Table 3. Treatment profile and efficacy ($n = 6$)

Patient no.	#02	#03	#04	#05	#06	#07
Dose reduction of SMILE (course)	L-Asp: 7→4 times (2nd)	L-Asp: reduced at a dose of 50% (2nd)	–	L-Asp: 7→4 times (1st)	–	–
Delay of the second course of SMILE	–	7 days	–	–	2 days	2 days
Planned treatment	Terminated	Completed	Terminated	Completed	Completed	Completed
Overall response	NE	CR	NR	PR	CR	CR
Additional treatment	–	SMILE × 1, SMI(L)E × 1 for PBSCH, HD-auto PBSCT	DeVIC × 1	SMILE × 1, HD-ETP for PBSCH, HD-auto PBSCT	MILD × 2, HD-ETP for PBSCH, MILD × 1, HD-auto PBSCT	SMILE × 4 (from 4th course, *E. coli* L-Asp was switched to *Erwinia* L-Asp)
Outcome	TRD, 2M	AND, 15M	DOD, 3M	AWD, 7M	AND, 7M	AND, 7M

AND, alive with no evidence of disease; AWD, alive with disease; CR, complete response; DeVIC, dexamethasone, etoposide, ifosfamide, carboplatin; DOD, died of disease; ETP, etoposide; HD, high dose; M, months after registration; MILD, methotrexate, ifosfamide, L-asparaginase, dexamethasone; NE, not evaluable; NR, no response; PBSCH, peripheral blood stem cell harvest; PBSCT, peripheral blood stem cell transplantation; PR, partial response; SMI(L)E, dexamethasone, methotrexate, ifosfamide, etoposide; SMILE, dexamethasone, methotrexate, ifosfamide, L-asparaginase, etoposide; TRD, treatment-related death.

lymphomas.[30] Furthermore, the addition of anthracyclines, which are important in B-cell lymphoma regimens, has not been shown to increase therapeutic efficacy in NK-cell malignancies.[30] Finally, the role of high-dose chemotherapy with HSCT remains controversial for NK-cell lymphomas because the ideal conditioning regimen for HSCT is currently unclear.[30] Therefore, the definition of a chemotherapeutic regimen with high treatment efficacy is an important goal. The SMILE regimen reported herein is currently the only regimen formulated specifically for NK/T-cell lymphomas.

The most common adverse event during SMILE chemotherapy was grade 4 neutropenia. At level 1, one treatment-related death occurred due to a delay in G-CSF administration. However, after the protocol revision that mandated G-CSF administration from day 6, severe infection was not observed. Three out of six patients were able to undergo high-dose chemotherapy with autologous HSCT. Based on these results and the fact that only six patients were evaluated in this trial, we considered that the hematological toxicities and severe infectious complications of SMILE in level 1 with G-CSF support should be evaluated further in the setting of a prospective clinical trial.

The most frequent non-hematological adverse event was hyponatremia. Hyponatremia might be related to the syndrome of inappropriate secretion of antidiuretic hormone or renal tubular damage, which was likely caused by chemotherapeutic agents in the SMILE protocol, particularly ifosfamide. Interestingly, hyponatremia was also observed in a recent clinical trial for localized ENKL.[14] Therefore, other disease-specific causes of hyponatremia would have to be examined in future studies.

The antitumor effect of two courses of SMILE, which resulted in a CR rate of 50% and ORR of 67%, was remarkable. To date, two promising results of nonanthracycline-containing regimens in nasal ENKL have been reported. In a Mexican prospective study of newly diagnosed advanced-stage ENKL with nasal involvement,[25] patients were given six courses of cyclophosphamide, methotrexate, etoposide, and dexamethasone chemotherapy, with sandwiched radiotherapy of 55 Gy after three courses in cases with facial involvement. Another Chinese study of nasal ENKL relapsing or refractory to anthracycline-containing chemotherapy used a salvage regimen containing L-asparaginase, vincristine, and dexamethasone followed by involved-field radiotherapy (median: 56 Gy).[23] Although the CR rates in these studies exceeded 55%, high-dose radiotherapy was used, making evaluation of the contribution of the efficacy of chemotherapeutic regimens difficult. Moreover, high-dose radiotherapy may not be an option for patients with advanced disseminated diseases, and for relapsed patients who have already received involved-field radiotherapy during primary treatment for nasal ENKL. The therapeutic efficacy of the SMILE protocol requires further evaluation in a larger number of patients with advanced ENKL.

