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The Prognostic Subgroups as Defined by the Patterns of Epstein-Barr Virus Infection in Patients with Hodgkin Lymphoma

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Background : The purpose of this study was to investigate the prognostic significance of the Epstein-Barr virus (EBV) infected non-neoplastic lymphocytes in patients with Hodgkin lymphoma (HL). **Methods :** Seventy-seven cases of HL were evaluated by immunohistochemical analysis and EBV-encoded RNA *in situ* hybridization. The cases were divided into three groups according to the EBV status. EBV was negative in 48 cases (group 1), EBV was located in the Hodgkin/Reed-Sternberg (HRS) cells, but not in the non-neoplastic lymphocytes in 20 cases (26%) (group 2) and EBV was located in both the HRS cells and the non-neoplastic lymphocytes in 9 cases (12%) (group 3). **Results :** The groups differed with respect to the age distribution, the clinical presentation and the prognosis. The median ages were 30 (group 1), 47.5 (group 2) and 23 years (group 3) ($p = 0.011$). B symptoms ($p = 0.007$) and the histologic subtype of mixed cellularity classical HL ($p = 0.001$) were more common in the EBV-positive patients than in their EBV-negative counterparts. Two patients from group 3 had associated chronic EBV infection syndrome. The five-year survival rate was 97.56% in group 1, 75.76% in group 2 and 100% in group 3 ($p = 0.0178$). **Conclusions :** HL with EBV located in both the HRS cells and the non-neoplastic lymphocytes is a distinct prognostic subgroup that has better survival than the HL with EBV located in only the HRS cells.

Key Words : Herpesvirus 4, human; Hodgkin disease; *In situ* hybridization

Epstein-Barr virus (EBV) is a member of the human herpes virus family and EBV was initially isolated from a cultured Burkitt lymphoma (BL) cell line by Epstein *et al.*[1] in 1964. It is the causative agent of infectious mononucleosis and it is associated with several B cell malignancies and particularly BL, post-transplant lymphoproliferative disease and Hodgkin lymphoma (HL).[2,3] EBV is present in malignant Hodgkin/Reed-Sternberg (HRS) cells in approximately 50% of the cases of HL, and especially in the cases of the mixed cellularity classical HL subtype.[4-7] HRS cells display a type-II form of latency with the viral antigen expression limited to EBV nuclear antigen 1, latent membrane protein (LMP)1, LMP2A and LMP2B.[8] With respect to the prognostic significance of EBV, some studies have demonstrated a negative correlation between the EBV status and the prognosis,[9-12] while others have reported favorable[13,14] or adverse[15] effects. Several studies have suggested that EBV positive HL in young patients carries a favorable prognosis, but it denotes poor survival in the elderly.[16,17] The previous studies regarding EBV have typically focused on EBV-infected neoplastic cells, but EBV is often located in non-neoplastic small lymphocytes as well as in HRS cells.[18-20] As the presence of EBV in the non-neoplastic lymphocytes in HL patients can be easily neglected in the routine process of making a diagnosis, its biologic significance has yet to be explored. Those patients with many infected non-neoplastic lymphocytes seem to have an immunologic defect that prohibits EBV eradication and these patients may show differences of their chemotherapeutic response or prognosis as compared to that of the patients with EBV-infected HRS cells alone. In order to further investigate the clinical significance of EBV-infected non-neoplastic lymphocytes in HL, we performed a clinicopathologic analysis of HL patients according to their EBV status.

MATERIALS AND METHODS

A total of 133 patients were enrolled in the present study. All of them were diagnosed with HL at Samsung Medical Center from January 1995 to December 2008. Of the 133 cases, 82 underwent EBV-encoded RNA (EBER) *in situ* hybridization

(ISH) during their routine diagnostic work up. The 5 cases with an equivocal staining pattern of EBV ISH were excluded. The clinical information, including age, gender, the site of involvement, the symptoms, the Ann Arbor stage, the outcome and overall survival (OS) from the remaining 77 cases was obtained from the electronic medical records.

