Advances in Brief

Diagnostic and Prognostic Implications of Circulating Cell-free Epstein-Barr Virus DNA in Natural Killer/T-Cell Lymphoma

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Abstract

Purpose: Natural killer/T-cell (NK/T-cell) lymphoma is a highly aggressive tumor for which no serological tumor marker has yet been established to be useful clinically. We investigated the potential of circulating EBV DNA as a tumor marker for this malignancy.

Experimental Design: A real-time quantitative PCR assay was used to measure circulating EBV DNA.

Results: Plasma EBV DNA levels were measured in 18 patients with NK/T-cell lymphoma at presentation and during therapy. Plasma EBV DNA was detected in 17 of the 18 patients (median, 659 copies/ml; interquartile range, 181–17,042 copies/ml) but in none of 35 control subjects ($p < 0.0001$). Serial measurements of plasma EBV DNA levels during therapy showed a close correlation between clinical response and changes in plasma EBV DNA levels. Clinically responding patients showed a fall of plasma EBV DNA levels to low or undetectable levels, whereas those who failed therapy showed a rapid increase in plasma EBV DNA levels. Most importantly, patients with high baseline plasma EBV DNA levels (≥ 600 copies/ml) demonstrated a significantly inferior survival than those with low baseline plasma EBV DNA (< 600 copies/ml; 21% *versus* 78%; $P = 0.024$).

Conclusions: Plasma EBV DNA, as measured by real-time quantitative PCR, is a useful tumor marker for diagnosis, disease monitoring, and prediction of outcome in patients with NK/T-cell lymphoma.

Introduction

NK[3]/T-cell lymphomas represent a distinct clinicopathological entity characterized by the presence of progressive necrotic lesions, the expression of NK cell-associated marker, CD56, in tumor cells, and its strong association with the EBV (1, 2). The disease is rare in the United States and Europe but much more common in Asians and South Americans. Most patients present with a midfacial destructive disease in the nasal cavity, nasopharynx, or palate (referred to as nasal NK/T-cell lymphoma), but a minority have tumors that arise from other extranodal sites, including the skin, upper airway, gastrointestinal tract, and testis. The disease generally pursues an aggressive clinical course that is associated with a poor outcome (2–5). Currently, no serological tumor marker has yet been identified for disease monitoring and prediction of outcome in this group of patients.

Circulating cell-free EBV DNA has been detected in the plasma/serum from patients with EBV-associated tumors, including Hodgkin's disease, posttransplant lymphoproliferative disease, AIDS-related lymphoma, and nasopharyngeal carcinoma (6–11). In our recent study, plasma EBV DNA was also found in four patients with "nasal-type" NK/T-cell lymphoma. In this small series, plasma EBV DNA levels appeared to correlate with therapeutic response. However, its impact on prognosis has not yet been determined (11). In the present study, we aimed to investigate the dynamics of plasma EBV DNA levels in patients with nasal and nonnasal types of NK/T-cell lymphoma prior to and in the course of therapy using real-time quantitative PCR to correlate the changes of plasma EBV DNA levels with clinical response during therapy and to evaluate the prognostic value of plasma EBV DNA levels.

Materials and Methods

Patients. Between December 1998 and March 2001, plasma samples of 18 patients of newly diagnosed or relapsed NK/T-cell lymphoma managed in the Department of Clinical Oncology, Prince of Wales Hospital, were enrolled in this study, including the 4 patients described in our previous study (11). The diagnosis of NK/T-cell lymphoma was established histologically using the criteria described by Jaffe *et al.* (1) and the Revised European American Lymphoma Classification (12). The presence of EBV in tumor cells was assessed by *in situ* hybridization on paraffin-embedded tissue sections using a fluorescein-conjugated oligonucleotide probe for EBERs (Novocastra, Newcastle upon Tyne, United Kingdom) as described

previously (13). Patients were staged according to the Ann Arbor Staging System (14). Investigations included a complete history, physical examination, full blood counts, blood chemistry, serum lactate dehydrogenase levels, bone marrow aspiration and biopsy, chest radiography, and computed tomographic scan of the thorax, abdomen, and pelvis. Endoscopic examination of the primary tumor was performed in patients with tumor involvement of the nasal/nasopharyngeal regions and gastrointestinal tract. All patients were treated with uniform departmental protocols according to the primary disease site. Blood samples were taken for EBV DNA analysis at presentation and serially during the course of therapy and subsequent follow-up visits.

