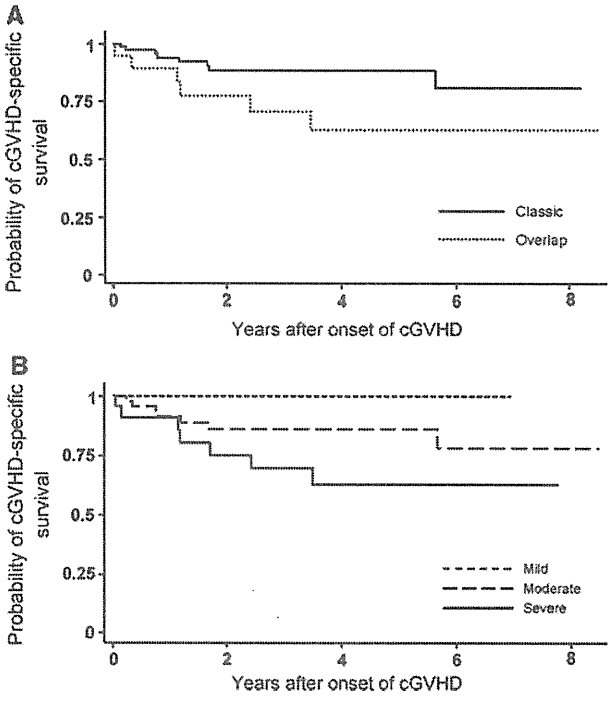

Fig. 1 Chronic GVHD-specific survival in patients with chronic GVHD diagnosed by the NIH consensus criteria. **a** Probability of chronic GVHD-specific survival (cGSS) among patients who developed classic chronic GVHD (*solid line*) and overlap syndrome (*dotted line*). **b** Probability of cGSS among patients who developed mild (*short dashed line*), moderate (*long dashed line*), and severe chronic GVHD (*solid line*)

improved grading scales for established cGVHD. A retrospective analysis of data on HLA-identical sibling transplantation reported to the International Bone Marrow Transplant Registry identified five variables independently associated with worse survival of those who developed historic cGVHD: low Karnofsky performance status at cGVHD diagnosis (<80), chronic diarrhea, weight loss, presence of cutaneous manifestation, and lack of oral involvement [15]. The Seattle group also proposed a revised classification for distinguishing limited and extensive cGVHD by the use of 16 clinical criteria [16]. Although these new classifications do not clearly discriminate between cGVHD and delayed onset GVHD with features resembling aGVHD, they have been shown to be at least useful for identifying patients at higher risk of NRM. Future studies are strongly warranted to compare the prognostic values of NIH cGVHD subcategories with those determined by other cGVHD grading system [21].

So far, several groups have reported the prognostic relevance of cGVHD severity graded by the NIH criteria and consistently found the inferior survival of patients with severe cGVHD [20–23], although such association was not observed in one earlier study [19]. While only a few of these studies focused on the significance of distinction between "overlap syndrome" and "classic cGVHD", our study revealed a trend toward worse survival in patients with overlap syndrome compared to those with classic GVHD, as was recently reported by Kim et al. [23]. In the present study, patients with overlap syndrome had a significantly shorter median time to the development of cGVHD than patients with classic cGVHD and were more likely to receive corticosteroid treatment for prior aGVHD at the onset of cGVHD. Intriguingly, these observations were very similar to the findings by Arora et al. [22], who reported that most of patients with overlap syndrome had a history of prior aGVHD and a progressive cGVHD onset, although they did not observe worse survival of this subgroup of patients compared to those with classic cGVHD. Given that nearly all patients who developed overlap syndrome had a prior history of aGVHD in our study cohort, NIH overlap syndrome in most instances could be considered as a flare of preexisting aGVHD, concomitant with development of classic cGVHD. In this context, it is important to note that early flare of cGVHD or early treatment change for exacerbation of cGVHD has been reported to be associated with increased NRM and inferior cGSS [34, 35]. It is also of note that a significantly higher proportion of patients with overlap syndrome had thrombocytopenia less than $100 \times 10^3/\mu L$ at cGVHD onset in our study. Since the progressive cGVHD onset and the presence of thrombocytopenia were consistently associated with an increased NRM across various studies [16, 36], more effective management of patients with overlap syndrome and thrombocytopenia might be needed.

Duration of systemic immunosuppressive therapy is suggested to be a useful surrogate endpoint to evaluate the response to specific treatment for cGVHD [26]. Although we could not find significant association of NIH cGVHD subtypes with duration of systemic IST, patients who had been given ongoing systemic corticosteroids at the onset of cGVHD were found to receive significantly prolonged systemic IST in multivariable analysis, consistent with the findings of Vigorito et al. [37]. In our study, the duration of systemic IST was also prolonged in patients who had high-risk underlying disease compared with those who had standard-risk disease. If the activity of cGVHD were likely to worsen in the high-risk subgroup of patients, one possible explanation might be the preference of physicians to taper systemic IST faster for patients at higher risk of relapse.

The present study, however, has several limitations; the retrospective study design, small cohort size, recording bias, and heterogeneity of underlying diseases and transplantation procedures might substantially influence the results. In addition, diagnostic cGVHD manifestations of affected organs or sites might have originated from other causes, including drug reactions, infection, and

Table 3 Univariable and multivariable analysis of factors potentially associated with chronic GVHD-specific survival among patients who developed chronic GVHD defined by the National Institutes of Health criteria

Variable	*n* (%)	Univariable analysis		Multivariable analysis	
		HR (95% CI)	*P* value	HR (95% CI)	*P* value
Patient age					
Less than 50 years	51 (53)	1.00		–	
50 years or more	45 (47)	1.40 (0.49–4.05)	0.53	–	
Donor/recipient sex combination					
Other than female/male	69 (72)	1.00		–	
Female/male	27 (28)	1.03 (0.32–3.28)	0.97	–	
Disease status at transplant					
Standard risk	51 (53)	1.00		1.00	
High risk	45 (47)	3.03 (0.95–9.68)	0.061	2.75 (0.86–8.80)	0.088
Donor/recipient HLA compatibility					
Matched	80 (83)	1.00		–	
Mismatched	16 (17)	0.33 (0.04–2.53)	0.29	–	
Conditioning regimen					
Myeloablative intensity	54 (56)	1.00		–	
Reduced intensity	42 (44)	1.04 (0.36–3.00)	0.95	–	
Stem cell source					
Bone marrow	67 (70)	1.00		–	
Peripheral blood	24 (25)	2.07 (0.69–6.19)	0.19	–	
Cord blood	5 (5)	1.63 (0.57–4.68)	0.37	–	
Prior aGVHD					
Grade 0–1	47 (49)	1.00		–	
Grade 2–4	49 (51)	1.16 (0.40–3.37)	0.78	–	
Subcategory of cGVHD					
Classic cGVHD	77 (80)	1.00		–	
Overlap syndrome	19 (20)	2.76 (0.96–7.97)	0.060	–	
Severity of cGVHD at onset					
Mild to moderate	73 (76)	1.00		1.00	
Severe	23 (24)	3.10 (1.09–8.86)	0.034	2.58 (0.90–7.39)	0.077
Platelet count at cGVHD onset					
$100 \times 10^3/\mu L$ or more	65 (68)	1.00		1.00	
Less than $100 \times 10^3/\mu L$	31 (32)	4.19 (1.40–12.5)	0.010	4.05 (1.35–12.1)	0.013
Eosinophil count at cGVHD onset					
Less than 500/μL	68 (71)	1.00		–	
500/μL or more	28 (29)	0.90 (0.28–2.88)	0.86	–	
Systemic corticosteroids at cGVHD onset					
Not received	63 (66)	1.00		–	
Received	33 (34)	1.74 (0.61–4.97)	0.30	–	

CI confidence interval, *aGVHD* acute graft-versus-host disease, *cGVHD* chronic graft-versus-host disease

comorbidity before transplantation. Furthermore, genital tract involvement might be underestimated because female patients do not always report about their genital symptoms to physicians.

