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REVIEW

Measuring Epstein–Barr virus (EBV) load: the significance and application for each EBV-associated disease

Hiroshi Kimura[1]*, Yoshinori Ito[2], Ritsuro Suzuki[3] and Yukihiro Nishiyama[1]

[1]*Department of Virology Nagoya University Graduate School of Medicine, Nagoya, Japan*
[2]*Department of Pediatrics Nagoya University Graduate School of Medicine, Nagoya, Japan*
[3]*Department of HSCT Data Management, School of Medicine, Nagoya University, Nagoya, Japan*

SUMMARY

Because Epstein–Barr virus (EBV) is ubiquitous and persists latently in lymphocytes, simply detecting EBV is insufficient to diagnose EBV-associated diseases. Therefore, measuring the EBV load is necessary to diagnose EBV-associated diseases and to explore EBV pathogenesis. Due to the diverse biology of EBV, the significance of measuring EBV DNA and the optimal type of specimen differ among EBV-associated diseases. Recent advances in molecular technology have enabled the EBV genome to be quantitated rapidly and accurately. Real-time polymerase chain reaction (PCR) is a rapid and reliable method to quantify DNA and is widely used not only as a diagnostic tool, but also as a management tool for EBV-associated diseases. However, each laboratory currently measures EBV load with its own "homebrew" system, and there is no consensus on sample type, sample preparation protocol, or assay units. The EBV real-time PCR assay system must be standardised for large-scale studies and international comparisons.

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INTRODUCTION

Epstein–Barr virus (EBV) belongs to the genus Lymphocryptovirus, subfamily Gammaherpesvirinae, family Herpesviridae [1]. In primary infection, EBV is predominantly asymptomatic but occasionally causes infectious mononucleosis in adolescents or young adults. Rarely, chronic active EBV infection develops in immunocompetent hosts [2,3]. Moreover, several malignancies, including Burkitt's lymphoma, Hodgkin's lymphoma, nasal natural killer (NK) cell lymphoma, nasopharyngeal carcinoma, and post-transplant lymphoproliferative disorder (PTLD) have been etiologically linked to EBV infection [2,3].

Because EBV is ubiquitous and establishes a lifelong persistent infection after primary infection, simply detecting EBV is insufficient to diagnose EBV-associated diseases. Measuring the EBV load is essential to follow and diagnose EBV-associated diseases and to explore the pathogenesis of EBV infection. Over the last decade, advances in molecular technology have enabled minimal amounts of DNA to be quantified rapidly and accurately, and such techniques have been used to diagnose viral infection. For EBV infections, a variety of methods, techniques, and protocols have been used to measure EBV loads at many institutions.

However, EBV detection techniques and viral load estimation values have not been standardised and results vary between different laboratories [4]. Furthermore, there is no consensus regarding

*Corresponding author: H. Kimura, Department of Virology, Nagoya University Graduate School of Medicine, 65 Turumai-cho, Showa-ku, Nagoya 466-8550, Japan.
E-mail: hkimura@med.nagoya-u.ac.jp

Abbreviations used
EBV, Epstein–Barr virus; NK, natural killer; PTLD, post-transplant lymphoproliferative disorder; PBMCs, peripheral blood mononuclear cells; CTL, cytotoxic T lymphocytes; EBER, EBV-encoded small RNA; EBNA, EBV nuclear antigen; BARTs, BamHI A rightward fragments; LMP, latent membrane protein; PCR, polymerase chain reaction; HIV, human immunodeficiency virus; NHL, non-Hodgkin's lymphoma

what type of samples should be tested: peripheral blood mononuclear cells (PBMCs), whole blood, plasma, or serum. In this review, we summarise the principles of measuring EBV load based on the biology of EBV infection and propose protocols for managing EBV-associated diseases.

BIOLOGY OF EBV INFECTION

Similar to other gammaherpesviruses, EBV establishes a life-long infection in B cells. Figure 1 shows a schematic representation of both primary and persistent EBV infections. In primary infection, cell-free EBV in the saliva infects naïve B cells in the oropharynx [5]. B cells are infected after the viral envelope glycoprotein, gp350/220, attaches to the cell surface protein CD21, the primary EBV receptor [6]. EBV initiates a latent growth-transforming infection, causing naïve B cells to transform into proliferating blasts. In immunocompetent hosts, both EBV-specific cytotoxic T lymphocytes (CTL) and NK cells control the outgrowth of EBV-transformed cells during primary infection [7]. Primary EBV infection is usually asymptomatic, but occasionally progresses to infectious mononucleosis, which resolves spontaneously after the emergence of EBV-specific immunity [7]. EBV then establishes a latent infection in memory B cells (Figure 1), which are non-permissive for viral replication [8,9]. After convalescence, EBV persists latently in these memory B cells in an episomal form. These virus-infected cells persist at a low level, approximately 1 in 10 000 to 100 000 memory B cells [5]. Occasionally, infected memory B cells differentiate into plasma cells that undergo lytic infection and produce virus (Figure 1). Newly infected naïve B cells have phenotypes of transformed cells, but are controlled by CTL unless immunity is suppressed. In immunocompromised hosts, transformed cells become proliferating blasts that can result in symptomatic disease, such as PTLD.

The epithelial cells of Waldeyer's ring are also infected by EBV and shed virus during primary infection [10,11]. EBV replicates in a permissive cell type in the oropharynx, probably specialised epithelial cells, that either binds virus directly or acquires virus by transfer from the surface of adjacent B cells (Figure 1) [12]. EBV infects epithelial cells through a CD21-independent mechanism, and the viral glycoprotein gH mediates EBV attachment to CD21-negative epithelial cells [13].

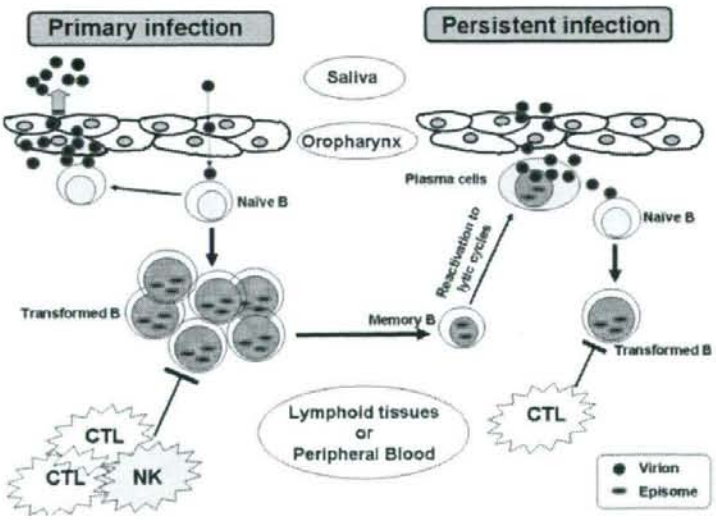

Figure 1. Schematic representation of Epstein–Barr virus (EBV) infection. In primary infection, EBV in the saliva directly infects naïve B cells in the oropharynx. EBV-infected B cells transform and proliferate as activated blasts but are finally controlled by cytotoxic T lymphocytes (CTL) or natural killer (NK) cells. After convalescence, EBV persists as a latent infection with episomal DNA in memory B cells. Occasionally, memory B cells differentiate into plasma cells that undergo lytic infection and produce virus. Newly infected naïve B cells become transformed, but are controlled by CTL unless cellular immunity is suppressed.

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Accumulating evidence suggests that EBV also infects T and NK cells during primary infection. Although T and NK cells do not typically express CD21, it is expressed by thymic T cells [14]. In the tonsils of acute infectious mononucleosis patients, EBV-positive T and NK cells are seen, although they are rare [15,16]. In addition, T and NK cells are detected in the peripheral blood during acute infectious mononucleosis [17]. Recently, Isobe *et al.* reported the *in vitro* infection of human NK cells by EBV [18]. Moreover, NK cells activated by EBV-infected B cells acquire CD21 by synaptic transfer, and these ectopic receptors allow EBV to bind to NK cells [19].

CLASSIFICATION OF EBV-ASSOCIATED DISEASES BY EBV LATENT GENES

In healthy individuals, EBV is latently maintained in memory B cells, which express only the transcripts for EBV-encoded small RNAs (EBERs) [20,21]; this state is termed latency 0 (Table 1) [1,22]. In EBV-associated diseases, viral gene expression is classified into one of the three other latency patterns [1,2]. In latency type I, which is found in Burkitt's lymphoma [23], EBV nuclear antigen (EBNA)-1 and *Bam*HI A rightward fragments (BARTs) are expressed in addition to EBERs (Table 1). In latency type II, characteristics of Hodgkin's lymphoma [24] and nasopharyngeal carcinoma [25], EBNA-1, latent membrane protein (LMP)-1, LMP-2, BARTs, and EBERs are expressed (Table 1). In latency type III, associated with lymphoproliferative disorders [26], all latency genes, including EBNA-2 and EBNA-3s, are expressed (Table 1). As EBNA-3s are dominant $CD8^+$ CTL targets [7], cells in latency type III are usually eliminated by CTL. Thus, latency type III is only maintained in immunosuppressed states, such as in post-transplant or AIDS patients. On the other hand, in latency types I and II, only a restricted number of less-antigenic EBV latent genes are expressed, allowing EBV-infected cells to evade CTL [7].

Although EBV latency patterns can be classified grossly into these four types, this classification is not very strict, and heterogeneous patterns are reported in EBV-associated diseases [27,28]. Patterns of viral gene expression can differ between different cell subsets in the same individual or even tissue. Moreover, both latent and lytic infections are observed within the same patient or tissue. For example, in infectious mononucleosis, EBV-transformed B cells undergo latency type III, while plasma cells or epithelial cells are in lytic infection and produce cell-free virus. In nasopharyngeal carcinoma, only a few cells may enter lytic viral replication [29], while the majority of nasopharyngeal carcinoma cells are in the latent II phase [25].

TECHNICAL ASPECTS IN MEASURING EBV LOAD IN PERIPHERAL BLOOD

Several methods have been developed to measure EBV load and are summarised in Table 2.

When possible, detecting virus-associated antigens using virus-specific antibodies is a direct

Table 1. Patterns of Epstein–Barr virus (EBV) infection and EBV-associated diseases

Pattern	EBV-related genes							
	EBNA-1	EBNA-2	EBNA-3s	LMP-1	LMP-2	BARTs	EBERs	
Latency 0	−	−	−	−	−	±	+	Healthy carrier
Latency I	+	−	−	−	−	+	+	Burkitt's lymphoma
Latency II	+	−	−	+	+	+	+	Hodgkin's lymphoma Nasal NK cell lymphoma Chronic active EBV infection Nasopharyngeal carcinoma
Latency III	+	+	+	+	+	+	+	Infectious mononucleosis Post-transplant or opportunistic lymphoproliferative disorder

EBNA, EBV nuclear antigen; LMP, latent membrane protein; BARTs, *Bam*HI A rightward transcripts; EBERs, EBV-encoded small RNAs; NK, natural killer.

