Table I. Patient profile by transplant type.

	Matched related BMT-PBSCT unrelated BMT	Mismatched related BMT-PBSCT	Unrelated BMT	Unrelated CBSCT
Patients number	11	10	21	15
Age at transplantation (median)	4·3*	5·7*	1·6*	1·1*
≥5 years	4	4	5	0
Body weight at transplantation (median)	12·4*	13·9*	10·0*	8·2*
Conditioning regimen				
BU + CY	6	1	4	2
BU + CY + ATG	2	1	11	6
Radiation-containing	1	3	6	4
Others	2	5	0	3
GVHD prophylaxis				
CsA + MTX	7	3	11	7
CsA + PSL	0	2	0	3
CsA	2	2	0	3
MTX	2	0	0	1
FK506 + MTX	0	1	7	0
FK506 + PSL	0	1	1	1
FK506	0	1	0	0
Others	0	0	2	0
Acute GVHD(≥gradeII)	3	4	7	7
Chronic GVHD	4	6	10	5
Rejection	3	2	2	2
Dead	2	5	4	3

BU, busulfan; CY, cyclophosphamide; ATG, antithymocyte globulin; GVHD, graft-*versus*-host disease; CsA, ciclosporin A; MTX, methotrexate; PSL, prednisolone; FK506, tacrolimus; CBSCT, cord blood stem cell transplantation; BMT, bone marrow transplantation; PBSCT, peripheral blood stem cell transplantation.
*$P < 0.05$.

Univariate analyses of OS and FFS grouped by patient characteristics and transplant variables are shown in Table II. Statistically significant univariate associations were noted for poorer OS with HLA-mismatched related donors and children aged more than 5 years at the time of transplantation, and for poorer OS and FFS with use of a conditioning regimen other than BU-CY and BU-CY-ATG. The overall 5-year survival rates for patients who received BM and CB from an unrelated donor were 80·0 ± 9·0% and 80·0 ± 10·3% respectively. Using multivariate analysis, a conditioning regimen other than BU-CY and BU-CY-ATG was found to be the only factor examined that was associated with transplantation failure (Table III).

Discussion

Our review of the outcomes of haematopoietic SCT for treatment of WAS in Japan since 1985 showed that in 57 WAS patients who underwent SCT, the overall 5-year survival rate

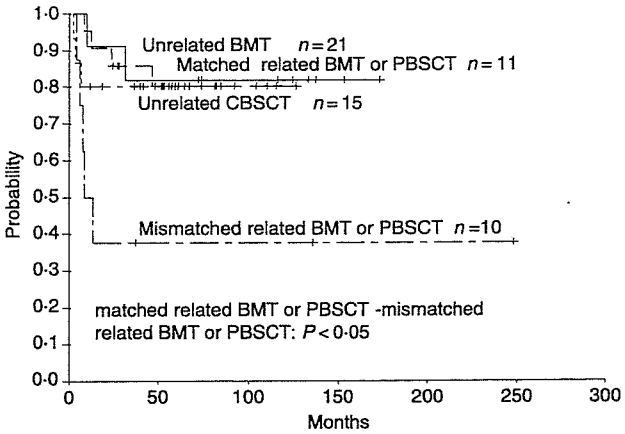

Fig 1. Overall survival (OS) rate of Wiskott–Aldrich syndrome (WAS) patients after transplantation. Kaplan–Meier estimates of OS for patients with WAS who received a transplant from a related matched donor (matched related), a related mismatched donor (mismatched related), an unrelated bone marrow donor (unrelated BMT), and an unrelated cord blood donor (unrelated CBSCT).

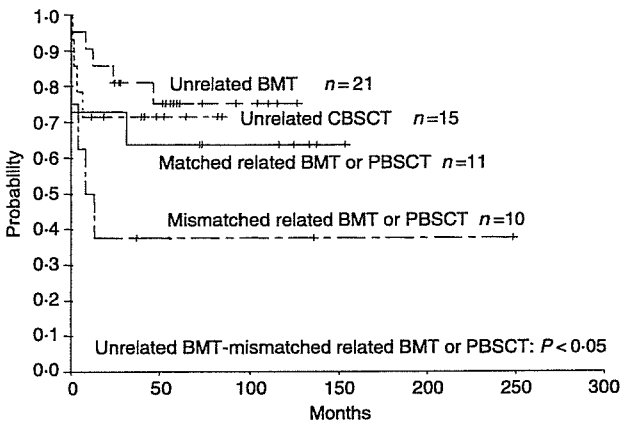

Fig 2. Failure-free survival (FFS) rate of Wiskott–Aldrich syndrome (WAS) patients after transplantation. Kaplan–Meier estimates of FFS for patients with WAS who received a transplant from a related matched donor (matched related), a related mismatched donor (mismatched related), an unrelated bone marrow donor (unrelated BMT), and an unrelated cord blood donor (unrelated CBSCT).

for those with an HLA-matched related donor was 81·8%, and the 5-year FFS rate was 64·3%. Surprisingly, the overall survival of patients who underwent transplantation from an HLA-matched unrelated BM or CB donor was 80·0%, and therefore did not differ from that of patients who received a transplant from a related HLA-matched donor. This result is quite different from past reports, in which transplant from an unrelated donor has been associated with a poor outcome (Fischer *et al*, 1994). The availability of a detailed HLA-matching system for unrelated donors for BMT may be a reason for the improved outcome in transplantation from HLA-matched unrelated donors. In CB transplantation, graft rejection is a common complication in many patients with

Table II. Survival and failure-free survival 5 years after stem cell transplantation for Wiskott–Aldrich syndrome (univariate analysis).

	n	OS at 5 years (%)	FFS at 5 years (%)
All patients	57	73·7 ± 6·1	65·7 ± 6·6
Age			
<5 years	41	79·8 ± 6·4*	72·0 ± 7·3
≥5 years	13	53·8 ± 13·8*	46·2 ± 13·8
Source and donor			
Related matched BMT, PBSCT	11	81·8 ± 11·6*	64·3 ± 14·5
Related mismatched BMT, PBSCT	10	37·5 ± 17·1*†	37·5 ± 17·1*
Unrelated BMT	21	80·0 ± 9·0†	75·2 ± 9·7*
Unrelated CBSCT	15	80·0 ± 10·3	71·4 ± 12·1
Conditioning regimen			
BU-CY and BU-CY-ATG	32	86·9 ± 6·1*	83·8 ± 6·7*
Other	23	55·8 ± 10·5*	40·4 ± 10·6*
Year of transplantation			
Till 1999	30	73·3 ± 8·1	60·0 ± 8·9
2000 and after	25	74·1 ± 9·2	72·9 ± 9·6

BU, busulfan; CY, cyclophosphamide; ATG, antithymocyte globulin; OS, overall survival; FFS, failure-free survival; CBSCT, cord blood stem cell transplantation; BMT, bone marrow transplantation; PBSCT, peripheral blood stem cell transplantation.
*†$P < 0·05$.

Table III. Multivariate analysis of failure-free survival after stem cell transplantation for Wiskott–Aldrich syndrome.