In conclusion, our present study suggests that both the subcategory and global severity of cGVHD proposed by NIH consensus criteria have effects on cGSS and the risk of NRM among patients who develop NIH cGVHD. Future prospective studies are warranted to more precisely characterize the clinical significance of the subcategory and severity of cGVHD evaluated by the NIH consensus criteria.

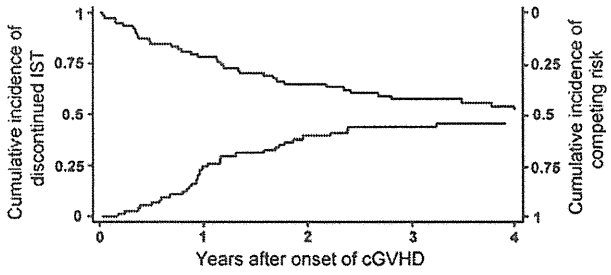

Fig. 2 Cumulative incidence of discontinued systemic immunosuppressive treatment. The *lower curve* shows the cumulative incidence of discontinued systemic immunosuppressive treatment (IST) in the absence of death, recurrent primary disease, or secondary malignancy among 81 patients who developed NIH cGVHD and received systemic IST (*left-hand scale*). The *upper curve* shows the competing risks of death or recurrent/secondary malignancy during systemic IST (*right-hand scale*). At the onset of cGVHD, 69 patients had been already given ongoing systemic IST consisting of calcineurin inhibitors alone ($n = 36$), calcineurin inhibitors plus corticosteroids ($n = 27$), corticosteroids alone ($n = 4$), or corticosteroids plus mycophenolate mofetil ($n = 2$)

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Conflict of interest The authors have no conflict of interest to declare.

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bjh research paper

Molecular lesions in childhood and adult acute megakaryoblastic leukaemia

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Summary

While acute megakaryoblastic leukaemia (AMKL) occurs in children with (DS-AMKL) and without (paediatric non-DS-AMKL) Down syndrome, it can also affect adults without DS (adult non-DS-AMKL). We have analysed these subgroups of patients (11 children with DS-AMKL, 12 children and four adults with non-DS-AMKL) for the presence of molecular lesions, including mutations and chromosomal abnormalities studied by sequencing and single nucleotide polymorphism array-based karyotyping, respectively. In children, AMKL was associated with trisomy 21 (somatic in non-DS-AMKL), while numerical aberrations of chromosome 21 were only rarely associated with adult AMKL. DS-AMKL was also associated with recurrent somatic gains of 1q (4/11 DS-AMKL patients). In contrast to trisomy 21 and gains of 1q, other additional chromosomal lesions were evenly distributed between children and adults with AMKL. A mutational screen found *GATA1* mutations in 11/12 DS-AMKL, but mutations were rare in paediatric non-DS-AMKL (1/12) and adult AMKL (0/4). *JAK3* (1/11), *JAK2* (1/11), and *TP53* mutations (1/11) were found only in patients with DS-AMKL. *ASXL1*, *IDH1/2*, *DNMT3A*, *RUNX1* and *CBL* mutations were not found in any of the patient group studied, while *NRAS* mutation was identified in two patients with paediatric non-DS-AMKL.

Keywords: acute megakaryoblastic leukaemia, children, Down syndrome, chromosome abnormality, single nucleotide polymorphism array.

Acute megakaryoblastic leukaemia (AMKL) is a heterogeneous subtype of acute myeloid leukaemia (AML) with diverse genetic and morphological characteristics. AMKL, a rare form of AML (3-14%), is more frequent in children than in adults. In Down syndrome (DS), AMKL predominates and is associated with somatic *GATA1* mutations (Wechsler *et al*, 2002; Hirose *et al*, 2003; Rainis *et al*, 2003). While most paediatric cases are *de novo* AMKL, adult AMKL is frequently observed as a secondary leukaemia after chemotherapy or leukaemic transformation of several chronic myeloproliferative neoplasms (MPNs) including chronic myeloid leukaemia (CML), polycythaemia vera (PV), essential thrombocytosis (ET), and idiopathic myelofibrosis (IMF) (Akahoshi *et al*, 1987; Radaelli *et al*, 2002; Mesa *et al*, 2005). Based on these findings, AMKL can be divided into three groups; i.e., AMKL with DS (DS-AMKL), paediatric AMKL without DS (paediatric non-DS-AMKL), and adult AMKL without DS (adult non-DS-AMKL).

Between 20% and 30% of children with DS-AMKL have a preceding history of transient abnormal myelopoiesis (TAM), in which the blasts are morphologically and phenotypically indistinguishable from those of AMKL (Zipursky *et al*, 1992). Somatic mutations of the *GATA1* gene are found in both TAM and DS-AMKL, suggesting that the acquisition of additional

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genetic alterations might be necessary for progression from TAM to AMKL (Ahmed *et al*, 2004). Although acquired mutations of *TP53, JAK3, JAK2* or *FLT3* have been found in patients with DS-AMKL, the incidence of those gene mutations was low, and *TP53* and *JAK3* gene mutations have been identified in both DS-AMKL and TAM (Malkin *et al*, 2000; Walters *et al*, 2006; De Vita *et al*, 2007; Kiyoi *et al*, 2007; Klusmann *et al*, 2007; Malinge *et al*, 2008). Mutations of *GATA1, JAK2, FLT3, KIT*, or *MPL* were also found in children with non-DS-AMKL (Malinge *et al*, 2008). *JAK3* and *TP53* mutations were reported in adult AMKL patients (Kiyoi *et al*, 2007). More recently, somatic mutations of genes, including those associated with proliferation signalling [*CBL* (Dunbar *et al*, 2008; Makishima *et al*, 2009; Sanada *et al*, 2009), *RUNX1* (Gaidzik *et al*, 2011)] and with modification of epigenetic status [*ASXL1* (Chou *et al*, 2010), *DNMT3A* (Ley *et al*, 2010)], have been found in various myeloid malignancies including AML, but not fully investigated in an AMKL cohort until now.

Recently, array-based comparative genomic hybridization (CGH-A) or single nucleotide polymorphism arrays (SNP-A) have been utilized to investigate pathogenetic lesions in haematological malignancies. Both technologies can detect microdeletions and microduplications, which are usually missed by conventional metaphase analysis. Additionally, SNP-A can detect loss of heterozygosity (LOH) due to acquired uniparental disomy (UPD), a common type of chromosomal lesion in myeloid malignancies, including AML(Gondek *et al*, 2007). Here we have analysed and compared the molecular lesions in AMKL, in particular in the three subtypes of this disease (DS-AMKL, paediatric non-DS-AMKL, and adult non-DS-AMKL).

Methods

Patients

Informed consent for sample collection from patients or their parents was obtained according to the institutional review board-approved procedures and protocols. We studied 11 patients with DS-AMKL and 16 with non-DS-AMKL (12 children and four adults) investigated at the Nagoya University Graduate School of Medicine, Nagoya, Japan and the Cleveland Clinic, Cleveland, Ohio, USA. Some of the paediatric patients (DS-AMKL-1-8, paediatric non-DS-AMKL-1-11) have been reported previously (Hama *et al*, 2008a,b). Characteristics of the patients with AMKL are summarized in Table I. The diagnosis of AMKL was based on morphology, histopathology, and the expression of megakaryocyte-specific antigens, CD41, CD42 or CD61. The diagnosis of DS was confirmed by conventional cytogenetic analysis. Bone marrow or peripheral blood samples were obtained from the patients with AMKL at the time of diagnosis. Molecular analysis of the mutational status was approved by the Ethics Committee of Nagoya University Graduate School of Medicine and Cleveland Clinic. In addition, we analysed a cohort of 642 adult and 232 paediatric patients with AML for the presence of trisomy 21 and frequency of AMKL, and a cohort of 798 various myeloid malignancies with SNP-A karyotyping data for the presence of a commonly gained region of 1q.

