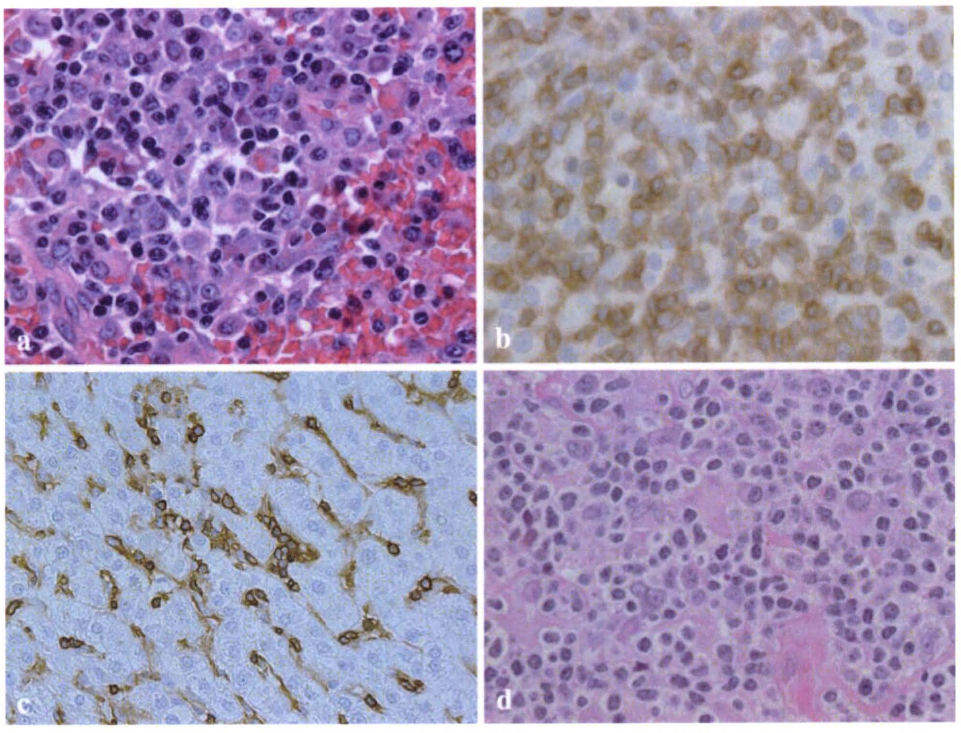

Fig. 2 Histologic pictures of the spleen, the liver, and a lymph node. **a** H&E staining of the resected spleen. Lymphoma cells with irregular nuclei and slightly dispersed chromatin are seen in the red pulp; ×400. **b** Lymphoma cells in the spleen are positive for CD3; ×400. **c** CD3 staining of a biopsy specimen of the liver. Lymphoma cells infiltrate the sinusoids; ×200. **d** H&E staining of a biopsied lymph node. The normal architecture is effaced by the diffuse infiltration of lymphoma cells; ×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 TCRγ chain. A southern blot analysis of DNA extracted from the spleen demonstrated gene rearrangements for TCR constant β chain, Jβ1 chain, and Jβ2 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, ifosfamide, 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^2), cytarabine (8 g/m^2), 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 $CD2^{+}$, $CD3^{+}$, $CD4^{-}$, $CD5^{-/+}$, $CD7^{+/-}$, $CD8^{+/-}$, $CD16^{-/+}$, $CD56^{+/-}$, and $CD57^{-/+}$. 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 γ-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.

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

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Brief report

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

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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 relapse; 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 mismatched 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.[1-3] However, there are few reports concerning HLA-haplotype loss in leukemia.[4,5]

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.

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.

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.[6] 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.[7]

Isolation of DNA and single nucleotide polymorphism analysis

The $CD13^+/CD34^+$ 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.[8]

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.[9]