Factor	RR of failure	*P*	CI
Conditioning (except BU-CY and BU-CY-ATG)	7·455	0·003	1·996–27·834
Age (≥5 years)	2·410	0·243	0·550–10·559
Donor (mismatched related donor)	1·774	0·516	0·315–9·994

non-malignant diseases, but the incidence of graft rejection in our subjects was lower than that for other transplant patients; this result suggests that CBSCT may be preferable in immunodeficiency disorders. On the other hand, patients receiving transplants from HLA-mismatched related donors had poorer OS, consistent with data in past reports. Our results suggest that WAS patients without an HLA-matched sibling should receive BM or CB transplantation from an unrelated donor, but not from an HLA-mismatched related donor. Boys older than 5 years also showed shorter survival and, because WAS patients become increasingly susceptible to episodes of infection and haemorrhage with aging, immediate transplantation should be performed after WAS diagnosis. Therefore, prompt diagnosis of WAS is extremely important. In addition, a benefit of CBSCT is that the period from registration to the transplant is short, suggesting that CBSCT should be actively considered for WAS patients.

For patients receiving transplantation from an HLA-matched sibling, a conditioning regimen including BU (16 mg/kg) and CY (200 mg/kg) has been shown to give relatively high survival rates, whereas total body irradiation plus cyclophosphamide has been associated with poorer survival (Fischer *et al*, 1994). In our study, patients who underwent a conditioning regimen other than BU-CY and BU-CY-ATG had poorer OS and FFS, and use of a BU-CY regimen was favourable for WAS patients for transplantation from both an HLA-matched sibling and from an unrelated donor. CY (200 mg/kg) was used in all 13 patients who received a BU-CY conditioning regimen, whereas a CY dose of 120 mg/kg was used in five of the 20 patients who received BU-CY-ATG. As all five of these patients are alive and did not have graft rejection, reduction of drug doses in conditioning may be possible. Recently, non-myeloablative transplantation for WAS patients has been reported (Longhurst *et al*, 2002), and several patients in our study received transplantation using a fludarabine-containing regimen. However, because this was used as conditioning in the second transplantation for most patients, we were unable to perform an accurate evaluation of the fludarabine-containing regimen. As it is important to evade conditioning-related sequelae, evaluation of the efficacy of reduced-intensity transplantation in WAS patients through the accumulation of cases will be of importance.

Unexpectedly, six out of seven patients who underwent second transplantation are alive. Moreover, all three patients receiving transplantation from a HLA-mismatched related donor are alive with complete donor chimaerism. These findings might justify the use of reduced-intensity conditioning regimens for the initial transplantation of patients with WAS, especially as the prognosis of the second transplantation of these patients was better than expected.

In conclusion, we recommend that patients diagnosed with WAS receive SCT as soon as possible after diagnosis. As donor

sources, unrelated but HLA-matched BM or CB donors are appropriate for transplantation, with a low associated risk similar to that with a related HLA-matched donor.

Acknowledgement

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Allogeneic Bone Marrow Transplantation from Unrelated Human T-Cell Leukemia Virus-I–negative Donors for Adult T-Cell Leukemia/Lymphoma: Retrospective Analysis of Data from the Japan Marrow Donor Program

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ABSTRACT

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) from an HLA-matched related donor has been suggested to improve the poor prognosis of adult T-cell leukemia/lymphoma (ATLL). However, the infusion of HTLV-I–infected cells from HTLV-I–positive related donors could lead to the development of donor-derived ATLL under immunosuppressive conditions. Although most ATLL patients lack a suitable HLA-matched related donor and require an HTLV-I–negative unrelated donor, little information is currently available regarding the outcome of unrelated bone marrow transplantation (UBMT) for ATLL. To evaluate the role of UBMT in treating ATLL, we retrospectively analyzed data from 33 patients with ATLL treated by UBMT through the Japan Marrow Donor Program (JMDP). Overall survival (OS), progression-free survival, and cumulative incidence of disease progression and progression-free mortality at 1 year after UBMT were 49.5%, 49.2%, 18.6%, and 32.3%, respectively. Multivariate analysis identified recipient age as an independent prognostic factor for OS (P = .044). Patients age ≥50 years who showed nonremission at transplantation tended to have higher rates of treatment-related mortality. Our observations suggest that UBMT could represent a feasible treatment option for ATLL patients and warrant further investigation based on these risk factors.

KEY WORDS

Adult T-cell leukemia/lymphoma • Allogeneic hematopoietic stem cell transplantation • Unrelated donor • Graft-versus-adult T-cell leukemia/lymphoma

INTRODUCTION

Adult T-cell leukemia/lymphoma (ATLL) is a peripheral T-cell neoplasm caused by human T-cell leukemia virus type I (HTLV-I) [1,2]. ATLL is generally classified into 4 clinical subtypes based on clinical and laboratory features: acute, chronic, smoldering, and lymphoma type. Clinically, acute- and lymphoma-type ATLL show an aggressive course, with tumor

burden, severe hypercalcemia, multiorgan failure, and poor performance status. ATLL has an extremely poor prognosis, with a median survival of about 6 months for the acute type and about 10 months for the lymphoma type; these patients are usually highly immunocompromised and develop various opportunistic infections. [3] Furthermore, their tumor cells are usually resistant to conventional chemotherapies, because overexpression of multidrug-resistance genes leads to intrinsic drug resistance. [4,5] Intensified chemotherapy [6,7] and autologous stem cell transplantation [8] likewise have failed to improve the prognosis. Thus, alternative treatment strategies for ATLL are needed.

Some cases of successful treatment with allogeneic stem cell transplantation (allo-HSCT) from an HLA-matched related donor have been reported, and a graft-versus-ATLL (GvATLL) effect has been implicated for improving treatments outcomes in transplant patients undergoing transplantation for ATLL. [9–11] However, more than 2/3 of patients with ATLL lack HLA-matched related donors. Furthermore, approximately 2/3 of the siblings of patients with ATLL are HTLV-I carriers [12], and allo-HSCT from an HTLV-I–positive donor may carry a risk of promoting the development of ATLL through the addition of a new HTLV-I load on the immunocompromised host. [13,14] Although most ATLL patients lack a suitable HLA-matched related donor and require an unrelated donor to benefit from allo-HSCT, few reports are available concerning the results of unrelated donor bone marrow transplantation (UBMT) for ATLL [9,11,15–18], and the number of patients in these few reports has been too small on which to base any solid conclusions. Therefore, to clarify the feasibility and efficacy of UBMT from an HTLV-I-negative donor for ATLL, we retrospectively analyzed registered data and clinical outcomes of UBMT for ATLL through the Japan Marrow Donor Program (JMDP).

