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Determination of Epstein-Barr Virus–Infected Lymphocyte Cell Types in Peripheral Blood Mononuclear Cells as a Valuable Diagnostic Tool in Hematological Diseases

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Background. High loads of Epstein-Barr virus (EBV) in peripheral blood mononuclear cells (PBMCs) can be indicative of a broad spectrum of diseases, ranging from asymptomatic infection to fatal cancers.

Methods. We retrospectively investigated the EBV-infected cell types in PBMCs among 291 patients. Based on EBV-infected cell types, the clinical features and prognoses of 93 patients with EBV-associated (EBV$^+$) T/natural killer (NK)–cell lymphoproliferative diseases (LPDs) T/NK-LPDs) were investigated over a 5-year period.

Results. Although B-cell-type infection was found in immunocompromised patients and patients with asymptomatic high EBV carriage, infectious mononucleosis, EBV$^+$ B-cell LPDs and B-cell lymphomas, T-cell, NK-cell or multiple-cell-type infection in immunocompetent hosts were highly suggestive of EBV$^+$ T/NK-LPDs, EBV$^+$ T/NK-cell lymphomas, and aggressive NK-cell leukemia. Patients with non–B-cell infection had a poorer prognosis than those with B-cell-type infection. In our cohort, 79.6% of patients with EBV$^+$ T/NK-LPDs were >18 years old, and NK cells were identified as EBV-infected cell type in 54.8%. Nearly half of patients with EBV$^+$ T/NK-LPDs had genetic defects associated with immunodeficiency. However, hemophagocytic lymphohistiocytosis, and not genetic defects, was the only parameter correlated with poor prognosis of EBV$^+$ T/NK-LPDs.

Conclusions. Determination of EBV-infected cell types among PBMCs is a valuable tool for the differential diagnosis of EBV$^+$ hematological diseases.

Key words: chronic active Epstein-Barr virus infection; Epstein-Barr virus; hemophagocytic lymphohistiocytosis; lymphoproliferative disorders.

Epstein-Barr virus (EBV) is a herpesvirus that infects >90% of the population, and its infection is strongly linked to a remarkable variety of diseases, ranging from asymptomatic infections to a number of hematological and nonhematological cancers [1–3]. Although most EBV-associated (EBV$^+$) diseases are asymptomatic, possible pathological responses include prolonged fever, lymphadenopathy, liver dysfunction, and hepatosplenomegaly [4]. It may be difficult to distinguish these diseases without detailed clinical information. These facts emphasize the need for a uniform approach to an aggressive analysis of EBV status and pathological consequences in the routine diagnosis of EBV$^+$ diseases.

Measurement of EBV DNA now plays an important role in the diagnosis and management of EBV$^+$ diseases [5–10]. Currently, whole blood, peripheral blood mononuclear cells (PBMCs), and plasma have been used to quantify EBV DNA [11, 12]. Although measurements in PBMCs represent EBV loads derived from cells circulating in the blood, measurements in plasma provide information distinct from measurements in the circulating cells [13]. Plasma was proposed to be a better specimen source than PBMCs for evaluating or tracking responses to therapy in EBV$^+$ diseases [5]. In contrast, because different lymphoproliferative diseases (LPDs) are associated with different EBV-infected lymphocyte cell types [14–19], determining the cell type could provide another valuable marker for the differential diagnosis of EBV$^+$ LPDs.

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The goal of the current retrospective study was to determine the relative utility of determining EBV-infected lymphocyte cell types in PBMCs as a tool for discriminating among EBV$^+$ hematological diseases. Based on the identification of EBV-infected cell types, the clinical features and prognoses of EBV$^+$ T/natural killer (NK)–cell LPDs (T/NK-LPDs) were investigated over a 5-year period.

METHODS

Patients

We retrospectively investigated the EBV-infected cell types among 291 patients with high EBV loads (EBV DNA in PBMCs, $>1 \times 10^4$ copies/10^6 cells); specimens were obtained and analyzed to determine EBV-infected lymphocyte cell types for diagnostic purposes between January 2013 and September 2018 in Tongji Hospital. Patient characteristics and outcomes were collected retrospectively, including EBV DNA levels in PBMCs and plasma at first admission,. The study was carried out in accordance with the Declaration of Helsinki and approved by the ethics committee of Tongji Medical College, Huazhong University of Science and Technology. Informed consent was waived because of the retrospective nature of the study.

All patients were grouped into immunodeficient and immunocompetent groups based on whether an identifiable cause of immunosuppression was present at the time of specimen collection. The immunodeficient group included patients who were in an immunosuppressed state due to autoimmune disease, had undergone transplantation, or had been using immunosuppressant drugs for >3 months. There were no human immunodeficiency virus–infected patients in our cohort. The immunocompetent group included the following 7 disease entities: asymptomatic high EBV carriage (ASHEBV), infectious mononucleosis (IM), EBV$^+$ B-cell LPDs (B-LPDs), EBV$^+$ T/NK-LPDs, EBV$^+$ B-cell lymphomas (BCLs), EBV$^+$ T/NK-cell lymphomas (T/NKCLs), and aggressive NK-cell leukemia (ANKL). The diagnostic criteria for these disease entities are presented in the Supplementary Materials.

We also collected clinical, laboratory, and treatment data from 93 patients with EBV$^+$ T/NK-LPDs, including mutational analysis of genes associated with immunodeficiency and functional analyses of NK cells. The methodological details are described in the Supplementary Materials. The outcome of EBV$^+$ T/NK-LPDs was classified as complete response (CR), partial response (PR), stable disease, or progressive disease, as described elsewhere [20, 21]. The details are presented in the Supplementary Materials.

