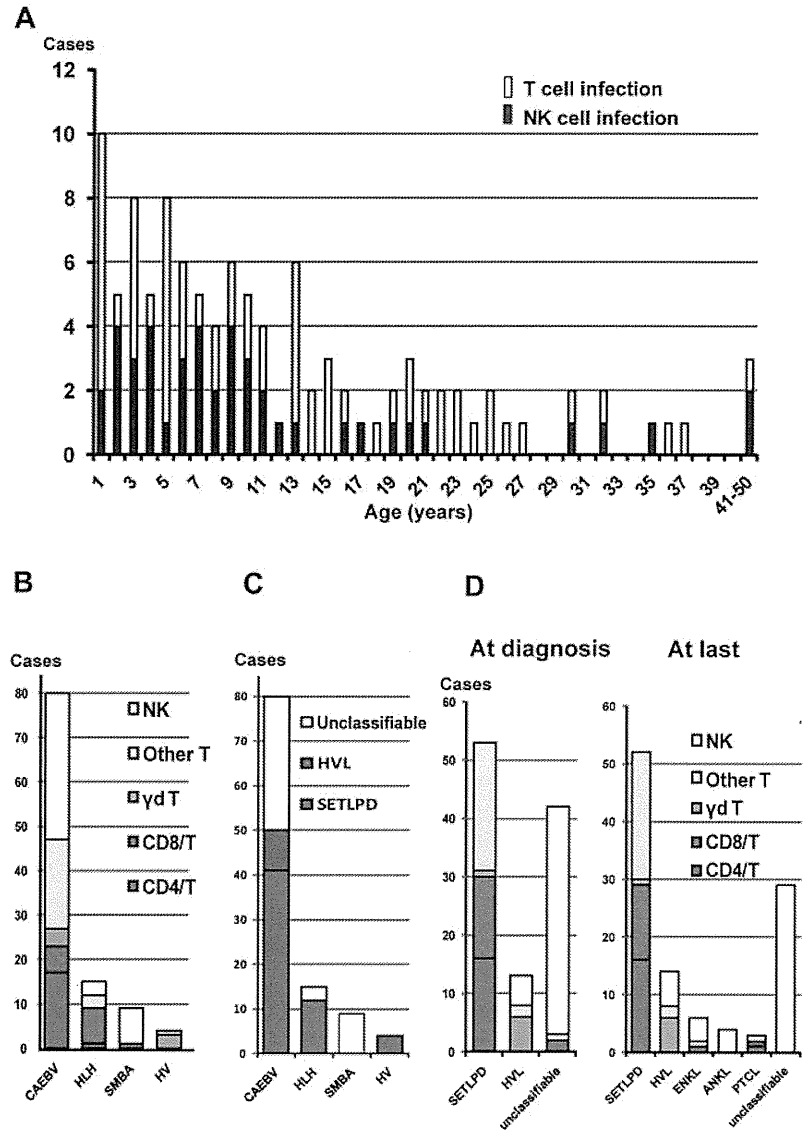

Figure 1. EBV-infected cell phenotypes of EBV⁺ T/NK lymphoproliferative diseases. (A) Age distribution of patients with T-cell and NK-cell types. (B) EBV-infected cells among categories of clinical groups. Infected T cells were further divided into CD4⁺ T cells, CD8⁺ T cells, γδ T cells, and "other T cells." The 25 cases of "other T cells" were defined as either phenotypically different T-cell subsets (2 patients were CD4⁻CD8⁻, 1 patient was CD4⁺CD8⁺, and 1 patient had 2 lineages consisting of CD4⁺CD8⁻ and CD4⁻CD8⁺ cells) or ill-defined T cells (n = 21). In the majority of the ill-defined T-cell patients, Abs against CD4 or CD8 could not be used to define their CD4/CD8 phenotype because the number of recovered PBMCs was not sufficient. SMBA indicates severe mosquito bite allergy; and HV, hydroa vacciniforme. (C) The 4th WHO pathologic classification of each clinical group at the time of diagnosis. SETLPD indicates systemic EBV⁺ T-cell lymphoproliferative disease of childhood; and HVL, hydroa vacciniforme–like lymphoma. (D) EBV-infected cells among categories of the pathologic classification at diagnosis and at the last follow-up or death. Patients in CR were classified according to the data and status before remission.

classification. Furthermore, prognostic factors and the efficacy of therapeutic interventions including hematopoietic stem cell transplantation (HSCT) were analyzed.

Methods

Eligibility criteria

Informed consent was obtained from all participants or their guardians in accordance with the Declaration of Helsinki. This study was approved by the institutional review board of Nagoya University Graduate School of Medicine. From 1998 to 2010, patients whose samples were sent to Nagoya University Graduate School of Medicine for determination of the EBV-infected cell phenotype and who fulfilled the following criteria were prospectively enrolled in this study: (1) EBV-associated T/NK-LPD suspected or diagnosed based on clinical and/or histopathological findings; (2) high EBV load detected in PBMCs by quantitative PCR ($\geq 10^{2.5}$ copies/μg of EBV-DNA)[12,32]; and (3) EBV infection in T or NK cells in the peripheral blood confirmed by either immunobead sorting followed by quantitative PCR[34-35] or FISH.[36] Exclusion criteria were: (1) pathologically defined ENKL,[5] ANKL,[37] or peripheral T-cell lymphoma (PTCL)[38]; (2) congenital immunodeficiency; (3) HIV positivity; and (4) other immunodeficiencies requiring immunosuppressive therapies or underlying diseases with potential immunosuppression. Patients were recruited through an announcement by the Japanese Association for Research on Epstein-Barr Virus and Related Diseases and on the homepage of our institute's website. Approximately 240 hematology units and 400 departments of pediatrics were included in the association.

On entry into the study, peripheral blood was collected and sent to Nagoya University Graduate School of Medicine to examine EBV-DNA quantification and EBV-infected cell determination along with detailed clinical data. Clonality analyses were also performed at this time if possible. Primary EBV infection was determined based on serological findings, detection of antiviral capsid Ag-IgM, and seroconversion of either antiviral capsid Ag-IgG or anti-EBV nuclear Ag. A total of 108 patients from 40 hospitals were enrolled in the study (25 from Nagoya University Hospital, 13 from Osaka Medical Center and Research Institute for Maternal and Child Health, 9 from Fukushima Medical University, and 61 from other hospitals). Each patient enrolled in the study was treated according to physician decision at each hospital. The physicians completed questionnaires regarding the administered treatment and outcome every 3 years (2001, 2004, and 2007); the final questionnaire was sent and collected in December 2010. Compared with data provided by previous national surveys for CAEBV and HLH,[29,31] we estimated that approximately 15%-20% of systemic EBV⁺ T/NK-LPD cases during the study period were recruited by this registry.

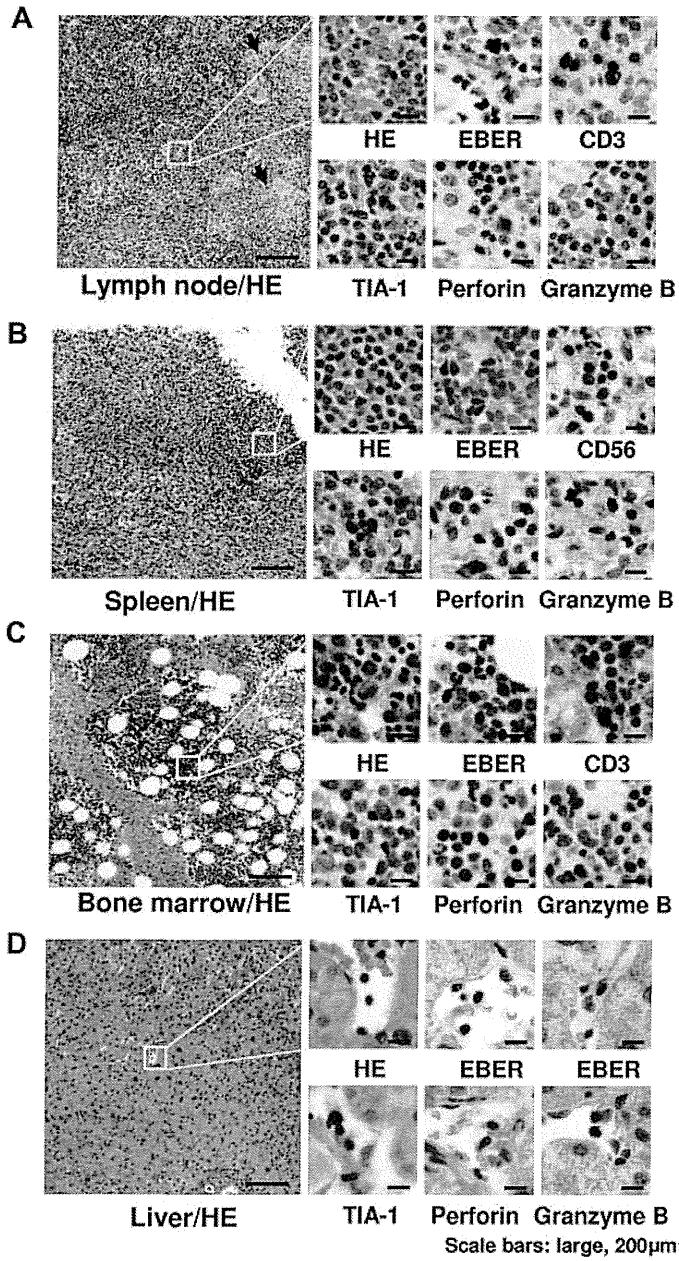

Figure 2. Histopathological findings of representative patients. (A) Cervical lymph node from a 6-year-old boy with chronic active EBV disease with T-cell infection (patient 3). Follicles and paracortical hyperplasia including a mild increase in transformed lymphocytes were seen. Focal epithelioid reactions were detected (arrows). Medium-sized transformed lymphocytes in the paracortex were positive for EBER. TIA-1 and perforin were positive, but granzyme B was negative. (B) Spleen from a 13-year-old boy with chronic active EBV disease with NK-cell infection (patient 6). White pulp was atrophic and red pulp showed congestion. Small lymphocytes infiltrating in the red pulp were positive for EBER. TIA-1 and perforin were positive, but granzyme B was negative. (C) BM from a 25-year-old female with chronic active EBV disease with T-cell infection (patient 17). In the mild hyperplastic BM, small lymphocytes were positive for EBER. TIA-1, perforin, and granzyme B were positive. (D) Liver from a 42-year-old female with chronic active EBV disease with NK-cell infection (patient 60). Small lymphocytes infiltrating in vessels and sinusoid were positive for EBER. TIA-1, perforin, and granzyme B were positive. HE indicates H&E staining. Images of sections were obtained by a microscopy (BX50, Olympus Corp) with CCD camera (D5-5M-L1, Nikon Corp). Each micrograph was represented at either a 100× or 400× magnification using 10× or 40× objective lens (UPlanFL, Olympus Corp), respectively.

Patient criteria

Patients were clinically divided into 4 groups according to the clinical categorization at the 2008 National Institutes of Health meeting: (1) CAEBV of T/NK–cell type, (2) EBV-associated HLH, (3) hydroa vacciniforme, and (4) severe mosquito bite allergy.[39] The clinical diagnosis was made at entry into the study. Definitions of each clinical category are listed in Table 1. CAEBV was defined according to previously proposed criteria.[16,29] HLH was defined based on the criteria proposed by an international treatment study group.[11] Severe mosquito bite allergy and hydroa vacciniforme were applied for cases with only skin symptoms and lacking systemic symptoms. In this study, "severe mosquito bite allergy" and "hydroa vacciniforme" were used as clinical categories, whereas "hypersensitivity to mosquito bites" and "hydroa vacciniforme–like eruptions" were used as terms for symptoms; "hydroa vacciniforme–like lymphoma" was used as a term for pathologic classification.