SNP-A karyotyping analysis

Mononuclear cells were isolated using Ficoll-Hypaque density gradient centrifugation and cryopreserved until use. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Chatsworth, CA). High-density Affymetrix single nucleotide polymorphism arrays (SNP-A) (6.0) (Affymetrix, Santa Clara, CA, USA) were applied as a karyotyping platform to identify LOH, microduplication and microdeletion as previously described (Gondek *et al*, 2007, 2008).

Bioinformatic analysis

Signal intensity was analysed and SNP calls determined using Gene Chip Genotyping Analysis Software Version 4.0 (GTYPE). Genotyping console v3.0 (Affymetrix) was used for analysis of 6.0 arrays as previously described (Gondek *et al*, 2007, 2008).

We excluded germ-line encoded copy number variants (CNVs) and non-clonal areas of gene copy number-neutral LOH from further analysis by utilizing a bioanalytic algorithm based on lesions identified by SNP-A in an internal control series (N = 1003) and reported in the Database of Genomic Variants (http://projects.tcag.ca/variation/). Through calculation of their average sizes, we defined a maximal size of germ line LOH in the controls and consequently excluded all defects of this type in patient samples; according to 95% confidence interval, stretches of UPD >25·8 Mb were considered unlikely to be of germline origin. In addition, all non-clonal areas of UPD seen in controls were interstitial.

Gene mutational screening

To screen for gene mutations, genomic DNA was amplified by polymerase chain reaction (PCR); sequenced genes included *GATA1* (exons 2, 3) (Hirose *et al*, 2003), *JAK2* (exon 14), *JAK3* (exon 2–24) (Kiyoi *et al*, 2007), *TP53* (exons 5-8) (Hirose *et al*, 2003), *FLT3* (exons 11, 12, and 17) (Hirose *et al*, 2003), *NRAS* (codons 12, 13 and 61) (Hirose *et al*, 2003), *ASXL1* (exon 13) (Sugimoto *et al*, 2010), *IDH1/IDH2* (exon 2/exon 4) (Yan *et al*, 2009), *DNMT3A* (exon 23) (Ley *et al*, 2010), *RUNX1* (exons 3–8) (Kohlmann *et al*, 2010), and *CBL* (exon 8, 9) (Makishima *et al*, 2009), as previously described.

Statistical analysis

For comparison of the frequency of mutations or other clinical features between disease groups, categorical variables were analysed using the Fisher's exact test and continuous variables were tested using Mann–Whitney U test. All reported P values were two-sided, with a significance level of α = 0·05 used.

Table I. Patient characteristics.

Disease	Sex	Age at Dx (years)	WBC at Dx ($\times 10^9$/l)	Induction failure	Relapse	HSCT	Survival	Cause of death	Results of metaphase cytogenetics
DS-AMKL-1	M	1	6·2	–	–	–	Alive	–	47,XY,der(7)t(1;7)(q23;q36),del(20)(q11q13·1),+21c
DS-AMKL-2	M	1	4·9	+	–	UR-BMT	Dead	Heart failure	48,X,der(X)t(X;1)(q28;q25),+11,+21c
DS-AMKL-3	M	2	5·3	–	–	–	Alive	–	47,XY,del(11)(p?),+21c [18/20]/46, XY [2/20]
DS-AMKL-4	F	1	16·0	–	–	–	Alive	–	47,XX,+21c [14/20]/90,idemx2,-3,-7,-9,del(11)(q?),-18 [6/20]
DS-AMKL-5	M	1	4·4	–	–	–	Alive	–	47,XY,add(7)(q22),+add(21)(q22) [2/20]/48,idem,+add(21) [6/20]/47,idem,del(6)(q?) [2/20]
DS-AMKL-6	F	2	107·0	–	–	–	Alive	–	47,XX,t(5;12)(p15;q21),+add(7)(p11),+21c,+add(22)(q13)
DS-AMKL-7	F	2	10·7	+	+	–	Dead	Leukaemia	47,XX,del(7)(p15),+8,del(13)(q12q32),-14,der(14;21)(q10;q10) c,del(17)(p11),+21c,+mar
DS-AMKL-8	F	1	16·1	–	–	–	Alive	–	47,XX,+21c
DS-AMKL-9	F	2	6·4	–	–	–	Alive	–	47,XX,+21c
DS-AMKL-10	M	2	10·8	–	–	–	Alive	–	48,XY,+21c,+21,der(22),t(1;22)(q2?;q13)
DS-AMKL-11	M	1	3·3	–	+	UR-CBT	Dead	Relapse	47,XY,add(7)(p11·2)
Paediatric non-DS-AMKL-1	F	2	42·3	–	–	Auto	Alive	–	46,XX,del(2)(q11),del(2)(q31),der(5)t(2;5)(q11;q22),der(5)t(5;?13) (q35;?q14),-13, add(16)(p13),+mar
Paediatric non-DS-AMKL-2	F	15	2·0	–	–	Auto	Alive	–	49,XX,+5,+8,i(17)(q10),+21
Paediatric non-DS-AMKL-3	F	2	48·7	–	+	Auto	Dead	Relapse	47,XX,+add(16)(p13),+21
Paediatric non-DS-AMKL-4	F	0	37·5	+	–	–	Dead	Leukaemia	46,XX,t(1;22)(p13;q13) [5/20]
Paediatric non-DS-AMKL-5	M	1	1·0	–	–	UR-CBT	Alive	–	46,XY
Paediatric non-DS-AMKL-6	F	1	14·4	–	–	–	Alive	–	58,XX,+X,+2,+2,+6,+7,+8,+10,+13,+15,+19,+19,+22
Paediatric non-DS-AMKL-7	M	3	2·2	+	+	UR-BMT	Dead	Relapse	46,XY,t(16;21)(p11;q22) [18/20]/46,idem,add(11)(q13), del(13)(q12q14) [2/20]
Paediatric non-DS-AMKL-8	M	0	26·0	+	–	R-BMT	Alive	–	46,XY,t(2;7)(p12;p22)
Paediatric non-DS-AMKL-9	F	0	17·9	+	–	R-BMT	Alive	–	46,XX,-7,-7,del(11)(p11),+2mar,inc
Paediatric non-DS-AMKL-10	M	1	12·3	–	–	–	Alive	–	51,XY,+der(1) t(1;22)(p13;q13),t(1;22)(p13;q13),+6,+7,+10,+19 [13/20]/53, idem,+6,+8 [3/20]
Paediatric non-DS-AMKL-11	M	1	5·7	+	–	–	Dead	Leukaemia	45,XY,-11,der(11)t(3;11)(q21;p15)
Paediatric non-DS-AMKL-12	F	1	26·0	–	+	R-BMT	Dead	Relapse	47,XX,t(1;22)(p13;q13),der(2),t(1;2)(q23;q35),+19
Adult non-DS-AMKL-1	M	54	19·4	Refused chemotherapy	–	–	Dead	Leukaemia	46,XY,add(6)(p23),del(11)(q13q23),del(13)(q14q22) [4/20]/46,idem,del(7)(q22) [16/20]
Adult non-DS-AMKL-2	M	48	0·9	+	–	–	Dead	Leukaemia	46,XY,t(2;12)(q32;q24),der(3)t(3;12)(q21;q12)
Adult non-DS-AMKL-3	F	43	2·2	–	+	UR-BMT	Alive	–	46,XX,t(3;3)(q21;q26)
Adult non-DS-AMKL-4	F	60	6·1	–	–	–	Alive	–	46,XX

DS-AMKL, acute megakaryoblastic leukaemia with Down syndrome; Dx, diagnosis; WBC, white blood cell count; IF, induction failure; HSCT, haematopoietic stem cell transplantation; UR-CBT, unrelated cord blood stem cell transplantation; UR-BMT, unrelated bone marrow transplantation; R-BMT, related bone marrow transplantation.

