Detection of Epstein-Bar virus genes in different types of lymphomas

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Abstract— Epstein–Barr virus (EBV) is enveloped double stranded DNA virus belongs to gammaherpesvirus subfamily that characterized by establishment of latency. EBV infection is linked to the development of several malignancies, primarily of B cell and epithelial-cell origin, including lymphoma and nasopharyngeal carcinoma. Nowadays, oncogenic potential of EBV has been intensively studied in a wide range of human neoplasms, including Hodgkin's lymphoma, non-Hodgkin's lymphoma, nasopharyngeal carcinoma, gastric carcinoma. Viral latency infection which is associated with development of cancer is characterized by the synthesis of 6 EBV nuclear antigens (EBNA-LP, EBNA-1, EBNA-3A, EBNA-3B and EBNA-3C) and 3 latent membrane protein (LMP1, LMP2A, LMP2B). LMP1 is an essential EBV encoded oncogene for the ability of EBV to immortalize B cells. The presence of LMP1 in tumor biopsies from patients with certain EBV associated malignancies such as Hodgkin disease, immunoblastic lymphomas and nasopharyngeal carcinoma suggests that may contribute to EBV-mediated tumor-genesis. This oncogenic ability of LMP1 can be attributed to upregulation of anti-apoptotic proteins, interfering with the machinery regulating cellular senescence and inducing the angiogenesis and metastasis in EBV associated tumours. Detection of EBV genes in pathology samples is relevant since its high prevalence in some cancers makes the virus a promising target of specific therapies.

Keywords: Epstein Barr virus, Latent Membrane Protein, Epstein–Barr nuclear antigen, Hodgkin Lymphoma, Non Hodgkin Lymphoma

INTRODUCTION

Epstein-Barr virus (EBV) is a ubiquitous human gamma herpesvirus involved in cellular transformation and malignancy and infects more than 90% of the adult population throughout the world. It is discovered by Sir Michael Anthony Epstein and Yvonner Barr in a cell line derived from Burkitt lymphoma in 1964 [1, 2]. EBV infection is associated with a variety of cancer. It is found in Burkitt lymphoma (BL), Hodgkin lymphoma (HL), non Hodgkin lymphoma (NHL), nasopharyngeal cancer, gastric cancers, and a variety of unusual cancers seen in people with acquired immunodeficiency syndrome (AIDS) or with transplanted organs. EBV is a human herpesvirus that establishes a life-long persistence in the host [3].

EBV is a double-stranded DNA virus of the gamma (γ) herpesviruses subfamily. γ herpesviruses are well-known as tumor viruses that express virus cancer genes and immortalize infected-lymphocytes [4]. The life cycle of all herpesviruses in their natural host can be divided into lytic (resulting in the production of infectious progeny) and latent (dormant) infections. The establishment of viral latency is a hallmark of all known herpesviruses [2].

The virus infects and replicates within epithelial cells, and most viral particles are cleared by cytotoxic T cells. However, EBV survives in a host cell by establishing latency as an episome (a double-stranded circular form of the genome) in memory B cells. In latent

infection, EBV uses only a limited number of genes to maintain its genome and evade the host immune reaction [5].

EBV infects not only B cells but also T or natural killer (NK) cells. EBV causes benign lymphoproliferative disease infectious mononucleosis, and is associated with various kinds of lymphoid malignancies [6].

EPSTEIN BARR VIRUS

EBV is a member of the Gamma herpesvirus (subfamily), Lymphocryptoviridae (genus) and human herpesvirus 4 (species).

EBV consists of a core, capsid, tegument, and envelope (**Fig. 1**). The core contains a linear double-stranded DNA genome that located inside an icosadeltahedral nucleocapsid, approx 100 nm in size and contains 162 capsomeres. Between the capsid and the viral envelope is an amorphous layer termed the tegument. It contains numerous proteins which is responsible for connecting the capsid to the envelope, and acting as a reservoir for viral proteins that are required during the initial stages of viral infection. The envelope is derived from cell nuclear membranes and contains several viral glycoproteins [2].

