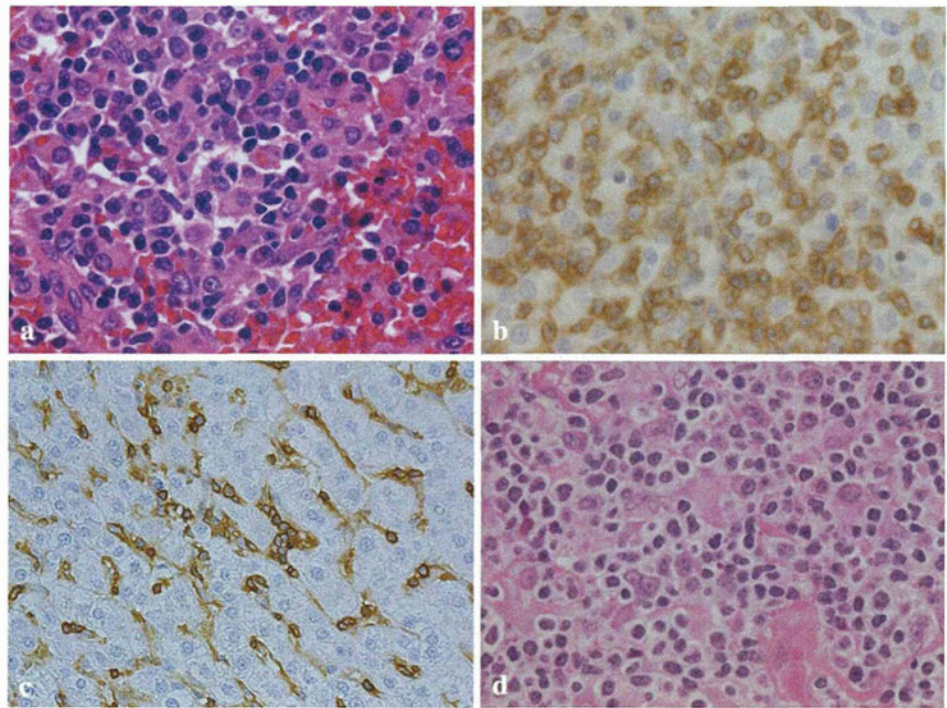


**Fig. 2** Histologic pictures of the spleen, the liver, and a lymph node. **a** H&E staining of the resected spleen. Lymphoma cells with irregular nuclei and slightly dispersed chromatin are seen in the red pulp;  $\times 400$ . **b** Lymphoma cells in the spleen are positive for CD3;  $\times 400$ . **c** CD3 staining of a biopsy specimen of the liver. Lymphoma cells infiltrate the sinusoids;  $\times 200$ . **d** H&E staining of a biopsied lymph node. The normal architecture is effaced by the diffuse infiltration of lymphoma cells;  $\times 400$



lymphocytes (Fig. 2). A bone marrow aspirate demonstrated infiltration of abnormal lymphocytes accounting for 10% of nucleated cells. Immunohistochemical staining of the spleen, liver, and lymph nodes showed that these lymphocytes were positive for CD3 but negative for CD20. In situ hybridization of Epstein-Barr virus-encoded RNA-1 (EBER-1) was negative. Regarding the expression of T-cell-associated antigens, these lymphocytes were positive for CD2, CD3, CD5, CD7, CD8, and TCR $\alpha\beta$ , but negative for CD4 and TCR $\gamma\delta$ . A polymerase chain reaction (PCR) analysis of DNA extracts from the spleen, liver, lymph node, and bone marrow cells showed a monoclonal gene rearrangement of the TCR $\gamma$  chain. A southern blot analysis of DNA extracted from the spleen demonstrated gene rearrangements for TCR constant  $\beta$  chain, J $\beta$ 1 chain, and J $\beta$ 2 chain. The karyotype of the spleen cells was 46,XY. We reevaluated the bone marrow smear in May 2007 and observed a small number of abnormal lymphocytes. From these findings, we made a diagnosis of hepatosplenic  $\alpha\beta$  T cell lymphoma in Stage IVB.

The pancytopenia immediately improved after the splenectomy, and he received chemotherapy with entecavir for the prophylaxis of activation of HBV hepatitis, including the CHOP (cyclophosphamide, adriamycin, vincristine, prednisolone) and DeVIC (dexamethasone, etoposide, ifosamide, carboplatin) regimens. These treatments brought about a minimal response of the lymphoma and improved his fever and liver dysfunction; however, the effect was temporary. We then performed three courses of ESHAP

(etoposide, cisplatin, cytarabine, methylprednisolone) chemotherapy. This treatment resulted in a durable partial remission; however, residual lymphoma cells accounting for 3% of nucleated marrow cells were observed. He did not have an HLA-matched sibling donor, and an adequate cord blood graft with sufficient cell numbers was not obtained. Therefore, we conducted an allogeneic bone marrow transplantation (BMT) from an HLA-haploidentical sibling donor as part of a clinical trial at Hyogo Medical College, Hyogo, Japan. The lymphoma, however, became refractory to ESHAP chemotherapy from January 2008, and salvage treatments such as Hyper CVAD (cyclophosphamide, adriamycin, vincristine, dexamethasone, high dose methotrexate, high dose cytarabine) chemotherapy induced minimal response. He was moved to Hyogo Medical College to receive the BMT in a state of progressive disease. The conditioning regimen consisted of fludarabine (120 mg/m<sup>2</sup>), cytarabine (8 g/m<sup>2</sup>), cyclophosphamide (120 mg/kg), and total body irradiation (800 cGy in four fractions). He died of sepsis with *enterococcus faecium* during conditioning for the transplantation.

## Discussion

$\gamma\delta$  HSTCL is a rare subtype of malignant lymphoma, accounting for less than 5% of peripheral T cell lymphomas. This type of lymphoma predominantly affects young adult males and is often associated with marked

hepatosplenomegaly, bone marrow involvement, B symptoms, and pancytopenia without superficial lymphadenopathy. Another rarer subtype of HSTCL with  $\alpha\beta$  type TCR shares most of the clinical features of  $\gamma\delta$  HSTCL, but differs in several points. For example, female predominance and older age distribution are seen in  $\alpha\beta$  type. Macon et al. [5] reported that  $\alpha\beta$  type often presents with superficial lymphadenopathy as observed in the present patient. The background of chronic immune suppression is often seen in  $\gamma\delta$  type, but not in  $\alpha\beta$  type. Since only 23 cases of  $\alpha\beta$  subtype have been reported to date [1–3, 5, 7–11], the etiology and clinical picture of this subtype are not fully understood. Thus, the accumulation of cases of  $\alpha\beta$  type is required to establish a clinical entity of this subtype of HSTL.

In  $\alpha\beta$  HSTCL, tumor cells are medium-sized with scant to moderate basophilic cytoplasm and have round to oval nuclei with slightly dispersed chromatin, containing conspicuous nucleoli. Characteristic features of  $\alpha\beta$  HSTCL include intact capsules and a homogeneous cut surface without nodulation in the spleen. The lymphoma cells infiltrate mainly in the red pulp, making white pulp atrophic. In the liver, tumor cells mainly infiltrate sinusoids and in some cases, the periportal region. Similarly in the bone marrow, interstitial or sinusoidal infiltration is seen. In affected lymph nodes, the normal architecture is effaced by a diffuse infiltration of lymphoma cells. Similar features and histologic findings were observed in the present patient.

The general pattern of T cell-related antigen expression on tumor cells of  $\alpha\beta$  HSTCL is CD2<sup>+</sup>, CD3<sup>+</sup>, CD4<sup>-</sup>, CD5<sup>-/+</sup>, CD7<sup>+/-</sup>, CD8<sup>+/-</sup>, CD16<sup>-/+</sup>, CD56<sup>+/-</sup>, and CD57<sup>-/+</sup>. The tumor cells are often positive for cytotoxicity-related intracellular protein TIA1 (T cell restricted intracellular antigen-1), but not surface perforin and granzyme B. Epstein-Barr (EB) virus is not detectable in tumor cells in a majority of cases as evaluated by Epstein-Barr virus-encoded RNA-1 (EBER-1) in situ hybridization. A mostly nonproductive  $\gamma$ -chain gene rearrangement is present [5]. Most of these findings were observed in lymphoma cells in the present patient. Tumor cells of  $\alpha\beta$  HSTCL frequently have an abnormal karyotype, that is, isochromosome 7q, trisomy 8, or both.

The prognosis of HSTCL, either  $\alpha\beta$  or  $\gamma\delta$  type, has been reported to be very poor with a median survival time of less than 1 year [5]. CHOP or CHOP-like chemotherapy rarely induces a complete remission, and salvage chemotherapy including treatment with ESHAP, hyper CVAD, pentostatin, or alemtuzumab has been reported to be only temporarily effective. Cytopenia often parallels disease progression in HSTCL, which makes it difficult to continue chemotherapy in the salvage course. The effect of splenectomy on the prognosis has not been

established; however, in the present case, the cytopenia improved immediately after the procedure, and the improvement continued even with the progression of the disease. Splenectomy, therefore, should be taken into consideration when intensive chemotherapy is needed regardless of severe cytopenia. Because of the transient effects of salvage chemotherapies such as ESHAP and hyperCVAD in the present patient, we planned an allogeneic BMT from an HLA-haploidentical sibling donor. However, he died of sepsis during the conditioning procedure for the transplantation 9 months after the diagnosis. Only 6 of 23 patients with  $\alpha\beta$  HSTCL reported in the literature could receive an allogeneic stem cell transplantation [3, 7–11]. Of these six patients, there have been two survivors free from relapse [7, 8]. It should be noted that these two patients received an allogeneic stem cell transplantation in a state of complete or partial remission and not with progressive disease. Donor lymphocyte infusion or the discontinuation of treatment with immunosuppressants has been reported to be effective against recurrent lymphoma [9, 10], indicating the involvement of a graft-versus-lymphoma effect. Although evidence is limited, it may be very instructive for hematologists that allogeneic stem cell transplantation should be considered at the beginning of treatment as a potentially curative therapeutic option for patients with either  $\alpha\beta$  or  $\gamma\delta$  HSTCL before the disease becomes refractory to chemotherapy.

**Conflict of interest statement** No author has any conflict of interest.

## References

1. Farcet JP, Gaulard P, Marolleau JP et al (1990) Hepatosplenic T-cell lymphomas: sinusal/sinusoidal localization of malignant cells expressing the T-cell receptor  $\gamma\delta$ . *Blood* 75:2213–2219
2. Suarez F, Wlodarska I, Rigal-Huguet F et al (2000) Hepatosplenic  $\alpha\beta$  T-cell lymphoma. *Am J Surg Pathol* 24:1027–1032
3. Lai R, Larratt LM, Etches W et al (2000) Hepatosplenic T-cell lymphoma of  $\alpha\beta$  lineage in a 16-year-old boy presenting with hemolytic anemia and thrombocytopenia. *Am J Surg Pathol* 24:459–463
4. Harris NL, Jaffe ES, Diebold J et al (2000) The World Health Organization classification of hematological malignancies report of the clinical advisory committee meeting. *Mod Pathol* 13:193–207
5. Macon WR, Levy NB, Kurtin PJ et al (2001) Hepatosplenic  $\alpha\beta$  T-cell lymphomas: a report of 14 cases and comparison with hepatosplenic  $\gamma\delta$  T-cell lymphomas. *Am J Surg Pathol* 25:285–296
6. Ozaki S, Ogasahara K, Kosaka M et al (1998) Hepatosplenic  $\gamma\delta$  T-cell lymphoma associated with hepatitis B virus infection. *J Med Invest* 44:215–217
7. Machino T, Okoshi Y, Kaneko S et al (2007) Hepatosplenic  $\alpha\beta$  T-cell lymphoma successfully treated with allogeneic bone marrow transplantation. *Bone Marrow Transplant* 39:513–514