We did not use the International Working Group (IWC) criteria to assess the response in this trial for the following reasons:[31] (1) ENKL and ANKL usually show extranodal involvement that is often difficult to measure bidimensionally; and (2) given that the SMILE phase I study is the first prospective, international, multicenter-based clinical trial for ENKL and ANKL in East Asia, simple and familiar response criteria were thought to be more appropriate. Recently, the IWC criteria have been revised, and 18-fluoro-deoxyglucose (FDG) positron emission tomography scanning was incorporated into the evaluation of lymphomatous involvement.[32] Moreover, a recent report suggested that ENKL is an FDG-avid lymphoma.[33] In future clinical trials on ENKL, the appropriateness of the revised IWC criteria for ENKL needs to be examined.

According to the criteria for DLT assessment stipulated in the protocol, we did not escalate the dose to level 2 and could not determine the MTD in this trial. However, all grade 3 non-hematological toxicities developing in patients who were enrolled after the protocol revision in level 1 were manageable and transient. In addition, no grade 4 hematological toxicities lasting 7 days or more were observed in patients who were enrolled after the protocol revision (Table 2). SMILE at dose level 1 was thought to be promising because three out of six patients in level 1 achieved a CR. From these results, we believe that dose level 1 of SMILE chemotherapy is appropriate for further clinical studies. Our findings are now further evaluated in a prospective phase II study of level 1 SMILE with G-CSF support, which has started since July 2007.

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Review Article

Extranodal NK/T-cell lymphoma: diagnosis and treatment cues

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Abstract

Extranodal NK/T-cell lymphoma, nasal type (ENKL) is mostly endemic to East Asia. It predominantly occurs in the nasal or paranasal areas and less frequently in the skin. Most of the tumours show NK-cell, but rarely T-cell, phenotypes. The Epstein–Barr virus (EBV) genome can be usually detected in lymphoma cells. Geographic localization of ENKL matches the endemic distribution of EBV, suggesting that EBV plays an important role in lymphomagenesis. Originally, NK-cell and T-cell types were believed to present the same clinicopathologic characteristics, but recent data suggest more aggressive characteristics for the NK-cell phenotype. Although ENKL is sensitive to radiotherapy, it shows a poorer response to chemotherapeutic agents than other lymphomas due to expression of p-glycoprotein. Therefore, new therapeutic approaches must be considered. Several new clinical trials are now being conducted in East Asia.

Keywords: **natural killer cell; azurophilic granule; Epstein–Barr virus; CD56; cytotoxic molecule**

Introduction

Extranodal NK/T-cell lymphoma (ENKL), nasal type most frequently affects the nose and paranasal area [1–3]. The immunophenotype of the lymphoma cells mostly reflects that of NK-cells, but sometimes is also characteristic of T-cells. In some cases differential diagnosis is difficult when only using paraffin embedded specimens. Therefore at present, the diagnostic term 'NK/T-cell lymphoma' is used. It should however be noted that no 'NK/T-cell' actually exists. This type of lymphoma shows a marked geographic preference for East Asia and Latin America. The incidence is also different within the endemic areas; in Asia, the rates of occurrence are: 3.3% in Japan [4], 5% in Taiwan [5], 6% in Hong Kong [6] and 8% in Korea [7]. In this review, we summarize the disease characteristics of ENKL of nasal type with special emphasis on diagnostic pitfalls.

