

34 例 (33 家系)、DKC 17 例、分類不能疾患 17 例 (12 家系)、CDA 16 例 (5 家系)、CSA 15 例 (5 家系)、AT 10 例、HLH 10 例の全エクソンシーケンスを行った (合計 325 例、204 家系)。全エクソンシーケンスでは症例あたり平均約 20,000 個のヒトゲノムレファレンス配列と異なる塩基配列がみつき、このうち dbSNP131、in-house (当研究室でこれまでに解析した 100 例のエクソンシーケンスから得られた) SNP データにあるものを SNP として除き、最終的に症例あたり平均 300 個の候補となる変異が同定された。これらのなかから家系の情報、遺伝形式などを考慮して候補遺伝子の絞り込みを行った。

これまでに得られた成果としては、先天性の血小板減少症では 13 家系の常染色体優性遺伝の家系の解析例のうち 6 家系で *ACTN1* 遺伝子が疾患原因遺伝子となっていることを発見し、新規の原因遺伝子として報告した。また、JMML では従来から知られていた RAS パスウェイの変異が約 90% の症例にみられることに加え、*SETBP1* 遺伝子が secondary mutation として病勢の進行に関わり、予後不良因子であると報告した。さらに、DBA でも既知の ribosome 関係の遺伝子の変異・欠失がみられなかった症例のエクソンシーケンスによりこれまでに報告がない RPS27、RPL27 などの ribosome 関係の遺伝子の変異を同定した。

#### D. 考察

次世代シーケンサーを用いた網羅的な全エクソンシーケンスは遺伝性疾患での原因遺伝子の検索に極めて有用な方法であり、特に家系を解析することが候補遺伝子を絞り込むうえで有効であると考えられた。

#### E. 結論

全エクソンシーケンスは遺伝性疾患の原因遺伝子の検索に極めて有用な方法であり、今後さらに解析を続けていく予定である。

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G. 知的財産権の出願・登録状況

なし。

### Ⅲ. 研究成果の刊行に関する一覧

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#### IV. 研究成果の刊行物・別刷り

To the editor:

## Rabbit antithymocyte globulin and cyclosporine as first-line therapy for children with acquired aplastic anemia

Horse antithymocyte globulin (hATG) and cyclosporine have been used as standard therapy for children with acquired aplastic anemia (AA) for whom an HLA-matched family donor is unavailable. However, in 2009, hATG (lymphoglobulin; Genzyme) was withdrawn and replaced by rabbit ATG (rATG; thymoglobulin; Genzyme) in Japan. Many other countries in Europe and Asia are facing the same situation.<sup>1</sup> Marsh et al recently reported outcomes for 35 adult patients with AA who were treated with rATG and cyclosporine as a first-line therapy.<sup>2</sup> Although the hematologic response rate was 40% at 6 months, several patients subsequently achieved late responses. The best response rate was 60% compared with 67% in a matched-pair control group of 105 patients treated with hATG. The overall and transplantation-free survival rates appeared to be significantly inferior with rATG compared with hATG at 68% versus 86% ( $P = .009$ ) and 52% versus 76% ( $P = .002$ ), respectively. These results are comparable to those from a prospective randomized study reported by Scheinberg et al comparing hATG and rATG.<sup>3</sup> Both studies showed the superiority of hATG over rATG.<sup>2,3</sup>

We recently analyzed outcomes for 40 Japanese children (median age, 9 years; range, 1-15) with AA treated using rATG and cyclosporine. The median interval from diagnosis to treatment was 22 days (range, 1-203). The numbers of patients with very severe, severe, and nonsevere disease were 14, 10, and 16, respectively. The ATG dose was 3.5 mg/kg/day for 5 days. The median follow-up time for all patients was 22 months (range, 6-38). At 3 months, no patients had achieved a complete response (CR) and partial response (PR) was seen in only 8 patients (20.0%). At 6 months, the numbers of patients with CR and PR were 2 (5.0%) and 17 (42.5%), respectively. After 6 months, 5 patients with PR at 6 months had achieved CR and 4 patients with no response at 6 months had achieved PR, offering a total best response rate of 57.5%. Two patients relapsed at 16 and 19 months without receiving any second-line treatments. Two patients with no re-

sponse received a second course of rATG at 13 and 17 months, but neither responded. Sixteen patients underwent hematopoietic stem cell transplantation (HSCT) from alternative donors (HLA-matched unrelated donors,  $n = 13$ ; HLA-mismatched family donors,  $n = 3$ ). Two deaths occurred after rATG therapy, but no patients died after HSCT. Causes of death were intracranial hemorrhage at 6 months and acute respiratory distress syndrome at 17 months. The overall 2-year survival rate was 93.8% and the 2-year transplantation-free survival rate was 50.3% (Figure 1).

In our previous prospective studies with hATG, the response rates after 6 months were 68% and 70%, respectively, with no increases in response rates observed after 6 months.<sup>4,5</sup> Our results support the notion that rATG is inferior to hATG for the treatment of AA in children. First-line HSCT from an alternative donor may be justified, considering the excellent outcomes in children who received salvage therapies using alternative donor HSCT.

