcinoma (103-107). The most common and outstanding genetic changes are the loss of chromosomal region 9p21 (p16, p15, and p14^{ARF}) and 3p (RASSF1A), which occur early in the progression of this tumor. The highest deletion frequencies were found on chromosome 3p (95%) and 9p (85%) in the invasive tumors (108-110). Bearing the aberrant target genes p16 and RASSF1A, the abnormal genetic changes in chromosomes 3p and 9p appear to predispose nasopharyngeal cells to sustain latent EBV infection (108-110). Such genetic alterations detected in nasopharyngeal epithelium may even precede EBV infection. EBV infection in premalignant nasopharyngeal epithelium may drive the clonal expansion of genetically altered NP cells, transforming them into malignant cells.

A unique feature of this unusual undifferentiated cancer is its universal association with the EBV that exists in a latent form exclusively in the cancer cells and not in the adjacent surrounding tissues (108-110). Higher EBV antibody titers, particularly of the IgA class, occur in NPC patients than in controls. These antibody levels rise with

the tumor burden, regardless of geographic location or ethnic group cancer (108,111).

Multiple copies of circular EBV-DNA and other footprints of this virus are regularly found in the carcinoma cells of virtually all low-grade differentiated or undifferentiated tumors (112,113). The strong link of this virus to NPC has led to the discovery that serum EBV DNA is a powerful tool in advising NPC patients on their clinical outcome in terms of early cancer detection, disease monitoring, tumor response to treatment, and relapse (114,115). Quantitative analysis of cell-free EBV DNA in plasma of patients with NPC is highly sensitive and specific (96% and 93%, respectively), providing the best tumor marker reported for any cancer (108).

Although nasopharyngeal carcinoma cells possess normal antigen processing and are effectively recognized by EBV-specific CTLs, these cells are not damaged (116). EBV-encoded viral IL-10 is increased in nasopharyngeal carcinoma and has been associated with increased production of IL-1 α and IL-1 β by epithelial cells and by CD4+ T cells, which may, in turn, contribute to the growth of the tumor and to immune evasion (117). Over expression of bcl-2 may also play a role in oncogenesis by allowing the cell to bypass apoptosis (118).

TREATMENT

In order to improve NPC diagnosis and lead to enhancement in treatment strategies, molecular mechanism by which EBV interacts with the critical genetic changes must be clarified (110). External radiotherapy is used as a successful treatment at the early-stage disease. However, the treatment results of the advanced disease are not as satisfactory due to a high rate of local relapse and distant metastases.

The disease being highly sensitive to platinum-based chemotherapy, efforts have been made to improve treatment results by integrating radiotherapy with some form of chemotherapy as primary treatment (119-127). There have been four randomized studies on the use of neoadjuvant chemotherapy, two of which showed positive results (120-122, 124). Neoadjuvant chemotherapy is only effective in reducing the tumor size. Whereas, concurrent cisplatin-

radiation with/without adjuvant chemotherapy using cisplatin and 5FU as standard treatment for locoregionally advanced NPC is the best way of sequencing the two modalities that consistently improves survival. Also, altered fractionation and intensity-modulated radiotherapy (IMRT) have shown to improve local control in NPC (128-129).

The neoadjuvant approach also provides a unique opportunity to test the efficacy of innovative agents and combinations (such as gemcitabine and taxanes). But, the efficacy testing of newer agents has been limited to phase II studies in locally advanced or metastatic disease, with innovative agents both achieving high response rates as single agents as well as in combination with cisplatin (130-131). Above mentioned conventional treatments for nasopharyngeal carcinoma (NPC) frequently fails and are accompanied by severe long-term side effects (132,133). As the T cell responses to EBV in healthy virus carriers are examined the possibility of using such responses to treat NPC have increased. Due to the successful results of adoptive T cell therapy for EBV-positive post-transplant lymphoproliferative disease, the researchers focused on exploring the development of T cell based therapies for EBV positive NPC (132-136).

Factors such as NPC expressing EBV proteins which are known targets for CD8 and/or CD4+ T Cells; antigen

processing pathways within the malignant cell being intact; T-cell responses to viral proteins being restricted via HLA alleles; and the CTL-CD8 cytotoxic T lymphocytes-responses being reactivated, are found to indicate the success of T cell based therapy for NPC (132-136). There is still a lot of debate for the usage of this therapy. If NPC is to be effective using CD8 T cells, the emphasis will be on whether LMP-2 protein is expressed in the tumor. If this protein is not expressed, the effort will be given to induce CTL responses to the other proteins. In addition, even though not yet proven, EBNA1-specific CD4+ T cells may induce antitumor function (132,135). EBV vaccine to protect against initial infection or to boost immunity in individuals with EBV-related tumors is another approach in the treatment efforts. The vaccines currently under investigation are using combinations of several defined EBV epitopes to induce EBV-specific CTL immunity (136,137).

After many years in production, there are now two candidate vaccines ready for trial, awaiting the consensus of the stakeholders on how and when these should be used. An alternative future approach would be to produce genetically engineered therapeutic vaccines increasing the specific immune responses to the viral gene products expressed in EBV positive NPC (137).

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