Bone marrow that is positive for Epstein-Barr virus encoded RNA-1 by *in situ* hybridization is related with a poor prognosis in patients with extranodal natural killer/T-cell lymphoma, nasal type

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Background and Objectives. Extranodal NK/T-cell lymphoma, nasal type is an aggressive lymphoma that is always associated with Epstein-Barr virus (EBV). This study was done to evaluate the use of EBV-encoded RNA-1 *in situ* hybridization (EBER-1 ISH) to detect occult micrometastasis in the bone marrow (BM) of patients with nasal NK/T-cell lymphoma.

Design and Methods. A total of 23 patients who underwent BM biopsy for routine pre-therapeutic evaluation were enrolled in the study. We used EBER-1 ISH to investigate the expression of EBER-1 in 30 BM specimens. The clinical correlation and therapeutic outcomes of these patients were analyzed. In addition, genomic analysis of EBV was performed in five patients.

Results. Conventional morphologic examinations failed to identify any lymphoma involvement in the 23 BM specimens obtained at initial staging. However, 10 of the 23 BM were positive for EBER-1. A lower survival rate was seen in patients with BM positive for EBER-1. Only the BM EBER-1 ISH result was shown to be an independent variable predicting overall survival in stage I and II patients (p=0.027; hazard ratio for death 0.066, 95% confidence interval, 0.006 to 0.733), suggesting that EBER-1 positivity in BM is the major determinant of a poor prognosis. However, discrepancies in the EBV strains between the primary tumor and BM existed in two of the five studied patients.

Interpretations and Conclusions. We suggest that EBER-1 ISH should be performed on BM specimens of patients with nasal NK/T-cell lymphoma to identify the presence of EBER-1 positive cells, which appears to carry a poor prognosis. Whether or not the EBER-1 positive cells in the BM of nasal NK/T-cell lymphoma patients are true tumor cells requires further study.

Key words: nasal NK/T-cell lymphoma, *in situ* hybridization, bone marrow, Epstein-Barr virus.

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Extranodal NK/T-cell lymphoma, nasal type is an aggressive lymphoma that frequently involves the midline facial structures. Alhough the majority of patients present with locoregional disease, less than 50% of these patients can be cured of the disease.[1-2] Compared with other types of lymphoma, there is a lack of good parameters to predict the outcome of this disease. Locoregional relapse is a major problem, but systemic failure has also been frequently encountered.[3-4] Thus, occult early dissemination of the lymphoma cells should be considered. According to the current concept, nasal NK/T-cell lymphoma is almost always associated with Epstein-Barr virus (EBV).[5-6] *In situ* localization of EBV encoded-RNA (EBER) has been shown to be a highly sensitive and specific technique for demonstrating EBV infection, and can be applied to archival histological material.[7-8] In order to evaluate the usefulness of EBER-1 *in situ* hybridization (ISH) in detecting minimal bone marrow (BM) involvement and to analyze the prognostic significance of finding such involvement, we retrospectively re-examined the results of EBER-1 ISH in the BM biopsies of 23 patients with nasal NK/T-cell lymphoma. The clinical correlation and therapeutic outcomes of these patients were also analyzed.

Design and Methods

Patients

From February 1989 to June 2004, 37 cases of histologically proven nasal NK/T-cell lymphoma were recorded at National Cheng Kung University Hospital. These patients had primary tumor localized to the nasal cavity or its adjacent tissues, and a

histopathological examination showing features typical of NK/T-cell lymphoma, including an angiocentric growth pattern, NK phenotypes, and EBV positivity by EBER-1 ISH. Of the 37 cases, 23 patients who had undergone BM biopsies for lymphoma staging at the time of diagnosis were analyzed in the present study. The clinical records and imaging studies of these 23 patients were reviewed. All patients were staged according to the Ann Arbor classification. Complete staging procedures included physical examination, chest X-ray, full blood count, blood biochemistry, BM aspiration, trephine biopsy, and computed tomography of the head and neck, thorax, abdomen, and pelvis.