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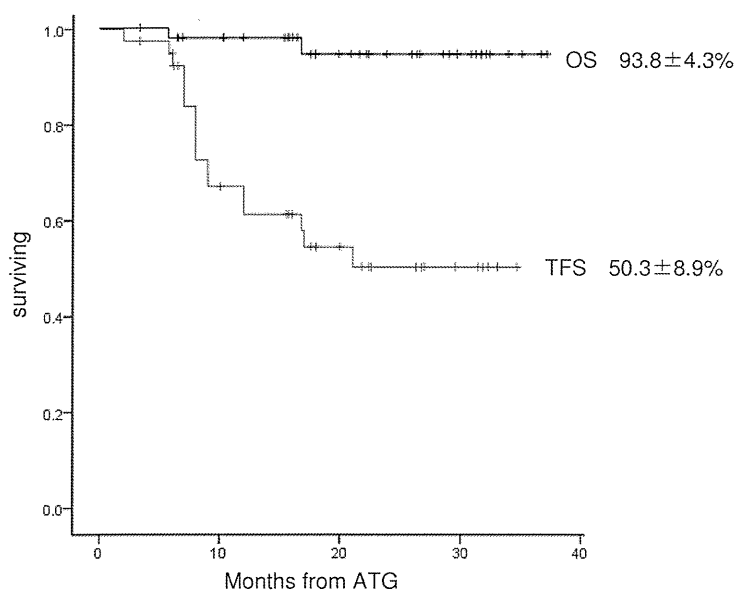


Figure 1. Kaplan-Meier estimates of overall survival (OS) and transplantation-free survival (TFS) in 40 Japanese children with AA. Survival was investigated using Kaplan-Meier methods. OS for all patients with AA after rATG and cyclosporine as first-line therapy included patients who later received HSCT for nonresponse to rATG. In the analysis of TFS for all patients treated with rATG and CSA, transplantation was considered an event.

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To the editor:

## Peripheral blood stem cells versus bone marrow in pediatric unrelated donor stem cell transplantation

The relative benefits and risks of peripheral blood stem cells (PBSCs) versus bone marrow (BM) for allogeneic hematopoietic stem cell transplantation (SCT) are still a matter of highly controversial debates.<sup>1-3</sup> The first randomized study comparing the 2 stem cell sources in unrelated donor SCT recently documented comparable overall and event-free survival, but indicated a higher risk for chronic graft-versus-host disease (GVHD) with PBSCs.<sup>4</sup> Only a few pediatric patients were included in this study even though the long-term sequelae of chronic GVHD are of particular concern in this patient group.

We retrospectively compared the long-term outcome of contemporaneous unrelated donor SCT in 220 children transplanted with BM (n = 102) or PBSCs (n = 118) for hematologic malignancies and reported to the German/Austrian pediatric registry for SCT. All patients had received myeloablative conditioning followed by unmanipulated SCT from HLA-matched unrelated donors. The PBSC and BM groups were comparable with regard to patient and donor age, sex, cytomegalovirus (CMV) serostatus, disease status at transplantation, GVHD prophylaxis, growth factor use, and degree of HLA matching. The groups differed with regard to disease category with slightly more myelodysplastic syndrome patients ( $P = .02$ ) and a higher CD34-cell dose ( $P = .001$ ) in the PBSC group.

Neutrophil and platelet engraftment were achieved significantly faster after PBSC than BM transplantation (Figure 1A-B). In this entirely pediatric cohort, the incidence of clinically relevant grade

II-IV acute GVHD (Figure 1C) did not differ. Most importantly, the incidence of chronic GVHD (PBSCs vs BM: 35% vs 33%, respectively;  $P = .9$ ) and extensive chronic GVHD (Figure 1D) proved low and was virtually identical in the 2 groups. With a median follow-up time of 3 years, overall survival (PBSCs vs BM: 50%  $\pm$  5% vs 46%  $\pm$  6%, respectively;  $P = .63$ ) and event-free survival (PBSCs vs BM: 45%  $\pm$  5% vs 44%  $\pm$  6%, respectively;  $P = .59$ ) were comparable (Figure 1E-F). In multivariable analysis, taking into account all parameters with  $P < .2$  in univariate analysis, the only significant independent risk factor for treatment failure was advanced disease status at the time of transplantation (relative risk = 2.4, 95% confidence interval, 1.5-3.8;  $P = .001$ ). In contrast, stem cell source (PBSCs vs BM) had no effect (relative risk = 1.1, 95% confidence interval, 0.7-1.6;  $P = .8$ ).

Our registry-based analysis provides evidence that in pediatric recipients of HLA-matched unrelated-donor transplantation with consistent antithymocyte globulin (ATG) use during conditioning, transplantation with PBSCs and BM results in comparable clinical outcomes without detectable differences in the risk of acute or, more importantly, chronic GVHD. Consistent with a recent study underscoring the role of ATG for the prevention of acute and chronic GVHD,<sup>5</sup> the use of ATG in 96% of our transplantation procedures compared with only 27% in the above-mentioned randomized study by Anasetti et al<sup>4</sup> might be one of the key factors responsible for the overall low and comparable incidence of



## Inherited bone marrow failure syndromes in 2012

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**Abstract** Inherited bone marrow failure syndromes (CBMFS) are a heterogeneous group of genetic disorders characterized by bone marrow failure, congenital anomalies, and an increased risk of malignant disease. The representative diseases with trilineage involvement are Fanconi anemia and dyskeratosis congenita, while the disease with the single lineage cytopenia is Diamond–Blackfan anemia. Recent advances in our understanding of these diseases have come from the identification of genetic lesions responsible for the disease and their pathways. Although recent studies have identified many causative genes, mutations of these genes have only been found in less than half of the patients. Next-generation sequencing technologies may reveal new causative genes in these patients. Also, induced pluripotent stem cells derived from patients with CBMFS will be useful to study the pathophysiology of the diseases. The only long-term curative treatment for bone marrow failure in patients with inherited bone marrow failure syndromes is allogeneic hematopoietic stem cell transplantation, although this procedure has a risk of severe adverse effects. Multicenter prospective studies are warranted to establish appropriate conditioning regimens aimed at reducing transplant-related mortality.

**Keywords** Inherited bone marrow failure syndrome · Fanconi anemia · Diamond–Blackfan anemia · Dyskeratosis congenita

### Introduction

Inherited bone marrow failure syndromes (IBMFS) are a heterogeneous group of genetic disorders characterized by bone marrow failure, congenital anomalies, and increased risk of malignant disease. Such bone marrow failure may affect all three hematopoietic cell lineages or single cell lineages individually. Diseases characterized by trilineage involvement include Fanconi anemia and dyskeratosis congenita, while Diamond–Blackfan anemia results in single-lineage cytopenia. Recent advances in our understanding of these diseases have arisen from the identification of genetic lesions responsible for such diseases and their pathogenic pathways. These investigations have further clarified both normal and pathological hematopoiesis. In this current review, we describe recent insights into three IBMFS: Fanconi anemia, Diamond–Blackfan anemia, and dyskeratosis congenita.