The hematoxylin and eosin-stained slides were reviewed for all the cases and the World Health Organization (WHO) clas-

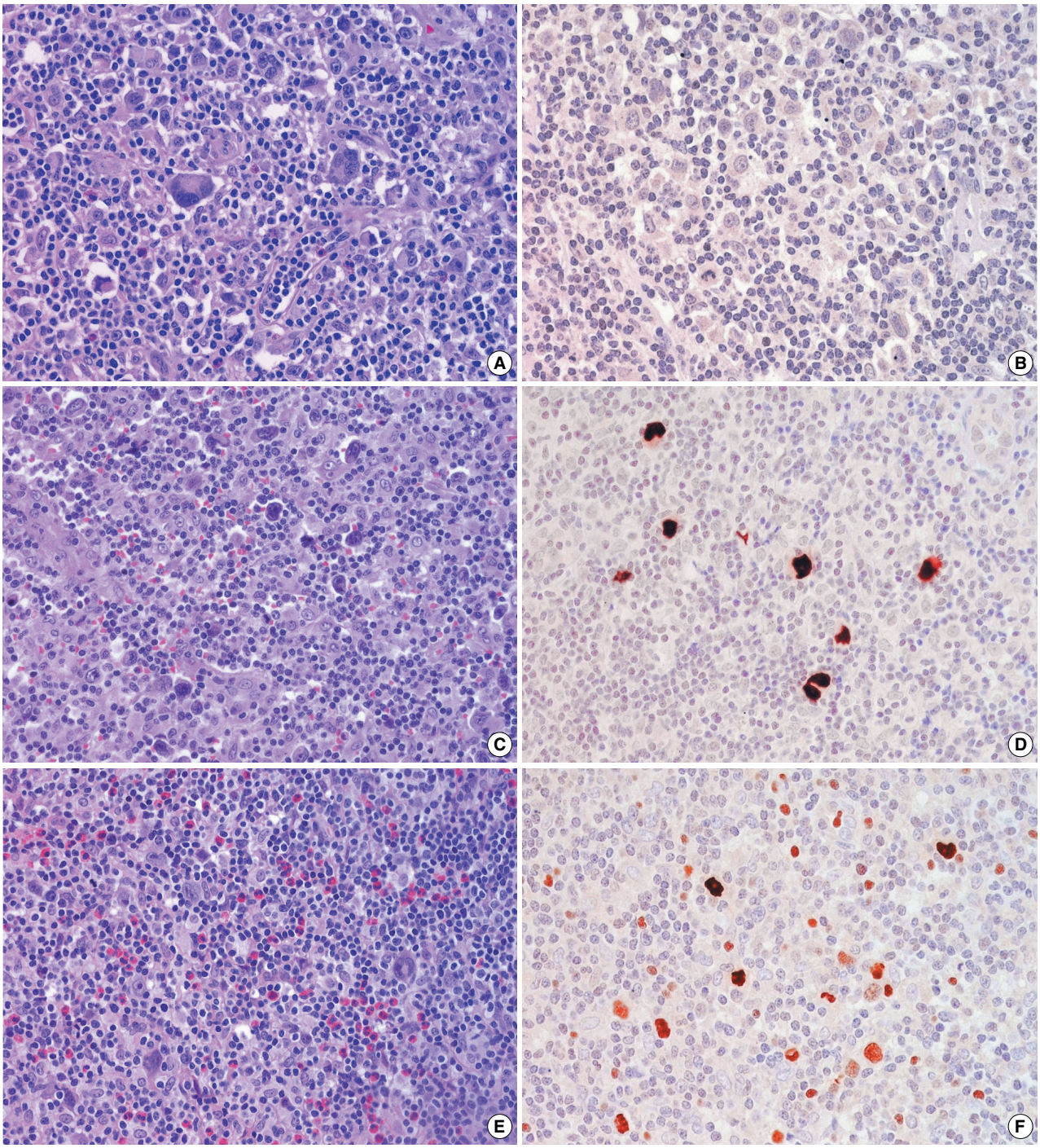

Fig. 1. The histology and the Epstein-Barr virus (EBV) *in situ* hybridization (ISH) staining of Hodgkin lymphoma (HL) according to EBV status: (A) Group 1 exhibiting mixed cellularity classical HL. (B) EBV ISH staining is negative in group 1. (C) Group 2 exhibiting mixed cellularity classical HL. (D) EBV ISH staining is positive for Hodgkin/Reed-Sternberg (HRS) cells only in group 2. (E) Group 3 exhibiting mixed cellularity classical HL. (F) EBV ISH staining is positive for HRS cells and many non-neoplastic lymphocytes in group 3.