Results

Cytogenetic and clinical characterization of AMKL patients

To enhance cytogenetic diagnosis, metaphase cytogenetics was complemented by SNP-A-based karyotyping using Affymetrix 6.0 arrays. Using conventional cytogenetics, somatic chromosomal aberrations were found in 23/27 patients. SNP-A-based karyotyping confirmed most unbalanced defects, including the germline gain of chromosome 21 in DS patients. Somatic gains, other than germline trisomy 21, were found in seven DS-AMKL patients, including recurrent duplication of 1q31q44 in 4 and 7q in 2 DS-AMKL (Fig 1A). Deletions were found in eight DS-AMKL patients, including deletion 5p and 7p in two patients each. UPD 3q and UPD 7p were found in another DS-AMKL patient. Somatic gains were found in seven paediatric non-DS-AMKL cases, including trisomy 8 in 2 and somatic trisomy 21 in two patients while deletions were present in five patients, including deletion 7q and 13q in two patients, respectively (Fig 1B). Deletions were found only in one adult non-DS-AMKL patient, including deletion 2q and 17q. An

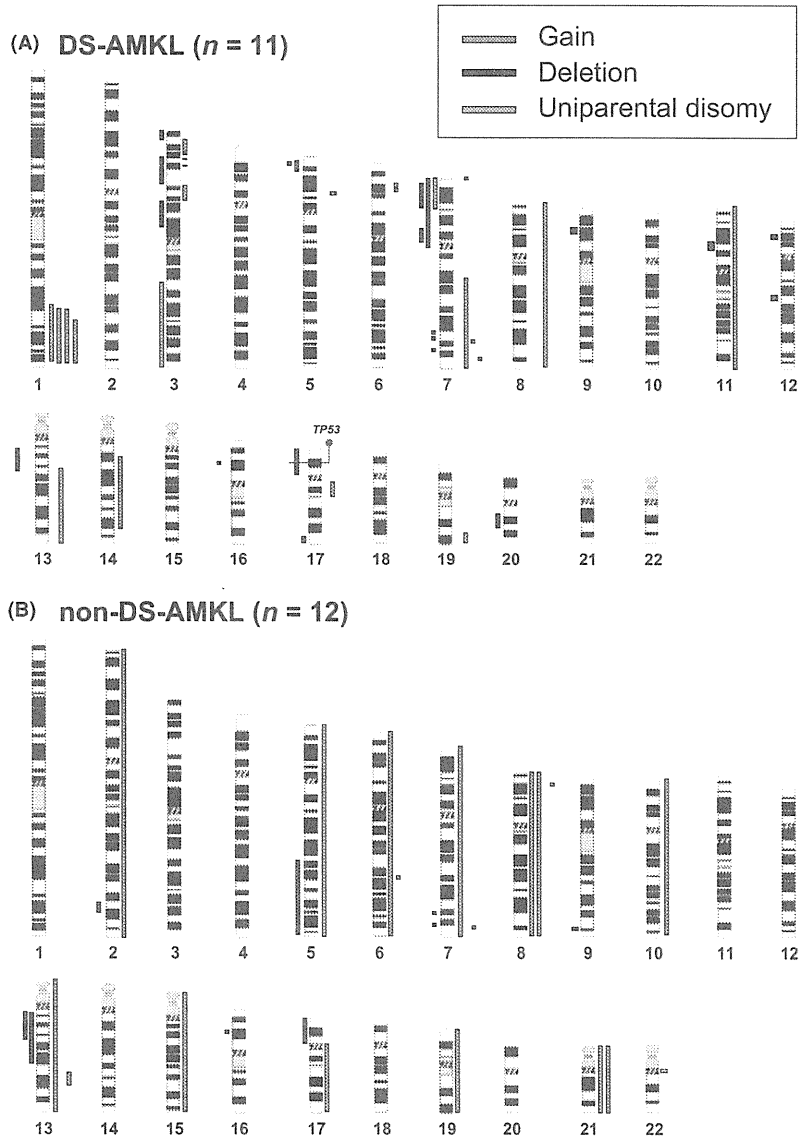

Fig 1. Single nucleotide polymorphism array-based karyotyping of AMKL. Genomic distribution and type of lesion identified by single nucleotide polymorphism array analysis in patients with DS-AMKL (n = 11) (A) and with non-DS-AMKL (n = 12) (B). Green bars represent gain, red bars indicate deletion, and blue corresponds to uniparental disomy. The fine red line pinpoints the locus of *TP53* gene mutated at that locus. Somatic gains other than trisomy 21 were found in seven DS-AMKL patients, including recurrent duplication of 1q in 4 and 7q in two patients.

Table II. Mutational status of pathogenic genes in DS-AMKL and non-DS-AMKL patients.

	Mutational status analysed gene										
Patient number	*GATA1*	*JAK3*	*JAK2*^{V617F}	*TP53*	*NRAS*	*ASXL1*	*FLT3*	*IDH1/2*	*DNMT3A*	*RUNX1*	*CBL*
DS-AMKL-1	■										
DS-AMKL-2	■										
DS-AMKL-3	■										
DS-AMKL-4	■										
DS-AMKL-5	■										
DS-AMKL-6	■										
DS-AMKL-7	■	■	■	■							
DS-AMKL-8	■										
DS-AMKL-9	■										
DS-AMKL-10											
DS-AMKL-11	■										
Paediatric non-DS-AMKL-1					■						
Paediatric non-DS-AMKL-2					■						
Paediatric non-DS-AMKL-3											
Paediatric non-DS-AMKL-4											
Paediatric non-DS-AMKL-5											
Paediatric non-DS-AMKL-6											
Paediatric non-DS-AMKL-7											
Paediatric non-DS-AMKL-8											
Paediatric non-DS-AMKL-9											
Paediatric non-DS-AMKL-10											
Paediatric non-DS-AMKL-11	■										
Paediatric non-DS-AMKL-12											
Adult non-DS-AMKL-1											
Adult non-DS-AMKL-2											
Adult non-DS-AMKL-3											
Adult non-DS-AMKL-4											
Total	11	1	1	1	2	0	0	0	0	0	0

Grey cells and white cells represent gene mutation and wild type, respectively.
DS, Down syndrome; AMKL, acute megakaryoblastic leukaemia.