### Fanconi anemia

Fanconi anemia (FA) is a rare autosomal recessive disease characterized by congenital abnormalities, progressive bone marrow failure, and cancer susceptibility. FA, which has an incidence of less than 10 per million live births, is the most frequent inherited cause of aplastic anemia [1]. FA is a genetically heterogeneous disease defined by complementation groups. To date, 15 genes have been identified as playing a causative in FA and these genes, FANCA to FANCP, have been cloned [2]. Children with FA often develop aplastic anemia during the first decade of life, with death often resulting from complications of bone marrow failure, such as severe infection or bleeding. FA

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patients also develop clonal chromosomal abnormalities in bone-marrow progenitor cells, such as monosomy 7, which are associated with myelodysplastic syndrome (MDS) and acute myeloblastic leukemia (AML) [3]. The gene FANCD1, which is responsible for complementation group FA-D1, is identical to the hereditary breast cancer susceptibility gene, BRCA2, and has been reported as affected in 3 % of patients with Fanconi anemia. As compared with children from other FA groups, more severe phenotypes are seen in FA-D1 patients, such as co-occurrence of multiple anomalies, development of multiple malignancies with earlier onset, and increased incidence of leukemia and solid tumors [4], including Wilms tumor, neuroblastoma, and brain tumors.

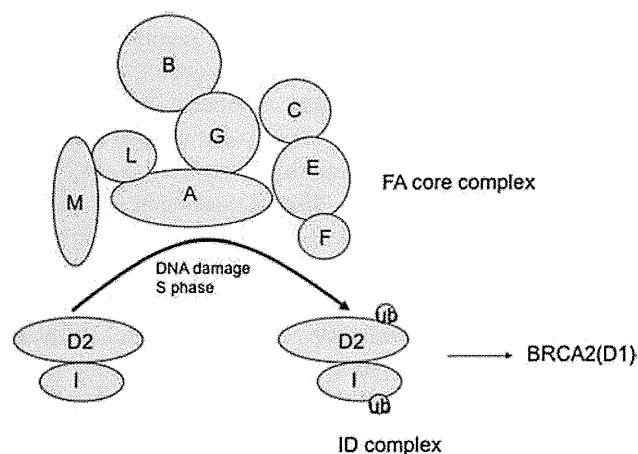
While the treatment of choice for FA patients remains allogeneic stem cell transplantation (SCT) from an HLA-matched sibling or unrelated donor, older patients may develop squamous-cell carcinomas (SCCs) of the head and neck or gynecological system. In particular, some studies have demonstrated there is a high incidence of SCC, such as esophageal cancer, in FA patients who have received SCT. The age-specific hazard of SCC has been shown to be 4.4-fold higher in patients who receive transplants and, in addition, SCCs occurred at significantly younger ages in the transplant group [5]. Thus, further investigations of the complete care of FA patients need to be undertaken.

Complementation groups and genes of FA

Since cells derived from FA patients are hypersensitive to DNA interstrand cross-linking (ICL) agents, such as diepoxybutane (DEB), mitomycin C (MMC), and cisplatin, it is expected that FA genes are involved in ICL repair. In 1993, FANCC was the first FA gene to be cloned by expression cloning [6, 7]. Subsequently, 15 other genes have been cloned (Table 1). At present, the FANCD genes, which range from FANCA to FANCP, and the FA pathway have been shown to resolve ICLs encountered during DNA replication. There are three primary groups of FA proteins, which include the FA core complex, the ID (FANCD2/I) complex, and the BRCA complex (Fig. 1). In these groups, there are eight FA proteins (FANCA/B/C/E/F/G/L/M) that form a multi-subunit ubiquitin E3 ligase complex, the FA core complex, which activates the monoubiquitination of the ID complex after genotoxic stress, such as ICL, or during the S phase (Fig. 1) [8, 9]. The monoubiquitinated ID complex forms foci on damaged DNA. FANCM is also a crucial gene, as it is a sensor for detecting stalled DNA replication. The BRCA complex, which is also referred to as homologous recombination (HR), consists of FANCD1, FANCI, FANCN, and FANCO, and is located downstream of the DI complex on the ICL repair pathway.

**Table 1** Genes mutated in patients with Fanconi anemia

	Other names	Chromosomal Locus	Population in FA patients (%)
FANCA		16q24.3	60–70
FANCB		Xp22.2	2
FANCC		9q22.3	14
FANCD1	BRCA2	13q13.1	3
FANCD2		3p25.3	3
FANCE		6p22-p21	3
FANCF		11p15	2
FANCG		9p13	10
FANCI		15q25-q26	1
FANCI	BACH1/BRIP1	17q22	2
FANCL		2p16.1	0.20
FANCM		14q21.3	0.20
FANCN	PALB2	16p12.3-p12.2	0.70
FANCO	RAD51C	17q22	0.20
FANCP	SLX4	16p13.3	0.20



**Fig. 1** Simplified scheme of the FA pathway. Depending on the FA core complex, FANCD2 and FANCI are monoubiquitinated after DNA damage or during the S-phase

Prognosis factors in FA patients

Although FANCD gene knockout mice models have been established, they differ from the hematological phenotypes of human FA patients [10]. With the exception of the lethal phenotype of the BRCA2/FANCD1 knockout mouse, the hematological parameters of the other FA groups show only a slightly decreased platelet count and a slightly increased erythrocyte mean cell volume in mice at a young age, which did not progress to aplastic anemia or leukemia. However, both male and female mice showed hypogonadism and impaired fertility, which is consistent with the human FA patient phenotypes.