sification was used for histological subtyping. Immunohistochemical staining was performed on the paraffin sections using antibodies that included CD3, CD20 (Novocastra, Newcastle upon Tyne, UK), CD15, and CD30 (DAKO, Glostrup, Denmark). EBER ISH (Novocastra) was performed using a fluorescein isothiocyanate-labeled oligonucleotide probe directed to the EBER-1. A positive reaction for the EBER ISH was defined as distinct nuclear staining on the HRS cells or the non-neoplastic cells. The cases were divided into three groups according to the EBV status. In group 1, EBV was negative in both the HRS cells and the non-neoplastic small lymphocytes (Fig. 1A, B). In group 2, EBV was located in the HRS cells, but not in the non-neoplastic small lymphocytes (Fig. 1C, D). In group 3, EBV was located in both the HRS cells and the non-neoplastic small lymphocytes (Fig. 1E, F). The clinicopathologic variables were compared among the three groups for statistical differences using a log-rank analysis. OS was estimated using the Kaplan-Meier product-limit method. OS was calculated from the date of diagnosis to the date of death from any cause or the date of the last follow-up visit. p-values less than 0.05 were considered statistically significant and all p-values correspond to two-sided significance tests.

Table 1. Clinical characteristics of all cases

Factor	EBV status			p-value	
	Negative	Positive		Group 1 vs 2 vs 3	Group 1 vs 2 + 3
	Group 1 (n = 48)	Group 2 (n = 20)	Group 3 (n = 9)		
Age				0.011	0.118
< 20	4	2	4		
≥ 20	44	18	5		
Sex				-	0.000
Male	20	17	9		
Female	28	3	0		
Primary site				0.949	0.819
Lymph node	39	17	8		
Mediastinum	6	2	1		
Others	3	1	0		
B symptom				0.022	0.007
Absent	38	10	5		
Present	10	10	4		
Ann arbor stage				0.701	0.704
I + II	27	14	5		
III + IV	19	4	4		
Not evaluated	2	2	0		
Recurrence				0.156	0.156
Present	6	2	0		
Absent	39	15	9		
NA	3	3	0		
Clinical outcome				0.017	0.043
Alive	47	16	9		
Death from HL	1	3	0		
Death from OC	0	1	0		
Survival time (range, mo)	51 (5-133)	38 (5-162)	63 (6-106)		

Group 1, EBV negative; Group 2, EBV positive in HRS cells and negative in non-neoplastic cells; Group 3, EBV positive in both HRS cells and non-neoplastic cells.
EBV, Epstein-Barr virus; NA, not available; HL, Hodgkin lymphoma; OC, other cause.

RESULTS

The clinicopathologic characteristics of the three groups are summarized in Table 1. A total of 77 patients (31 women and 46 men) with a median age of 32 years (range, 7 to 83 years) were included in the analysis. The patients presented with a mass (55%), B symptoms (14%), cough and pain. Group 1 (EBV-negative HL) consisted of 48 cases (62%). EBV was positive in 29 cases (38%), and nine of which showed EBV positivity in both the HRS cells and the non-neoplastic lymphocytes.

The median age of the patients was 30 years (range, 13 to 83 years) in group 1, 47.5 years (range, 18 to 71 years) in group 2 and 23 years (range, 7 to 62 years) in group 3. Fig. 2 outlines the age distribution according to the EBV status. The patients in group 3 were significantly younger than those of the other groups (p = 0.011). The majority of EBV-positive patients (groups 2 and 3) showed B symptoms while only 10 out of the

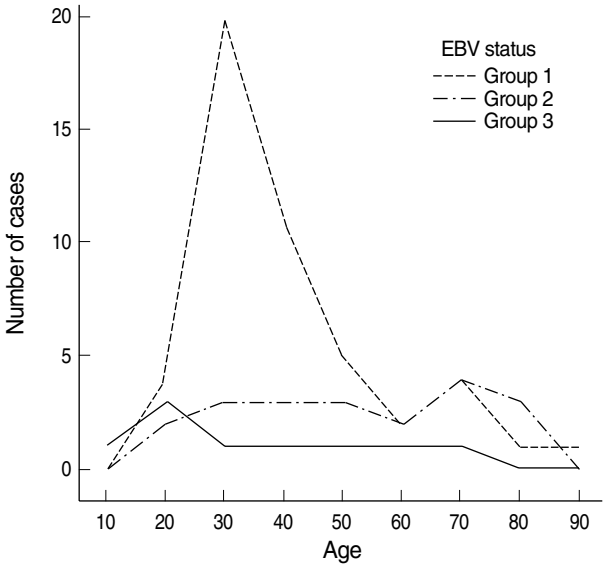

Fig. 2. Age distributions according to Epstein-Barr virus (EBV) status. Group 1, EBV negative; Group 2, EBV positive in Hodgkin/Reed-Sternberg (HRS) cells and negative in non-neoplastic cells; Group 3, EBV positive in both HRS cells and non-neoplastic cells.