Determination of Dominant EBV-Infected Lymphocyte Cell Types

Briefly, B, T, and NK cells were isolated from PBMCs by means of magnetic-activated cell sorting (MACS) and analyzed with real-time polymerase chain reaction (PCR) using primers for the EBV gene *EBNA1*, as described elsewhere [7, 20] or fluorescent in situ hybridization (FISH) with EBV-encoded small nuclear RNA (EBER) probes (Agilent; catalog no. Y5200) (details in the Supplementary Materials). The dominant infected cell types were identified as those with higher EBV DNA levels than the unfractionated PBMCs based on real-time PCR and/or ≥0.2% EBER-positive cells by FISH, as described elsewhere [21]. Multiple-cell-type infections were defined as those in which >2 cell types met the above criteria.

Statistical Analysis

Statistical analyses were performed using SPSS (IBM; version 19) and GraphPad Prism (GraphPad; version 7) software. Overall survival (OS) was analyzed using the Kaplan-Meier method and the log-rank test. The Cox proportional hazard model of OS was used for univariate analyses, and variables with a P value <.05 in the univariate analysis were further included in a multivariate analysis, with χ^2 and Fisher exact tests used for categorical variables, and the Mann-Whitney U test for quantitative variables. The EBV DNA levels were log-transformed before the correlation and regression analyses. The Pearson test was used for correlations. Differences were considered statistically significant at $P <. 05$ (2 sided).

RESULTS

Determination of EBV-Infected Cell Types

To determine EBV-infected cell types, PBMCs isolated from patients were fractionated into B, T, and NK cells using MACS, purities of which were confirmed by flow cytometry to be 97%–99% for B and T cells and 91%–95% for NK cells (Figure 1A). The resulting cells were then analyzed with real-time PCR to amplify the genomic *EBNA1* as a surrogate marker for EBV and with FISH assay with probes against EBER (Figure 1B and 1C). Twenty-six patients were examined with both real-time PCR and FISH, and the EBV-infected cell types identified by real-time PCR were highly consistent with FISH results. Furthermore, the EBV DNA copy number determined by real-time PCR was correlated with the number of EBER-positive cells by FISH (Figure 1D). Therefore, in the subsequent studies, we used real-time PCR to determine the EBV-infected cell types for better clinical feasibility.

Dominant EBV-Infected Cell Types in Different EBV Disease Entities

A total of 291 patients were ultimately enrolled in this study. In the immunodeficiency group, 45 patients were identified as having dominant B-cell-type infection, including posttransplantation lymphoproliferative disorder (PTLD) (n = 2), posttransplantation status (n = 13), the use of immunosuppressant drugs (n = 23), or the presence of an autoimmune disease (n = 7), and 1 patient with PTLD after renal transplantation was identified as having NK-cell-type infection. The other 245 patients in the immunocompetent group exhibited various EBV disease entities, summarized in Table 1.