Recent studies have revealed a relationship between the acetaldehyde and FA pathways. Acetaldehyde is an organic chemical compound that is naturally present in coffee, bread, and ripe fruit, which is produced as product of a plant's normal metabolism. It is also produced by the oxidation of ethylene. In the liver, the enzyme alcohol dehydrogenase (ADH) oxidizes ethanol into acetaldehyde, which is then further oxidized into harmless acetic acid by acetaldehyde dehydrogenase (ALDH). These oxidation reactions are coupled with the reduction of  $\text{NAD}^+$  to NADH. ALDH2, an isozyme of ALDH, contains the functional polymorphism, *ALDH2* Glu487Lys. An association between this polymorphism and squamous cell carcinomas, such as esophageal cancer in alcoholics, has been reported. A recent study reported that exposure of cells to acetaldehyde results in a concentration-dependent increase in FANCD2 monoubiquitination [11]. Acetaldehyde also stimulates BRCA1 phosphorylation at Ser1524 and increases the level of H2AX, a marker of homologous recombination. Both modifications occur in a dose-dependent manner.

Another report showed that ALDH2 is essential for the development of FANCD2 (−/−) embryos [12]. Nevertheless, mothers with AA enzyme (ALDH 2(+/−)) can support the development of double-mutant (ALDH2 (−/−)FANCD2(−/−)) mice. These embryos are unusually sensitive to ethanol exposure in utero, with ethanol consumption by postnatal double-deficient mice rapidly precipitating bone marrow failure. ALDH2 (−/−)FANCD2(−/−) mice also spontaneously develop acute leukemia.

This previous study also provided the first evidence of the factors responsible for driving the FA hematological phenotype in mice. DNA damage caused by acetaldehyde may contribute critically to the genesis of fetal alcohol syndrome in fetuses, as well as to abnormal development, bone marrow failure, and cancer predisposition in FA patients. This research group also focused on hematopoietic stem cells (HSCs) in another study [13]. They reported finding that some aged ALDH2(−/−)FANCD2(−/−) mutant mice that did not develop leukemia spontaneously developed aplastic anemia, with a concomitant accumulation of damaged DNA within the hematopoietic stem and progenitor cell (HSPC) pool. Only HSPCs and not the more mature blood precursors require *Aldh2* for protection against acetaldehyde toxicity. There is more than a 600-fold reduction in the HSC pool of mice deficient in both FA pathway-mediated DNA repair and acetaldehyde detoxification. This study data indicated that the emergence of bone marrow failure in FA was probably due to aldehyde-mediated genotoxicity restricted to the HSPC pool.

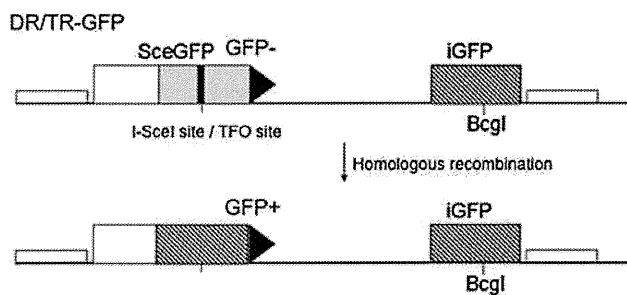
All of the ALDH data suggest that ALDH2 polymorphism is critical to the prognosis of FA patients.

## Intercrosslink repair

DNA ICLs are toxic to dividing cells, as they induce mutations, chromosomal rearrangements, and cell death. In order to survive, organisms have developed strategies for dealing with DNA damage. As such, specialized repair pathways have evolved for specific kinds of DNA damage, including double-strand break (DSB) and ICL. Inducers of ICLs are important drugs in cancer treatment and include the well-known chemotherapeutic agents mitomycin C, cisplatin, cyclophosphamide, and their respective derivatives. While cells derived from most individuals with FA are hypersensitive to ICLs, they are generally not hypersensitive to inducers of DSBs such as ionizing radiation, indicating that the ICL repair pathway is distinct from that of DSB.

Homologous recombination is a DNA repair pathway that utilizes strand exchange in a gene conversion reaction involving a single-strand and a DNA duplex. In mammalian cells, this is a major repair pathway for DNA damage such as DSBs. The strand exchange protein RAD51 and the products of the hereditary breast cancer susceptibility genes BRCA1 and BRCA2 [14, 15] are critical proteins in HR in mammalian cells.

In 2005, a cellular study in humans showed that mutation of either the FA core complex members or the FANCD2 monoubiquitination site resulted in HR defects [16]. These defects, however, are mild compared with those resulting from a BRCA2 deficiency. HR measurements in these previous studies were performed with the widely used DR-GFP reporter system, in which a DSB formed by I-SceI endonuclease results in green fluorescent protein-expressing (GFP+) cells repaired by HR (Fig. 2). Further studies have reported on the mechanisms of ICL repair, particularly in terms of the replication-coupled manner. A 2008 study using a cell-free system based on *Xenopus* egg extracts found that ICL is repaired in a replication-dependent manner [17]. Another study in the *Xenopus* egg showed that ubiquitinated FANCI-FANCD2 is essential for replication-dependent ICL repair and that it is able to control the incision step [18]. Development and use of a TR-GFP assay, a modified version of the DR-GFP HR assay system, demonstrated that ICL repair in mammalian cells is dependent on DNA replication. The TR-GFP assay uses a DNA template with a site-specific ICL at sequences that are complemented to triplex-forming oligonucleotide conjugated with psoralen (pso-TFO) [19]. The construct also contains an origin of replication from the Epstein–Barr virus (EBV), enabling replication in human cells. Their results showed that ICL-induced HR was substantially compromised in the absence of FA proteins, suggesting that the FA pathway is specifically involved in replication-coupled HR repair. Use of direct assays for ICL-induced HR in vivo, along with studies that



**Fig. 2** DR-GFP assay. In the *DR-GFP* substrate, an I-SceI site is inserted into the GFP gene on *sceGFP*. GFP is inactivated by the stop-codon in the I-SceI site. To restore functionality of the *GFP* gene, the *iGFP* gene has .8 kb of sequence homology to direct the repair of an I-SceI-cleaved *SceGFP* gene

have demonstrated the involvement of the FA pathway overall, may facilitate delineation of the mechanisms and factors involved in this process.