Table 2. Five-year survival rate according to EBV status

Factor	EBV status			p-value	
	Negative	Positive		Group 1 vs 2 vs 3	Group 1 vs 2 + 3
	Group 1	Group 2	Group 3		
5YSR (%)	97.56	75.76	100	0.0178	0.0850

Group 1, EBV negative; Group 2, EBV positive in HRS cells and negative in non-neoplastic cells; Group 3, EBV positive in both HRS cells and non-neoplastic cells.
EBV, Epstein-Barr virus; 5YSR, 5-year survival rate.

Table 3. Histological characteristics of all cases

Factor	EBV status			p-value	
	Negative	Positive		Group 1 vs 2 vs 3	Group 1 vs 2 + 3
	Group 1 (n = 48)	Group 2 (n = 20)	Group 3 (n = 9)		
Histologic subtype				0.005	0.001
Nodular sclerosis cHL	31	8	1		
Mixed cellularity cHL	11	12	6		
Lymphocyte-rich cHL	3	0	2		
Nodular lymphocyte predominant HL	2	0	0		
Undetermined	1	0	0		
IHC findings					
CD15 +/-/ND	31/9/8	14/3/3	7/1/1	0.803	0.555
CD30 +/-/ND	36/2/10	17/0/3	6/0/3	0.544	0.270

Group 1, EBV negative; Group 2, EBV positive in HRS cells and negative in non-neoplastic cells; Group 3, EBV positive in both HRS cells and non-neoplastic cells.
EBV, Epstein-Barr virus; cHL, classical Hodgkin lymphoma; IHC, immunohistochemistry; ND, not done.

38 EBV-negative patients (group 1) presented with B symptoms (p = 0.007). Quantitative polymerase chain reaction for EBV and using whole blood was performed in only two cases from group 3 during the clinical course following chemotherapy and the EBV copy number was 225.9 and 2,649 copies/5 μL whole blood, respectively. The EBV viral capsid antigen IgM was negative and the IgG was positive in both patients. Of note, one of the two patients had a deep scar derived from a mosquito allergy on his thigh. There was no significant association between the Ann Arbor stage and the EBV status (p = 0.701). Follow-up was available for 71 patients and this revealed disease recurrence in eight patients (10%). Four patients died of HL and one patient died secondary to lung cancer.

The median survival time of all the patients was 49 months (range, 5 to 162 months). The five-year survival rate was 97.56 % in group 1 and 75.76% in group 2 (p = 0.0178) (Table 2, Fig. 3). The patients from group 3 had an excellent prognosis and at the conclusion of the study, all the patients were alive and they were without recurrence. No significant differences in survival were observed according to age, gender, the Ann Arbor stage and the presence or absence of B symptoms.

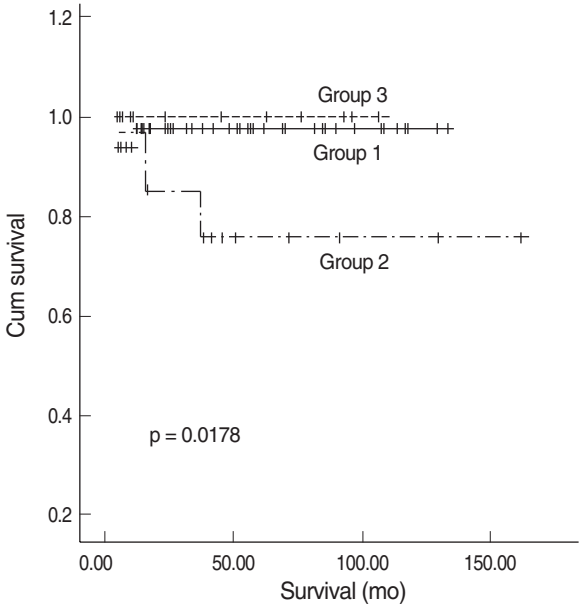

Fig. 3. Survival curve of Hodgkin lymphoma patients according to Epstein-Barr virus (EBV) status. Group 1, EBV negative; Group 2, EBV positive in Hodgkin/Reed-Sternberg (HRS) cells and negative in non-neoplastic cells; Group 3, EBV positive in both HRS cells and non-neoplastic cells.