PATIENTS AND METHODS

Patients and Transplantation Procedure

The subjects of this retrospective study consisted of 33 patients with ATLL (acute type, n = 20; lymphoma type, n = 7; not described, n = 6) who received UBMT from a donor mediated and recruited through the JMDP between September 1999 and January 2004. The clinical indications for UBMT were determined by each individual institution. The median time from diagnosis of ATLL to UBMT was 8 months (range, 5–28 months). At the time of transplantation, 13 patients were in complete remission (CR), 2 patients were in partial remission (PR), and 14 patients were in nonremission (NR); disease status at the time of transplantation was not described in 4 patients. CR status was reported in detail for 13 patients, with 11 patients in first CR (CR1) and 2 patients in second CR (CR2) (Table 1). All unrelated donors were HTLV-I antibody–negative. Serologic typing for HLA-A, -B, and -DR was performed using a standard 2-stage complement-dependent test of microcytotoxicity. [19] Alleles at the HLA-A, -B, and -DRB1 loci were identified by high-resolution DNA typing as described previously. [20] Serologic typing revealed that 22 patients were matched at the HLA-A, -B, and -DR loci. Four patients were mismatched at 1 HLA-DR locus, and 1 patient was mismatched at 2 loci of HLA-A and -DR. DNA typing revealed that 13 patients were matched at HLA-A, -B and -DRB1 loci. Ten patients were mismatched at 1 locus; 9 patients were mismatched at the HLA-DRB1 locus, and the remaining patient was mismatched at 1 HLA-A locus. Another 4 patients were mismatched at 2 loci. HLA typing data were not described in 6 patients. Patient and donor characteristics are summarized in Table 2.

Table 1. *Patient characteristics*

Characteristic	Value
Median age at transplantation, years (range)	49 (range, 24–59)
Sex, n	
Male	18
Female	15
Performance status, n	
0–1	21
2–4	4
ND	8
Subtypes of ATLL, n	
Acute	20
Lymphoma	7
ND	6
Disease status at transplantation, n	
CR or PR	15
NR	14
ND	4
Duration from diagnosis to UBMT, n	
Within 1 year	21
Beyond 1 year	11
ND	1
Conditioning, n	(TBI-containing, 22; non–TBI-containing, 11)
CST	27
RIST	6
Cell dose, n	
$< 3.0 \times 10^8$/kg	16
$\geq 3.0 \times 10^8$/kg	14
ND	3
GVHD prophylaxis, n	
CsA + MTX	13
TCR + MTX	20

ND indicates not described; CR, complete remission; PR, partial remission; NR, nonremission; UBMT, unrelated bone marrow transplantation; TBI, total body irradiation; CST, conventional stem cell transplantation; RIST, reduced-intensity stem cell transplantation; GVHD, graft-versus-host disease; CsA, cyclosporine; MTX, methotrexate; TCR, tacrolimus.

Table 2. *Patient and donor characteristics*

Characteristic	Value
HLA-A, -B, and -DRB1 allele mismatches, n	
0	13
1	10
2	4
ND	6
Sex of donor/patient, n	
Male/male	13
Female/female	8
Female/male	5
Male/female	7
Extent of ABO match, n	
Match	19
Minor mismatch	4
Major mismatch	7
Major/minor	2
ND	1

ND indicates not described.

Transplantation was performed according to the protocol of each institution; therefore, conditioning regimens and prophylaxis against graft-versus-host disease (GVHD) differed among patients. Conditioning regimens were myeloablative in 27 patients; total body irradiation (TBI) was incorporated in 22 patients. Reduced-intensity conditioning regimens were used in 6 patients. GVHD prophylaxis included cyclosporine (n = 13) and tacrolimus (n = 20) combined with methotrexate. All recipients received bone marrow transplantation, which was not manipulated.

Assessment of Engraftment, GVHD, Survival, and Progression-Free Mortality

The day of sustained engraftment was defined as the first of 3 consecutive days with an absolute neutrophil count exceeding 0.5×10^9/L. Acute GVHD was diagnosed and graded according to the standard criteria described previously. [21,22] Chronic GVHD was evaluated according to standard criteria [23] in patients who survived more than 100 days after transplantation. Overall survival (OS) was defined as the duration (in days) from transplantation to death from any cause. Progression-free survival (PFS) was defined as days from transplantation to disease progression or death from any cause. Progression-free mortality was defined as death without disease progression.

Data Management and Statistical Considerations

Data were collected by the JMDP using a standardized report form. Follow-up reports were submitted at 100 days, 1 year, and every subsequent year after transplantation. The cumulative incidence of disease progression and progression-free mortality were evaluated using Gray's method, [24] considering each other risk as a competing risk. OS and PFS were estimated using the Kaplan-Meier method. Potential confounding factors considered in the analysis were age, sex, disease status, duration from diagnosis to transplantation, Eastern Cooperative Oncology Group (ECOG) performance status, [25] conditioning regimen, number of bone marrow cells transplanted, and presence of grade II–IV acute GVHD. Proportional hazard modeling was used to evaluate any influence of these factors on OS, treating development of acute GVHD as a time-dependent covariate. Factors associated with at least borderline significance ($P < .05$) in univariate analyses were subjected to multivariate analyses using backward-stepwise proportional hazards modeling. P values $P < .10$ were considered statistically significant.

RESULTS

Engraftment and GVHD

Transplantation outcomes are summarized in Table 3. The median number of cells transplanted was 2.44×10^8 nucleated cells/kg of recipient body weight (range, $0.58–3.58 \times 10^8$ nucleated cells/kg of recipient body weight). Five patients (15%) died within 20 days. Neutrophil engraftment was achieved in 28 patients. Late graft failure occurred in 1 of these 28 patients, although the patient showed engraftment on

Table 3. *Transplantation outcome*

	Value
Alive/dead, n	19/14
Median follow-up for survivors, days (range)	139 (87–600)
Cause of death	
Progression, n	2
Death without progression, n	9
Median days after transplantation (range)	32 (10–71)
Late graft failure, n	1
GVHD, n	1
Infection, n	3
TMA, n	2
VOD, n	1
Arrhythmia, n	1
Not described, n	3
Disease progression, n	5
Median days after transplantation (range)	122 (61–223)
Engraftment, n	
Engraftment	28
Death within 20 days	5
Late graft failure	1
Acute GVHD, n	
None	3
Grade I	8
Grade II	12
Grade III	3
Grade IV	2
Chronic GVHD, n	
None	14
Limited	1
Extensive	3

GVHD indicates graft-versus-host disease; TMA, thrombotic microangiopathy; VOD, venooculusive disease.

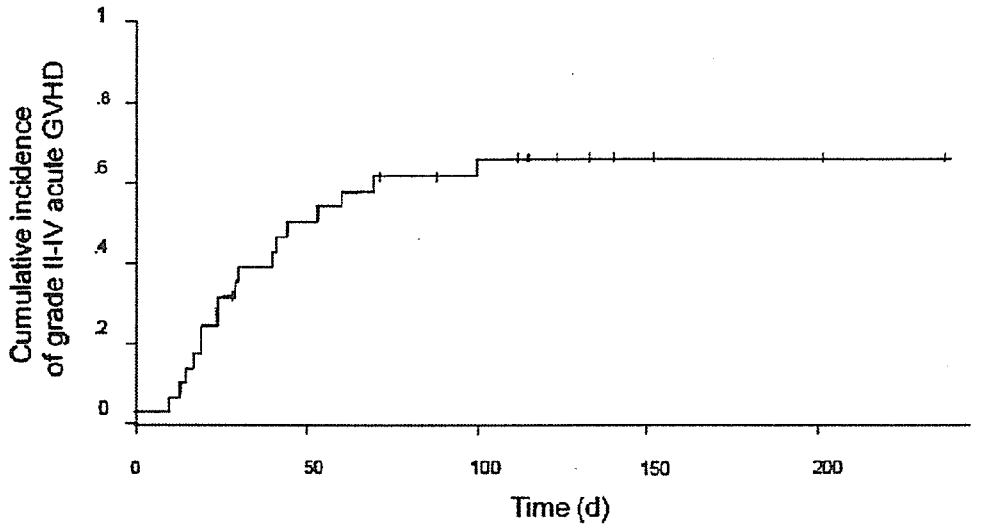

Figure 1. Cumulative incidence of grade II–IV acute GVHD in patients who achieved neutrophil engraftment.

day 14. Acute GVHD developed in 25 of the 28 patients who achieved engraftment (89%): grade I GVHD in 8 patients, grade II in 12 patients, grade III in 3 patients, and grade IV in 2 patients. The cumulative incidence of grade II–IV acute GVHD was 61% (Figure 1). Chronic GVHD developed in 4 of 18 patients, with limited disease in 1 patient and extensive disease in the other 3 patients.