Ontogeny of NK-cells

NK-cells were first defined as a functional subset of lymphocytes that mediate major histocompatibility complex-nonrestricted cytotoxicity [8]. They were later recognized to have large granular lymphocyte (LGL) morphology, germline configurations of T-cell receptor (TCR) and immunoglobulin genes and a surface CD3 (sCD3)-negative and CD56-positive phenotype [9]. From these findings, NK-cells are now regarded as a third lineage of lymphocytes that is distinct from T- and B-cells. Because NK-cells develop from T/NK bi-potential common progenitors (Figure 1) [10,11], they share many similarities with T-cells, particularly with cytotoxic T-cells. Therefore, the phenotypes of NK-cell and T-cell lymphoma/leukaemia also have much in common, which makes it difficult to perform differential diagnosis [12–14].

Pathological transformation of NK-cells

Myeloid antigen-positive T/NK bi-potential progenitors are believed to develop by transformation into myeloid/NK cell precursor acute leukaemia [15,16]. NK-cell lineage committed progenitors are also hypothesized to transform to blastic NK-cell lymphoma (BNKL) or precursor NK-cell acute lymphoblastic leukaemia/lymphoma (NK-ALL) [17,18]. Previously, CD4-positive and CD4-negative types of BNKL/NK-ALL were identified [18]. Although there remain several controversies regarding CD56 expression and dendritic cell lineage, the $CD4^+$ $CD56^+$ type of this tumour is somehow related to plasmacytoid dendritic cells or to the monocytic lineage [19,20], The CD4-negative type probably represents the true BNKL/NK-ALL. Two mature NK-cell neoplasms, ENKL [1] and aggressive NK-cell leukaemia [18d], are transformed from functionally

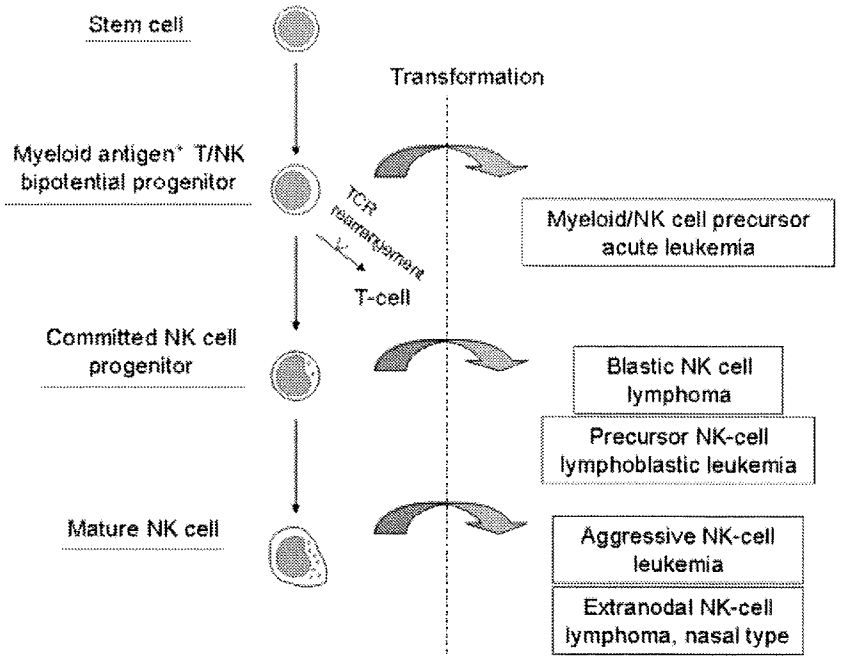

Figure 1. Ontogeny of NK-cells and transformation to NK-cell malignancies. NK-cells are differentiated from stem cells through myeloid-antigen positive NK/T bi-potential progenitors and lineage-committed progenitors. Myeloid/NK cell precursor acute leukaemia is transformed from the myeloid antigen-positive progenitor. Blastic NK-cell lymphoma and precursor NK-cell lymphoblastic leukaemia are derived from a relatively mature, NK-cell lineage committed progenitor. Two mature NK-cell neoplasms, aggressive NK-cell leukaemia and extranodal NK-cell lymphoma, nasal type, are transformed from mature NK-cells

mature NK-cells. Aggressive NK-cell leukaemia is a distinct leukaemic form of mature NK-cell malignancy with frequent hepatosplenic involvement [21,22]. Although these two diseases share many features, several clinicopathologic and phenotypic differences have been reported [23]. Therefore, aggressive NK-cell leukaemia remains as a distinct disease entity in the forthcoming World Health Organization classifications. A summary of the clinicopathologic characteristics of NK-cell lineage neoplasms is given in Table 1.