**Diamond–Blackfan anemia**

Diamond–Blackfan anemia (DBA) is a rare congenital bone marrow failure syndrome characterized by severe normochromic macrocytic anemia and reticulocytopenia, with selective hypoplasia of erythroid precursors in the bone marrow. Up to 50 % of affected individuals have physical abnormalities including short stature, craniofacial dysmorphism, heart defects, and anomalies of the thumbs and genitourinary tract [20]. Increased risk of malignant disease, such as acute myeloid leukemia and osteogenic sarcoma, has also been reported to occur in this syndrome [21]. The incidence of DBA has been estimated to be 5–7 per million live births in Europe and North America. In a national study conducted in Japan between 2006 and 2010, 65 new DBA patients were registered. During this study period, the mean number of live births per year in Japan was reported to be 1.08 million, putting the incidence of DBA at 12 per each million live births, as most of these patients were diagnosed as DBA during infancy. The majority of these patients are sporadic, with the percentages of patients with autosomal dominant inheritance reported to be less than 10 %. Corticosteroids are recommended as a first line therapy, as these have been reported to improve erythropoiesis in approximately 80 % of DBA patients. In patients refractory to corticosteroids or who develop other forms of cytopenia, HSC transplantation has been suggested as a viable alternative [22].

**Molecular pathogenesis**

The first DBA gene (RPS19) was identified in 1999 and was found in approximately 25 % of the probands in

western countries [23]. Since then, a total of nine genes encoding large (RPL) or small (RPS) ribosomal subunit proteins were found to be mutated in DBA patients, including RPL5 (6.6 %), RPL11 (4.8 %), RPL35A (3 %), RPS24 (2 %), RPS17 (1 %), RPS7 (1 %), RPS10 (6.4 %), and RPS26 (2.6 %) [24] (Table 2). Collectively, mutations in at least one of these nine genes have been detected in approximately 50–60 % of DBA patients. Of 68 Japanese been examined, mutations in RPS 19, RPL5, RPL11, RPS17, RPS26 were identified in 10 (14.7 %), six (8.8 %), three (4.4 %), one (1.5 %), one (1.5 %), and one (1.5 %), respectively. These mutations have subsequently been determined to occur in 32.4 % of Japanese patients [25, 26]. A low incidence of mutations in the RPS19 gene may account for the overall lower incidence of total mutations in the Japanese population.

As conventional gene sequencing cannot identify large gene deletions, there have been only a few reports of patients with allelic losses in the RPS19 and RPL35A genes. Kuramitsu et al. [26] investigated large deletions of the RP genes using gene copy number variation analysis based on a quantitative-PCR and a single-nucleotide polymorphism (SNP) array. This study used sequencing to screen for large gene deletion in 27 patients without gene mutations. The PCR-based gene copy number assay identified a large deletion in seven (25.9 %) of 27 patients. Of these, three patients had RPS17, two had RPS19, one had RPL5, while one had RPL35A deletions. The SNP array confirmed six of the seven large deletions. Based on these new methods, the frequency of RP gene abnormalities in the DBA patients increased to 42.6 %. All patients with large deletions in DBA genes exhibited malformation with growth retardation. However, half of the patients with a mutation due to sequencing had growth retardation, while all seven patients with a large deletion exhibited growth retardation. While four of seven patients responded to corticosteroids, there were no phenotypic

**Table 2** Ribosomal protein gene mutations and deletions in 68 Japanese patients with Diamond–Blackfan anemia

Gene	Mutation	Deletion	Total (%)
RPS 19	10	2	12 (17.6 %)
RPL 5	6	1	7 (10.3 %)
RPS 17	3	0	3 (4.4 %)
RPL 11	3	1	4 (5.9 %)
RPS 10	1	0	1 (1.5 %)
RPS 26	1	0	1 (1.5 %)
RPS 35A	0	1	1 (1.5 %)
RPS 24	0	0	0
RPS 14	0	0	0
Total	22	7	29 (42.6 %)

differences noted between patients with and without large deletions, including response rate to corticosteroids and other malformations.

Farrar et al. [27] also identified RP gene deletions in nine (17 %) of 51 patients without any identifiable mutation by SNP array. Of these nine patients, three had RPS17, two had RPS26, two had RPS19, and two had RPL35A deletions. Clinically, five of the nine patients responded to corticosteroids. Two exhibited short stature. These two studies suggested that genomic deletions may be detected in 4–10 % of DBA patients, which is more common than has been previously suspected. Thus, in addition to conventional gene sequencing, molecular studies of suspected DBA cases should also include either a SNP array or PCR-based gene copy number assay.

Despite extensive sequencing of all the RP genes, at present mutations have only been found in approximately half of DBA patients examined, which raises the question whether other genes are responsible for DBA. Recent advance in genomic sequencing have made it possible to search for new candidate genes. Sankaran et al. [28] performed exome sequencing on two siblings without RP gene mutations. Both affected siblings satisfied the diagnostic criteria for DBA and both parents had normal blood values, suggesting X-linked or autosomal recessive inheritance. During sequencing, at least 10-fold coverage was obtained in more than 93 % of the target bases. After filtering, a total of 74 variants were identified as being shared by the three affected siblings. Of these 74 mutations, 31 were found in two affected siblings but not in the unaffected sibling. No variants were identified that would fit an autosomal recessive model of inheritance. Only the GATA1 gene showed appropriate segregation for an X-linked disease with full penetrance. The mutation in the GATA1 gene is a G-C transversion at position 48,649,736 on the X chromosome and results in a substitution of leucine for valine at amino acid 74 of the GATA1 protein. This mutation impaired production of the full-length form of the exon 2 protein. After screening 62 additional male DBA patients without known mutations for the GATA1 mutation, the study also identified one patient with a mutation in GATA1 at the exon 2-intron 2 junction. It was predicted that this would result in impaired splicing and a frameshift of the full-length GATA1 open reading frame. Overall, this study has opened new avenues for studying the molecular pathogenesis of DBA.