Survival and disease progression

The 1-year OS and PFS were 49.5% (95% confidence interval [CI], 31.2%–78.5%) and 49.2% (95% CI, 33.6%–72.1%), respectively (Figure 2). Disease progression was observed in 5 patients, and the median number of days from transplantation to disease progression was 122 (range, 61–223 days). As of the last follow-up, 14 deaths had been reported. Primary cause of death was disease progression in 2 patients and was not described in 3 patients, but the other 9 deaths were not due to disease progression (see Table 3). Primary causes of transplantation-related death within 100 days after transplantation were late graft failure in 1 patient, GVHD in 1 patient, infection in 3 patients (with methicillin-resistant *Staphylococcus aureus*–positive sepsis in 1 patient and pulmonary infection in 2 patients), thrombotic microangiopathy (TMA) in 2 patients, veno-occlusive disease (VOD) in 1 patient, and arrhythmia in 1 patient.

Univariate and Multivariate Analyses for OS

Pretransplantation and posttransplant factors were calculated for OS (Table 4). In univariate analyses, OS was not significantly associated with sex, duration from diagnosis to transplantation, ECOG performance status, conditioning regimen, number of bone marrow cells transplanted, or presence of grade II–IV acute GVHD. On the other hand, patient age and disease status at transplantation were identified as significant independent risk factors. In multivariate analyses, only patient age at transplantation was identified as exerting a significant independent risk impact on OS (≥50 years vs <50 years; relative risk, 3.47; 95% CI, 1.03–11.6; $P = .044$). Disease status at transplantation exerted a marginally significant impact on OS (NR vs CR or PR; relative risk, 3.17; 95% CI, 0.96–10.5; $P = .059$) (Figure 3).

Influence of Pretransplantation Factors on Disease Progression and Progression-Free Mortality

The cumulative incidence of disease progression and progression-free mortality at 1 year were 18.6% and 32.3%, respectively (Figure 4). To clarify how age and disease status at transplantation affected OS, we evaluated the relationship between these factors and the incidence of progression-free mortality. The cumulative incidence of progression-free mortality was significantly higher in patients age ≥50 years at transplantation (50% vs 18%; $P = .048$; Figure 5A). NR at transplantation exerted a marginally significant effect on increased progression-free mortality (54% vs 20%; $P = .070$; Figure 5B).

DISCUSSION

This study analyzed the data and evaluated treatment outcomes for 33 patients with ATLL who received UBMT. Two important findings were identified regarding UBMT for ATLL. First, UBMT from HTLV-I–negative donors for ATLL represents a feasible treatment. Second, recipient age (≥50 years) and NR disease status at transplantation were independent risk factors for OS, and patients with ATLL displaying these risk factors tended to exhibit higher frequencies of treatment-related mortality.

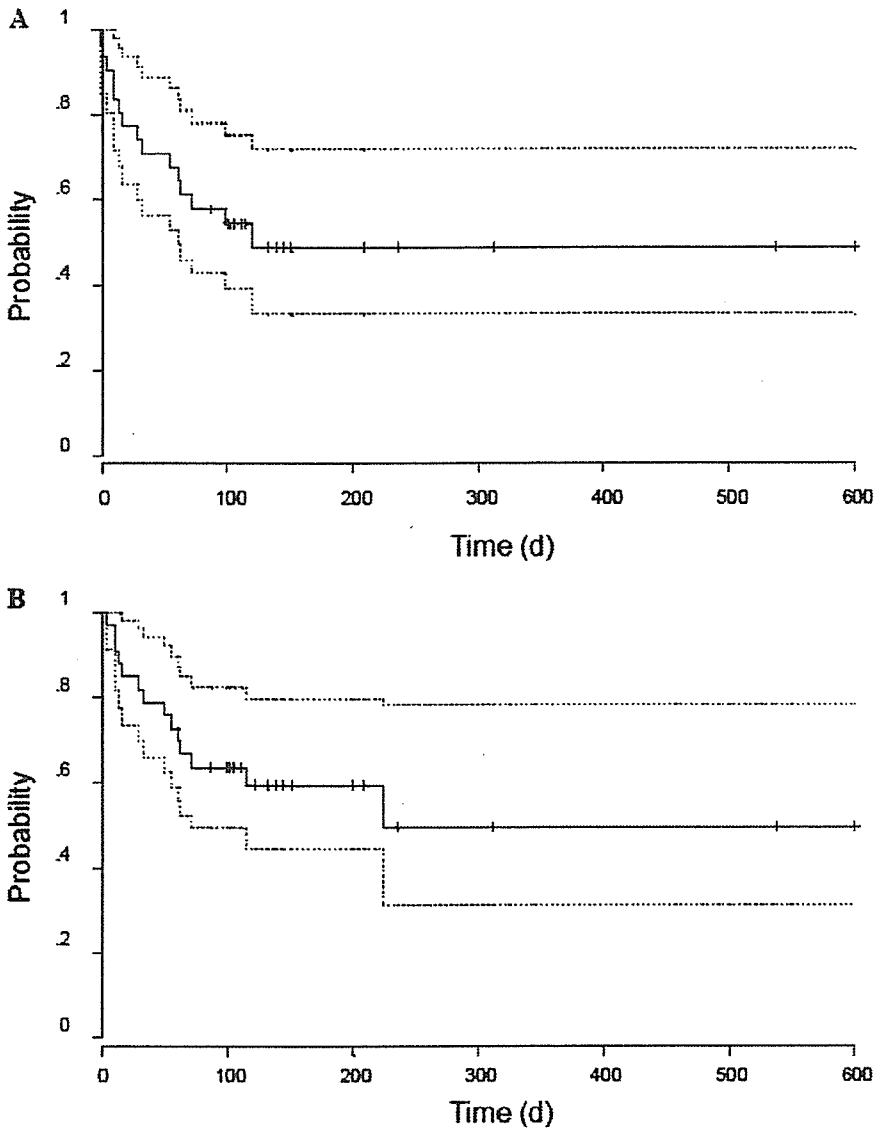

Figure 2. Probability of progression-free survival (A) and overall survival (B) after unrelated bone marrow transplantation for adult T-cell leukemia/lymphoma. Dashed lines represent 95% confidence intervals.