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Table 2. Representative methods to measure Epstein–Barr viral load

Methods	Targets	Characteristics				Disadvantages
		Sensitivity	Rapidity	Handling	Quantitativeness	
Immunofluorescence	Antigen	Low	Moderate	Moderate	Fair	Low sensitivity No good antibodies available
EBER-1 *in situ* hybridisation	RNA	Moderate	Slow	Difficult	Fair	Only applicable for cells Requires specialised skills
Semi-quantitative PCR	DNA	High	Moderate	Moderate	Poor	Inaccurate quantification
Quantitative-competitive PCR	DNA	High	Moderate	Moderate	Fair	Requires time and labour
Real-time PCR	DNA	High	Rapid	Easy	Fair	Needs special equipment

EBER-1, Epstein-Barr virus encoded small RNA 1; PCR, polymerase chain reaction.

and easy way to measure viral load in the peripheral blood. However, there are currently no good monoclonal antibodies or suitable antigens for EBV. Only one antigen, EBNA-1, is expressed in all EBV-associated diseases. As the EBNA-1 protein is expressed at low levels in EBV-infected cells, an anti-complement immunofluorescence method is required to enhance the fluorescent signal. However, the sensitivity of this method is too low to be applied routinely in clinical settings (Table 2).

EBER-1, one of the EBERs, is detectable in virtually all EBV-infected cells and is expressed at very high levels, reaching 10^7 molecules per cell, although no protein is apparently translated. Previous studies have used *in situ* hybridisation with an EBER-1 probe to detect and count EBV-infected cells [30,31]. This technique is widely used to detect EBV in tissue specimens [32]. Although EBER-1 *in situ* hybridisation is a specific and direct method to detect EBV-infected cells, it is only applicable to infected cells and does not detect cell-free virus (Table 2). Furthermore, specialised skills are needed to handle RNA. This technique is not currently used to measure EBV load in the peripheral blood, and *in situ* hybridisation has been replaced by methods that detect amplified DNA, such as polymerase chain reaction (PCR).

PCR is a sensitive and rapid DNA detection method that has been used to measure EBV loads. Semi-quantitative PCR, by endpoint detection of diluted samples or by quantifying amplified products, was first developed in the mid-1990s [33–37]. However, the linear range of such semi-quantitative PCR is too narrow to measure a variety of samples because the amount of amplified product reaches a plateau after the log phase of the reaction [38]. This inaccuracy limits this method to the detection of only very large differences (Table 2) [39]. To overcome this problem, quantitative-competitive PCR, which uses the presence of co-amplified PCR targets of known concentrations, was developed in the late 1990s [40,41]. The competitor acts as a standard and as a control for differences in amplification efficiency and enables quantitative-competitive PCR to determine EBV loads within two- to four-fold differences [39]. However, quantitative-competitive PCR requires both time and skill to complete as this assay includes gel electrophoresis and Southern blot hybridisation steps (Table 2). Thus, this method has not been widely used.

Real-time PCR is a rapid and reproducible method for quantifying DNA that was first introduced for EBV in 1999 [31,42–44]. Real-time PCR measures the accumulation of PCR products with either a fluorogenic probe or SYBR green I dye, coupled with real-time laser scanning. In the former system, a dual-labelled fluorogenic hybridisation probe (a "TaqMan" probe) is commonly used. One fluorescent dye serves as a reporter and its emission spectrum is quenched by the second fluorescent dye. Nuclease degradation of the hybridisation probe releases the quenching of the

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reporter fluorescent emission, resulting in an increase in peak fluorescence [45]. In the latter system, SYBR green I dye is used as a marker for product accumulation. This system is less expensive, but less specific for EBV than a hybridisation probe strategy [46].

Real-time PCR has a large dynamic range for target molecule determination because real-time measurement of the PCR product enables the amplified products to be quantified in the log phase of the reaction [45]. Furthermore, because the reaction is performed and measured in sealed wells, the system does not require the many precautions that are taken with amplified products to avoid contamination. This is a great improvement over conventional PCR assays, which have considerable risks of carry-over contamination. With its speed, accuracy, and ability to handle many samples, the real-time PCR assay has replaced other quantitative PCR methods and is now widely used for measuring EBV load (Table 2). One disadvantage of real-time PCR is the need of specialised and relatively expensive equipment for real-time laser scanning, although the cost is decreasing. Like other EBV methods, there is currently no standardised real-time PCR protocol for measuring EBV load [4]. To date, all real-time PCR assays used to measure EBV load have been "in house" or "homebrew" systems, and primers and probes differ across many laboratories. Most important, the real-time PCR assay requires a standard, usually a plasmid containing the target gene. As these standards are made and serially diluted in individual laboratories, EBV values from each system cannot be compared, even when the same system is used.

APPLICATION OF EBV LOAD MEASUREMENTS FOR EACH EBV-ASSOCIATED DISEASE

The biology of EBV infection is complex and differs across the EBV-associated diseases. For example, in PTLD, blast B cells in latency type III proliferate and migrate into the peripheral blood. Most EBV genomes in the peripheral blood are cell-associated (Figure 2). In contrast, in nasopharyngeal carcinoma, malignant cells proliferate in tissues and rarely migrate into the peripheral blood, and most EBV genomes in peripheral blood are cell-free. Therefore, determining the EBV load depends on whether and how much cell-associated or cell-free EBV exists in the peripheral blood, and the most desirable specimens differ among the different EBV-associated diseases (Table 3). In this chapter, the significance and application of techniques to measure EBV load are discussed based on the biology of each EBV-associated disease.

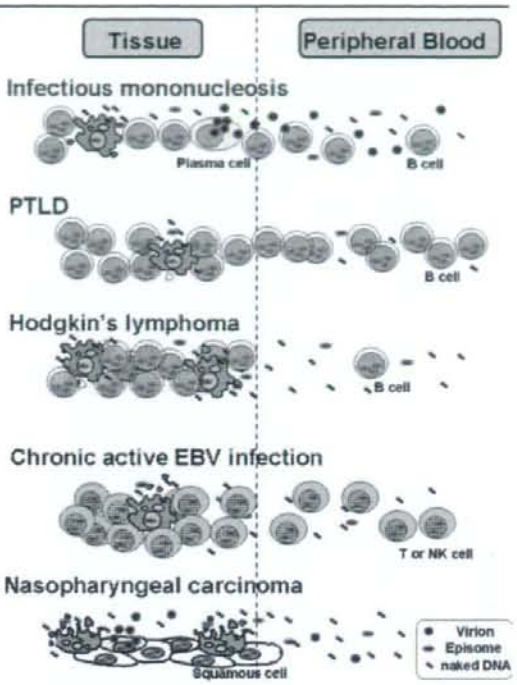

Figure 2. How Epstein–Barr virus (EBV) persists in tissues and peripheral blood. Infectious mononucleosis: Virion and naked (degraded) EBV DNA derived from plasma cells in lytic infection or from apoptotic cells, respectively, passes into the peripheral blood. Some transformed, latently infected B cells leak into the bloodstream. Both cell-free and cell-associated EBV exist in the peripheral blood. Post-transplant lymphoproliferative disorder (PTLD): Transformed B cells proliferate vigorously in lymphoid tissues and flow into the peripheral blood. Most of the EBV DNA in the blood is cell-associated. Hodgkin's lymphoma: Episomal or naked EBV DNA, derived from apoptotic cells, passes into the peripheral blood. Some latently EBV-infected tumour cells (Hodgkin cells) may leak into the bloodstream. Chronic active EBV infection: EBV-infected T and NK cells migrate into the peripheral blood. Cell-free EBV DNA, derived from apoptotic cells in affected organs, also exists in the peripheral blood. Nasopharyngeal carcinoma: Episomal or naked EBV DNA, derived from apoptotic tumour cells (squamous cells), passes into the peripheral blood. Virions derived from a very restricted subset of tumour cells in lytic infection may leak into the blood.

Table 3. Optimal specimens for measuring viral load in each Epstein–Barr virus (EBV)-associated disease

Disease	Infected cells	Infection pattern	Specimens for measuring viral load		
			Plasma or serum	Mononuclear cells	Whole blood
Infectious mononucleosis	Plasma cells B cells	Lytic infection Latency III	Desirable	Not recommended	Not recommended
Post-transplant lymphoproliferative disorder	B cells	Latency III	Controversial	Desirable	Preferable
Hodgkin's lymphoma	Hodgkin cells (B cell origin)	Latency II	Desirable	Not recommended	ND
Chronic active EBV infection	T or NK cells	Latency II	Useful for prognosis	Desirable for diagnosis	ND
Nasopharyngeal carcinoma	Squamous cells	Latency II (Lytic infection)	Desirable	Not recommended	ND

NK, natural killer; ND, no or little data available.

Infectious mononucleosis/EBV-associated haemophagocytic syndrome

In primary EBV infection, such as infectious mononucleosis, both cell-associated and cell-free EBV exist in the peripheral blood (Figure 2) [47]. Some transformed B cells in latent infection leak into the bloodstream. Encapsidated viral genomes (virions), which are produced from plasma cells in lytic infection, flow into the peripheral blood [48]. Fragmented or naked DNA from apoptotic cells may also be detected as cell-free EBV DNA in the peripheral blood. Thus, EBV DNA can be detected in both cell-free blood (serum or plasma) and PBMCs.

In primary EBV infection, EBV DNA is detected in the blood of virtually all patients [35]. After the appearance of EBV-specific immunity, EBV is controlled [49], and the EBV DNA load decreases gradually in PBMCs but disappears rapidly from the plasma [50–52]. Memory B cells, in which EBV is latent in an episomal form, remain in the peripheral blood. As EBV DNA is detected infrequently in the serum or plasma of healthy seropositive carriers [35,53,54], the presence of cell-free EBV DNA signifies a primary infection or reactivation of EBV. These observations indicate that plasma and serum are the most desirable specimens for identifying infectious mononucleosis or primary EBV infection (Table 3).

As infectious mononucleosis is a self-limiting disease that is usually diagnosed by clinical symptoms and serology, measuring EBV DNA is not necessary for the diagnosis. However, EBV load in the serum or plasma correlates with disease severity in infectious mononucleosis [35,52,55]. Primary EBV infection occasionally causes haemophagocytic syndrome. This disease, also called haemophagocytic lymphohistiocytosis, is rare in Western countries, but is common in eastern Asia [1,56]. As EBV-associated haemophagocytic syndrome can be a severe and even life- threatening disease, early diagnosis and intensive therapy are necessary [56]. Extremely high viral loads are seen in both PBMCs and the serum of patients with EBV-associated haemophagocytic syndrome [35,50,55, 57]. Monitoring EBV DNA in serum is useful for evaluating the therapeutic response [55,57].

Post-transplant lymphoproliferative disorder

In immunosuppressed transplant patients, EBV-transformed B cells become proliferating blasts and occasionally progress to PTLD. Transformed B cells proliferate vigorously in lymphoid tissues and migrate into the peripheral blood. Most EBV is cell-associated and EBV DNA is detected in high copy numbers in PBMCs (Figure 2).