8. Mansour MR, Dogan A, Morris EC et al (2005) Allogeneic transplantation for hepatosplenic  $\alpha\beta$  T-cell lymphoma. *Bone Marrow Transplant* 35:931–934
9. Chanan-khan A, Islam T, Alam A et al (2004) Long-term survival with allogeneic stem cell transplant and donor lymphocyte infusion following salvage therapy with anti-CD52 monoclonal antibody (Campath) in a patient with  $\alpha\beta$  hepatosplenic T-cell non-Hodgkin's lymphoma. *Leuk Lymphoma* 45:1673–1675
10. Takaku T, Miyazawa K, Sashida G et al (2005) Hepatosplenic  $\alpha\beta$  T-cell lymphoma with myelodysplastic syndrome. *Int J Hematol* 82:143–147
11. Minauchi K, Nishio M, Itoh T et al (2007) Hepatosplenic  $\alpha\beta$  T cell lymphoma presenting with cold agglutinin disease. *Ann Hematol* 86:155–157

# blood

2010 115: 3158-3161  
Prepublished online Feb 1, 2010;  
doi:10.1182/blood-2009-11-254284

## Relapse of leukemia with loss of mismatched HLA resulting from uniparental disomy after haploidentical hematopoietic stem cell transplantation

Itzel Bustos Villalobos, Yoshiyuki Takahashi, Yoshiki Akatsuka, Hideki Muramatsu, Nobuhiro Nishio, Asahito Hama, Hiroshi Yagasaki, Hiroh Saji, Motohiro Kato, Seishi Ogawa and Seiji Kojima

---

Updated information and services can be found at:

<http://bloodjournal.hematologylibrary.org/cgi/content/full/115/15/3158>

Articles on similar topics may be found in the following *Blood* collections:

Immunobiology (4316 articles)  
Transplantation (1579 articles)  
Free Research Articles (1045 articles)  
Myeloid Neoplasia (389 articles)  
Brief Reports (1354 articles)  
Clinical Trials and Observations (3038 articles)

---

Information about reproducing this article in parts or in its entirety may be found online at:

[http://bloodjournal.hematologylibrary.org/misc/rights.dtl#repub\\_requests](http://bloodjournal.hematologylibrary.org/misc/rights.dtl#repub_requests)

Information about ordering reprints may be found online at:

<http://bloodjournal.hematologylibrary.org/misc/rights.dtl#reprints>

Information about subscriptions and ASH membership may be found online at:

<http://bloodjournal.hematologylibrary.org/subscriptions/index.dtl>

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

Copyright 2011 by The American Society of Hematology; all rights reserved.



## Brief report

# Relapse of leukemia with loss of mismatched HLA resulting from uniparental disomy after haploidentical hematopoietic stem cell transplantation

Itzel Bustos Villalobos,<sup>1</sup> Yoshiyuki Takahashi,<sup>1</sup> Yoshiki Akatsuka,<sup>2,3</sup> Hideki Muramatsu,<sup>1</sup> Nobuhiro Nishio,<sup>1</sup> Asahito Hama,<sup>1</sup> Hiroshi Yagasaki,<sup>1</sup> Hiroh Saji,<sup>4</sup> Motohiro Kato,<sup>5</sup> Seishi Ogawa,<sup>6</sup> and Seiji Kojima<sup>1</sup>

<sup>1</sup>Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya; <sup>2</sup>Division of Immunology, Aichi Cancer Center Research Institute, Nagoya; <sup>3</sup>Department of Hematology and Oncology, Fujita Health University, Aichi; <sup>4</sup>Human Leukocyte Antigen Laboratory, Nonprofit Organization, Kyoto; <sup>5</sup>Department of Pediatrics, Graduate School of Medicine, University of Tokyo, Tokyo; and <sup>6</sup>21st Century Center of Excellence Program, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

We investigated human leukocyte antigen (HLA) expression on leukemic cells derived from patients at diagnosis and relapse after hematopoietic stem cell transplantation (HSCT) using flow cytometry with locus-specific antibodies. Two of 3 patients who relapsed after HLA-haploidentical HSCT demonstrated loss of HLA alleles in leukemic cells at re-

lapse; on the other hand, no loss of HLA alleles was seen in 6 patients who relapsed after HLA-identical HSCT. Single-nucleotide polymorphism array analyses of sorted leukemic cells further revealed the copy number-neutral loss of heterozygosity, namely, acquired uniparental disomy on the short arm of chromosome 6, resulting in the total loss of the mis-

matched HLA haplotype. These results suggest that the escape from immunosurveillance by the loss of mismatched HLA alleles may be a crucial mechanism of relapse after HLA-haploidentical HSCT. Accordingly, the status of mismatched HLA on relapsed leukemic cells should be checked before donor lymphocyte infusion. (*Blood*. 2010;115(15):3158-3161)

## Introduction

Human leukocyte antigen (HLA) molecules expressed on the cell surface are required in presenting antigens to T cells. The HLA class I antigens are vital in the recognition of tumor cells by tumor-specific cytotoxic T cells. The loss of HLA class I molecules on the cell surface membrane may lead to escape from T-cell immunosurveillance and the relapse of leukemia. Previously, loss of HLA class I haplotype has been described in solid tumors.<sup>1-3</sup> However, there are few reports concerning HLA-haplotype loss in leukemia.<sup>4,5</sup>

We examined HLA class I expression in leukemic blasts from patients who relapsed after hematopoietic stem cell transplantation (HSCT) to analyze whether the loss of HLA on leukemic cells was related to the relapse after HLA-identical or haploidentical HSCT.

## HLA class I expression on leukemic cells

Samples were collected at diagnosis and post-transplantation relapse. HLA expression of leukemic blasts and normal cells was analyzed by flow cytometry as previously reported.<sup>6</sup> Anti-HLA A2-FITC (cloneBB7.2) and anti-HLA A24-FITC (clone17a10) monoclonal antibodies were purchased from Medical & Biological Laboratories; HLA-A11 (IgM), HLA-A30, HLA-31 (IgM), HLA-25, HLA-26 (IgM), HLA-Bw6 (IgG3), and HLA-Bw4 (IgG3) antibodies were purchased from One Lambda. For leukemic cell markers, CD13-PE (IgG1) were purchased from Immunotech and CD34-APC (IgG1) were purchased from BD Biosciences. Samples were analyzed with FACSCalibur cytometer and CellQuest software. The method of genomic HLA typing was previously reported.<sup>7</sup>

## Isolation of DNA and single nucleotide polymorphism analysis

The CD13<sup>+</sup>/CD34<sup>+</sup> leukemic blasts were sorted by flow cytometry from bone marrow cells at the time of diagnosis and of relapse. Genomic DNA was extracted from leukemic cells sorted by a fluorescence-activated cell sorter as well as from phytohemagglutinin-stimulated patient-derived T cells and subjected to single nucleotide polymorphism (SNP) array analysis using GeneChip NspI arrays (Affymetrix) according to the manufacturer's protocol. Allele-specific copy number was detected using Copy Number Analyzer for GeneChip software as previously described.<sup>8</sup>

## Limiting dilution-based CTLp frequency assay

The frequencies of cytotoxic T-lymphocyte precursor (CTLp) specific for the recipient-mismatched HLA molecules were analyzed using a standard limiting dilution assay.<sup>9</sup>

## Methods

### Patients and transplantation procedure

We identified 9 children with acute leukemia who relapsed after HSCT. Their leukemic samples were cryopreserved both at the time of the initial diagnosis and of relapse. The patients' characteristics are summarized in supplemental Table 1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Three patients received HSCT from an HLA-haploidentical family donor, and the other 6 patients received HSCT from an HLA-matched donor (4 siblings and 2 unrelated donors).

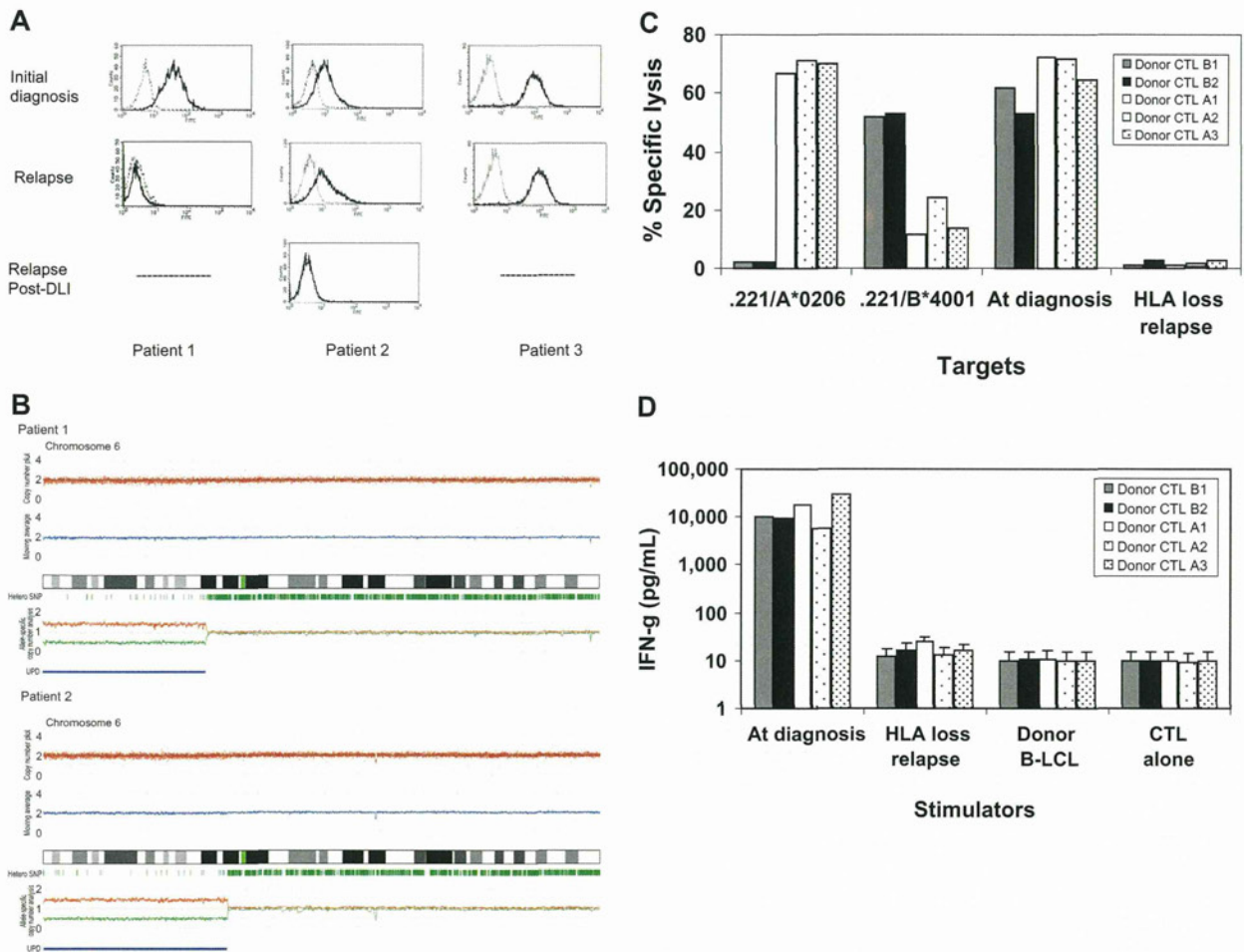
Written informed consent was given by the parents according to the protocol approved by the ethics committee of Nagoya University Graduate School of Medicine in accordance with the Declaration of Helsinki.

Submitted November 15, 2009; accepted December 22, 2009. Prepublished online as *Blood* First Edition paper, February 1, 2010; DOI 10.1182/blood-2009-11-254284.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2010 by The American Society of Hematology



**Figure 1. The loss of mismatched HLA expression on leukemic blasts caused by uniparental disomy on chromosome 6p impaired recognition and killing of donor's alloreactive cytotoxic T lymphocytes.** (A) Leukemic blasts at the time of initial diagnosis and at the time of relapse after hematopoietic stem cell transplantation (HSCT) and donor lymphocyte infusion (DLI) were gated by CD34<sup>+</sup> and CD13<sup>+</sup>, and then the surface expression of mismatched human leukocyte antigen (HLA) alleles was examined with anti-HLA-A2 antibodies. In 3 patients with acute myelogenous leukemia (AML) who experienced relapse after HLA-haploidentical HSCT, HLA-A2 expression was lost in patient 1 at relapse 15 months after HSCT and lost in patient 2 at second relapse 6 months after DLI. (B) Single nucleotide polymorphism (SNP) array analyses of sorted leukemic cells with the loss of an HLA allele revealed that the short arm of chromosome 6 shows copy number-neutral loss of heterozygosity or acquired uniparental disomy as detected by dissociated allele-specific copy number plots (red and blue lines at the bottom), resulting in the total loss of the mismatched HLA haplotype in both patient 1 and patient 2. The presence of acquired uniparental disomy is also indicated by normal total copy numbers with missing heterozygous SNPs (green bars) in the distal part of the short arm. (C) Recipient alloantigen-specific cytotoxic T-lymphocyte (CTL) clones were generated by a conventional cloning method from cytotoxicity-positive wells obtained in the limiting dilution assays using the donor CD8<sup>+</sup> cells as responders. Donor CTL clones A1, A2, and A3 were specific for HLA-A\*0206. Donor CTL clones B1 and B3 were specific for HLA-B\*4001, all of which recognize mismatched HLA alleles between the donor and recipient. Those 5 representative CTL clones were tested for HLA specificity and recognition of leukemic blasts obtained at the time of the initial diagnosis and at the time of HLA loss relapse after DLI by a standard <sup>51</sup>Cr-release assay at the effector/target ratio of 30:1. (D) Their interferon- $\gamma$  production was also assessed against leukemic blasts collected at the time of diagnosis and at the time of HLA-loss relapse.