Treatment

Radiation was delivered using a 6-MV linear accelerator in daily fractions of 1.8-2.0 Grays (Gy), 5 days per week, for a total dose of 45-64 Gy over 5-7 weeks. The radiation fields included the primary tumor area with adequate margins. The chemotherapy regimens included CEOP (cyclophosphamide epirubicin, vincristine, and prednisolone), ProMACE-CytaBOM (prednisolone, epirubicin, cyclophosphamide, etoposide, cytarabine, bleomycin, vincristine, and methotrexate), ESHAP (etoposide, solumedrol, cytarabine, and cisplatin), DeVIC (dexamethasone, etoposide, ifosfamide, cisplatin), and weekly cisplatin.

BM specimens, morphologic evaluation, EBER-1 ISH and immunohistochemistry

All smear and trephine biopsy specimens of the BM were retrieved for review and further studies. In addition to the NK/T-cell lymphoma cases, BM specimens from patients with other diseases were also included as controls; these specimens came from patients with EBV-negative lymphoma (n=10), and non-lymphoma diseases (n=10). The specimens were fixed in 10% neutral formalin or B5 solution. After fixation for 2 to 6 hours, the specimens were decalcified for 45 to 60 minutes, using the formic acid-sodium citrate method. This decalcifying solution was composed of equal amounts of component A (50g sodium citrate [Sigma, St. Louis, MO, USA] in 250 mL distilled water) and component B (250 mL 90% formic acid [Merck, Rahway, NJ, USA] in 250 mL distilled water). The ISH study for EBER-1 was performed using polymerase chain reaction (PCR)-derived, digoxigenin-labeled DNA probes specific for EBER-1, as previously described.[9] Immunohistochemical studies with the streptavidin-biotin-peroxidase method were performed on paraffin-embedded tissues using the following markers: CD3 (PC3/188A; Dako, Denmark), CD5 (4C7; Cell Marque, Hot springs, USA), CD56 (1B6; Ventana, Tucson, USA), and latent membrane protein 1 (LMP-1; Glostrup, Dako, Denmark).The morphologic evaluation was carried out on hematoxylin-eosin-stained sections. A trephine biopsy specimen was considered positive only if it could be unequivocally diagnosed as containing neoplastic cells. An atypical large granular lymphoid cell (LGLC) in the marrow smear was characterized by a moderate amount of cytoplasm, large cytoplasmic azurophilic granules, and an irregular nucleus (Figure 1). BM was interpreted as positive for EBER-1 ISH when there were cells showing unequivocal nuclear staining with EBV oligonucleotide accompanied by nucleuar atypia, i.e., nuclei larger than small lymphocytes, and showing some irregular outline (Figure 2). Numbers of EBER-1$^+$ cells in BM were counted under a high-power field (HPF, 400X) for all section areas, and the result was expressed as an average number per HPF.

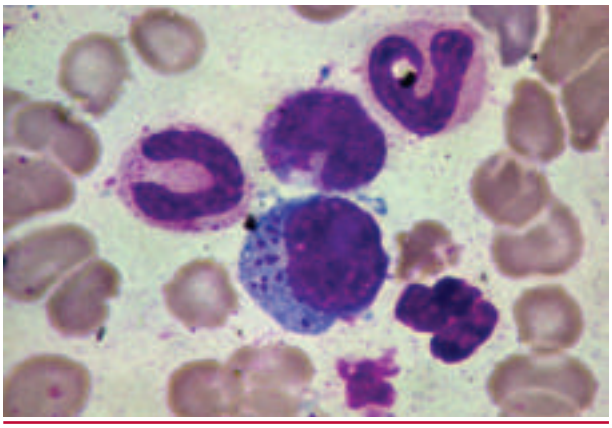

Figure 1. An atypical lymphoid cell with large azurophilic granules in a bone marrow aspirate smear from a patient with nasal NK/T-cell lymphoma.

Sample DNA preparation and analysis of the EBV subtype

Tissue DNA was extracted from specimens of nasal NK/T-cell lymphoma and the trephine biopsy of the BM. All the specimens were paraffin-embedded. Approximately 100 ng of DNA from each sample were used for each PCR reaction. To study the Ff polymorphism of EBV in these samples, the EBV BamHI F region was amplified with a pair of primers (forward primer 5'- GCCCAATGGGGTAGTAGGTT, and reverse primer 5'- TCAGGCGACAGTAACACAGG). The PCR was performed in a total volume of 50 μL, which contained 0.12 nM of both primer pairs, 100 ng of the DNA sample, and 25 μL of Hotstart Master Mix containing 2.5 U of Hotstart Taq DNA polymerase, 200 nM of each dNTP, and 1.25 mM $Mg2^+$ (Qiagen, Hilden, Germany). The amplification reaction was performed for 35 cycles in a thermocycler (Model 9700, Perkin-Elmer Corporation), under the following conditions: initially, 95°C for 15 min for activation, followed by a denaturation phase at 95°C for 30 s, an annealing phase at 55°C for 30 s, and an extension phase at 72°C for 1