Cell-free, non-encapsidated EBV DNA is also detected in the blood, indicating fragmented or naked DNA [48]. Riddler *et al.* were the first to show that molecular testing for EBV in the peripheral blood could be used to non-invasively monitor PTLD [34]. Measuring EBV DNA has since been studied extensively and is now an indispensable tool for controlling PTLD. This method is used not only for diagnosis, but also for disease prediction [33,34,58–60], therapeutic efficacy estimation [61,62], and prevention [63–66].

There has been debate regarding which specimen(s) should be used to identify PTLD. Earlier studies used PBMCs because EBV DNA is detected in high copy numbers in these cells [33,34,36]. Cell-free EBV DNA is also present, although at lower quantities [67]. Recent studies have used plasma or serum because they are readily obtained and handled. Several studies reported that quantifying cell-free EBV DNA predicted the development of PTLD [59,68,69]. However, serum or plasma samples lack cell-associated virus and therefore plasma loads are not correlated with PBMC values [70]. Stevens *et al.* reported that the increased EBV DNA loads in PTLD patients were restricted to the cellular compartment, as parallel serum samples were below the cut-off value [67]. Clave *et al.* also reported that viral DNA was detected only in the cellular compartment in some patients and that measuring EBV load in the plasma could provide a false negative result [62]. More recently, unfractionated whole blood has been used because whole blood can be obtained readily and contains all blood compartments that may harbour EBV. There have been several reports that whole blood is better than plasma/serum when testing PTLD patients [67,70–73]. Based on these observations, whole blood is the preferred specimen for PTLD Table 3), although more thorough studies are needed to resolve this controversy.

PTLD pathogenesis differs between stem cell and solid organ transplants. In stem cell transplantation, donor-derived B cells are the origin of PTLD. Immunosuppression and delayed immune reconstitution, both of which are severe just after conditioning but are subsequently cleared, are the major causes of the disease. Increases in EBV load are seen 2–3 months after stem cell transplantation [60]. Two-thirds of PTLD cases occur within 80 days after stem cell transplantation [74]. The overall incidence of PTLD is relatively low in stem cell transplantation (<1%), although T-cell-depleted transplants or the use of anti-thymocyte globulin greatly increases the risk of PTLD. The high EBV load has some positive predictive and a very good negative predictive value, particularly if the graft was T-cell depleted [59,75]. Most transplantation facilities now monitor EBV load with real-time PCR for high-risk patients undergoing stem cell transplantation [3]. In contrast, recipient-derived B cells are the origin of PTLD in solid organ transplantation, although primary EBV infection from donors occurs occasionally through grafts. Immunosuppression, which is necessary to control rejection, is the main cause of PTLD. Immunosuppression usually must be maintained for the lifetime of the patient. Therefore, the risk of developing PTLD continues for life, although early PTLD (in the first year after transplantation) occurs in cases of primary EBV infection [76]. On the otherhand asymptomatic EBV reactivation with high viral load is observed during the extended post-transplant follow-up [77]. The predictive value of the EBV load in solid organ transplantation is less clear, because some recipients have high EBV loads and remain stable for months or years without developing PTLD [78,79]. Measuring viral load appears to be most useful in monitoring patients who were EBV-seronegative before transplantation but are at high risk of developing PTLD [76]. The incidence of PTLD also depends on the type of transplantation: multivisceral has the highest incidence (13–33%), followed by intestinal (7–11%), heart–lung (9.4%), lung (1.8–7.9%), heart (3.4%), liver (2.2%), and kidney (1%) [76].

Differences in the management of PTLD between stem cell and solid organ transplantations are summarised in Table 4. Many laboratories and facilities have proposed that measuring EBV load is a valuable diagnostic and prognostic tool for monitoring PTLD. However, as different systems are used for different samples and patients, it is difficult to determine which EBV load value should be used to identify high-risk patients. For example, in stem cell transplantation, some authors recommend that an EBV load of >300 copies/10^5 PBMCs is indicative of intervention [66], while others have reported that an EBV load >50 000 copies/mL of serum predicts the development of PTLD [69]. We also proposed that an EBV load >10 000 copies/μg PBMC DNA is

Table 4. Management of post-transplant lymphoproliferative disorder: difference between stem cell and solid organ transplantation

	Stem cell transplantation	Solid organ transplantation
Risk factors	T cell-depleted grafts Anti-thymocyte/lymphocyte globulin	Primary infection (seropositive donor/seronegative recipient) Anti-thymocyte/lymphocyte globulin Multivisceral, intestinal, heart–lung, or lung transplantation
EBV load monitoring		
Period	Early after transplantation (~3 months)	Early after transplantation (~1 year) but risk continues for life
Predictive value	Good	Poor
Therapy		
First line	Anti-CD20 monoclonal antibody	Reduction or withdrawal of immunosuppression
Second line	Cell therapy Donor leucocyte infusion EBV-specific CTL	Anti-CD20 monoclonal antibody

EBV, Epstein–Barr virus; CTL, cytotoxic T lymphocytes.

indicative of developing PTLD [60]. This indicates that quantitative values cannot be compared between laboratories; therefore, we have not shown any representative PTLD values in Table 4. Recent studies have indicated that changes in viral load kinetics, rather than single viral load measurements, show a better correlation with organ involvement [80].

AIDS-related lymphoma

Many different EBV-associated diseases develop in patients infected with human immunodeficiency virus (HIV), including not only diseases of lymphocyte origin but also those of epithelial cell origin, such as oral hairy leucoplakia. For diseases of lymphocyte origin, opportunistic B lymphoproliferative disorders, Hodgkin's lymphoma, and non-Hodgkin's lymphoma (NHL) are more common in HIV-infected patients [81]. Three principal types of NHL are recognised in the HIV setting: (1) sporadic Burkitt's lymphoma, which develops relatively early in disease, (2) peripheral NHL, which occurs at the late stages, and (3) primary central nervous system lymphoma, which predominantly occurs in profoundly immunocompromised late-stage patients [4]. EBV appears to play a pivotal role in the development of AIDS-associated primary central nervous system lymphoma and is frequently associated with virus in the cerebrospinal fluid [81]. Importantly, EBV is rarely detected in the cerebrospinal fluid in HIV-infected patients without primary central nervous system lymphoma.

The EBV copy number in PBMCs increases rapidly after HIV infection, and this increase precedes the decrease in $CD4^+$ T cell counts [47]. The presence of EBV in the blood is significantly associated with lower $CD4^+$ T cell counts, but the EBV load is not correlated with $CD4^+$ T cell counts [82]. Piriou *et al.* suggested that inter-individual differences in EBV load are maintained after HIV infection, providing evidence for the existence of an individual EBV set point. Thus, currently the significance of measuring EBV load in the blood is unclear. Some authors have suggested that EBV loads may be a useful marker to diagnose EBV-associated NHL [83], but longitudinal studies of EBV load in both PBMCs and serum samples from HIV-infected patients have indicated no specific correlation with the development of NHL [84].

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Hodgkin's lymphoma

Approximately 40–50% of Hodgkin's lymphoma patients are EBV-positive and the disease is etiologically linked to EBV in Western countries [2,3], although the role of EBV in the pathogenesis of the disease is unclear. EBV is maintained in Hodgkin and Reed–Sternberg cells in latency type II with episomal DNA [24]. Hodgkin and Reed Sternberg cells are thought to originate from germinal centre B cells. Compared to blast B cells in PTLD, Hodgkin and Reed–Sternberg cells rarely migrate into the peripheral blood. EBV exists predominantly in the serum or plasma as episomal or naked EBV DNA derived from apoptotic lymphoma cells (Figure 2) [47,85]. Indeed, cell-free EBV DNA is detected in the serum of most patients with EBV-associated Hodgkin's lymphoma [42]. EBV load in the serum or plasma is correlated with therapeutic responses [86], and EBV positivity in post-treatment samples indicates a poor prognosis [42]. Thus, serum and plasma are optimal samples to monitor Hodgkin's lymphoma (Table 3).

Nasal NK cell lymphoma

Nasal NK cell lymphoma, while rare in Western countries, is relatively common in East Asia. The primary site of involvement is the nasal cavity, but sometimes similar neoplasms develop in extranasal sites [87]. Nasal NK cell lymphoma is almost always associated with EBV [88]. Similar to Hodgkin's lymphoma, nasal NK cell lymphoma patients have increased amounts of circulating EBV DNA in the plasma or serum [89], potentially because apoptotic proliferating tumour cells release EBV DNA [90]. Before treatment, circulating EBV DNA increases from 10^5 to 10^6 copies/mL, and EBV DNA is correlated with the clinical staging and prognosis [91,92]. These results indicate that plasma EBV DNA is a useful tumour biomarker for the initial evaluation of nasal NK cell lymphoma. As shown in PTLD, unfractionated whole blood may be used instead of plasma, although there is currently no comparative data on plasma and whole blood.

Chronic active EBV infection

Chronic active EBV infection is a rare, life-threatening disease that occurs in children or young adults. This disease is characterised by chronic or recurrent infectious mononucleosis-like symptoms, such as fever, hepatosplenomegaly, persistent hepatitis, and extensive lymphadenopathy [2,3]. There is accumulating evidence that the clonal expansion of EBV-infected T or NK cells plays a central role in the pathogenesis of chronic active EBV infection [93–95]. We proposed that this disease consists of a T cell- or NK cell-type disease, based on the results of PBMC fractionation and subsequent quantitative PCR [96–98]. EBV-infected T or NK cells with a latency type II pattern can evade the host cellular immune system due to the limited expression of viral proteins with reduced antigenicity [27,98,99]. Together with its poor prognosis, some investigators have recommended calling this disease EBV-associated T/NK lymphoproliferative disorder [100].

Patients with chronic active EBV infection have much higher viral loads in their peripheral blood than latently infected individuals [31]. Both PBMCs and plasma (or serum) have been used to estimate viral loads [96,101–103]. EBV-infected T and NK cells migrate into the peripheral blood. Cell-free EBV DNA, derived from apoptotic cells in affected organs, is also present in the serum or plasma. Such cell-free EBV DNA is sensitive to deoxyribonuclease digestion, indicating that it is episomal or naked DNA [98]. Interestingly, some patients do not have cell-free EBV DNA [96]. Compared to Hodgkin's lymphoma, EBV DNA is more cell-associated in the peripheral blood during chronic active EBV infection (Figure 2). Thus, PBMCs are desirable specimens for diagnostic purposes (Table 3), and in fact PBMCs from most patients with chronic active EBV infection have more than $10^{2.5}$ copies/μg EBV DNA [96]. A higher viral load in the plasma has been associated with deteriorating clinical status [35,102]. Recently, we analysed chronic active EBV infection patients with stem cell transplantation and found that the plasma EBV load at diagnosis, but not PBMC load, was significantly higher in deceased patients than in living patients [104]. The plasma viral load indicates the amount of EBV-infected cells that are infiltrating organs and may reflect organ damage and therefore prognosis (Table 3).