Table 4. *Prognosis factors in univariate and multivariate analyses*

	Univariate		Multivariate	
	Relative risk (95% CI)	*P*	Relative risk (95% CI)	*P*
Age ≥50 versus <50 years	4.03 (1.23–13.3)	.022	4.03 (1.23–13.3)	.022
Male versus female	0.97 (0.34–2.80)	.95		
PS 0–1 versus 2–4	0.44 (0.11–1.70)	.23		
NR versus CR or PR	3.37 (1.03–11.0)	.044		.059
UBMT within 1 year versus beyond 1 year	0.54 (0.15–2.00)	.35		
RIST versus CST	0.71 (0.19–2.59)	.60		
TBI versus non-TBI	1.35 (0.45–4.04)	.59		
Cell dose < 3.0 × 10^8/kg versus ≥ 3.0 × 10^8/kg	0.98 (0.31–3.05)	.97		
GVHD II–IV present versus absent	1.91 (0.50–7.26)	.34		

CI indicates confidence interval; PS, performance status; NR, nonremission; CR, complete remission; PR, partial remission; UBMT, unrelated bone marrow transplantation; RIST, reduced-intensity stem cell transplantation; CST, conventional stem cell transplantation; TBI, total body irradiation; GVHD, graft-versus-host disease.

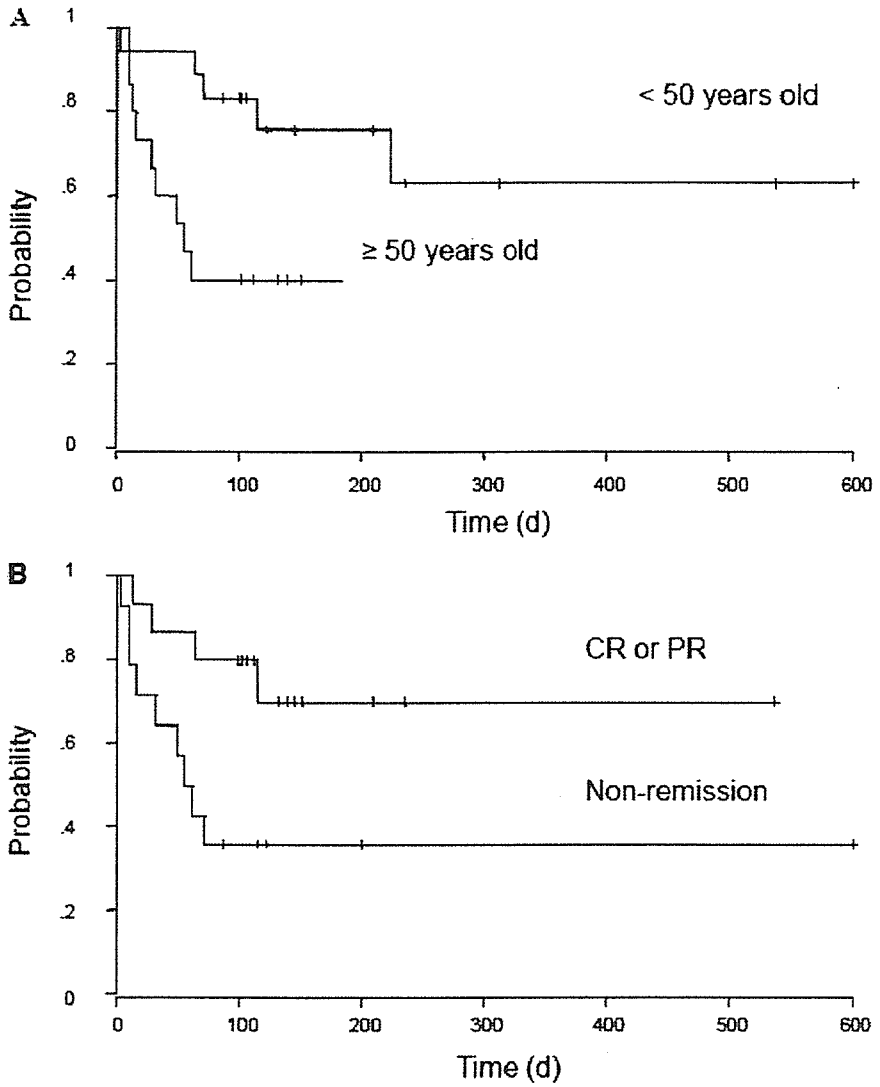

Figure 3. Overall survival according to pretransplantation factors, age (A) and disease status at transplantation (B).

ATLL has an extremely poor prognosis, with projected 2- and 4-year survival rates of 16.7% and 5.0% for the acute type and 21.3% and 5.7% for the lymphoma type, respectively. [3] Neither intensified chemotherapy nor autologous stem cell transplantation have improved the prognosis. Encouraging results for allo-HSCT for ATLL from HLA-matched related donors have been reported by several groups; thus, allo-HSCT may improve the poor prognosis of ATLL. However, the number of patients in most reports has been too small to allow evaluation of the efficacy of allo-HSCT for ATLL. The present results were derived from a large number of patients who underwent transplantation (33 patients) performed through the JMDP. Longer follow-up is, of course, needed to confirm the curative potential of allo-HSCT for ATLL. However, the good survival rates noted here suggest that allo-HSCT is an effective treatment for ATLL, and that patients with ATLL will benefit from allo-HSCT through HTLV-I–negative unrelated donors, because the OS and PFS rates at 1 year after UBMT were 49.5% and 49.2%, respectively. Compared with the results for patients with non-Hodgkin's lymphoma in the National Marrow Donor Program, the incidence of grade III–IV acute GVHD in the present study was low (18% vs 30%). [26] The outcome in the present study appears to be favorable, possible due to the lower incidence of grade III–IV acute GVHD. This observation is compatible with previous studies showing a lower incidence of acute GVHD in Japanese patients compared with Western patients, which might reflect the less diverse genetic background of in the Japanese population. [27,28]

Frequency of relapse after transplantation differs between autologous and allo-HSCT for ATLL. The use of high-dose chemotherapy with autologous HSCT has been reported in only 9 patients, all of whom relapsed or died from transplantation-related mortality. [8] In contrast, the cumulative incidence of

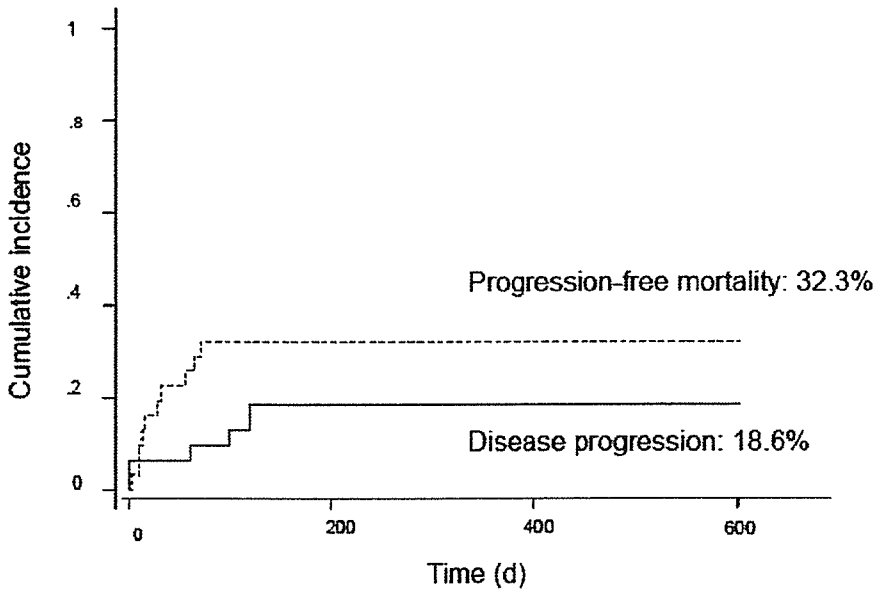

Figure 4. Cumulative incidence of disease progression (—) and progression-free mortality (---) after transplantation.