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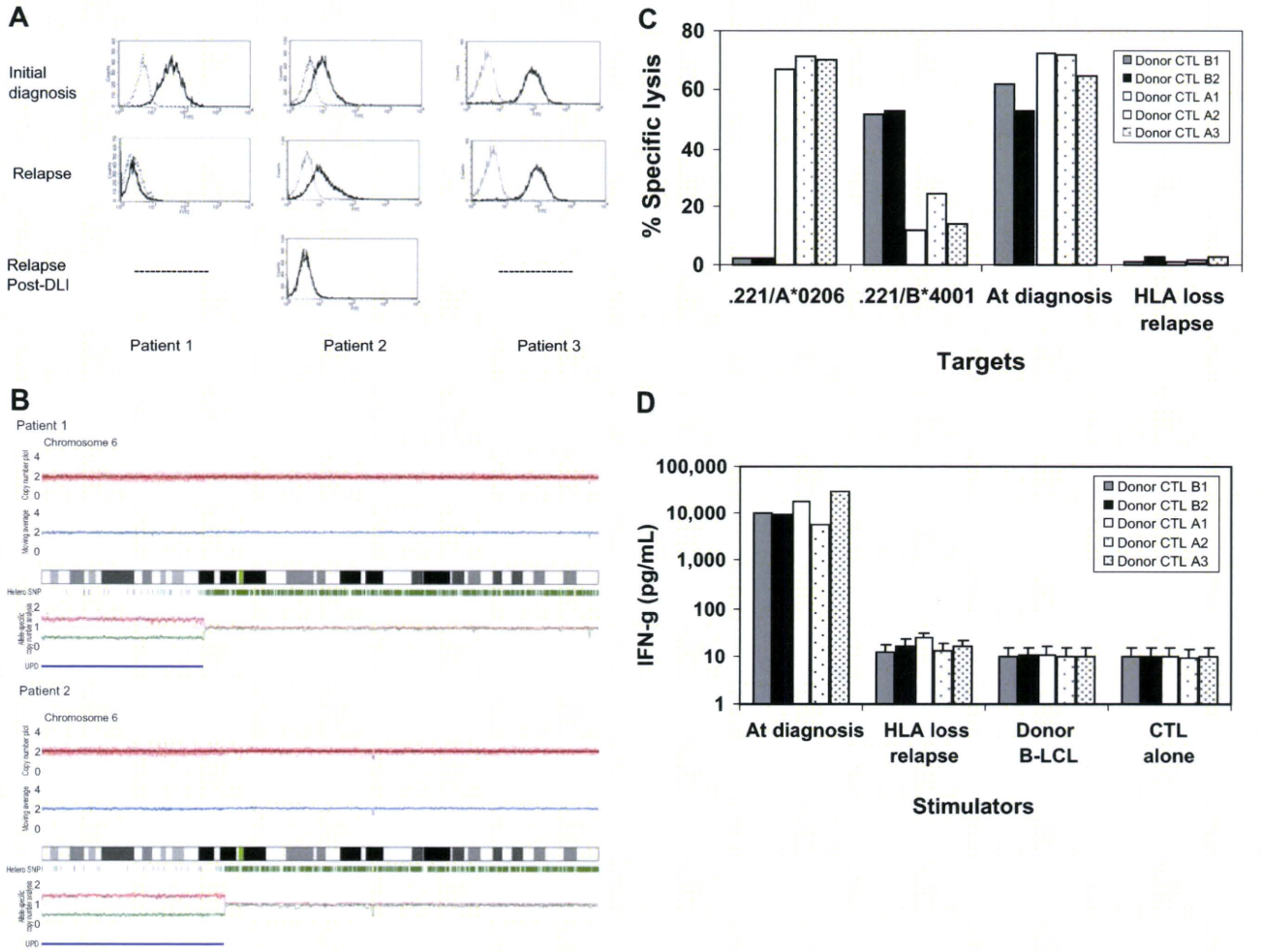

Figure 1. The loss of mismatched HLA expression on leukemic blasts caused by uniparental disomy on chromosome 6p impaired recognition and killing of donor's alloreactive cytotoxic T lymphocytes. (A) Leukemic blasts at the time of initial diagnosis and at the time of relapse after hematopoietic stem cell transplantation (HSCT) and donor lymphocyte infusion (DLI) were gated by $CD34^+$ and $CD13^+$, 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 $CD8^+$ 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 ^{51}Cr-release assay at the effector/target ratio of 30:1. (D) Their interferon-γ 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.[10,11]

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.[12]

The cytotoxicity of CTL clones against target cells was analyzed by conventional chromium 51 (^{51}Cr) release assay as previously reported.[13]

CTL clones (10^4 cells/well) were mixed with the indicated stimulator cells (10^4 cells/well) in 96-well, round-bottom polypropylene plates and spun at 1200*g* for 3 minutes before overnight incubation in 200 μL of RPMI 1640 medium supplemented with 10% fetal bovine serum. On the next day, 50 μL of supernatant was collected and interferon-γ 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 (10^7 $CD3^+$/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 $CD13^+/CD34^+$ 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-α or interferon-γ 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 $CD13^+/CD34^+$ 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.[14]

In patient 2, we examined whether the number of CTLp had changed during the posttransplantation course. Limiting dilution analysis with a split-well ^{51}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 $CD8^+$ 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.[15] 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.[16] Relapsed leukemic cells may possess genomic instability that elicits genetic diversity.[17] 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.[18,19] 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-matched donor (supplemental Table 2). Because UPD does not 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[16] 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.[20] However, DLI is effective even for the relapse of AML after haploidentical HSCT.[21] 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.