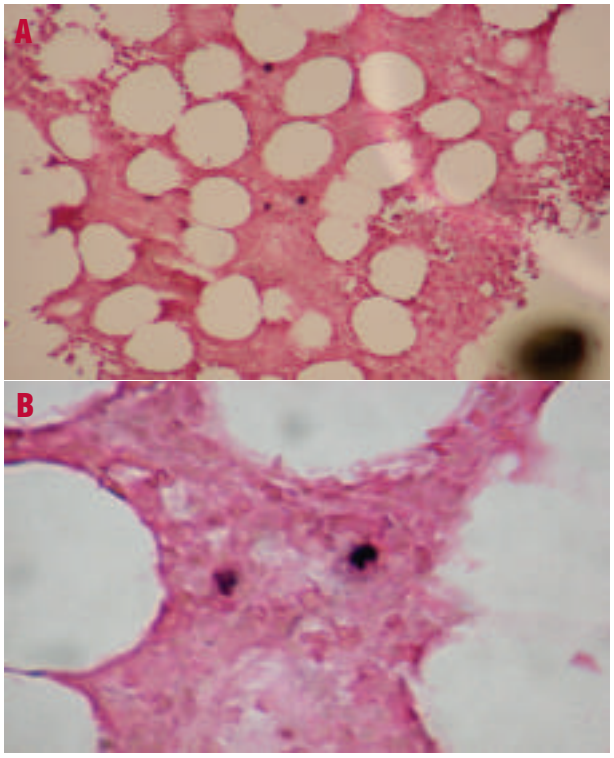

Figure 2. Bone marrow trephine biopsy specimen in a case of nasal NK/T-cell lymphoma. A. Low power view showing scattered EBER-1 positive cells. B. Some of the EBER-1 positive cells have irregular-shaped nuclei (nuclear atypia).

min. Another 10 min extension phase at 72°C was performed at the end of each round of PCR. The specific PCR product was 221-bp long. DNA from B95-8 cells was used as a positive control, and double-distilled water (ddH_2O) was used as a negative control for each reaction. After PCR, the amplified products were purified and eluted with 10 μL ddH_2O, using the MinElute PCR Purification kit (Qiagen). Two microliters of eluted DNA were then restriction-enzyme digested using 10 U BamHI at 37°C for 2 h, in a total reaction volume of 20 μL. Ten microliters of the digested products were subjected to electrophoresis (100 V, 25 min) on a 2% agarose gel stained with ethidium bromide. The presence or absence of specific PCR targets was observed under UV illumination. The f variant was recognized as featuring an extra-BamHI site in the BamHI F region. Thus, if a sample contained the f variant of EBV, the 221-bp PCR product would be digested by the BamHI enzyme into 143-bp and 78-bp fragments.

Statistical analysis

Demographic data of the subgroups were compared using the χ^2 test for categorical data, or Student's t test for continuous variables. Overall survival was measured from diagnosis to death or last follow-up. Survival curves were obtained by the Kaplan-Meier method and compared using the log rank test. Univariate and multivariate analyses were conducted using a Cox proportional-hazards model to evaluate the variables: gender, age, disease stage, B symptoms, International Prognostic Index, and bone marrow EBER-1 expression for their prognostic value in predicting survival. All p values were two-tailed, with a value of ≤ 0.05 considered to be significant.

Results

Patients and treatment modalities

There were 12 men and 11 women, with a median age of 57 years (range, 25 to 77 years). Most of the patients had early-stage disease (stage I/II, 91%; stage III/IV, 9%). The other characteristics of these 23 patients are listed in Table 1. Patients were usually treated with combined modalities if they developed B symptoms (Table 2). Eleven patients (48%) received initial treatment with radiotherapy alone, and nine patients (39%) received combined chemoradiotherapy. One patient was treated with chemotherapy only and two did not receive anti-cancer treatment because of their poor general condition.