Nasopharyngeal carcinoma

Nasopharyngeal carcinoma is prevalent in southern China, northern Africa, and among Alaskan

Eskimos. Nearly 100% of anaplastic or poorly differentiated nasopharyngeal carcinomas contain EBV genomes and express EBV proteins [2]. The EBV genome is present in transformed epithelial cells, but not in tumour lymphocytes.

In nasopharyngeal carcinoma patients, EBV DNA is detected in the serum or plasma, but not in PBMCs [43,90]. Deoxyribonuclease sensitivity indicates that most of the cell-free EBV DNA is episomal or naked with some encapsidated DNA [90,105], suggesting that DNA is released primarily not only from apoptotic tumour cells but also from cells undergoing lytic infection (Figure 2). Viral copy number in cell-free blood is an important adverse prognostic factor, as is the persistence or reappearance of high copy numbers of EBV DNA in the serum or plasma [106–108]. Based on these reports, serum or plasma is the preferred sample type to measure EBV DNA in nasopharyngeal carcinoma patients (Table 3).

FUTURE PERSPECTIVES

Measuring EBV load is a routine procedure in high-risk patients undergoing stem cell or solid organ transplantation. Real-time PCR is the easiest and most reliable way to measure EBV load and is, therefore, the most widely used method. However, each transplantation facility monitors EBV load with its own "homebrew" system and there is no consensus on the sample type, sample preparation protocol, or assay units used. Each facility uses different primer/probe designs, standards, and equipment. However, standardisation of these materials is necessary for large-scale studies and international comparisons. Ideally, a standardised kit for measuring EBV DNA will be developed and become commercially available. In contrast to "commercially interesting" viruses, such as HIV type 1, hepatitis B virus, and hepatitis C virus, for which commercial kits are available, EBV is classified as a non-commercially interesting viral target [109]. In the era of expanding transplantation medicine, however, the number of transplants and the intensity of immunosuppression are increasing. Furthermore, effective PTLD treatments, such as anti-CD20 monoclonal antibodies, have been developed. Therefore, the importance of measuring EBV load is increasing. In the near future, we will propose the development of a standardised real-time PCR kit for measuring EBV load to permit large-scale studies and international comparisons.

Obviously, measuring EBV load alone is not sufficiently informative to assess a patient's status. Additional information on viral gene expression would provide a better assessment of each patient's condition [39]. Qu *et al.* examined the expression of EBV-associated genes in solid organ transplant recipients and found that persistent low-load carriers expressed only LMP-2, whereas high-load carriers expressed both LMP-1and LMP-2 [77]. They used qualitative reverse-transcription PCR to detect EBV-associated genes. Very recently, quantitative methods using real-time reverse-transcription PCR have been used to analyse the expression of EBV-associated genes [110,111]. These quantitative methods will help not only to clarify the pathogenesis of EBV-associated diseases but also to manage patients with high viral loads.

Finally, evaluating EBV-specific cellular immunity is helpful to manage EBV-associated diseases. Human leucocyte antigen class I tetramer analysis is a rapid and direct way to quantify EBV-specific CTL [112]. Using tetramer assays, a number of investigators have combined quantitating the EBV load with serial monitoring of EBV-specific CTL in transplant patients [62,113,114]. High viral loads are predictive of PTLD development only when CTL responses are low or undetectable. Such dual monitoring of EBV load and CTL could improve the clinical predictions of PTLD, although the complexity and cost would also increase [76].

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REFERENCES

1. Rickinson AB, Kieff E. Epstein-Barr Virus. In *Virology* (5th edn, Vol. 2), Knipe DM, Howly PM (eds). Wolters Kluwer/Lippincott Williams & Wilkins: Philadelphia, 2006; 2655–2700.

2. Cohen JI. Epstein-Barr virus infection. *N Engl J Med* 2000; **343**: 481–492.
3. Williams H, Crawford DH. Epstein-Barr virus: the impact of scientific advances on clinical practice. *Blood* 2006; **107**: 862–869.
4. Macsween KF, Crawford DH. Epstein-Barr virus-recent advances. *Lancet Infect Dis* 2003; **3**: 131–140.
5. Thorley-Lawson DA, Gross A. Persistence of the Epstein-Barr virus and the origins of associated lymphomas. *N Engl J Med* 2004; **350**: 1328–1337.
6. Fingeroth JD, Weis JJ, Tedder TF, Strominger JL, Biro PA, Fearon DT. Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2. *Proc Natl Acad Sci USA* 1984; **81**: 4510–4514.
7. Hislop AD, Taylor GS, Sauce D, Rickinson AB. Cellular responses to viral infection in humans: lessons from Epstein-Barr virus. *Annu Rev Immunol* 2007; **25**: 587–617.
8. Niedobitek G, Hamilton-Dutoit S, Herbst H, *et al*. Identification of Epstein-Barr virus-infected cells in tonsils of acute infectious mononucleosis by in situ hybridization. *Hum Pathol* 1989; **20**: 796–799.
9. Tierney RJ, Steven N, Young LS, Rickinson AB. Epstein-Barr virus latency in blood mononuclear cells: analysis of viral gene transcription during primary infection and in the carrier state. *J Virol* 1994; **68**: 7374–7385.
10. Borza CM, Hutt-Fletcher LM. Alternate replication in B cells and epithelial cells switches tropism of Epstein-Barr virus. *Nat Med* 2002; **8**: 594–599.
11. Tugizov SM, Berline JW, Palefsky JM. Epstein-Barr virus infection of polarized tongue and nasopharyngeal epithelial cells. *Nat Med* 2003; **9**: 307–314.
12. Shannon-Lowe CD, Neuhierl B, Baldwin G, Rickinson AB, Delecluse HJ. Resting B cells as a transfer vehicle for Epstein-Barr virus infection of epithelial cells. *Proc Natl Acad Sci USA* 2006; **103**: 7065–7070.
13. Molesworth SJ, Lake CM, Borza CM, Turk SM, Hutt-Fletcher LM. Epstein-Barr virus gH is essential for penetration of B cells but also plays a role in attachment of virus to epithelial cells. *J Virol* 2000; **74**: 6324–6332.
14. Fischer E, Delibrias C, Kazatchkine MD. Expression of CR2 (the C3dg/EBV receptor, CD21) on normal human peripheral blood T lymphocytes. *J Immunol* 1991; **146**: 865–869.
15. Anagnostopoulos I, Hummel M, Kreschel C, Stein H. Morphology, immunophenotype, and distribution of latently and/or productively Epstein-Barr virus-infected cells in acute infectious mononucleosis: implications for the interindividual infection route of Epstein-Barr virus. *Blood* 1995; **85**: 744–750.
16. Hudnall SD, Ge Y, Wei L, Yang NP, Wang HQ, Chen T. Distribution and phenotype of Epstein-Barr virus-infected cells in human pharyngeal tonsils. *Mod Pathol* 2005; **18**: 519–527.
17. Kasahara Y, Yachie A, Takei K, *et al*. Differential cellular targets of Epstein-Barr virus (EBV) infection between acute EBV-associated hemophagocytic lymphohistiocytosis and chronic active EBV infection. *Blood* 2001; **98**: 1882–1888.
18. Isobe Y, Sugimoto K, Yang L, *et al*. Epstein-Barr virus infection of human natural killer cell lines and peripheral blood natural killer cells. *Cancer Res* 2004; **64**: 2167–2174.
19. Tabiasco J, Vercellone A, Meggetto F, Hudrisier D, Brousset P, Fournie JJ. Acquisition of viral receptor by NK cells through immunological synapse. *J Immunol* 2003; **170**: 5993–5998.
20. Babcock GJ, Decker LL, Volk M, Thorley-Lawson DA. EBV persistence in memory B cells in vivo. *Immunity* 1998; **9**: 395–404.
21. Hochberg D, Middeldorp JM, Catalina M, Sullivan JL, Luzuriaga K, Thorley-Lawson DA. Demonstration of the Burkitt's lymphoma Epstein-Barr virus phenotype in dividing latently infected memory cells in vivo. *Proc Natl Acad Sci USA* 2004; **101**: 239–244.
22. Kis LL, Takahara M, Nagy N, Klein G, Klein E. Cytokine mediated induction of the major Epstein-Barr virus (EBV)-encoded transforming protein, LMP-1. *Immunol Lett* 2006; **104**: 83–88.
23. Tao Q, Robertson KD, Manns A, Hildesheim A, Ambinder RF. Epstein-Barr virus (EBV) in endemic Burkitt's lymphoma: molecular analysis of primary tumor tissue. *Blood* 1998; **91**: 1373–1381.
24. Deacon EM, Pallesen G, Niedobitek G, *et al*. Epstein-Barr virus and Hodgkin's disease: transcriptional analysis of virus latency in the malignant cells. *J Exp Med* 1993; **177**: 339–349.
25. Brooks L, Yao QY, Rickinson AB, Young LS. Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts. *J Virol* 1992; **66**: 2689–2697.
26. Young L, Alfieri C, Hennessy K, *et al*. Expression of Epstein-Barr virus transformation-associated genes in tissues of patients with EBV lymphoproliferative disease. *N Engl J Med* 1989; **321**: 1080–1085.
27. Yoshioka M, Ishiguro N, Ishiko H, Ma X, Kikuta H, Kobayashi K. Heterogeneous, restricted patterns of Epstein-Barr virus (EBV) latent gene expression in patients with chronic active EBV infection. *J Gen Virol* 2001; **82**: 2385–2392.
28. Xue SA, Labrecque LG, Lu QL, *et al*. Promiscuous expression of Epstein-Barr virus genes in Burkitt's lymphoma from the central African country Malawi. *Int J Cancer* 2002; **99**: 635–643.