Patients were also classified according to the 4th WHO classification for tumors of hematopoietic and lymphoid tissues.[7] The definitions of pathologic classification are listed in Table 1. The classification was made both at the diagnosis and at the last follow-up or death. Patients diagnosed with ENKL, ANKL, or PTCL were excluded from the study, but some developed these diseases during the follow-up period. Of 108 patients, 54 were biopsied (liver, n = 15; skin, n = 15; lymph nodes, n = 10; intestine, n = 3; spleen, n = 2; muscle, n = 2; others, n = 7), and 6 were autopsied. For differential diagnosis, BM examination was performed in most patients (79%), even though there were no hematologic abnormalities of the peripheral blood. When abnormal findings were detected in BM or peripheral blood, EBER/immunohistochemical staining was performed. Histopathology was reviewed by the Central Pathology Review Board (Shigeo Nakamura, Nagoya University and Koichi Ohshima, Kurume University).

Disease status was defined as follows: stable disease, partial remission (PR), and complete remission (CR). Patients with PR had no symptoms but had significant EBV loads in PBMCs (EBV-DNA $\geq 10^{2.5}$ copies/μg of DNA).[12,32] CR patients had no symptoms and continuously low or no EBV loads in PBMCs (EBV-DNA $< 10^{2.5}$ copies/μg DNA). Disease activity was assessed before HSCT and was classified as either active or inactive as described previously.[40] Active disease was defined by the existence of symptoms and signs such as fever, persistent hepatitis, lymphadenopathy,

Table 2. Comparison of characteristics based on EBV-infected cell type in 108 patients with EBV$^+$ T/NK-LPD

	Total cells (n = 108)	T cells (n = 64)	NK cells (n = 44)	P*
Sex (male/female)	50/58	27/37	23/21	NS
Age at disease onset, y	12.1 ± 10.6	12.7 ± 10.3	11.3 ± 11.0	NS
Clinical category at diagnosis, n				
CAEBV	80	47	33	NS
HLH	15	12	3	.066
Severe mosquito bite allergy	9	**1**	**8**	**.003**
Hydroa vacciniforme	4	4	0	NS
Past history of infectious mononucleosis, n (%)	37 (34)	24 (22)	13 (12)	NS
Primary infection at diagnosis, n (%)	19 (18)	**16 (15)**	**3 (3)**	**.012**
EBV DNA quantity in peripheral blood at diagnosis				
Mononuclear cells, log copies/μg DNA, mean	4.3 ± 0.9	4.2 ± 0.9	4.5 ± 0.8	NS
Plasma, log copies/mL, mean	3.3 ± 1.7	3.5 ± 1.6	3.1 ± 2.0	NS
EBV clonality, monoclonal/oligoclonal/polyclonal	64/8/4	36/4/3	28/4/1	NS
TCR rearrangement, any rearrangement/none	42/48	**36/20**	**6/28**	**< .001**
Chromosomal aberration (abnormal/normal cases)	6/84	4/50	2/34	NS
Symptoms and signs at diagnosis, n (%)				
Fever	98 (91)	59 (92)	39 (89)	NS
Liver dysfunction	83 (77)	49 (77)	34 (77)	NS
Splenomegaly	64 (59)	39 (61)	25 (57)	NS
Thrombocytopenia	47 (44)	26 (41)	21 (48)	NS
Anemia	46 (43)	29 (45)	17 (39)	NS
Lymphadenopathy	41 (38)	27 (42)	14 (32)	NS
Hemophagocytic syndrome	38 (36)	23 (36)	15 (34)	NS
Hypersensitivity to mosquito bites (HMB)	32 (30)	**3 (5)**	**29 (43)**	**< .001**
Hydroa vacciniforme-like eruption (HV-LE)	15 (14)	8 (13)	7 (16)	NS
HMB$^+$HV$^-$LE$^+$	5 (5)	**0 (0)**	**5 (11)**	**.001**
HMB$^-$HV$^-$LE$^+$	10 (9)	**8 (13)**	**2 (5)**	NS
Chemotherapy, n (%)	70 (65)	45 (70)	25 (57)	NS
HSCT, n (%)	59 (55)	32 (50)	27 (61)	NS
Outcome, n (%)				
Dead	47 (44)	27 (42)	20 (45)	NS
Alive	61 (57)	37 (58)	27 (61)	NS
Stable disease	11 (10)	8 (13)	3 (7)	NS
Complete remission	46 (43)	26 (41)	20 (20)	NS
Partial remission	4 (4)	3 (5)	1 (2)	NS

NS indicates not significant.
*$P < .10$ are shown; $P < .05$ (shown in bold) are statistically significant.

hepatosplenomegaly, pancytopenia, or progressive skin lesions along with an elevated EBV load in the peripheral blood. Liver dysfunction was defined as an increase in alanine transaminase levels to 2 times above the upper limit of normal on at least 2 consecutive occasions.

Analyses of EBV and determination of EBV-infected cells

DNA was extracted from 1×10^6 PBMCs or 200 μL of plasma and real-time quantitative PCR was then performed as described previously.[12,32] EBV clonality was assessed by Southern blotting with a terminal repeat probe, as described previously.[12,41] To determine which cell population harbored EBV, either immunobead sorting followed by quantitative PCR or FISH assay was performed. For the former method, PBMCs were fractionated into CD3$^+$, CD4$^+$, CD8$^+$, CD16$^+$, CD19$^+$, CD56$^+$, TCRαβ$^+$, and TCRγδ$^+$ cells using an immunobead method (IMag Cell Separation System; BD Biosciences) that resulted in 97%-99% purity.[34-35] Purified cells were analyzed by real-time quantitative PCR. The infected-cell phenotypes were determined in comparison with unfractionated (whole) PBMCs, as described previously.[34-35] For example, patients were defined as CD3$^+$ when CD3$^+$ cells contained higher amounts of EBV DNA than whole PBMCs. The FISH assay was performed as described previously.[36] Briefly, PBMCs were stained with fluorescence labeled mAbs against surface marker, fixed, permeabilized, and hybridized with EBER-specific PNA Probe/FITC (Y5200; Dako). After enhancing fluorescence, stained cells were analyzed using a FACSCalibur flow cytometer and CellQuest Version 5.1.1 software (BD Biosciences). More than 0.1% of EBER$^+$ cells was considered to be significant and such subset was designated EBV$^+$. This frequency was chosen based on previous data using EBV$^+$ cell lines.[36]

TCR gene rearrangement

TCR gene rearrangement was determined by multiplex PCR using the T-cell Gene Rearrangement/Clonality assay (InVivoScribe Technologies), which was developed and standardized in a European BIOMED-2 collaborative study.[42]

Histopathology

Immunostaining was performed using an avidin-biotin peroxidase complex method with mAbs against CD3 (Dako), CD56 (Novocastra Laboratories), perforin (Novocastra Laboratories), T cell–restricted intracellular Ag 1 (TIA-1; Immunotech), and granzyme B (Monosan).[43] FISH was performed using the EBER probe (Dako) as described previously.[43] Hybridization was detected using mouse monoclonal anti-FITC Ab (Dako) and a Vectastain ABC kit (Vector).

Statistical analysis

Statistical analysis was performed using SPSS for Windows Version 18.0. For univariate analysis, either the χ^2 or the Fisher exact test (single-sided) was used to compare categorical variables. The Mann-Whitney U test was used to compare quantitative variables. Logistic regression analysis was used for multivariate analysis. Comparison between quantities of EBV-DNA in PBMCs and plasma was performed by regression analysis. The Kaplan-Meier method and the log-rank test were used for survival analysis. $P < .05$ was considered statistically significant for all analyses.

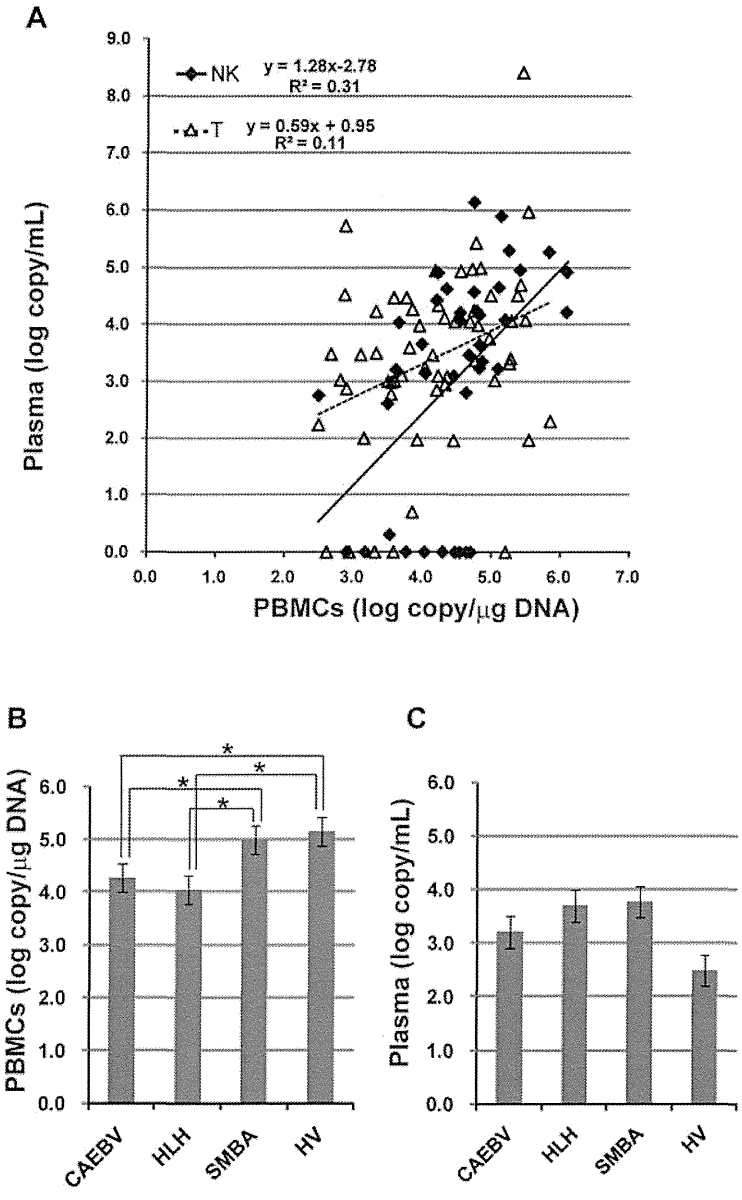

Figure 3. Viral load in the peripheral blood at the time of diagnosis. EBV-DNA was quantified by real-time PCR. (A) Correlation of viral load between PBMCs and plasma. The correlation was separately estimated in patients with T-cell infection and those with NK-cell infection. (B) Quantity of EBV-DNA in PBMCs among categories of clinical groups. *$P < .05$. (C) Quantity of EBV-DNA in plasma among categories of clinical groups. SMBA indicates severe mosquito bite allergy; and HV, hydroa vacciniforme.

Results

Characteristics of patients with EBV$^+$T/NK-LPD

A total of 108 patients (50 men and 58 women) were enrolled in this study. Detailed characteristics of each patient are shown in supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Age at diagnosis ranged from 1 to 51 years (median, 14 years). At the time of diagnosis, the main phenotypes of EBV-infected cells in the peripheral blood were T cells and NK cells in 64 and 44 patients, respectively. Onset age ranged from 1 to 50 years (median, 9 years). Most patients (91%) were children and young adults less than 30 years of age, but there were some middle-aged patients (age range, 30-50 years) also existed (Figure 1A). There was no difference in onset age between patients with the T-cell type and those with the NK-cell type. The former were further subdivided into the CD4$^+$ T-cell type (n = 18), the CD8$^+$ T-cell type (n = 14), the γδ T-cell type (n = 7), and other or ill-defined T-cell type (n = 25). In 2 patients (patients 92 and 100, supplemental Table 1), 2 lineages of cells were infected with EBV.