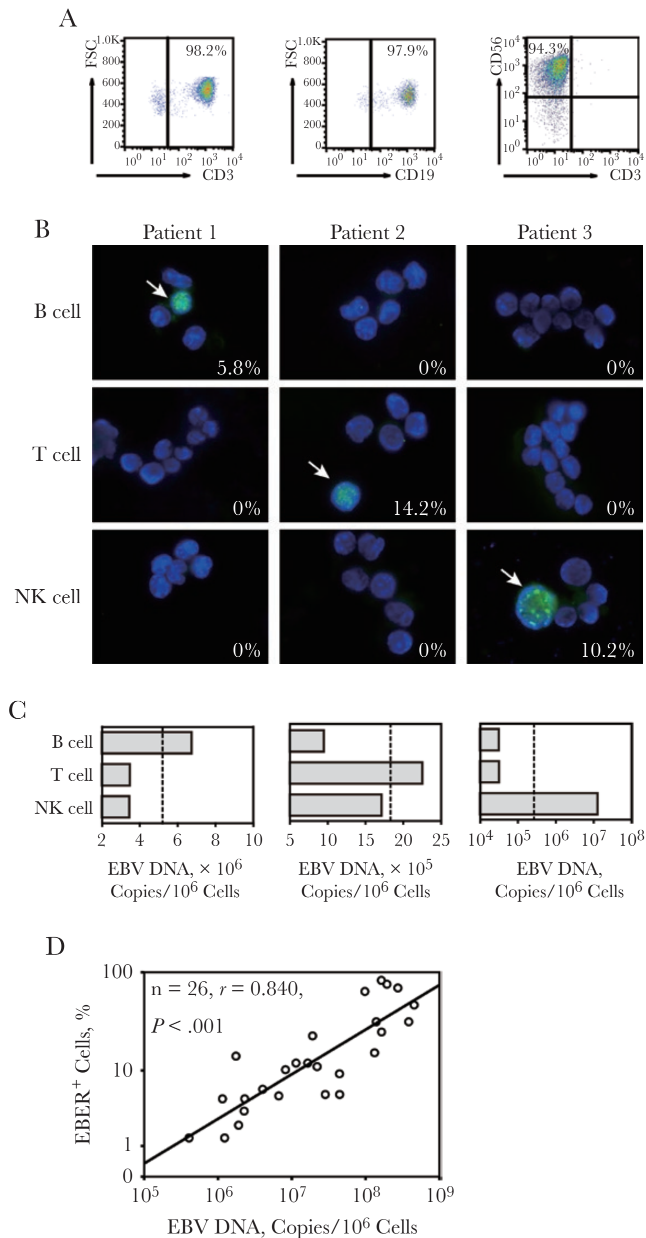

Figure 1. Validation of magnetic-activated cell sorting (MACS) and real-time polymerase chain reaction (PCR) for determining Epstein-Barr virus (EBV)–infected lymphocyte cell types. Patient peripheral blood mononuclear cells (PBMCs) were fractionated into B, T, and natural killer (NK) cells using MACS. *A–C,* The purities of the postsorting B, T, and NK cells were confirmed with flow cytometry and subsequent fluorescent in situ hybridization (FISH) analysis, using EBV-encoded small nuclear RNA (EBER) as a probe (*B*) as well as real-time PCR for amplifying genomic *EBNA1* (*C*) to identify the infected cell types. The dominant EBV-infected cell type was defined as the type with >0.2% EBER-positive cells by FISH or a higher EBV DNA copy number than that in unfractionated PBMCs by real-time PCR. *B,* Typical FISH images from 3 patients are shown. White arrows indicate EBER-positive cells, and numbers represent the percentage of EBER-positive cells. *C,* Corresponding results of real-time PCR analysis from the same 3 patients are shown. Dotted lines indicate EBV DNA levels detected in unfractionated PBMCs. *D,* Correlation between percentage of EBER-positive cells and EBV DNA level in 26 patients with EBV-positive PBMCs. Abbreviations: FSC, forward scatter; EBER, EBV-encoded small nuclear RNA; NK, natural killer.

In the immunocompetent group, B-cell-type EBV infection was detected in 107 patients (44%), including ASHEBV (n = 30), IM (n = 22), EBV$^+$ B-LPDs (n = 40), EBV$^+$ BCLs (n = 8) and EBV$^+$ T/NKCLs (n = 6). The disease entities associated with T-cell, NK-cell, or multiple-cell-type infection included EBV$^+$ T/NK-LPDs (n = 93), EBV$^+$ T/NKCLs (n = 29), and ANKL (n = 16). In the EBV$^+$ lymphomas, the dominant EBV-infected cell types were typically consistent with the cell type origin of the lymphoma, with the exception of 6 cases of angioimmunoblastic T-cell lymphoma in which B-cell-type infection was dominantly detected.

High EBV DNA Load in PBMCs Predictive of Non–B-Cell Infection

In PBMCs, EBV DNA levels were much higher in patients with non–B-cell infections than in those with B-cell infections (P < .001; Figure 2A). A similar difference was also found in plasma samples between the patients with non–B-cell infections and those with B-cell infections (P < .01; Figure 2B).

In PBMCs and plasma samples, EBV DNA levels were significantly higher among patients with EBV$^+$ T/NK-LPDs and ANKL than among those with immunodeficiency, ASHEBV, IM, or EBV$^+$ B-LPDs (P < .01; Figure 2C and 2D). There was no significant difference in PBMCs or plasma EBV DNA levels between immunodeficiency, IM, EBV$^+$ B-LPDs, and EBV$^+$ BCLs. Interestingly, there was no difference in plasma EBV DNA levels between patients with EBV$^+$ T/NK-LPDs or ANKL and those with EBV$^+$ BCLs (Figure 2D), which indicated that the EBV DNA level in PBMCs is a better marker for distinguishing non–B-cell-type from B-cell-type EBV$^+$ diseases.

Dominant EBV Infection of T/NK Cells as an Inferior Prognostic Factor

The patients with B-cell-type infections had a profoundly favorable OS compared with patients with T-cell, NK-cell, or multiple-cell-type EBV infections (Figure 3A; P < .001). However, there was no significant difference in OS among the patients with T-, NK, and multiple-cell-type infections (P > .05). The patients with EBV$^+$ T/NK-LPDs had an inferior prognosis compared with EBV$^+$ B-LPDs and an OS similar to that in the patients with either EBV$^+$ BCLs or T/NKCLs, which indicated that EBV$^+$ T/NK-LPDs have a lymphomalike prognosis (Figure 3B). As expected, the patients with ANKL (n = 16), which involves systemic neoplastic proliferation of NK cells, exhibited a fulminant and extremely aggressive clinical course compared with that seen in patients with other EBV$^+$ T/NKCLs or LPDs.

Clinical Features and Treatment Outcome of EBV$^+$ T/NK-LPDs

Of the 139 patients with non–B-cell infection, 93 (56 male and 37 female) had EBV$^+$ T/NK-LPDs diagnosed. Age at disease onset ranged from 2.7 to 85.4 years (median, 37.1 years); 74 patients (79.6%) were >18 years old, and only 6 (6.5%) were <10 years old. Notably, NK cells were infected in a significant proportion of the patients with EBV$^+$ T/NK-LPDs (51 of 93

Table 1. Summary of Epstein-Barr Virus (EBV)–Infected Cell Types by Disease Group in Patients with EBV-Positive Peripheral Blood Mononuclear Cells