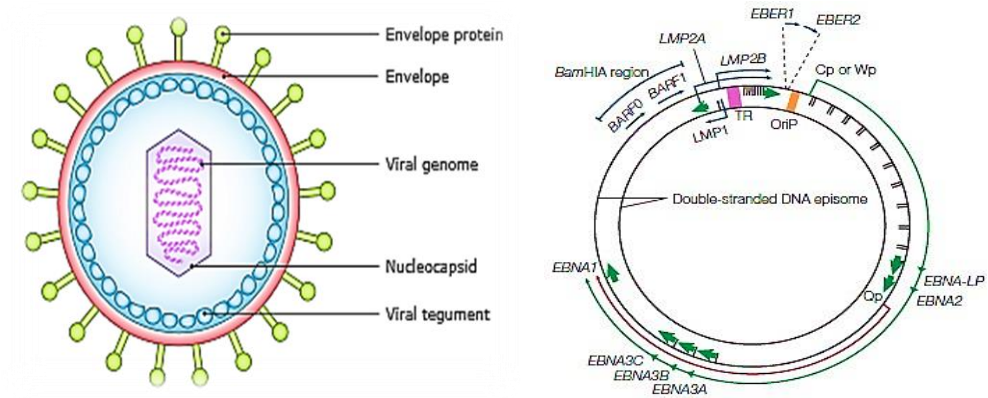

Figure 1 : The EBV and genomic structure

The EBV genome is composed of linear double stranded DNA, approximately 172 kilobase pairs (kb) in length and contains about 85 genes. EBV characterized by three patterns of latent gene expression: latency I (latency programme), II (default programme) and III (growth programme). Latency III is characterized by expression of all the latent genes (Epstein-Barr Nuclear Antigens EBNAs, Latent Membrane Proteins LMPs, and Epstein-Barr Encoded small RNA EBERS) [7]. (Table 1)

Table 1: Latent Viral Genes Expressed in Tumour Cells [2]

	Latency III	**Latency II**	**Latency I**
EBV genes expressed:	EBNA-1, EBNA-2 EBNA-3A, EBNA-3B EBNA-3C, EBNA LP LMP-1, LMP-2A	EBNA-1, LMP-1 LMP-2A, LMP-2B	EBNA-1
Phenotypes:	PTLD LCL AIDS NHL	HD NPC	BL

*** EBNA,** Epstein–Barr nuclear antigen; **LMP,** latent membrane protein; **PTLD,** posttransplant lymphoproliferative disease; **LCL,** lymphoblastic cell line; **NHL,** Non Hodgkin's lymphoma; **NPC,** nasopharyngeal carcinoma; **HD,** Hodgkin's disease; **BL,** Burkitt's lymphoma

LIFE CYCLE OF EPSTEIN-BARR VIRUS

EBV is transmitted by saliva and oral contact in most cases with rare cases of transmission by transfusion. In primary infection EBV infects and replicates within oropharyngeal epithelium, then infects circulating B lymphocytes, the virus entry to cells through the complement receptor (Cluster of Differentiation CD21) and the viral envelope glycoprotein gp350/220.8). For the penetration of the virus into cell membrane, the viral glycoprotein complex gH-gL-gp42 and co-receptor HLA class II are necessary [8]. (Fig 2)

It is supposed that the peripheral EBV-infected memory B cells can return to Waldeyer's ring, undergo reactivation and produce infectious virus to be shed into saliva[1].

In immunocompetent hosts, both humoral and cellular immune responses are evoked by primary infection of EBV. Antibodies (e.g. IgG, IgM, IgA) against EBV viral capsid antigen or early antigen neutralize the viruses and EBV specific cytotoxic T lymphocytes (CTLs) destroying most infected cells expressing viral proteins[9]. Primary EBV infection is usually asymptomatic, but occasionally progresses to infectious mononucleosis, which resolves spontaneously after the emergence of EBV-specific immunity [10].

However immune system can't eliminate the virus completely. EBV eventually enters memory B cells and infects nearly 1 in 10,000 to 100,000 memory B cells. In this condition, EBV is non pathogenic and invisible to the immune system of the host [8, 10].