After entry into the study, patients were clinically categorized into 4 groups based on clinical symptoms and diagnostic criteria: CAEBV (n = 80), EBV-associated HLH (n = 15), severe mosquito bite allergy (n = 9), and hydroa vacciniforme (n = 4; Figure 1B). The CAEBV group consisted of 47 patients with the T-cell type (59%) and 33 with the NK-cell type (41%); the former were further subdivided into the CD4$^+$ T-cell type (21%), the CD8$^+$ T-cell type (8%), and the γδ T-cell type (5%). Eight of 15 (53%) EBV-associated HLH patients had EBV-harboring CD8$^+$ T cells, in contrast to their low occurrence in the other clinical groups. In addition, most patients (89%) with severe mosquito bite allergy had EBV-infected NK cells, whereas many (75%) with hydroa vacciniforme had EBV-infected γδ T cells (Figure 1B). Therefore, clinical profiles were closely linked with the EBV$^+$ cell immunophenotype.

Between 1 and 349 months from the onset of disease (median, 46 months), 47 patients had died, whereas 61 patients were alive for follow-up periods of 13-263 months (median, 82 months). The

main causes of death were multiple organ failure (n = 10), hepatic failure (n = 6), heart failure (n = 5), pulmonary failure (n = 5), sepsis (n = 5), intracranial hemorrhage (n = 5), intestinal hemorrhage or perforation (n = 3), hemophagocytic syndrome (n = 2), and other (n = 6). Of the 47 patients who died, 20 (42%) died after transplantation. Of the 61 surviving patients, 41 were in CR and 4 were in PR without any symptoms, whereas 16 remained in stable disease at the last follow-up.

Clonality analysis

At the time of diagnosis, viral clonality was analyzed by Southern blot analysis using EBV terminal repeat. Of 76 patients with available DNA, EBV-infected cells were monoclonal in 64 (84%) and oligoclonal in 8 (11%). Polyclonal EBV-infected cells were detected in only 4 patients (5%). TCR rearrangement was analyzed in 90 patients at the time of diagnosis, 42 of whom had monoclonal rearrangements. Six patients with NK-cell infection demonstrated TCR rearrangement. Because this analysis uses a PCR-based method, erroneous detection of a seemingly clonal cell population (pseudoclonality) or reduced TCR diversity caused by the prevalence of a few Ag-selected subclones, which are often seen in EBV infection, may occur.[42] Chromosomal aberrations were detected in the peripheral blood or lymph nodes at diagnosis in 6 patients, whereas an additional 6 patients later developed chromosomal aberrations in their clinical course of 1-9 years (median, 5 years). Patterns of chromosomal aberrations in each patient are shown in supplemental Table 2. These results provided additional support to the assertion that patients with EBV$^+$ T/NK-LPDs had clonality at early stages and subsequently developed overt lymphoma or leukemia with an increase of chromosomal aberrations in their clinical course.

Pathologic categories based on the 4th WHO classification

At the time of diagnosis, based on the 4th WHO classification, 53 and 13 patients were classified into systemic EBV$^+$ T-LPD of childhood and hydroa vacciniforme–like lymphoma, respectively. The proportion of these pathologic categories in each clinical group is shown in Figure 1C. Four patients clinically categorized to hydroa vacciniforme without any cellular atypia or systemic symptoms were classified into hydroa vacciniforme–like lymphoma based on the monoclonality of cells with TCR rearrangements. In systemic EBV$^+$ T-cell LPD, T-cell subsets of EBV-infected cells were variable (Figure 1D). In hydroa vacciniforme–like lymphoma, 6 of 13 patients had γδ T-cell infection. Conversely, 42 patients were not classified into either of these pathologic categories because they failed to correspond to criteria in the current WHO classification. Classification of each patient is shown in supplemental Table 1.

At the last follow-up or death, there were 29 patients who were unclassifiable, most of whom had CAEBV of the NK-cell type and severe mosquito bite allergy with NK-cell infection (Figure 1D). In the clinical course, ENKL developed in 6 patients (patients 2, 5, 20, 34, 60, and 81 in supplemental Table 1) after 9 months to 12 years of follow-up after onset (median, 1.5 years), whereas ANKL developed in 4 patients (patients 8, 43, 66, and 80) after 2-17 years of follow-up (median, 12 years); most of these patients had NK-cell infection. EBV$^+$ PTCL developed in 3 patients after 1 year (patient 83), 5 years (patient 93), and 20 years (patient 53) of follow-up. The EBV$^+$ PTCL patients in this study were characterized by their expression of cytotoxic molecules, nodal manifestation, lack of CD56 expression, and TCR gene rearrangement. These features

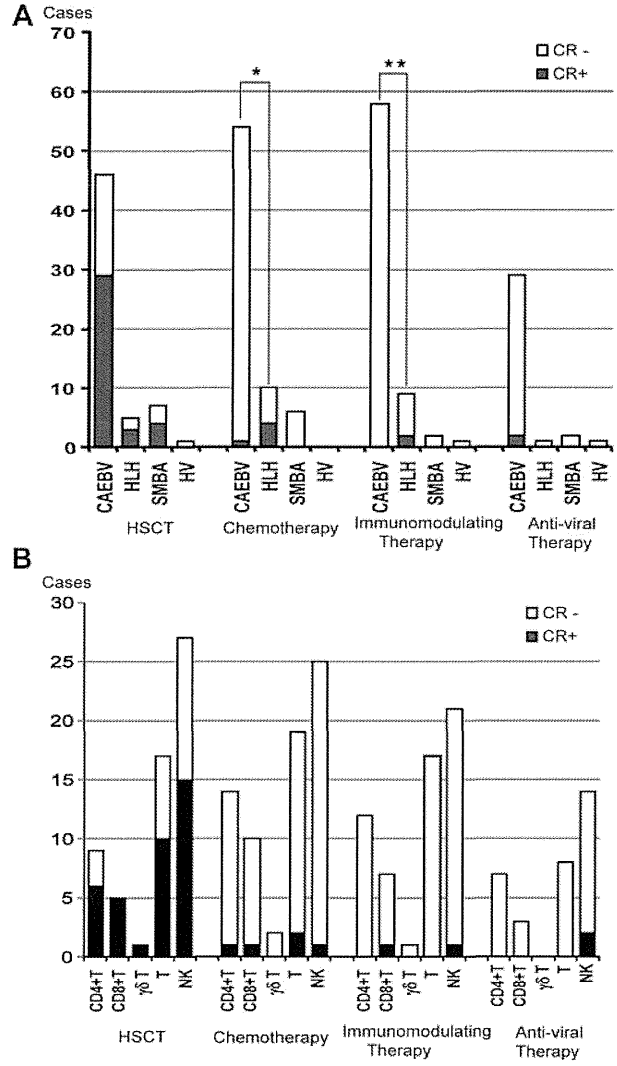

Figure 4. Efficacy of therapeutic interventions. (A) Number of patients treated with each therapy and patients who maintained CR are shown among categories of clinical groups. SMBA indictes severe mosquito bite allergy; and HV, hydroa vacciniforme. *P = .002; **P = .02. (B) Numbers of patients who received each therapy and those who maintained sustained CR are shown among categories of EBV-infected cells.

suggest a pathologic distinction between these EBV$^+$ PTCL and extranasal ENKL.

Representative results of histological examinations are shown in Figure 2. Histological findings and the number of EBER$^+$ cells varied among patients. EBER$^+$ lymphocytes were detected at various frequencies. Infiltrating cells (presumably EBV-infected) expressed cytotoxic molecules such as TIA-1, perforin, and granzyme B. BM aspirations showed various findings, but most patients had normocellular BM without any abnormal findings. Patients with EBV-associated HLH showed normoplastic or hyperplastic BM with mild or moderate hemophagocytosis. In all patients, however, BM findings showed an absence of hematologic malignant disorders at the time of diagnosis.

Differences between patients with T-cell and NK-cell infection

We compared clinical and virological differences between T- and NK-cell infections (Table 2). T-cell infection was characterized by higher rates of primary EBV infection and TCR rearrangement,

Table 3. Univariate and multivariate analyses of factors associated with mortality in 108 patients with EBV+ T/NK-LD

	Univariate analysis		Multivariate analysis†	
	OR (95% CI)	P*	OR (95% CI)	P‡
Female sex	**1.40 (0.98-1.97)**	**.048**	1.26 (0.48-3.31)	.64
Age at disease onset (≥ 8 years)§	**1.63 (1.17-2.28)**	**.003**	**4.43 (1.61-12.2)**	**.004**
Past history of infectious mononucleosis	0.62 (0.35-1.11)	.093	0.36 (0.12-1.02)	.054
Primary infection at diagnosis	0.47 (0.18-1.20)	.079	0.32 (0.08-1.25)	.10
Clinical entity at diagnosis				
CAEBV	1.12 (0.90-1.39)	NS		
HLH	0.65 (0.24-1.77)	NS		
Severe mosquito bite allergy	1.04 (0.30-3.65)	NS		
Hydroa vacciniforme	0.43 (0.05-4.03)	NS		
T-cell infection	1.13 (0.69-1.71)	NS		
NK-cell infection	0.95 (0.69-1.30)	NS		
EBV DNA in mononuclear cells (≥ $10^{4.5}$ copies/μg DNA)	1.16 (0.79-1.71)	NS		
EBV DNA in plasma (≥ $10^{3.5}$ copies/mL)	1.23 (0.84-1.72)	NS		
EBV monoclonality	1.08 (0.89-1.31)	NS		
TCR rearrangement	1.13 (0.73-1.76)	NS		
Chromosomal aberration	1.92 (0.34-10.9)	NS		
Symptoms and signs at diagnosis				
Fever	1.10 (0.98-1.24)	NS		
Liver dysfunction	**1.33 (1.09-1.63)**	**.006**	**4.25 (1.23-14.7)**	**.022**
Splenomegaly	**1.38 (1.01-1.88)**	**.033**	¶	
Anemia	**1.84 (1.18-2.88)**	**.005**	1.36 (0.31-6.01)	.68
Thrombocytopenia	**1.75 (1.13-2.71)**	**.009**	1.80 (0.44-7.33)	.41
Lymphadenopathy	1.24 (0.77-2.00)	NS		
Hemophagocytic syndrome	1.30 (0.72-2.32)	NS		
Hypersensitivity to mosquito bites	0.89 (0.69-1.15)	NS		
Hydroa vacciniforme-like eruption	0.86 (0.34-1.97)	NS		
Chemotherapy	0.84 (0.53-1.34)	NS		
HSCT	**0.67 (0.045-0.98)**	**.022**	**0.34 (0.12-0.96)**	**.041**
T-cell infection group	**0.54 (0.30-0.97)**	**.021**		
NK-cell infection group	0.83 (0.51-1.34)	NS		

NS indicates not significant.
*$P < .10$ are shown; $P < .05$ (shown in bold) are statistically significant.
†For multivariate analysis, factors with $P < .10$ were included.
‡$P < .05$ (shown in bold) are statistically significant.
§Stratified onset ages were analyzed in advance, and ≥ 8 years was chosen as the age factor.
¶Splenomegaly was excluded from multivariate analysis, because this factor was closely associated with anemia, thrombocytopenia, and liver dysfunction.

whereas a significant number (43%) of patients with NK-cell infection had hypersensitivity to mosquito bites (Table 2). Interestingly, 5 patients had both hypersensitivity to mosquito bites and hydroa vacciniforme–like eruptions; these patients all had NK-cell infection (Table 2). Conversely, 8 of 10 patients with hydroa vacciniforme–like eruptions but without hypersensitivity to mosquito bites had T-cell infections (Table 2).

A comparison of viral load in the peripheral blood between patients with T- and NK-cell infections detected similar levels of EBV-DNA in both PBMCs and plasma (Table 2). Correlation of viral loads between PBMCs and plasma was estimated (Figure 3A). The quantity of EBV-DNA in PBMCs was significantly correlated with that in plasma in both T-cell and NK-cell infections, although EBV-DNA was not detected in the plasma from 15 patients. We also compared viral load among clinical groups (Figure 3B-C). Interestingly, the quantity of EBV-DNA in PBMCs was significantly higher in patients with severe mosquito bite allergy and hydroa vacciniforme, but these patients did not have any systemic symptoms.

