Pretreatment EBV-DNA copy number is predictive of response and toxicities to SMILE chemotherapy for extranodal NK/T-cell lymphoma, nasal type

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Running title: EBV-DNA can predict of response/toxicity of SMILE
Category: Regular Articles, Clinical trials and observations
Word counts: Translational relevance, 142 words
Abstract, 245 words
Text, 2799 words
Figure/table counts: 3 figures; 3 tables
Reference count: 32 references
Abstract

Purpose: Extranodal NK/T-cell lymphoma, nasal type (ENKL), is an Epstein-Barr virus (EBV)-associated lymphoma for which a new chemotherapeutic regimen called SMILE (steroid, methotrexate, ifosfamide, L-asparaginase and etoposide) recently showed promising results.

Experimental design: The amount of EBV-DNA was prospectively measured in whole blood and plasma samples by real-time quantitative polymerase chain reaction from 26 patients registered in the SMILE phase II study.

Results: Before treatment, the EBV-DNA was detected in 22 samples of whole blood with a median number of 3,691 copies/mL (range: 0-1.14 x 10^7), but 15 samples of plasma with a median of 867 copies/mL (range: 0-1.27 x 10^7). Results of these 2 measurements of EBV-DNA well correlated (R^2 = 0.994, P < 0.001). The overall response rate to SMILE was significantly higher in patients with less than 10^5 copies/mL of EBV-DNA in whole blood at enrollment (90% vs. 20%, P = 0.007), and in patients with less than 10^4 copies/mL of EBV-DNA in plasma (95% vs. 29%, P = 0.002). The incidence of grade 4 toxicity of SMILE other than leukopenia/neutropenia was significantly higher in patients with 10^5 copies/mL of EBV-DNA or more in whole blood (100% vs. 35%, P = 0.007) than that of others, and in patients with 10^4 copies/mL or more in plasma (86% vs. 26%, P = 0.002).

Conclusions: These findings suggest that whole blood is more sensitive for clinical use than plasma. The EBV-DNA amount in whole blood was useful for predicting tumor response, toxicity and prognosis after SMILE chemotherapy for ENKL.
Translational relevance

Peripheral blood of patients with extranodal NK-cell lymphoma, nasal type (ENKL) contains fragmented Epstein-Barr virus (EBV)-DNA. The amount of EBV-DNA can be a good marker for estimating the tumor burden and prognosis of ENKL patients. We recently developed a novel chemotherapeutic regimen SMILE, comprising steroid, methotrexate, ifosfamide, L-asparaginase and etoposide. The tumor response rate and survival rate was dramatically improved. However, it is known that the prognostic significance of certain factors may vary when the treatment modality changes. Therefore, the significance of EBV-DNA was analyzed in this study. Consequently, pretreatment whole blood and plasma EBV-DNA were predictive of response and prognosis. Multivariate analysis showed that plasma EBV-DNA was a significant prognostic factor. Furthermore, the EBV-DNA load was also predictive of adverse events by chemotherapy. Prediction of toxicity is particularly important for the SMILE regimen because it is excessively toxic for some patients.
Introduction

Epstein-Barr virus (EBV) causes a variety of benign and neoplastic diseases, including infectious mononucleosis, post-transplantation lymphoproliferative disorder (PTLD) and EBV-associated malignancies such as lymphomas, including extranodal NK/T-cell lymphoma, nasal type (ENKL), Hodgkin lymphoma, Burkitt lymphoma, age-associated large B-cell lymphoma and several other T-cell lymphomas. ENKL is a rare subtype of non-Hodgkin lymphoma, mainly occurs in the nasal/paranasal area, skin or gastrointestinal tract, and is much more common in Asia and Latin America than in Western countries. The prognosis of ENKL was poor under conventional radiotherapy and/or chemotherapy, but has recently improved by concurrent chemoradiotherapy or newly developed SMILE chemotherapy, comprising the steroid dexamethasone, methotrexate, ifosfamide, L-asparaginase and etoposide.

This type of lymphoma is invariably associated with episomal infection of EBV in the tumor cells, which implies its tumorigenic role. The presence of EBV-DNA in peripheral blood has been used as a surrogate marker for estimating tumor amount in several EBV-associated malignancies. In particular, after organ transplantation, increasing loads of EBV in whole blood, lymphocytes and plasma are associated with corresponding increases in the risk of PTLD. For nasopharyngeal carcinoma (NPC), plasma EBV-DNA load is known to be useful for monitoring disease activity and predicting the outcome of treatment. The disease activity and prognosis of ENKL can also be monitored by measuring circulating EBV-DNA in plasma. For patients registered to the SMILE phase II study, we simultaneously conducted a prospective research.
observational study, SMILE-EBV study, in which the amounts of EBV-DNA in whole blood and plasma were evaluated for ENKL.