BM findings on conventional examination

Six of the 23 patients underwent two or more BM examinations because of disease relapse or for therapeutic response evaluation. Overall, 30 BM specimens were included for study. Of the 30 BM specimens, only one obtained from a patient while she was experiencing disease relapse was positive for lymphoma involvement using the conventional histological examination. Scattered atypical LGLC were noted in the marrow blood preparations of five patients before treatment, and in another five preparations obtained from four patients while they were experiencing disease relapse. The atypical LGLC disappeared after combined chemoradiotherapy in one patient (Table 1).

EBER-1 ISH study of BM

Ten out of the 23 BM specimens (43%) obtained before treatment were positive for EBER-1, including all five bone marrow specimens that contained atypical LGLC. The numbers of EBER-1$^+$ cells in the BM of the NK/T-cell lymphoma cases ranged from 1.2/HPF to 17.0/HPF (median 6.7/HPF). Except for two cases each of cutaneous T-cell lymphoma (4.1/HPF) and aplastic anemia (4.2/HPF), BM specimens in all other cases in the control group were completely negative for EBER-1 ISH. Five specimens obtained from four patients during disease relapse were positive for EBER-1 ISH. Only one specimen was negative for EBER-1 ISH while the patient was experiencing a relapse. Follow-up EBER-1 ISH in one patient converted from positive to negative after chemoradio-

Table 1. Clinicopathologic findings in the 23 patients with nasal NK/T-cell lymphoma.

Patient No./ Age(y)/ Gender	Stage	IPI	BM findings			Treatment	Response	Outcome
			EBER-1 ISH	H&E	Atypical LGLC			
1/25/F	II	0	–	–	–	R/T	CR	Alive NOD 175 months+
2/58/F	I	0	–	–	–	R/T	CR	Alive NOD 68 months+
3/77/M	I	1	–	–	–	R/T	CR	Alive NOD 56 months+
4/45/M	IB	0	–	–	–	RT	CR	Alive NOD 50 months+
5/ 30/ M	I	0	–	–	–	R/T	CR	Alive NOD 43 months+
6/ 60/ F	IIB	1	–	–	–	R/T	CR	Alive NOD 39 months+
7/ 65/ F	IB	2	–	–	–	C/T+ R/T	CR	Alive NOD 24 months+
8/ 72/ F	I	1	–	–	–	R/T	CR	Alive NOD 14 months+
9/ 56/ F	I	0	–	–	–	R/T	CR	DOD 45 months, loco-regional relapse
10/ 69/ M	II	1	–→ –*	– → –	– → –	R/T	CR	DOD 37 months, loco-regional relapse
11/58/M	IB	0	– → +**	– → –	– → +	C/T+ R/T	CR	DOD 18 months, systemic relapse (liver)
12/ 52/ F	I	0	- → +	– → +	– → +	R/T	CR	DOD 9 months, systemic relapse (spleen, bone marrow)
13/ 45/ M	IB	0	+ → –	– → –	+ → –	CCRT+ C/T	CR	Alive NOD 40 months+
14/ 51/ M	IB	2	+	–	+	C/T+ R/T	CR	Alive NOD 35 months+
15/ 38/ F	IB	0	+	–	–	C/T+ CCRT	CR	Alive NOD 4 months+
16/ 45/ M	IB	0	+	–	+	C/T+ R/T	CR	DOD 24 months, loco-regional relapse
17/ 39/ M	I	0	+ → +	– → +	– ? +	C/T+ R/T	CR	DOD 12 months, systemic relapse (lung)
18/ 49/ F	IB	1	+ → +	– → –	– ? +	C/T+ R/T	CR	DOD 10 months, systemic relapse (liver)
19/ 61/ M	I	1	+	–	–	R/T	CR	DOD 3 months, systemic relapse (liver)
20/ 25/ M	IB	0	+	–	–	Nil	PD	DOD 2 months
21/ 36/ M	IB	1	+	–	+	C/T+ R/T	PD	DOD 1 month
22/ 67/ F	IIIB	4	–	–	–	C/T	PD	DOD 2 month
23/ 67/ F	IVB	5	+	–	+	Nil	PD	DOD 1 month

*BM: bone marrow; ISH: in situ hybridization; IPI: International Prognostic Index; LGLC: large granular lymphoid cells; F: female; M: male; R/T: radiotherapy; C/T: chemotherapy; CCRT: concurrent chemoradiotherapy; CR: complete remission; PD: progressive disease; NOD: no evidence of disease; DOD: died of disease; * EBER-1 ISH in the subsequent bone marrow specimen obtained during disease relapse remained negative; **EBER-1 ISH in the subsequent bone marrow specimen obtained during disease relapse changed from negative to positive.*

therapy. All ten BM specimens that contained atypical LGLC and the one BM positive for lymphoma involvement by conventional examination were positive for EBER-1 ISH (Table 1).