Efficacy of therapeutic interventions

Each patient received a variety of therapies. HSCT was administered to 59 patients, which induced sustained CR in 63% of patients with CAEBV, 60% of HLH patients, and 57% of severe mosquito bite allergy patients (Figure 4A). Seventy patients received chemotherapy such as etoposide/cyclosporine A/dexamethasone, cyclophosphamide/doxorubicin/vincristine/prednisolone (CHOP), CHOP plus etoposide, and high-dose cytosine arabinoside therapy. Chemotherapy was effective in some patients, but the effect was usually transient and failed to induce sustained CR in most cases. Chemotherapy induced sustained CR in only 5 patients, 4 of whom had HLH (Figure 4A). Immunomodulating therapies such as prednisolone, cyclosporine A, high-dose IV immunoglobulin, and methyl prednisolone pulse therapy were administered to 58 patients. The immunomodulating therapies induced sustained CR in 2 patients with HLH (Figure 4A). In patients with HLH, both chemotherapy and immunomodulating therapy induced sustained CR more frequently compared with those with CAEBV ($P = .002$ and $P = .02$, respectively). Antiviral therapies such as acyclovir, adenine arabinoside, and ganciclovir were administered to 32 patients. In 2 patients (patients 11 and 45 in supplemental Tale 1), sustained CR was achieved during oral acyclovir therapy and weekly IV administration of adenine arabinoside (Figure 4A). However, because antiviral therapies had been administered for a long time, it was not clear whether CR was induced by them or if it was spontaneously achieved.

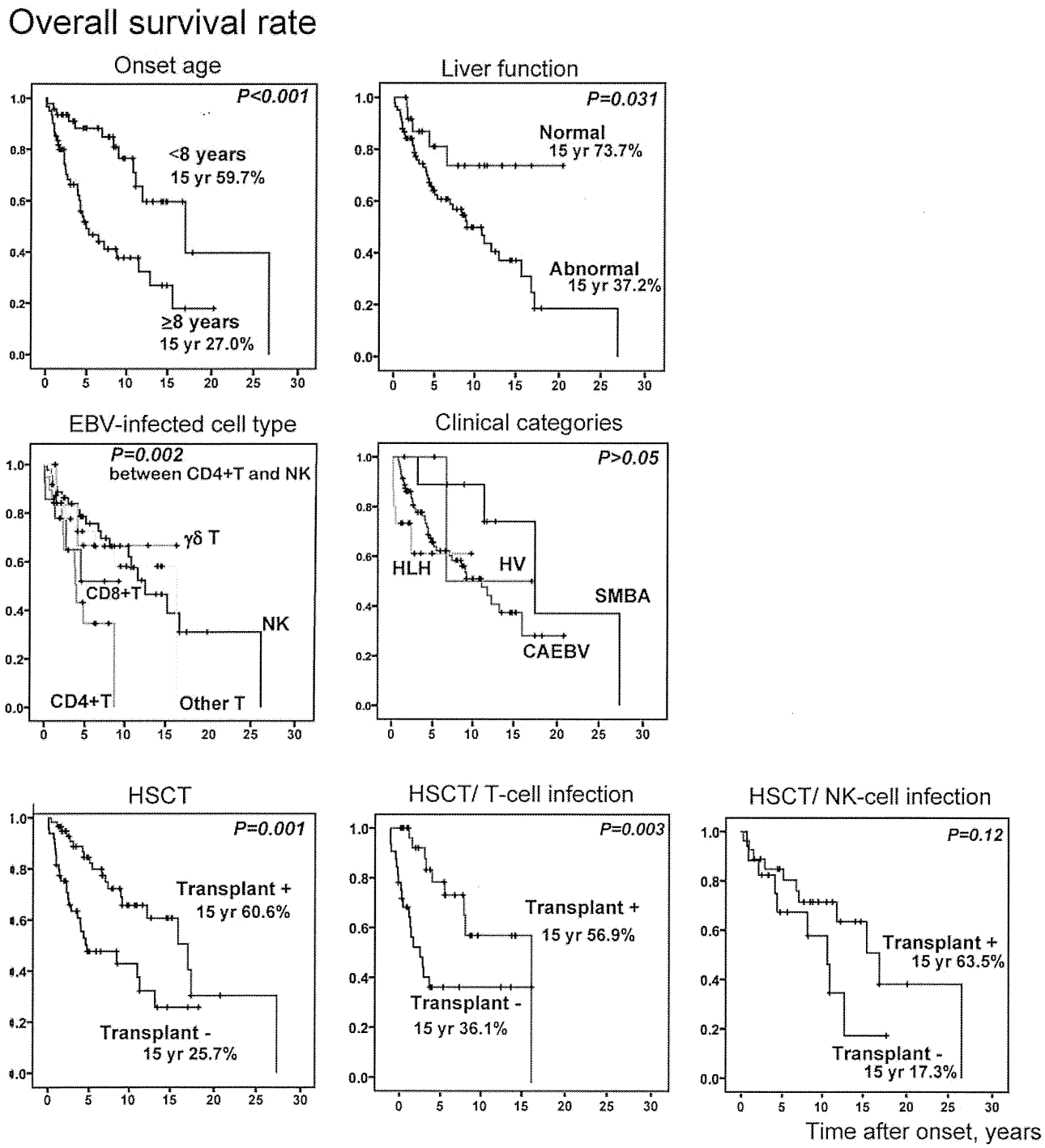

Figure 5. Probability of survival rates from time of disease onset. Overall survival rates from onset (n = 108) were calculated from Kaplan-Meier estimates between each subgroup (onset age ≥ 8 years or < 8 years, with or without liver dysfunction, EBV-infected cell types, clinical categories, and with or without HSCT). HSCT patients were divided into groups based on T-cell infection (n = 64) and NK-cell infection (n = 44) and independently analyzed. SMBA indicates severe mosquito bite allergy; and HV, hydroa vacciniforme.

The effects of each therapy among cell types are shown in Figure 4B. There was no statistical difference in the CR rate of each therapy among cell types.

Factors associated with mortality

The factors associated with mortality were analyzed (Table 3), and univariate analysis showed that sex (female), onset age (≥ 8 years), liver dysfunction, splenomegaly, anemia, and thrombocytopenia were significantly associated with mortality. Conversely, HSCT was inversely correlated with mortality rate (odds ratio, 0.67), and this was statistically significant only in patients with T-cell infection. Multivariate analysis using factors for which $P < .10$ revealed that onset age and liver dysfunction were independently significant factors that increased mortality (Table 3); again, HSCT was an independent factor that decreased mortality rate.

Table 4. Comparison of characteristics based on outcome in 59 patients after transplantation

	Total (n = 59)	Alive (n = 39)	Dead (n = 20)	P*
Sex, male/female	29/30	22/17	7/13	NS
Age at disease onset, y	11.8 ± 9.2	11.0 ± 9.0	13.6 ± 9.5	NS
Clinical category at diagnosis, n				
CAEBV	46	32	14	NS
HLH	5	3	2	NS
Severe mosquito bite allergy	7	4	3	NS
Hydroa vacciniforme	1	0	1	NS
EBV DNA quantity in peripheral blood at diagnosis				
Mononuclear cells, log copies/μg DNA	4.5 ± 0.8	4.4 ± 0.9	4.5 ± 0.89	NS
Plasma, log copies/mL	3.3 ± 1.6	3.3 ± 1.3	3.3 ± 2.0	NS
T-cell infection, n	32	23	9	NS
NK-cell infection, n	27	16	11	NS
Age at HSCT, y	17.5 ± 9.23	**15.6 ± 9.1**	**21.2 ± 8.3**	**.034**
Time from onset to HSCT, mo	65.0 ± 68.2	52.2 ± 54.7	90.0 ± 84.8	.059
Disease status at transplantation, active/inactive	25/34	**13/26**	**12/8**	**.046**
Preceded chemotherapy, n (%)	42 (71)	27 (69)	15 (75)	NS
Stem cell source, BM/peripheral blood/cord blood	35/11/13	22/8/9	13/3/4	NS
Donor, MRD/MUD/MMRD/MMUD	18/11/4/26	10/9/3/17	8/2/1/9	NS
No of mismatched HLA	0.76 ± 0.9	0.76 ± 0.9	0.75 ± 0.9	NS
Preconditioning regimen, myeloablative/reduced	21/38	11/28	10/10	.086

NS indicates not significant; MRD, matched related donor; MUD, matched unrelated donor; MMRD, mismatched related donor; and MMUD, mismatched unrelated donor.
*$P < .10$ are shown; $P < .05$ (shown in bold) are statistically significant.

We compared overall survival rates between each subgroup to confirm association of the above factors with mortality (Figure 5). Overall survival rate in patients whose onset was more than 8 years was significantly low ($P < .001$). Patients with liver dysfunction at the time of diagnosis had lower survival rate ($P = .031$). When patients were divided into 5 groups based on EBV-infected cells, patients with $CD4^+$ T-cell infection had a significantly lower survival rate compared with those with NK-cell infection ($P = .002$). However, there was no statistical difference in survival rate among clinical groups, although the numbers in some groups were small. Patients who received HSCT survived longer ($P = .001$) and, again, this was statistically significant only in patients with T-cell infection ($P = .003$).

Characteristics of patients after HSCT

Of 59 patients who underwent HSCT, 39 patients (66%) survived 1-144 months after transplantation (median, 35.5 months). Conversely, 20 patients (34%) died 1 day to 48 months after transplantation (median, 1.8 months). Detailed characteristics of each patient are shown in supplemental Table 3. Main causes of death were multiple organ failure (n = 5), intracranial hemorrhage (n = 5), sepsis (n = 2), and other (n = 8). Of the 20 deaths, 15 were considered to be treatment related. We compared various factors between patients who lived and those who died after HSCT (Table 4). Univariate analysis showed that age at HSCT was higher and patients with active disease status at the time of HSCT died more frequently after HSCT (Table 4). Time from disease onset to HSCT and intensity of the conditioning regimen (either myeloablative or reduced) were marginally associated with death ($P = .059$ and $P = .086$, respectively). To determine independent risk factors, we performed multivariate analysis using factors for which $P < .10$, and found that none was an independent risk factor for death (data not shown).

We compared overall survival rates (Figure 6A) and event-free survival rates (Figure 6B) of transplanted patients between each subgroup. Although disease status at HSCT was not an independent risk factor by multivariate analysis, overall survival rate was significantly higher in patients with inactive disease at the time of HSCT ($P = .014$); however, its significance diminished for the event-free survival rate. Patients who received HSCT at an age less than 15 years had significantly higher overall ($P = .013$) and event-free survival rates ($P = .015$). Patients whose time from onset to HSCT was less than 30 months also had significantly higher overall ($P = .036$) and event-free survival rates ($P = .033$). Interestingly, these were statistically significant only in patients with T-cell infection.

Discussion

Determining the phenotype of EBV-infected cells is mandatory for our further understanding of the pathogenesis of EBV^+ T/NK-LPDs and related biologic behaviors. In the present study, we used unfixed peripheral blood to determine the phenotypes of EBV-infected cells. One caveat of this study is that we may have missed EBV-associated T/NK-LPDs if EBV-infected cells failed to migrate into the peripheral blood.[33] Furthermore, EBV-infected cells in the peripheral blood might be different from those existing in tissues, although there was no discordant result between tissue biopsy and peripheral blood.