### Cytotoxic assay of CTL clones against leukemic blasts and a mismatched HLA cDNA-transfected B-lymphoblastoid cell line

The remaining cells of several cytotoxicity-positive wells used for the CTLp assay for the donor were used to obtain allo-HLA-restricted CTLs. CTL clones were isolated by standard limiting dilution and expanded as previously described.<sup>10,11</sup>

The HLA class I-deficient 721.221 B-lymphoblastoid cell line was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, and 1mM sodium pyruvate. Retroviral transduction was conducted as previously described.<sup>12</sup>

The cytotoxicity of CTL clones against target cells was analyzed by conventional chromium 51 (<sup>51</sup>Cr) release assay as previously reported.<sup>13</sup>

CTL clones (10<sup>4</sup> cells/well) were mixed with the indicated stimulator cells (10<sup>4</sup> cells/well) in 96-well, round-bottom polypropylene plates and spun at 1200g for 3 minutes before overnight incubation in 200  $\mu$ L of RPMI 1640 medium supplemented with 10% fetal bovine serum. On the next day, 50  $\mu$ L of supernatant was collected and interferon- $\gamma$

was measured by enzyme-linked immunosorbent assay with 3,3',5,5'-tetramethylbenzidine substrate (Sigma-Aldrich).

### Results and discussion

Three children with high-risk acute myelogenous leukemia (AML) received haploidentical grafts from their parents but relapsed 8, 14, and 15 months after HSCT. Patient 2 received 3 courses of donor lymphocyte infusion (DLI) for relapsed leukemia after haploidentical HSCT. After the third unmanipulated DLI (10<sup>7</sup> CD3<sup>+</sup>/kg), she experienced acute grade-III graft-versus-host disease and achieved complete remission. However, she experienced a second relapse 6 months later. To monitor residual disease in those patients, we used flow cytometric analysis with antibodies specific for the mismatched HLA alleles between the donor and patient. Surprisingly, we found total loss of

HLA-A2 expression on CD13<sup>+</sup>/CD34<sup>+</sup> leukemic cells from bone marrow in 2 of 3 patients who underwent HLA-haploidentical HSCT, whereas microscopic analysis showed relapse (Figure 1A). To test whether HLA class I molecules could be up-regulated, samples were cultured for 48 hours in medium supplemented with tumor necrosis factor- $\alpha$  or interferon- $\gamma$  and measured again; however, no restoration was observed (data not shown).

Next, to examine the potential loss of genes encoding the undetectable HLA alleles, we sorted CD13<sup>+</sup>/CD34<sup>+</sup> leukemic blasts and performed DNA genotyping. We found that, in addition to the HLA-A locus, the HLA-B, -C, and -DR loci were not encoded; only the mismatched haplotype was lost in both patients (supplemental Table 2). We then questioned whether this phenomenon would also occur in HLA-matched HSCT settings using anti-HLA class I antibodies. We did not observe any loss of HLA class I expression in any of the patients at the time of relapse (supplemental Figure 1). These results suggest that loss of HLA class I haplotype at the time of posttransplantation relapse is uncommon in HLA-matched HSCT.

To elucidate the mechanism of the loss of the mismatched HLA haplotype, we performed an SNP array analysis of genomic DNA extracted from leukemic blasts at the time of diagnosis and of relapse. Genomic DNA from patient-derived T cells was used as a reference. Leukemic cells at the time of relapse showed copy number-neutral loss of heterozygosity or an acquired uniparental disomy (UPD) of the short arm of chromosome 6 encompassing the HLA locus, whereas no allelic imbalance was identified at the time of diagnosis (Figure 1B). Loss of one allele from one parent and duplication of the remaining allele from the other parent led to UPD.<sup>14</sup>

In patient 2, we examined whether the number of CTLp had changed during the posttransplantation course. Limiting dilution analysis with a split-well <sup>51</sup>Cr-release assay was carried out to compare the CTLp frequencies specific for the mismatched antigens between the recipient and donor. Interestingly, the CTLp frequencies were recovered after DLI (Table 1). Restoration of CTLp after 3 DLIs could eradicate such leukemic cells, lasting for 6 months thereafter.

Next, we generated allo-HLA-restricted CTLs from CD8<sup>+</sup> cells obtained at day 520 in patient 2 and tested with the 721.221 B-lymphoblastoid cell line transfected with 1 of 3 mismatched HLA alleles (Figure 1C-D).

Despite high transplantation-related mortality resulting from severe graft-versus-host disease and posttransplantation infections, haploidentical HSCT has been widely used with the expectation of a strong graft-versus-leukemia effect.<sup>15</sup> However, our observation provides a possible limitation of this strategy. Indeed, 2 of 3 patients showed genomic loss of the recipient-specific HLA-haplotype, which led to escape from the graft-versus-leukemia effect and relapse of the disease.

Vago et al also reported a similar observation in 5 of 17 (29.4%) patients whose disease relapsed after haploidentical HSCT.<sup>16</sup> Relapsed leukemic cells may possess genomic instability that elicits genetic diversity.<sup>17</sup> Immunologic pressure by alloreaction to major HLA antigens may select leukemic variants of HLA class I loss, which results in the survival and proliferation of these variants.

In haploidentical HSCT, the importance of natural killer (NK)-cell alloreactivity is emphasized to achieve the graft-versus-leukemia effect.<sup>18,19</sup> HLA loss on leukemic blasts may in turn enhance the NK-cell alloreactivity. Our 2 patients with HLA loss had a group 1 homozygous HLA-C locus that is a suppressive killer immunoglobulin-like receptor (KIR) for NK cells and a KIR-mismatched donor (supplemental Table 2). Because UPD does not

**Table 1. The CTLp frequency reactive to the recipient alloantigen in the recipient after transplantation and the donor**

Samples	Maximum CD8 <sup>+</sup> input*	No. of growing wells†	CTLp frequency <sup>-1</sup> (95% confidence interval)
Donor	33 300	8	$8.6 \times 10^5$ ( $1.49 \times 10^5$ - $5.0 \times 10^5$ )
Day 100	35 500	0	UD
Day 180	17 700	0	UD
Day 300‡	86 000	0	UD
Day 520§	95 000	7	$4.3 \times 10^5$ ( $7.2 \times 10^5$ - $2.5 \times 10^5$ )

Purified CD8<sup>+</sup> T cells from the peripheral blood mononuclear cells obtained after transplantation from patient 2 and her donor were cultured at 2- or 3-fold serial dilutions with 33 Gy-irradiated  $3 \times 10^4$  leukemic blasts cryopreserved at the time of initial diagnosis in 96-well, round-bottom plates in advanced RPMI 1640 medium supplemented with 4% pooled human serum, interleukin-6 (IL-6), and IL-7 (10 ng/mL; both from R&D Systems). The IL-2 (50 U/mL) was added on day 7 with a half medium change. For each dilution, there were at least 12 replicates. On day 14 of culture, a split-well analysis was performed for recipient-specific cytotoxicity against <sup>51</sup>Cr-labeled recipient T-cell blasts, donor T-cell blasts, and leukemic blasts harvested at the time of initial diagnosis and at the time of relapse after DLI if indicated. The supernatants were measured in a  $\gamma$  counter after 4-hour incubation. The wells were considered to be positive for cytolytic activity if the total counts per minute released by effector cells was more than 3 SD above the control wells (mean counts per minute released by the target cells incubated with irradiated stimulator cells alone). The CTLp frequency was calculated using L-Calc software (StemCell Technologies). The CTLp frequencies reactive with recipient T-cell blasts in CD8<sup>+</sup> T cells obtained around days 100, 180, and 300 (4 months before relapse) were undetectable, whereas the CTLp frequency obtained at day 520 (1 month after the third DLI or 2 weeks after remission confirmed by bone marrow aspirate) was close to the CTLp frequency in the donor CD8<sup>+</sup> cells. Complete remission and more than 99% donor chimerism were confirmed on those days.

CTLp indicates CTL precursor; and UD, undetermined because no growing wells are present.

\*Number of input CD8<sup>+</sup> T cells seeded at the highest number per well.

†Number of wells out of 12 wells that received the highest CD8<sup>+</sup> cells and showed detectable growth.

‡Corresponds to 4 months before relapse.

§Corresponds to 1 month after the third DLI or 2 weeks after complete remission was confirmed by bone marrow aspirate.

change the total copy number of the gene, donor NK cells should have been suppressed even after UPD occurred in these patients. Interestingly, the remaining patient who experienced relapse without HLA loss after HLA-haploidentical HSCT had a KIR-mismatched donor, so alloreactive NK cells were possibly enhanced to kill leukemic blasts with HLA loss.

Although one limitation of our study is an insufficient number of cases, our results combined with those in a recent report<sup>16</sup> suggest that leukemic cells occasionally escape from immunosurveillance through the loss of the mismatched HLA haplotype by the mechanism of UPD after haploidentical HSCT. DLI for relapsed AML is less effective than that for chronic myelogenous leukemia after HLA-matched HSCT.<sup>20</sup> However, DLI is effective even for the relapse of AML after haploidentical HSCT.<sup>21</sup> Evaluation of loss or down-regulation of HLA on relapsed leukemic blasts after HLA-haploidentical HSCT should be considered because DLI would probably be ineffective in patients whose leukemic cells lose HLA class I antigen.

## Acknowledgments

This work was supported in part by the Ministry of Education, Culture, Science, Sports, and Technology, Japan (Grant for Scientific Research on Priority Areas; B01 no. 17016089); Grants for Research on the Human Genome, Tissue Engineering Food Biotechnology, and the Second and Third Team Comprehensive 10-year Strategy for Cancer Control (no. 26) from the Ministry of Health, Labor, and Welfare, Japan; Core Research for Evolutional Science and Technology of Japan

(Grant-in-Aid); the College Women's Association of Japan (scholarship award, I.B.V.); a grant from Foundation for Promotion of Cancer Research; and a grant from Morinaga Hoshikai and Grant-in-Aid for Scientific Research (c) No. 20591252.

## Authorship

Contribution: I.B.V. performed experiments and wrote the manuscript; Y.T. designed the research, analyzed data, and wrote

the manuscript; Y.A., H.S., M.K., and S.O. performed experiments, analyzed data, and wrote the manuscript; S.K. supervised this work and wrote the manuscript; and all other authors were responsible for clinical work and critically reviewed the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Seiji Kojima, Department of Pediatrics, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, 466-8550, Japan; e-mail: kojimas@med.nagoya-u.ac.jp.