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

Samples	Maximum $CD8^+$ input*	No. of growing wells†	CTLp frequency^{-1} (95% confidence interval)
Donor	33 300	8	8.6×10^5 (1.49×10^6-5.0×10^5)
Day 100	35 500	0	UD
Day 180	17 700	0	UD
Day 300‡	86 000	0	UD
Day 520§	95 000	7	4.3×10^5 (7.2×10^5-2.5×10^5)

Purified $CD8^+$ 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 3×10^4 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 ^{51}Cr-radiolabeled 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 γ 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 $CD8^+$ 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 $CD8^+$ 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 $CD8^+$ T cells seeded at the highest number per well.

†Number of wells out of 12 wells that received the highest $CD8^+$ 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.

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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.

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

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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.

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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 ≤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

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(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 -DR 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 (≤10 years vs ≥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 (≤2 vs ≥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) (≤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 ≥2 antigen mismatches, unrelated CB donors with ≥2 antigen mismatches, and unrelated BM donors with ≥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 ≥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
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

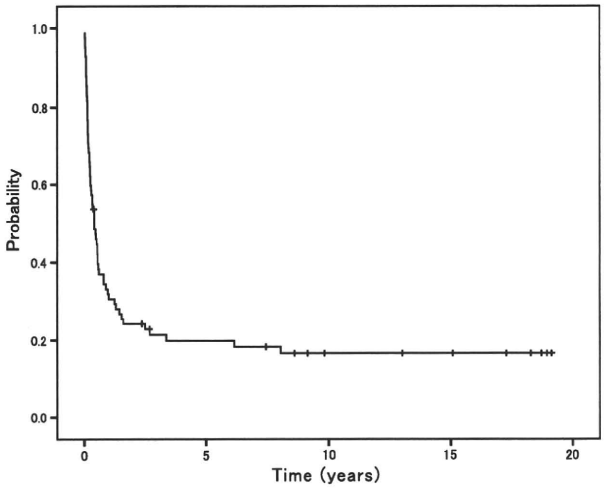

Figure 1. Kaplan-Meier curve for DFS.

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

			Multivariate		
Variable	5-Year DFS, %	Univariate *P* Value	HR	95% CI	*P* Value
All recipients					
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				
BM/PB recipients					
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.

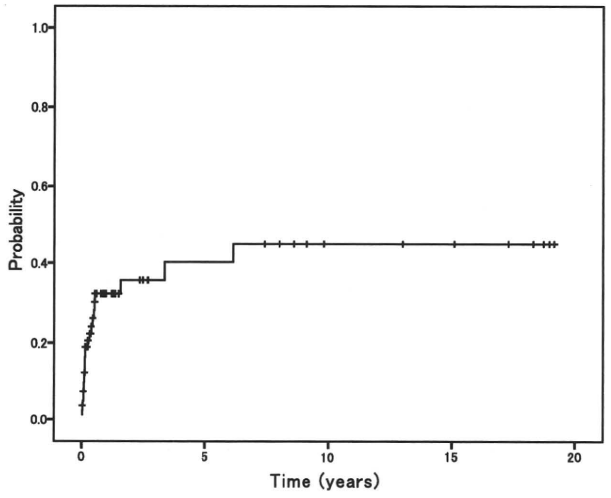

Figure 2. Time to NRM for 82 patients with refractory or relapsed acute leukemia.

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 ≥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

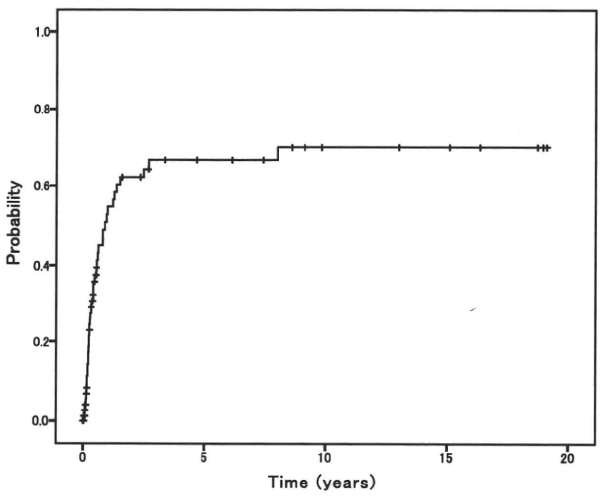

Figure 3. Time to relapse for 82 patients with refractory or relapsed acute leukemia.

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 cytoreduction 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 significantly 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
All recipients					
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				
BM/PB recipients					
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 actual significance of each additional abnormality should be investigated in a larger number of patients.