#### Role of p53 in the pathophysiology of DBA

Although current evidence suggests that impaired ribosomal biogenesis should affect all blood cell lineages, one question remains as to why it affects only the erythroid progenitors. Several animal models have demonstrated the

role of p53 in the pathophysiology of DBA. The RPS19-deficient zebrafish model has been shown to have many features of DBA and is accompanied by the up-regulation of the p53 family [29]. Suppression of p53 in the RPS19-deficient zebrafish alleviated the phenotype and improved survival. RPS19 knockdown mouse fetal liver cells, which were created by retrovirus-infected siRNA, showed reduced proliferation but normal differentiation of erythroid cells and an increased level of p53 and p21 [30]. Dutt et al. [31] have examined the accumulation and activity of p53 in different hematopoietic lineages after a partial knockdown of the RPS19 gene in primary human bone marrow-derived CD34 cells. Their study showed that p53 accumulates selectively in erythroid progenitors, resulting in lineage-specific p53 target gene expression, cell cycle arrest, and apoptosis. While pifithrin- $\alpha$  has been shown to inhibit the activity of p53, nutlin-3 activates p53 through the inhibition of HDM2. In addition, nutlin-3 selectivity impairs erythropoiesis, whereas inhibition of p53 by pifithrin- $\alpha$  rescues the erythroid defect. To directly examine whether p53 accumulation is operative in patients with DBA, bone marrow biopsies from eight patients with DBA were stained with anti-human p53 antibody and shown to have strong nuclear staining in two patients and weak nuclear staining in six patients. The erythroid lineage has a low threshold for the induction of p53, which accounts for the selective impaired erythropoiesis in patients with DBA.

#### Alternative therapies for DBA

Although approximately 80 % of DBA patients initially respond to corticosteroid, half of the responders are steroid-dependent. Only 20 % of these patients achieve remission. Although historically many alternative drugs have been tried, there has been no agreement on a second-line therapy. L-leucine is an essential amino acid and is known to be an activator of mRNA and stimulate protein synthesis through the mammalian target of rapamycin (mTOR) pathway. L-leucine treatment of the RPS19-deficient zebrafish model results in a striking improvement of anemia and developmental defects. These findings were reproduced in primary human CD34 cells after knockdown of the RPS19 gene [32]. Therapeutic effect of L-leucine has also been confirmed in the mouse model for RPS19-deficient DBA and shown to be associated with reduced p53 activity in hematopoietic progenitors [33]. Recently, leucine has been used on an investigational basis in one patient with DBA and is reported to have achieved a remission [34].

These findings support commencement of a clinical trial with L-leucine as an alternative therapy for DBA.

## Dyskeratosis congenita

### Clinical features of patients with dyskeratosis congenita

Dyskeratosis congenita (DC) is a rare inherited disease characterized by the classical mucocutaneous triad of abnormal skin pigmentation, nail dystrophy, and mucosal leucoplakia in approximately 80–90 % of patients [34]. Patients with DC are unable to maintain the telomere complex that protects the chromosome ends and consequently have very short telomeres [35]. Shortened telomeres can cause a wide variety of clinical features across a phenotypic spectrum consisting not only of mucocutaneous abnormalities but also multisystem symptoms including bone marrow failure, pulmonary fibrosis, hepatic fibrosis, and predisposition to malignancy [36, 37]. Indeed, non-mucocutaneous features, such as bone marrow failure and pulmonary fibrosis, occasionally precede mucocutaneous abnormalities, making it difficult to diagnose patients with DC based on clinical features alone. The incidence of DC is estimated to be one per million live births.

The diagnostic criteria for DC proposed by Vulliamy [38] include one or more of the three classic mucocutaneous features combined with hypoplastic bone marrow and at least two other somatic features known to occur in DC. The primary causes of mortality in patients with DC are bone marrow failure syndrome (60–70 %), pulmonary complications (10–15 %), and malignancy (particularly MDS and AML) (10 %) [36, 37].

### Genetic background of DC

DC is a genetically heterogeneous disorder, showing autosomal recessive, autosomal dominant, and X-linked inheritance. The *DKC1* gene on chromosome (chr) Xq28, which encodes dyskerin, was the first gene identified in the X-linked DC patients [39]. Dyskerin has a close association with the RNA component of telomerase (*TERC*), and mutations in dyskerin cause a reduction in accumulation of *TERC* and reduced telomere length [35]. In addition to its role in the biogenesis of telomerase RNA dyskerin is involved in ribosomal RNA biogenesis. Dyskerin catalyzes uridine to pseudouridine, which is a critical step for ribosomal RNA maturation and function. These findings imply that both telomere and ribosomal defects may occur in patients with *DKC1* mutations. Subsequently, heterozygous *TERC* mutations have also been found in autosomal dominant DC patients [40]. Genetic screening has identified mutations of other components of the telomerase complex, including *TERT* (chr 5p15) [41, 42], *NOP10* (chr 15q14-q15) [43], and *NHP2* (chr 5q35) [44] in patients with rare autosomal recessive DC. Mutations of *TERT* have also been reported in the autosomal dominant family [45].

Moreover, heterozygous mutations of *TINF2* (chr 14q12) that encode TIN2, which is the main component of shelterin and which protects telomeres, have been identified in <11 % of DC patients [46, 47].

More recently, mutations of *TCAB1* (chr 17p13) were identified in patients with DC as autosomal recessive forms [48]. Venteicher et al. [49] found that *TCAB1* associates with *TERT*, dyskerin and *TERC*, and small Cajal body RNAs (scaRNAs) that are involved in modifying splicing RNAs to control telomerase trafficking. *TCAB1* defects prevented *TERC* from associating with the Cajal bodies, which disrupted the telomerase–telomere association. A recent case report described biallelic mutations of the *CTCI* gene (chr 17p13) in a patient with DC [50]. This gene was originally described as causative gene of the Coats plus syndrome, which is a form of cerebroretinal microangiopathy with calcifications and cysts (CRMCC). The mutation frequencies of these new genes for DC remain unknown.