## References

- Garrido F, Ruiz-Cabello F, Cabrera T, et al. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. *Immunol Today*. 1997;18(2):89-95.
- Seliger B, Cabrera T, Garrido F, Ferrone S. HLA class I antigen abnormalities and immune escape by malignant cells. *Semin Cancer Biol*. 2002;12(1):3-13.
- Marincola FM, Jaffee EM, Hicklin DJ, Ferrone S. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Adv Immunol*. 2000;74:181-273.
- Wetzler M, Baer MR, Stewart SJ, et al. HLA class I antigen cell surface expression is preserved on acute myeloid leukemia blasts at diagnosis and at relapse. *Leukemia*. 2001;15(1):128-133.
- Masuda K, Hiraki A, Fujii N, et al. Loss or down-regulation of HLA class I expression at the allelic level in freshly isolated leukemic blasts. *Cancer Sci*. 2007;98(1):102-108.
- Takahashi Y, McCoy JP Jr, Carvallo C, et al. In vitro and in vivo evidence of PNH cell sensitivity to immune attack after nonmyeloablative allogeneic hematopoietic cell transplantation. *Blood*. 2004;103(4):1383-1390.
- Sasazuki T, Juji T, Morishima Y, et al. Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor: Japan Marrow Donor Program. *N Engl J Med*. 1998;339(17):1177-1185.
- Nannya Y, Sanada M, Nakazaki K, et al. A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. *Cancer Res*. 2005;65(14):6071-6079.
- Torikai H, Akatsuka Y, Miyazaki M, et al. A novel HLA-A\*3303-restricted minor histocompatibility antigen encoded by an unconventional open reading frame of human TMSB4Y gene. *J Immunol*. 2004;173(11):7046-7054.
- Akatsuka Y, Nishida T, Kondo E, et al. Identification of a polymorphic gene, BCL2A1, encoding two novel hematopoietic lineage-specific minor histocompatibility antigens. *J Exp Med*. 2003;197(11):1489-1500.
- Riddell SR, Greenberg PD. The use of anti-CD3 and anti-CD28 monoclonal antibodies to clone and expand human antigen-specific T cells. *J Immunol Methods*. 1990;128(2):189-201.
- Kondo E, Topp MS, Kiem HP, et al. Efficient generation of antigen-specific cytotoxic T cells using retrovirally transduced CD40-activated B cells. *J Immunol*. 2002;169(4):2164-2171.
- Takahashi Y, Harashima N, Kajigaya S, et al. Regression of human kidney cancer following allogeneic stem cell transplantation is associated with recognition of an HERV-E antigen by T cells. *J Clin Invest*. 2008;118(3):1099-1109.
- Tuna M, Knuutila S, Mills GB. Uniparental disomy in cancer. *Trends Mol Med*. 2009;15(3):120-128.
- Koh LP, Rizzieri DA, Chao NJ. Allogeneic hematopoietic stem cell transplant using mismatched/haploidentical donors. *Biol Blood Marrow Transplant*. 2007;13(11):1249-1267.
- Vago L, Perna SK, Zanussi M, et al. Loss of mismatched HLA in leukemia after stem-cell transplantation. *N Engl J Med*. 2009;361(5):478-488.
- Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nat Immunol*. 2002;3(11):999-1005.
- Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. 2002;295(5562):2097-2100.
- Velardi A, Ruggeri L, Mancusi A, et al. Clinical impact of natural killer cell reconstitution after allogeneic hematopoietic transplantation. *Semin Immunopathol*. 2008;30(4):489-503.
- Kolb HJ. Graft-versus-leukemia effects of transplantation and donor lymphocytes. *Blood*. 2008;112(12):4371-4383.
- Huang XJ, Liu DH, Liu KY, Xu LP, Chen H, Han W. Donor lymphocyte infusion for the treatment of leukemia relapse after HLA-mismatched/haploidentical T-cell-replete hematopoietic stem cell transplantation. *Haematologica*. 2007;92(3):414-417.



## Prognostic Factors for Outcomes of Pediatric Patients with Refractory or Relapsed Acute Leukemia Undergoing Allogeneic Progenitor Cell Transplantation

Nobuhiro Watanabe,<sup>1</sup> Yoshiyuki Takahashi,<sup>2</sup> Kimikazu Matsumoto,<sup>1</sup> Asahito Hama,<sup>2</sup> Hideki Muramatsu,<sup>2</sup> Sayoko Doisaki,<sup>2</sup> Keizo Horibe,<sup>3</sup> Koji Kato,<sup>1</sup> Seiji Kojima<sup>2</sup>

Allogeneic stem cell transplantation (SCT) is the only curative therapy for patients with refractory or relapsed acute leukemia, although the prognosis remains poor. Few reports have described outcomes of SCT in pediatric patients with refractory acute leukemia. To identify prognostic factors for these patients, we retrospectively evaluated SCT outcomes for advanced acute leukemia in 82 pediatric patients from 3 transplant units in Nagoya City between 1990 and 2008. Median age at transplantation was 8 years (range, 0.5-17 years). Transplantation was performed in the first refractory relapse for 53 patients (64.6%), in the second or subsequent relapse for 16 patients (19.5%), and during primary induction failure for 13 patients (15.9%). Only 4 patients (4.9%) underwent transplantation in the untreated first relapse, and 39 patients (47.6%) received unrelated donor progenitor cells. Of the 82 patients, 61 died (77.9%), with a median survival of 7.1 months (95% confidence interval [CI], 4.2-10.0 months). Median disease-free survival (DFS) was 4.7 months (95% CI, 2.6-6.9 months). In multivariate analysis, peripheral blood blasts, cord blood transplantation, and more than 3 courses of previous salvage chemotherapy were predictive of DFS. These results support the notion that allogeneic SCT offers only a small chance of cure for most pediatric patients with refractory or relapsed acute leukemia, and suggest that reduction of the leukemia burden and earlier optimal timing of transplantation are essential for long-term survival even in patients with refractory acute leukemia.

*Biol Blood Marrow Transplant* ■: 1-8 (2010) © 2010 American Society for Blood and Marrow Transplantation

**KEY WORDS:** Allografting, Pediatric patients, Acute leukemia, Refractory or relapsed

### INTRODUCTION

Although advances in chemotherapy have improved the prognosis for patients with acute leukemia, outcomes remain poor in patients with refractory or relapsed disease [1-7]. Moreover, much of the literature has reported results for pediatric and adult patients together, making outcomes in pediatric patients alone difficult to determine. The present study sought to identify prognostic factors influencing outcomes after

stem cell transplantation (SCT) in children with acute leukemia who had not achieved remission with chemotherapy.

### PATIENTS AND METHODS

Three transplant units in Nagoya City were asked to provide data on all pediatric patients (aged  $\leq 17$  years at the time of transplantation) who underwent allogeneic SCT for acute lymphoblastic leukemia (ALL;  $n = 48$ ), acute myelogenous leukemia (AML;  $n = 31$ ), or acute undifferentiated leukemia (AUL;  $n = 3$ ) after failing to achieve remission between 1990 and October 2008. Remission was defined as morphologically normal bone marrow (BM) without cytogenetic evidence of leukemia, and a morphologically normal peripheral blood (PB) smear with recovery of PB hematologic values, including a platelet count  $>100 \times 10^9/L$  and an absolute neutrophil count  $>1.5 \times 10^9/L$ . Chromosomal abnormalities classified as good prognostic features included AML with translocation 8;21 ( $n = 3$ ) and ALL with hyperdiploid karyotype ( $n = 2$ ). Karyotypes considered to have a poor prognosis included AML and ALL with abnormalities of chromosome 7

From the <sup>1</sup>Division of Hematology and Oncology, Children's Medical Center, Japanese Red Cross Nagoya First Hospital, Nagoya, Japan; <sup>2</sup>Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya, Japan; and <sup>3</sup>Clinical Research Center, National Hospital Organization Nagoya Medical Center, Nagoya, Japan.

*Financial disclosure:* See Acknowledgments, page XXX.

Correspondence and reprint requests: Nobuhiro Watanabe, MD, Division of Hematology and Oncology, Shizuoka Children's Hospital of Shizuoka Prefecture, 860 Urushiyama, Aoi-ku, Shizuoka 420-8660, Japan (e-mail: nobuhiro.watanabe@sch.pref.shizuoka.jp).

Received May 17, 2010; accepted July 21, 2010

© 2010 American Society for Blood and Marrow Transplantation  
1083-8791/\$36.00

doi:10.1016/j.bbmt.2010.07.019

( $n = 4$ ), those involving the long arm of chromosome 11 ( $n = 12$ ), and translocation 1;19 ( $n = 2$ ), t4;11 ( $n = 4$ ), or t9;22 ( $n = 10$ ). The other 46 patients (including 14 patients with normal karyotype) were assigned to the intermediate-risk category.

The results of HLA testing performed using standard serologic methods for HLA-A, -B, -DR, and -DQ identity were confirmed by high-resolution molecular typing. The conditioning regimens used for transplantation and protocols for graft-versus-host disease (GVHD) prophylaxis were those used at our institutions during the period when the patients underwent transplantation. Conditioning regimens were classified as total body irradiation (10-12 Gy, divided into 4 fractions)-based myeloablative (MA), high-dose chemotherapy-based MA, or reduced-intensity (RIC) [8]. Patients and donors provided written informed consent, and unrelated donor cells were procured under the auspices of the Japanese Marrow Donor Program following the applicable current guidelines.

### Statistical Analysis

Data were collected to allow study of the following subgroups: age at SCT ( $\leq 10$  years vs  $\geq 11$  years), year of transplantation (1990-2000 vs 2001-2008), disease status (primary induction failure [PIF] vs first or later refractory relapse, untreated relapse, or chemoresistant relapse), type of leukemia (ALL vs AML/AUL), number of salvage treatment courses before transplantation ( $\leq 2$  vs  $\geq 3$ ), cytogenetics (good vs other), conditioning regimen (total body irradiation vs chemotherapy only, MA vs RIC), donor source (BM/PB vs cord blood [CB]; HLA disparity), duration of previous first complete remission (CR1) ( $\leq 1$  year vs  $> 1$  year), high disease burden (% BM blasts before SCT or presence of circulating blasts), acute GVHD (aGVHD; grade 0-I vs grade II-IV), and chronic GVHD (cGVHD; none vs limited vs extensive). HLA disparity was considered a trinary variable for risk factor analysis. The HLA-higher mismatched group included patients undergoing transplantation from a related donor with  $\geq 2$  antigen mismatches, unrelated CB donors with  $\geq 2$  antigen mismatches, and unrelated BM donors with  $\geq 1$  antigen mismatches. The HLA-middle mismatched group included related donors with 1 antigen mismatch, CB donors with 1 antigen mismatch, and unrelated matched BM donors. The HLA-less mismatched group included HLA-matched related donors.

Unadjusted survival probabilities were estimated using the Kaplan-Meier method. Comparisons of unadjusted between-group survival rates were made using the log-rank test. Univariate analyses were performed for various pretransplantation and transplantation variables related to disease-free survival (DFS), nonrelapse mortality (NRM), and relapse rate at 5 years posttransplantation. Cox proportional hazards

regression modeling was used to assess the ability of patient characteristics and treatment-related variables to predict survival. All variables showing a probable association ( $P < .10$ ) with DFS, NRM, or relapse rate in univariate analyses were included in the Cox proportional hazards model. Time-dependent covariates were used to study aGVHD and cGVHD. All statistical tests were two-sided, and differences were considered statistically significant at  $P < .05$ . Associations between discrete variables were assessed by Fisher's exact and generalized exact tests. All analyses were performed using SPSS software (SPSS, Chicago, IL).

## RESULTS

### Patient Characteristics

Demographic data and disease characteristics are shown in Table 1. Median patient age at transplantation was 8 years (range, 0.5-17 years). Transplantation was performed during the first refractory relapse in 53 patients (64.6%), during a second or subsequent relapse in 16 patients (19.5%), and during PIF in 13 patients (15.9%). Four patients (4.9%) underwent transplantation during an untreated first relapse, and the remaining 78 patients were unable to attain CR despite induction chemotherapy. Fifty-four patients (65.8%) had received  $\geq 3$  courses of salvage chemotherapy, and 49 patients (59.7%) had experienced early relapse after a remission lasting less than 1 year. For the 43 patients who received an allograft from a related donor, 33 (40.2%) were HLA-identical and 10 (12.2%) were HLA-mismatched. Of the 39 unrelated donors, 20 (24.4%) were HLA-identical and 19 (23.1%) were HLA-mismatched. The transplant source was BM in 65 patients (79.3%), mobilized PB stem cells in 9 patients (11.0%), and CB in 8 patients (9.8%). Three patients (3.7%) received an RIC regimen, whereas 79 patients (96.3%) received an MA conditioning regimen. GVHD prophylaxis consisted primarily of tacrolimus combination therapy (37 [45.1%]), cyclosporine combination therapy (21 [25.6%]), or methotrexate alone (21 [25.6%]).