Methods

Study design

The aim of this study is to evaluate the copy numbers of EBV-DNA from whole blood and plasma in patients with ENKL who received SMILE chemotherapy. The predictive value of EBV-DNA for tumor response, toxicity and prognosis were analyzed, as well as the preference of samples from whole blood or plasma. The eligibility criteria, treatment and response were described in the report of the phase II study 11. EBV-encoded small RNA (EBER) in situ hybridization positivity was counted in accordance with our previous study 18. A total of 38 patients were enrolled: 26 from Japan, 6 from Hong Kong and 6 from South Korea. The amounts of EBV-DNA were measured in whole blood and plasma samples from patients participating in this phase II study at 3 time points: before the treatment, after two courses of SMILE and after a series of treatments. Because of the lack of an international standardized method for quantification of EBV-DNA, the 26 patients from Japan were the subjects for this study. All samples were measured in a central laboratory. Registration onto the study was conducted by facsimile from the participating institutes to the C-SHOT Data Center (Nagoya, Japan), simultaneously with the entry into the SMILE study. The study was approved by both the Protocol Review Committee and the institutional review board of each institution in Japan. Written informed consent was obtained from all of the patients.
The study was registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR number, UMIN000000741), as an associated but separate study of SMILE phase II (UMIN-CTR number, UMIN000000712).

**Response and toxicity criteria**

CR was defined as the complete disappearance of all objective signs of disease, including enlarged lymph nodes or hepatomegaly and splenomegaly at re-staging. Partial response (PR) was defined as at least a 50% reduction of tumor volume without the occurrence of new lesions at re-staging. Progressive disease (PD) was defined as a greater than 25% increase in the sum of tumor lesions or the emergence of one or more new lesions or clinical symptoms that indicate disease progression. No response (NR) was defined as any response that did not fall into the categories defined above. If a patient died of any cause before day 42 of the second course of SMILE and could not undergo the defined re-staging procedure, the patient’s response was recorded as early death (ED). The overall response rate (ORR) was defined as the proportion of all patients who could be evaluated for response who experienced CR or PR. Toxicity was graded according to the Common Terminology Criteria for Adverse Events v3.0.

**Quantification of EBV-DNA**

A 5-ml patient peripheral blood was obtained, sent to the central laboratory (Nagoya University Graduate School of Medicine), and divided into whole blood and plasma samples. DNA was extracted from 200 μL of either whole blood or plasma, using
QIAamp DNA blood kits (QIAGEN K.K., Tokyo, Japan). A real-time quantitative polymerase chain reaction (PCR) assay was performed and the result was expressed as copies per 1 ml of sample, as previously described\textsuperscript{19,20}. The minimum detection level was 2 copies per reaction, which was equivalent to 100 copies/mL for whole blood or plasma.

**Statistical analysis**

Regression analysis compared the copy numbers in whole blood and plasma. Fisher’s exact test was used to compare the responses or toxicities to the SMILE chemotherapy. Mann-Whitney’s $U$ test and Kruskal-Wallis test were used to compare the levels of EBV-DNA between patient groups. Cutoff value of the categorization by EBV-DNA levels were determined by the receiver operating characteristic (ROC) analysis. Patient survival data were analyzed by the method of Kaplan and Meier, and were compared by log-rank test. Univariate and multivariate analyses were performed using Cox proportional hazard model. Data were analyzed with STATA version 11 (College Station, TX) and SPSS (SPSS, Somers, NY) software.

**Results**

**Patient characteristics**

The baseline characteristics of 26 eligible patients are listed in Table 1. The median age was 46.5 (range: 17-67) years, and the male:female ratio was 14:12. Twelve patients (46%) had newly diagnosed stage IV disease, 11 were in first relapse, and 3 were in the
primary refractory status. EBER in situ hybridization was positive in all specimens, with a median positivity of 68% (range: 12% to 96%) of lymphoma cells.