Clinical characteristics of extranodal NK-cell lymphoma, nasal type

The nose and paranasal area including the upper aerodigestive tract contains the origin of more than 80% of extranodal NK-cell lymphomas, nasal type. Macroscopic findings by nasal endoscope are shown in Figure 2. Initial complaints of ENKL, nasal type include local symptoms such as nasal obstruction, discharge and bleeding. Thereafter, as the disease extends, necrosis, swellingor bony destruction of the nasal area develops. However, such extreme local progressions are currently rare because of early disease recognition and reference to specialized physicians. The skin is the second most frequent organ of origin, accounting for approximately 10% of cases [19]. Cases originating from the liver and/or spleen account for 5% of ENKLs, nasal type. More rare organs of onset include the lung, gastrointestinal tract, kidney, pancreas, testis and brain. Nasal lymphomas more frequently present as a localized disease (ratio 4:1), whereas lymphomas at other sites are more frequently detected at an advanced stage (ratio 2:3) [24–35]. Because this lymphoma essentially presents an extranodal origin, clinical stage III is rare and most of the advanced stage cases are in stage IV. Some cases show long-term limitation to the original site. However, once the tumour develops outside the original site, the disease rapidly progresses and disseminates. Fever, haemophagocytosis and disseminated intravascular coagulation are not rare in this situation. Several cases of stage IV or aggressive NK-cell leukaemia could not be treated because of the progression of the disease and poor status of the patient [36].

Diagnosis of extranodal NK-cell lymphoma, nasal type

Diagnosis of ENKL, nasal-type is based on histopathologic examination of biopsy specimens, but is sometimes difficult because of the existence of wide necrosis around the tumour (Figure 3A) that is characterized by expression of Fas and Fas ligand on the tumour cells [37]. Selection of appropriate sites for biopsy is important for prompt diagnosis, as are repeated approaches in case the specimens only include necrotic tissue.

Histologically, tumour cells from ENKL generally show angiocentric growth pattern (Figure 3A) [1,38]. The growth pattern is such a notable feature of this lymphoma that the diagnostic term used to be 'angiocentric lymphoma' [39]. In clinical practice, sampling error can prevent recognition of angiocentricity; therefore this finding is currently not mandatory for diagnosis [38]. The presence of cucumber-

Hematol Oncol (2008)
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Table 1. Clinicopathologic characteristics of NK-cell lineage neoplasms

	Myeloid/NK cell precursor acute leukaemia	Blastic NK-cell lymphoma/Precursor NK-cell lymphoblastic leukaemia	Aggressive NK-cell leukaemia	Extranodal NK cell lymphoma, nasal type	
				Limited stage	Advanced stage
Morphology	Blastic	Blastic	LGL	LGL	
Azurophilic granule	−	−	+	+	
Lymph node involvement	+	+	+	−	+/−
Extranodal involvement	Bone marrow, blood, mediastinum	Skin, bone marrow	Bone marrow, blood, liver, spleen	Nose, skin	Nose, skin, bone marrow, blood
B-symptom	Rare	Rare	Frequent	Rare	Frequent
Surface marker	CD7+, CD33+, CD34+, CD56+	CD4+/−, CD7+, CD56+, TdT+	CD2+, CD16+, CD56+	CD2+, cyCD3+, CD56+	
EBV	−	−	+/−	+	
Clinical course	Aggressive	Aggressive	Aggressive	Sometimes indolent	Aggressive
Therapy	AML chemotherapy	Chemotherapy for lymphoid neoplasms	No standard therapy	Radiotherapy followed by chemotherapy	No standard therapy
Prognosis	Relapse is frequent, and the prognosis is poor.	Relapse is frequent, and the prognosis is poor.	Very poor	Fair	Very poor

LGL, large granular lymphocyte; EBV, Epstein–Barr virus; AML, acute myeloid leukaemia.