In addition to this cohort, we also searched for additional plasma samples of NK/T-cell lymphomas stored in our serum bank between 1995 and 1998. Eight cases of NK/T-cell lymphoma collected at presentation were identified and included for survival analysis. Circulating EBV DNA levels were determined.

As controls, blood samples from 35 healthy subjects were also analyzed. The study was approved by the Ethics Committee of the Chinese University of Hong Kong, and informed consent was obtained from all subjects.

DNA Extraction from Plasma Samples.   Plasma samples were collected from patients and healthy subjects according to the protocol described previously (10, 15). The samples were stored at $-20°C$ until further processing. DNA from the plasma samples was extracted using a QIAamp Blood kit (Qiagen, Hilden, Germany), according to the "blood and body fluid protocol" as recommended by the manufacturer (16, 17).

Real-Time Quantitative EBV DNA PCR.   Circulating EBV DNA concentrations were measured using a real-time quantitative PCR system toward the *Bam*HI-W fragment region of the EBV genome as described previously (10). All plasma DNA samples were also subjected to real-time quantitative PCR analysis for the β-*globin* gene (17), which served as a control for amplifiability of plasma DNA. Multiple negative water blanks were included in every analysis.

A calibration curve was run in parallel and in duplicate with each analysis, using DNA extracted from an EBV-positive cell line Namalwa (American Type Culture Collection no. CRL-1432; Ref. 18) as standard. Results were expressed as copies of EBV genomes/ml of plasma.

Amplification data were collected using an ABI Prism 7700 sequence Detector (PE Applied Biosystems, Foster City, CA) and were analyzed using the Sequence Detection System software developed by PE Applied Biosystems. The mean quantity of each duplicate was used for further concentration calculation. The concentration expressed in copies/ml was calculated using the following equation (19):

$$ C = Q \times \frac{V_{\text{DNA}}}{V_{\text{PCR}}} \times \frac{1}{V_{\text{ext}}} $$

where C is target concentration in plasma (copies/ml); Q is target quantity (copies) determined by sequence detector in a PCR; V_{DNA} is total volume of DNA obtained following extraction, typically 50 μl/Qiagen extraction; V_{PCR} is volume of DNA solution used for PCR, typically 5 μl; and V_{ext} is volume of plasma extracted, typically 0.4–0.8 ml.

Table 1   Clinical characteristics and plasma EBV DNA levels in a cohort of 18 patients with NK/T-cell lymphoma

	NK/T-cell lymphoma ($n = 18$)
Number of patients	
Previously untreated	16
Relapsed	2
Age	
Median, years (range)	61 (34–94)
Sex	
Male	12
Female	6
Karnofsky performance status	
Median (range)	100 (60–100)
Site	
Nasal cavity	12
Small bowel	2
Colon	1
Skin	1
Testis	1
Lymph node	1
Stage	
I	10
II	7
III	
IV	1
Plasma lactate dehydrogenase concentration	
Median, units/L (interquartile range)	161 (139–244)
EBER	
Positive	13/13
Unknown	5
Plasma EBV DNA	
Detectable	17
Median level, copies/ml (interquartile range)	659 (181–17042)

Statistical Analysis.   CR was defined as an absence of detectable disease on clinical and radiological criteria. PR was defined as reduction of 50% of tumor by clinical and radiological criteria. Comparison of parameters between groups was performed using the Mann-Whitney U test or Fisher's exact test as appropriate. Overall survival was measured from the date of diagnosis to the last day of follow-up or death from any cause. Survival curves were constructed using the Kaplan-Meier method. The difference in survival curves was assessed by the log-rank test. Statistical analysis was performed using the software STATISTICA version 5.5.

