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Review
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Epstein-Barr Virus-Associated Classical Hodgkin Lymphoma and Its Therapeutic Strategies

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Abstract

Over the past few decades, our understanding of the epidemiology and immunopathogenesis of Hodgkin lymphoma (HL) has made enormous advances. Consequently, the treatment of HL has changed significantly, rendering this disease of the most curable human cancers. To date, about 80% of patients achieve long-term disease-free survival. However, therapeutic challenges still remain, particularly regarding the salvage strategies for relapsed and refractory disease, which need further identification of better prognostic markers and novel therapeutic schemes. Although the precise molecular mechanism by which Epstein-Barr virus (EBV) contributes to the generation of malignant cells present in HL still remains unknown, current increasing data on the role of EBV in the pathobiology of HL have encouraged people to start developing novel and specific therapeutic strategies for EBV-associated HL. This review will provide an overview of therapeutic approaches for acute EBV infection and the classical form of HL (cHL), especially focusing on EBV-associated HL cases.

Key Words: Classical Hodgkin lymphoma, EBV, Hodgkin and Reed-Sternberg cells

INTRODUCTION

Lymphoma is a type of cancer involving cells of the immune system, and has two major categories: Hodgkin lymphoma (HL) and all other lymphomas (non-HL). Although the two types may display the same symptoms, they are readily distinguishable via microscopic examination, and differ in the way they develop, spread and are treated. HL is a unique clinicopathological disorder characterized by the rare presence of malignant cells (usually accounting for 0.1% to 10% of the cells in the total tumor mass) in a background of a non-neoplastic cellular microenvironment comprising T- and B-lymphocytes and other cell types (Weiss *et al.*, 1999). In the United States, about 8,500 new cases of HL were expected to be diagnosed in 2010, and the overall incidence is increasing each year (Maggioncalda *et al.*, 2011).

Although the pathogenesis of HL is still largely unknown, the association of HL with Epstein-Barr virus (EBV) infection has been demonstrated in many reports. For examples, HL patients show elevated antibody titers to EBV antigens (Levine *et al.*, 1971), and the risk of developing HL is increased up to three-fold in population that have experienced infectious mononucleosis caused by primary EBV infection (Gutensohn and Cole, 1980), and EBV DNA/RNA can be detected in HL tissues (Brink *et al.*, 1997), suggesting that as a primary event in the development of this disease, EBV infection may play a very crucial role in the pathogenesis of HL.

According to a population-based cohort study, the frequency of EBV associated HL among total HL cases varies from 30 to 50% (in certain cases even higher; >90% in developing and underdeveloped countries, and >95% in HIV associated cases), and most of them appear in classical Hodgkin lymphoma (cHL), the major type of HL (Herbst *et al.*, 1991; Pallesen *et al.*, 1991; Ambinder *et al.*, 1993; Leoncini *et al.*, 1996). Since EBV is an oncogenic virus that is able to subvert cellular processes supporting growth and survival, the approach to unravel the function of latent EBV genes expressed in cHL tissues will provide strategies for more novel and targeted therapies for cHL patients. Thus, this review, based on considerable progress recently made regarding the pathways related to the generation of cHL over the past years, presents a summary of EBV antiviral drugs, the main characteristics of the disease entities, the data on the possible role of EBV in the pathogenesis of EBV-positive HL, and current therapeutic options including immunotherapy.

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Table 1. Antiviral therapeutic candidates targeting EBV replication

Target	Structure	Drug (prodrug)	Mode of action	References
EBV DNA Pol	Acyclic nucleoside or nucleotide analogue	Acyclovir (valaciclovir)	Competitive substrates or DNA chain terminator	(Purifoy *et al.*, 1993)
		Ganciclovir (valganciclovir)		(Jung and Dorr, 1999)
		Penciclovir (famiciclovir)		(Boyd *et al.*, 1986)
		Adefovir		(Friedrichs *et al.*, 2004)
		Cidofovir		(Friedrichs *et al.*, 2004)
	Pyrophosphate analogue	Foscarnet, a phosphonoformic acid	Pyrophosphate-binding site	(Datta and Hood, 1981) (Yajima *et al.*, 1976)
		Phosphonoacetic acid		
	Non-nucleoside	4-oxo-dihydroquinolines	V823 of EBV DNA Pol	(Hartline *et al.*, 2005)
Molecules except for EBV DNA Pol	L-ribonucleoside	Maribavir	EBV ED-A	(Gershburg *et al.*, 2004) (Zacny *et al.*, 1999) (Gershburg and Pagano, 2002)
	L-nucleoside	β-L-5-indodioxolane uracil	nk	(Kira *et al.*, 2000)
		Indolocarbazole (NGIC-1)	nk	(Gershburg *et al.*, 2004)

Pol: polymerase, V823: valine at amino acid position 823, PK: protein kinase, nk: not known.

EBV INFECTION AND ANTIVIRAL DRUGS

EBV, a human γ herpes virus, is widely spread in human populations, and more than 95% of adults are EBV seropositive (Young and Rickinson, 2004). Primary EBV infection is usually asymptomatic in childhood, but the infection in adolescence frequently results in infectious mononucleosis (IM), and is associated with a variety of human malignancies including Burkitt's lymphoma, nasopharyngeal carcinoma, post-transplant lymphoproliferative disease, as well as HL (Rowe *et al.*, 1986; Rickinson *et al.*, 1987; Izumi and Kieff, 1997; Timms *et al.*, 2003).