Figure 2. Naso-endoscopic findings of nasal NK/T-cell lymphoma. (A) Naso-endoscopic findings of a patient who presented with nasal discharge. Tumour formation and tissue swelling by necrosis can be observed. (B) Surface ulcerations can be seen, and a part of the nasal cartilage was destroyed

like cells with elongated nuclei is particular to ENKL, and is helpful for diagnosis (Figure 3B). If the biopsy specimen is small, a touch imprint smear with Giemsa staining is sometimes useful for diagnosis because of the presence of azurophilic granules in the tumour cells (Figure 4). The accumulation of NK-cells or cytotoxic T-cells does not directly indicate a malignant condition; however, since it is not usually recognized in the nasal mucosa, the assemblage of NK-cells (or rarely cytotoxic T-cells) is still important evidence. Epstein–Barr virus (EBV) is harboured in ENKLs of nasal type, and detection by *in situ* hybridization can be achieved for paraffin-embedded tissues or touch imprint smears (Figure 5). EBV is rarely observed in

Figure 3. Biopsy specimen of nasal NK/T-cell lymphoma. (A) In a vast necrotic region, there exist several atypical medium-to-large lymphocytes. Tumour cells show an angiocentric growth pattern. (B) The nuclei of several tumour cells are elongated, and present a cucumber-like morphology (Arrow)

Hematol Oncol (2008)
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Figure 4. Touch imprint smear of ENKL. There are many atypical NK-cells with prominent azurophilic granules

lymphocytes residing in normal or inflammatory nasal mucosa or adjacent tissue; therefore detection is particularly important for specimens that mostly consist of necrotic tissue.

For ENKLs, histopathologic diagnosis of bone marrow involvement is occasionally difficult. Detection of EBV is also helpful in this situation [40]. Recently, the prognostic significance of such occult or minute involvement has been shown for early stage patients [41]. Routine examination of bone marrow involvement by using EBV *in situ* hybridization is now recommended.

Immunophenotype of extranodal NK-cell lymphoma, nasal type

Phenotypic markers expressed in ENKL include CD2, cytoplasmic CD3 (cyCD3), CD7 and CD56, which also represent the phenotype of normal NK-cells [25,42,43]. Cytotoxic molecules such as TIA-1, granzyme B and perforin, are also positive in ENKL [37,44]. Table 1 shows the differential diagnosis of mature NK-cell tumours. If lymphoma cells are negative for these cytotoxic molecules and show a T-cell phenotype, diagnosis of another type of T-cell lymphoma should be considered. For differentiation of NK-cell from T-cell lymphoma, expression of sCD3, CD5 or TCRs on the lymphoma cells can be evaluated [31,43,45], in addition to the rearrangement of TCR genes. However, routine diagnostic use of these procedures is sometimes difficult and unavailable. Previously, the clinical features and prognosis of true T-cell nasal lymphoma were regarded to be similar to that of NK-cell type, resulting in the adoption of the term 'NK/T-cell lymphoma'. It should be noted, however, that this nomenclature falsely suggests existence of 'NK/T-cells'. Another point to be noted is that the use of the term 'NK/T' is restricted to lymphomas occurring in the nasal/paranasal area, and is not applied to lymphomas originated from extra-nasal sites. Differential diagnosis from other types of T-cell lymphomas is therefore required for the extra-nasal type of extranodal NK-cell lymphoma.