Results

Clinical Characteristics and Baseline Plasma EBV DNA.   The clinical characteristics, EBV status in tumor cells, and pretreatment plasma EBV DNA concentrations of the 18 patients are summarized in Table 1. Of the 18 patients, 16 had newly diagnosed NK/T-cell lymphoma, and 2 had local recurrence of nasal NK/T-cell lymphoma. One patient had NK/T-cell lymphoma of the skin presenting with a leg ulcer and classified as stage IV disease. The remaining 17 patients had early stage disease (stage I/II). Tissue samples were available in 13 patients for evaluation of EBV status. All exhibited EBER positivity in the tumor cells.

Plasma EBV DNA was detected in 17 of the 18 (94%) patients. The median plasma EBV DNA level in these 17

patients was 659 copies/ml (interquartile range, 181–17,042 copies/ml). In contrast, plasma EBV DNA was not detected in any of the 35 control subjects ($P < 0.0001$). Similarly, plasma EBV DNA was also detected in the 8 cases of NK/T-cell lymphoma obtained from the serum bank, ranging from 709 copies/ml to 62,004,600 copies/ml. Taken together, the median plasma EBV DNA level in all 25 patients of NK/T-cell lymphoma was 1,670 copies/ml (interquartile range, 307–22,503 copies/ml). Detection of plasma EBV DNA as a tumor marker for NK/T-cell lymphoma using real-time quantitative PCR yielded a high sensitivity and specificity of 96 and 100%, respectively.

Clinical Response and Changes in Plasma EBV DNA with Therapy. Of the 18 cohort patients, 15 had serial measurements of plasma EBV DNA during the course of therapy and the subsequent follow-up. Three patients were excluded for serial measurements: 1 patient who had no detectable plasma EBV DNA at presentation; 1 died of gastrointestinal bleeding and sepsis before therapy; and 1 died of sepsis shortly after commencement of chemotherapy. Clinical response and changes in plasma EBV DNA levels of the 15 patients are summarized in Table 2. Clinical response was observed in 10 patients (9 CRs and 1 PR). One patient had static disease, 1 had progressive disease, and 3 were not evaluable for clinical response. All 10 patients who responded to therapy demonstrated a significant reduction of plasma EBV DNA levels after therapy, whereas those who failed therapy showed a rapid increase in plasma EBV DNA levels. Changes in plasma EBV DNA levels after therapy between responders and nonresponders were significantly different ($P = 0.03$). Fig. 1, *A–D,* illustrates the dynamic changes of plasma EBV DNA levels in 4 complete responders (patients 6, 7, 8, and 9) during the course of therapy. In the 9 patients who achieved complete remission, 7 had no detectable plasma EBV DNA at the time of response assessment. Two patients (patients 8 and 9) had residual plasma EBV DNA of 250 copies/ml and 484 copies/ml, respectively, while in apparent clinical remission. They subsequently developed recurrence and died of disease at 12.6 and 7.5 months, respectively. In both patients, recurrence was preceded by a >40-fold increase in plasma EBV DNA levels (Fig. 1, *C* and *D*).

Patient 10, who had stage II primary NK/T-cell lymphoma of the colon, showed static disease after four cycles of m-BACOD chemotherapy. However, there was an associated increase in plasma EBV DNA level from a baseline value of 29,237 to 446,479 copies/ml. Despite treatment with surgery (panprotocolectomy), the disease progressed with development of malignant ulceration in the hard palate 2 months after surgery. The patient continued to deteriorate and died 8.1 months after initial diagnosis (Fig. 1*E*). Patient 12, who had local recurrence of nasal NK/T-cell lymphoma, progressed rapidly despite two courses of chemotherapy with DHAP. The tumor extended to the contralateral nasal cavity, nasopharynx, and oropharynx. At the time of progression, the plasma EBV DNA level increased from a baseline of 67 to 23,117 copies/ml. Treatment with RT resulted in resolution of tumor and a rapid decrease in plasma EBV DNA to 40 copies/ml by the end of therapy (Fig. 1*F*). The patient remained well 22 months after initial recurrence with a plasma EBV DNA level of 86 copies/ml.