**Amount of EBV-DNA and correlation between whole blood and plasma**

EBV-DNA was detected in 22 samples of whole blood (median: $3.7 \times 10^3$, range: $0 - 1.1 \times 10^7$ copies/mL) and 15 samples of plasma (median: $8.7 \times 10^2$, range: $0 - 1.3 \times 10^7$ copies/mL). The level of EBV-DNA was not different among the 3 disease state (newly-diagnosed, relapsed or refractory) groups at enrollment both in whole blood ($P = 0.19$ by Kruskal-Wallis test) and in whole blood ($P = 0.22$). An inconsistent result was seen in 9 patients. EBV-DNA was positive in whole blood but was negative in plasma in 8 patients. Conversely, in another patient, the EBV-DNA was only detected in plasma. EBV-DNA was not detected in either whole blood or plasma in 3 patients (nos. 9, 23 and 25). The concordance rate between whole blood and plasma was 65% (17/26). The viral DNA copy numbers were compared between whole blood and plasma before SMILE chemotherapy. A strong correlation was found between the amounts in whole blood and those in plasma ($r = 0.997$, $P < 0.001$, Figure 1). No differences were found for the EBV-DNA level among patients with newly diagnosed stage IV, relapsed and refractory status ($P = 0.19$ for whole blood, and $P = 0.24$ for plasma). No significant correlation was found between EBER positivity and plasma or whole blood EBV-DNA level (Supplemental Figure S1).

**Dynamic changes of EBV loads in whole blood and plasma before and after**
**treatment**

EBV loads in whole blood or plasma from the 16 patients (8 with CR, 7 with PR and 1 with PD) were measured before the treatment, after two courses of SMILE chemotherapy and after a series of treatments. Viral load declined in most patients with CR or PR after two courses of SMILE chemotherapy and/or after a series of treatments (Figure 2). However, 5 patients with CR or PR did not show the decrease of viral load. Of these, 2 patients experienced disease recurrence, and another patient died of transplant-related mortality in CR. Other 2 patients maintained response at the time of last follow-up.

**Correlation of the amount of EBV-DNA in blood samples and response or toxicities to the therapy**

Among the 26 patients, there were 12 patients with CR, 8 with PR, 1 with NR, 3 with PD and 2 with ED (Table 2), and the ORR was 77%. For patients with less than $10^5$ copies/mL of EBV-DNA in whole blood, the ORR was 90% (19/21), but was 20% (1/5) in patients with $10^5$ copies/mL or more ($P=0.005$). In addition, the ORR was 95% (18/19) in patients with less than $10^4$ copies/mL of EBV-DNA in plasma, but was 29% (2/7) in patients with $10^5$ copies/mL or more ($P=0.002$). All 3 patients without detectable EBV-DNA in either whole blood or plasma attained CR. The amounts of EBV-DNA before treatment were not significantly different between patients with CR and those with PR (whole blood, $P=0.82$; plasma, $P=0.68$).

Grade 4 leukopenia (77%) and neutropenia (88%) were commonly observed.
Grade 4 anemia was encountered in one patient and thrombocytopenia was seen in 9 patients. The non-hematologic grade 4 toxicities included infection (n = 2), alanine aminotransferase elevation (n=1) and encephalopathy (n=1); three patients experienced grade 4 somnolence, which was complicated by a grade 3 infection in one patient and by grade 4 encephalopathy in another patient. One patient experienced grade 2 pancreatitis and had complications from grade 4 hyponatremia, hyperamylasemia and appetite loss. Grade 4 toxicity other than leukopenia/neutropenia was significantly higher in patients with $10^5$ copies/mL of EBV-DNA or more in whole blood (100% vs. 35%, $P=0.007$). Grade 4 toxicity other than leukopenia/neutropenia was also significantly higher in patients with $10^4$ copies/mL of EBV-DNA or more in plasma (86% vs. 26%, $P=0.002$) (Table 2).

**Prognostic significance of EBV-DNA**

Patients with $10^5$ copies/mL of EBV-DNA or more in whole blood showed significantly lower survival than those with less than $10^5$ copies/mL (Figure 3A, $P < 0.0001$). Similarly, the prognosis of patients with $10^4$ copies/mL of EBV-DNA or more in plasma was significantly worse than that in those with less than $10^4$ copies/mL (Figure 3B, $P < 0.0001$). EBER positivity of more than 75% was also a factor associated with poor prognosis (Figure 3C). Plasma and whole blood EBV-DNA before SMILE chemotherapy were significant prognostic factors for overall survival by univariate analysis, as well as serum lactate dehydrogenase (LDH) elevation, B symptom and EBER positivity (Table 3). Multivariate analysis showed that LDH elevation [hazard
ratio (HR): 8.5, 95% confidence interval (CI): 1.9-38.0] and pretreatment whole blood EBV-DNA (HR: 65.5, 95% CI: 5.3-813.7) were significant prognostic factors. Plasma EBV-DNA was not prognostic (HR: 3.90, 95% CI: 0.70-21.8) if adjusted by LDH elevation using multivariate analysis. EBER positivity showed marginal significance (HR: 3.3, 95% CI: 0.95-11.8) if included in the model with LDH elevation.