For the treatment of acute EBV infection, many antiviral drugs have been suggested as potent therapeutic candidates, all of which inhibit EBV replication. From a target perspective, they fall into two groups (Table 1). The first group includes acyclic nucleoside/nucleotide analogues and pyrophosphate analogues, the target of which is the EBV DNA polymerase. Despite their efficient inhibition of the viral polymerase *in vitro* in tissue culture experiments, they have displayed limitations by toxic side effects, poor oral bioavailability, and emergence of drug-resistant virus strains in *in vivo* treatment (Pagano *et al.*, 1983; Purifoy *et al.*, 1993). Recently, through further searching for new therapeutic compounds with the enhanced specificity in their antiviral action, a second group demonstrating unique modes of action has become available, which contains compounds of a mixed nature with divergent structures. Among them, maribavir inhibits phosphorylation of the EBV DNA-processivity factor, EA-D indirectly by an EBV protein kinase, BGLF4. Despite yet unspecified modes of action in many cases, the common feature of this group is that their targets are located at molecules other than EBV DNA polymerase itself. However, in all cases, these anti-EBV drugs targeting the virus's own replication process have limited use in the majority of EBV-associated malignancies, in which EBV viruses are latently infected, because those drugs can function only when EBV replicates actively (van der Horst *et al.*, 1991). In this respect, it is noteworthy that several groups have focused on the development of a potential method to induce a switch from latent to lytic infection with the subsequent treatment of those anti-EBV drugs as an option for the therapy of EBV-positive malignancies (Westphal *et al.*, 2000; Daigle *et al.*, 2011).

EBV LATENCY AND HL

During primary infection, EBV is transmitted by acutely infecting B cells in the oropharyngeal epithelium. This process results in a localized replicative infection through lytic cycles, some of which undergo latency by permanent infection of circulating B cells. Most EBV associated malignancies including HL are characterized by such viral latency, in which viral genomes are replicated by host DNA polymerase only when cells divide, and propagate into progeny cells indefinitely. Therefore, the antiviral drugs aimed at viral replication processes listed in Table 1 would not affect EBV in the latent phase, as demonstrated by their lack of effect on latently EBV-infected cell lines or tumors (Pagano, 1995). This fact subsequently emphasizes the needs of special strategies for EBV-associated tumors distinct from those for the acute infection.

In latent infection, viral gene expression is tightly regulated with expression of only a limited number of EBV latency genes. Thus, only 12 genes of EBV can be expressed in different combinations during latent viral infection whereas about 70 major open reading frames are expressed during the lytic cycle (Kieff, 1996). The EBV latency genes are expressed in 4 programs: latency 0, I, II and III (Shah and Young, 2009). In latency III, all of the 12 latency genes are expressed, including 6 nuclear proteins (EBNA 1, 2, LP, 3A, 3B, 3C), 3 membrane proteins (LMP-1, LMP-2A and LMP-2B), BART, and 2 small RNAs (EBER 1 and 2). In latency II, the viral genes for EBNA-1, the three membrane proteins, and the EBERs are expressed while in latency state 0/I, none or only EBNA-1 is expressed. B-lymphocytes in latency III are proliferating but highly immunogenic, while the remaining latency forms are seen in non-proliferating, resting cells. Among the 4 programs,

HL has been reported to be closely associated with latency II, expressing only 6 viral genes: EBNA1, EBER1 and 2, LMP1, and LMP2A and 2B (Murray *et al.*, 1992; Deacon *et al.*, 1993; Grasser *et al.*, 1994; Chiang *et al.*, 1996).

According to its histopathological and clinical characteristics, HL is categorized into 2 types: cHL, and the nodular lymphocyte predominance HL (NLPHL). About 95% of all HL cases represent cHL, while the rest are NLPHL (Niedobitek *et al.*, 1997). As described, one of the most unique features of HL is the rarity of malignant cells in the tumor mass. The two types of HL have their own unique malignant cells, designated as mononuclear Hodgkin's cells and multinucleated Reed-Sternberg (HRS) cells in cHL whereas they are called LP (lymphocyte predominant) cells in NLPHL, respectively (Poppema *et al.*, 2008). Unlike the LP cells of NLPHL, EBV DNA genomes are frequently detected in HRS cells of cHL patients, in which the viruses display the type II form of latency expressing all of the type II-related 6 viral genes (Weiss *et al.*, 1989; Weiss *et al.*, 1999; Thomas *et al.*, 2004; Kuppers, 2009). Since EBV is an oncogenic virus, this high association rate with EBV in HRS cells strongly suggests a possible role of EBV latent gene expression in the generation of the malignancies. Since the latent viral antigens are abundantly present in EBV-associated HRS cells, but not in the non-EBV-associated ones, providing extra targets for immunotherapy, the latent viral genes expressed in HRS cells and the related pathways have gained attention as additional therapeutic options in the case of EBV-associated cHL.