Disease Entity	Total, No. (n = 291)	EBV-Infected Lymphocyte Cell Types, No. (%)			
		B Cell (n = 152)	T Cell (n = 21)	NK Cell (n = 88)	Multiple (n = 30)
Immunodeficient	46	45 (98)	0 (0)	1 (2)	0 (0)
Immunocompetent	245	107 (44)	21 (9)	87 (36)	30 (12)
ASHEBV	30	30 (100)	0 (0)	0 (0)	0 (0)
Infectious mononucleosis	22	22 (100)	0 (0)	0 (0)	0 (0)
EBV$^+$ B-LPDs	41	41 (100)	0 (0)	0 (0)	0 (0)
EBV$^+$ T/NK-LPDs	93	0 (0)	19 (20)	51 (55)	23 (25)
EBV$^+$ BCLs	8	8 (100)	0 (0)	0 (0)	0 (0)
Hodgkin lymphoma	5	5 (100)	0 (0)	0 (0)	0 (0)
Diffuse large BCL, not otherwise specified	3	3 (100)	0 (0)	0 (0)	0 (0)
EBV$^+$ T/NKCLs	35	6 (17)	2 (6)	21 (60)	6 (17)
AITL	6	6 (100)	0 (0)	0 (0)	0 (0)
Extranodal NK/T-cell lymphoma, nasal type	26	0 (0)	0 (0)	21 (81)	5 (19)
Peripheral TCL	3	0 (0)	2 (67)	0 (0)	1 (33)
ANKL	16	0 (0)	0 (0)	15 (94)	1 (6)

Abbreviations: AITL, angioimmunoblastic T-cell lymphoma; ANKL, aggressive NK-cell leukemia; ASHEBV, asymptomatic high EBV carriage; BCL, B-cell lymphoma; B-LPDs, B-cell lymphoproliferative diseases; EBV, Epstein-Barr virus; EBV$^+$, EBV-associated; NK, natural killer; TCL, T-cell lymphoma; T/NKCLs, T/NK-cell lymphomas; T/NK-LPDs, T/NK-cell lymphoproliferative diseases.

[54.8%]). Among the patients with multiple-cell-type infection, 95.7% (22 of 23) had EBV DNA in NK cells in addition to other lymphocyte cell types. The clinical characteristics of the patients are summarized in Supplementary Table 1, according to infected cell types. In general, patients with different cell types exhibited similar clinical and laboratory characteristics, with only a few exceptions. Those with NK-cell-type infection had lower platelet levels and higher lactate dehydrogenase levels, and those with T-cell-type infection had lower EBV DNA levels in plasma.

The patients with EBV$^+$ T/NK-LPDs had chronic active EBV infection (n = 56), EBV$^+$ hemophagocytic lymphohistiocytosis (HLH) (n = 33), hydroa vacciniforme (n = 3), and severe mosquito bite allergy (n = 1), based on diagnostic criteria. Between 0.8 and 44.7 months of follow-up (median, 11.2 months), chronic active EBV infection in 17 patients and hydroa vacciniforme in 1 developed into HLH. During this period, 41 patients (44.1%) with EBV$^+$ T/NK-LPDs died. Of surviving patients at the last follow-up, 5 were in CR and 19 in PR

Sixty-two patients had received chemotherapy or immunotherapy, including 35 patients treated with the HLH-2004 regimen [22] or DEP regimen (doxorubicin, etoposide, and methylprednisolone) [23] regimens, 18 with P-GEMOX regimen (pegaspargase, gemcitabine, and oxaliplatin) [24], 3 with CHOP regimen (cyclophosphamide, doxorubicin, vincristine, and prednisolone) or CHOP-like regimens, and 6 with anti–programmed death 1 antibody. The other 31 patients had received only steroid and/or antiviral therapies. After the start of chemotherapy or immunotherapy, 72.6% of the patients (45 of 62) exhibited a transient response within 8 weeks, including 6 in CR and 39 in PR, of whom 9 received allogeneic hematopoietic stem cell transplantation (allo-HSCT) after the termination of chemotherapy or immunotherapy and 36 did not. Of the 36 patients without allo-HSCT, only 11 had sustained PR, 5 patients had relapse, 18 died of progression of EBV$^+$ T/NK-LPDs, and 2 died of infection. Of the 9 patients who received allo-HSCT, 7 achieved sustained CR or PR, 1 had relapse, and 1 died of transplantation-related complications.

Mutations in Genes Associated With Immunodeficiency in EBV$^+$ T/NK-LPDs

Sixty-nine patients with EBV$^+$ T/NK-LPDs underwent mutational analysis using targeted sequencing panel that covered genes associated with immunodeficiency. Overall, 47.8% of the patients (33 of 69) were found to have deleterious mutations (Figure 4A). Among those with mutations, 1 patient had hemizygous mutations with *XIAP*, and 1 was compound heterozygotes with *UNC13D*; 26 patients harbored monoallelic mutation in 1 gene, and 4 had monoallelic mutation in 2 genes simultaneously. All mutations were either single-base substitutions or frame-shift mutations (Supplementary Table 2).

Mutations were detected at a higher frequency of 60.0% (9 of 15) among patients <18 years old at onset compared with 44.4% (24 of 54) among older patients. Mutations were detected in 12 of 23 patients (52.2%) with an initial diagnosis of EBV$^+$ HLH, in 10 of 15 (66.7%) with EBV$^+$ T/NK-LPDs that developed into HLH during the follow-up period, and in 11 of 31 (35.5%) with EBV$^+$ T/NK-LPDs but not HLH. There were no significant differences in mutation frequencies according to EBV-infected cell type ($P = .23$).