29. Trumper PA, Epstein MA, Giovanella BC, Finerty S. Isolation of infectious EB virus from the epithelial tumour cells of nasopharyngeal carcinoma. *Int J Cancer* 1977; **20**: 655–662.
30. Nakazawa Y, Chisuwa H, Ikegami T, *et al.* Efficacy of quantitative analysis of Epstein-Barr virus-infected peripheral blood lymphocytes by in situ hybridization of EBER1 after living-related liver transplantation: a case report. *Transplantation* 1997; **63**: 1363–1366.
31. Kimura H, Morita M, Yabuta Y, *et al.* Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. *J Clin Microbiol* 1999; **37**: 132–136.
32. Middeldorp JM, Brink AA, van den Brule AJ, Meijer CJ. Pathogenic roles for Epstein-Barr virus (EBV) gene products in EBV-associated proliferative disorders. *Crit Rev Oncol Hematol* 2003; **45**: 1–36.
33. Savoie A, Perpete C, Carpentier L, Joncas J, Alfieri C. Direct correlation between the load of Epstein-Barr virus-infected lymphocytes in the peripheral blood of pediatric transplant patients and risk of lymphoproliferative disease. *Blood* 1994; **83**: 2715–2722.
34. Riddler SA, Breinig MC, McKnight JL. Increased levels of circulating Epstein-Barr virus (EBV)-infected lymphocytes and decreased EBV nuclear antigen antibody responses are associated with the development of posttransplant lymphoproliferative disease in solid-organ transplant recipients. *Blood* 1994; **84**: 972–984.
35. Yamamoto M, Kimura H, Hironaka T, *et al.* Detection and quantification of virus DNA in plasma of patients with Epstein-Barr virus-associated diseases. *J Clin Microbiol* 1995; **33**: 1765–1768.
36. Rooney CM, Loftin SK, Holladay MS, Brenner MK, Krance RA, Heslop HE. Early identification of Epstein-Barr virus-associated post-transplantation lymphoproliferative disease. *Br J Haematol* 1995; **89**: 98–103.
37. Lucas KG, Burton RL, Zimmerman SE, *et al.* Semiquantitative Epstein-Barr virus (EBV) polymerase chain reaction for the determination of patients at risk for EBV-induced lymphoproliferative disease after stem cell transplantation. *Blood* 1998; **91**: 3654–3661.
38. Wang AM, Mark DF. Quantitative PCR. In *PCR Protocols*, Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds). Academic Press, Inc.: San Diego, 1990: 70–75.
39. Rowe DT, Webber S, Schauer EM, Reyes J, Green M. Epstein-Barr virus load monitoring: its role in the prevention and management of post-transplant lymphoproliferative disease. *Transpl Infect Dis* 2001; **3**: 79–87.
40. Rowe DT, Qu L, Reyes J, *et al.* Use of quantitative competitive PCR to measure Epstein-Barr virus genome load in the peripheral blood of pediatric transplant patients with lymphoproliferative disorders. *J Clin Microbiol* 1997; **35**: 1612–1615.
41. Stevens SJ, Vervoort MB, van den Brule AJ, Meenhorst PL, Meijer CJ, Middeldorp JM. Monitoring of epstein-barr virus DNA load in peripheral blood by quantitative competitive PCR. *J Clin Microbiol* 1999; **37**: 2852–2857.
42. Gallagher A, Armstrong AA, MacKenzie J, *et al.* Detection of Epstein-Barr virus (EBV) genomes in the serum of patients with EBV-associated Hodgkin's disease. *Int J Cancer* 1999; **84**: 442–448.
43. Lo YM, Chan LY, Lo KW, *et al.* Quantitative analysis of cell-free Epstein-Barr virus DNA in plasma of patients with nasopharyngeal carcinoma. *Cancer Res* 1999; **59**: 1188–1191.
44. Niesters HG, van Esser J, Fries E, Wolthers KC, Cornelissen J, Osterhaus AD. Development of a real-time quantitative assay for detection of Epstein-Barr virus. *J Clin Microbiol* 2000; **38**: 712–715.
45. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res* 1996; **6**: 986–994.
46. Fan H, Gulley ML. Epstein-Barr viral load measurement as a marker of EBV-related disease. *Mol Diagn* 2001; **6**: 279–289.
47. Ambinder RF, Lin L. Mononucleosis in the laboratory. *J Infect Dis* 2005; **192**: 1503–1504.
48. Ryan JL, Fan H, Swinnen LJ, *et al.* Epstein-Barr Virus (EBV) DNA in plasma is not encapsidated in patients with EBV-related malignancies. *Diagn Mol Pathol* 2004; **13**: 61–68.
49. Hoshino Y, Morishima T, Kimura H, Nishikawa K, Tsurumi T, Kuzushima K. Antigen-driven expansion and contraction of CD8+-activated T cells in primary EBV infection. *J Immunol* 1999; **163**: 5735–5740.
50. Kimura H, Hoshino Y, Hara S, *et al.* Viral load in Epstein-Barr virus-associated hemophagocytic syndrome. *Microbiol Immunol* 2002; **46**: 579–582.
51. Fafi-Kremer S, Morand P, Brion JP, *et al.* Long-term shedding of infectious epstein-barr virus after infectious mononucleosis. *J Infect Dis* 2005; **191**: 985–989.
52. Balfour HH Jr., Holman CJ, Hokanson KM, *et al.* A prospective clinical study of Epstein-Barr virus and host interactions during acute infectious mononucleosis. *J Infect Dis* 2005; **192**: 1505–1512.
53. Gan YJ, Sullivan JL, Sixbey JW. Detection of cell-free Epstein-Barr virus DNA in serum during acute infectious mononucleosis. *J Infect Dis* 1994; **170**: 436–439.

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54. Hara S, Kimura H, Hoshino Y, *et al.* Detection of herpesvirus DNA in the serum of immunocompetent children. *Microbiol Immunol* 2002; **46**: 177–180.
55. Kimura H, Nishikawa K, Hoshino Y, Sofue A, Nishiyama Y, Morishima T. Monitoring of cell-free viral DNA in primary Epstein-Barr virus infection. *Med Microbiol Immunol* 2000; **188**: 197–202.
56. Rouphael NG, Talati NJ, Vaughan C, Cunningham K, Moreira R, Gould C. Infections associated with haemophagocytic syndrome. *Lancet Infect Dis* 2007; **7**: 814–822.
57. Teramura T, Tabata Y, Yagi T, Morimoto A, Hibi S, Imashuku S. Quantitative analysis of cell-free Epstein-Barr virus genome copy number in patients with EBV-associated hemophagocytic lymphohistiocytosis. *Leuk Lymphoma* 2002; **43**: 173–179.
58. Green M, Cacciarelli TV, Mazariegos GV, *et al.* Serial measurement of Epstein-Barr viral load in peripheral blood in pediatric liver transplant recipients during treatment for posttransplant lymphoproliferative disease. *Transplantation* 1998; **66**: 1641–1644.
59. van Esser JW, van der Holt B, Meijer E, *et al.* Epstein-Barr virus (EBV) reactivation is a frequent event after allogeneic stem cell transplantation (SCT) and quantitatively predicts EBV-lymphoproliferative disease following T-cell–depleted SCT. *Blood* 2001; **98**: 972–978.
60. Hoshino Y, Kimura H, Tanaka N, *et al.* Prospective monitoring of the Epstein-Barr virus DNA by a real-time quantitative polymerase chain reaction after allogenic stem cell transplantation. *Br J Haematol* 2001; **115**: 105–111.
61. Holmes RD, Orban-Eller K, Karrer FR, Rowe DT, Narkewicz MR, Sokol RJ. Response of elevated Epstein-Barr virus DNA levels to therapeutic changes in pediatric liver transplant patients: 56-month follow up and outcome. *Transplantation* 2002; **74**: 367–372.
62. Clave E, Agbalika F, Bajzik V, *et al.* Epstein-Barr virus (EBV) reactivation in allogeneic stem-cell transplantation: relationship between viral load, EBV-specific T-cell reconstitution and rituximab therapy. *Transplantation* 2004; **77**: 76–84.
63. Yang J, Tao Q, Flinn IW, *et al.* Characterization of Epstein-Barr virus-infected B cells in patients with posttransplantation lymphoproliferative disease: disappearance after rituximab therapy does not predict clinical response. *Blood* 2000; **96**: 4055–4063.
64. Gustafsson A, Levitsky V, Zou JZ, *et al.* Epstein-Barr virus (EBV) load in bone marrow transplant recipients at risk to develop posttransplant lymphoproliferative disease: prophylactic infusion of EBV-specific cytotoxic T cells. *Blood* 2000; **95**: 807–814.
65. van Esser JW, Niesters HG, van der Holt B, *et al.* Prevention of Epstein-Barr virus-lymphoproliferative disease by molecular monitoring and preemptive rituximab in high-risk patients after allogeneic stem cell transplantation. *Blood* 2002; **99**: 4364–4369.
66. Cesaro S, Murrone A, Mengoli C, *et al.* The real-time polymerase chain reaction-guided modulation of immunosuppression enables the preemptive management of Epstein-Barr virus reactivation after allogeneic haematopoietic stem cell transplantation. *Br J Haematol* 2005; **128**: 224–233.
67. Stevens SJ, Verschuuren EA, Pronk I, *et al.* Frequent monitoring of Epstein-Barr virus DNA load in unfractionated whole blood is essential for early detection of posttransplant lymphoproliferative disease in high-risk patients. *Blood* 2001; **97**: 1165–1171.
68. Wagner HJ, Wessel M, Jabs W, *et al.* Patients at risk for development of posttransplant lymphoproliferative disorder: plasma versus peripheral blood mononuclear cells as material for quantification of Epstein-Barr viral load by using real-time quantitative polymerase chain reaction. *Transplantation* 2001; **72**: 1012–1019.
69. Aalto SM, Juvonen E, Tarkkanen J, *et al.* Epstein-Barr viral load and disease prediction in a large cohort of allogeneic stem cell transplant recipients. *Clin Infect Dis* 2007; **45**: 1305–1309.
70. Wadowsky RM, Laus S, Green M, Webber SA, Rowe D. Measurement of Epstein-Barr virus DNA loads in whole blood and plasma by TaqMan PCR and in peripheral blood lymphocytes by competitive PCR. *J Clin Microbiol* 2003; **41**: 5245–5249.
71. Stevens SJ, Pronk I, Middeldorp JM. Toward standardization of Epstein-Barr virus DNA load monitoring: unfractionated whole blood as preferred clinical specimen. *J Clin Microbiol* 2001; **39**: 1211–1216.
72. Wada K, Kubota N, Ito Y, *et al.* Simultaneous quantification of Epstein-Barr virus, cytomegalovirus, and human herpesvirus 6 DNA in samples from transplant recipients by multiplex real-time PCR assay. *J Clin Microbiol* 2007; **45**: 1426–1432.
73. Bakker NA, Verschuuren EA, Veeger NJ, *et al.* Quantification of Epstein-Barr virus-DNA load in lung transplant recipients: a comparison of plasma versus whole blood. *J Heart Lung Transplant* 2008; **27**: 7–10.
74. Gratama JW. Epstein-Barr virus infections in bone marrow transplantation recipients. In *Bone Marrow Transplantation*, Forman SJ, Blume KG, Thomas ED (eds). Blackwell Scientific Publications: Boston, 1994; 429–442.