In latent infection, the EBV genome establishes as a multicopy circular episome in the host cell or by integrating the viral DNA into the host genome. The expression of EBV genome is restricted in order to escape the immune system of the host. According to the patterns of expression of EBV genome, latency has been classified into three types (type III latency, type II latency, and type III latency).

EBV infected naïve B cells in the lymphoid tissue of Waldeyer's ring, which express the full spectrum of latent gene products, show type III latency (growth program). The products include 6 EBV nuclear antigens (EBNA1, 2, 3A, 3B, 3C, and LP), 3 latent membrane proteins (LMP1, 2A, and 2B). LMP-2B is a splice variant of LMP-2A which lacks the N-terminal tail with its kinase-interacting domains. EBV activates B cells to become proliferating blasts through by the growth program. [11]

The naïve infected B cells enter the germinal center (GC) where they proliferate and clonally expand. The germinal center infected cells exhibit type II latency (default program), which characterized by a restricted EBV gene expression pattern (limited to EBNA1, LMP1, LMP 2A and 2B, and EBERs). Through the process of the germinal center reaction, these infected GC cells differentiate into memory B cells to exit from the cell cycle and enter the peripheral circulation. The EBV-infected memory B cells in periphery expressing only EBERs, so they rarely detected by the immune system. However, some of them that express EBNA-1 protein divide occasionally to maintain the long-term reservoir of EBV, which is referred to type III latency [8].

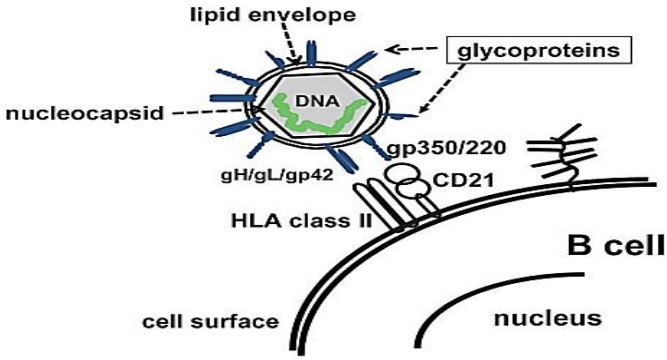

Figure (2): Attachment of EBV to B cells through viral glycoproteins and cellular receptors[10]

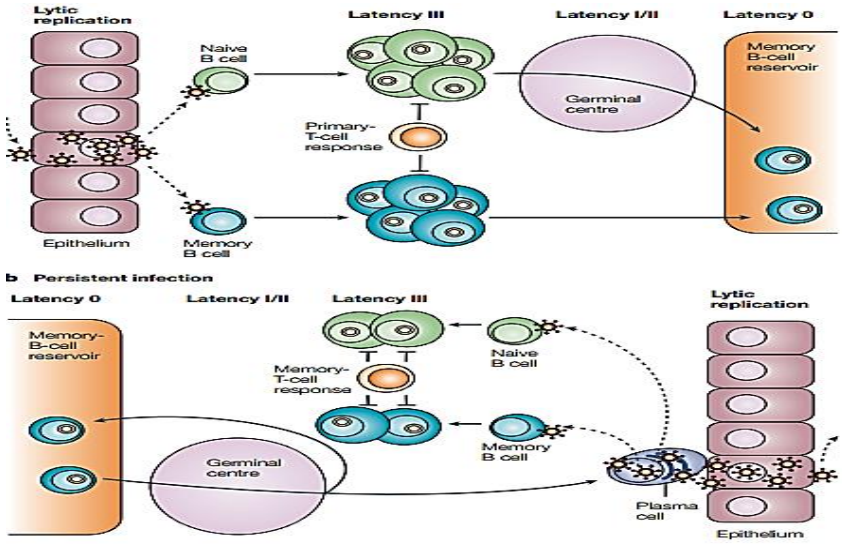

Figure (3): Life Cycle of Epstein Barr virus [12]

Table 2: Epstein–Barr virus latency gene function [9]