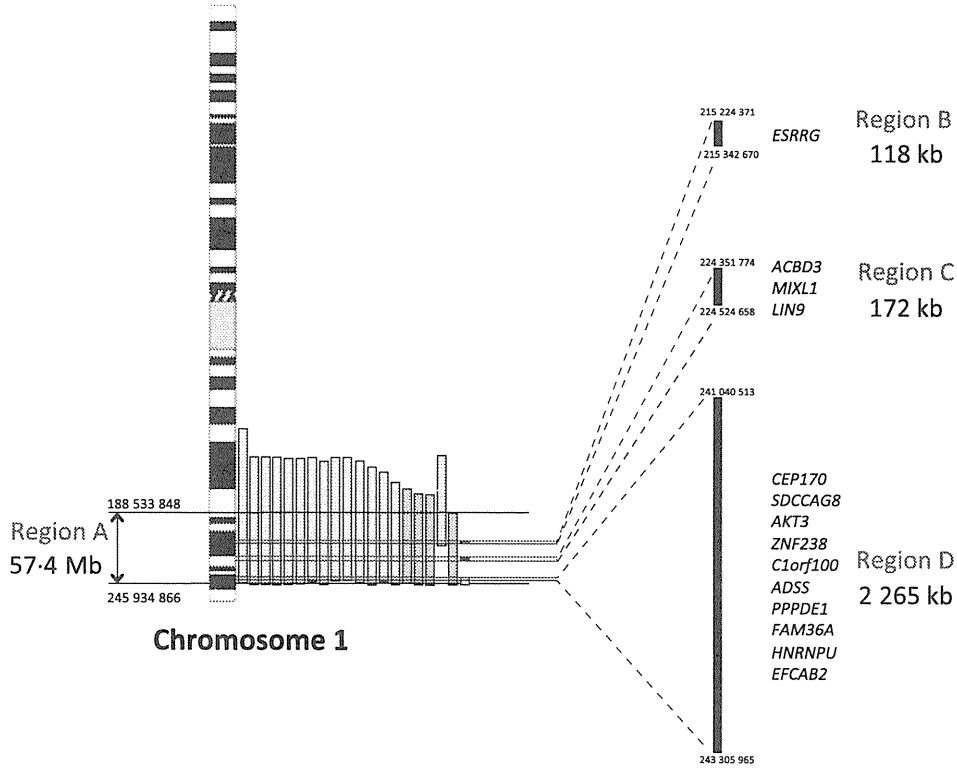

Fig 2. Summary of 1q gain lesions within the common region found in the DS-AMKL cohort: detected by single nucleotide polymorphism array (SNP-A) karyotyping in 22 of 798 patients with haematological disease. Analysis of the SNP-A karyotyping data of 798 patients with various haematological diseases identified 22 patients who harboured 1q gains that overlapped a region commonly duplicated in 4 DS-AMKL patients [Region A (57·4 Mb)]. Region B (118 kb), including only one gene (*ESRRG*) was shared by 91% (20/22) of patients. Region C (172 kb) was shared by 86% (19/22) patients, in which three genes (*ACBD3*, *MIXL1*, and *LIN9*) were located. Region D (2265kb) was shared by 86% (19/22) patients, and contained 10 genes including *AKT3*. Purple and pale blue bars indicate 1q gains found in DS-AMKL (n = 4) and other haematological diseases (n = 18), respectively. The diagnosis of 22 patients with 1q gains are as follows: DS-AMKL (n = 4), chronic myelomonocytic leukaemia (n = 5), secondary AML (n = 5), myelodysplastic syndrome (MDS) (n = 3), Fanconi anaemia (n = 2), idiopathic myelofibrosis (n = 1), chronic myeloid leukaemia (n = 1), and aplastic anaemia-derived MDS (n = 1).

overall complex karyotype (three or more abnormalities) was found in 6/11 DS-AMKL and 4/12 paediatric non-DS-AMKL.

Mutational analysis of patients with AMKL

Gene mutational status was analysed in 11 patients with DS-AMKL and 16 with non-DS-AMKL (12 children and four adults with AMKL, Table II). *GATA1* mutations were found in 10/11 (91%) with DS-AMKL, but only in one paediatric non-DS-AMKL and not found in adult patients. *NRAS* mutations (Q61R; G12S) were found in two paediatric non-DS-AMKL patients. *JAK2* (V617F), *JAK3* (Q501H), *TP53* (V157D) mutations were found in a single DS-AMKL patient (DS-AMKL-7). *ASXL1*, *IDH1/2*, *DNMT3A*, *RUNX1* and *CBL* mutations were not found in AMKL. With the exception of a hemizygous *TP53* mutation (microdeletion on 17p), all of the other mutations studied were heterozygous.

Perhaps of interest, we have identified a non-synonymous amino acid change in *ASXL1* (K888T) in a child with non-DS-AMKL. While this alteration was never reported as a SNP and not found in over 100 normal individuals from Japan, the nucleotide change was confirmed to be not of somatic origin, rather is it likely to represent a rare non-synonymous SNP found in a complete remission following successful chemotherapy.

Comparison of pathogenetic lesions among subgroups of AMKL

GATA1 mutations were more frequently found in DS-AMKL (10/11) compared to non-DS-AMKL (1/16) ($P < 0{\cdot}001$), consistent with previous reports. Additional mutations screened were only rarely found in DS-AMKL and non-DS-AMKL and thus the statistical comparisons were not warranted.

However, when the cytogenetic defects detected by SNP-A analysis were analysed, the frequency of 1q gain was significantly higher in DS-AMKL as compared to non-DS-AMKL

Table III. Comparison of somatic lesions detected by SNP-A analysis between DS-AMKL and non-DS-AMKL.

Type of somatic lesion	DS-AMKL (n = 11)	Non-DS-AMKL (n = 12)	P-value
Somatic 1q gain			
Yes	4	0	0·02
No	7	12	
Somatic gain lesion other than germline +21			
Yes	6	7	NS
No	5	5	
Somatic loss lesion			
Yes	8	5	NS
No	3	7	
Somatic UPD lesion			
Yes	1	0	NS
No	10	12	
Any somatic lesion			
Yes	8	8	NS
No	3	4	

SNP-A, single nucleotide polymorphism array; DS, Down syndrome, AMKL, acute megakaryoblastic leukaemia, UPD; uniparental disomy, NS; not significant.

[36% (4/11) vs. 0%, P = 0·02]. We also screened the SNP-A karyograms of 798 patients with myeloid malignancies (including paediatric and adult leukaemias, Fig 2) and found a commonly affected region on 1q (57·4 Mb, 188 533 848–245 934 866) in 2·8% (22/798) patients (Fig 2, region A), defined by patients with DS-AMKL. In addition to these four DS-AMKL patients, gain of 1q was also found in chronic myelomonocytic leukaemia (n = 5), secondary AML (n = 5), myelodysplastic syndrome (MDS, n = 3), Fanconi anaemia (n = 2), idiopathic myelofibrosis (n = 1), CML (n = 1), and aplastic anaemia-derived MDS (n = 1). We were also able to determine that an alternate commonly affected region on 1q (region B, 118 kb) was shared by 20/22 patients. This region was defined by a somatic microduplication in a patient with idiopathic myelofibrosis and contained only the *ESRRG* gene. Regions C (172 kb) and D (2·2 Mb) were shared by (19/22) each and harboured 3 and 10 genes, respectively (Fig 2). The frequencies of all other somatic lesions (gains, losses, UPDs) were not statistically different between patient groups (Table III).

Correlation between somatic trisomy 21 and megakaryoblastic phenotype

In addition to germline-encoded trisomy 21 of DS, somatic trisomy 21 was found in two additional patients with paediatric non-DS-AMKL. To establish whether there was an association between trisomy 21 and megakaryoblastic morphology, we analysed a cohort of paediatric AML patients without DS; AMKL was found in 23/232 of them. AMKL was found in 9/642 cases of adult AML without DS. Somatic trisomy 21 was present in 8 paediatric AML patients that showed megakaryoblastic phenotype (P < 0·001), while no association was found in adults (trisomy 21 was found in 21 patients, of whom none had AML with megakaryoblastic morphology; P > 0·999). To confirm our results, we also analysed 6009 adult AML without DS registered in the Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer (Mitelman *et al*, 2011) and found non-DS-AMKL in 130 patients. Somatic trisomy 21 was present in 264 adult AML patients, of whom only seven had non-DS-AMKL (P = 0·515, Table IV).

Discussion

In AML, distinct molecular lesions may lead to a similar clinical phenotype; conversely, specific morphological features could be a result of recurrent lesions. For instance, AMKL is frequently seen in children with DS and is characterized by the presence of *GATA1* mutations; thus, we posed a question whether AMKL seen in children and adults share common molecular abnormalities. As expected, *GATA1* mutations were rare in non-DS-AMKL and found in only 1/11 children with this condition. To date, a few children with *GATA1* mutations in non-DS-AMKL have been reported (Rainis *et al*, 2003; Malinge *et al*, 2008). Interestingly, all of them had acquired trisomy 21 in their leukaemic cells. Our non-DS-AMKL

Table IV. Association between somatic trisomy 21 and megakaryoblastic morphology in paediatric and adult AML patients without DS.