Immunological analyses revealed defective NK-cell cytotoxicity in 90.6% of 53 patients and defective degranulation (Figure 4B), as defined by the protein level of CD107 of activated NK cells, in 14 (26.9%) of 52 patient (Figure 4C), both of which was not closely correlated with genetic findings. Moreover, 24 (52.2%) of 46 patients were found to have perforin expression lower than the normal range despite a lack of

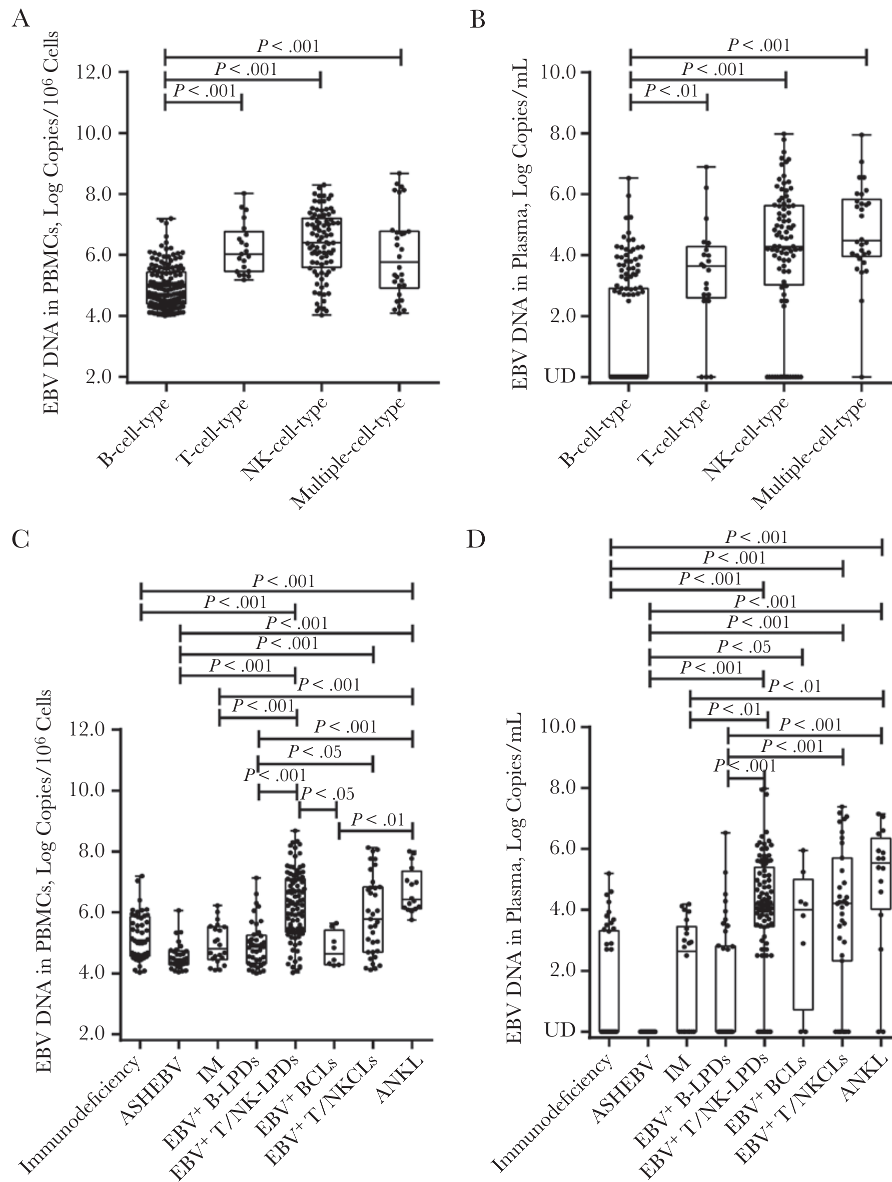

Figure 2. Box-and-whisker plots of Epstein-Barr virus (EBV) DNA levels in peripheral blood mononuclear cells (PBMCs) and plasma by EBV-infected cell type and disease entity. *A, B,* EBV DNA levels at time of diagnosis in PBMCs (*A*) or plasma (*B*) among patient groups with different EBV-infected cell types, including B (n = 152), T (n = 21), natural killer (NK) (n = 88), and multiple cell types (n = 30). *C, D,* EBV DNA levels at time of diagnosis in PBMCs (*C*) or plasma (*D*) among patient groups with different EBV-associated (EBV$^+$) disease entities, including immunodeficiency (n= 46), asymptomatic high EBV carriage (ASHEBV) (n = 30) infectious mononucleosis (IM) (n = 22), EBV$^+$ B-cell lymphoproliferative diseases (B-LPDs) (n = 41), EBV$^+$ T/NK-LPDs (n = 93), EBV$^+$ B-cell lymphomas (BCLs) (n = 8), EBV$^+$ T/NK-cell lymphomas (T/NKCLs) (n = 35), and aggressive NK-cell leukemia (ANKL) (n = 16). For all graphs, median levels (*lines*), 25th to 75th percentiles (*box*), and range (*whiskers*) are shown for each patient group. Significant differences between groups are shown with the corresponding *P* values.

mutations in *PRF1,* except in 1 patient of *PRF1* heterozygotes (Supplementary Figure 1*A*). Abnormal granzyme levels were identified in 13 (28.3%) of 46 patients (Supplementary Figure 1*B*). Nearly all the patients with EBV$^+$ T/NK-LPDs were found to have ≥1 defect in NK-cell function, regardless of the mutational status of genes associated with lymphocyte cytotoxicity.

