Diagnostic Approaches for Epstein–Barr Virus

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Epstein–Barr virus (EBV) is the causative agent of many diseases including infectious mononucleosis (IM), and it is associated with different subtypes of lymphoma, sarcoma and carcinoma such as Hodgkin's lymphoma, non-Hodgkin's lymphoma, nasopharyngeal carcinoma, and gastric carcinoma. With the advent of improved laboratory tests for EBV, a timelier and accurate diagnosis could be made to aid better prognosis and effective treatment. For histopathological lesions, the in situ hybridization (ISH) of EBV-encoded RNA (EBER) in biopsy tissues remains the gold standard for detecting EBV.

Keywords: Epstein–Barr virus ; laboratory diagnostic techniques ; carcinoma ; exosome

1. Introduction

Epstein–Barr virus (EBV) is a member of the Herpesviridae family and is a ubiquitous pathogen that is persistently harbored by people throughout the world. The viral genome is about 170 kb and comprises a linear double stranded DNA molecule that encodes >85 genes. It is encased within a capsid which is surrounded by the viral envelope [1][2]. EBV is found in approximately 95% of the total population. Primary infection with EBV is more frequent during childhood and causes a mild disease. The disease is typically asymptomatic in 20%–80% of individuals by the age of two-to-three years [1][3]. When uninfected teenagers and young adults are exposed to EBV, approximately 30%–70% will develop infectious mononucleosis (IM) [3].

EBV can infect a wide range of cells and tissues including T and B lymphocytes, nasopharynx and oropharynx squamous epithelial cells, salivary and stomach glands, thyroid glandular epithelial cells, smooth muscle, and follicular dendritic cells [4]. However, EBV primarily infects and replicates in the stratified squamous epithelium of the oropharynx, followed by a latent infection of B lymphocytes [4]. It has been suggested that the EBV infection of B lymphocytes occurs in the oropharyngeal lymphoid organs [2]. In normal carriers, the virus persists in circulating memory B cells and initiates the production of immunoglobulins [1][2]. Following EBV's infection of B cells, a specific set of latency-related genes and transcripts are expressed, and the virus could remain dormant in resting memory B cells, from which it intermittently reactivates at any mucosal site where B cells are present (**Table 1**) [4][5]. The reactivation of EBV poses a great and difficult challenge to infected hosts [3]. In healthy adults, it is estimated that for every million B cells in circulation, approximately 1 to 50 are infected with EBV, with the number of latently-infected cells in each individual remaining stable for several years [6]. Therefore, EBV coexists with most human hosts without obvious outcomes. However, in some people, the virus is associated with the development of certain malignancies [2].

Table 1. Epstein–Barr virus (EBV) in infected B-cells with EBV latency pattern and associated malignancy.

	Infected Cells				
	Native B-cells	Germinal Center B-cells	Peripheral Memory B-cells	Dividing Peripheral Memory B-cells	Plasma Cells
Transcription program	Latency III	Latency II	Latency 0	Latency I	Lytic

	Infected Cells				
	Native B-cells	Germinal Center B-cells	Peripheral Memory B-cells	Dividing Peripheral Memory B-cells	Plasma Cells
Viral proteins	All EBNAs, EBERs, LMP-1, LMP-2A and LMP-2B	EBNA-1, EBERs, LMP-1 and LMP-2A	EBERs	EBNA-1 and EBERs.	All lytic genes
Function of viral proteins	Activate B-cell	Differentiate activated B-cell into memory B-cell	Allow for lifetime persistence	Allow for the virus in latency-programmed cell to divide	Assist viral replication in plasma cells
Associated malignancies	IM and post-transplant lymphoproliferative disorder	Nasal NK cell lymphoma, Hodgkin's lymphoma, chronic active EBV infection, NPC and peripheral NK/T cell lymphoma	Healthy carrier	Burkitt lymphoma and gastric carcinoma	IM and NPC
Specimens for measuring viral load	Plasma or serum, MNCs and WBC	Plasma or serum, MNCs (for chronic active EBV infection), tissue biopsy	Plasma or serum, WBC	Plasma or serum	Plasma or serum

EBV, Epstein–Barr virus; EBNA, Epstein–Barr virus nuclear antigen; LMP, latent membrane protein; EBERs, EBV-encoded small RNAs; NK cells, natural killer cells; NK/T cell, nasal natural killer (NK)/T-cell; MNCs, mononuclear cells; WBC, white blood cell; IM, infectious mononucleosis; NPC, nasopharyngeal carcinoma.