Baseline Plasma EBV DNA Levels and Survival. Because of the small number of patients in the prospective cohort, the eight cases of NK/T-cell lymphomas identified in the serum bank were included in the survival analysis. At the time of analysis, the median follow-up of NK/T-cell patients who remained alive was 13 months (range, 4.5–24.3 months). On the basis of the median plasma EBV DNA level of 659 copies/ml detected at presentation in the prospective cohort, the 26 NK/T-cell lymphoma patients were divided into high (≥600 copies/ml) and low (600 copies/ml) plasma EBV DNA groups for survival analysis. Using univariate analysis, patients with high plasma EBV DNA levels at presentation showed a significantly poorer overall survival at 20 months, as compared with those with low plasma EBV DNA levels (21% *versus* 78%; $P = 0.024$; Fig. 2). No significant difference was observed in the age, Karnovsky performance status, disease stage, and serum LDH between patients with high plasma EBV DNA and those with low plasma EBV DNA levels (data not shown). Comparison of overall survival was also performed according to age (≥60 years *versus* <60 years), LDH levels (elevated *versus* normal), and disease stage (early stage *versus* advanced stage) using univariate analysis. Only disease stage demonstrated an influence on survival, showing a significantly better survival in patients with early stage as compared with those with advanced stage disease ($P = 0.015$). Importantly, even among patients with early-stage disease, significantly inferior survival was also found in patients with high baseline plasma EBV DNA levels as compared with those with low baseline plasma EBV DNA levels ($P = 0.03$). Multivariate analysis was not performed because of the small number of patients.

Discussion

In the present study, we investigated the diagnostic value, dynamic changes, and prognostic effect of plasma EBV DNA levels in a cohort of patients with NK/T-cell lymphoma during therapy and follow-up. The results underline the clinical importance of plasma EBV DNA measurement in the diagnosis, disease monitoring, and prediction of outcome.

NK/T-cell lymphoma is a rare but highly aggressive disease that commonly poses great difficulty in diagnosis and classification. Morphologically, the disease is characterized by a broad cytological spectrum. Distinction from reactive or inflammatory conditions can be difficult, especially when the predominant cells are small or medium-sized lymphoid cells or there is an admixture of inflammatory cells (1–3). In contrast to B-cell lymphomas, clonality of NK/T-cell lymphoma cannot be readily determined by immunohistochemical studies or molecular analysis for T-cell receptor gene rearrangements. In addition, histological diagnosis can often be hampered by extensive necrosis and the small size of biopsy samples obtained. In such situations, detection of plasma EBV DNA by quantitative real-time PCR assay may be helpful in diagnosis.

Our results demonstrate a close correlation between plasma EBV DNA levels and therapeutic response. All patients who responded to treatment showed a marked reduction of plasma EBV DNA to low or undetectable levels. In contrast, patients who failed treatment had a rapid increase in plasma EBV DNA levels. Thus, rapid decline in plasma EBV DNA levels early in

Table 2 Change in plasma EBV DNA levels and response to therapy in 15 cohort patients with NK/T-cell lymphoma