**Discussion**

For EBV-associated malignancies, the significance placed on EBV-DNA in peripheral blood as a biomarker has increased in recent decades. Previous studies have reported that the level of EBV-DNA in a peripheral blood compartment is a useful biomarker in EBV-associated malignancies. Lei et al. found a significant reduction of plasma EBV-DNA in patients with EBV-associated lymphoid malignancies (Hodgkin lymphoma, nasal NK/T cell lymphoma, PTLD and Burkitt lymphoma), during the course of effective therapy. In addition, disease progression was associated with a rapid increase in plasma EBV-DNA levels in patients with ineffective therapy. Gandhi et al. showed that EBV-DNA is specifically detected in plasma of EBV-positive Hodgkin lymphoma patients before treatment. Viral DNA was undetectable following therapy in responsive patients and patients with long-term remission. Patients who experienced relapse had a significantly higher plasma EBV-DNA concentration before treatment. The plasma DNA concentration was persistently low or undetectable in patients with complete clinical remission. Overall survival and relapse-free survival were significantly higher for patients with a pretreatment plasma EBV-DNA level of less than
1500 copies/mL. Au et al. reported that plasma EBV-DNA level is valuable as a tumor biomarker and for prognostication in EBV-positive lymphoma. EBV-DNA in plasma became undetectable for patients in remission, but was elevated for those with refractory disease. A high level of EBV-DNA was significantly associated with inferior overall survival by multivariate analysis. Subgroup analysis of NK cell lymphoma showed that the level of EBV-DNA was also correlated with disease stage. Presentation of a high level of EBV-DNA was also significantly associated with inferior overall survival by multivariate analysis in their cohort. Prognostic factors of lymphoma may change when the treatment modality changes. In the present study, however, EBV-DNA copy number in plasma or whole blood was also predictive of response and survival of ENKL patients who received SMILE chemotherapy, in agreement with other observations in the literature.

Another novel finding is that severe adverse events of the chemotherapy were also predictable using the EBV-DNA amount, which has not been identified by other studies in the literature. This analysis is only possible by examining patients who receive exactly the same treatment, ideally subjects of prospective studies. Because the level of EBV-DNA was not different by the 3 disease status groups (newly-diagnosed, relapsed or refractory), we examined the patients together in this study. As an interpretation of this finding, patients with higher tumor burden may experience more severe toxicity due to poor general condition or tissue damage by the tumor. Another hypothesis is that the toxicity by chemotherapy is mediated by certain toxic substances in tumor cells. Since NK cells possess cytotoxic activities, almost all ENKL have
cytotoxic molecules such as perforin or granzymes. In several EBV-associated malignancies, the high viral load may be explained by the tumor releasing viral components. Toxic substances that are released from tumor cells degraded by chemotherapy like SMILE, although they may not be cytotoxic molecules, may contribute to the high rate of adverse reactions after chemotherapy. Whatever the reason, measurement of EBV-DNA may be helpful for patient stratification to avoid excessive toxicity because the myelosuppressive adverse reaction of SMILE is rather profound for a part of patients.