Patient cohort	Morphology	Somatic trisomy 21		P-value
		Present	Absent	
Paediatric AMLwithout DS (N = 232) (Nagoya University)	AMKL (n = 23)	8	15	<0·001
	Other AML (n = 209)	6	203	
Adult AML without DS (N = 642) (Cleveland Clinic)	AMKL (n = 9)	0	9	>0·999
	Other AML (n = 633)	21	612	
Adult AML without DS (N = 6009) (Mitelman database)	AMKL (n = 130)	7	123	0·515
	Other AML (n = 5879)	257	5622	

patient with *GATA1* mutation did not have acquired trisomy 21 in his leukaemic cells. Recently, mutations in *JAK2*, *JAK3*, *TP53*, *FLT3*, *ASXL1*, *DNMT3A*, *IDH1*, and *IDH2* have been found in various myeloid malignancies, including AML; some of these genes could be involved in signalling along the megakaryoblastic differentiation pathway. In this series of experiments we analysed a large cohort of AMKL for the presence of distinctive or shared chromosomal and genetic defects.

In addition to genes previously reported as mutated in AMKL (*JAK2*, *JAK3*, *TP53*), we identified *NRAS* mutation in two children with non-DS-AMKL but genes recently identified in various myeloid malignancies (*ASXL1*, *DNMT3A*, *IDH1*, *IDH2*, *RUNX1*, and *CBL*) were not found to be mutated in our AMKL cohort. While having only a few cases precluded systematic analysis, it appears that adult and paediatric cases of AMKL do not display distinctive mutational patterns.

In addition to the germline trisomy 21 in DS, somatic duplications of chromosome 21 were found in two cases of paediatric non-DS-AMKL. In paediatric AML, trisomy 21 is strongly linked to megakaryoblastic AML phenotype, as further analysis of AML with this lesion revealed six additional cases of AMKL, and as result, 8/23 cases of somatic trisomy 21 were AMKL. Conversely, there is no significant association between trisomy 21 and adult AMKL in both the Cleveland Clinic cohort and Mitelman Database. It is speculated that the presence of trisomy 21 contributes to megakaryoblastic phenotype only in the paediatric population.

A distinctive feature of DS-AMKL is the presence of a gain of 1q, found in 4/11 cases and absent in all other cases of non-DS-AMKL. Gain of 1q through duplication, isochromosome formation or unbalanced translocations, is one of the most frequent acquired cytogenetic abnormalities in human neoplasia and has also been found in various haematological malignancies, including B-lineage acute lymphoblastic leukaemia (B-ALL) (Johansson *et al*, 2004), multiple myeloma (Nilsson *et al*, 2003), and non-Hodgkin lymphomas (Johansson *et al*, 1995). Expression analyses implicated several upregulated genes associated with this amplification in ALL (Davidsson *et al*, 2007). Similarly, 1q gain was also found in DS-AMKL patients (Hayashi *et al*, 1988; Silva *et al*, 2009); a recent international Berlin-Frankfurt-Munster (iBFM) study of the cytogenetic metaphase analysis reported significantly higher incidence of 1q gains in DS-AML (16%) compared to non-DS-AML (2%) (Forestier *et al*, 2008). In 2/4 patients, metaphase cytogenetics showed the presence of unbalanced translocations involving 1q but an associated gain was detected only by SNP-A. Two out of four patients showed a previously cryptic 1q gain by SNP-A but not by metaphase cytogenetics. As a result the overall frequency of this lesion was found to be higher than previously reported using only routine cytogenetics (Forestier *et al*, 2008). By comparison, 2·8% (22/798) of patients with haematological disease harboured somatic 1q gains within the regions shared by four patients with DS-AMKL. We speculate that the commonly affected region of chromosome 1q contains a putative gene involved in pathogenesis of DS-AMKL. Potential candidate genes include *AKT3* (Nakatani *et al*, 1999; Stahl *et al*, 2004) and *ESRRG* (Ijichi *et al*, 2011), both reported to be overexpressed in association with cancer development and cell proliferation.

In summary, this study comprehensively analysed the molecular lesions in AMKL using SNP-A and mutational analysis of the selected genes. High frequency of 1q gain as well as *GATA1* mutation in DS-AMKL suggests that leukemogenesis mechanisms are diverse among subgroups of AMKL.

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Authorship

A. Hama, H. Muramatsu, H. Makishima designed research, performed research, analysed data, and wrote the paper. Y.S., H.S., M.J. performed research. C.O. designed research, analysed data, and wrote the paper. Y.T., H.S., S.D., A.S., N.W., K.M., K.K. designed research. S.K. designed research and wrote the paper. J.P.M. designed research, performed research, analysed data, and wrote the paper.

Conflict of interest disclosure

The authors declare no competing financial interests.

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EBV-associated T/NK–cell lymphoproliferative diseases in nonimmunocompromised hosts: prospective analysis of 108 cases

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EBV-associated T/NK–cell lymphoproliferative disease (T/NK-LPD) is defined as a systemic illness characterized by clonal proliferation of EBV-infected T or NK cells. We prospectively enrolled 108 nonimmunocompromised patients with this disease (50 men and 58 women; median onset age, 8 years; age range, 1-50 years) evidenced by expansion of EBV$^+$ T/NK cells in the peripheral blood; these were of the T-cell type in 64 cases and of the NK-cell type in 44, and were clinically categorized into 4 groups: 80 cases of chronic active EBV disease, 15 of EBV-associated hemophagocytic lymphohistiocytosis, 9 of severe mosquito bite allergy, and 4 of hydroa vacciniforme. These clinical profiles were closely linked with the EBV$^+$ cell immunophenotypes. In a median follow-up period of 46 months, 47 patients (44%) died of severe organ complications. During the follow-up, 13 patients developed overt lymphoma or leukemia characterized by extranodal NK/T-cell lymphoma and aggressive NK-cell leukemia. Fifty-nine received hematopoietic stem cell transplantation, 66% of whom survived. Age at onset of disease (≥ 8 years) and liver dysfunction were risk factors for mortality, whereas patients who received transplantation had a better prognosis. These data depict clinical characteristics of systemic EBV$^+$ T/NK-LPD and provide insight into the diagnostic and therapeutic approaches for distinct disease. (*Blood*. 2012;119(3): 673-686)

Introduction

EBV-associated lymphoproliferative diseases (LPDs) have a vast spectrum from reactive to neoplastic processes in the transformation and proliferation of lymphocytes spanning B, T, and NK cells,[1-3] and are clinically complicated by the interaction between the biologic properties of EBV$^+$ lymphocytes and the host immune status. Our understanding of these diseases is now evolving and has led to the recognition of a variety of EBV$^+$ diseases, including Burkitt lymphoma,[3] age-related EBV$^+$ B-cell LPD,[4] extranodal NK/T-cell lymphoma of nasal type (ENKL),[5] aggressive NK-cell leukemia (ANKL),[6] classic Hodgkin lymphoma,[3] and immunodeficiency-associated lymphoproliferative disorders.[1] EBV-associated T- and NK-cell LPD (T/NK-LPD) was first incorporated into the 4th World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissues, in which systemic EBV$^+$ T-cell LPD of childhood and hydroa vacciniforme–like lymphoma are proposed as distinct entities.[7-8] Historically, based on their broad clinical manifestations, these diseases have been described under various nosological terms from indolent (eg, severe mosquito bite allergy[9] and hydroa vacciniforme[10]) to aggressive or fulminant forms (eg, EBV-associated hemophagocytic lymphohistiocytosis [HLH],[11] chronic active EBV disease [CAEBV] of the T/NK–cell type,[12] fulminant EBV$^+$ T-cell LPD of childhood,[13] and fatal infectious mononucleosis[3]).