The histological subtypes included nodular sclerosis classical HL (40 cases, 51.9%), mixed cellularity classical HL (29 cases, 37.7%), lymphocyte-rich classical HL (5 cases, 6.5%), nodular lymphocyte predominant HL (2 cases, 2.6%) and the undetermined type (1 case, 1.3%) (Table 3). The mixed cellularity type accounted for 11 out of 48 cases in group 1 (23%), 12 out of 20 cases in group 2 (60%) and 6 out of 9 cases in group 3 (67%) and the mixed cellularity type occurred frequently in EBV-positive HL patients (p = 0.001). There was no difference of the immunohistochemical findings among the three groups. No significant differences in survival were present according to the histologic subtype.

DISCUSSION

EBV is an important infectious agent that is associated with the pathogenesis of Hodgkin lymphoma. Of the EBV latent genes, LMP1 stimulates the overexpression of B-cell activation markers and it upregulates antiapoptotic genes, including Bcl-2, Mcl-1 and A20.[21] EBV-positive HRS cells may be rescued from apoptotic death by activation of nuclear factor (NF) κB,[22]

which has antiapoptotic properties, or by promoting turnover of its inhibitor IκBα.[23] Aside from inhibiting apoptosis, the activated NF κB pathway plays a crucial role in the induction of proliferation of HRS cells.[24]

The rate of detecting EBV in HL depends on ethnicity, age, the histologic type and the immunologic status. Higher rates of EBV positivity were observed in the mixed cellularity subtype, young children, elderly patients, Asians and human immunodeficiency virus (HIV)-positive patients.[25,26] In the present study, the three subgroups of HL, which were defined by the pattern of EBV infection, showed a distinct age distribution. In the youngest group, EBV was found in both the HRS cells and the non-neoplastic lymphocytes. EBV was negative in young adults and it was positive in the HRS cells, but it was negative in the non-neoplastic lymphocytes in the oldest subgroup. A similar age distribution has been previously described and this provides biological support that EBV has a pathogenetic role in Hodgkin disease in children and the older age groups.[27-29] The strong association of EBV with HL in elderly patients is thought to be due to a decline in EBV-specific cellular immunity with age or alternatively, with increased viral reactivation.

In the present study, the EBV-positive elderly patients carried the worst prognosis. Jarrett *et al.*[29] recently reported that the prognostic impact of EBV status varied with the age at the time of diagnosis. For patients aged 16 to 34 years, the EBV-associated cases had a survival advantage compared with the EBV-negative cases, while for the patients aged 50 years or older, EBV positivity was associated with significantly poorer outcomes. These results suggest that an impaired immune status may contribute to the development of EBV^+ cHL in older patients.

The presence of EBV in HL is not exclusive to the HRS cells. Khan *et al.*[18] reported detecting EBV in the small lymphocytes of patients with HL by *in situ* hybridization. Faumont *et al.*[19] analyzed the LMP1 gene from HRS cells and bystander B lymphocytes and they reported that both cell types are infected by different, but related EBV strains. Conversely, Garcia-Cosio *et al.*[20] showed that the two viral types coexist in the majority of HIV-positive cases. However, HRS cells are more frequently infected by type 1 EBV, and the bystander B lymphocytes are co-infected by types 1 and 2. They further suggested that the higher frequency of dual-infection in the bystander B lymphocytes is likely associated with an immunodeficient status.

EBV-positive HL that occurs in childhood develops following a primary EBV infection. The incidence of EBV-positive HL in children is higher for the patients who suffer from infectious mononucleosis than in those patients who do not. In the present study, HL with EBV-positive non-neoplastic lymphocytes was more common in children. A high EBV load was detected in the blood of two of such patients, one of whom had mosquito-bite hypersensitivity. Mosquito-bite hypersensitivity is a cutaneous manifestation of chronic EBV infection, and this is caused by an innate T-cell immunologic defect for EBV and it is commonly associated with a hydroa-vacciniforme-like skin eruption, chronic active EBV infection and chronic natural killer lymphocytosis.[30] Although we were unable to find evidence for immune deficiency from the clinical records of the remaining patients, it is conceivable that the young children with EBV positivity in both the HRS cells and non-neoplastic lymphocytes have a common EBV-specific immune defect, and some may have a subclinical chronic active EBV infection. In the current study, the prognosis for the patients in this group was excellent and it was comparable with that of the EBV-negative patients. The superior prognosis, as compared to patients with EBV positivity confined to the HRS cells, cannot yet be explained with our current knowledge, but it may ultimately be ascribed to the younger age of the patient population. In conclusion, we identified three distinct biologic subgroups via the EBV staining patterns and these differed according to both the age distribution and prognosis. Careful observation and interpretation of the EBV staining pattern are important for making the diagnosis of HL and arriving at a prognosis.

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