Pretreatment EBV-DNA Copy Number Is Predictive of Response and Toxicities to SMILE Chemotherapy for Extranodal NK/T-cell Lymphoma, Nasal Type


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 PCR 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 × 10⁷), but 15 samples of plasma with a median of 867 copies/mL (range: 0–1.27 × 10⁷). Results of these 2 measurements of EBV-DNA well correlated (R² = 0.994, P < 0.001). The overall response rate to SMILE was significantly higher in patients with less than 10⁵ copies/mL of EBV-DNA in whole blood at enrollment (90% vs. 29%, P = 0.007) and in patients with less than 10⁴ 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 10⁵ copies/mL of EBV-DNA or more in whole blood (100% vs. 29%, P = 0.007) than that of others and in patients with 10⁴ 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. Clin Cancer Res; 18(15); 4183–90. ©2012 AACR.

Introduction

Epstein–Barr virus (EBV) causes a variety of benign and neoplastic diseases, including infectious mononucleosis, posttransplantation 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 (1–3). ENKL is a rare subtype of non-Hodgkin lymphoma, mainly occurs in the nasal/paranasal area, skin, and Latin America than in Western countries (4–6). The prognosis of ENKL was poor under conventional radiotherapy and/or chemotherapy (4, 7) but has recently improved by concurrent chemoradiotherapy (8, 9) or newly developed SMILE chemotherapy, comprising the steroid dexamethasone, methotrexate, ifosfamide, L-asparaginase, and etoposide (10, 11).

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.
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.

Materials and 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 was 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 useful for monitoring disease activity and predicting the outcome of treatment (15, 16). The disease activity and prognosis of ENKL can also be monitored by measuring circulating EBV-DNA in plasma (17). For patients registered to the SMILE phase II study, we simultaneously conducted a prospective observational study, SMILE-EBV study, in which the amounts of EBV-DNA in whole blood and plasma were evaluated for ENKL.

Statistical analysis

Regression analysis compared the copy numbers in whole blood and plasma. Fisher exact test was used to compare the responses or toxicities to the SMILE chemotherapy. Mann–Whitney U test and Kruskal–Wallis test were used to compare the levels of EBV-DNA between patient groups. Cut-off value of the categorization by EBV-DNA levels were determined by the receiver operating characteristic 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 carried out using Cox proportional hazard model. Data were analyzed with STATA version 11 and SPSS (SPSS) software.

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.). A real-time quantitative PCR assay was carried out and the result was expressed as copies per 1 mL of sample, as previously described (19, 20). The minimum detection level was 2 copies per reaction that was equivalent to 100 copies/mL for whole blood or plasma.
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%–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: $3.7 \times 10^4$, range: $0–1.1 \times 10^5$ copies/mL) and 15 samples of plasma (median: $8.7 \times 10^4$, range: $0–1.3 \times 10^7$ copies/mL). The level of EBV-DNA was not different among the 3 disease stages (newly diagnosed, relapsed or refractory) 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 of 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 ($r = 0.997$, $P < 0.001$, Fig. 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 (Supplementary Fig. 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 complete response, 7 with partial response, and 1 with progressive disease) were measured before the treatment, after 2 courses of SMILE chemotherapy, and after a series of treatments. Viral load declined in most patients with complete response or partial response after 2 courses of SMILE chemotherapy and/or after a series of treatments. Viral load declined in most patients with complete response or partial response after 2 courses of SMILE chemotherapy and/or after a series of treatments (Fig. 2). However, 5 patients with complete or partial response did not show the decrease of viral load. Of these, 2 patients experienced disease recurrence, and another patient died of transplant-related mortality in complete response. 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 complete response, 8 with partial response, 1 with no response, 3 with progressive disease, and 2 with early death (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 of 21), but was 20% (1 of 5) in patients with 10^5 copies/mL or more (P = 0.005). In addition, the ORR was 95% (18 of 19) in patients with less than 10^4 copies/mL of EBV-DNA in plasma, but was 29% (2 of 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 complete response. The amounts of EBV-DNA before treatment were not significantly different between patients with complete response and those with partial response (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 nonhematologic grade 4 toxicities included infection (n = 2), alanine aminotransferase elevation (n = 1), and encephalopathy (n = 1); 3 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. 29%, 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 (Fig. 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 (Fig. 3B, P < 0.0001). EBER positivity of more than 75% was also a factor

---

**Table 2.** Correlation of the levels of EBV-DNA and response/adverse events to SMILE chemotherapy for newly diagnosed stage IV, relapsed or refractory ENKL

<table>
<thead>
<tr>
<th>Whole blood EBV-DNA</th>
<th>Plasma EBV-DNA (copies/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;10^5 copies/mL</td>
<td>&lt;10^5 copies/mL P</td>
</tr>
<tr>
<td>Response</td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>1</td>
</tr>
<tr>
<td>PR</td>
<td>0</td>
</tr>
<tr>
<td>NR</td>
<td>0</td>
</tr>
<tr>
<td>PD</td>
<td>2</td>
</tr>
<tr>
<td>ED</td>
<td>2</td>
</tr>
<tr>
<td>Adverse event</td>
<td></td>
</tr>
<tr>
<td>Any grade 4^a</td>
<td>5</td>
</tr>
<tr>
<td>No grade 4</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: CR, complete response; ED, early death; PD, progressive disease; PR, partial response; NR, No response.