CAEBV originally referred to chronic or recurrent infectious mononucleosis-like symptoms.[14-16] A severe form of CAEBV was found to be prevalent in east Asian countries and was characterized by clonal expansion of the EBV-infected T or NK cells,[12,17-18] whereas in Western countries CAEBV is mostly associated with EBV-infected B cells.[19-20] The term EBV-associated HLH was coined to describe hemophagocytosis involving BM or other organs and resulting in pancytopenia in the peripheral blood. This disease is also frequently seen in east Asian countries,[11] and involves a clonal expansion of EBV$^+$ T or NK cells, which produce inflammatory cytokines that induce the activation of macrophages and hemophagocytosis.[21-23] Apart from these systemic diseases, accumulating evidence indicates that 2 cutaneous diseases, hydroa vacciniforme and severe mosquito bite allergy, are closely associated with EBV$^+$ T or NK cells. Hydroa vacciniforme is characterized by recurrent vesiculopapules usually occurring on sun-exposed areas and seen in children and adolescents.[10] In some of these patients, systemic symptoms including fever, wasting, lymphadenopathy, and hepatosplenomegaly have been recorded.[24-26] Severe mosquito bite allergy was determined to be associated with EBV$^+$ NK cells, but rarely with EBV$^+$ T cells, and to progress into overt lymphoma or leukemia in the long-standing clinical course.[9,27] These EBV$^+$ cutaneous diseases had the same geographic distribution as the other EBV$^+$ T/NK–cell lymphomas and LPDs among

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Table 1. Definitions of EBV$^+$ T/NK-LPDs in this study

Disease	Eligibility criteria	Exclusion criteria	Lineages/clonality	References
Clinical category				
CAEBV of T/NK–cell type	(1) Illness ≥ 3 mo in duration (EBV-related illness or symptoms including fever, persistent hepatitis, lymphadenopathy, hepatosplenomegaly, pancytopenia, uveitis, interstitial pneumonia, hydroa vacciniforme-like eruptions, and hypersensitivity to mosquito bites)*	(1) No evidence of previous immunological abnormalities or other recent infection that might explain the observed condition	T/NK cell	12
	(2) Increased amounts of EBV detected by Southern blot hybridization or EBER$^+$ cells in affected tissues or peripheral blood; ≥ $10^{2.5}$ copies/μg of EBV DNA in PBMCs	(2) Congenital immunodeficiency including X-linked lymphoproliferative disorders	Polyclonal, oligoclonal, monoclonal	16,29
HLH	(1) Clinical criteria (fever and splenomegaly)	(1) Hemophagocytic syndrome in accelerated phase of CAEBV of T/NK cell type	T/NK cell	11
	(2) Laboratory criteria (cytopenia affecting 2 of 3 lineages in the peripheral blood, hypertriglyceridemia, and/or hypofibrinogenemia) (3) Histological criteria (hemophagocytosis in the BM, spleen, or lymph nodes)	(2) Congenital immunodeficiency including familial HLH	Polyclonal, oligoclonal, monoclonal	
Severe mosquito bite allergy*	Hypersensitivity to mosquito bites characterized by high fever after bites, ulcers, necrosis, and scarring*	Any systemic symptoms in addition to the cutaneous lesions were categorized to CAEBV of T/NK cell type	T/NK cell, polyclonal, oligoclonal, monoclonal	9,39
Hydroa vacciniforme*	Recurrent vesiculopapules with central umbilication and crust formation mimicking herpetic vesicles usually occurring on sun-exposed areas	Any systemic symptoms in addition to cutaneous lesions categorized as CAEBV of T/NK cell type	T/NK cell, polyclonal, oligoclonal, monoclonal	10,39
Pathological classification				
Systemic EBV$^+$ T-cell LPD	(1) Illness or symptoms including fever, persistent hepatitis, lymphadenopathy, hepatosplenomegaly, hemophagocytosis, and interstitial pneumonia (2) Can occur shortly after primary EBV infection or in the setting of CAEBV (3) Monoclonal expansion of EBV-infected T cells with an activated cytotoxic phenotype in tissues or peripheral blood	Other overt leukemia and lymphoma such as extranodal NK/T-cell lymphoma, aggressive NK-cell leukemia, and peripheral T-cell lymphoma	T-cell, monoclonal	7
Hydroa vacciniforme-like lymphoma	(1) Recurrent vesiculopapules with central umbilication and crust formation usually occurring on sun-exposed areas with or without systemic symptoms including fever, wasting, lymphadenopathy, and hepatosplenomegaly (2) Monoclonality of EBV-infected cells	Other overt leukemia and lymphoma such as extranodal NK/T-cell lymphoma, aggressive NK-cell leukemia, and peripheral T-cell lymphoma	T/NK cell, monoclonal	7

*"Severe mosquito bite allergy" and "hydroa vacciniforme" were used as clinical categories, whereas "hypersensitivity to mosquito bites" and "hydroa vacciniforme-like eruptions" were used to designate symptoms.

east Asians and Native Americans in Central and South America and Mexico,[8] and were encountered as a part of the initial and accompanying symptoms of the systemic EBV$^+$ T/NK-LPDs.[28-30] However, the mutual relationship and clinicopathologic distinctiveness of these EBV$^+$ T/NK-LPDs are unfounded, posing diagnostic and therapeutic problems for pathologists and hematologists, respectively. These patients appear to exist in the gray zone between systemic EBV$^+$ T-cell LPD of childhood and hydroa vacciniforme–like lymphoma according to the 4th WHO classification. The former encompasses CAEBV of T-cell type, EBV$^+$ HLH, and EBV$^+$ T-cell lymphomas with prodromal phase, whereas the latter may include all cases with EBV$^+$ hydra vacciniforme despite the presence or absence of the systemic disease in the patient's history.

The aim of the present study was to clarify the clinicopathologic characteristics of these EBV$^+$ T/NK-LPDs and the biologic properties of the proliferating cells by analyzing a large number of patients. We previously performed a nationwide survey for CAEBV of T/NK–cell type and determined its prognostic factors.[29] Similarly, a nationwide study for HLH was recently performed in Japan.[31] However, these studies were retrospective and lacked the precise diagnosis of the current level because of their study design. In 1998, we established an EBV-DNA quantification system using real-time PCR,[32-33] which allowed for the determination of the phenotype of EBV-infected cells in the peripheral blood with the combination of fractionation to the lymphocyte subset.[12,34-35] More recently, we developed the simultaneous staining method for surface antigens and nuclear EBV-encoded small RNA (EBER) to more precisely determine EBV-infected cell phenotypes.[36] Using these techniques, we enrolled and prospectively followed patients with definitive cases of EBV$^+$ T/NK-LPDs in 1998. In this study, 108 nonimmunocompromised patients with EBV$^+$ T/NK-LPDs were analyzed for clinical and virological characteristics to obtain an understanding of their pathogenesis and for refining their