Factors Correlated With Survival in EBV$^+$ T/NK-LPDs

Kaplan-Meier analysis revealed that the patients >60 years old at onset of disease and those who had hypoimmunoglobulinemia, interstitial pneumonia, or HLH in the natural course of disease had a significantly lower survival rate (Figure 5). Interestingly, there were no significant differences between = patients with different EBV-infected cell types and between those with or without detected mutations (Supplementary Figure 2). The clinical characteristics of the patients with EBV$^+$ T/NK-LPDs were analyzed using a Cox proportional hazard model (Figure 6). Age >60 years at onset of disease, hepatomegaly, hypoimmunoglobulinemia, EBV DNA levels in plasma $\geq 1 \times 10^5$ copies/mL, interstitial pneumonia, and the presence of HLH were further included in multivariate

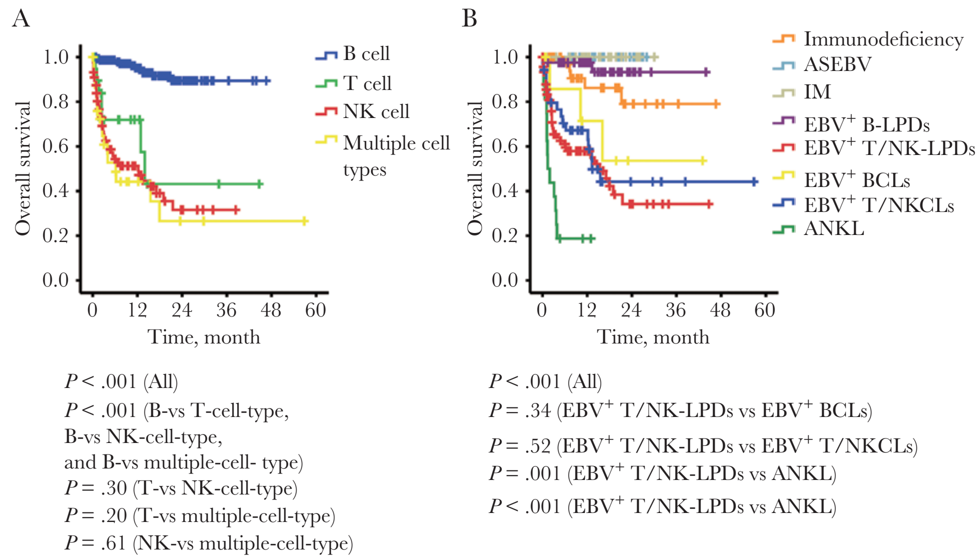

Figure 3. Survival analysis in patients with Epstein-Barr virus–associated (EBV+) hematological diseases. *A,* Kaplan-Meier survival curves for overall survival (OS) were compared by EBV-infected cell type, including B (n = 152), T (n = 21), natural killer (NK) (n = 88) and multiple cell types (n = 30). *B,* Kaplan-Meier survival curves for OS of patients were compared by EBV+ disease entity, including immunodeficiency (n= 46), asymptomatic high EBV carriage (ASHEBV) (n = 30), infectious mononucleosis (IM) (n = 22), EBV+ B-cell lymphoproliferative diseases (LPDs) (B-LPDs) (n = 41), EBV+ T/NK-cell LPDs (T/NK-LPDs) (n = 93), EBV+ B-cell lymphomas (BCLs) (n = 8), EBV+ T/NK-cell lymphomas (T/NKCLs) (n = 35), and aggressive NK-cell leukemia (ANKL) (n = 16).

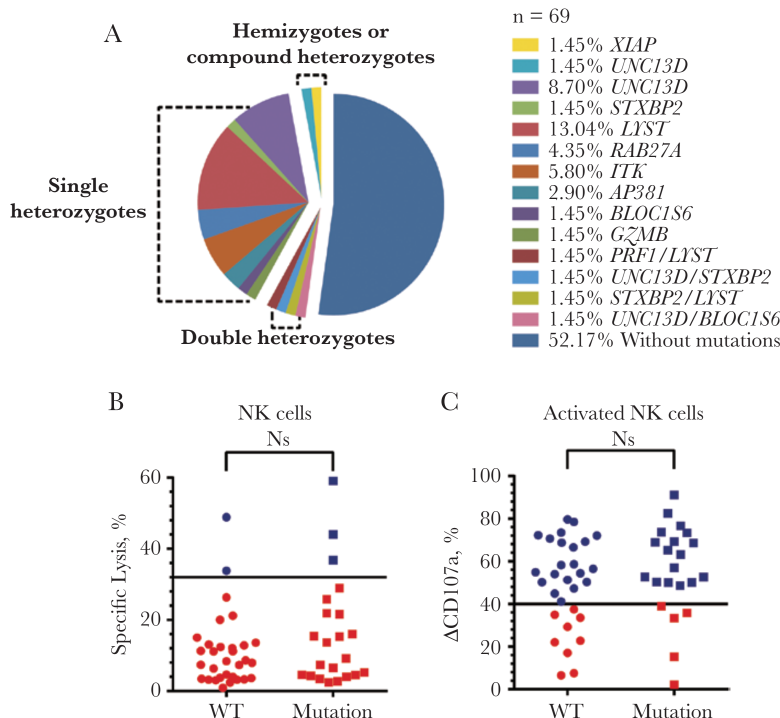

Figure 4. Mutations in genes associated with immunodeficiency and natural killer (NK)–cell functions in Epstein-Barr virus–associated (EBV+) T/NK-cell lymphoproliferative diseases (T/NK-LPD)s. *A,* Mutational analysis was carried out in 69 patients with EBV+ T/NK-LPDs using a next-generation targeted sequencing panel covering genes associated with immunodeficiency. A 12-gene panel was tested in 30 patients and a 26-gene panel in 39. The percentage of patients with mutations detected in EBV+ T/NK-LPDs are shown. *B,* Comparison of NK cell cytotoxicity to K562 cells in patients with EBV+ T/NK-LPDs without (n = 31) or with (n = 22) detection of mutations. *C,* CD107a degranulation assays were performed using interleukin 2 (IL-2)–activated NK cells derived from patients with EBV+ T/NK-LPDs without (n = 30) or with (n = 22) detection of mutations. ΔCD107a represents the net increase in the percentage of CD107a+ cells after stimulating K562 cells with IL-2. The lines in the graphs represent the lower limit of the normal range established with healthy donors. Each dot represents the corresponding mean of triplicates for an individual patient. Abbreviations: NS, not significant (*P*> .05); WT, wild type.