In the present study, EBV-infected cells in EBV^+ T/NK-LPDs were immunophenotypically divided into $CD4^+$ T cells, $CD8^+$ T cells, γδ T cells, and NK cells, the variable proportions of which were observed in each of the clinical categories. Kasahara et al reported that CAEBV and EBV-associated HLH were largely caused by $CD4^+$ T or NK cells and $CD8^+$ T cells, respectively.[22] We demonstrated that CAEBV was caused by not only $CD4^+$ T and NK cells but also by $CD8^+$ T and γδ T cells. We also demonstrated that EBV-infected cells in nearly half of hydroa vacciniforme or hydroa vacciniforme–like lymphoma patients were γδ T cells, which is in agreements with our previous observations.[36] Interestingly, all of these cells express molecules characteristic of cytotoxic cells. In fact, $EBER^+$ lymphocytes in EBV^+ T/NK-LPDs usually express cytotoxic molecules including perforin, granzyme B, and TIA-1, as shown in this study and in previous studies.[7,44]

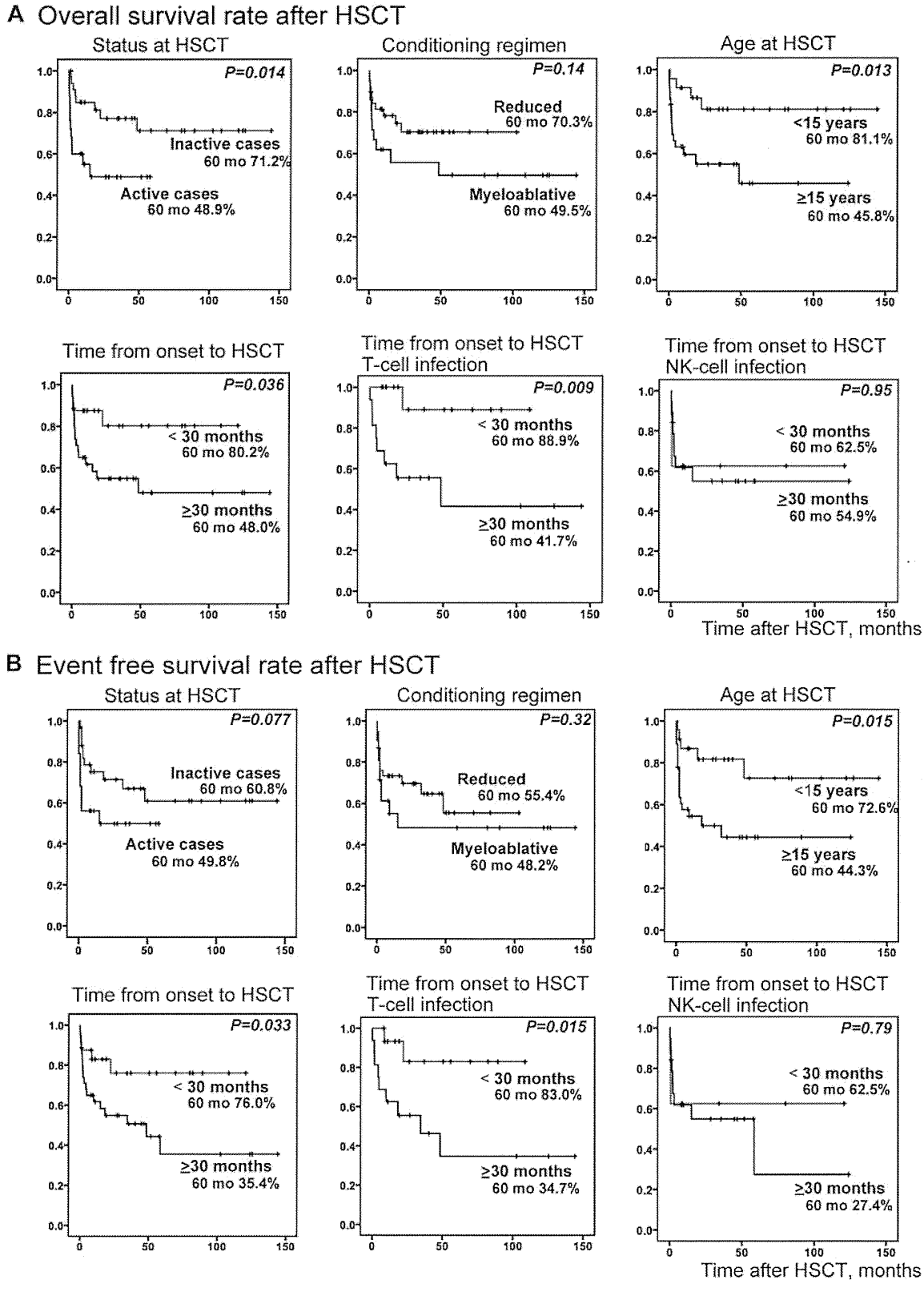

Figure 6. Probability of survival rates after HSCT. Survival rates after HSCT were calculated from Kaplan-Meier estimates between each subgroup (inactive or active cases at HSCT, reduced or myeloablative conditioning, age ≥ 15 years or < 15 years at HSCT, and time from onset to HSCT ≥ 30 months or < 30 months). Stratified ages were analyzed in advance, and ≥ 15 years was chosen as the age factor. Similarly stratified times from onset to HSCT were analyzed in advance, and ≥ 30 months was chosen as the time factor. (A) Overall survival rate after HSCT (n = 59). (B) Event-free survival rate after HSCT (n = 59). For time from onset to HSCT, patients were divided into T-cell infection (n = 32) and NK-cell infection (n = 27) groups and independently analyzed.

The mechanism underlying EBV infection of T and NK cells, which do not express CD21, remains unresolved. It has been shown that NK cells activated by EBV-infected B cells acquire CD21 by synaptic transfer, and these ectopic receptors allow EBV binding to

NK-cell hosts.[45] It is plausible that killer cells in close contact with EBV-infected B cells may acquire EBV infection directly and then proliferate with clonality.

In the present study, we evaluated prognostic factors among patients with EBV$^+$ T/NK-LPDs. Multivariate analysis showed that age at onset of disease ($\geq$ 8 years) and liver dysfunction were independent risk factors for mortality, and that patients receiving transplantations had a better prognosis. We found previously that older onset age ($\geq$ 8 years) was associated with mortality in patients with CAEBV.[29] Furthermore, a recent report demonstrated that adult patients with CAEBV had progressive and more aggressive courses than those of childhood onset cases.[46] Interestingly, patients with CD4$^+$ T-cell infection had shorter survival rates than those with NK infection, whereas clinical categories were not correlated with survival rates. Onset age of patients with CD4$^+$ T-cell infection was high (median, 14.5 years). These results suggest that adult patients with CD4$^+$ T-cell infection may have more aggressive features and are likely to develop multiple organ failure. Although the reason is unclear, we should be cautious about rapid progression in patients with CD4$^+$ T-cell infection.

We surveyed administered therapies based on physician questionnaire responses. A potential limitation of this study design was the use of retrospective questionnaires; therefore, we should be cautious about the evaluation of treatment efficacy. Nevertheless, it seems that only HSCT induced CR in patients with EBV-associated T/NK-LPDs except for HLH. Some EBV-associated HLH patients responded well to chemotherapy and immunomodulating therapies,[47] but patients with CAEBV were generally refractory to chemotherapy. Similar findings were reported in patients with CAEBV in the United States.[20] Furthermore, Kaplan-Meier estimates indicated that shorter time from onset to HSCT ($<$ 30 months) and inactive disease at HSCT resulted in long survival times, suggesting that earlier HSCT in patients in good condition is preferred. Patients with CAEBV have a higher risk of transplantation-related complications.[41,48] Recently, Kawa et al reported excellent outcome of HSCT with reduced-intensity conditioning.[40] Although the superiority of reduced-intensity conditioning over myeloablative conditioning did not reach statistical significance in that study, it appears that a reduced-intensity regimen is sufficient to prevent transplantation-related deaths.[40,49]

The concept of EBV$^+$ T/NK-LPD was initially proposed by Kawa et al, and then examined by other researchers.[27,44] This umbrella term encompasses specific clinical diseases of the CAEBV T/NK–cell type, EBV-associated HLH, severe mosquito bite allergy, and hydroa vacciniforme, the distinction of which are differentiated based on clinical manifestations. However, if the clinical data are absent regarding the prodromal phase of expansion of EBV$^+$ T/NK–cells with variable clonality, we cannot discriminate systemic diseases such as ANKL and extranasal ENKL from EBV$^+$ NK-LPDs, because EBV$^+$ proliferating cells are indistinguishable in morphology and phenotype. Recently, this issue was highlighted by Takahashi et al.[50] Interestingly, 4 patients of the present series developed ANKL in their clinical course, 2 of whom had only skin symptoms categorized as severe mosquito bite allergy at the time of the diagnosis. In addition, 6 patients who were clinically categorized as CAEBV NK-cell type (4 cases) and T-cell type (2 cases) developed ENKL; the major clinical difference from de novo ENKL was its early onset (median age, 8.5 years). Three patients had hypersensitivity to mosquito bites. There were no differences in pathologic features between these patients and de novo ENKL patients.[50] Furthermore, new development of chromosomal aberrations was seen in 6 patients during follow-up. In this study, most of the patients with EBV$^+$ T/NK-LPDs had clonality of EBV-infected cells. These results indicate that patients with clonally expanding EBV-infected T or NK cells in EBV$^+$ T/NK-LPD eventually develop overt leukemia and lymphoma, the clinicopathologic findings of which are in keeping with those well documented in extranasal ENKL, ANKL, and PTCL, with additional mutations in cancer genes or tumor-suppressor genes.

In 2008, an international meeting was organized at the National Institute of Health to better define the pathogenesis, classification, and treatment of EBV-associated LPDs in nonimmunocompromised hosts.[39] At that meeting, acute and chronic EBV syndromes of T cells and NK cells were clarified to have a broad spectrum, in which hydroa vacciniforme, hydroa vacciniforme–like lymphoma, severe mosquito bite allergy, and systemic EBV$^+$ T-LPD of childhood were listed as EBV$^+$ T/NK-LPDs under an umbrella term of CAEBV of T/NK–cell type.[39] In the present study, EBV$^+$ T/NK-LPD is characterized by the systemic distribution of EBV$^+$ clones beyond the clinical categorization currently proposed as CAEBV, HLH, severe mosquito bite allergy, and hydroa vacciniforme. Furthermore, we also shed light on the clinicopathologic distinctiveness of patients with NK-cell infection, which has not been well addressed in the past even though these patients comprise approximately 40% of EBV$^+$ T/NK-LPD cases. This phenotype was more closely associated with hypersensitivity to mosquito bite and a relatively indolent clinical course, the biologic significance of which should be clarified in the future.

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Authorship

Contribution: H.K. designed the study, followed the patients, analyzed the data, and wrote the manuscript; Y.I. contributed to the study design, followed the patients, and helped to edit the manuscript; S. Kawabe, K.G., and S.E. performed the experiments; Y.T., S. Kojima, and T.N. followed the patients, collected the clinical data, and helped to edit the manuscript; A.K., A.S., and K.K. followed the patients and collected the clinical data; K.O. performed the experiments and helped to edit the manuscript; and S.N. contributed to the study design, performed the experiments, and wrote the manuscript.

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INTERNAL MEDICINE

CASE REPORT

A Case of Congenital Dyserythropoietic Anemia Type 1 in a Japanese Adult with a *CDAN1* Gene Mutation and an Inappropriately Low Serum Hepcidin-25 Level

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Abstract

We describe the first case of genetically diagnosed congenital dyserythropoietic anemia (CDA) type 1 in a Japanese man. The patient had hemolytic anemia since he was a child, and he developed diabetes, hypogonadism, and liver dysfunction in his thirties, presumably from systemic iron overload. When he was 48 years old a diagnosis was finally made by genetic analysis that revealed a homozygous mutation of *CDAN1* gene (Pro1129Leu). His serum hepcidin-25 level was inappropriately low. We conclude that physicians should be aware of the possibility of CDA in a patient with anemia and systemic iron overload at any age.