75. Wagner HJ, Cheng YC, Huls MH, *et al.* Prompt versus preemptive intervention for EBV lymphoproliferative disease. *Blood* 2004; **103**: 3979–3981.
76. Preiksaitis JK. New developments in the diagnosis and management of posttransplantation lymphoproliferative disorders in solid organ transplant recipients. *Clin Infect Dis* 2004; **39**: 1016–1023.
77. Qu L, Green M, Webber S, Reyes J, Ellis D, Rowe D. Epstein-Barr virus gene expression in the peripheral blood of transplant recipients with persistent circulating virus loads. *J Infect Dis* 2000; **182**: 1013–1021.
78. Hopwood PA, Brooks L, Parratt R, *et al.* Persistent Epstein-Barr virus infection: unrestricted latent and lytic viral gene expression in healthy immunosuppressed transplant recipients. *Transplantation* 2002; **74**: 194–202.
79. Carpentier L, Tapiero B, Alvarez F, Viau C, Alfieri C. Epstein-Barr virus (EBV) early-antigen serologic testing in conjunction with peripheral blood EBV DNA load as a marker for risk of posttransplantation lymphoproliferative disease. *J Infect Dis* 2003; **188**: 1853–1864.
80. Funk GA, Gosert R, Hirsch HH. Viral dynamics in transplant patients: implications for disease. *Lancet Infect Dis* 2007; **7**: 460–472.
81. Navarro WH, Kaplan LD. AIDS-related lymphoproliferative disease. *Blood* 2006; **107**: 13–20.
82. Ling PD, Vilchez RA, Keitel WA, *et al.* Epstein-Barr virus DNA loads in adult human immunodeficiency virus type 1-infected patients receiving highly active antiretroviral therapy. *Clin Infect Dis* 2003; **37**: 1244–1249.
83. Fan H, Kim SC, Chima CO, *et al.* Epstein-Barr viral load as a marker of lymphoma in AIDS patients. *J Med Virol* 2005; **75**: 59–69.
84. Van Baarle D, Wolthers KC, Hovenkamp E, *et al.* Absolute level of Epstein-Barr virus DNA in human immunodeficiency virus type 1 infection is not predictive of AIDS-related non-Hodgkin lymphoma. *J Infect Dis* 2002; **186**: 405–409.
85. Jahr S, Hentze H, Englisch S, *et al.* DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res* 2001; **61**: 1659–1665.
86. Lei KI, Chan LY, Chan WY, Johnson PJ, Lo YM. Quantitative analysis of circulating cell-free Epstein-Barr virus (EBV) DNA levels in patients with EBV-associated lymphoid malignancies. *Br J Haematol* 2000; **111**: 239–246.
87. Oshimi K. Progress in understanding and managing natural killer-cell malignancies. *Br J Haematol* 2007; **139**: 532–544.
88. Harabuchi Y, Yamanaka N, Kataura A, *et al.* Epstein-Barr virus in nasal T-cell lymphomas in patients with lethal midline granuloma. *Lancet* 1990; **335**: 128–130.
89. Kwong YL. Natural killer-cell malignancies: diagnosis and treatment. *Leukemia* 2005; **19**: 2186–2194.
90. Chan KC, Zhang J, Chan AT, *et al.* Molecular characterization of circulating EBV DNA in the plasma of nasopharyngeal carcinoma and lymphoma patients. *Cancer Res* 2003; **63**: 2028–2032.
91. Au WY, Pang A, Choy C, Chim CS, Kwong YL. Quantification of circulating Epstein-Barr virus (EBV) DNA in the diagnosis and monitoring of natural killer cell and EBV-positive lymphomas in immunocompetent patients. *Blood* 2004; **104**: 243–249.
92. Lei KI, Chan LY, Chan WY, Johnson PJ, Lo YM. Diagnostic and prognostic implications of circulating cell-free Epstein-Barr virus DNA in natural killer/T-cell lymphoma. *Clin Cancer Res* 2002; **8**: 29–34.
93. Jones J, Shurin S, Abramowsky C, *et al.* T-cell lymphomas containing Epstein-Barr viral DNA in patients with chronic Epstein-Barr virus infections. *N Engl J Med* 1988; **318**: 733–741.
94. Kikuta H, Taguchi Y, Tomizawa K, *et al.* Epstein-Barr virus genome-positive T lymphocytes in a boy with chronic active EBV infection associated with Kawasaki-like disease. *Nature* 1988; **333**: 455–457.
95. Kawa-Ha K, Ishihara S, Ninomiya T, *et al.* CD3-negative lymphoproliferative disease of granular lymphocytes containing Epstein-Barr viral DNA. *J Clin Invest* 1989; **84**: 51–55.
96. Kimura H, Hoshino Y, Kanegane H, *et al.* Clinical and virologic characteristics of chronic active Epstein-Barr virus infection. *Blood* 2001; **98**: 280–286.
97. Kimura H, Morishima T, Kanegane H, *et al.* Prognostic factors for chronic active Epstein-Barr virus infection. *J Infect Dis* 2003; **187**: 527–533.
98. Kimura H, Hoshino Y, Hara S, *et al.* Differences between T cell-type and natural killer cell-type chronic active Epstein-Barr virus infection. *J Infect Dis* 2005; **191**: 531–539.
99. Kimura H. Pathogenesis of chronic active Epstein-Barr virus infection: is this an infectious disease, lymphoproliferative disorder, or immunodeficiency? *Rev Med Virol* 2006; **16**: 251–261.
100. Kawa K, Okamura T, Yagi K, Takeuchi M, Nakayama M, Inoue M. Mosquito allergy and Epstein-Barr virus-associated T/natural killer-cell lymphoproliferative disease. *Blood* 2001; **98**: 3173–3174.
101. Maeda A, Wakiguchi H, Yokoyama W, Hisakawa H, Tomoda T, Kurashige T. Persistently high

Epstein-Barr virus (EBV) loads in peripheral blood lymphocytes from patients with chronic active EBV infection. *J Infect Dis* 1999; **179**: 1012–1015.
102. Kanegane H, Wakiguchi H, Kanegane C, Kurashige T, Miyawaki T, Tosato G. Increased cell-free viral DNA in fatal cases of chronic active Epstein-Barr virus infection. *Clin Infect Dis* 1999; **28**: 906–909.
103. Ohga S, Nomura A, Takada H, *et al*. Epstein-Barr virus (EBV) load and cytokine gene expression in activated T cells of chronic active EBV infection. *J Infect Dis* 2001; **183**: 1–7.
104. Gotoh K, Ito Y, Shibata-Watanabe Y, *et al*. Clinical and virologic characteristics of 15 patients with chronic active Epstein–Barr virus infection treated with hematopoietic stem cell transplantation. *Clin Infect Dis* 2008; **46**: 1525–1534.
105. Shotelersuk K, Khorprasert C, Sakdikul S, Ponthanakasem W, Voravud N, Mutirangura A. Epstein-Barr virus DNA in serum/plasma as a tumor marker for nasopharyngeal cancer. *Clin Cancer Res* 2000; **6**: 1046–1051.
106. Lo YM, Chan LY, Chan AT, *et al*. Quantitative and temporal correlation between circulating cell-free Epstein-Barr virus DNA and tumor recurrence in nasopharyngeal carcinoma. *Cancer Res* 1999; **59**: 5452–5455.
107. Lo YM, Chan AT, Chan LY, *et al*. Molecular prognostication of nasopharyngeal carcinoma by quantitative analysis of circulating Epstein-Barr virus DNA. *Cancer Res* 2000; **60**: 6878–6881.
108. Lin JC, Wang WY, Chen KY, *et al*. Quantification of plasma Epstein-Barr virus DNA in patients with advanced nasopharyngeal carcinoma. *N Engl J Med* 2004; **350**: 2461–2470.
109. Niesters HG. Molecular and diagnostic clinical virology in real time. *Clin Microbiol Infect* 2004; **10**: 5–11.
110. Bell AI, Groves K, Kelly GL, *et al*. Analysis of Epstein-Barr virus latent gene expression in endemic Burkitt's lymphoma and nasopharyngeal carcinoma tumour cells by using quantitative real-time PCR assays. *J Gen Virol* 2006; **87**: 2885–2890.
111. Kubota N, Wada K, Ito Y, *et al*. One-step multiplex real-time PCR assay to analyse the latency patterns of Epstein-Barr virus infection. *J Virol Methods* 2008; **147**: 26–36.
112. Kuzushima K, Hayashi N, Kudoh A, *et al*. Tetramer-assisted identification and characterization of epitopes recognized by HLA A*2402-restricted Epstein-Barr virus-specific CD8+ T cells. *Blood* 2003; **101**: 1460–1468.
113. Meij P, van Esser JW, Niesters HG, *et al*. Impaired recovery of Epstein-Barr virus (EBV)–specific CD8+ T lymphocytes after partially T-depleted allogeneic stem cell transplantation may identify patients at very high risk for progressive EBV reactivation and lymphoproliferative disease. *Blood* 2003; **101**: 4290–4297.
114. Annels NE, Kalpoe JS, Bredius RG, *et al*. Management of Epstein-Barr virus (EBV) reactivation after allogeneic stem cell transplantation by simultaneous analysis of EBV DNA load and EBV-specific T cell reconstitution. *Clin Infect Dis* 2006; **42**: 1743–1748.

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Autologous Hematopoietic Stem Cell Transplantation in Extranodal Natural Killer/T Cell Lymphoma: A Multinational, Multicenter, Matched Controlled Study

Jeeyun Lee,[1] Wing-Yan Au,[2] Min Jae Park,[1] Junji Suzumiya,[3] Shigeo Nakamura,[4] Jun-Ichi Kameoka,[5] Chikara Sakai,[6] Kazuo Oshimi,[7] Yok-Lam Kwong,[2] Raymond Liang,[2] Harry Yiu,[8] Kam-Hung Wong,[8] Hoi-Ching Cheng,[8] Baek-Yeol Ryoo,[9] Cheolwon Suh,[10] Young Hyeh Ko,[11] Kihyun Kim,[1] Jae-Won Lee,[12] Won Seog Kim,[1] Ritsuro Suzuki[13]

Extranodal natural killer (NK)/T cell lymphoma, nasal type, is a recently recognized distinct entity and the most common type of non–B cell extranodal lymphoma in Asia. This retrospective analysis studied the potential survival benefits of hematopoeitic stem cell transplantation (HSCT) compared with a historical control group. A total of 47 patients from 3 previously published series of HSCT were matched according to NK/T cell lymphoma International Prognostic Index (NKIPI) risk groups and disease status at transplantation with 107 patients from a historical control group for analysis. After a median follow-up of 116.5 months, the median survival time was not determined for the HSCT group, but it was 43.5 months for the control group (95% confidence interval [CI] = 6.7 to 80.3 months; $P = .127$, log-rank test). In patients who were in complete remission (CR) at the time of HSCT or at surveillance after remission, disease-specific survival rates were significantly higher in the HSCT group compared with the control group (disease-specific 5-year survival rate, 87.3% for HSCT vs 67.8% for non-HSCT; $P = .027$). In contrast, in subgroup analysis on non-CR patients at the time of HSCT or non-HSCT treatment, disease-specific survival rates were not significantly prolonged in the HSCT group compared with the control group (1-year survival rate, 66.7% for HSCT vs 28.6% for non-HSCT; $P = .141$). The impact of HSCT on the survival of all patients was significantly retained at the multivariate level with a 2.1-fold (95% CI = 1.2- to 3.7-fold) reduced risk of death ($P = .006$). HSCT seems to confer a survival benefit in patients who attained CR on postremission consolidation therapy. These findings suggest that, in particular, patients in CR with high NKIPI risk scores at diagnosis should receive full consideration for HSCT.