### Survival, NRM, and Relapse Rate

Sixty-one of the 82 patients died (77.9%), with a median survival of 7.1 months (95% confidence interval [CI], 4.2-10.0 months). A total of 66 patients (83.4%) either died or displayed disease progression. Median DFS was 4.7 months (95% CI, 2.6-6.9 months) (Figure 1). Median follow-up for the disease-free survivors was 8.6 years (range, 0.37-19 years). On univariate analysis, significant prognostic factors for DFS were stem cell type, presence of PB blasts before SCT, more than 3 courses of previous salvage chemotherapy, and cGVHD (Table 2).

NRM occurred in 25 patients (30.5%), including 9 from respiratory failure, including interstitial

Table 1. Patient Characteristics

	n	%
Number of patients	82	
Sex		
Male	52	63.4
Female	30	36.4
Diagnosis		
ALL	48	58.5
AML	31	37.8
AUL	3	3.7
Age		
Median (range), years	8 (0.5-17)	
≤10 years	55	67.1
≥11 years	27	32.9
Year of transplantation		
1990-2000	44	53.7
2001-2008	38	46.3
Disease status before SCT		
Primary induction failure	13	15.9
First relapse refractory	53	64.6
>First relapse refractory	16	19.5
Number of chemotherapy cycles before SCT		
Untreated first relapse	4	4.9
1 cycles	8	9.8
2 cycles	16	19.5
≥3 cycles	54	65.8
Cytogenetic subgroup		
Good	5	6.1
Intermediate	44	53.7
Bad	30	36.6
Missing	2	2.4
Stem cell type		
BM	65	79.3
PB	9	11
CB	8	9.8
Donor type		
BM/PB		
Matched related	33	40.2
Mismatched related	10	12.2
One locus mismatched	3	3.7
Two loci mismatched	7	8.5
Matched unrelated	20	24.4
Mismatched unrelated		
One locus mismatched	11	13.4
CB	8	9.8
One locus mismatched	3	3.7
Two loci mismatched	5	6.1
Donor sex		
Male	38	46.3
Female	44	53.7
Conditioning regimen		
Reduced-intensity conditioning		
Yes	3	3.7
No	79	96.3
Total body irradiation		
Yes	76	92.7
No	6	7.3
Use of ATG	11	13.4
Use of busulfan	47	57.3
Use of cyclophosphamide	14	17.1
Use of melphalan	66	80.5
GVHD prophylaxis		
MTX alone	21	25.6
Cyclosporine + MTX	19	23.2
Cyclosporine + MTX + PSL	1	1.2
Cyclosporine + PSL	1	1.2
Tacrolimus + MTX	36	43.9
Tacrolimus + MMF	1	1.2
None	3	3.7
Leukemia burden at SCT		
Presence of circulating blasts		
Yes	44	53.7

(Continued)

Table 1. (Continued)

	n	%
No	38	46.3
≥25% marrow blasts		
Yes	57	69.5
No	20	24.4
Missing	5	6.1
Duration of CR1, months		
<6	33	40.2
6-12	16	19.5
≥12	31	37.8
Missing	2	2.4

ALL indicates acute lymphoblastic leukemia; AML, acute myelogenous leukemia; AUL, acute undifferentiated leukemia; SCT, stem cell transplantation; BM, bone marrow; PB, peripheral blood; CB, cord blood; ATG, antithymocyte globulin; MMF, mycophenolate mofetil; MTX, methotrexate; PSL, prednisolone.

pneumonia, diffuse alveolar hemorrhage, and bronchiolitis obliterans; 6 from infection; 5 from severe aGVHD; 3 from multiorgan failure, including veno-occlusive disease; and 2 from encephalopathy (Figure 2). On univariate analysis, no risk factors were associated with higher NRM rates.

A total of 41 patients (50%) relapsed, with a median relapse time from SCT of 10.7 months (95% CI, 3.2-18.1 months) (Figure 3). Risk factors associated with relapse rate included stem cell type, presence of PB blasts before SCT, the number of salvage chemotherapy, and cGVHD (Table 3). In contrast, age, sex, diagnosis (ALL vs AML/AUL), cytogenetic subgroup, and duration of CR1 were not significantly associated with clinical outcomes.

### Multivariate Analysis

Cox regression analysis showed that the number of treatment courses before SCT, stem cell type, and the presence of PB blasts before SCT were predictive of DFS (Table 2). No prognostic factors for NRM were identified, whereas the number of treatment courses before SCT, stem cell type, and presence of PB blasts were associated with relapse rate (Table 3). In patients receiving a BM or PB graft, HLA disparity also was associated with DFS and relapse rate (Tables 2 and 3).

Figure 4 shows the manner in which the predicted DFS probability under the Cox regression model summarized in Table 2 varies with PB blasts and number of treatment courses before SCT. In the 54 patients receiving ≥3 courses of treatment before transplantation, DFS at 5 years was 28.0% (range, 19.0%-37.0%) in 25 patients without circulating PB blasts and 3.4% (range, 0%-6.8%) in 29 patients with PB blasts ( $P = .004$ ).

### DISCUSSION

The recent development and implementation of more aggressive chemotherapy protocols and better

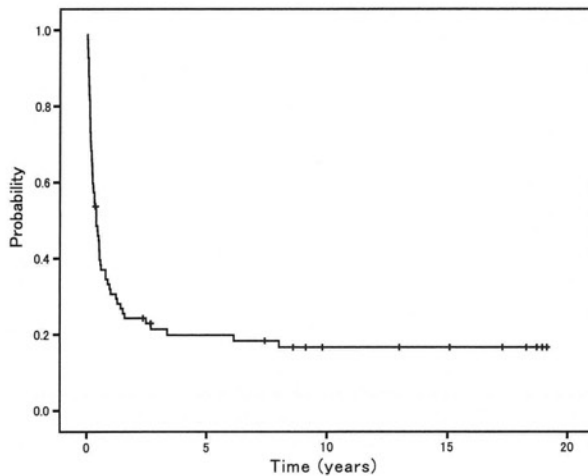


Figure 1. Kaplan-Meier curve for DFS.

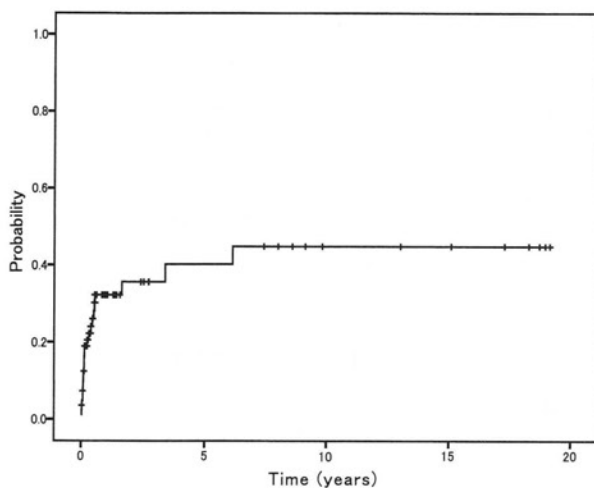
supportive care following rigorous clinical trials have led to improving survival rates in children with acute leukemias, including lymphoid and myelogenous malignancies [9-14]. Nonetheless, current therapeutic

results remain unsatisfactory, particularly for patients with primary refractory or relapsed disease despite salvage chemotherapy. In patients with refractory or relapsed acute leukemia treated with allogeneic SCT, long-term survival is generally poor [1-7]. Previous studies of outcomes of allogeneic SCT for advanced acute leukemia have been limited by the small number of patients and the inclusion of a range of disease statuses, from remission to refractory or relapsed disease. Most studies also have included both pediatric and adult patients, and few have examined pediatric patients with acute leukemia who never achieved CR [4]. In the present study, we evaluated a large number of pretreatment variables for effects on transplantation outcomes in pediatric patients. We found that the number of treatment courses before SCT, stem cell type, and the presence of PB blasts before SCT were independently associated with DFS in pediatric patients with refractory or relapsed acute leukemia, as has been reported for adult patients [4-6,15-18]. In our study, two-thirds of patients had received more than 3 courses of chemotherapy, making this a heavily pretreated

Table 2. Prognostic Factors Associated with DFS

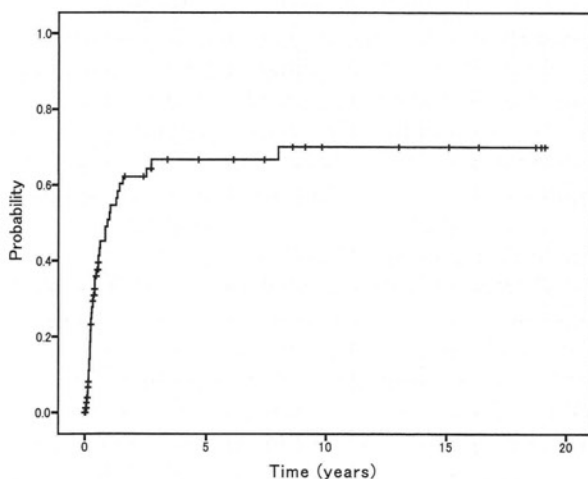
Variable	5-Year DFS, %	Univariate P Value	Multivariate		
			HR	95% CI	P Value
<b>All recipients</b>					
Year of transplantation					
1990-2000	29.5 ± 6.9	.098			
2001-2008	13.2 ± 5.5				
Number of previous therapies					
≤2	35.7 ± 9.1	.024	1		
≥3	14.8 ± 4.8		3.62	1.62-8.08	.002
Stem cell type					
BM/PB	24.3 ± 5.0	.015	1		
CB	0		5.69	1.35-24.03	.018
% BM blasts before SCT					
<25% (n = 20)	35.0 ± 10.7	.097			
≥25% (n = 57)	19.3 ± 5.2				
PB blast before SCT					
Blast-negative	36.8 ± 7.8	<.001	1		
Blast-positive	9.1 ± 4.3		3.24	1.61-6.54	.001
Chronic GVHD					
None	20.3 ± 6.8	.013			
Limited/extensive	42.3 ± 9.7				
<b>BM/PB recipients</b>					
Year of transplantation					
1990-2000	31.7 ± 7.3	.091			
2001-2008	15.2 ± 6.2				
Number of previous therapies					
≤2	43.5 ± 10.3	.014	1		
≥3	15.7 ± 5.7		4.98	2.02-12.26	<.001
PB blast before SCT					
Blast-negative	38.9 ± 8.1	.004	1		
Blast-positive	10.5 ± 5.0		2.53	1.19-5.41	.016
HLA disparity					
Match	30.2 ± 6.3	.013	1		
Mismatch	9.5 ± 6.4		2.38	1.05-5.35	.037
Chronic GVHD					
None	21.9 ± 7.3	.014			
Limited/extensive	44.0 ± 9.9				

DFS indicates disease-free survival; BM, bone marrow; PB, peripheral blood; CB, cord blood; SCT, stem cell transplantation; GVHD, graft-versus-host disease.



**Figure 2.** Time to NRM for 82 patients with refractory or relapsed acute leukemia.

group of patients. In multivariate analysis, fewer courses of pretransplantation chemotherapy was associated with better DFS and relapse rate. Similar observations have been reported previously [3,15]. As Schmid et al. [15] reported, this difference is not attributable to a higher NRM, as might be expected given the higher cumulative organ toxicity in heavily pretreated patients. These authors also reported that disease stage at the time of transplantation did not differ between patients pretreated with 2 or  $\geq 3$  courses of salvage chemotherapy. These observations might offer clues to guide the selection of chemoresistant leukemic cells during repeated reinduction attempts and possibly favor earlier timing of allogeneic SCT once acute leukemia appears to be refractory to chemotherapy. Prompt transplantation using HLA-mismatched/haploidentical blood and BM grafts might be warranted for patients without a suitable related matched donor [19]. Patients who



**Figure 3.** Time to relapse for 82 patients with refractory or relapsed acute leukemia.

do not achieve remission after multiple salvage therapies with circulating PB blasts might not be good candidates for SCT.

We hypothesized that the leukemic burden, as measured by the PB blast count and BM leukemia infiltrate, would be important prognostic factors for outcome. The results of our univariate and multivariate analyses suggest that PB blasts can be predictive of DFS. Our analysis confirms an association between high disease burden and poor survival, as reported previously [16-18]. The high relapse rate after SCT suggests that current high-dose chemoradiation regimens are often inadequate to eradicate leukemia. Multiple preparative regimens have been tested in the phase II setting in an attempt to improve outcomes in patients with advanced leukemia, but none has demonstrated superiority over other regimens [20]. This finding supports the current practice of attempting cytorreduction before proceeding with transplantation in patients showing circulating blasts or extensive BM disease. This argues in favor of further attempts at blast reduction in patients with high blast counts, inevitably increasing the time to transplantation.