THERAPEUTIC STRATEGIES AGAINST cHL

Based on differences in histological features and cellular composition, cHL is further divided into 4 morphological subtypes (Pileri *et al.*, 2002; Eberle *et al.*, 2009): nodular sclerosis (NS) which accounts for the majority of cases, mixed cellularity (MC), lymphocyte-rich (LR), and lymphocyte-depleted (LD) HL. LRHL and LDHL each comprises less than 5% of all cHL cases, and the majority is the NSHL subtype (Table 2). The detection rate of EBV in cHL varies depending on multifactorial etiological factors such as country, sex, ethnicity, and the age of patients. For example, EBV-positive cHL is less common in developed populations compared to underdeveloped countries (20-50% in North American and European countries vs. 60-100% in Peru and Kenya) (Weiss *et al.*, 1991; Herbst *et al.*, 1992; Hummel *et al.*, 1992; Chang *et al.*, 1993; Weinreb *et al.*, 1996). Among the subtypes of cHL, EBV genomes or proteins more commonly appear in the MC subtype with an increased incidence but less frequently in the other subtypes (Pallesen *et al.*, 1991; Murray *et al.*, 1992; Pinkus *et al.*, 1994). Despite the complexity in its histological and cellular characteristics, the primary therapeutic strategies against the all subtypes in cHL cases are similar because the immunophenotypic and general features of the malignant cells of cHL, the HRS cells, are surprisingly alike irrespective of the subtype or the presence of EBV.

With conventional chemotherapy, more than 80% of patients suffering from cHL are cured, which is otherwise a fatal disease with 90% of untreated patients dying within 2 to 3 years. However, up to 30% of patients with advanced HL will progress or relapse even after the therapeutic treatment (Connors *et al.*, 2001); thus the development of new and more potent regimens with improved outcomes has become more necessary. In this respect, in addition to the general targets of HRS cells, the presence of EBV latent genes that are expressed in malignant cells provides an excellent opportunity for targeted therapy.

Chemotherapy for early-stage cHL

Regardless of its type, data of patients with early-stage HL show that present therapies result in high expectations of cure for HL patients, and more than 80% of patients have had their cancer successfully eradicated. Currently, treatment options of HL are tailored to type, stage, patient age, and an assessment of the risk of resistance (Diehl *et al.*, 2003; Klimm *et al.*, 2005). The induction chemotherapy regimens given as the initial treatment for HL are shown in Table 3. Although ABVD is the gold standard for all early HL cases, there are several data suggesting improved outcomes using the aggressive escalated BEACOPP regimen in advanced-stage HL (Carbone *et al.*,

Table 2. Epidemiology of Hodgkin lymphoma and their EBV association

REAL/WHO classification (% cases/total HL)	Subtypes	Malignant cell	% cases of subtypes	Association level with EBV*	References
cHL (95%)		HRS cell		Positive or negative	(Jarrett *et al.*, 2005)
	NSHL		60-80% of cHL	++	(Flavell *et al.*, 2000) (Herbst *et al.*, 1992)
	MCHL		15-30% of cHL	+++ (up to 96%)	(Spitz *et al.*, 1986) (Cleary *et al.*, 1994)
	LDHL		<5% of cHL	++	(Slack *et al.*, 2009)
	LRHL		<5% of cHL	+/− (less than 10%)	(Shimabukuro-Vornhagen *et al.*, 2005)
NLPHL (5%)		LP cell		Negative	(Chan, 1999)

REAL/WHO: Revised European American Lymphoma (REAL)/World Health Organization, EBV: Epstein–Barr virus; cHL, classical Hodgkin lymphoma, NLPHL: nodular lymphocyte-predominant Hodgkin lymphoma, NS: nodular sclerosis, MC: mixed cellularity, LD: lymphocyte depletion, LR: lymphocyte-rich, HRS: Hodgkin/Reed–Sternberg, LP: lymphocyte predominant.
*varies depending on genetic and environmental factors of the affected patients.

Table 3. Conventional combination chemotherapeutic strategies for HL

Therapy regimen	Combination of agents	Description	References
MOPP	Mechlorethamine Vincristine Procarbazine Prednisone	Developed in1964. Derived by replacing methotrexate with procarbazine in MOMP, the first combination chemotherapy for HL.	(Longo *et al.*, 1986)
ABVD	Adriamycin [doxorubicin] Bleomycin Vinblastine Dacarbazine	Developed in the early 1970's as an alternative to MOPP. Currently is the standard chemotherapy regimen for treating HL.	(Boleti and Mead, 2007)
Stanford V	Doxorubicin Vinblastine Mustard Bleomycin Vincristine Etoposide Prednisone	Developed in 1985 at Stanford University. Characterized by frequent administration over a shorter period of time than the above regimens.	(Hoppe *et al.*, 1985; Hoppe *et al.*, 1989)
BEACOPP	Bleomycin Etoposide Doxorubicin Cyclophosphamide Vincristine Procarbazine Prednisone	Developed in 1997 for advanced-stage HL	(Diehl *et al.*, 1997)
Escalated BEACOPP	Higher doses of etopo side and doxorubicin and cyclophospha mide and the addition of granulocyte colony-stimulating factor for neutrophil support	Modified to improve treatment results with unfavorable and advanced-stage HL.	(Diehl *et al.*, 1998a; Diehl *et al.*, 1998b; Engert *et al.*, 2009)

2011). Although it has been reported that chemotherapy alone may be more efficient for controlling some disease cases (Meyer *et al.*, 2004), combined-modality therapy is currently the most common treatment of HL, which consists of an abbreviated course of chemotherapy and involved field radiation.