Key words: congenital dyserythropoietic anemia, iron metabolism, hemochromatosis, hepcidin, growth differentiation factor-15

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Introduction

Congenital dyserythropoietic anemia (CDA) is a rare congenital erythropoietic disorder with characteristic morphological abnormalities of the bone marrow cells, ineffective erythropoiesis and systemic iron overload (1). Three types of CDA are known: types 1, 2 and 3. The genes responsible for types 1 and 2 have recently been identified as *CDAN1* and *SEC23B*, respectively (2, 3). Both CDA types 1 and 2 are inherited recessively. The incidence of CDA is very rare, and in a recent pan-European survey, only 124 CDA type 1 cases were recorded (4). To date, several Japanese CDA type 1 cases have also been reported (5-7), but none of them has been genetically proven. Here, we describe in a Japanese adult a case of CDA type 1 with systemic iron overload that was genetically diagnosed in his late forties.

Case Report

A Japanese man was referred to the Kyoto University Hospital for hyperglycemia when he was 38 years old. He had had hemolytic anemia since he was a child, but its etiology had not been determined. He had undergone splenectomy when he was 36 years old, which ameliorated his anemia to some extent. At his first visit to our hospital, his white blood cell count was 6,400/μL; red blood cell count, 1.93×10^6/μL; hemoglobin (Hb) level, 7.5 g/dL; hematocrit level, 21.0%; mean corpuscular volume, 108.8 fL; platelet count, 365×10^3/μL; and reticulocyte count, 53×10^3/μL (Ta-

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Table 1. Laboratory Data

Features	Laboratory data at the first visit (38 years old)	Laboratory data at the time of diagnosis (48 years old)
White blood cells (per μL)	6.4×10^3	5.3×10^3
Red blood cells (per μL)	1.93×10^6	2.41×10^6
Hemoglobin (g/dL)	7.5	8.5
Hematocrit (%)	21.0	24.1
Reticulocytes (per μL)	53×10^3	—
Platelet counts (per μL)	365×10^3	312×10^3
Total bilirubin (mg/dL)	1.5	1.6
Direct bilirubin (mg/dL)	0.7	0.1
Haptoglobin (mg/dL)	<7.9	—
AST (IU/L)	49	26
ALT (IU/L)	68	16
LDH (IU/L)	302	277
Ferritin (ng/mL)	4058	186

— indicates that the tests were not performed. Abbreviations; AST, aspartate aminotransferase (reference range, 13-29 IU/L); ALT, alanine aminotransferase (reference range, 8-28 IU/L); LDH, lactate dehydrogenase (reference range, 129-241 IU/L).

ble 1). He had hepatic dysfunction, with a slightly elevated serum alanine aminotransferase level (68 IU/L), hyperglycemia (blood sugar level of 146 mg/dL and HbA1c level of 6.9%) with very low insulin secretion (serum c-peptide level, <0.1 ng/mL), and hypogonadism with a serum testosterone level lower than 0.2 ng/mL, i.e., very low (reference range, 2.7-10.7 ng/mL). His blood test results also suggested iron overload (transferrin saturation of 95.3% and serum ferritin level of 4,058 ng/mL), and the liver biopsy results revealed marked accumulation of iron in the parenchymal cells. Thus, hemochromatosis, along with liver dysfunction, diabetes and hypogonadism, was diagnosed. Insulin therapy was then started. Occasional phlebotomy was also started to remove excess iron and to gradually decrease his serum ferritin and alanine aminotransferase levels to within the reference ranges (Table 1). When he was 46 years old, a series of intensive diagnostic examinations were started. The findings of the biochemical analyses for erythrocyte membrane disorders or unstable hemoglobinopathies were all negative. The bone marrow examination revealed marked erythroid hyperplasia (the myeloid to erythroid ratio of 0.34) and remarkable dysplastic features in the erythroid cells, with megaloblastoid changes and multinuclear cells (Fig. 1A-E). However, no significant dysplasia was observed in the granulocytic or megakaryocytic series (Fig. 1A, B), and no ring sideroblasts were observed in the iron staining. When he was 48 years old, we obtained his written informed consent and approval by the ethics committee of Kyoto University to perform a genetic analysis for indicators of hereditary iron disorders in his peripheral blood cells. The results of the genetic analyses for pyruvate kinase deficiency and thalassemia syndromes were all negative. There were no mutations in the exons and the exon-intron borders of hereditary hemochromatosis genes including *HFE*, *TFR2*, *HJV*, *HAMP*, and *SLC40A1*. However, a homozygous mutation in *CDAN1 ex26 c.3503 C>T* (Pro1129Leu) was detected, consistent with CDA type 1 (Fig. 2). When we reviewed his bone marrow specimen, internuclear bridges that connected two separate erythroblasts were occasionally observed (7 bridges in 500 erythroblasts, Fig. 1F-J). His serum hepcidin-25 level was 0.8 ng/mL [reference range, 2.3-37 ng/mL; analyzed with a quantitative liquid chromatography coupled with tandem mass spectrometry method (8)]. The growth differentiation factor-15 (GDF15) level was 8,469 pg/mL (reference range, 215-835 pg/mL; analyzed with a commercial ELISA kit from R&D, Minneapolis, MN). The patient had two siblings, a brother and a sister; both were in good health. There was no significant family history except that his mother had anemia of undetermined etiology, and his paternal grandfather had diabetes. He declined genetic analysis of his family for the *CDAN1* gene.

Discussion

We encountered an adult patient with hemolytic anemia with various symptoms caused by systemic iron overload, who turned out to have a genetic mutation consistent with CDA type 1. To our knowledge, this is the first documented case of CDA type 1 in a Japanese with *CDAN1* gene mutation. Dgany et al. identified the same *CDAN1* gene mutation as the current case in a French Polynesian family (2). *CDAN1* is located on chromosome 15q15.1-15q15.3, and it codes for a nuclear protein, codanin-1, the human homolog of discs lost (dlt) which is required for cell survival and cell cycle progression in *Drosophila* (9). The diagnosis of CDA type 1 has usually been made from clinical features together with characteristic morphological features of the bone marrow cells such as binucleated erythroblasts, and internuclear bridges between the erythroid cells. As codanin-1 is essential for proper cellular trafficking of the heterochromatin

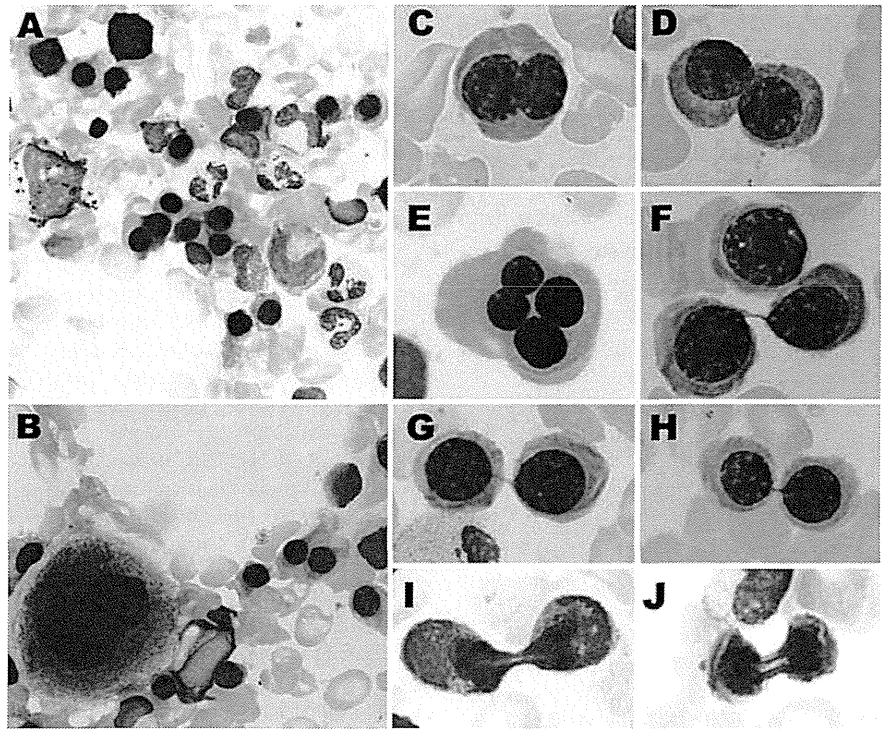

Figure 1. **Bone marrow cell morphology (May-Grünwald-Giemsa staining, original magnification ×1,000; C-J, images were further magnified by photographic enlargement). A and B: Erythroid hyperplasia. No significant dysplasia was observed in the granulocytic or megakaryocytic series. C and D: Binucleated erythroblasts. These cells were found in approximately 12% of the erythroblasts. E: A few tetranucleated erythroblasts were found. F-J: Internuclear bridges between the erythroblasts were found after careful inspection.**

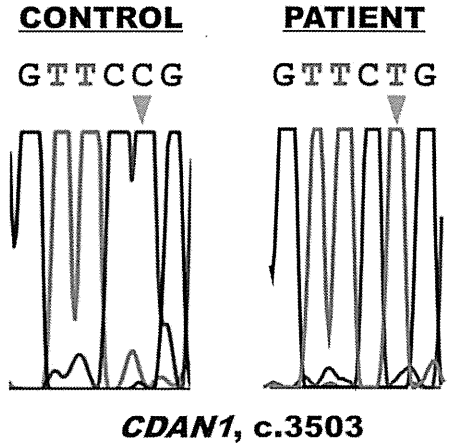

Figure 2. **The homozygous mutation in *CDAN1* (*ex26 c.3503 C>T*, Pro1129Leu) detected in the patient.**

protein HP1-α (10), defects of this protein may result in such morphological abnormalities. In the current case, the bone marrow examination results showed numerous binucleated erythroblasts, but the internuclear bridges, which are much more specific features of this disorder, were observed in less than 3% of the erythroblasts and were overlooked in the first inspection (Fig. 1). Therefore, making a definitive diagnosis of CDA from bone marrow cell morphology alone can sometimes be difficult.

CDA types 1 and 2 are known to be accompanied by iron overload. Similar to hereditary hemochromatosis, inappropriately low production of hepcidin, the central regulator of systemic iron homeostasis, has been proposed as the etiology of iron overload in CDA (11). As the main function of hepcidin is to downregulate the expression of ferroportin, the only known cellular iron exporter of mammals, downregulation of hepcidin results in an increase in ferroportin expression, thereby increasing iron absorption from the intestine and causing systemic iron overload. A previous report demonstrated marked increases of GDF15 in the serum of CDA type 1 patients (12). GDF15, a humoral factor belonging to the transforming growth factor-β superfamily, has been shown to suppress hepatic production of hepcidin (13). Consistent with the previous reports, systemic iron overload was induced in the current case without repeated red cell transfusions, the serum GDF15 level was remarkably elevated, and the serum hepcidin-25 level was inappropriately low. Thus, we postulate that serum hepcidin-25 and GDF15 are useful markers for CDA.

CDA is generally regarded as a pediatric disease because the initial symptoms, such as anemia, jaundice, and splenomegaly, usually appear in the first decade. However, the current case was diagnosed when the patient was in his late forties, and in the pan-European survey, CDA was diag-

nosed in a substantial proportion of patients who were middle-aged or older (4). Early diagnosis of CDA is important because iron chelation therapy (or phlebotomy if anemia is mild) should be started as early as possible to avoid iron overload, which can cause irreversible tissue damage. In addition, interferon-α is known to be effective for ameliorating anemia and iron accumulation in patients with CDA type 1, although the precise mechanism is still unknown (14). The survey data and our findings of the current case suggest that we should be aware of the possibility of CDA in patients with anemia and systemic iron overload at any age.

The authors state that they have no Conflict of Interest (COI).