Patients	Age/Sex	Therapy	Plasma EBV DNA levels (copies/ml)		ρ	Response	Remarks
			Pretreatment	Posttreatment			
1	40/F	m-BACOD + HDT	107	0	−107	CR	Nasal NK/T-cell NHL,[a] stage I. CR after 3 cycles of m-BACOD. Receiving RT at the time of analysis. Remained well 9.7 months from diagnosis.
2	73/F	CHOP + RT	181	0	−181	CR	Nasal NK/T-cell NHL, stage I. CR after 3 cycles of CHOP. Remained well 22 months from diagnosis.
3	63/M	m-BACOD	289	0	−289	CR	NK/T-cell NHL of small bowel, stage I. CR after 4 cycles of m-BACOD but refused further therapy. Remained well 6.5 months from diagnosis.
4	37/M	m-BACOD + HDT + RT	308	0	−308	CR	Nasal NK/T-cell NHL, stage I. CR after 3 cycles of m-BACOD. Remained well 20.1 months from diagnosis.
5	43/M	m-BACOD + RT	1318	0	−1318	CR	Nasal NK/T-cell NHL, stage I. CR after 3 cycles of m-BACOD. Remained well 11.6 months from diagnosis.
6	59/M	m-BACOD + RT	1670	0	−1670	CR	Nasal NK/T-cell NHL, stage I. CR after 3 cycles of m-BACOD. Remained well 14.5 months from diagnosis.
7	49/M	m-BACOD + RT	8379	0	−8379	CR	Relapsed nasal NK/T-cell NHL, stage I. CR after 2 cycles of m-BACOD. Failed to yield enough stem cells for HDT. Received RT and remained well 24.3 months after diagnosis of recurrence.
8	63/M	m-BACOD	22503	250	−22253	CR	NK/T-cell NHL of right testis, stage II. CR after 3 cycles of m-BACOD. While awaiting for RT, developed recurrence in skin, associated with an increase in plasma EBV DNA of 11,476 copies/ml. Died of NHL and pneumonia 12.6 months from diagnosis.
9	66/M	m-BACOD + RT	41300	494	−40806	CR	Nasal NK/T-cell NHL, stage II. CR after 3 cycles of m-BACOD. Developed pancytopenia and increase in plasma EBV DNA of 12,618,740 copies/ml after RT. Bone marrow studies revealed RHS and NHL infiltration. Died of NHL 7.5 months from diagnosis.
10	62/M	m-BACOD	134	0	−134	PR	NK/T-cell NHL of lymph node, stage II. PR after 3 cycles of m-BACOD, receiving the 6th cycle of m-BACOD at the time of analysis. Remained well 4.5 months after diagnosis.
11	46/M	m-BACOD	29237	446479	+417242	SD	NK/T-cell of colon, stage II. SR after 4 cycles of m-BACOD. Underwent panprotocolectomy but disease progressed subsequently. Died of disease 8.1 months after initial diagnosis.
12	34/F	DHAP	66	23117	+23050	PD	Nasal NK/T-cell NHL in 1994, stage I. Local relapse in 1998, CR2 after m-BACOD and HDT. Second local relapse in 1999. Developed rapid progression after 2 cycles of DHAP, showing extension to contralateral nasal cavity, nasopharynx, and oropharynx. Received RT and demonstrated CR3 and a fall in plasma EBV DNA to 40 copies/ml. Remained well 21.5 months after 2nd recurrence.
13	82/F	Dex + RT	523	341	−182	NE	NK/T-cell NHL of skin in leg, stage IV. Patient died of sepsis shortly after commencement of RT, survived 2.7 months from diagnosis. Not evaluable for response.
14	55/M	CHOP	659	35	−624	NE	NK/T-cell NHL of small bowel, stage I. Died of sepsis after 2nd cycle of CHOP, survived 2.9 months from diagnosis. Not evaluable for response.
15	94/F	CVP	270898	55568	−215330	NE	Nasal NK/T-cell NHL, stage II. Refused further therapy after 1st cycle of CVP and subsequently died of disease 20 days after diagnosis.