At present, eight of the mutated genes in DC have been shown to be associated with the telomerase holoenzyme (*TERT*, *TERC*, *DKC1*, *NOP10*, *NHP2*, *TCAB1*, and *CTCI*) or the shelterin complex (*TINF2*), accounting for approximately 50 % of DC patients. Mutations in telomerase and telomere components have also been identified in patients with aplastic anemia, pulmonary fibrosis, and liver diseases that did not have any mucocutaneous manifestations [45, 46, 51–59]. These findings suggest that defective telomere maintenance causes not only classical DC, but also a broad spectrum of diseases previously thought to be idiopathic and thus this has led to a new concept of diseases termed “syndromes of telomere shortening”.

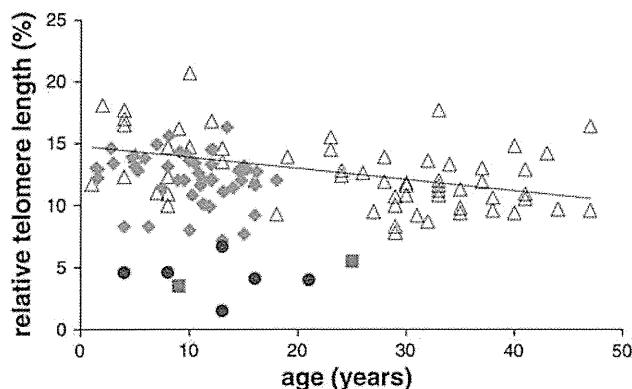
### Cryptic DC patients in aplastic anemia

Patients with DC have been shown to have disease diversity in terms of age at onset, symptoms, and severity. This diversity occurs even among the patients with the same gene mutation. Bone marrow failure sometimes precedes mucocutaneous manifestations in patients with DC, and a substantial proportion of patients with aplastic anemia have shorter telomeres compared with normal individuals [60, 61]. These observations have prompted screening for gene mutations responsible for telomere maintenance in patients with aplastic anemia and other bone marrow failure syndromes. This screening identified mutations in *TERC* and *TERT* in 3 % of the aplastic anemia patients [54, 55]. Our research group conducted a study in Japanese children with aplastic anemia and identified two of 96 as having the *TERT* mutations, although none of the patients had a *TERC* mutation [53]. Patients with *TERC* or *TERT* mutations have been shown to have very short telomeres in their blood cells. Recently, Du et al. [52] found that 6 (5.5 %) of 109

pediatric patients with severe aplastic anemia had mutations of *TINF2*. In an unpublished study, our research group screened for mutations of *TINF2* and found that of the 96 pediatric patients with aplastic anemia that were examined, none exhibited any mutations of this gene.

Three methods are commonly used for measuring telomere length, including Southern blot, real-time polymerase chain reaction, and flow cytometry and fluorescence in situ hybridization (flow-FISH). Of these, the flow-FISH has been shown to be the most appropriate when undertaking “prospective” screening [62, 63]. As shown in Fig. 3, patients with DC and aplastic anemia with the *TERT* mutation were all found to have very short telomeres as compared with the idiopathic aplastic anemia patients and normal individuals. As a small subset of patients with apparently idiopathic aplastic anemia have been shown to carry telomere gene mutations, identification of such patients is critical for informing treatment decisions. Aplastic anemia patients should be routinely screened for telomere gene mutations prior to starting any treatment. However, because screening of gene mutations can be both laborious and time consuming, we have adopted the screening of telomere length in blood cells rather than screening of gene mutations.

It should be noted that short telomeres are not specific for patients with DC, as they are also seen in patients with other bone marrow failure syndromes. Although short telomeres have also been found in patients with other congenital bone marrow failure syndromes, such as Shwachman–Diamond syndrome and Fanconi anemia, telomere lengths in patients with DC have been demonstrated to be



**Fig. 3** Relative telomere length in peripheral blood lymphocytes from patients with dyskeratosis congenita (*filled circles*), patients with aplastic anemia harboring *TERT* mutations (*filled squares*), patients with idiopathic aplastic anemia (*filled argyles*) and normal individuals (*open triangles*). Telomere lengths were measured by flow cytometry-fluorescent in situ hybridization (flow-FISH). Relative telomere length was calculated as the ratio between the telomere signal of each sample and the telomere signal of the control cell line (cell line 1301). These data were provided by the Department of Pediatrics, Nagoya University Graduate School of Medicine

shorter than those in all other bone marrow failure syndromes. In fact, telomere length in most patients with DC is below the first percentile of telomere length found in healthy controls [64].

Family members of patients with DC should receive genetic counseling to rule out if they are silent carriers. In particular, genetic counseling is necessary during the proband search for a donor for HSC transplantation. Studies on telomere length analyses in families with DC have shown that mutated carriers with clinical signs of bone marrow failure have short telomeres. Even so, telomere length cannot predict the presence or absence of a mutation in family members with bone marrow failure. In addition, there have been rare cases that show normal telomere length, even though the subject harbors the same mutation as the proband. This suggests that mutation alone does not sufficiently explain the reduction of telomere length [51].

#### Clinical management for DC

Bone marrow failure and immune deficiency are the most common causes of death in up to 60–70 % of patients with DC. Androgen (e.g. oxymetholone) has been used to improve cytopenia in patients with DC since the 1960s. However, the mechanism of action of androgen has not been well understood until recently. Calado et al. [65] showed that in vitro exposure of normal peripheral blood cells to androgen produced higher *TERT* mRNA levels. When these patients were treated with cells from patients who had a heterozygous mutation of the telomerase, it was possible to restore their low baseline telomerase activity to normal levels. Thus, as telomere shortening is closely associated with malignant disease, androgen therapy might be able to prevent or postpone the development of various types of cancers. Erythropoietin and/or G-CSF combined with androgen has occasionally provided transient hematopoietic recovery to poor responders to androgen alone [66]. However, this combination should be used with caution, as severe splenic peliosis and fatal rupture have been reported in two patients with DC who received simultaneous administration of androgen and G-CSF [67].