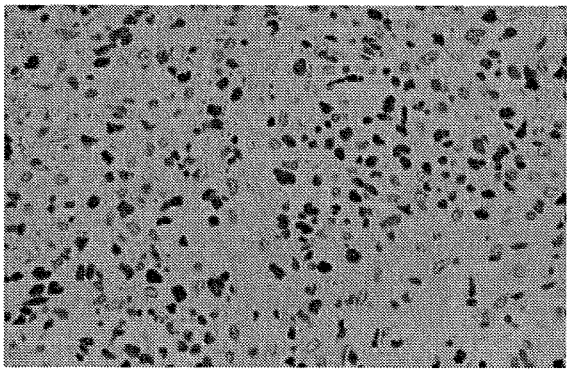

Figure 5. Epstein–Barr virus small RNA (EBER) in-situ hybridization (ISH). Because lymphoma cells harbour the EBV, they are positive for EBER-ISH

Recently, studies with large numbers of patients showed that the prognosis of nasal NK-cell lymphoma is significantly poorer than that of nasal 'T-cell' lymphoma [29,46]. Differential diagnosis of NK-cell lymphoma from genuine T-cell lymphoma may therefore be required in the future. A search for a diagnostic marker is therefore warranted.

Diagnostic pitfalls for extranodal NK/T-cell lymphoma

Because CD56 is also expressed in a part of acute myeloid leukaemia (AML) [47], differential diagnosis of NK-cell malignancies from CD56-positive AML is occasionally difficult, particularly for those with extramedullary or cutaneous involvement. $CD4^+$ $CD56^+$ haematodermic neoplasm also frequently shows cutaneous/subcutaneous involvement [18,19]. In this context, CD56-positive AML and $CD4^+$ $CD56^+$ haematodermic neoplasm are unexceptionally negative for EBV, which is useful for the differential diagnosis. Positive EBV status is thus required for the diagnosis of NK/T-cell lymphomas.

Treatment of extranodal NK/T-cell lymphoma

Limited stages

For limited stages of usual aggressive non-Hodgkin lymphoma, three to four courses of a chemotherapy regimen that includes anthracycline, such as CHOP, supplemented with involved field irradiation is regarded as the standard therapy [48]. However, for nasal NK/T-cell lymphoma, the overall 5-year survival rate using this strategy is less than 50% [49,50]. Reasons include the expression of the multidrug-resistant p-glycoprotein in NK/T-cell lymphoma cells [51,52]. P-glycoprotein actively exports doxorubicin and vincristine, which are the main components of CHOP chemotherapy. Radiotherapy remains effective but cannot prevent recurrence of the disease outside the radiation field. The overall 5-year survival rate therefore remains limited to 40–50% when using radiotherapy alone [31,53,54].

Ribrag *et al.* treated eight patients in the limited stage of nasal NK/T-cell lymphoma with radiotherapy followed by chemotherapy and reported an excellent result (10 years overall survival: 100%) [55]. They have concluded that a sufficient dose of radiotherapy immediately after diagnosis

Hematol Oncol (2008)
DOI: 10.1002/hon

is desirable for treatment of this disease. At present, radiotherapy followed by chemotherapy is regarded as a standard strategy for limited stage ENKL [31,56].

Yamaguchi *et al.* also reported excellent control of the disease by simultaneous chemoradiotherapy using radiation therapy and DeVIC chemotherapy (RT-DeVIC) [50]. Based on this finding, the Japanese Clinical Oncology Group has conducted a phase I/II study of RT-DeVIC chemoradiotherapy. The study is now closed with sufficient numbers of patients registered. Its results are anticipated.

Advanced stages

The prognosis of advanced stage ENKL, nasal type, as well as that of aggressive NK-cell leukaemia, is extremely poor when using any chemotherapeutic regimen [22]. Aviles *et al.* from Mexico reported the utility of sandwich chemoradiotherapy, which consisted of three courses of cyclophosphamide, methotrexate, etoposide and dexamethasone (CMED), radiotherapy and additional three courses of CMED. The 5-year overall survival rate using this method was reported as 65% [57]. This was an excellent result, but the reported toxicities were surprisingly low despite the relatively high dose of chemotherapeutic drugs used. Therefore, confirmation through replication is required.