Table 2. Comparisons of the characteristics of stage I and II patients, based on EBER-1 ISH results.

Characteristics	*EBER-1⁺ n=9*	*EBER-1⁻ n=12*	*p value*
Age, year			
Median (range)	45 (25-61)	56 (25-77)	NS
Sex			
Male/Female	7/2	5/7	NS
Systemic B symptoms	8	4	0.032
Fever	6	2	0.030
Atypical LGLC in BM	4	0	0.021
Ann Arbor stage			
I	9	9	NS
II	0	3	NS
IPI			
0/1	8	11	NS
2	1	1	NS
Radiotherapy alone	1	10	0.002
Chemotherapy+ Radiotherapy	7	2	0.009
Interferon	1	0	NS

LGLC: large granular lymphoid cell; IPI: International Prognostic Index; NS: not significant.

Bone marrow findings on immunohistochemistry

Immunohistochemical studies using CD3, CD5, CD56 and LMP-1 monoclonal antibodies were performed in the ten EBER-1⁺ BM specimens obtained before treatment. CD3⁺ cells outnumbered EBER-1⁺ cells in these specimens, and the distribution of cells positive for both markers was not fully compatible. CD56 stained scattered plasma cells and paratrabecular stromal cells, but no other cells were highlighted. Staining for CD5 and LMP-1 was also negative in these BM specimens.

BamHI "f" variant analysis

The *f* variant analysis was based on the presence of an extra BamHI restriction enzyme site in the F fragment region of the EBV DNA. Among the patients with EBER-1⁺ BM, DNA was successfully extracted from five patients and used for comparison with their primary tumor specimens. Among the ten EBER-1⁺ specimens, the *f* variant existed in only one primary tumor and one BM specimem from two different patients (Figure 3).

Clinical outcome and survival analysis

The median follow-up for living patients was 40 months (range, 4 to 175 months). Two patients, one with stage III and one with stage IV disease, died 9 days and 2 months after diagnosis, respectively. Of the 21 patients with loco-regional disease (stages I, II), 19 achieved complete remission after induction treatment. Ten of the 21 patients died of disease, including five who had systemic relapse (Figure 4). To clarify the

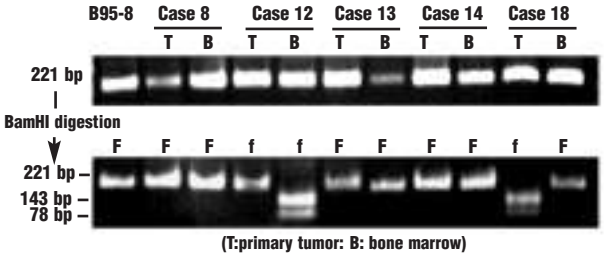

Figure 3. The *f* variant was identified based on the presence of an extra BamHI restriction enzyme site in the F fragment region of EBV DNA. In the ten specimens, the *f* variant existed in the primary tumor of case #18 and the BM of case #12. (B95-8: prototype F fragment; T: primary tumor; B: bone marrow).

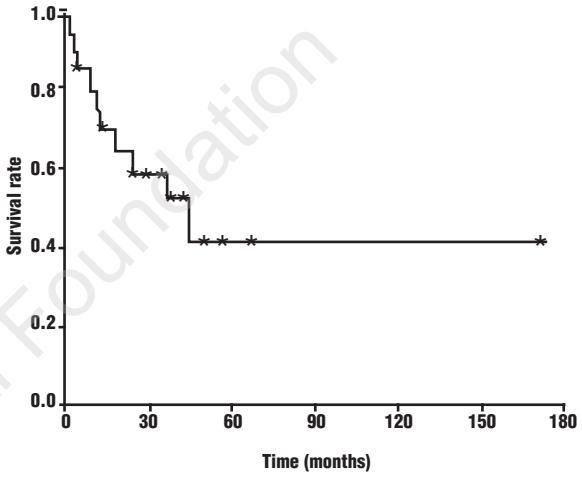

Figure 4. Overall survival of 21 patients with stage I or II nasal NK/T-cell lymphoma.