Treatment strategies for the patients with relapsed cHL

Although most HL patients attain a remission after induction chemotherapy, relapse rates range from 10-15% in the early stage (Specht *et al.*, 1998) to 30-40% in the advanced stage (Oza *et al.*, 1993). For patients with relapsed HL, high dose chemotherapy plus autologous hematopoietic stem cell transplantation (HDC/HSCT) has become recognized as the most effective treatment. However, the fact that more than 15% of the relapsed patients are still dying of progressive lymphoma after HDC/HSCT treatment stresses an obvious need for more successful therapeutic strategies.

Currently, one of the most promising types of treatment for HL is immunotherapy targeting the malignant HRS cells of cHL. Since HRS cells display virtually the same immunopathological characteristics among different HL subtypes, drug development in HL has been based on the general understanding of cellular pathways altered in HRS cells and of interactions between HRS cells and the tumor microenvironment (summarized in Fig. 1).

HRS cells: The most unique characteristic of HL compared to other tumors is the presence of a small number of malignant HRS cells in a background of non-neoplastic reactive lymphoid cell population. Although the origin of the HRS cells had been an issue of intense debate due to no obvious normal cellular counterpart to the phenotype of HRS cells, the presence of non-functional somatic mutations in rearranged immunoglobulin genes of HRS cells confirmed that they originate from pre-apoptotic B cells that lost the capacity to express a high-affinity B-cell receptor, which were then rescued from apoptosis by transforming events (Kuppers *et al.*, 1994; Kuppers, 2003). To date, many cases of aberrant activation of signaling pathways and transcription factors involved in the rescue of HRS cells from apoptosis have been identified, knowledge from which has been applied to the development of novel therapeutic agents for relapsed HL after primary treatment.

Studies on signaling pathways of HRS cells have implicated deregulated activation of a variety of intercellular proteins (NF-κB, Jak/STATs, Akt/mTOR, Notch-1, and ERK) as well as surface receptors (CD30 and CD40) (Clodi and Younes, 1997; Fiumara *et al.*, 2001; Younes *et al.*, 2003; Zheng *et al.*, 2003),

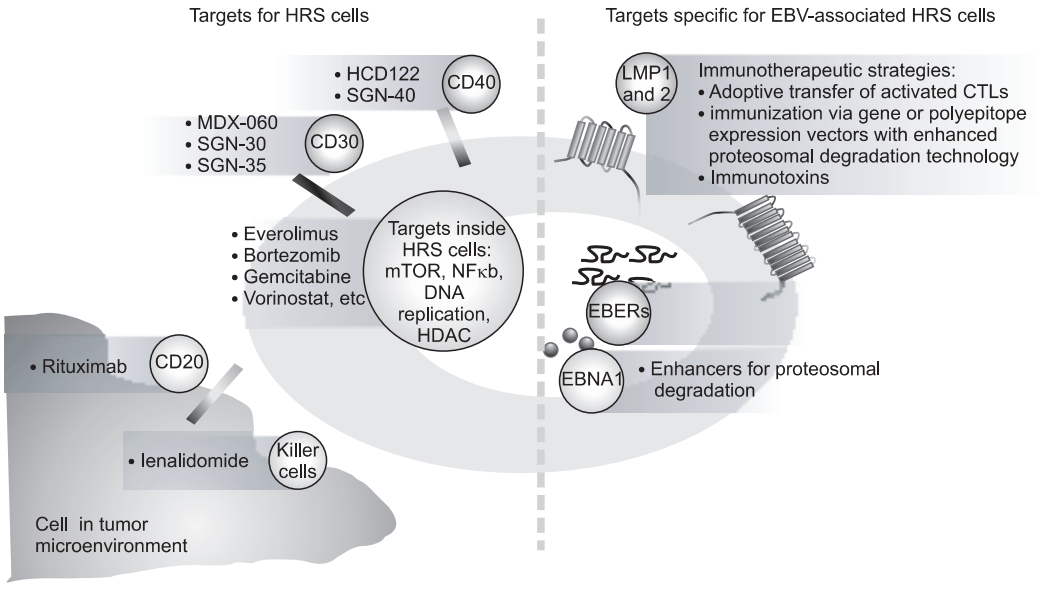

Fig. 1. Current and future therapeutic targets for HRS cells. A number of clinical trial targets for HRS cells have been identified and several novel treatments for HL therapy are under investigation. Many current approaches to handle relapsed HL aim to control specific target molecules that are related to the survival of HRS cells. In this respect, viral specific antigens expressed from EBV, a possible etiological agent for malignant HRS cells, are very attractive targets for development of a novel therapy. On the left of the figure are illustrated general targets for HRS cells, irrespective of the presence of EBV, with their related therapeutic options. On the right are targets specific for EBV-associated HRS cells. Targets expressed on the cells in tumor microenvironment are shown on the bottom left side. Gray circles and lightly shaded text boxes denote targets and their responding therapeutic option partners, respectively.