disease progression was lower after UBMT in this study. Interestingly, patients with ATLL displaying acute or chronic GVHD reportedly did not relapse. [9] In another report, patients with ATLL who relapsed after allo-HSCT reachieved CR after tapering or discontinuation of immunosuppressive agents and donor lymphocyte infusions. [10,11] Reactivation in tax-specific CD8-positive cytotoxic T lymphocytes (CTLs), which has been recently shown in patients with ATLL after allo-HSCT, may indicate a potential contribution of CTLs to anti-ATLL immunity and induction of a GvATLL effect. [29] These results strongly suggest that a GvATLL effect could work on some patients with ATLL to prevent relapse after allo-HSCT. In the present study, neither univariate nor multivariate analysis showed a survival benefit for acute GVHD. We were unable to analyze the relationship between chronic GVHD and relapse, because of the low number of patients with chronic GVHD. In fact, the number of patients may have been insufficient to confirm GvATLL in this study. On the other hand, the absence of benefit from GVHD in preventing relapse suggests that a GvATLL effect could occur in patients with ATLL after allo-HSCT without clinically obvious GVHD. [11]

Transplantation-related mortality was a significant problem in this study. Five patients (15%) died within 20 days, from infection in 3 patients and TMA in 2 patients. Nine patients (27%) died within 100 days, due to infection in 3 patients, TMA in 2 patients, and VOD in 1 patient. Patients with ATLL might have an increased risk of frequent opportunistic infection, because they have an associated T-cell immunodeficiency. Furthermore, ATLL is usually systemic in distribution, and the accumulated organ damages as a result of repeated cytotoxic chemotherapy seen in patients before transplantation may have contributed to the onset of TMA. In univariate and multivariate analysis, recipient age (≥50 years) and NR disease status at transplantation represented significant risk factors for OS. The multivariate analyses were limited by the small number of patients in each subgroup; however, patients displaying these risk factors tended to have a higher rate of treatment-related mortality than patients without these factors, and it can be assumed that these risk factors have a significant relationship with outcome clinically. In this study, mostly myeloablative conditioning regimens were used before transplantation. Given that conventional allo-HSCT is designed to eradicate tumor cells with myeloablative intensity using maximally tolerated doses of high-dose chemotherapy and radiotherapy, the desirable effects often may be offset by overwhelming toxicity in patients age ≥50 years. Moreover, the number of patients with ATLL who are eligible for allo-HSCT with myeloablative conditioning is limited, because the typical patient with ATLL has a relatively advanced age at presentation (about 60 years). To reduce treatment-related mortality, allo-HSCT with reduced-intensity conditioning offers a new treatment option for patients with ATLL who are ineligible for allo-HSCT with myeloablative conditioning due to advanced age or medical infirmity. [30,31] Okamura et al [32] reported on 16 patients age > 50 years with ATLL who underwent allo-HSCT with reduced-intensity conditioning from HLA-matched related donors and found that treatment-related mortality was acceptable and that allo-HSCT with reduced-intensity conditioning was a feasible treatment for ATLL. Given these findings, UBMT

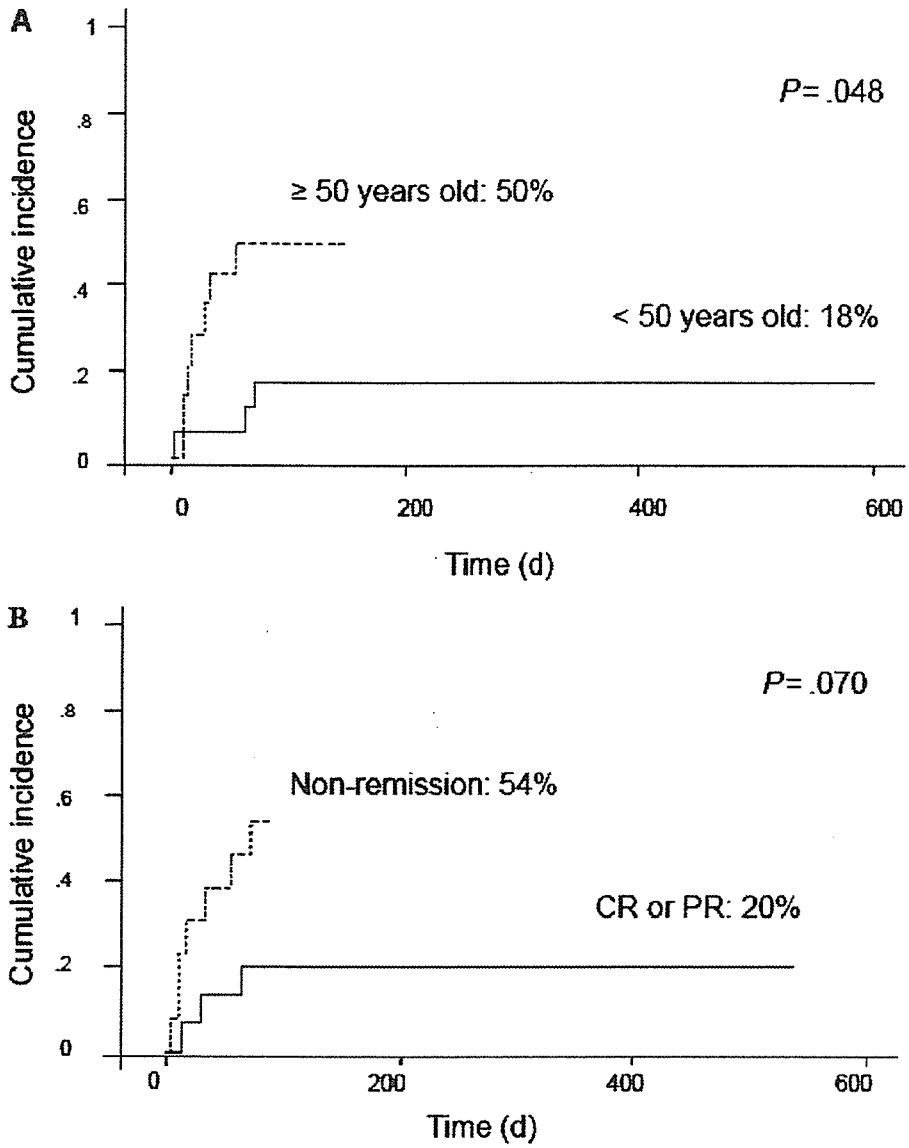

Figure 5. Cumulative incidence of progression-free mortality grouped according to pretransplantation factors, age (A) and disease status at transplantation (B).

with reduced-intensity conditioning should be considered for elderly patients with ATLL.