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Extranodal natural killer (NK)/T cell lymphoma, nasal type, is a recently recognized distinct entity in the World Health Organization (WHO) classification of lymphoid tumors [1]. This lymphoma occurs more

From the [1]Division of Hematology-Oncology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea; [2]Department of Medicine, Queen Mary Hospital, Hong Kong; [3]First Department of Internal Medicine, Fukuoka University School of Medicine, Fukuoka, Japan; [4]Department of Pathology, Nagoya University School of Medicine, Nagoya, Japan; [5]Department of Rheumatology and Haematology, Tohoku University School of Medicine, Sendai, Japan; [6]Department of Hematology and Oncology, Chiba Cancer Center, Chiba, Japan; [7]Department of Hematology, Juntendo University, Tokyo, Japan; [8]Department of Clinical Oncology, Queen Elizabeth Hospital, Hong Kong; [9]Department of Hematology-Oncology, Korea Cancer Center Hospital, Seoul, Korea; [10]Department of Internal Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea; [11]Department of Pathology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea; [12]Department of Statistics, Korea University, Seoul, Korea; and [13]Department of Hematopoietic Stem Cell Transplantation Data Management, Nagoya University School of Medicine, Nagoya, Japan.

J. Lee and W.Y. Au contributed equally as first authors.

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Correspondence and reprint requests: Won Seog Kim, MD, PhD, Division of Hematology/Oncology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Ilwon-dong Kangnam-Gu, Seoul 135-710 Korea (e-mail: wskimsmc@skku.edu) or Ritsuro Suzuki, MD, PhD, Department of Hematopoietic Stem Cell Transplantation Data Management, Nagoya University School of Medicine, 1-1-20 Daiko-Minami, Higashi-Ku, Nagoya 461-0047, Japan (e-mail: r-suzuki@med.nagoya-u.ac.jp).

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frequently in Asia than in Western countries and is the most common type of non–B cell extranodal lymphoma in Asia [2]. The treatment outcome for NK/T cell lymphomas depends on disease stage. Overall, long-term survival in these lymphomas, reported as 30% to 40% [3-6], tends to be inferior to that for other aggressive lymphomas. Even in localized NK/T cell lymphomas, primary chemotherapy and/or radiotherapy (RT) results in complete remission (CR) rates of 40% to 60%, with 5-year overall survival (OS) rates of 42% to 83% [3,4,7-11], with high systemic failure rates of 25% to 30% [7-9,12].

In an effort to identify strategies for improving these low success rates in treating NK/T cell lymphoma, the use of high-dose chemotherapy (HDC) and autologous hematopoietic stem cell transplantation (HSCT) has been investigated [13-17]. Determining the survival benefit of HSCT based on the results of these studies is difficult, however, because of the small size as well as the heterogeneous nature of the patient cohorts.

Recently, a prognostic model specific for NK/T cell lymphoma (NK/T cell lymphoma International Prognostic Index [NKIPI]) has been proposed and validated [6,18]. Clinical variables included in the NKIPI risk scoring system are B symptoms, stage, lactic dehydrogenase (LDH) level, and regional lymphadenopathies. Owing to its extranodal characteristics, the prognostic impact of the IPI has been controversial in this particular subtype of non-Hodgkin lymphoma (NHL). Similar to other prognostic models, the NKIPI has the major aim of identifying high-risk patients and thereby provide better risk-based stratification for optimal treatment.

To explore the potential benefits of autologous HSCT, we have pooled and reanalyzed data from 3 previously published series [6,13,14]. To critically evaluate the role of autologous HSCT, we compared those results with those for a matched control group identified from historical data.

PATIENTS AND METHODS

Patients and Data Collection

Our cohort comprises 59 patients with NK/T cell lymphomas who underwent autologous HSCT reported in 3 previous studies from Korea, Hong Kong, and Japan [6,13,14]. From these 59 patients, 48 were selected for reanalysis (Korea, n = 16 [6]; Hong Kong, n = 16 [13]; Japan, n = 16 [14]). Patient selection was based on availability from a historical control group of patients who were matched according to NKIPI risk group (risk score 0-1 vs 2-4) and disease status at transplantation (first CR [CR1], second CR [CR2] vs partial remission [PR]/no response [NR]), at a ratio of 1:3. In cases where the NKIPI score was not available at the time of analysis, only disease status was considered for matching criteria. The sources of the matching control group were the lymphoma data registry for each study group. The order of priority in selection criteria for matched control cases was NKIPI risk score, followed by disease status at transplantation or conventional treatment/observation. The matched control cases (n = 107) received conventional chemotherapy with or without RT (n = 34), RT alone (n = 5) as salvage therapy, or observation (n = 68) at CR1 or CR2 as postremission care instead of HDC/autologous HSCT. All patients had pathologically confirmed NK/T cell lymphoma according to the WHO classification [1]. One patient with negative Epstein-Barr virus (EBV) in situ hybridization from the HSCT group was excluded from the final analysis; thus, the group from Japan included 15 patients.

Extranasal NK/T cell lymphoma was defined as described previously [3]. In brief, upper aerodigestive tract NK/T cell lymphoma (UNKTL) was defined as that involving the nasal cavity, nasopharynx, and the upper aerodigestive tract, whereas extra-upper aerodigestive tract NK/T cell lymphoma (EUNKTL) included lymphomas occurring at all other sites [3]. The following clinical data were collected from the medical records: demographic information, LDH level at diagnosis, initial Ann Arbor stage, IPI at diagnosis, NKIPI at diagnosis, presence or absence of B symptoms, performance status, date of diagnosis, date of autologous HSCT, disease status at transplantation, transplantation outcome, salvage treatment type and outcome, date of last follow-up, and cause of death. The study design was approved by the Samsung Medical Center's Institutional Review Board.

Chemotherapy

Each patient received 1 of the following initial treatment modalities: (1) an anthracycline-containing chemotherapeutic regimen with or without RT (n = 125), (2) a non–anthracycline-containing chemotherapeutic regimen with or without RT (n = 15), (3) involved-field RT (IFRT) as the primary treatment (n = 13), or (4) surgery plus RT (n = 1). Anthracycline-based regimens used included CHOP (cyclophosphamide, (Cy) doxorubicin, vincristine, and prednisolone; n = 68), dose-escalated CHOP (deCHOP; n = 1), velCHOP (velcade plus CHOP; n = 1), CEOP (Cy, epirubicin, vincristine, and prednisolone; n = 14), CEOP/ProMACE (CEOP followed by Cy, doxorubicin, etoposide, and prednisone; n = 4), MACOP B (methotrexate [MTX], doxorubicin, Cy, vincristine, prednisone, and bleomycin; n = 2), CHOEP (Cy, doxorubicin, vincristine, etoposide, and prednisolone; n = 6), ProMace (n = 3), ProMace/Cytabom (ProMace plus cytabarabine, bleomycin, vincristine, MTX, and leucovorin; n = 21), COPBLAM (Cy, vincristine, prednisone, bleomycin, doxorubicin, and procarbazine; n = 2), EPOCH (etoposide, doxorubicin, vincristine, Cy, and prednisolone; n = 1),

cisplatin/Cy/adriamycin/vindesine/prednisolone (n = 1), and epi-COP (epirubicin, Cy, vincristine, prednisolone; n = 1). The non–anthracycline-containing regimens used were IMEP (ifosfamide, MTX, and etoposide; n = 3), ESHAP (etoposide, methylprednisolone, cisplatin, and cytarabine; n = 1), DHAP (dexamethasone, cytarabine, and cisplatin; n = 1), DeVIC (carboplatin, etoposide, ifosfamide, and dextamethasone; n = 1), IMVP-16 (ifosfamide, MTX, and etoposide; n = 2), and VIPD (etoposide, ifosfamide, cisplatin, and dexamethasone; n = 7). In patients with localized disease, IFRT was given at the physician's discretion after chemotherapy. Treatment response was assessed according to standard response criteria [19].

HDC/Autologous HSCT

The procedures for HDC and autologous HSCT have been described previously [13,14,20]. In brief, the following conditioning regimens were used: CBV (etoposide, carmustine, and Cy; n = 14), BEAM (carmustine, etoposide, cytarabine, and melphalan (Mel); n = 12), MCEC (ranimustine, Cy, etoposide and carboplatin; n = 8), BEAC (carmustine, etoposide, cytarabine, and Cy; n = 2), Cy/TBI (Cy and total body irradiation; n = 2), VCT (etoposide, Cy and TBI; n = 2), and others (n = 7).

Statistical Analysis

Disease-specific survival and relapse-free survival (RFS) were estimated using the Kaplan-Meier method. Disease-specific survival was calculated from the date of diagnosis to the date of death from the disease or the last follow-up. RFS was calculated from the date of CR to the first documented relapse in patients who attained CR. Survival rates were compared for statistical differences using log-rank analysis. Survival rates were compared for statistical differences by using log-rank analysis. Continuous biological variables were dichotomized for log-rank analysis. A backward-stepwise Cox regression analysis was performed to delineate prognostic factors at the multivariate level, and all hazard ratios (HRs) were adjusted for age. P values $< .05$ were considered statistically significant, and all P values correspond to 2-sided significance tests.

RESULTS

Patient Characteristics

A total of 47 patients who underwent autologous HSCT were compared with 107 matched controlled cases. Baseline characteristics are summarized in Table 1. All clinical parameters except age and initial Ann Arbor stage were relatively well balanced between the control group and the study group. The median time from diagnosis to transplantation was 8.8 months (range, 2.1 to 86.3 months). The proportion of patients under age 60 years was significantly higher in the autologous HSCT group compared with the control group (95.7% in the autologous HSCT group vs 72.0% in the control group; $P = .001$). In addition, the proportion of patients with localized disease was lower in the autologous HSCT group (66.0%) than in the non-HSCT group (82.2%) ($P = .038$). Otherwise, there were no significant differences in distributions of sex, performance status, LDH level, IPI risk group, presence of B symptoms, anatomic category, NKIPI risk group, or primary treatment modality between the 2 groups.

Autologous HSCT Outcome

More than 90% of the patients in each group received primary chemotherapy with or without IFRT (Table 1). Approximately 2/3 of the patients received CBV, BEAM, or MCEC as the conditioning regimen before HSCT. Of the HSCT group, 30% (n = 14) were in CR1, 28% (n = 13) were in CR2, 28% (n = 13) were in PR/SD, and 15% (n = 7) were in PD at the time of transplantation. Using an intent-to-treat analysis, 66.0% (n = 31) attained CR after HSCT, 4.3% (n = 2) attained PR, and 19.1% (n = 9) had PD. Four fatal toxicities were observed, with a treatment-related mortality (TRM) rate of 8.5% (septic shock, n = 1; pneumonia, n = 1; unspecified, n = 2). Of the 31 patients who attained CR after HSCT, 13 (41.9%) experienced relapse; the median RFS from the date of CR to the first documented relapse or follow-up was 23.3 months (range, 0.2 to 180.3 months). Of the 13 patients who experienced relapse, 5 received salvage chemotherapy, 4 received RT, 2 underwent allogeneic HSCT, and 2 received palliative treatment. After a median follow-up of 99.8 months post-HSCT (range, 23.4 to 180.9 months), the median survival time after HSCT has not yet been reached. There was no significant difference in survival between the HSCT and control groups (56.2% vs 47.6%; $P = .127$) (Figure 1B).