Gene	Function
EBER-1 and EBER-2	Block apoptosis and induce IL-10 production
EBNA-1	Maintains EBV episome copy number
EBNA-2	Enhances expression of viral and cellular genes
EBNA-3A	Limits EBNA-2 mediated transactivation
EBNA-3B	Blocks p53 and Rb activity
EBNA-3C	Limits EBNA-2 mediated transactivation
EBNA-LP	Cooperates with EBNA-2 in transactivation
LMP-1	Activates signaling pathways and blocks apoptosis
LMP-2A	Blocks lytic activation
LMP-2B	Enhances lytic activation

LATENT MEMBRANE PROTEIN 1

The oncogenic mechanisms of EBV are thought to be attributable predominantly to LMP1 (Figure 4). LMP1 (molecular weight, 63 kDa) is an integral membrane protein that consists of 3 different domains. The short N-terminal cytoplasmic domain orientates LMP1 protein and binds to the plasma membrane . It composed of 25 amino acids.[13] The long C-terminal cytoplasmic tail contains signaling activity (200 amino acid). A 6-transmembrane loop between the C- and N-terminal domains provides a platform for self-aggregation and oligomerization. The C-terminal tail has 2 distinct domains: C-terminal activation regions 1 and 2 (CTAR1 and 2 / TES 1and 2), which activate the NF-kB pathway.[5] LMP1 aggregates provide a platform for CTAR1 and CTAR2 to interact with downstream molecules. LMP1 can also activate the C-Jun N-terminal kinase (JNK)/AP-1, MAPK, and phosphatidylinositol 3-kinase (PI3K)-Akt pathways[13].

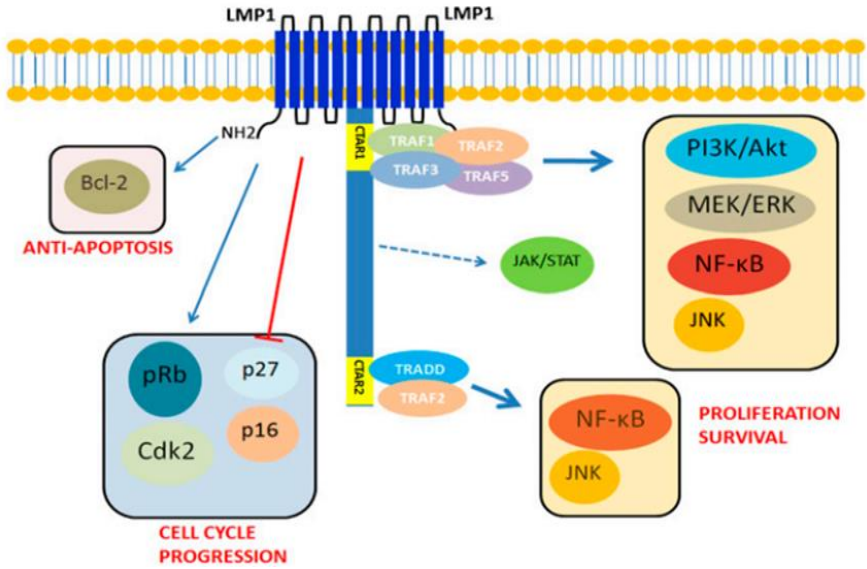

Figure (4): Latent membrane protein-1 (LMP1)-mediated activation of natural factor-κB (NF-κB) signaling**.** Latent Membrane Protein 1 (LMP1) ; ERK, extracellular signal-regulated kinases; JAK3, Janus kinase 3; JNK, Jun amino-terminal kinases; MEK, MAPK/ERK kinase; PI3K, phosphatidylinositol 3-kinase; TRADD, tumor necrosis factor receptor type 1–associated DEATH domain; TRAF, tumor necrosis factor receptor–associated factor.

ROLE LMP-1 AS ONCOGENE

Firstly, transient expression of LMP1 in primary resting B cells induces DNA synthesis, a process reminiscent of antigen-mediated B cell-activation. LMP1 can also suppress cell death induced by a variety of stimuli through the up-regulation of anti-apoptotic proteins, such as Bcl-2, Mcl-1, Bfl-1, A20 and cIAPs. LMP1 promotes the growth of primary embryo fibroblasts by interfering with the machinery regulating cellular senescence. Finally, expression of this EBV-encoded protein in carcinoma cell lines induces IL-8 production and upregulation of matrix metalloproteinases, suggesting that in addition to its transforming potential, LMP1 may influence angiogenesis and metastasis in EBV associated tumours[14]