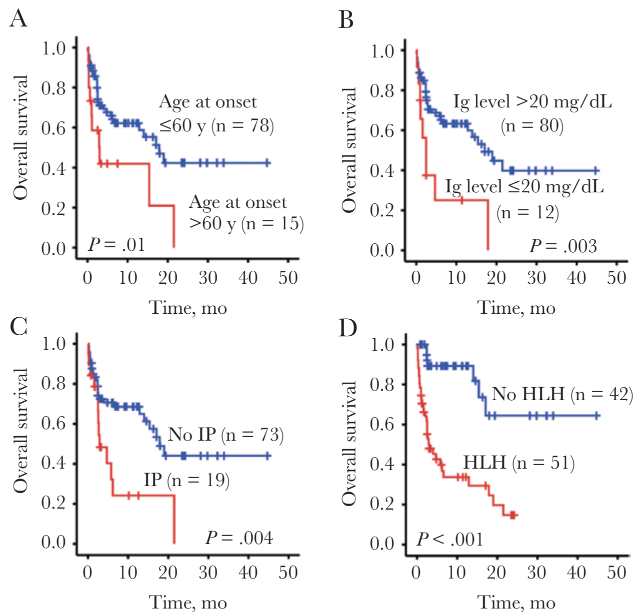

Figure 5. Probability of survival rates in patients with Epstein-Barr virus–associated T/natural killer–cell lymphoproliferative diseases, according to different prognostic factors. Kaplan-Meier estimates of overall survival were calculated among subgroups by age at onset (≤60 or >60 years) (*A*) and by the presence or absence of hypoimmunoglobulinemia (immunoglobulin [Ig] level ≤20 mg/dL) (*B*), interstitial pneumonia (IP) (*C*), or hemophagocytic lymphohistiocytosis (HLH) (*D*). Significant differences between groups are shown, with *P* values. Abbreviation: mo, month.

analysis, whereas anemia, thrombocytopenia, ferritin levels >500 μg/L, and fibrinogen levels ≤1.5 g/L were excluded because these factors were key elements of HLH. In multivariate analysis, HLH was the only parameter that was significantly correlated with a worse OS (hazard ratio, 3.56; 95% confidence interval, 1.43–8.87; $P = .007$).

DISCUSSION

To date, the relationship between the EBV-infected cell type in PBMCs and disease entity has not been comprehensively investigated. In the current study, we used MACS and real-time PCR techniques to explore EBV-infected cell types in PBMCs from 291 patients, and we summarized the diseases entities involved. Double-labeling techniques in tissue specimens for the simultaneous detection of EBER with in situ hybridization and cell lineage markers with immunohistochemistry have been used for some malignant EBV$^+$ diseases [25, 26]. However, determination of EBV-infected cell types is not performed routinely in the diagnosis of EBV$^+$ hematological diseases because tissue biopsy specimens are is not always obtained owing to the invasive nature of biopsies, lack of specific sites, or difficulty of access. In the current study, we overcame these restrictions by using a noninvasive and rapid method to determine EBV-infected cell types in PBMCs.

B-cell-type infection was observed in immunocompromised patients and in patients with ASHEBV, IM, EBV$^+$ B-LPDs, and EBV$^+$ BCLs. With the exception of EBV$^+$ BCLs, and PTLD, most of these B-cell-type diseases merely reflected the self-limited reactivation of EBV infection related to the EBV life cycle. The prevailing clinical treatment options include watching/waiting and steroid and/or antiviral therapies. In contrast, T-cell, NK-cell, and multiple-cell-type EBV infections in immunocompetent hosts are highly suggestive of EBV$^+$ T/NK-cell diseases, which require immediate diagnosis and prompt treatment. EBV levels in PBMCs are much higher in patients with T-cell, NK-cell, and multiple-cell-type EBV infections than in those with B-cell infection, suggesting that patients with high EBV levels in PBMCs are more likely to have non–B-cell infection. We thus concluded that individuals with persistent high EBV loads in PBMCs, especially >1 million copies/10^6 cells, should be examined for dominant EBV-infected lymphocyte cell types in PBMCs as the first step in the differential diagnosis of EBV$^+$ hematological diseases.

EBV$^+$ T/NK-cell diseases consist of a broad spectrum of lymphoproliferative disorders characterized by persistent EBV infection in NK and/or T cells, including EBV$^+$ T/NK-LPDs, EBV$^+$ T/NKCLs, and ANKL, which are mainly prevalent in East Asia and Latin America [27]. Many aspects of EBV$^+$ T/NK-LPDs remain unknown, especially in adult patients, in whom only very limited knowledge has been gained from a small series of case reports [28–32].

In the current study, EBV$^+$ T/NK-LPDs were diagnosed in 93 patients, 79.6% of whom were >18 years old. The study was therefore able to provide a comprehensive analysis of the clinical, genetic, and prognostic aspects of adult EBV$^+$ T/NK-LPDs. First, NK cells were identified as the dominant EBV infection target in our adult study cohort, whereas EBV$^+$ T/NK-LPDs among children in Japan are mainly related to EBV-infected T cells [14, 21, 33, 34]. Second, EBV$^+$ T/NK-LPDs exhibit a lymphomalike prognosis. They initially present with persistent or recurrent symptoms similar to those of IM but often develop into HLH or undergo malignant transformation. The majority of patients with EBV$^+$ T/NK-LPDs responded to HLH-2004 regimen or DEP regimen but were unable to sustain remission without allo-HSCT consolidation.