which are highly associated with pro-survival signals. Each of these proteins can be a therapeutic target of selective small molecule inhibitors. The AKT/mTOR pathway is an intracellular signaling pathway associated with the apoptotic process. In many malignant cells, this pathway is overactivated, and upon blockage of the pathway, cell cycle arrest and apoptosis of the cells subsequently take place. A preclinical study demonstrated that inhibition of mTOR, the mammalian target of rapamycin, caused apoptosis in HL cells. In addition, everolimus, a rapamycin-derived mTOR inhibitor, verified antitumor activity in relapsed HL patients in the phase I clinical study (Jundt *et al.*, 2005; Yee *et al.*, 2006), suggesting mTOR inhibitors as excellent therapeutic candidates for HL. Since NF-κB plays a central role in the regulation of various gene expression involved in cell survival, apoptosis, carcinogenesis, and inflammation, this molecule has been regarded as a very potential therapeutic target for many cancers (Baud and Karin, 2009). Bortezomib, a tripeptide with pyrazinoic acid, phenylalanine, and leucine with boronic acid, is a proteasome inhibitor that hinders activation of NF-κB by inhibiting cytoplasmic IκBα degradation (O'Connor, 2005). In preclinical studies, it inhibited proliferation of HL cell lines, and induced cell cycle arrest as well as apoptosis (Adams, 2003; Zheng *et al.*, 2004). Not only the intracellular proteins described above but also surface receptor molecules can be specific targets for HL therapy. HRS cells characteristically express high levels of CD30 and CD40, both of which play a role in the increased NF-κB activity observed in HL as a member of the TNF receptor family. Immunotherapeutic agents targeting CD30 and CD40 are currently being evaluated for potential use in HL. Since they are surface receptor antigens that contain extracellular moieties, various monoclonal antibodies (mAbs) and mAb-drug conjugates are easily accessible to their target molecules. Initially, unconjugated mAbs targeting CD30 (MDX-060 and SGN-30) were evaluated in relapsed HL clinical cases. The antibodies failed to show efficient antitumor activity (Ansell *et al.*, 2007; Forero-Torres *et al.*, 2009; Blum *et al.*, 2010), but a next-generation drug-antibody conjugate, SGN-35 (brentuximab vedotin), improved the modest clinical activity of the unconjugated mAbs. This potent immunotoxin contains the antitubulin agent monomethyl auristatin E attached to SGN-30 (Oflazoglu *et al.*, 2008). The mAb-drug conjugate is rapidly internalized on CD30 binding. Recently, the U.S. Food and Drug Administration granted fast-track designation for SGN-35 for the treatment of HL. In case of anti-CD40 mAbs, HCD122 and SGN-40 had been evaluated in patients with CD40-expressing lymphoid malignancies (Law *et al.*, 2005; Luqman *et al.*, 2008; Robak, 2008; Younes, 2009).

In addition to the drugs acting on specific target molecules, anticancer drugs that broadly inhibit the signaling pathways activated in HRS cells are often used for patients with refractory HL. These wide-ranging inhibitors, such as systemic chemotherapeutic drugs, histone deacetylase (HDAC) inhibitors, and proteasome inhibitors, can modulate several unrelated signaling molecules (Bolden *et al.*, 2006; Brogdon *et al.*, 2007; Gediya *et al.*, 2008; Tarasenko *et al.*, 2008). Most systemic chemotherapeutic drugs with antitumor activity are DNA replication inhibitors. For patients with relapsed HL, systemic chemotherapeutic drugs, such as gemcitabine (a nucleoside analog), vinblastine (an anti-microtubule drug), fludarabine (a purine analogue), and melphalan (an alkylating agent) have been used as a single agent or in combination regimens (Friedberg *et al.*, 2003; Straus *et al.*, 2011).

HDACs in cooperation with histone acetyltransferases (HATs) play an important role in epigenetic regulation of broad gene expression via post-transcriptional modification of his-

tone proteins. The balance between the two enzymes is critical for regulating the expression and the functional status of a variety of proteins that are involved in cell proliferation, survival, and immunity (Glozak *et al.*, 2005; Bolden *et al.*, 2006; Brogdon *et al.*, 2007). Unbalanced activities between HDAC and HAT have been reported in several malignancies, including HL (Glozak *et al.*, 2005), implying HDAC inhibitors as a potential therapeutic target for HL. HDAC inhibitors, such as vorinostat (a suberoylanilide hydroxamic acid), mocetinostat (a benzamide), panobinostat (a hydroxamic acid), entinostat (a benzamide), are currently under phase I or II trials for relapsed HL (Dickinson *et al.*, 2009; Boumber *et al.*, 2011; Jona *et al.*, 2011; Kirschbaum *et al.*, 2011).

Tumor microenvironment in HL: The nature of the aberrant immune response in the vicinity of HRS cells is very unique. Pathologic examination of infiltrating cells around HRS cells demonstrated that CD4 T cells, eosinophils, B cells, and macrophages, are abundantly present favorably producing TH2 and immunosuppressive chemokines/cytokines, whereas CD8 T cells and NK cells are generally sparse. This tumor microenvironment plays a significant role in the maintenance of ineffective immunity against HRS cells (Poppema *et al.*, 1999; Lamprecht *et al.*, 2008; Aldinucci *et al.*, 2010), providing a favorable milieu for HRS cells to proliferate and escape from apoptosis and host antitumor defenses.

Therapeutic strategies targeting the tumor microenvironment had been evaluated by targeting CD20 in healthy B cells using rituximab (Coiffier *et al.*, 1998), an anti-CD20 mAb, in patients with relapsed HL. CD20 is highly expressed on reactive B cells present in the tumor infiltrates whereas its surface expression on HRS cells is very low (Foss *et al.*, 1999). The treatment of rituximab eliminates CD20-positive reactive B cells that are supportive to HRS cells, subsequently depriving the malignant cells of survival signals. Promising results in relapsed cHL therapy have been reported in clinical trials testing rituximab (Oki and Younes, 2010).