prognostic predictors of the stage I and II patients, univariate and multivariate analyses were performed. In the Cox proportional-hazards model, only the BM EBER-1 ISH result was shown to be an independent variable predicting overall survival (p=0.027; hazard ratio for death, 0.066, 95% confidence interval, 0.006 to 0.733), suggesting that BM positive for EBER-1 ISH is the major determinant of a poor prognosis (Table 3). As shown in Figure 5, EBER-negative patients had significantly better overall survival than those who were positive for EBER-1 ISH (not reached vs. 12 months, respectively; p=0.013).

Discussion

Although lymphomatous infiltration of the BM can be classified into several patterns: nodular, diffuse, interstitial, para-trabecular, and so-called packed marrow, trivial lymphoma involvement is difficult to diagnose on morphologic bases alone. Several methods have been

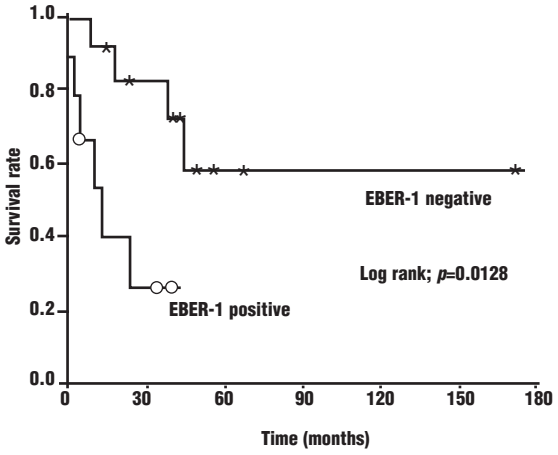

Figure 5. Overall survival of stage I and II patients, showing a significant difference in favor of EBER-1-negative patients.

Table 3. Multivariate Cox proportional-hazards analysis for potential prognostic factors of overall survival.

Variables (95% CI)	*Hazard ratio for death*	*p value*
Age	1.004 (0.916–1.100)	0.936
Sex (female vs. male)	0.617 (0.125-3.045)	0.554
Stage (I vs. 2)	1.047 (0.076-14.462)	0.972
B symptoms (negative vs. positive)	0.902 (0.167-4.862)	0.904
IPI (0 vs 1,2)	0.884 (0.088-8.861)	0.916
EBER-1 ISH (negative vs. positive)	0.066 (0.006-0.733)	0.027
Atypical LGLC (negative vs. positive)	7.279 (0.617-85.849)	0.115

used as adjuncts to the morphologic evaluation of lymphoma involvement in BM, such as immunohistochemical stains, flow cytometry, cytogenetics studies, and other molecular analyses.[10,11] However, the clinical significance of these findings is largely unknown in most types of lymphomas. It is well known that EBV exists in almost all nasal NK/T-cell lymphoma cells. The diagnostic usefulness of EBER-1 ISH on paraffin wax embedded material is well established. In fact, this technique is part of a routine panel of diagnostic investigations in certain neoplasms such as nasopharyngeal carcinoma, post-transplantation lymphoproliferative disease, and nasal NK/T- cell lymphoma.[8,12,13] Compared with CD56, EBER is more reliable and sensitive for the diagnosis of NK/T-cell lymphoma.[14] This is why we applied EBER-1 ISH to detect trivial BM involvement in nasal NK/T-cell lymphoma. Detection of submicroscopic BM involvement in nasal NK/T-cell lymphoma using EBER-1 ISH has been previously reported, but the BM was noted to be involved by lymphoma cells in less that 10% of patients.[15,16] In a study by Wong *et al.*,[2] two of 25 patients were interpreted to have positive findings at initial diagnosis, and another three during or after the systemic relapse.[15] Among the five BM specimens positive for EBER ISH, two lymphomatous infiltrates could not be identified on morphologic examination. All five patients died a short time after the diagnosis of marrow involvement by the lymphoma cells. In the study by Sung *et al.*, which included 33 patients with nasal NK/T-cell lymphoma, only one BM specimen was noted to be positive for EBER expression, and the conventional examination failed to identify any lymphoma cells.[16] The patient positive for EBER expression died of disease 214 days after diagnosis. In contrast to the above results, EBER-1 ISH was positive in 10 of the 23 patients in our series, and conventional morphologic examination had failed to identify them, although atypical LGLC were noted in four marrow blood preparations. Three patients with overt existing EBER-1$^+$ cells in the BM experienced disease-free survival for 4$^+$, 35$^+$, 40$^+$ months after receiving aggressive sequential or concurrent chemoradiotherapy. One of the three patients underwent a follow-up BM EBER-1 ISH study and the result converted from positive to negative. The EBER-1 ISH of two patients changed from negative to positive, and another two patients showed persistent EBER-1$^+$ cells in their BM while experiencing disease relapse. All these four patients died of disease. In the studied population, of the 21 patients with stage I or II nasal NK/T-cell lymphoma, 10 of 12 patients negative for EBER-1 received irradiation alone, in contrast to only one patient in the EBER-1$^+$ group. However, the more aggressive treatment did not lead to a better outcome in the EBER-1$^+$ group, although the one patient who received radiotherapy alone in this group died of systemic relapse after a short period. Thus, we concluded that BM positive for EBER-1 correlated with inferior survival in patients with loco-regional nasal NK/T-cell lymphoma, whose staging was determined by conventional morphologic examination methods. However, if aggressive, combined modality treatment was given, the patients had a chance of being cured of their disease. In addition, tumor fever was noted in 7 of these 11 EBER-1$^+$ patients (64%), while only two of patients negative for EBER-1 experienced tumor fever (p= 0.03) (Table 2). Thus, occult BM involvement should be considered if tumor fever occurs in patients with this disease entity. Another important question raised is why the frequency of BM EBER-1$^+$ in this study was much higher than that in the other two studies. In our series, even one BM cell positive for