Allogeneic HSC transplantation is the only curative treatment for bone marrow failure in patients with DC. However, the outcome in previous reports has been disappointing due to unacceptable transplant-related toxicities, including severe pulmonary/liver complications, especially in transplants from an alternative donor [68, 69]. To avoid these complications, non-myeloablative conditioning regimens have been recently used in several cases. Dietz et al. [70] reported encouraging results of six patients with DC who received a fludarabine-based non-myeloablative regimen. Of the four surviving patients, three were recipients of unrelated grafts. Non-myeloablative

transplants are expected to provide improvement in the short-term survival. At our institute, three patients with DC underwent allogeneic bone marrow transplantation following non-myeloablative conditioning from 2003 to 2009. Successful engraftment was achieved in all patients with only a few regimen-related toxicities, and at the present time all continue to survive without any symptoms [71]. However, due the late effects of conditioning agents and allogeneic immune responses within the recipient's organs, such as the lung and liver, longer-term follow-ups are necessary to definitively clarify the present results.

## Conclusion

Although recent studies have identified many causative genes, mutations of these genes have only been found in half of the patients with DBA or DKC. Next-generation sequencing (or massive parallel sequencing) technologies have led to a tremendous revolution in genomics, with their effects currently becoming increasingly widespread. This new strategy may soon be able to reveal the remaining unknown causative genes in IBMFS.

Recently, Agarwal et al. [72] established induced pluripotent stem cells (iPSCs) derived from a patient with DC and showed that the reprogrammed DC cells overcame a critical limitation in TERC levels to restore the telomere maintenance and self-renewal. These findings indicate that drugs or gene therapy that upregulate TERC activity may show therapeutic potential in patients with DC. These same strategies may also be applicable for other IBMFS.

The only long-term curative treatment for bone marrow failure in patients with IBMFS is allogeneic HSC transplantation, although this procedure has a risk of severe adverse effects. Multicenter prospective studies are needed to establish appropriate conditioning regimens aimed at reducing transplant-related mortality. Future studies must aim to improve short-term outcomes, such as hematological recovery, and to decrease the incidence of late adverse effects.

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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<sup>+</sup> 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<sup>+</sup> 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 he-**

**matopoietic stem cell transplantation, 66% of whom survived. Age at onset of disease ( $\geq 8$  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<sup>+</sup> 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,<sup>1-3</sup> and are clinically complicated by the interaction between the biologic properties of EBV<sup>+</sup> 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<sup>+</sup> diseases, including Burkitt lymphoma,<sup>3</sup> age-related EBV<sup>+</sup> B-cell LPD,<sup>4</sup> extranodal NK/T-cell lymphoma of nasal type (ENKL),<sup>5</sup> aggressive NK-cell leukemia (ANKL),<sup>6</sup> classic Hodgkin lymphoma,<sup>3</sup> and immunodeficiency-associated lymphoproliferative disorders.<sup>1</sup> 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<sup>+</sup> T-cell LPD of childhood and hydroa vacciniforme-like lymphoma are proposed as distinct entities.<sup>7-8</sup> Historically, based on their broad clinical manifestations, these diseases have been described under various nosological terms from indolent (eg, severe mosquito bite allergy<sup>9</sup> and hydroa vacciniforme<sup>10</sup>) to aggressive or fulminant forms (eg, EBV-associated hemophagocytic lymphohistiocytosis [HLH],<sup>11</sup> chronic active EBV disease [CAEBV] of the T/NK-cell type,<sup>12</sup> fulminant EBV<sup>+</sup> T-cell LPD of childhood,<sup>13</sup> and fatal infectious mononucleosis<sup>3</sup>).

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

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**Table 1. Definitions of EBV<sup>+</sup> T/NK-LPDs in this study**

Disease	Eligibility criteria	Exclusion criteria	Lineages/clonality	References
<b>Clinical category</b>				
CAEBV of T/NK-cell type	(1) Illness $\geq$ 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)* (2) Increased amounts of EBV detected by Southern blot hybridization or EBER <sup>+</sup> cells in affected tissues or peripheral blood; $\geq 10^{2.5}$ copies/ $\mu$ g of EBV DNA in PBMCs	(1) No evidence of previous immunological abnormalities or other recent infection that might explain the observed condition (2) Congenital immunodeficiency including X-linked lymphoproliferative disorders	T/NK cell  Polyclonal, oligoclonal, monoclonal	12  16,29
HLH	(1) Clinical criteria (fever and splenomegaly) (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)	(1) Hemophagocytic syndrome in accelerated phase of CAEBV of T/NK cell type (2) Congenital immunodeficiency including familial HLH	T/NK cell  Polyclonal, oligoclonal, monoclonal	11
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
<b>Pathological classification</b>				
Systemic EBV <sup>+</sup> 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,<sup>8</sup> and were encountered as a part of the initial and accompanying symptoms of the systemic EBV<sup>+</sup> T/NK-LPDs.<sup>28-30</sup> However, the mutual relationship and clinicopathologic distinctiveness of these EBV<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> HLH, and EBV<sup>+</sup> T-cell lymphomas with prodromal phase, whereas the latter may include all cases with EBV<sup>+</sup> hydroa 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<sup>+</sup> 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.<sup>29</sup> Similarly, a nationwide study for HLH was recently performed in Japan.<sup>31</sup> 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,<sup>32-33</sup> 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.<sup>12,34-35</sup> 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.<sup>36</sup> Using these techniques, we enrolled and prospectively followed patients with definitive cases of EBV<sup>+</sup> T/NK-LPDs in 1998. In this study, 108 nonimmunocompromised patients with EBV<sup>+</sup> T/NK-LPDs were analyzed for clinical and virological characteristics to obtain an understanding of their pathogenesis and for refining their