[a] NHL, non-Hodgkin's lymphoma; SR, static response; HDT, high-dose therapy; CVP, cyclophosphamide, vincristine, prednisolone; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisone, dacarbazine; RHS, reactive hemophagocytic syndrome; SD, stable disease; PD, progressive disease; NE, not evaluable.
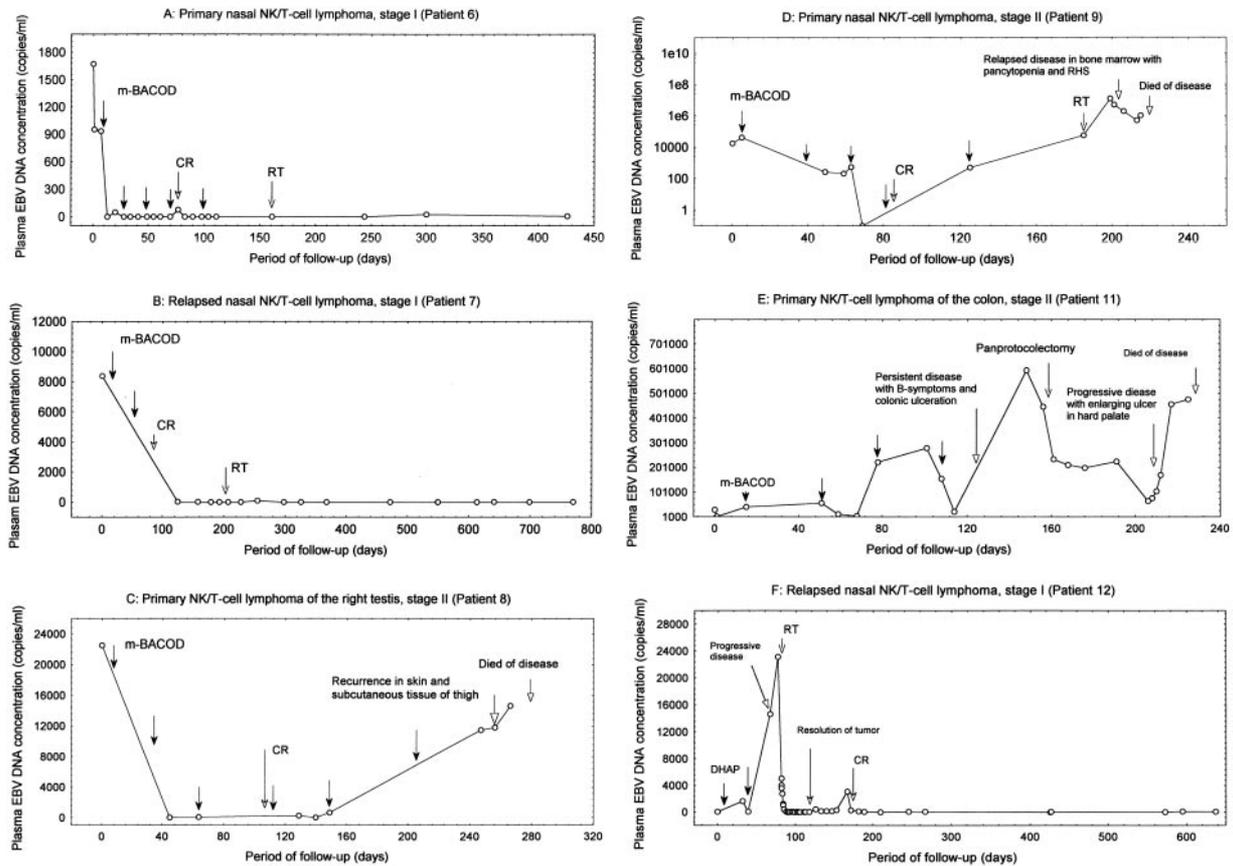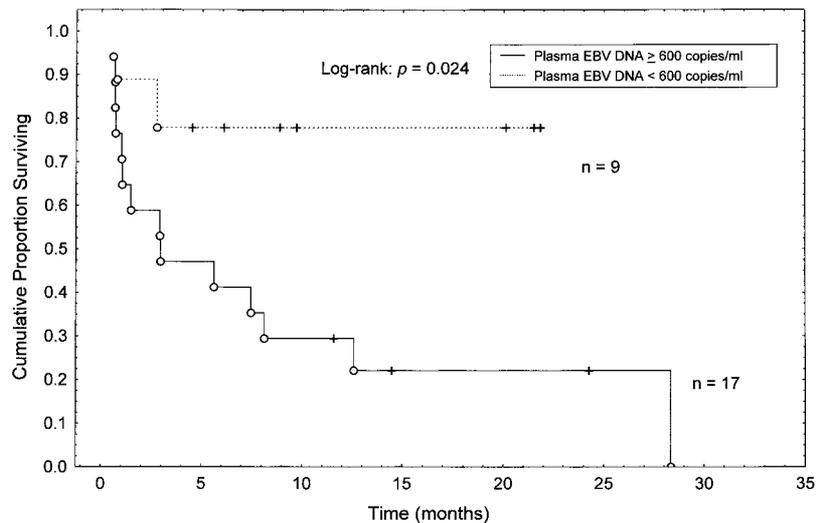
Fig. 1 Changes of plasma EBV DNA levels and therapeutic response from patients with NK/T-cell lymphoma during therapy and subsequent follow-up visits. *A,* patient with primary nasal NK/T-cell lymphoma. *B,* patient with relapsed nasal NK/T-cell lymphoma. *C,* patient with primary NK/T-cell lymphoma of right testis. *D,* patient with primary nasal NK/T-cell lymphoma. *E,* patient with primary NK/T-cell of the colon. *F,* patient with relapsed nasal NK/T-cell lymphoma. *RHS,* reactive hemophagocytic syndrome.