Andrew et al. [21] reported an association between a graft-versus-leukemia (GVL) effect and GVHD after allogeneic SCT for refractory or relapsed acute leukemia. However, in our study, cGVHD was significantly associated with DFS and relapse rate in the univariate analysis, but not in multivariate Cox regression model. In the study of Andrew et al., the leukemic burden before transplantation was not included as a pretransplantation variable in the analysis of factors that had a significant effect on clinical outcomes. A European report of a large number of patients receiving cyclosporine and methotrexate as GVHD prophylaxis documented a greater GVL effect associated with aGVHD in CR1 compared with relapse [22]. High leukemic burden and selection of chemoresistant leukemic cells during repeated reinduction attempts may cause the failure of complete donor chimerism, preventing the development of cGVHD or a GVL effect in these patients.

We found no difference in DFS, NRM, or relapse rate between patients with ALL and those with AML/AUL. A study based on data from the International Bone Marrow Transplant Registry reported that the GVL effect was greatest in AML, was of borderline significance in chronic myelogenous leukemia, and was absent in ALL [23]. However, these patients underwent BM transplantation while in CR1. Ringden et al. [22] noted that the GVL effect is most obvious in early disease. Our findings demonstrate that leukemic burden is a more important factor in DFS than the difference in disease between ALL and AML/AUL in patients receiving SCT with refractory leukemia.

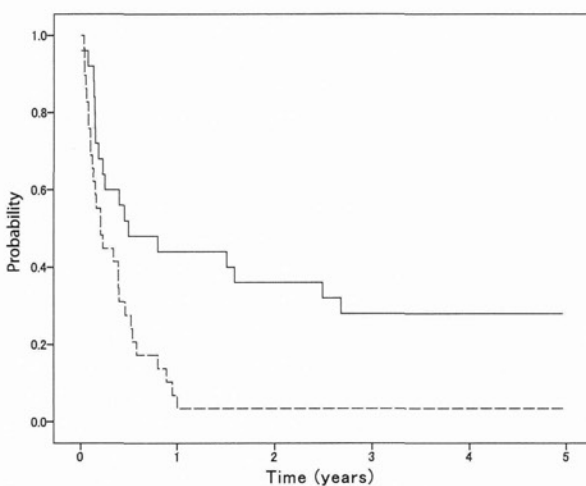
As in previous reports [16,21], we found a nonsignificant trend in survival associated with classical

**Table 3. Risk Factors Associated with Relapse Rate**

Variables	5-Year Relapse Rate, %	Univariate P Value	Multivariate		
			HR	95% CI	P Value
<b>All recipients</b>					
Number of previous therapies					
≤2	59.6 ± 12.5	.076	1		
≥3	70.7 ± 7.4		3.01	1.29-7.03	.011
Stem cell type					
BM/PB	67.7 ± 7.0	.034	1		
CB	100		4.78	1.18-19.42	.029
PB blast before SCT					
Blast-negative	53.4 ± 11.0	<.001	1		
Blast-positive	88.2 ± 6.3		3.19	1.55-6.56	.002
Chronic GVHD					
None	78.2 ± 7.4	.004			
Limited/extensive	53.0 ± 12.6				
<b>BM/PB recipients</b>					
Number of previous therapies					
≤2	53.6 ± 13.9	.059	1		
≥3	75.7 ± 7.3		3.66	1.51-8.83	.004
PB blast before SCT					
Blast-negative	51.9 ± 11.2	.002	1		
Blast-positive	86.8 ± 7.0		2.33	1.05-5.16	.038
HLA disparity					
Match	61.5 ± 8.3	.041	1		
Mismatch	83.2 ± 10.5		2.51	1.09-5.77	.031
Chronic GVHD					
None	76.0 ± 8.1	.009			
Limited/extensive	53.1 ± 12.6				

BM indicates bone marrow; PB, peripheral blood; CB, cord blood; SCT, stem cell transplantation; GVHD, graft-versus-host disease.

prognostic cytogenetic features. Other groups have reported a low relapse rate and better long-term survival after SCT in patients with *inv16* and *t15;17*, even in non-complete remission [24,25]. In our study, there were no patients with *inv16* and *t15;17*, and few patients with a good-risk chromosomal abnormality. Given the limited number of patients with good-risk abnormalities, the



**Figure 4.** Effect of previous therapy and the presence of PB blasts pre-transplantation on DFS at 5 years. Outcomes for 54 patients who received ≥3 courses of therapy before transplantation are shown. DFS at 5 years was 28.0% (range, 19.0%-37.0%) in 25 patients without circulating PB blasts (solid line) and 3.4% (range, 0%-6.8%) in 34 patients with PB blasts (dashed line) ( $P = .004$ ).

actual significance of each additional abnormality should be investigated in a larger number of patients.

In our study, CB transplantation was significantly associated with DFS and relapse rate. The diminished GVHD after CB transplantation reported by some investigators raises the concern that CB-derived cells might not be capable of generating a sufficient GVL response. However, the incidence of recurrent leukemia after CB transplantation does not differ from that reported in BM or PB transplantation [26]. Because of the limited number of patients in our subgroup analyses and the possibility of an unidentified bias in stem cell source selection, our findings should be verified by further analysis in a larger population.

In our BM and PB recipients, HLA disparity was significantly associated with DFS and relapse. This finding suggests that there may be several reasons for the increased risk of relapse in the HLA-mismatched group, such as selection bias and more heavy immunosuppressive therapy. Increased immunosuppression might decrease the GVL effect [27].

Despite all of the recent advances in SCT, survival of children with refractory or relapsed acute leukemia has not changed significantly since the first reports of BM transplantation more than 3 decades ago [28]. Our results suggest that innovative strategies are justified if allografting is to be contemplated in most pediatric patients with refractory acute leukemia. Recent sequential use of intensive chemotherapy, RIC transplantation, and prophylactic donor lymphocyte

transfusions might represent steps forward in the treatment of refractory myelogenous malignancies [15,29].

Realistically, increasing survival to any significant degree in pediatric patients with refractory acute leukemia will require novel approaches to overcoming the intrinsic resistance of leukemia cells to high-dose chemoradiotherapy. The risk factors for mortality and relapse identified in the present study may help guide clinicians in making recommendations for allogeneic SCT in pediatric patients with refractory acute leukemia. Our data suggest that earlier optimal timing of transplantation will be associated with better clinical outcomes in patients with refractory or relapsed acute leukemia, regardless of other factors. Conversely, transplantation might not be indicated for patients with persistent PB blasts after 3 courses of salvage chemotherapy.

## ACKNOWLEDGMENTS

This work was supported by a grant from the Ministry of Health, Labor, and Welfare of Japan.

*Financial disclosure:* The authors have no conflicts of interest to disclose.

## REFERENCES

1. Estey EH. Treatment of relapsed and refractory acute myelogenous leukemia. *Leukemia*. 2000;14:476-479.
2. Brown RA, Wolff SN, Fay JW, et al. High-dose etoposide, cyclophosphamide and total body irradiation with allogeneic bone marrow transplantation for resistant acute myeloid leukemia: a study by the North American Marrow Transplant Group. *Leuk Lymphoma*. 1996;22:271-277.
3. Biggs JC, Horowitz MM, Gale RP, et al. Bone marrow transplants may cure patients with acute leukemia never achieving remission with chemotherapy. *Blood*. 1992;80:1090-1093.
4. Nemecek ER, Gooley TA, Woolfrey AE, et al. Outcome of allogeneic bone marrow transplantation for children with advanced acute myeloid leukemia. *Bone Marrow Transplant*. 2004;34:799-806.
5. Goldman FD, Rumelhart SL, DeAlacron P, et al. Poor outcome in children with refractory/relapsed leukemia undergoing bone marrow transplantation with mismatched family member donors. *Bone Marrow Transplant*. 2000;25:943-948.
6. Greinix HT, Reiter E, Keil F, et al. Leukemia-free survival and mortality in patients with refractory or relapsed acute leukemia given marrow transplants from sibling and unrelated donors. *Bone Marrow Transplant*. 1998;21:673-678.
7. Oyekunle AA, Kroger N, Zabelina T, et al. Allogeneic stem-cell transplantation in patients with refractory acute leukemia: a long-term follow-up. *Bone Marrow Transplant*. 2006;37:45-50.
8. Champlin R, Khouri I, Shimoni A, et al. Harnessing graft-versus-malignancy: non-myeloablative preparative regimens for allogeneic haematopoietic transplantation, an evolving strategy for adoptive immunotherapy. *Br J Haematol*. 2000;111:18-29.
9. Schrappe M, Reiter A, Ludwig WD, et al. Improved outcome in childhood acute lymphoblastic leukemia despite reduced use of anthracyclines and cranial radiotherapy: results of trial ALL-BFM 90. German-Austrian-Swiss ALL-BFM Study Group. *Blood*. 2000;95:3310-3322.
10. Gaynon PS, Trigg ME, Heerema NA, et al. Children's Cancer Group trials in childhood acute lymphoblastic leukemia: 1983-1995. *Leukemia*. 2000;14:2223-2233.
11. Pui CH, Boyett JM, Rivera GK, et al. Long-term results of Total Therapy studies 11, 12 and 13A for childhood acute lymphoblastic leukemia at St. Jude Children's Research Hospital. *Leukemia*. 2000;14:2286-2294.
12. Webb DK, Harrison G, Stevens RF, et al. Relationships between age at diagnosis, clinical features, and outcome of therapy in children treated in the Medical Research Council AML 10 and 12 trials for acute myeloid leukemia. *Blood*. 2001;98:1714-1720.
13. Woods WG, Neudorf S, Gold S, et al. A comparison of allogeneic bone marrow transplantation, autologous bone marrow transplantation, and aggressive chemotherapy in children with acute myeloid leukemia in remission. *Blood*. 2001;97:56-62.
14. Creutzig U, Ritter J, Zimmermann M, et al. Improved treatment results in high-risk pediatric acute myeloid leukemia patients after intensification with high-dose cytarabine and mitoxantrone: results of Study Acute Myeloid Leukemia-Berlin-Frankfurt-Munster 93. *J Clin Oncol*. 2001;19:2705-2713.
15. Schmid C, Schleuning M, Schwerdtfeger R, et al. Long-term survival in refractory acute myeloid leukemia after sequential treatment with chemotherapy and reduced-intensity conditioning for allogeneic stem cell transplantation. *Blood*. 2006;108:1092-1099.
16. Wong R, Shahjahan M, Wang X, et al. Prognostic factors for outcomes of patients with refractory or relapsed acute myelogenous leukemia or myelodysplastic syndromes undergoing allogeneic progenitor cell transplantation. *Biol Blood Marrow Transplant*. 2005;11:108-114.
17. Hosing C, Saliba RM, Shahjahan M, et al. Disease burden may identify patients more likely to benefit from second allogeneic hematopoietic stem cell transplantation to treat relapsed acute myelogenous leukemia. *Bone Marrow Transplant*. 2005;36:157-162.
18. Sierra J, Storer B, Hansen JA, et al. Transplantation of marrow cells from unrelated donors for treatment of high-risk acute leukemia: the effect of leukemic burden, donor HLA-matching, and marrow cell dose. *Blood*. 1997;89:4226-4235.
19. Huang XJ, Liu DH, Liu KY, et al. Haploidentical hematopoietic stem cell transplantation without in vitro T-cell depletion for the treatment of hematological malignancies. *Bone Marrow Transplant*. 2006;38:291-297.
20. Aurer I, Gale RP. Are new conditioning regimens for transplants in acute myelogenous leukemia better? *Bone Marrow Transplant*. 1991;7:255-261.
21. Grigg AP, Szer J, Beresford J, et al. Factors affecting the outcome of allogeneic bone marrow transplantation for adult patients with refractory or relapsed acute leukaemia. *Br J Haematol*. 1999;107:409-418.
22. Ringden O, Labopin M, Gluckman E, et al. Graft-versus-leukemia effect in allogeneic marrow transplant recipients with acute leukemia is maintained using cyclosporin A combined with methotrexate as prophylaxis. Acute Leukemia Working Party of the European Group for Blood and Marrow Transplantation. *Bone Marrow Transplant*. 1996;18:921-929.
23. Horowitz MM, Gale RP, Sondel PM, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood*. 1990;75:555-562.
24. Kuwatsuka Y, Miyamura K, Suzuki R, et al. Hematopoietic stem cell transplantation for core binding factor acute myeloid leukemia: t(8;21) and inv(16) represent different clinical outcomes. *Blood*. 2009;113:2096-2103.
25. Bourquin JP, Thornley I, Neuberg D, et al. Favorable outcome of allogeneic hematopoietic stem cell transplantation for relapsed or refractory acute promyelocytic leukemia in childhood. *Bone Marrow Transplant*. 2004;34:795-798.
26. Eapen M, Rubinstein P, Zhang MJ, et al. Outcomes of transplantation of unrelated donor umbilical cord blood and bone marrow in children with acute leukaemia. *Lancet*. 2007;369:1947-1954.
27. Remberger M, Storer B, Ringden O, et al. Association between pretransplant thymoglobulin and reduced non-relapse mortality

- rate after marrow transplantation from unrelated donors. *Bone Marrow Transplant.* 2002;29:391-397.
28. Thomas ED, Buckner CD, Banaji M, et al. One hundred patients with acute leukemia treated by chemotherapy, total body irradiation, and allogeneic marrow transplantation. *Blood.* 1977;49:511-533.
29. Schmid C, Schleuning M, Ledderose G, et al. Sequential regimen of chemotherapy, reduced-intensity conditioning for allogeneic stem-cell transplantation, and prophylactic donor lymphocyte transfusion in high-risk acute myeloid leukemia and myelodysplastic syndrome. *J Clin Oncol.* 2005;23:5675-5687.