Prognostic Analysis for Autologous HSCT

The following clinical factors predicted poor survival of patients undergoing autologous HSCT in univariate analysis: advanced Ann Arbor stage (stage III/IV; $P = .045$) and disease status at the time of transplantation (non-CR; $P < .001$) (Table 2). For RFS after autologous HSCT, advanced Ann Arbor stage (stage III/IV; $P = .021$), elevated LDH level ($P = .026$), non-CR at the time of transplantation ($P = .001$), and high IPI risk group (high-intermediate/high; $P = .005$) predicted relapse after HSCT. In multivariate analysis with stage, the presence of B symptoms, anatomic category, and disease status at HSCT, only disease status at HSCT retained its statistical significance for RFS ($P < .001$; HR = 3.5; 95% confidence

Table 1. Patient and Treatment Characteristics

	All Patients	HSCT	Controls	P Value
Total cases, n (%)	154 (100)	47 (30.5)	107 (69.5)	
Median age, years (range)	47 (17 to 80)	42 (17 to 62)	52 (17 to 80)	
Age, years, n (%)				
≤ 60	122 (79.2)	45 (95.7)	77 (72.0)	.001
> 60	32 (20.8)	2 (4.3)	30 (28.0)	
Sex, n (%)				
Male	111 (72.1)	34 (72.3)	77 (72.0)	.962
Female	43 (27.9)	13 (27.7)	30 (28.0)	
Performance status, n (%)				
ECOG 0-1	139 (90.3)	43 (91.5)	96 (89.7)	.733
ECOG 2-4	15 (9.7)	4 (8.5)	11 (10.3)	
Ann Arbor stage, n (%)				
Limited (I-II)	118 (77.1)	31 (66.0)	87 (82.2)	.038
Advanced (III-IV)	36 (23.5)	16 (34.0)	20 (17.8)	
LDH (n = 150), n (%)				
≤ Upper limit of normal	77 (51.3)	23 (51.1)	54 (51.4)	.972
> Upper limit of normal	73 (48.7)	22 (48.9)	51 (48.6)	
IPI risk group (n = 151), (%)				
Low/low-intermediate	127 (84.1)	37 (82.2)	90 (84.9)	.680
High-intermediate/high	24 (15.9)	8 (17.8)	16 (15.1)	
B symptoms, n (%)				
Positive	97 (63.0)	27 (57.4)	70 (65.4)	.345
Negative	57 (37.0)	20 (42.6)	37 (34.6)	
Anatomic category, n (%)				
UNKTL	141 (91.6)	42 (89.4)	99 (92.5)	.516
EUNKTL	13 (8.4)	5 (10.6)	8 (7.5)	
NKIPI risk group (n = 145), n (%)				
Low risk (group 1-2)	80 (55.2)	23 (54.8)	57 (55.3)	.949
High risk (group 3-4)	65 (44.8)	19 (45.2)	46 (44.7)	
Primary treatment, n (%)				
Anthracycline-based chemotherapy ± RT	125 (81.2)	41 (87.2)	84 (78.5)	
Non-anthracycline-based chemotherapy ± RT	15 (9.7)	3 (6.4)	12 (11.9)	.600
RT only	13 (8.4)	3 (6.4)	10 (10.2)	
Surgical excision + RT	1 (0.6)	0 (0.0)	1 (0.8)	
Disease status at treatment, n (%)				
CR1	61 (39.6)	14 (29.8)	47 (43.9)	
CR2	34 (22.1)	13 (27.7)	21 (19.6)	.232
PR/NR/PD	59 (38.3)	20 (42.6)	39 (36.4)	

NKIPI indicates natural killer/T cell lymphoma International Prognostic Index; UNKTL, upper aerodigestive NK/T cell lymphoma; EUNKTL, extra-upper aerodigestive NK/T cell lymphoma; RT, radiotherapy; CR1, first complete response; CR2, second complete response; PR, partial response; NR, no response; PD.

interval [CI] = 1.6 to 7.9) and disease-specific survival (P <.001; HR = 7.2; 95% CI = 4.4 to 1.6). Thus, disease status at autologous HSCT was the most important prognostic factor for survival and RFS.

Impact of HSCT on Survival in NK/T Cell Lymphoma

After a median follow-up of 116.5 months (range, 13.2 to 234.0 months), the median survival was 47.3

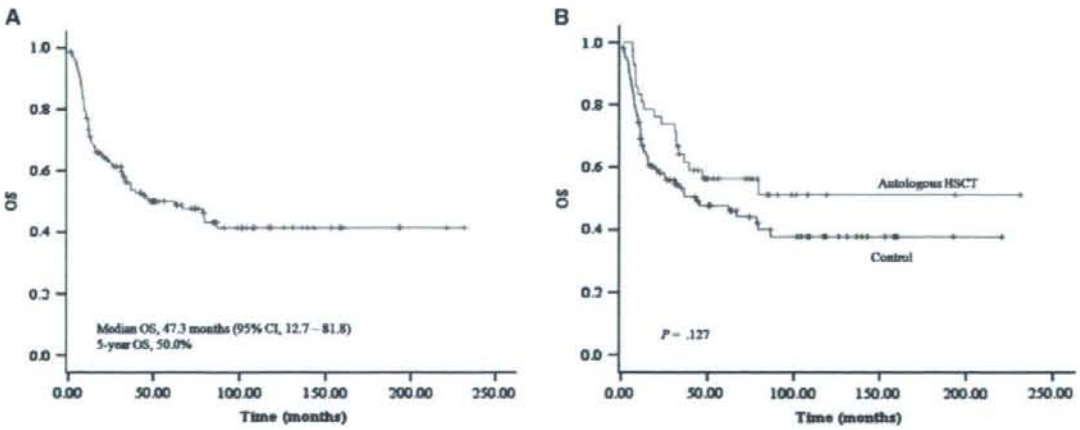

Figure 1. Survival of all patients (A) and survival according to HSCT (B).

Table 2. Univariate Analysis for the Patients with HSCT

Parameters	Relapse-Free Survival		Disease-Specific Survival	
	Median (95% CI), Months	P Value	Median (95% CI), Months	P Value
Age, years				
≤ 60	13.7 (0.0 to 30.8)	.600	NA	NA
> 60	NA			
Ann Arbor stage				
Limited (I/II)	NR	.021	NR	.045
Advanced (III/IV)	4.2 (0.0 to 9.3)		36.5 (11.0 -62.0)	
LDH				
≤ Upper limit of normal	NR	.026	NR	.145
> Upper limit of normal	6.2 (0.0 to 29.8)		31.8 (0.0 to 104.4)	
B symptoms				
Positive	16.8 (0.0 to 44.4)	.654	NR	.536
Negative	19.3 (0.0 to 43.6)		NR	
Anatomic category				
UNKTL	16.8 (0.0 to 34.6)	.527	NR	.152
EUNKTL	2.2 (1.3 to 3.2)		36.5 (0.0 to 73.5)	
Disease status at HSCT				
CR	NR	.001	NR	<.001
Non-CR	2.5 (1.7 to 3.4)		19.2 (0.0 to 56.5)	
IPI risk group				
Low/low-intermediate	21.0	.005	NR	.223
High-intermediate/high	1.8 (1.2 to 2.4)		19.2 (0.0 to 81.0)	
NKIPI risk group				
Low risk (group 1-2)	NR	.086	NR	.066
High risk (group 3-4)	6.2 (0.0 to 20.1)		31.1 (0.0 to 70.7)	

NA indicates not applicable; ; NKIPI indicates natural killer/T cell lymphoma International Prognostic Index; UNKTL, upper aerodigestive NK/T cell lymphoma; EUNKTL, extra-upper aerodigestive NK/T cell lymphoma; RT, radiotherapy; CR, complete response; NR, no response; LDH, lactate dehydrogenase; HSCT, hematopoietic stem cell transplantation.

months (95% CI = 12.7 to 81.8) for all patients (Figure 1A). The median survival time had not yet been reached for the HSCT group, but it was 43.5 months for the control group (95% CI = 6.7 to 80.3 months; $P = .127$, log-rank test) (Figure 1B). For all patients in both groups, the clinical factors significantly predicting unfavorable survival in univariate analysis were performance status (Eastern Cooperative Oncology Group [ECOG] 2 to 4; $P = .042$), advanced Ann Arbor stage (stage III/IV; $P = .02$), elevated LDH level ($P = .010$), anatomic category (EUNKTL; $P = .017$), disease status at treatment (non-CR; $P < .001$), IPI (high-intermediate/high; $P = .027$), and NKIPI (group 3-4; $P = .003$) (Table 3).

Clinical parameters included in the multivariate analysis were performance status (0 to 1 vs ≥ 2), Ann Arbor stage (I/II vs III/IV), LDH level (normal vs elevated), anatomic category (UNKTL vs EUNKTL), disease status at treatment (CR1/CR2 vs non-CR), and HSCT versus non-HSCT. A backward-conditional Cox regression model was used. Significant prognostic factors for survival in all patients were LDH level ($P = .005$; HR = 2.0; 95% CI = 1.2 to 3.2), disease status at treatment ($P = < .001$; HR = 7.8; 95% CI = 4.6 to 13.0), and HSCT ($P = .006$; HR = 2.1; 95% CI = 1.2 to 3.7) (Table 4).

Influence of Autologous HSCT on Survival in Subgroup Analyses

We performed subgroup analyses in an attempt to identify patients who would potentially benefit from HSCT. In those patients who were in CR1 or CR2 at the time of HSCT or surveillance after remission, disease-specific survival rates were significantly higher in the HSCT group compared with the control group (disease-specific 5-year survival rate, 87.3% for HSCT vs 67.8% for non-HSCT; $P = .027$, log-rank test) (Figure 2A). We also performed subgroup analyses according to NK-IPI risk group (low risk vs high risk) (Figure 2B, C). In the low-risk group (group 1-2), there was no significant difference in survival between the HSCT and control groups (disease-specific 5-year survival rate, 86.7% for HSCT [n = 16] vs 69.1% for non-HSCT [n = 38]; $P = .291$, log-rank test) (Figure 2B). In the high-risk group (group 3-4), however, the HSCT group (n = 6) seemed to have more favorable clinical course compared with the control group (n = 27) in terms of survival with marginal statistical significance (disease-specific 5-year survival rate, 100% vs 51.2%; $P = .053$, log-rank test) (Figure 2C). For those patients who were in PR at the time of HSCT or other treatment, there was no difference in survival between the HSCT group and the control group (disease-specific 5-year survival rate, 29.6% vs 22.2%; $P = .472$, log-rank test) (data not shown).

Subgroup analyses on non-CR patients at the time of HSCT or non-HSCT treatment (chemotherapy with or without RT or RT alone) revealed no significant difference in disease-specific survival rates between the HSCT and control groups (1-year survival rate, 66.7% vs 28.6%; $P = .141$, log-rank test) (Figure 3A). Further subgroup analyses demonstrated