Another potential therapeutic agent targeting the tumor microenvironment of HL is lenalidomide, a thalidomide-derivative. Lenalidomide functions at multiple steps, including direct induction of apoptosis in tumor cells as well as activation of killer cells (Bartlett *et al.*, 2004). An international phase I/II study with lenalidomide for elderly HL patients had been initiated (Boll *et al.*, 2010).

Table 4. Functions of six EBV genes expressed in HRS cells

EBV genes expressed in HRS cells	Functions (target cellular proteins)	Therapeutic strategies	References
EBER 1 and 2	Cell growth (IGF-1, IL-6, IL-9) Modulation of immune response (IL-10, TLR3, RIG-1) Inhibition of apoptosis (PKR)	Nt	(Ho *et al.*, 1999; Komano *et al.*, 1999; Kitagawa *et al.*, 2000; Yamamoto *et al.*., 2000; Iwakiri *et al.*, 2003; Iwakiri *et al.*, 2005; Samanta *et al.*, 2006; Samanta *et al.*, 2008; Iwakiri *et al.*, 2009; Iwakiri and Takada, 2010)
EBNA1	Modulation of EBV DNA replication Maintenance of EBV episome Inhibition of apoptosis (survivin)	Enhanced proteosomal degradation (enhancement of immunogenicity)	(Tellam *et al.*, 2001)
LMP1	Inhibition of apoptosis (NF-κB, PI3K/Akt/mTOR, Jak/STAT) Immunesuppressor (IL-10)	Enhanced proteosomal degradation (enhancement of immunogenicity, and rescue from oncogenic phenotypes) Adoptive immunotherapy (transfer of LMP1-specific CTLs, or primed DCs) Immunization with LMP1 gene or polyepitope expression vectors	(Duraiswamy *et al.*, 2003; Tellam *et al.*, 2003; Taylor *et al.*, 2004; Smith *et al.*, 2006; Pan *et al.*, 2009; Lutzky *et al.*, 2010; Chia *et al.*, 2011)
LMP2A	Prosurvival signal (Ras/PI3K/Akt, Notch and β-catenin/Wnt) Modulation of innate immune response	Adoptive immunotherapy (transfer of LMP2A-specific CTLs, or primed DCs) Immunization with LMP2A gene or polyepitope expression vectors	(Taylor *et al.*, 2004; Smith *et al.*, 2006; Pan *et al.*, 2009; Shah *et al.*, 2009; Lutzky *et al.*, 2010; Chia *et al.*, 2011)
LMP2B	Modulation of innate immune response	nt	(Shah *et al.*, 2009)

TLR: Toll-like receptor, RIG: retinoic acid-inducible gene, PKR: RNA-dependent protein kinase, Nt: not tested, CTL: cytotoxic T lymphocyte, DC: dendritic cells.

Treatment strategies for the patients with EBV-associated cHL

The incidence of high relapse rates in EBV-associated HL compared to non-EBV-associated HL after primary treatment is still conflicting. However, in population-based studies have consistently shown a noticeable survival disadvantage in EBV-positive HL especially in the case of older patients (Enblad *et al.*, 1999; Clarke *et al.*, 2001; Stark *et al.*, 2002), indicating the influence of EBV existence in HRS cells on clinical outcome. Furthermore, the fact that an EBV transforming protein, LMP1, is expressed almost 100% in HRS cells of HL in HIV-infected patients suggests that EBV may play as a crucial etiological agent in the generation of HRS cells, at least in certain circumstances (Thompson *et al.*, 2004; Carbone *et al.*, 2009).

EBV infection in HRS cells exhibits the pattern of the type II latency, expressing only a limited number of viral genes: EBERs, EBNA1, LMP1, LMP2A and 2B. Thus, the viral genes expressed during the type II latency are hypothetically the most excellent candidates for the therapeutic target against the malignant HRS cells of HL (Table 4).

EBERs: EBV-encoded small RNA 1 and 2 (EBER1 and EBER2) are nonpolyadenylated and noncoding RNAs of 167 and 172 nucleotides, respectively (Rosa *et al.*, 1981). They form stem-loop double-stranded RNA (dsRNA) structures by intermolecular base-pairing, which enable them to interact with several cellular proteins (Ho *et al.*, 1999). It has been demonstrated that EBERs play a role in EBV-mediated oncogenesis in EBV latently-infected cells. First, EBERs affect membrane signaling by initiating the production of various interleukins (ILs) that are involved in cell growth, such as IL-6, IL-9, or insulin-like growth factor-1 (Ho *et al.*, 1999; Kitagawa *et al.*, 2000; Iwakiri *et al.*, 2003; Iwakiri *et al.*, 2005). They also play a role in regulation of apoptosis by binding the RNA-dependent protein kinase (PKR), subsequently inhibiting PKR-mediated apoptosis in the EBV infected cells (Komano *et al.*, 1999; Yamamoto *et al.*, 2000). In addition, EBERs can modulate interferon (IFN)/antiviral response. It was demonstrated that EBERs induce expression of type-I IFNs by binding to the retinoic acid-inducible gene I (RIG-I) product that is a cytosolic protein that recognizes viral dsRNA inside the cell (Samanta *et al.*, 2006). Another evidence of a role of EBERs in innate immunity is their binding to Toll-like receptor 3 after secreted from EBV-infected cells (Iwakiri *et al.*, 2009; Iwakiri and Takada, 2010), triggering innate immunity that might explain immunopathologic diseases caused by active EBV infection. However, EBERs also induce an anti-inflammatory cytokine, IL-10, through RIG-1-mediated interferon regulatory factor (IRF) 3 (Samanta *et al.*, 2008), suggesting that modulation of innate immune signaling by EBERs may contribute to EBV-mediated oncogenesis.