EBER-1 ISH unequivocally accompanied with nuclear atypia was interpreted as a positive finding. Perhaps this explains the difference between our study and previous ones. Though latent EBV infection may be found in B lymphoid cells, EBER-1$^+$ cells were rarely noted in the BM in our previous and current studies, and in other reports.[17,18] In spite of this, whether or not the EBER-1$^+$ cell is the true malignant cell is still a concern. Hardarson *et al*. used the EBER ISH method to show that a variable extent of EBV infection occurred in benign and malignant lymphoid lesions.[19] Teramoto *et al*. found, when they studied EBV in the neoplastic and non-neoplastic cells of lymphoma tissues, that non-neoplastic lymphocytes were infected with EBV more frequently than were lymphoma cells. In addition, they observed that T-cell lymphoma might provide favorable conditions for the EBV infection of non-neoplastic lymphocytes.[20] The genomic analysis of EBV in our patients was investigated by exploring the existence of an extra restriction enzyme site in the Bam HI-F region in the *f* variant of EBV DNA. Wu *et al*. found that the EBV genomes in nasal and peripheral T-cell lymphoma were distinct from those in nasopharyngeal carcinoma in their infrequency of the *f* variant.[21] Not surprisingly, the *f* variant existed in only two of the ten specimens analyzed in our study. However, discrepancies in the EBV genome between the BM and primary nasal tumor specimen existed in two of the five cases. Thus, we suggest that not all EBER-1$^+$ cells in the BM of nasal NK/T-cell lymphoma patients are true tumor cells. This means that the ISH method may offer a better method for clinical use, since the morphologic correlation afforded by this technique makes it possible to determine whether the virus is localized in the tumor cells. In addition, immunohistochemical studies are neither sensitive nor specific for identifying the occult tumor cells in BM of nasal NK/T-cell lymphoma patients.

In conclusion, based on our results, we recommend the routine use of EBER-1 ISH in BM specimens from patients with nasal NK/T-cell lymphoma. Those patients whose BM contains EBER-1$^+$ atypical lymphoid cells, i.e. possible lymphoma involvement, should receive a more aggressive, combined modality of treatment, although the best treatment regimen for this disease entity has yet to be identified.

W-TH: conception, design, interpretation of results and draft of this article; K-CC, S-SC: EBER-1 in situ hybridization and immunohistochemisty; J-RH: EBV genome analysis, G-CH, H-HWC: patient data collection, interpretation, database and statistical analysis, T-YC, W-CS: patient follow-up, data collection and interpretation; C-JT: substantial contributions to conception, design, acquisition of data, interpretation of results. All authors reviewed the article critically and approved it for publication.

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