The EBV infection of B lymphocytes results in two outcomes with respect to the physiological impacts of antigen stimulation. The first outcome leads to the production of memory B cells that persist for a long period, which is subsequently associated with dormant viral persistent. Meanwhile, the second outcome results in the differentiation of B cells into plasma cells that are programmed to die [3][7]. This results in lytic replication, which is accompanied by the expression of several viral proteins, including the trans-activator protein BZLF1 (otherwise called ZEBRA) and viral protein complexes that are collectively known as early antigen (EA) and viral capsid antigen (VCA), leading to the elicitation of the humoral immune response [4][8]. In the course of the lytic cycle, regulatory proteins such as immediately early antigen (IEA) and EA groups are sensitized to permit the production of viral DNA (EBV-DNA), VCA and membrane proteins (MAs) [9].

Furthermore, an in vitro study demonstrated that from the approximately 100 viral genes that are expressed during replication, only ten are expressed in latently-infected B cells [10]. There are different types of RNA and proteins expressed in the latently-infected B cells. They include non-coding RNAs (EBV-encoded small RNA 1 (EBER1) and EBER2, small non-coding RNAs, microRNAs, EBV-stable intronic-sequence RNAs (EBV-sisRNAs), EBV small nucleolar RNAs (EBV-snoRNAs) and RPMS1 messenger RNA), six nuclear proteins (Epstein–Barr virus nuclear antigen 1 (EBNA1), EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA5) and two latent membrane proteins (latent membrane protein 1 (LMP1) and LMP2) [5][11]. The diverse expression programs of EBV-encoded proteins apparently rely on the type, differentiation, and activation status of the infected B cells [10]. During the latency phase, viral proteins are reduced to evade the recognition of infected cells by cytotoxic T cells [4][5][10].

2. EBV-Associated Diseases

EBV was first discovered through its relationship with African Burkitt lymphoma. It is a causative agent for IM (commonly known as kissing disease) and has also been detected in oral hairy leukoplakia [12]. Previous reports have shown that

particular latent EBV-transcription programs are exhibited in numerous human tumors, including immunoblastic lymphoma in immunosuppressed patients, Burkitt lymphoma, Hodgkin's lymphoma, and nasopharyngeal carcinoma (NPC) [2][4][13][14]. These typical expression patterns act as rough guidelines to aid in the clinicopathological diagnosis of every type of EBV [4][13]. The investigation of patients with EBV-infected tumors has provided a reasonable degree of proof that EBV was present before neoplastic transformation, which highlights the need to further elucidate how much EBV contributes to tumorigenesis [4]. EBV is also associated with autoimmune diseases, including rheumatoid arthritis, Sjogren's syndrome, systemic lupus erythematosus, and multiple sclerosis [15][16][17]. Furthermore, the virus is associated with a wide variety of benign and neoplastic diseases including posttransplant lymphoproliferative disorder (PTLD) and NPC (which are almost exclusively EBV-related), Hodgkin's and non-Hodgkin's lymphomas, and gastric carcinoma (**Table 2**). On the other hand, many other types of sarcoma are less consistently EBV-related [18].

Table 2. Diseases associated with EBV infection.

Tumor	Subtypes	Association with EBV (% cases)	References
Autoimmune disease	Multiple sclerosis	99	[19]
	Systemic lupus erythematous	99	[19]
	Rheumatoid arthritis	88	[19]
	Sjogren's syndrome	57	[16]
XLP	XLP1 and XLP2	65	[20]
Benign reactive infection	Infectious mononucleosis	>99	[21]
	Oral hairy leukoplakia	>95	[21]
	Chronic active EBV infection	100	[21]
Nasopharyngeal carcinoma	Non-keratinizing	100	[22]
	Keratinizing	30–100	[22]
Gastric carcinoma	UCNT	100	[22]
	Adenocarcinoma	5–15	[22]
Non-Hodgkin's Lymphoma and Related Neoplasms			
Burkitt lymphoma	Endemic	100	[23]
	Sporadic	10–80	[23]
	AIDS-associated	30–40	[23]

Tumor	Subtypes	Association with EBV (% cases)	References
B-lymphoproliferative disease	Post-transplant	>90	[23]
	HIV-related	>90	[23]
Diffuse large B cell lymphoma	NOS	10	[23]
	PAL	100	[23]
	HIV-related	20–60	[23]
Rare immunocompromised B lymphomas	Plasmablastic lymphoma	75–90	[23]
	Primary effusion lymphoma	75–90	[23]
T/NK lymphoproliferative disease	CAEBV	100	[23]
	Extra-nodal T/NK lymphoma	100	[23]
	Aggressive NK lymphoma	100	[23]
Hodgkin's Lymphoma			
NLPHL	-	<4 (usually absent)	[24]
Classical Hodgkin's lymphoma	All subtypes	40	[25]
	Nodular sclerosis	10–40 (variably present)	[23][26]
	Mixed cellularity	70–80 (usually present)	[23][26]
	Lymphocyte depleted	10–50 (variably present)	[23][26]
	Lymphocyte rich	30–60 (variably present)	[23][26]
	HIV-related	>90	[23][26]