Fig. 2 Overall survival in 26 patients with NK/T-cell lymphoma according to baseline plasma EBV DNA levels. ○, complete data; +, censored data.

the course of therapy appears to be a strong predictor of therapeutic response. Indeed, similar observation has also been observed in patients with other EBV-associated lymphoid malignancies and that changes of plasma EBV DNA correlate well

with therapeutic response and disease status after therapy (11, 20–22) Interestingly, two patients who achieved a complete clinical response but demonstrated residual circulating EBV DNA developed early recurrence in association with a marked

increase in plasma EBV DNA concentrations. These observations suggest that circulating EBV DNA levels are related to tumor burden and can serve as an invaluable tumor marker for assessment of treatment response and disease monitoring. This clinical application will be particularly useful in the follow-up of nasal NK/T-cell lymphoma patients because postradiation or inflammatory changes after therapy often pose great difficulty in evaluation.

On the basis of age, disease stage, number of extranodal sites, performance status, and the LDH level, the IPI has been developed to predict outcome of aggressive nodal lymphomas (23). However, in the case of NK/T-cell lymphoma, the majority of patients run an aggressive course, despite their low IPI at presentation. Patients with nasal NK/T-cell lymphoma have a 5-year disease-free survival of only 25.1% (4), whereas patients with disease at other extranodal sites have a median survival of ~3.5 months (5). Thus, IPI alone is insufficient for predicting the outcome of such patients. In contrast, our results indicate that plasma EBV level at presentation is a strong predictor for outcome; patients with high baseline plasma EBV DNA levels ($\geq$600 copies/ml) had a significantly poorer overall survival than those with levels above this range. A similar prognostic impact of plasma EBV DNA has also been observed in patients with nasopharyngeal carcinoma (24). The biological explanation for the prognostic effect is unclear. It is possible that measurement of plasma EBV DNA levels provides a better estimate of tumor burden in the body than that offered by conventional staging modalities (24). Because plasma EBV DNA can be measured quickly and easily using a small amount of plasma before the start of therapy, patients with high baseline plasma EBV DNA levels can be selected for more intensive treatment strategies that may improve outcome.

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