Plasma is used as samples in most studies for evaluating EBV-DNA as a biomarker in EBV-associated disease. However, controversies exist as to which blood compartment should be used for measuring EBV because several compartments of blood, whole blood, peripheral blood mononuclear cells, plasma and serum can be used in the studies. Our previous study compared the usefulness of plasma and mononuclear cells for detecting EBV-DNA in ENKL patients, although the treatment was not unified. For the diagnosis of EBV-associated PTLD, earlier studies used peripheral blood mononuclear cells because EBV infection occurs in this cell compartment. Plasma or serum samples are readily obtained and widely used for diagnosing EBV-associated PTLD; however, the sensitivity appeared to be low. Several reports have revealed that whole blood, containing both cellular and humoral compartments, is better than plasma/serum when testing patients with PTLD. Recently, Spacek et al. reported that plasma is better than whole blood for the monitoring and estimation of prognosis for Hodgkin lymphoma. Plasma samples may
be recommended as a biomarker of disease activity rather than peripheral blood mononuclear cells in patients with Hodgkin lymphoma, as shown in another study. However, comparison among each blood compartment has not been well investigated. Useful compartments may differ among diseases. In the present study, the levels of EBV-DNA in plasma were compared with those in whole blood. Although strong correlation was detected between the viral copy numbers in whole blood and those in plasma, EBV-DNA was more frequently detected in whole blood samples before treatment. Notably, EBV-DNA was only detected in whole blood in 8 patients, while it was only positive in plasma in one patient. This suggests that whole blood is more suitable than plasma to examine the EBV-DNA for ENKL. The reason for the phenomenon that EBV-DNA was only detected in whole blood remains undetermined. Among such 8 patients in this study, only 4 patients showed bone marrow involvement, and none accompanied leukemic presentation. Only the possible explanation is that EBV-DNA might be lost or degraded in the fractionation procedure. Another point of interest is that EBV-DNA was not detected in either whole blood or plasma in 3 patients, although EBER was positive in tissue samples. Therefore, EBV-DNA detection in peripheral blood cannot be used as an alternative to the histological detection of EBV or the diagnosis of ENKL. Moreover, the levels of $10^5$ copies/mL of EBV-DNA in whole blood and $10^4$ copies/mL of EBV-DNA in plasma appear to be cut-off values: the patients with copy numbers lower than these showed significantly better outcome. These two copy numbers also showed clinical value to predict severe adverse events.

In conclusion, our study indicates that the level of EBV-DNA in plasma or whole
blood can predict response and adverse events of SMILE chemotherapy for newly diagnosed stage IV, relapsed or refractory ENKL. Whole blood samples were more suitable for this purpose, although plasma was preferable for other purposes such as diagnosis of EBV infection.

Acknowledgements

The authors would like to thank staff at all participating institutions in this study: Tokyo Medical and Dental University, Kanagawa Cancer Center, Yokohama City University, Okayama University, Obihiro Kosei Hospital, Saga University, Yamanashi University, Shinshu University, NTT Medical Center Tokyo, Fukushima Prefectural Medical College, Kurashiki Central Hospital, Niigata University, Kyushu Medical Center, Nagano Red Cross Hospital, Tsukuba University and Juntendo University.

We thank the members of Central Pathology Review Board (Drs. Koichi Ohshima at Kurume University and Kengo Takeuchi at Cancer Institute), Central Imaging Review Board (Drs. Takao Kodama and Takanori Yano at Miyazaki University, and Yosuke Kakitsubata at Miyazaki Konan Hospital), and Data and Safety Monitoring Committee (Drs. Jin Takeuchi at Nihon University, Keizo Horibe at Nagoya Medical Center, and Keitaro Matsuo at Aichi Cancer Center). We also thank Ms. Fumiyo Ando for excellent technical support for real-time quantitative PCR assay.

Authorship

Contributions: Y. Ito and H. Kimura performed experiments and wrote the manuscript;
K.I., M.Y., J.S., K.K., K.O and R.S. designed the study and wrote the manuscript; R.H. and R.S. collected data and undertook statistical analysis; Y.M., C.H., F.I., K.I., E.S., Y. Isobe, J.T., Y.H. Hajime Kobayashi., S.O. and Hikaru Kobayashi prepared patient data; S.N. conducted central pathological review, and all authors participated in interpretation of data and approval of the final manuscript.

Conflict-of-interest disclosure:

RS received honoraria from Kyowa-Hakko Kirin Company. KO is currently an employee of Eisai Pharmaceutical Co., Ltd. (Tokyo, Japan). The other authors declare that they have no potential conflicts of interest.
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patients. APMIS. 2010;119(1):10-16.
Table 1. Baseline patient characteristics (N = 26)

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<th>Characteristic</th>
<th>No. of Patients</th>
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<td>Female</td>
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<td>46</td>
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<td><strong>Disease state</strong></td>
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<td>46</td>
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<td>2</td>
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<td>19</td>
</tr>
<tr>
<td><strong>Detection of EBV-DNA in blood samples</strong></td>
<td></td>
<td></td>
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<tr>
<td>before treatment</td>
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<tr>
<td>Whole blood +, Plasma +</td>
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</tr>
<tr>
<td>Whole blood +, Plasma -</td>
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<td>31</td>
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<td>4</td>
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<tr>
<td>Whole blood -, Plasma -</td>
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<td>12</td>
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Abbreviation: LDH, lactate dehydrogenase
Table 2. Correlation of the levels of EBV-DNA and response/adverse events to SMILE chemotherapy for newly diagnosed stage IV, relapsed or refractory extranodal NK/T-cell lymphoma, nasal type