Recently, several reports from East Asia suggest the efficacy of L-asparaginase for treatment of mature NK/T-cell lymphoma [58–60]. L-asparaginase is an enzyme that digests serum L-asparagine and acts as an anti-tumour agent through asparagine starvation of tumours with low expression levels of asparagine synthetase [61,62]. Because L-asparaginase specifically acts on lymphoid cells, myelosuppression by L-asparaginase is minimal. L-asparaginase has long been regarded as a key drug for paediatric acute lymphoblastic leukaemia. A Chinese group treated nasal NK/T-cell lymphoma patients refractory to CHOP-like chemotherapy with a chemotherapy regimen that consisted of L-asparaginase, vincristine and dexamethasone supplemented by local radiotherapy. They reported good results with a 5-year overall survival rate of 55.6% [63]. Likewise, L-asparaginase is effective for this type of lymphoma but has many adverse reactions such as haemostatic complications, allergy and pancreatitis. These findings suggest a need for the establishment of safe and effective chemotherapeutic regimens. The NK-cell Tumour Study Group is now conducting clinical studies of a novel L-asparaginase-containing chemotherapy for initial stage IV, relapsed or refractory NK/T-cell leukaemia/lymphoma [36]. This regimen is termed SMILE, and consists of methotrexate, ifosfamide, etoposide, steroid and L-asparaginase. A phase I dose finding study has been completed [64] and we are now designing a subsequent phase II study.

Haematopoietic stem cell transplantation (HSCT)

Because the prognosis of ENKL is poor, there exist several reports of upfront autologous HSCT. In large-scale reports from Japan and Korea, long-term survival ranges from 50 to 70% [65–67]. However, retrospective analysis might be biased by patient selection. Prospective clinical trials are thus warranted before concluding that autologous HSCT is effective for ENKL.

On the other hand, allogeneic HSCT can also be applied for the treatment and is the only curative strategy for advanced stage or nonremission patients. Two large-scale analyses from Japan included high-risk patients and reported a long-term survival rate ranging from 30 to 40% [66,68]. The second study included patients who received reduced intensity stem cell transplants (RIST), and both reports indicated the absence of late recurrence at 2 years post-transplantation. These findings suggest the curative potential of allogeneic HSCT, but patient selection bias is also possible. Since many types of stem cell sources are now utilized for HSCT including cord blood and mismatched donors, further accumulation of data and prospective evaluations are also required.

Clinical significance of the Epstein–Barr virus

It is well-known that patient sera from EBV-positive malignancies contain fragmented viral DNA [69,70]. Measurement of the circulating viral DNA load in peripheral blood is useful for diagnosis, monitoring and prognostication of the disease. However, detection is sometimes misunderstood as the presence of viral particle itself; rather, the detected DNA is derived from dead tumour cells. For these reasons, most detected fragments are less than 500 bp in length, and longer fragments or the entire EBV genome are never detected [71]. EBV-DNA can therefore be used as a marker to predict the tumour burden [72,73], but prediction can potentially be affected by the presence of EBV unrelated to the lymphoma. There are several choices of source tissue for analysis including plasma, total blood and mononuclear cells, and each choice represents a different outcome [74]. The significance of the viral load in peripheral blood and the choice of source tissue used for analysis should be examined prospectively.

Conclusion

Several new insights have been recently developed for extranasal NK/T-cell lymphoma, nasal type. Diagnosis is thus becoming easier. However, the prognosis is particularly poor in both the limited and advanced stages. Appropriate therapeutic strategies should be explored by prospective studies.

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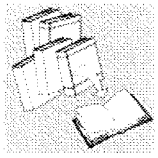
REVIEW



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

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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

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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].