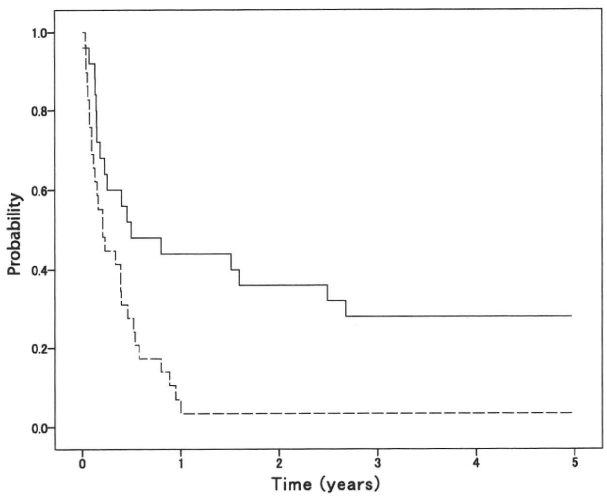

Figure 4. Effect of previous therapy and the presence of PB blasts pretransplantation on DFS at 5 years. Outcomes for 54 patients who received ≥3 courses of therapy before transplantation are shown. DFS at 5 years was 28.0% (range, 19.0%-37.0%) in 25 patients without circulating PB blasts (solid line)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

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.

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

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

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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 analyses 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.[1-4]

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.[5-7] 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,[8-14] which is essential for normal development of erythroid and megakaryocytic cells.[15-18] 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.[19,20] Mice harboring a heterozygous *GATA1* knockdown allele frequently develop erythroblastic leukemia.[21] 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.[22] 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.

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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.[13] 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.[23]

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.[13] 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 χ^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.[5-7] 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 GATA1s 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 GATA1s 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 GATA1s 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, GATA1s 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 GATA1s 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
1[13,24]	F	63.9	CR	207 C>G	Tyr69stop	PTC 1-3′
2[13]	F	89.0	Early death	199 G>T	Glu67stop	PTC 1-3′
3[13]	F	NA	NA	174 ins 19 bp CAGCCACCGCTGCAGCTGC	Frame shift at codon58, stop at codon 73	PTC 1-3′
4[13]	F	128.8	CR	IVS1 to IVS2 del 1415 bp	Splice mutant	Splicing error
5[13]	F	NA	NA	49 C>T	Gln17stop	PTC 1-5′
6[13]	F	248.6	NA	Loss of 2nd exon	Splice mutant	Splicing error
7[13]	F	31.2	CR	Loss of 2nd exon	Splice mutant	Splicing error
8[13]	M	199.6	CR	−11 to +33 del 44 bp	No translation from Met1	Loss of 1st Met
9[13]	M	44.9	Early death	45 ins C	Frame shift at codon15, stop at codon 39	PTC 1-5′
10[13]	M	50.9	CR	37 G>T	Glu13stop	PTC 1-5′
11[13]	F	103.0	Early death	90-91 del AG	Frame shift at codon 30, stop at codon 38	PTC 1-5′
12[13]	F	14.6	Evolved to ML-DS	116 del A	Frame shift at codon 39, stop at codon 136	PTC 2
13[13]	M	423.0	CR	185 ins 22 bp GCTGCAGCTGCGGCACTGGCCT	Frame shift at codon 62, stop at codon 74	PTC 1-3′
14[13]	M	201.2	CR	189 C>A	Tyr63stop	PTC 1-3′
15[13]	M	NA	NA	1 A>G	No translation from Met1	Loss of 1st Met
16[13]	F	28.3	CR	189 C>A	Tyr63stop	PTC 1-3′
17[13]	M	203.0	Evolved to ML-DS	38-39 del AG	Frame shift at codon 13, stop at codon 38	PTC 1-5′
18[13]	M	31.3	CR	189 C>A	Tyr63stop	PTC 1-3′
19[13]	M	NA	NA	90-91 del AG	Frame shift at codon 30, stop at codon 38	PTC 1-5′
20[13]	F	114.0	Early death	187 ins T	Frame shift at codon 63, stop at codon 67	PTC 1-3′
21[25]	F	26.0	Evolved to ML-DS	194 ins 20 bp GGCACTGGCCTACTACAGGG	Frame shift at codon 65, stop at codon 143	PTC 2
22[25]	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[24]	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[24]	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[24]	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[24]	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[24]	F	220.0	Early death	90-91 del AG	Frame shift at codon 30, stop at codon 38	PTC 1-5′
35[24]	M	166.0	Early death	IVS2 5′ boundary GT>CT	Splice mutant	Splicing error
36[24]	M	57.6	Early death	193-199 GACGCTG>TAGTAGT	Asp65stop	PTC 1-3′
37[24]	M	247.6	Early death	Exon2 to IVS2 del 218 bp	Splice mutant	Splicing error
38[24]	M	93.3	Early death	IVS1 3′ boundary AG>AA	Splice mutant	Splicing error
39[24]	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[24]	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 AGCAGCTTCCTCCACTGCCC	Frame shift at codon 50, stop at codon 143	PTC 2
45[24]	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.