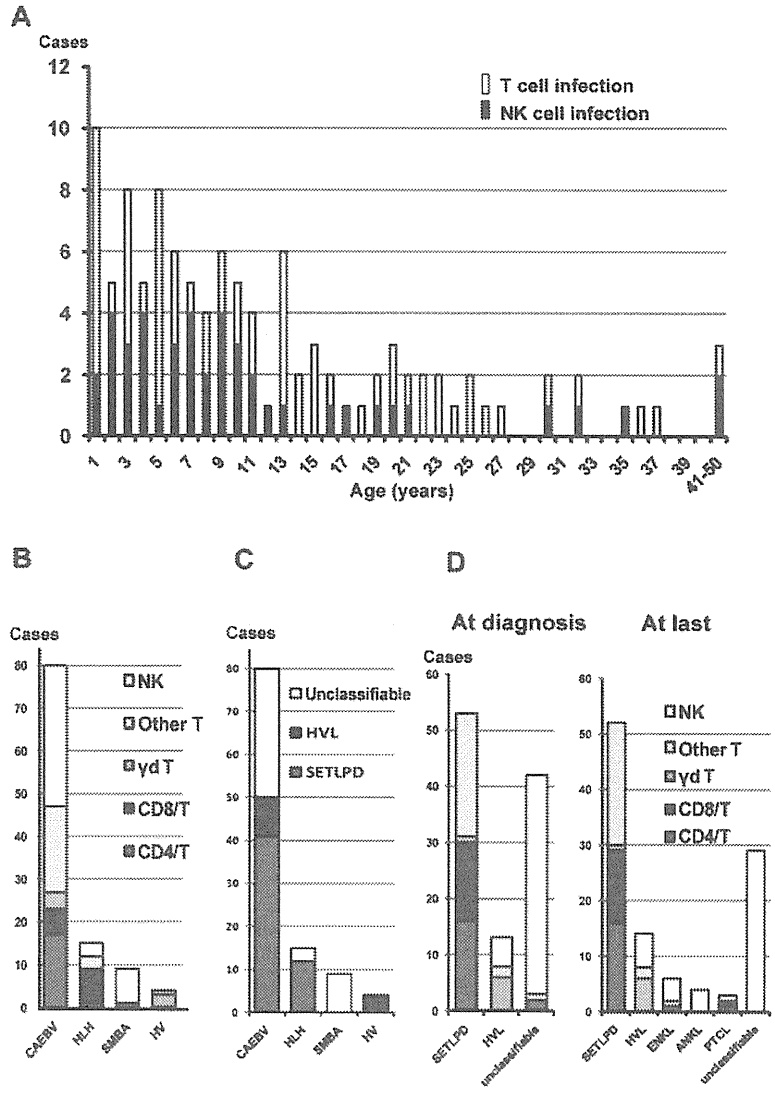

Figure 1. EBV-infected cell phenotypes of EBV⁺ T/NK lymphoproliferative diseases. (A) Age distribution of patients with T-cell and NK-cell types. (B) EBV-infected cells among categories of clinical groups. Infected T cells were further divided into $CD4^+$ T cells, $CD8^+$ T cells, γδ T cells, and "other 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.

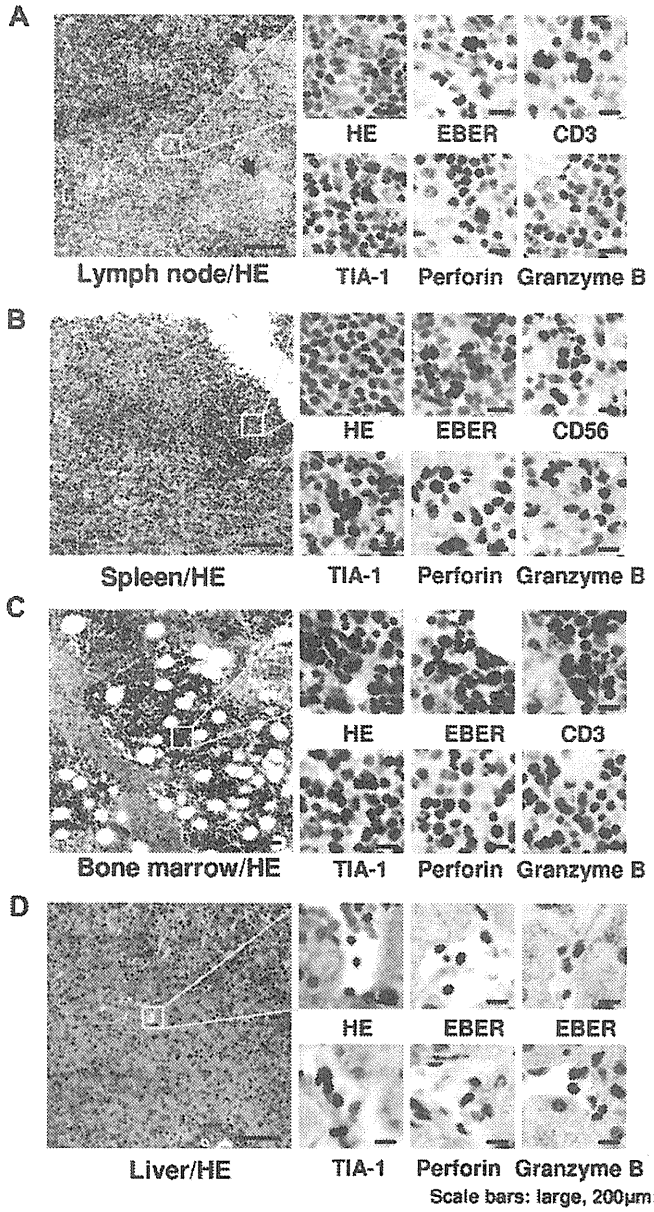

Figure 2. Histopathological findings of representative patients. (A) Cervical lymph node from a 6-year-old boy with chronic active EBV disease with T-cell infection (patient 3). Follicles and paracortical hyperplasia including a mild increase in transformed lymphocytes were seen. Focal epithelioid reactions 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 $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+$, $CD19^+$, $CD56^+$, $TCR\alpha\beta^+$, and $TCR\gamma\delta^+$ 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.

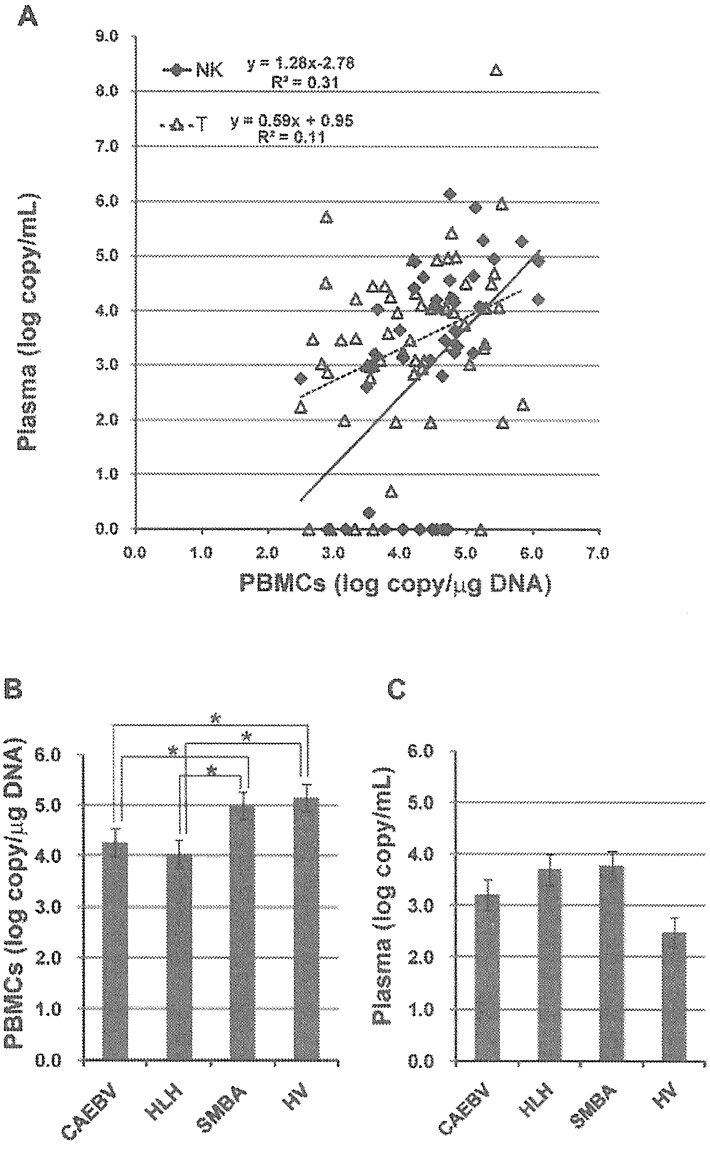

Figure 3. Viral load in the peripheral blood at the time of diagnosis. EBV-DNA was quantified by real-time PCR. (A) Correlation of viral load between PBMCs and plasma. The correlation was separately estimated in patients with T-cell infection and those with NK-cell infection. (B) Quantity of EBV-DNA 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