In all types of EBV-associated cancers, EBERs are abundantly expressed (Forte and Luftig, 2011). This fact makes EBERs a potentially important target for novel therapies for EBV-associated cancers including HL. However, other studies have found that EBERs are not essential for primary infection, viral replication, or B-cell immortalization (Swaminathan *et al.*, 1991). Furthermore, deletion of EBERs showed little effect on the EBV transformation frequency of primary B cells (Gregorovic *et al.*, 2011). Thus, to develop novel therapeutic approaches to EBV-associated cancers based on their EBER expression, clear mechanisms of EBERs should be identified by further studies.

EBNA1: EBNA1 is an indispensable protein for viral DNA replication and maintenance of the viral episome in infected cells (Hsieh *et al.*, 1993). It has been shown that EBNA1 not only promotes the efficiency of immortalization of human primary B cells *in vivo*, but also inhibits apoptosis by up-regulating the apoptosis suppressor protein, survivin (Humme *et al.*, 2003; Lu *et al.*, 2011), suggesting that it may play a direct role in the pathogenesis of EBV-associated malignancies.

Since EBNA1 is consistently expressed not only in chronic active EBV infection, but also in all EBV-associated malignancies (Yoshioka *et al.*, 2003), the viral antigen has been suggested as a very potent target for immunotherapy against HL. However, it is not easy to detect EBNA1-specific cytotoxic T lymphocytes (CTLs) from blood taken during primary infection or from healthy virus carriers (Masucci *et al.*, 1992; Rickinson *et al.*, 1992; Steven *et al.*, 1996), because EBNA1 is protected from processing and presentation via the MHC class I pathway due to its internal Glycine-Alanine repeat (GAr) domain (Levitskaya *et al.*, 1995), which acts as a inhibitory signal to prevent proteasome dependent degradation of this antigen (Levitskaya *et al.*, 1997). Nevertheless EBNA1 is a very attractive viral antigen for a therapeutic target due to its ubiquitous presence in all EBV infected cells, and novel strategies to avoid the restricted class I processing of EBNA1 have been reported by several groups. In the experiment of Tellam and his collegues, the EBNA1 gene was covalently linked with ubiquitin, and subject to targeting to the N-end rule pathway, in which the stability level of a protein *in vivo* can be dramatically changed by the identity of its N-terminal residue (Varshavsky, 1996; Tobery and Siliciano, 1999; Dantuma *et al.*, 2000). These modifications dramatically enhanced intracellular degradation of the protein and restored CD8+ T cell recognition, demonstrating that GAr-mediated proteosomal blockade on EBNA1 can be reversed (Tellam *et al.*, 2001). Moreover, it was demonstrated that CD8 T cells from patients with HL were successfully stimulated *in vitro* with a construct containing a GAr-deleted EBNA1, reversing the functional T cell impairment as well as responding to tumor cells expressing EBNA1 (Smith *et al.*, 2006).

Currently, it is not easy to discover a therapeutic drug that specifically modulates the degradation of EBNA1. However, if fine pharmacologic manipulation of the ubiquitin proteasome system that could alter the outcome of many diseases become possible, one might be able to develop highly specific drugs that target the degradation pathways of a single or a few proteins with no side effect on other proteins (Reinstein and Ciechanover, 2006).

EBV-encoded latent membrane proteins, LMP1, LMP2A, and 2B: LMP1 acts as a constitutively active receptor in a ligand-independent manner, mimicking CD40 (Gires *et al.*, 1997). It activates the majority of signaling pathways that are known to be activated in HRS cells, including NF-κB, PI3K/Akt/mTOR, and Jak/STAT pathways, thereby induces various antiapoptotic proteins and cytokines (Chen *et al.*, 2003; Lambert and Martinez, 2007; Kung *et al.*, 2011). Since normal germinal center B cells that lack BCR in their cell surfaces are eliminated by apoptosis, the HRS precursor cell is assumed to be rescued by LMP1 that plays a critical role in the protection of B cells from apoptotic death by up-regulating several anti-apoptosis genes (Asso-Bonnet *et al.*, 1998; Lee *et al.*, 2003).

The LMP2 gene encodes two protein isoforms, LMP2A and LMP2B. They are identical except for an additional 119 aa-

long cytoplasmic region at the amino-terminus of the LMP2A isoform (Longnecker and Kieff, 1990). LMP2A mimics BCR, thereby provides an essential prosurvival signal for B cells. In EBV-infected B cells, LMP2A functions to promote viral latency, providing signals to ensure cell survival in the absence of BCR signaling (Longnecker, 2000). It can enhance cell growth, survival, and cellular differentiation through activation of the Ras/PI3K/Akt, Notch and β-catenin/Wnt signaling pathways (Scholle *et al.*, 2000; Morrison *et al.*, 2003; Anderson and Longnecker, 2008). Compared to LMP2A, the role of LMP2B are relatively unknown. Recently, it was reported that LMP2B as well as LMP2A modulate signaling from receptors involved in innate immune responses (Shah *et al.*, 2009). Overall, the two viral proteins, LMP1 and LMP2A, are considered the major players in the generation of EBV-associated HRS phenotypes (Bechtel *et al.*, 2005).