EPSTEN BARR VIRUS AND LYMPHOMA

Cancer of the lymphatic system can be primary or secondary. Lymphoma refers to cancer that arises from lymphatic tissue. Lymphoid leukemias and lymphoma are now considered to be tumors of the same type of cell lineage. They are called "leukemia" when in the blood or marrow and "lymphoma" when in lymphatic tissue [15]. They are grouped together under the name "lymphoid malignancy". Lymphoma is generally considered as either Hodgkin lymphoma or non-Hodgkin lymphoma (Table 3).

Disease entity	Association to EBV	Infected cells	Latency type	Population at high risk
Burkitt lymphoma, endemic	100%	B	I	Equatorial Africa, New Guinea
Burkitt lymphoma, sporadic	30%	B	I	
Hodgkin lymphoma, mixed cellularity	60–80%	B	II	
Hodgkin lymphoma, nodular sclerosis B II	20–40%	B	II	
Lymphomatoid granulomatosis	100%	B	II	Western countries
EBV+ diffuse large B cell lymphoma of elderly	100%	B	III	
Post-transplant lymphoproliferative disorders B III	>90%	B	III	
Lymphoma associated with HIV infection	40%	B	I-III	
Primary effusion lymphoma	70-80%	B	III	
Extra nodal NK/T cell lymphoma, nasal type	100%	NK, T	II	East Asia

HODGKIN LYMPHOMA

Hodgkin's lymphoma is a distinct disorder accounting for 30% of lymphoid malignancies worldwide [8].

The main feature for Hodgkin lymphoma is the presence of Reed-Sternberg cells (HRS cells are malignant cells derived from B cells that have undergone a germinal center (GC) reaction and lack a functional B-cell receptor (BCR)) [16]. HL has been classified into several types based on the histologic differences and EBV-associated mixed cellularity which is most frequently (70%), followed by lymphocyte depletion (50%), nodular sclerosis (20%), and lymphocyte predominant subtypes (<5%) [16].

Percentage of EBV incidence observed in HL patients of developed countries is 30%~50%, whereas the percentage is nearly 100% in children of developing countries [8].

Variable rates of detection of EBV in H/RS cells have been found in various parts of the world with a range of 30-96% of HL cases [9].

EBV has been shown to affect the cell cycle and regulation of apoptosis. HRS cells exhibit type II latency (expressing LMP1, LMP2A and 2B, EBNA1, and EBERs) provides some clues for the oncogenic potential of EBV in the transforming events of HL which remains poorly understood. LMP1 mimics an immanently active CD40 receptor by self-aggregation and oligomerization, resembling the cellular growth signal that normally results from the binding of CD40 ligand [8, 17].

LMP1 induces many of the phenotypic changes seen in EBV infected B cells, including expression of the B cell activation markers, CD23
and CD40; IL-10 production; upregulation of cell adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), lymphocyte
function associated antigen 1 (LFA1) and LFA3; and downregulation of CD99. LMP1 also protects B cells from cell death by the upregulation of several anti-apoptosis genes including bcl-2, mcl-1, and A20 [8, 18].

The presence of LMP1 in tumour biopsies from patients with certain EBV associated malignancies, such as Hodgkin's disease, immunoblastic lymphomas and nasopharyngeal carcinomas suggests that it may contribute to EBV-mediated tumourigenesis [14].

NON HODGKIN LYMPHOMA

Latent Epstein-Barr virus (EBV) infection is associated with a heterogeneous group of non-Hodgkin lymphoma (NHL), including Burkitt's lymphoma, NK-T lymphomas and lymphoproliferative disease (LPD). Also diffuse large B-cell lymphoma (DLBCL) is the most common lymphoid neoplasm worldwide, accounting for 30% to 40% of all non-Hodgkin's lymphoma (NHL), and EBV infection is only associated with DLBCL in about 10% of cases among immunocompetent hosts [19].

BURKITT LYMPHOMA

Burkitt lymphoma is a B cell lymphoma, named after Denis Parsons Burkitt, a surgeon who first described the disease in 1958 when was working in equatorial Africa [2].