^aGrade 4 adverse events other than leukopenia and neutropenia.
associated with poor prognosis (Fig. 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 [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 (14, 21). Lei and colleagues 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 (21). In addition, disease progression was associated with a rapid increase in plasma EBV-DNA levels in patients with ineffective therapy. Gandhi and colleagues showed that EBV-DNA is specifically detected in plasma of EBV-positive Hodgkin lymphoma patients before treatment (22). 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 1,500 copies/mL. Au and colleagues reported that plasma EBV-DNA level is valuable as a tumor biomarker and for prognostication in EBV-positive lymphoma (17). 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 (23). In this 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 because of poor general condition or tissue damage by the tumor. Another hypothesis is that the
Clinical Cancer Research

Table 3. Prognostic factors affecting overall survival

<table>
<thead>
<tr>
<th>Variables</th>
<th>Unfavorable factors</th>
<th>Hazard ratio (CI)</th>
<th>P</th>
<th>Hazard ratio (CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>&gt;50 years</td>
<td>0.5 (0.2–1.9)</td>
<td>0.33</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>LDH level</td>
<td>Elevated</td>
<td>8.6 (2.4–30.4)</td>
<td>0.001</td>
<td>8.5 (1.9–38.1)</td>
<td>0.005</td>
</tr>
<tr>
<td>B symptom</td>
<td>Present</td>
<td>5.0 (1.3–19.0)</td>
<td>0.02</td>
<td>–</td>
<td>–</td>
</tr>
<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>
<td>66.5 (5.3–813.7)</td>
<td>0.001</td>
</tr>
<tr>
<td>Plasma EBV-DNA</td>
<td>≥10^5 copies/mL</td>
<td>10.3 (2.9–36.3)</td>
<td>&lt;0.001</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EBER</td>
<td>&gt;75%</td>
<td>4.0 (1.2–13.7)</td>
<td>0.03</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Final model.

Disclosure of Potential Conflicts of Interest

R. Suzuki received honoraria from Kyowa-Hakko Kirin Company. K. Oshimi is currently an employee of Eisai Pharmaceutical Co., Ltd. (Tokyo, Japan). No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: M. Yamaguchi, J. Suzumiya, K. Kawa, K. Oshimi, R. Suzuki
Development of methodology: H.Kimura, K. Oshimi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Ito, Y. Maeda, C. Hashimoto, F. Ishida, K. Izutsu, N. Fukushima, Y. Isobe, Y. Hasegawa, S. Okamura, H. Kobayashi, R. Hyo

Toxicity by chemotherapy is mediated by certain toxic substances in tumor cells. Because 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 (24, 25). Toxic substances that are released from tumor cells degraded by chemotherapy such as 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 (13–17, 22). 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 (16). For the diagnosis of EBV-associated PTLD, earlier studies used peripheral blood mononuclear cells because EBV infection occurs in this cell compartment (26, 27). Plasma or serum samples are readily obtained and widely used for diagnosing EBV-associated PTLD; however, the sensitivity seemed to be low (28, 29). Several reports have revealed that whole blood, containing both cellular and humoral compartments, is better than plasma/serum when testing patients with PTLD (20, 30, 31). Recently, Spacek and colleagues reported that plasma is better than whole blood for the monitoring and estimation of prognosis for Hodgkin lymphoma (32). 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 (20). However, comparison among each blood compartment has not been well investigated. Useful compartments may differ among diseases (14). In this 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, whereas 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. The only 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 histologic detection of EBV or the diagnosis of ENKL. Moreover, the levels of 10^5 copies/mL of EBV-DNA in whole blood and 10^6 copies/mL of EBV-DNA in plasma seem to be cut-off values: the patients with copy numbers lower than these showed significantly better outcome. These 2 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.

Downloaded from clincancerres.aacrjournals.org on April 14, 2017. © 2012 American Association for Cancer Research.
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Ito, F. Ishida, M. Yamaguchi, K. Oshimi, R. Suzuki

Writing, review, and/or revision of the manuscript: Y. Ito, H. Kimura, Y. Ito, K. Kawa, K. Oshimi, R. Suzuki

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Ito, H. Kimura, J. Takizawa, S. Nakamura, K. Oshimi, R. Suzuki

Study supervision: K. Kawa, K. Oshimi, R. Suzuki

Acknowledgments

The authors thank staff at all participating institutions in this study: Tokyo Medical and Dental University, Kanagawa Cancer Center, Yokohama City University, Okayama University, Shizuoka University, Gunma 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, Tuskuba University, and Juntendo University.

The authors also thank the members of Central Pathology Review Board (Drs. Koichi Oshimia at Kumamoto University and Kengo Takahashi at Canary 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 Takachib at Nihon University, Keizo Hohrie at Nagoya Medical Center, and Keiitro Matsu at Aichi Cancer Center) and Ms. Fumiyto Ando for excellent technical support for real-time quantitative PCR assay.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 30, 2012; revised May 16, 2012; accepted May 17, 2012; published OnlineFirst June 6, 2012.

References


27. Riddler SA, Breinig MC, Mc Knight JL. Increased levels of circulating Epstein-Barr virus (EBV)-infected lymphocytes and decreased EBV nuclear antigen antibody responses are associated with the development of posttransplant lymphoproliferative disease in solid-organ transplant recipients. Blood 1994;84:972–84.

PCR and in peripheral blood lymphocytes by competitive PCR. J Clin Microbiol 2003;41:5245–9.


Pretreatment EBV-DNA Copy Number Is Predictive of Response and Toxicities to SMILE Chemotherapy for Extranodal NK/T-cell Lymphoma, Nasal Type


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

Cited articles
This article cites 30 articles, 21 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/18/15/4183.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/18/15/4183.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.