Another concern related to allo-HSCT for ATLL involves the use of HTLV-1–positive carrier donors. About 2/3 of siblings of patients with ATLL are HTLV-I carriers. From the perspective of HTLV-I–positive donor risk, granulocyte colony–stimulating factor (G-CSF) can reportedly stimulate the proliferation of ATLL cells [33], and HTLV-I–positive donors may be at increased risk of developing ATLL due to the administration of G-CSF in the setting of allogeneic peripheral blood stem cell transplantation. From the perspective of patients with ATLL, allo-HSCT from an HTLV-I–positive donor may carry a risk of HTLV-I–associated disease after allo-HSCT [34] or a risk of promoting the future development of ATLL due to the new HTLV-I load on immunocompromised recipients [13,14]. On the other hand, to date there is no evidence in the JMDP or the literature that ATLL can develop from infected HTLV-I–negative donor cells due to the HTLV-I load of the recipient. The HTLV-I proviral load dramatically decreased to an undetectable level after transplantation, especially after transplantation from HTLV-I–negative donors. [18, 32] This decreased HTLV-I proviral load was observed after both myeloablative and reduced-intensity conditioning. Transplantation from an HTLV-I–positive donor is reportedly associated with a higher frequency of relapse compared with transplantation from an HTLV-I–negative donor. [11] Therefore, the uninfected normal donor T cells might overwhelm infected HTLV-I recipient T cells due to a GvATLL response and might act as an antiviral therapy. However, an HTLV-I–positive do-

nor might avoid clonal expansion of HTLV-I–infected T lymphocytes after allo-HSCT through the provision of cytotoxic T cells. Thus, it is currently difficult to determine whether an HTLV-I–positive or–negative donor should be selected. Longer follow-up is needed to resolve this issue. In the meantime, a prudent clinical attitude toward both HTLV-I–positive donors and recipients with ATLL is warranted.

In conclusion, allo-HSCT from an HTLV-I–negative unrelated donor appears to be an feasible alternative treatment for patients with ATLL for whom an HLA-matched related donor is unavailable. Further prospective controlled studies are needed to assess the efficacy of allo-HSCT for ATLL and to define the clinical indications of allo-HSCT for ATLL, taking into account donor selection, the conditioning regimen, and the prognostic factors identified in this study.

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APPENDIX: PARTICIPATING INSTITUTIONS

The following centers in Japan participated in this study: Hokkaido University Hospital, Sapporo University Hospital, Sapporo Hokuyu Hospital, Japanese Red Cross Asahikawa Hospital, Asahikawa Medical College Hospital, Hirosaki University Hospital, Tohoku University Hospital, Yamagata University Hospital, Akita University Hospital, Fukushima Medical College, National Cancer Center Central Hospital, Institute of Medical Science at the University of Tokyo, Toho University Hospital, Omori Hospital, Tokyo Metropolitan Komagome Hospital, Nihon University Hospital, Itabashi Hospital, Jikei University Hospital, Keio University Hospital, Tokyo Medical College Hospital, Tokyo Medical and Dental University Hospital, Tokyo University Hospital, Yokohama City University Hospital, Kanagawa Children's Medical Center, Kanagawa Cancer Center, Tokai University Hospital, St Marianna University Hospital, Chiba University Hospital, Chiba Children's Hospital, Matsudo Municipal Hospital, Kameda General Hospital, Saitama Children's Medical Center, Saitama Cancer Center Hospital, Saitama Medical School Hospital, Ibaraki Children's Hospital, Jichi Medical School Hospital, Dokkyo University Hospital, Fukaya Red Cross Hospital, Saiseikai Maebashi Hospital, Gunma University Hospital, Niigata University Hospital, Niigata Cancer Center Hospital, Shinshu University Hospital, Saku Central Hospital, Hamamatsu University Hospital, Hamamatsu Medical Center, Shizuoka General Hospital, Shizuoka Children's Hospital, Japanese Red Cross Nagoya First Hospital, Nagoya Daini Red Cross Hospital, Meitetsu Hospital, Nagoya University Hospital, Nagoya Ekisaikai Hospital, National Nagoya Hospital, Aichi Medical School Hospital, Nagoya City University Hospital, Showa Hospital, Anjo Kousei Hospital, Fujita Health University Hospital, Mie University Hospital, Kanazawa University Hospital, Kanazawa Medical University Hospital, Toyama Prefectural Central Hospital, Fukui Medical School Hospital, Shiga University of Medical Science, Center for Adult Disease in Osaka, Kinki University Hospital, Osaka University Hospital, Osaka Medical Center and Research Institute for Maternal and Child Health, Matsushita Memorial Hospital, Hyogo College of Medicine Hospital, Hyogo Medical Center for Adults, Kobe City General Hospital, Kobe University Hospital, Kyoto University Hospital, Kyoto Prefectural University of Medicine Hospital, Social Insurance Kyoto Hospital, Tottori Prefectural Central Hospital, Tottori University Hospital, Hiroshima Red Cross Hospital and Atomic-Bomb Survivors Hospital, Yamaguchi University Hospital, Ehime Prefectural Central Hospital, Okayama National Hospital, Kurashiki Central Hospital, Kyushu University Hospital, Harasanshin General Hospital, Hamanomachi General Hospital, National Kyushu Cancer Center, St Mary's Hospital, Kokura Memorial Hospital, Saga Prefectural Hospital, Nagasaki University Hospital, Miyazaki Prefectural Hospital, Kumamoto National Hospital, Kumamoto University Hospital, Oita Medical University Hospital, Kagoshima University Hospital, and Imamura Bun-in Hospital.

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Mutations in p53 cDNA sequence introduced by retroviral vector

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Abstract

The high mutation rates of retroviruses are a potential problem with retroviral vectors. We studied the mutation rates and spectra of p53 sequences transduced with a retroviral vector in a cancer gene therapy model. When p53-deficient H358 non-small cell lung cancer cells were treated with a retroviral vector carrying normal p53 cDNA, most of transduced cells were killed by apoptosis. However, a small number of clones escaped p53-mediated apoptosis. We examined the p53 cDNA structure in these resistant clones. PCR-based analysis showed that 88/102 clones had detectable mutations in p53, including gross rearrangements, deletions/insertions, and base substitutions. To study the mutation rate of the p53 sequence in all transduced clones, the retroviral vector containing the non-functional p53 gene and the Neo-resistant marker gene was introduced into H358 cells. Only one of 95 isolated clones showed a base substitution. These results indicate that the mutation rate of p53 is not particularly high, but there is a significant risk that cancer cells will resist p53 gene therapy as a result of retroviral replication errors.

Keywords: Mutation; Retroviral vector; p53 gene; Cancer gene therapy

Recombinant retroviral vectors have been widely used in clinical gene therapy protocols. However, there remain unanswered safety concerns relating to the properties of the wild-type retrovirus. The recent occurrence of leukemia in X-linked severe combined immunodeficiency (X-SCID) patients who received retroviral vector-mediated gene therapy indicates that insertional oncogenesis caused by retroviral integration occurs with a much higher frequency than previously expected [1]. Consequently, the safety and utility of retroviral vectors must be comprehensively reevaluated for further development of gene therapy.

One potential problem of retroviral vectors is instability of the provirus sequence [2–4]. Retroviruses mutate at very high frequencies in order to meet the changing requirements of their environments [5–9]. For example, with human immunodeficiency virus type-1 (HIV-1), the genetic diversity of which has been extensively studied, each virus isolated from an individual consists of multiple genomic subclasses known as quasispecies [10,11]. The mutation rates of retroviruses are thought to be 10^{-6}–10^{-4} per base per cycle [8,9], although the actual numbers can be affected by differences in vector backbone, target sequence studied, and assay method used [9].