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Loss of heterozygosity in 7q myeloid disorders: clinical associations and genomic pathogenesis

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Loss of heterozygosity affecting chromosome 7q is common in acute myeloid leukemia and myelodysplastic syndromes, pointing toward the essential role of this region in disease phenotype and clonal evolution. The higher resolution offered by recently developed genomic platforms may be used to establish more precise clinical correlations and identify specific target genes. We analyzed a series of patients with myeloid disorders using recent genomic technologies (1458 by single-nucleotide polymorphism arrays [SNP-A], 226 by next-generation sequencing, and 183 by expression microarrays). Using SNP-A, we identified chromosome 7q loss of heterozygosity segments in 161 of 1458 patients (11%); 26% of chronic myelomonocytic leukemia patients harbored 7q uniparental disomy, of which 41% had a homozygous *EZH2* mutation. In addition, we describe an SNP-A–isolated deletion 7 hypocellular myelodysplastic syndrome subset, with a high rate of progression. Using direct and parallel sequencing, we found no recurrent mutations in typically large deletion 7q and monosomy 7 patients. In contrast, we detected a markedly decreased expression of genes included in our SNP-A defined minimally deleted regions. Although a 2-hit model is present in most patients with 7q uniparental disomy and a myeloproliferative phenotype, haploinsufficient expression of defined regions of 7q may underlie pathogenesis in patients with deletions and predominant dysplastic features. (*Blood*. 2012;119(25):6109-6117)

Introduction

Complete loss of chromosome 7 (monosomy 7) or partial deletion involving its long arm [del(7q)] are highly recurrent chromosomal aberrations in myeloid disorders, including myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), and juvenile myelomonocytic leukemia (JMML).[1,2] The International Prognostic Scoring System (IPSS), the most validated score for predicting the evolution of patients with MDS, does not discriminate among chromosome 7 anomalies, uniformly assigning these patients to the poor-risk karyotype group.[3] Other metaphase cytogenetic (MC) studies have consistently associated lesions involving the long arm of chromosome 7 with inferior survival in AML cases.[1,4] However, there is a contention that monosomy 7 and del(7q) are not equivalent in prognosis and disease phenotype spectrum.[5,6]

In the traditional genetic view, loss of heterozygosity (LOH) for 1 tumor suppressor gene (TSG) allele increases the chance of inactivation of the remaining allele and total loss of function for a cancer-protective locus. In accordance to this 2-hit model, we and other groups found loss-of-function hypomorphic homo- and hemizygous mutations in a variety of genes, including *TP53*, *CBL*, or *TET2*.[7-10] However, there is growing evidence that haploinsufficient TSGs also lead to hastened tumorigenesis, showing dramatic phenotypes with loss of only a single allele.[11,12] The haploinsufficient model is supported by recent studies in the context of myeloid disorders harboring a deletion of the long arms of chromosome 5 or chromosome 20,[13-15]and it is possible that monosomy 7/del(7q) cases are associated with a similar mechanism.

To better address the genomic and clinical complexity of myeloid malignancies associated with 7q abnormalities, we analyzed a large series of cases with single nucleotide polymorphism array (SNP-A)–based karyotyping, direct and next-generation sequencing (NGS), and microarray expression platforms to (1) examine the association of different SNP-A 7q lesions with certain clinical features and other genomic aberrations, (2) define a commonly deleted region or regions (CDRs) and search for recurrent tumor suppressor mutations, and (3) test the haploinsufficiency hypothesis.

Methods

Patients

Informed consent was obtained following the Declaration of Helsinki according to protocols approved by the review boards and ethics committees of the participating institutions. Presentation bone marrow (BM)

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aspirates from 1458 patients with myeloid malignancies were studied using SNP-A, including 200 AML cases analyzed by SNP-A and NGS 300 through The Cancer Genome Atlas project (TCGA; http://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp). Microarray expression data were available on a cohort of 183 patients with MDS and 17 healthy controls.[16]

Diagnosis of hypocellular myelodysplastic syndrome (hMDS) was made based on the presence of dysplastic features and the overall clinical presentation, including the presence of cytopenias, the absence of an excess of blasts (5% in BM or 2% in blood), and a decreased cellularity of the marrow of less than or equal to 20%. When indicated based on clinical suspicion, immunohistochemical staining for CD34 was performed to rule out, or find, collections of immature cells.

Metaphase cytogenetics

Chromosome preparations were G-banded using trypsin and Giemsa, and karyotypes were described according to the International System for Human Cytogenetic Nomenclature.[17]

SNP-A analysis

Genome-Wide Human SNP 6.0 and GeneChip Human Mapping 250K arrays (Affymetrix) were used for SNP-A analysis of bone marrow DNA as described previously.[18] Germ-line encoded copy number variants and nonclonal areas of uniparental disomy (UPD) were excluded from further analysis by a bioanalytic algorithm, based on lesions identified by SNP-A karyotyping in an internal control series (n = 1003) and reported in the Database of Genomic Variants (http://projects.tcag.ca/variation/). Size and location criteria (telomeric > 8.7 Mb and interstitial, > 25 Mb) were used for identification of somatic UPD. In 11 patients, a 7q microdeletion (median size, 0.3 Mb; range, 0.1-0.7 Mb) was detected but testing of germ line DNA was not possible because of lack of appropriate samples. These patients were excluded from analysis as the pathophysiologic significance of such small lesions is not clear. None of those 7q microdeletions was included in any of the CDRs described subsequently.

Direct sequencing

Sanger technique was used for sequencing all exons of 36 candidate genes, included in our SNP-A–defined CDRs, screening a subset of 50 7q LOH patients [UPD(7q), n = 7; del(7q), n = 31; monosomy 7, n = 12]. Samples from this cohort were not used for NGS.

Next-generation sequencing

Two NGS approaches were used in this study. We generated exome chromosome 7 libraries that were enriched for the content of chromosome 7 coding sequences using the SureSelect capture synthetic biotinylated RNA probes from Agilent Technologies, tiling all the coding regions from chromosome 7. Libraries were subjected to high-throughput sequencing on a Genome Analyzer IIx (Illumina) and applied to 11 7q LOH patients [del(7), n = 6; del(7q), n = 2; UPD(7q), n = 3].

The second approach involved the sequencing of 15 paired bone marrow mononuclear cells and $CD3^+$ lymphocytes (used as germ line controls) from 15 patients with different myeloid disorders and SNP-A findings. Among them, we included 2 patients with 7q LOH [UPD(7q) and del(7q)]. A rational bioanalytic algorithm was applied to identify candidate nonsynonymous alterations. First, nonredundantly mapped reads were used for whole exome assembly using the reference genome hg19. Next, the software algorithm called all the positions that vary from the reference genome. The candidate alterations were subtracted by the results of $CD3^+$ lymphocyte-derived DNA (double-checked by direct and simultaneous visualization using DNAnexus Site) and subsequently validated using Sanger sequencing. Moreover, gene mutations affecting 7q LOH were screened using whole exome sequencing results available through TCGA.

Microarray data analysis

Previously published microarray expression data were obtained on a cohort of 183 MDS patients [monosomy 7/del(7q), n = 9].[16] Cell intensity calculation and scaling was performed using GeneChip Version 1.40 operating software (Affymetrix). Affymetrix CEL files were preprocessed using robust multiarray average. Data from 17 healthy controls were used to obtain patient and control expression ratios.

Statistical analysis

Comparisons of proportions and ranks of variables between groups were performed by the χ^2 test, Fisher exact test, Student t test or Mann-Whitney U test, as appropriate. We used the Kaplan-Meier and the Cox method to analyze overall survival (OS) and progression-free survival, with a 2-sided P less than or equal to .05 determining significance. In Cox models, examination of log (−log) survival plots and partial residuals was performed to assess that the underlying assumption of proportional hazards was met.

Results

Patient cohorts

Using SNP-A karyotyping, LOH segments involving 7q were identified in 161 of 1458 patients (11%), consisting of 9% MDS, 28% MDS/myeloproliferative neoplasms (MDS/MPN), 11% AML, 14% JMML, and 16% Fanconi anemia subsets (Figure 1A). MC identified 7q LOH in each of the cases detected by SNP-A except for 26 UPD cases and 11 patients in whom no interpretable metaphases were obtained (Figure 1B). In addition, in 7 cases a balanced translocation with 7q material was noted by MC; in all instances, SNP-A analyses detected a small deletion (> 1 and < 5 Mb) affecting the boundaries of the translocation. In 16 of 67 monosomy 7 cases by MC, SNP-A detected retained chromosome 7 material, probably contributing to marker chromosomes found by MC analysis. With increased resolution, there was a shift toward identification of more complex karyotypes and of additional lesions among the patients with isolated MC 7q aberrations (Figure 1C). By SNP-A, previously cryptic lesions were identified in 45% of the patients who otherwise showed a singular 7q LOH lesion by MC.

The 7q LOH cohort included men (57%) and women (43%) with a median age of 65 years (interquartile range, 59-73 years). The distribution of disease subsets and associated genomic lesions among the 3 classes of chromosome 7 lesions [UPD7q, del(7q) and monosomy 7] is shown in Figure 2, and Table 1 shows clinical characteristics at baseline.

Clinical and genomic correlates of monosomy 7/del(7q) patients

Compared with cases of partial deletions, those patients with del(7) were characterized by a lower number of genomic lesions per patient (1.2 vs 4.8; $P < .001$); the most remarkable the absence of 17p LOH cases among MDS patients.

Of 26 patients with monosomy 7 by SNP-A and a diagnosis of MDS, 20 (77%) fulfilled the diagnostic criteria of hMDS. Of note, these 20 patients had no other lesion detectable by SNP-A (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). That strong correlation between hMDS and the presence of an isolated monosomy 7 could not be established by MC, because no-growth was obtained in 8 hMDS-MC analyses and 4 high-risk MDS patients were described to harbor an isolated monosomy 7 by MC, whereas SNP-A found additional lesions in all of them. When patients with hMDS with or without monosomy 7 were compared, those with monosomy 7 showed a worse prognosis, with a higher transformation to leukemia ($P = .02$; hazard ratio, 3.4, 95%