BL cells are monomorphic medium sized cells with round nuclei, many nucleoli and abundant cytoplasm. Tumours show a 'starry sky' pattern due to presence of high numbers of macrophages, that have ingested apoptotic tumour cells. BL tumour cells usually express IgM, B-cell markers such as CD19, CD20 and CD22. The cancer has
one of the highest cell proliferation rates of any human tumour (short doubling time of tumour 24–48 h).

Burkitt's lymphoma cells evade the immune system by expressing type I latency (EBNA-1) and also by downregulating the expression of cell adhesion molecules and major histocompatibility class (MHC) class I molecules [19].

Burkitt's lymphoma is classified into three types depending on different geographic distribution and EBV association: endemic Burkitt's lymphoma, sporadic Burkitt's lymphoma and HIV associted Burkitt's lymphoma.

Endemic Burkitt's lymphoma is the high incidence type in children in equatorial Africa and New Guinea where malaria is hyperendemic. This type associated with EBV about 85%, but in the sporadic Burkitt's lymphoma only has 15% association. Sporadic Burkitt's lymphoma can seen in children and young adults in the developed world. Immunodeficiency associated Burkitt's lymphoma incidences in adults with HIV infection, are EBV positive in 30-40% of these tumours [10].

Endemic Burkitt's lymphoma infects jaw and facial bones, but also occur in the abdomen, ovaries, kidney and other extra nodal sites. On the other hand, sporadic Borkitt's lymphoma are more frequently in the abdominal masses [10].

Almost all cases of both endemic and sporadic Burkitt's lymphoma are characterized by translocation between the *myc* gene and one of the three immunoglobulin genes: the immunoglobulin heavy chain gene (IgH, IGH), and the kappa (IGK) or lambda light chain (IGL) genes. In 80% of cases, the translocation occurs with the IGH gene. The remaining 20% of cases are split between the translocations with the IGK and IGL resulting in deregulation of the myc gene into the IgH, suggesting that this genetic change is critical for oncogenesis. [19].

As described by Rowe et al, EBV contributes to the pathogenesis of Burkitt's lymphoma (BL) by providing the antiapoptotic signals necessary to override c-myc-induced cell death. The *myc* proto-oncogene plays a critical role in regulating cell proliferation, differentiation and apoptosis depending on the type of cell or other situations [20].

DIAGNOSIS

Diagnosis of EBV infection is carried by detection of antibodies against Epstein-Barr virus. The specific antibody produced following EBV infection are IgM to viral capsid antigen (VCA) and IgG antibodies to Epsein Barr Nuclear Antigen (EBNA) using Enzyme Linked Immunosorbent Assay (ELISA).

An accurate diagnosis for EBV may require the immunostaining of lymphoid tissues for EBV latent membrane protein (LMP-1) or molecular analyses that are more specific and sensitive methods for detecting EBV infection. It is based on in situ hybridization (ISH), Southern blotting and polymerase chain reaction (PCR) [2].

RNA-ISH (RISH) for detecting EBERs (EBV transcripts highly expressed in latently infected cells) is the standard procedure for EBV diagnosis allowing identification and distinction of infected cell types, but it is not permit type specific identification of EBV [21].

PCR-based methods are used for strain determination (type-1 or 2). Real-time quantitative PCR, has been proposed as a sensitive and specific tumour marker for diagnosis, disease monitoring, and prediction of outcome for several of the EBV-associated diseases. qPCR may prove useful in screening children with persistent lymphadenopathy. A positive result may prompt early lymph node biopsy to diagnose EBV-associated HL, as benign lymphadenopathy would be unlikely [22].

SUMMARY

EBV is a linear DNA double stranded viruses that is characteristics by latency cycle belongs to herpesviruses. It contains of many latent genes that responsible for many cancers include Burkitts lymphoma, Hodgkin lymphoma and nasopharyngeal carcinoma. The latent genes causes antiapopotosis and many changes of pathways in the cell. EBV maintains a long time by latent genes. Many molecular techniques were develop to detect of EBV, such as PCR and in situ hybridization (ISH). PCR-based methods of EBV DNA detection allows the identification of different types of EBV.

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