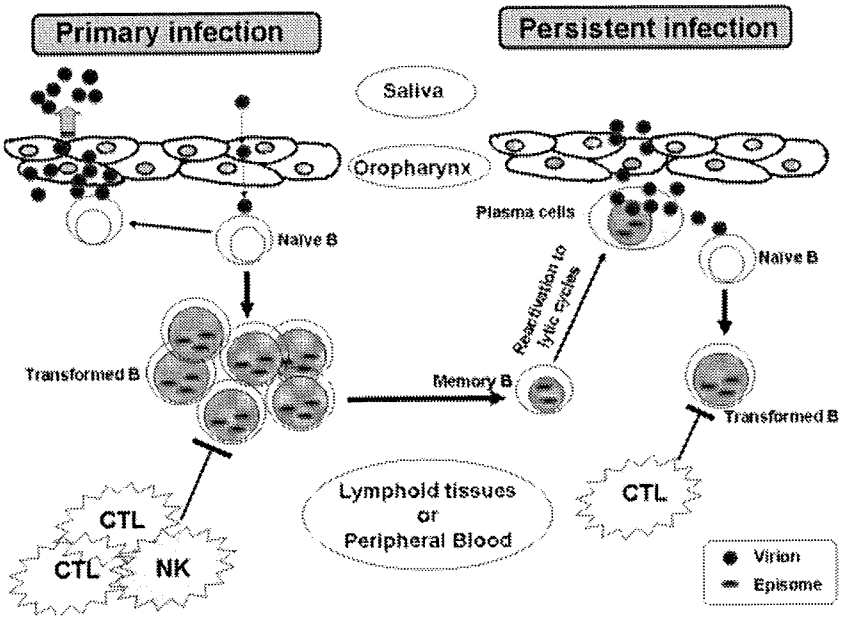

Figure 1. Schematic representation of Epstein–Barr virus (EBV) infection. In primary infection, EBV in the saliva directly infects naïve B cells in the oropharynx. EBV-infected B cells transform and proliferate as activated blasts but are finally controlled by cytotoxic T lymphocytes (CTL) or natural 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.

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.

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.

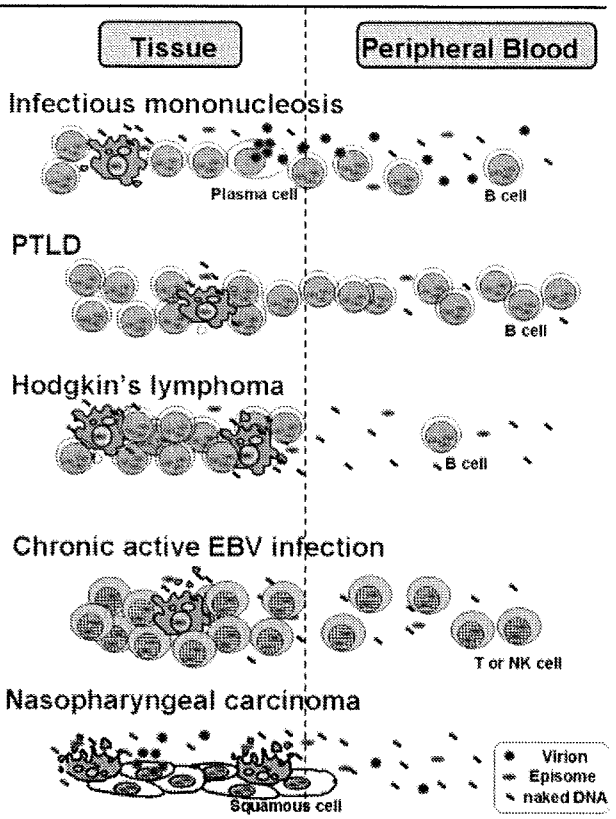

Figure 2. How Epstein–Barr virus (EBV) persists in tissues and peripheral blood. Infectious mononucleosis: Virion and naked (degraded) EBV DNA derived from plasma cells in lytic infection or from apoptotic cells, respectively, passes into the peripheral blood. Some transformed, latently infected B cells 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.

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.

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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

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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	Primary infection (seropositive donor/seronegative recipient)
	Anti-thymocyte/lymphocyte globulin	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	Anti-CD20 monoclonal antibody
	Donor leucocyte infusion	
	EBV-specific CTL	

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].

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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