Use of mAbs as immunotoxins against the upregulated surface molecules of HRS cells, such as CD30 and CD40, for targeted immunotherapy was stated in the previous section. In order to function as a potent target molecule against immunotoxins, the presence of an extracellular domain and its easy accessibility are essential, which are lacking in LMP1 as well as LMP2A (Flanagan *et al.*, 2003). These LMPs contain relatively large cytoplasmic domains with extremely short extracellular loops connecting transmembrane segments, which are not easily accessible by antibodies (Gires *et al.*, 1997; Panousis and Rowe, 1997; Lynch *et al.*, 2002). Moreover, the extracellular loop regions marginally elicit antibodies in the course of natural infection and tumorigenesis (Paramita *et al.*, 2011). Thus, despite their abundance, antibody-based targeting of LMP1 or LMP2 on EBV-positive HRS cells has low therapeutic possibilities.

Recently, it was shown that conformational peptides mimicking two adjacent loops of LMP1 induce high-affinity antibodies with antitumor activities in mice (Delbende *et al.*, 2009). However, unlike the *in vivo* experimental animal settings, the induction of effective CTL responses against those proteins has showed difficulties *per se* due to unique characteristics of HRS cells and their microenvironment as described (Marshall *et al.*, 2003). For example, *in vitro* HRS cells that present epitopes from LMP1 and LMP2A are subject to lysis by EBV-specific CTLs, but EBV-infected HRS cells survive *in vivo* (Sing *et al.*, 1997; Chapman *et al.*, 2001; Su *et al.*, 2001). Thus, although EBV-associated HL patients initially achieve an effective anti-EBV response after the period of first EBV infection, they do not completely eradicate EBV-infected HRS cells. In addition, despite the presence of EBV-specific CTLs in the peripheral blood, the CTLs are not found in the immediate surrounding area of EBV-positive HRS cells (Frisan *et al.*, 1995). Thus, for successful CTL therapy for EBV-positive HL, not only effective EBV-specific CTLs, but possibly also the modulation of the tumor microenvironment to eliminate the barriers that inhibit CTL function will be required.

Nevertheless, these LMP molecules still draw attention as major targets for therapeutic purposes. Since patients with HL display functional impairment of CTL, adoptive cellular immunotherapy based on efficient EBV-specific CTL cells may provide an answer to success. Development of an epitope-based vaccination strategy to augment EBV-specific cytolytic activity of CTLs is currently paid attention to as one of the preferred approaches for the treatment of EBV-associated relapsed HL. Firstly, efficient antigen (epitope) presentation by antigen presenting cells may improve the CTL activities. The therapeutic potentials of dendritic cell (DC) vaccine transduced by a recombinant vaccinia or adeno-associated virus carrying LMP1 and LMP2 CTL epitope DNA were evaluated, and the treatment of the vaccine was effective on eliminating tumors of syngeneic animals (Taylor *et al.*, 2004; Pan *et al.*, 2009). And a phase II study on vaccination of autologous DCs transduced with an adenovirus encoding a truncated LMP1 and full-length LMP2 is under evaluation for the safety and efficacy for patients with metastatic EBV-positive nasopharyngeal carcinoma (Lutzky *et al.*, 2010; Chia *et al.*, 2011). In this case, the truncated form of LMP1 was utilized because the full-length protein has both oncogenic and immunosuppressive properties (Gottschalk *et al.*, 2003). Secondly, antigen presentation by polyepitope vaccines may enhance CTL activities. A polyepitope comprises at least one recombinant protein including multiple CTL epitopes from one or more pathogens. A recombinant poxvirus vaccine encoding a polyepitope derived from LMP1, called as an LMP1 polyepitope vaccine, displayed stronger anti-LMP1 responses (Duraiswamy *et al.*, 2003). In addition, an exposure to a replication-deficient adenoviral system with polyepitopes from LMP1 or LMP2 induced effective expanding of specific T cells. HL patients with this adenoviral construct in combination with IL-2, were sufficient to reverse the functional T cell impairment and restored cytolytic function (Smith *et al.*, 2006). Like EBNA1, LMP1 molecules can be efficiently presented via cotranslational ubiquitination combined with N-end rule targeting. Since this method is involved with enhanced degradation of LMP1, this strategy completely abrogates cellular signaling pathways associated with the oncogenic phenotype, and helps in enhancing immunogenicity (Tellam *et al.*, 2003), suggesting that the proteasomal targeting strategy could be therapeutically utilized for various tumor-associated oncogenes.

CONCLUSIONS

In the early twentieth century, cHL was incurable in most cases. Although cHL is currently considered one of the most curable forms of cancer with conventional chemotherapy, therapeutic challenges still remain, especially in finding novel strategies to control patients with relapsed cHL, albeit in small numbers. Since immunopathogenic features of HRS cells are very similar in both EBV-positive cHL and EBV-negative cHL, efforts to find cellular targets for drug development to treat cHL have been mainly focused on common phenotypes of HRS cells regardless of the presence of EBV. However, the presence of EBV-latent antigens abundantly expressed in the malignant HRS cells represents an attractive therapeutic option for targeted immunotherapy. Through continued researches of existing and new treatment options for cHL, advances should continue to be made.

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