Third, in contrast to the previous finding that only 10% of pediatric patients with EBV$^+$ HLH in China were found to have genetic defects [35], 47.8% of the patients in our cohort were found to carry genetic defects. The differences in the infected cell types and the higher rate of deleterious mutations in adult patients suggest that the pathogenesis underlying adult EBV$^+$ T/NK-LPDs is significantly different from that in children. However, there were no significant differences in OS or clinical manifestations between the patients with and those without mutations. This finding strongly suggests that the EBV infection of T/NK cells in immunocompetent hosts is a more valuable surrogate marker than the targeted sequencing panel that covers only a limited number of genes. Moreover, HLH was the

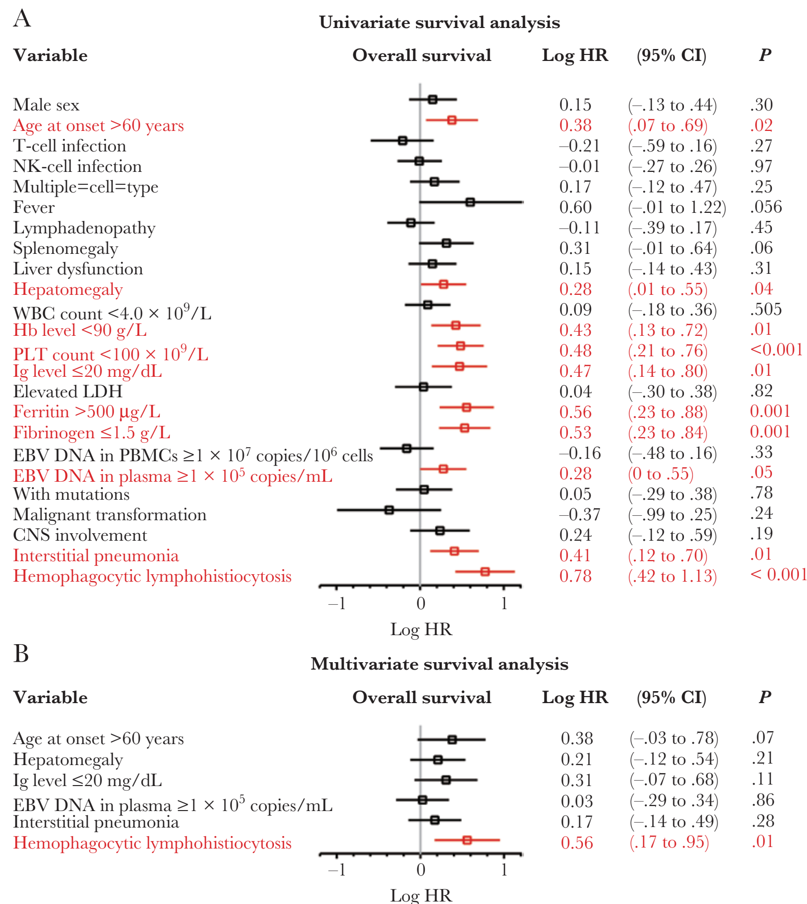

Figure 6. Forest plot of log hazard ratios (HRs) for overall survival according to clinical characteristics. Univariate (*A*) and multivariate (*B*) survival analysis of patients with Epstein-Barr virus–associated (EBV$^+$) T/natural killer (NK)–cell lymphoproliferative diseases based on Cox proportional hazards models. In multivariate analysis, the following factors were excluded because they were key elements of hemophagocytic lymphohistiocytosis: hemoglobin level <90 g/L, platelet count <100 × 10^9/L, ferritin level >500 µg/L, and fibrinogen level, ≤1.5 g/L. Liver dysfunction was defined as an increase in alanine transaminase level to twice the normal upper limit. Malignant transformation was defined as the presence of extranodal NK/T-cell lymphoma, aggressive NK-cell leukemia, or peripheral T-cell lymphoma during the follow-up period. Abbreviations: CI, confidence interval; CNS, central nervous system; Hb, hemoglobin; HR, hazard ratio; Ig, immunoglobulin; LDH, lactate dehydrogenase; PBMCs, peripheral blood mononuclear cells; PLT, platelet; WBC, white blood cell.

only parameter that was significantly correlated with a worse OS in the multivariate analysis, which demonstrates that HLH is the most important indication for allo-HSCT consolidation in patients with EBV$^+$ T/NK-LPDs.

Taken together, the determination of EBV-infected lymphocyte cell types among PBMCs is a valuable diagnostic tool for the discrimination of EBV$^+$ hematological diseases. Based on the EBV-infected lymphocyte cell types and the host's immune state (immunocompetent or immunocompromised), EBV$^+$ hematological diseases can be feasibly subclassified for further differential diagnosis. T/NK-cell-type EBV infection in immunocompetent hosts is suggestive of either T/NK cancers or lymphoproliferative disorders that require close monitoring and aggressive therapy.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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Author contributions. M. X. and Jianfeng Zhou proposed the conception of the work and designed the study; P. Z., C. Z., J. C., Jing Zhou, J. G., X. M., W. Z., Y. C., H. L., B. X., and Q. L. contributed to the collection of data; P. Z. and C. Z. performed data analysis; P. Z. wrote the manuscript; M.X. and Jianfeng Zhou supervised the manuscript; and all authors approved the final manuscript.

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