# blood

2010 116: 4631-4638  
Prepublished online Aug 20, 2010;  
doi:10.1182/blood-2010-05-282426

## **Down syndrome and GATA1 mutations in transient abnormal myeloproliferative disorder: mutation classes correlate with progression to myeloid leukemia**

Rika Kanazaki, Tsutomu Toki, Kiminori Terui, Gang Xu, RuNan Wang, Akira Shimada, Asahito Hama, Hirokazu Kanegane, Kiyoshi Kawakami, Mikiya Endo, Daisuke Hasegawa, Kazuhiro Kogawa, Souichi Adachi, Yasuhiko Ikeda, Shotaro Iwamoto, Takashi Taga, Yoshiyuki Kosaka, Seiji Kojima, Yasuhide Hayashi and Etsuro Ito

---

Updated information and services can be found at:

<http://bloodjournal.hematologylibrary.org/cgi/content/full/116/22/4631>

Articles on similar topics may be found in the following *Blood* collections:

*Myeloid Neoplasia* (389 articles)

---

Information about reproducing this article in parts or in its entirety may be found online at:

[http://bloodjournal.hematologylibrary.org/misc/rights.dtl#repub\\_requests](http://bloodjournal.hematologylibrary.org/misc/rights.dtl#repub_requests)

Information about ordering reprints may be found online at:

<http://bloodjournal.hematologylibrary.org/misc/rights.dtl#reprints>

Information about subscriptions and ASH membership may be found online at:

<http://bloodjournal.hematologylibrary.org/subscriptions/index.dtl>

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

Copyright 2011 by The American Society of Hematology; all rights reserved.



## Down syndrome and *GATA1* mutations in transient abnormal myeloproliferative disorder: mutation classes correlate with progression to myeloid leukemia

\*Rika Kanazaki,<sup>1</sup> \*Tsutomu Toki,<sup>1</sup> Kiminori Terui,<sup>1</sup> Gang Xu,<sup>1</sup> RuNan Wang,<sup>1</sup> Akira Shimada,<sup>2</sup> Asahito Hama,<sup>3</sup> Hirokazu Kanegane,<sup>4</sup> Kiyoshi Kawakami,<sup>5</sup> Mikiya Endo,<sup>6</sup> Daisuke Hasegawa,<sup>7</sup> Kazuhiro Kogawa,<sup>8</sup> Souichi Adachi,<sup>9</sup> Yasuhiko Ikeda,<sup>10</sup> Shotaro Iwamoto,<sup>11</sup> Takashi Taga,<sup>12</sup> Yoshiyuki Kosaka,<sup>13</sup> Seiji Kojima,<sup>3</sup> Yasuhide Hayashi,<sup>2</sup> and Etsuro Ito<sup>1</sup>

<sup>1</sup>Department of Pediatrics, Hirosaki University Graduate School of Medicine, Hirosaki, Japan; <sup>2</sup>Department of Hematology/Oncology, Gunma Children's Medical Center, Gunma, Japan; <sup>3</sup>Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya, Japan; <sup>4</sup>Department of Pediatrics, Graduate School of Medicine, University of Toyama, Toyama, Japan; <sup>5</sup>Department of Pediatrics, Kagoshima City Hospital, Kagoshima, Japan; <sup>6</sup>Department of Pediatrics, Iwate Medical University, Morioka, Japan; <sup>7</sup>Department of Pediatrics, St Luke's International Hospital, Tokyo, Japan; <sup>8</sup>Department of Pediatrics, National Defense Medical College, Tokorozawa, Japan; <sup>9</sup>Department of Pediatrics, Kyoto University Graduate School of Medicine, Kyoto, Japan; <sup>10</sup>Department of Pediatrics, Aomori City Hospital, Aomori, Japan; <sup>11</sup>Department of Pediatrics, Mie University Graduate School of Medicine, Tsu, Japan; <sup>12</sup>Department of Pediatrics, Shiga University of Medical Science, Ohtsu, Japan; and <sup>13</sup>Department of Hematology and Oncology, Hyogo Children Hospital, Kobe, Japan

Twenty percent to 30% of transient abnormal myelopoiesis (TAM) observed in newborns with Down syndrome (DS) develop myeloid leukemia of DS (ML-DS). Most cases of TAM carry somatic *GATA1* mutations resulting in the exclusive expression of a truncated protein (GATA1s). However, there are no reports on the expression levels of GATA1s in TAM blasts, and the risk factors for the progression to ML-DS are unidentified. To test whether the spectrum of transcripts

derived from the mutant *GATA1* genes affects the expression levels, we classified the mutations according to the types of transcripts, and investigated the modalities of expression by in vitro transfection experiments using *GATA1* expression constructs harboring mutations. We show here that the mutations affected the amount of mutant protein. Based on our estimates of GATA1s protein expression, the mutations were classified into GATA1s high and low groups. Phenotypic analy-

ses of 66 TAM patients with *GATA1* mutations revealed that GATA1s low mutations were significantly associated with a risk of progression to ML-DS ( $P < .001$ ) and lower white blood cell counts ( $P = .004$ ). Our study indicates that quantitative differences in mutant protein levels have significant effects on the phenotype of TAM and warrants further investigation in a prospective study. (*Blood*. 2010;116(22):4631-4638)

### Introduction

In children with Down syndrome (DS), the risk of developing acute megakaryocytic leukemia (AMKL) is estimated at 500 times higher than in children without DS. Interestingly, neonates with DS are at a high risk of developing a hematologic disorder referred to as transient abnormal myelopoiesis (TAM). It has been estimated that 5% to 10% of infants with DS exhibit the disorder, and in most cases, it resolves spontaneously within 3 months. However, approximately 20% of the severe cases are still subject to fatal complications and 20% to 30% of patients who escape from early death develop AMKL referred to as myeloid leukemia of DS (ML-DS) within 4 years.<sup>1-4</sup>

Recent studies found that high white blood cell (WBC) count, failure of spontaneous remission, early gestational age (EGA) and liver fibrosis or liver dysfunction are significantly associated with early death.<sup>5-7</sup> Most of the same covariates were found in all of the reports. However, the risk factors for the progression to ML-DS remain elusive.

Blast cells in most patients with TAM and ML-DS have mutations in exon 2 of the gene coding the transcription factor *GATA1*,<sup>8-14</sup> which is essential for normal development of erythroid and megakaryocytic cells.<sup>15-18</sup> The mutations lead to exclusive expression of a truncated *GATA1* protein (referred to as GATA1s)

translated from the second methionine on exon 3. These findings strongly suggest that the qualitative deficit of *GATA1* contributes to the genesis of TAM and ML-DS. The analysis of megakaryocyte-specific knockdown of *GATA1* in vivo has revealed a critical role for this factor in megakaryocytic development. Reduced expression (or complete absence) of *GATA1* in megakaryocytes leads to increased proliferation and deficient maturation as well as a reduced number of circulating platelets.<sup>19,20</sup> Mice harboring a heterozygous *GATA1* knockdown allele frequently develop erythroblastic leukemia.<sup>21</sup> These observations indicate that the expression levels of *GATA1* are crucial for the proper development of erythroid and megakaryocytic cells and compromised *GATA1* expression is a causal factor in leukemia.<sup>22</sup> Nevertheless, the impact of a quantitative deficit of the factor on the pathogenesis of TAM and ML-DS has not been examined.

In this study, we classified the *GATA1* mutations observed in TAM patients according to the types of transcripts, and investigated the modalities of gene expression by in vitro transfection assays using *GATA1* expression constructs. We report here that the spectrum of the transcripts derived from the mutant genes affects protein expression and the risk of progression from TAM to ML-DS.

Submitted April 30, 2010; accepted August 2, 2010. Prepublished online as *Blood* First Edition paper, August 20, 2010; DOI 10.1182/blood-2010-05-282426.

\*R.K. and T.T. contributed equally to this work.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2010 by The American Society of Hematology

## Methods

### Patients

This study was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine, and all clinical samples were obtained with informed consent from the parents of all patients with TAM, in accordance with the Declaration of Helsinki. The following clinical data were collected: sex, gestational age, birth weight, time of diagnosis, symptom at diagnosis, and clinical presentation. The following laboratory data were obtained: a complete blood cell count at diagnosis including WBC and the percentage of blasts in the peripheral blood, coagulation parameters, liver enzymes (alanine aminotransferase and aspartate aminotransferase), and total bilirubin. The procedure for the detection of *GATA1* mutations was described previously.<sup>13</sup> Genomic DNA was directly extracted from peripheral blood or bone marrow with the QIAamp blood mini kit (QIAGEN). Total RNA was extracted from white blood cells prepared by removal of erythrocytes by hypotonic buffer treatment of peripheral blood. Clinical features, outcomes, and characteristics of *GATA1* mutations are indicated in Table 1.

### Construction of *GATA1* expression vectors

To construct *GATA1* minigene expression vectors, fragments of the normal human *GATA1* gene from a part of intron 1 to the stop codon located on exon 6 were amplified by polymerase chain reaction (PCR; Prime STAR HS; Takara Bio) and subcloned to mammalian expression vector pcDNA3.1 (+)/Neo (Invitrogen). To introduce mutations identical to those observed in TAM patients into the expression vector, the regions containing mutations were amplified by PCR from patient samples and inserted into the expression plasmid. To construct expression vectors carrying cDNA, we performed PCR using cDNA derived from baby hamster kidney 21 (BHK-21) cells transfected with *GATA1* minigene vectors. The PCR products were subcloned to pcDNA3.1(+)/Neo. Details of the sequence of each expression construct are described in Table 2.

### Transfection

BHK-21, a baby hamster kidney fibroblast cell line, was cultured with Dulbecco modified Eagle medium supplemented 10% fetal bovine serum. *GATA1* expression vectors were transfected into BHK-21 cells using FuGENE HD transfection reagent (Roche Diagnostics) according to the manufacturer's methods. After 24 hours, protein and total RNA were extracted.

### Western blot analysis

Lysates of transfected BHK-21 cells were transferred to Hybond-P (GE Healthcare) and processed for reaction with anti-*GATA1* antibody M-20 (Santa Cruz Biotechnology) or anti-neomycin phosphotransferase II (NeoR) antibody (Millipore) as described previously.<sup>23</sup>

### Northern blot analysis

Two micrograms of total RNA were transferred to Hybond-N+ (GE Healthcare) and hybridized with *GATA1* or *NeoR* DNA probe. Hybridization and detection were performed with the Gene Images AlkPhos Direct Labeling and Detection System (GE Healthcare) according to the manufacturer's instructions.