3. Diagnoses of EBV-Associated Diseases

The physical presence of EBV inside a given neoplasm suggests that it may be implicated in the pathogenesis of clonal expansion in EBV-associated diseases [4]. As such, EBV can be used as a biomarker to diagnose and assess tumor spread as well as to monitor treatment. For this reason, the laboratory testing of EBV and the identification of viral gene products have become essential because EBV is considered a helpful tumor marker [21]. Currently, there are several diagnostic methods for EBV detection, including serological and molecular diagnostic methods, although each has their own limitations (**Table 3**).

Table 3. Advantages and disadvantages of various EBV diagnostic methods.

Method	Advantages	Disadvantages
Molecular methods (PCR and other nucleic amplification methods)	(1) Ability to differentiate between healthy carriers and patients with EBV-related disease based on viral load (low or high) (2) Low risk of contamination and reduced labor costs and turnaround time in qPCR (3) Allow for quantitative EBV DNA detection to monitor disease status. (4) Rapid (within 1 to 2 days) (5) More reliable than serological methods in terms of evaluating EBV status in immunocompromised patients (6) For early intervention, it is useful in screening high-risk populations and in monitoring EBV reactivation (7) Sensitive and specific across a wide dynamic range	(1) Could generate false-positive results due to improper blood sample storage and false-negative results due to the presence of nucleases (2) Lack of standardization (3) Expensive (4) Require special equipment
ISH	(1) Ability to identify EBV DNA or EBER transcripts in EBV–associated tumors. (2) Highly reliable confirmatory test for EBV (gold standard for EBV diagnosis)	(1) Only applicable to cells (2) Requires special skills (3) Could get counterproductive due to the histological interference between non-Hodgkin's and Hodgkin's lymphoma (4) EBER is downregulated in oral hairy leukoplakia
Heterophile antibody test	(1) Can measure heterophile antibodies released against serum viral proteins (2) Can differentiate between late primary infection and reactivation (3) Cost effective and easy to perform	(1) Less sensitive and less specific (especially in children) (2) Possibility of false-positive result in some cases of autoimmune disease (3) Possibility of false negative is high in young children
IFA (immunofluorescence assay)	(1) Gold standard reference method (2) Highly specific (3) Allows for the staging of EBV infections	(1) A high degree of variability (2) Lacks standardization (3) Equivocal diagnosis of acute EBV infection

Method	Advantages	Disadvantages
EIAs and ELISA	(1) Rapid method (2) More sensitive than the IFA (3) Suitable for automation (4) Inexpensive (5) Less hands-on time	(1) Less specific (2) Difficulty in the staging of EBV infection (single patient's serum) (3) Lack of standardization (4) Equivocal diagnosis of acute EBV infection
CLIA (chemiluminescence immunoassay)	Sensitive and specific in distinguishing primary infection (transient) from past infection	Requires further validation
Immunoblotting analysis	(1) Highly specific (2) Confirmatory method (3) Possibility of detecting the stage of EBV infection from serum (4) Detection of EBV-specific antibodies against several antigens	(1) Lack of the standardization of buffer conditions, the combination of recombinant antigens and the lysates from cell lines (2) Expensive
Immunoglobulin G (IgG) avidity testing	(1) Confirmatory test for intermediate results (2) Specifies the period of primary infection (3) Distinguishes active from past infections	(1) Depends on the individual maturation rates of antibodies (2) Not useful in newborns (due to maternal antibodies)
Viral cell culture	A precise and semi-quantitative method	(1) Expensive and time consuming (4–8 weeks) (2) Performed only in special laboratories (3) Requires trained personnel

Despite the fact that in situ hybridization (ISH) is the gold standard method for detecting EBV-associated carcinoma with a sensitivity of 100%, the molecular determination of viral DNA, RNA and EBV viral load is currently being utilized in the clinical assessment of tumor-associated EBV infections [21][27]. While viral culture may be used as an alternative semi-quantitative method, it is not preferable in clinical laboratories due to its high cost, slow turnaround time, and the need for trained personnel [4]. However, accurate laboratory tests to detect EBV are important in fundamental and epidemiological research. From a clinical perspective, tests for EBV will help to determine correct diagnoses for patients [28]. Moreover, with various diagnostic methods available, the detection of EBV also aids during treatment monitoring and the prognosis of EBV-associated diseases [27][28].

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