<table>
<thead>
<tr>
<th>Whole blood EBV-DNA</th>
<th>Plasma EBV-DNA (copies/mL)</th>
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<tbody>
<tr>
<td>≥ 10^5 copies/mL</td>
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<td>&lt; 10^5 copies/mL</td>
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<td>Response</td>
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</tr>
<tr>
<td></td>
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<td></td>
<td>0.007</td>
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<td>No grade 4</td>
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<td>0.007</td>
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*Grade 4 adverse events other than leukopenia and neutropenia.
Table 3. Prognostic factors affecting overall survival

<table>
<thead>
<tr>
<th>Variables</th>
<th>Unfavorable factors</th>
<th>Univariate</th>
<th>Multivariate *</th>
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<td>Hazard ratio (CI)</td>
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<tr>
<td>LDH level</td>
<td>Elevated</td>
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<td>B symptom</td>
<td>Present</td>
<td>5.0 (1.3-19.0)</td>
<td>0.02</td>
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<tr>
<td>WB EBV-DNA</td>
<td>≥ 10^5 copies/mL</td>
<td>53.2 (5.9-482.0)</td>
<td>&lt; 0.001</td>
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<tr>
<td>Plasma EBV-DNA</td>
<td>≥ 10^4 copies/mL</td>
<td>10.3 (2.9-36.3)</td>
<td>&lt; 0.001</td>
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<tr>
<td>EBER &gt; 75%</td>
<td>4.0 (1.2-13.7)</td>
<td>0.03</td>
<td>-</td>
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</tbody>
</table>

Abbreviations: LDH, lactate dehydrogenase; EBV, Epstein-Barr virus; WB, whole blood; CI, confidence interval

*: Final model
Figure legends

Figure 1. Comparison of EBV-DNA copies between whole blood and plasma in patients with extranodal NK/T-cell lymphoma, nasal type, who received SMILE chemotherapy

The EBV-DNA concentrations in whole blood or in plasma from the patients were measured using real-time PCR assay before SMILE chemotherapy. Dotted lines show the detection limits indicating 100 copies/mL of plasma or whole blood.

Figure 2. Serial analysis of EBV loads in blood samples from the patients with extranodal NK/T-cell lymphoma, nasal type

The EBV-DNA concentrations in whole blood or in plasma from the patients were measured using real-time PCR assay before SMILE chemotherapy, after two courses of SMILE chemotherapy and after a series of treatments. (A) Viral loads in whole blood in patients with complete response (CR). (B) Viral loads in plasma in patients with CR. (C) Viral loads in whole blood in patients with partial response (PR). (D) Viral loads in plasma in patients with PR. Dotted lines show the detection limits indicating 100 copies/mL of plasma or whole blood.

Figure 3. Survival of patients with extranodal NK/T-cell lymphoma, nasal type, who received SMILE chemotherapy by EBV parameters

(A) Overall survival was significantly lower for patients with a whole blood EBV-DNA
level of $10^5$ copies/mL or more ($P < 0.0001$). (B) Overall survival was significantly lower for patients with a plasma EBV-DNA level of $10^4$ copies/mL or more ($P < 0.0001$). (C) Overall survival was significantly lower for patients with EBER positivity of more than 75% ($P = 0.02$).
Below detection limit

Plasma EBV DNA [copies/mL]

Whole blood EBV DNA [copies/mL]

R² = 0.994
P < 0.001

Figure 1
Figure 2
**Figure 3**

(A)

Whole blood

- EBV-DNA < $10^5$ copies/mL
- EBV-DNA > $10^5$ copies/mL

(B)

Plasma

- EBV-DNA < $10^4$ copies/mL
- EBV-DNA > $10^4$ copies/mL

(C)

EBER

- EBER ≤ 75%
- EBER > 75%

(months)
Pretreatment EBV-DNA copy number is predictive of response and toxicities to SMILE chemotherapy for extranodal NK/T-cell lymphoma, nasal type


Cancer Res  Published OnlineFirst June 6, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-12-1064

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2012/06/06/1078-0432.CCR-12-1064.DC1

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