### RT-PCR

To detect alternatively spliced transcripts derived from *GATA1* minigene constructs or from patients' peripheral blood mononuclear cells (obtained by Ficoll-Hypaque fractionation), we performed reverse transcription (RT)-PCR using primers T7: 5' AATACGACTCACTATAG 3' and *GATA1* AS1, and *GATA1* S1 and *GATA1* AS1, respectively.<sup>13</sup> Densitometric analyses were performed by the Quantity-One software (Version 4.5.2; Bio-Rad Laboratories).

### Statistical analysis

The cumulative incidence of the progression to ML-DS was analyzed with the Gray test. Differences in the distribution of individual parameters among patient subsets were analyzed using the Pearson  $\chi^2$  test or Fisher exact test for categorized variables and the Mann-Whitney *U* test for continuous variables. The univariate Cox proportional hazards model was used to obtain the estimates and the 95% confidence interval of the relative risk for prognostic factors.

## Results

### Patient characteristics and outcomes

From 2003 to 2008, we screened *GATA1* mutations in clinical samples obtained from 78 patients with TAM upon request from referring hospitals. Acquired *GATA1* mutations were detected in a total of 72 (92.3%) patients among them. Of the 72 patients, 6 harbored multiple *GATA1* mutant clones and were excluded from this study because we could not determine a dominant clone in these patients. Those 6 have not progressed to ML-DS. For the remaining 66 patients (32 male and 34 female), the clinical characteristics and laboratory data at diagnosis are described in Table 1 and summarized in Table 3. Early death within the first 6 months of life occurred in 16 patients (24.2%). The covariates correlated with early death were as follows: EGA, low birth weight, high WBC count at diagnosis, high percentage of peripheral blast cells, complication of effusions, and bleeding diatheses. These prognostic factors were identified in previous studies.<sup>5-7</sup> Eleven (16.7%) cases subsequently developed ML-DS. The median age at diagnosis of ML-DS was 396 days (range 221-747 days). Univariate analysis revealed no covariates correlated with progression to ML-DS except the low total bilirubin level at diagnosis ( $P = .023$ ).

### *GATA1* mutations affect expression levels of *GATA1*s protein

We first asked whether the spectrum of transcripts derived from the mutant *GATA1* genes affected the expression levels of the translation products. The transcripts coding *GATA1*s protein were categorized into 3 groups as follows: loss of the first methionine, splicing errors, and premature termination codon (PTC). Furthermore, the PTC group was divided into 2 subcategories by the location of introduced PTC. In this report, we refer to the mutation that causes PTC before the second methionine at codon 84 as PTC type 1, and after codon 84 as PTC type 2. We constructed cDNA expression vectors for each class of mutations observed in TAM patients, and transfected these constructs into BHK-21 cells (Figure 1). The details of the *GATA1* mutations are described in Table 2. Western blot analysis revealed that *GATA1*s proteins were most abundantly expressed in mutants with splicing errors. The transcripts from mutants that had lost the first methionine were also efficiently translated. In contrast, in the cells expressing PTC type 1 or type 2 constructs, *GATA1*s expression levels were uniformly low. Note that the translation efficiency of the PTC type 2 transcripts was lowest among them.

To test the possibility that mutations in *GATA1* have an effect on the quantity of the transcripts, we next prepared human *GATA1* minigene expression vectors, and assessed the expression levels. Consistent with the results using cDNA expression vectors, Western blot analysis showed that the expression levels of *GATA1*s were lower in cells expressing PTC type 2 mutations, whereas the expression levels of the proteins from PTC type 1 mutations were not uniformly low (Figure 2Ai). Northern blot analysis revealed that the lowest expression levels of *GATA1* mRNAs were observed

**Table 1. Clinical features and mutation characteristics in TAM patients with GATA1 mutations**

Patient No.	Sex	WBC, $\times 10^9/L$	Outcome	GATA1 mutation*	Consequence of mutation	Mutation type
113 <sup>24</sup>	F	63.9	CR	207 C>G	Tyr69stop	PTC 1-3'
2 <sup>13</sup>	F	89.0	Early death	199 G>T	Glu67stop	PTC 1-3'
3 <sup>13</sup>	F	NA	NA	174 ins 19 bp CAGCCACCGCTGCAGCTGC	Frame shift at codon58, stop at codon 73	PTC 1-3'
4 <sup>13</sup>	F	128.8	CR	IVS1 to IVS2 del 1415 bp	Splice mutant	Splicing error
5 <sup>13</sup>	F	NA	NA	49 C>T	Gln17stop	PTC 1-5'
6 <sup>13</sup>	F	248.6	NA	Loss of 2nd exon	Splice mutant	Splicing error
7 <sup>13</sup>	F	31.2	CR	Loss of 2nd exon	Splice mutant	Splicing error
8 <sup>13</sup>	M	199.6	CR	-11 to +33 del 44 bp	No translation from Met1	Loss of 1st Met
9 <sup>13</sup>	M	44.9	Early death	45 ins C	Frame shift at codon15, stop at codon 39	PTC 1-5'
10 <sup>13</sup>	M	50.9	CR	37 G>T	Glu13stop	PTC 1-5'
11 <sup>13</sup>	F	103.0	Early death	90-91 del AG	Frame shift at codon 30, stop at codon 38	PTC 1-5'
12 <sup>13</sup>	F	14.6	Evolved to ML-DS	116 del A	Frame shift at codon 39, stop at codon 136	PTC 2
13 <sup>13</sup>	M	423.0	CR	185 ins 22 bp GCTGCAGCTGCGGCACTGGCCT	Frame shift at codon 62, stop at codon 74	PTC 1-3'
14 <sup>13</sup>	M	201.2	CR	189 C>A	Tyr63stop	PTC 1-3'
15 <sup>13</sup>	M	NA	NA	1 A>G	No translation from Met1	Loss of 1st Met
16 <sup>13</sup>	F	28.3	CR	189 C>A	Tyr63stop	PTC 1-3'
17 <sup>13</sup>	M	203.0	Evolved to ML-DS	38-39 del AG	Frame shift at codon 13, stop at codon 38	PTC 1-5'
18 <sup>13</sup>	M	31.3	CR	189 C>A	Tyr63stop	PTC 1-3'
19 <sup>13</sup>	M	NA	NA	90-91 del AG	Frame shift at codon 30, stop at codon 38	PTC 1-5'
20 <sup>13</sup>	F	114.0	Early death	187 ins T	Frame shift at codon 63, stop at codon 67	PTC 1-3'
21 <sup>25</sup>	F	26.0	Evolved to ML-DS	194 ins 20 bp GGCACTGGCCTACTACAGGG	Frame shift at codon 65, stop at codon 143	PTC 2
22 <sup>25</sup>	F	25.0	Evolved to ML-DS	194 ins 20 bp GGCACTGGCCTACTACAGGG	Frame shift at codon 65, stop at codon 143	PTC 2
23	F	49.9	CR	3 G>T	No translation from Met1	Loss of 1st Met
24	F	46.2	NA	IVS1 3' boundary AG>AA	Splice mutant	Splicing error
25	F	10.5	CR	194 ins 19 bp GCACTGGCCTACTACAGGG	Frame shift at codon 65, stop at codon 73	PTC 1-3'
26 <sup>24</sup>	F	244.0	Evolved to ML-DS	1 A>G	No translation from Met1	Loss of 1st Met
27	F	38.3	CR	Loss of 2nd Exon	Splice mutant	Splicing error
28 <sup>24</sup>	F	34.6	CR	IVS1 to exon2 del 148 bp	Splice mutant	Splicing error
29	M	25.9	Evolved to ML-DS	160 ins TC	Frame shift at codon 54, stop at codon 137	PTC 2
30	F	52.3	Evolved to ML-DS	187 ins CCTAC	Frame shift at codon 63, stop at codon 138	PTC 2
31 <sup>24</sup>	F	221.0	CR	183-193 del 11 bp CTACTACAGGG	Frame shift at codon 62, stop at codon 63	PTC 1-3'
32	M	149.7	CR	2 T>G	No translation from Met1	Loss of 1st Met
33 <sup>24</sup>	M	132.3	Evolved to ML-DS	101-108 del 8 bp TCCCCTCT	Frame shift at codon 34, stop at codon 36	PTC 1-5'
34 <sup>24</sup>	F	220.0	Early death	90-91 del AG	Frame shift at codon 30, stop at codon 38	PTC 1-5'
35 <sup>24</sup>	M	166.0	Early death	IVS2 5' boundary GT>CT	Splice mutant	Splicing error
36 <sup>24</sup>	M	57.6	Early death	193-199 GACGCTG>TAGTAGT	Asp65stop	PTC 1-3'
37 <sup>24</sup>	M	247.6	Early death	Exon2 to IVS2 del 218 bp	Splice mutant	Splicing error
38 <sup>24</sup>	M	93.3	Early death	IVS1 3' boundary AG>AA	Splice mutant	Splicing error
39 <sup>24</sup>	M	290.8	Early death	186 ins 12 bp GGCACTGGCCTA	Tyr62stop	PTC 1-3'
40	F	7.8	CR	2 T>C	No translation from Met1	Loss of 1st Met
41 <sup>24</sup>	M	136.6	Early death	IVS2 5' boundary GT>GC	Splice mutant	Splicing error
42	M	33.1	Early death	187 ins 8 bp TGGCCTAC	Frame shift at codon 63, stop at codon 139	PTC 2
43	M	9.0	CR	22 ins G	Frame shift at codon 8, stop at codon 39	PTC 1-5'
44	M	24.1	Evolved to ML-DS	149 ins 20 bp AGCAGCTTCTCCACTGCC	Frame shift at codon 50, stop at codon 143	PTC 2
45 <sup>24</sup>	F	53.3	CR	173 C>TGCTGCAGTGTAGTA	Frame shift at codon 58, stop at codon 141	PTC 2
46	F	119.0	CR	1 A>C	No translation from Met1	Loss of 1st Met
47	M	33.0	CR	189 C>A	Tyr63stop	PTC 1-3'
48	M	178.2	Early death	188 ins 22 bp GCAGCTGCGGCACTGGCCTACT	Frame shift at codon 63, stop at codon 74	PTC 1-3'
49	F	73.6	CR	3 G>A	No translation from Met1	Loss of 1st Met
50	F	12.9	CR	158 ins 7 bp AGCACAG	Frame shift at codon 53, stop at codon 69	PTC 1-5'
51	M	13.0	CR	154-161 del 8 bp ACAGCCAC	Frame shift at codon 52, stop at codon 64	PTC 1-5'
52	M	105.5	Early death	4 G>T	Glu2stop	PTC 1-5'
53	F	98.3	CR	4 G>T	Glu2stop	PTC 1-5'
54	F	356.9	CR	219 A>C	Splice mutant	Splicing error
55	F	25.8	Evolved to ML-DS	157 ins CA	Frame shift at codon 53, stop at codon 137	PTC 2
56	M	97.4	Evolved to ML-DS	185-188 del 4 bp ACTA	Frame shift at codon 62, stop at codon 135	PTC 2
57	F	97.3	Early death	3 G>A	No translation from Met1	Loss of 1st Met
58	M	NA	CR	3 G>A	No translation from Met1	Loss of 1st Met
59	M	20.2	CR	150 ins 5 bp TGGCT	Frame shift at codon 50, stop at codon 52	PTC 1-5'
60	M	133.4	CR	174 ins 19 bp CAAAGCAGCTGCAGCGGTG	Frame shift at codon 58, stop at codon 73	PTC 1-3'
61	M	NA	CR	220 G>T	Splice mutant	Splicing error
62	M	120.2	CR	220 G>A	Splice mutant	Splicing error
63	F	39.0	CR	97-139 del 43 bp	Frame shift at codon 33, stop at codon 122	PTC 2
64	F	NA	NA	156 ins C	Frame shift at codon 52, stop at codon 67	PTC 1-5'
65	F	32.4	CR	174 ins 7 bp CTGCAGC	Frame shift at codon 58, stop at codon 69	PTC 1-3'
66	M	69.4	Early death	174-177 GGCA>TGCGGTGG	Frame shift at codon 58, stop at codon 68	PTC 1-3'

We previously reported the GATA1 mutations of the indicated patients.

F indicates female; M, male; CR, complete remission; NA, not available; and IVS, intervening sequence.

\*For cDNA nucleotide numbering, nucleotide number 1 corresponds to the A of the ATG translation initiation codon in the reference sequence.