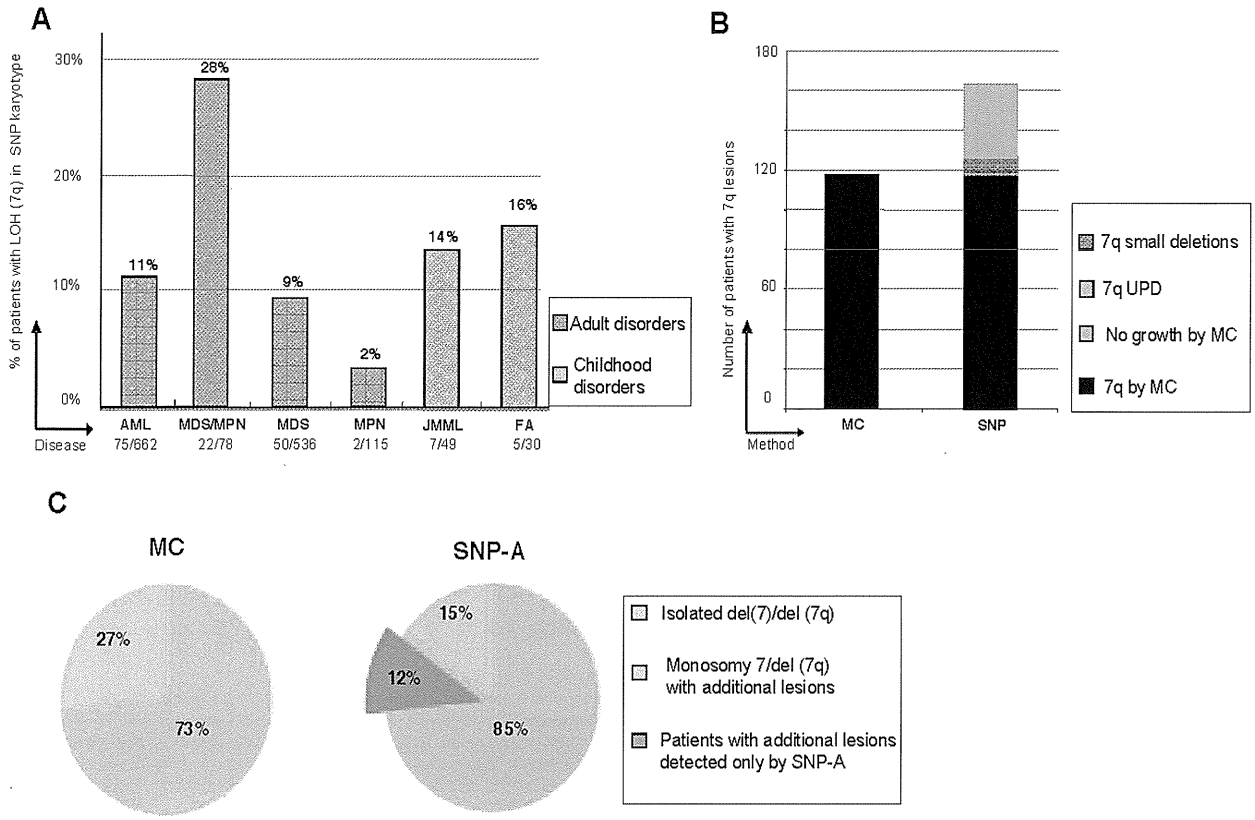

Figure 1. Frequency of detection of 7q and additional abnormalities by SNP-A. (A) Distribution of 7q LOH among the 1458 SNP-A–tested patients with myeloid malignancies, according to World Health Organization disease classification. (B) Number of patients with 7q LOH seen on MC and SNP-A. Lesions were observed in 117 of 1458 and 161 of 1458 patients when using MC and SNP-A, respectively. The additional 7q lesions found by SNP-A included those found in patients with no growth of MC cultures, small deletions affecting balanced translocation boundaries,[11] and UPD undetectable by MC.[26] (C) Percentage of patients with a sole 7q lesion versus accompanied by other abnormalities as identified by MC and SNP-A. SNP-A indicates single nucleotide polymorphism array;MDS/MPN, myelodysplastic syndrome/myeloproliferative neoplasm; AML, acute myeloid leukemia; MPN myeloproliferative neoplasms; JMML, juvenile myelomonocytic leukemia; FA, Fanconi anemia; UPD, uniparental disomy; MC, metaphase cytogenetics; monosomy 7, deletion of whole chromosome 7; and del(7q), partial deltion involving 7q.

confidence interval, 1.2%-9.7%; Figure 3A). Interestingly, 3 cases of monosomy 7 AML had a history of antecedent aplastic anemia. All the chromosome 7 lesions detected in 79 pediatric patients were monosomies; monosomy 7 was detected in 14% and 16% of patients with JMML and FA, respectively.

The subset of patients with del(7q) presented with an elevated percentage of high-risk disease (88% had AML or high-risk/intermediate-2 IPSS MDS) and a higher number of associated genomic lesions per patient. Del(5q) was the most common del(7q)-associated lesion found, occurring in 35 of 72 patients, all of which had AML or higher risk MDS. Of note, all del(5q) segments, except in 4 cases, involved either the centromeric or the telomeric extremes of the long arm of chromosome 5. The high frequency of del(5q) was followed closely by LOH 17p, seen in 14 of 72 patients, of which 5 of 14 were UPDs. Similar to patients with 5q, all patients had advanced stages of MDS or AML at diagnosis. All patients with LOH 17p spanned TP53; somatic mutations were present in 77% of cases tested.

The del(7q) MDS and MDS/MPN cohort had a shorter OS and time to leukemia transformation compared with patients with UPD(7q) or monosomy 7 (Figure 3B). In contrast, OS was similar in AML cases with monosomy 7 and del(7q) (Figure 3C), with both showing a significantly worse survival than in those patients with AML but without 7q LOH.

Clinical and genomic correlates of UPD(7q) patients

The UPD(7q) subset consisted of 26 patients, of which 17 were diagnosed with chronic myelomonocytic leukemia (CMML). Interestingly, 2 cases of AML and UPD(7q) also had history of antecedent CMML. The number of associated genomic lesions in the UPD(7q) cohort was lower than in the monosomy 7 and del(7q) subsets ($P = .03$ and $P < .001$, respectively), with a predominant presence of other regions of somatic copy neutral LOH rather than unbalanced defects. UPD(7q) was not associated with 5q or 17p LOH segments.

Comparing the 17 CMML patients to 55 CMML patients without UPD(7q) by SNP-A analysis, we found a trend toward worse survival. Those CMML patients with UPD(7q) showed a trend toward a shorter median OS (460 vs 730 days ($P = .2$; Figure 3D) and a higher rate of transformation to leukemia; whereas 26% of UPD(7q) patients progressed to higher-risk MDS or AML, advanced disease was observed in 13% of CMML patients without UPD(7q) ($P = .001$).

To test the prognostic validity and independence from known clinical variables of chromosome 7 SNP-A findings in patients' MDS and CMML, we developed a multivariate model for each cohort (Table 2). In the MDS model, the absence or presence of del(7q) or monosomy 7 kept the independent prognostic value when analyzed controlling for the clinical variables from the IPSS, ie, bone marrow blast percentage and number of cytopenias retained, whereas in the CMML model, the presence or absence of UPD 7q showed a trend toward statistical significance ($P = .1$) when tested together with the variables included in the score described by Onida et al,[19] that is, hemoglobin level below 12 g/dL, presence of circulating immature myeloid cells,

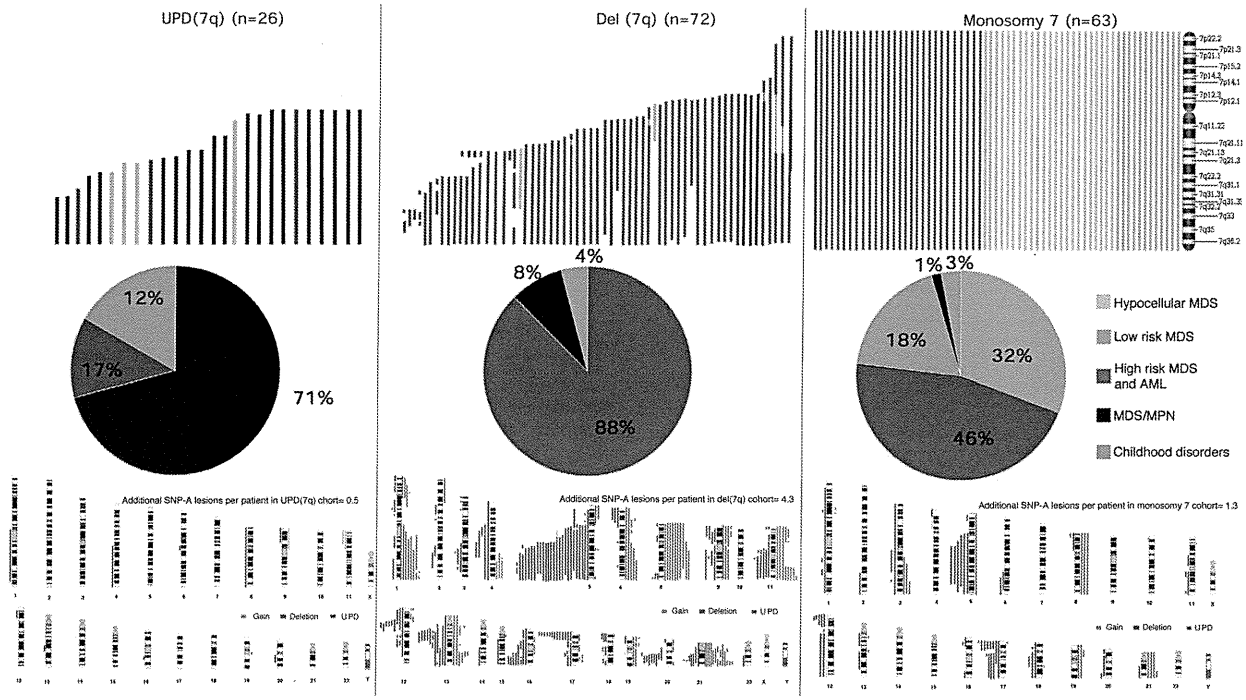

Figure 2. Distribution of disease subsets and associated genomic lesions among the 3 classes of chromosome 7 lesions. (Top) Distribution of LOH detected by SNP-A in the cohort, separated according to the nature of the lesion ([UPD(7q), del(7q), monosomy 7]. Patients have been grouped as follows: red, AML + high risk and intermediate-2 MDS; gray, low risk and intermediate-1 MDS; blue, hypocellular MDS; black, MDS/MPN; and green, Fanconi anemia and JMML. (Middle) Distribution of disease status in patients with 7 LOH separated according the nature of the lesion. (Bottom) Additional SNP-A–detected genomic lesions separated according the same criteria as stated herein. MDS indicates myelodysplastic syndrome; AML, acute myeloid leukemia; UPD, uniparental disomy; monosomy 7, deletion of whole chromosome 7; del(7q), partial deletion involving 7q; and MDS/MPN, myelodysplastic syndrome/myeloproliferative neoplasm.

absolute lymphocyte count greater than 2.5 × 10/L,[9] and marrow blasts greater than 10%.

Comparative analysis of SNP-A and MC

To provide a more detailed analysis about the added information that these karyotyping techniques could offer, Figure 4 illustrates how the distribution of disease subsets and outcome associations would be according to the lesion found by MC. Leaving aside those 44 patients with 7q LOH not detected by MC (26 UPD, 11 no growth, 7 small deletions in balanced translocations), the allocation of entities among MC del(7q) and monosomy 7 did not show a significant change. In fact, when only MC informative cases were considered, a multivariate model including both SNP-A and MC 7q lesions in MDS resulted in the variables cancelling each other ($P = .7$). No SNP-A–defined monosomy 7 was defined as a partial deletion by MC, because of which the strong association among monosomy 7 and hMDS and its high rate of transformation compared with hMDS without monosomy 7 remains unaltered. However, we must remark that this subgroup of MDS patients showed a higher frequency of no-growth MC analysis (in 8 hMDS patients, half of them harbored a monosomy 7 by SNP-A).

As a result of SNP findings, 16 patients were erroneously assigned to the monosomy 7 group by MC. SNP-A revealed that these samples instead had partial deletions and thus had been misclassified as high-risk patients. Probably, "relocation" of patients led to the lack of statistical difference noted in survival between MC-defined del(7q) and monosomy 7 patients (although a trend is still noted, $P = .07$).

Table 1. Comparative of clinical characteristics of patients at baseline according to the SNP-A–detected lesion nature

	Deletion(7), n = 63 (A)	Deletion(7q), n = 72 (B)	UPD(7q), n = 26 (C)	*P* (only significant comparisons reported)
Median age, y (range)	58 (27-70)	64 (56-72)	68 (63.2-77)	A vs B ($P = .01$) A vs C ($P = .009$)
Sex				
Male, %	59	56	57	
Female, %	41	44	43	
White blood cell count, × 10^9/L, median (IQR)	5.5 (2.7-25.7)	4.3 (2.1-13.5)	10.5 (8.1-37.9)	C vs A ($P = .028$) C vs B ($P = .01$)
Hemoglobin, g/dL, mean ± SD	9.1 ± 1.9	9.2 (8.9-10)	9.1 ± 2.1	
Mean corpuscular volume, median (IQR)	91 (85-99)	90 (86-104.8)	90 (85-103)	
Platelets, × 10^9/L, median (IQR)	47 (22-71)	50 (24-86)	46 (20.2-146.2)	
BM cellularity, median (%)	35 (22-61)	75 (45-90)	76(55-100)	A vs B ($P = .03$) A vs C ($P = .036$)

IQR indicates interquartile range; BM, bone marrow; UPD, uniparental disomy; and CMML, chronic myeloid leukemia..