The relatively high mutation rate of retroviral vectors could be a problem in certain clinical protocols, including cancer gene therapy using tumor suppressor genes. Unwanted mutations of the tumor suppressor gene may render the therapy ineffective or even induce the generation of a new cancer. p53 is the most commonly used gene in the therapies aimed at treating a number of malignant diseases [12]. In the present study, in order to assess the effects of retroviral vector instability on p53-mediated cancer gene therapy, we examined the mutation frequencies and spectra of p53 sequences introduced into p53-deficient H358 non-small cell lung cancer (NSCLC) cells.

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Materials and methods

Construction of recombinant retroviral vectors. A 1.45-kb cDNA fragment containing wild-type p53 cDNA (GenBank Accession No. K03199) was isolated from plasmid pC53-SN3 [13] using *Eco*RI/*Sma*I and was then subcloned into pBluescriptIIKS+ (Stratagene, La Jolla, CA), yielding pB1-p53. A 1.08-kb TK-NeoR cDNA, which contained TK promoter (TK-P) and neomycin-resistant gene (NeoR), was obtained from plasmid pMC1Neo (Stratagene) using *Xhe*I/*Bam*HI and was then ligated into plasmid pG1 (Genetic Therapy, Gaithersburg, MD), yielding pG1/TKNeo. The 1.6-kb p53 cDNA was isolated from pB1-p53 using *Hin*dII/*Sma*I, ligated with *Not*I linker, and was then subcloned into the *Not*I site of the retroviral vector pG1/TKNeo, in which the NeoR was driven by the TK promoter, and expression of p53 was driven by the long terminal repeat (LTR) promoter of the Moloney leukemia virus. This vector was designated pRVp53. Another retroviral vector, pRVmp53, containing mutant p53 cDNA with a stop codon immediately downstream of the initiation codon was generated using a QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The primers used to construct the p53 mutant were p53-94Xs (5′-CTCCTGGCCCCTGTGATCTTCTGTCCCTTC-3′) and p53-94Xas (5′-GAAGGGACAGAAGATCACAGGGGCCAGGAG-3′).

Cell culture. The H358 human NSCLC cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and grown in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) (Gibco-BRL, Gaithersburg, MD). NIH-3T3 cells were also purchased from the ATCC and grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% FCS. The amphotropic packaging cell line GP + env AM12 was obtained from the ATCC and grown in DMEM supplemented with glucose, 10% FCS, 15 μg/ml hypoxanthine (Sigma), 250 μg/ml xanthine sodium salt (Sigma), 25 μg/ml mycophenolic acid (Gibco), and 200 μg/ml hygromycine B (Sigma).

Transfection and transduction. Ten micrograms of vector plasmid DNA was transfected into GP + env AM12 cells (2×10^6) in a 10-cm dish using the calcium-phosphate method, as described previously [14], after which the cells were incubated for 6 h at 37 °C in a CO_2 incubator. The medium was then replaced with fresh DMEM containing 10% FCS, and the supernatant was harvested after 24 h. To determine retroviral vector titers, NIH-3T3 cells were transduced with supernatant produced by GP + env AM12 cells in the presence of 8 μg/ml of polybrene (Sigma). After 48 h, the cells were split at ratios of 1:10 and 1:20, and were then selected for 8–10 days in 500 μg/ml G418 (Calbiochem, Darmstadt, Germany). As described previously [15], titers were determined based on the number of G418-resistant colonies, virus volume, and fraction of infected cells plated. H358 cells were transduced at the same multiplicity of infection (MOI = 0.1), split and selected for 15–18 days in 250 μg/ml G418. The numbers of clones were counted, after which G418-resistant colonies were harvested at random and cultured. At appropriate times, Genomic DNA was extracted from the colonies using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). To prevent the same clones from being selected from the same plate, 2–5 clones were harvested from each 10-cm dish.

Apoptosis analysis. About 4×10^5 H358 cells were cultured in one well of a 2-well Lab-Tek Chamber Slide (Nalge Nunc International, Naperville, IL) coated with 0.02% polyethylenimine (Sigma) before being transduced with the retroviral vectors. Forty-eight hours later, cells were examined under a BX60 microscope (Olympus, Tokyo, Japan), and the incidence of apoptosis was determined using the DeadEnd Fluorometric TdT-mediated-X-dUTP nick end labeling (TUNEL) System (Promega, Madison, WI) according to the manufacturer's instructions.

Mutation analysis of p53. Polymerase chain reaction (PCR) detection of NeoR gene was performed as described previously [16]. The primers used were Neo (U) (5′-TTGTCACTGAAGCGGGAAGGG-3′) and Neo (L) (5′-ATATTCGGCAAGCAGGCATC-3′). Non-radioactive polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) was used to detect mutations within the p53 cDNA. Each coding region of the p53 transgene was amplified using a PCR protocol that entailed initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The primers used were designed to span the splicing sites of p53 genomic DNA and are shown in Table 1. The entire p53 cDNA was covered by the PCR products, which were heat-denatured, applied to GeneGel Excel 12.5/24 gels (Amersham Biosciences, Sweden), and electrophoresed with GenePhor (Amersham Biosciences, San Francisco, CA). Gels were then stained using a Hoefer Automated Gel Stainer (Amersham Biosciences) and a DNA Silver Staining Kit (Amersham Biosciences). When abnormal electrophoretic patterns were seen on SSCP analysis, the amplified PCR products were labeled using an ABI PRISM DNA sequencing kit (ABI, Foster City, CA) and sequenced using ABI PRISM310 (ABI).

Southern blot analysis. Genomic DNA was prepared from H358 cell clones using a QIAmp DNA Mini Kit (Qiagen) according to the manufacturer's instructions, and Southern blotting was carried out as described previously [17]. Briefly, 15 μg genomic DNA was digested with *Kpn*I, separated on a 1% agarose gel, transferred to a Hybond-N+ membrane (Amersham Biosciences) by capillary transfer, and then hybridized with a ^{32}P-labeled p53 probe, which was the full-length p53 cDNA excised from RVp53 using *Not*I (Fig. 1).

Results

Retroviral-mediated p53 gene transfer

The structure of the retroviral vector carrying the NeoR marker gene and the functional p53 gene (p53) is shown in

Table 1
Primers used for amplification of p53 cDNA

	Sequence	PCR product name and size (bp)	
A11-S	5′-GTGACACGCTTCCCTGGATT-3′	A fragment	345
A21-AS	5′-GAAGGGACAGAAGATGACAG-3′		
B1-S	5′-CTGGATTGGCAGCCAGACTG-3′	B fragment	329
B21-AS	5′-TGGCCAGTTGGCAAAACATC-3′		
C1-S	5′-ACTTGCACGTACTCCCCTGC-3′	C fragment	230
C2-AS	5′-CCTTCCACTCGGATAAGATG-3′		
D1-S	5′-CAGATAGCGATGGTCTGGCC-3′	D fragment	291
D21-AS	5′-TCCCAGGACAGGCACAAACA-3′		
E11-S	5′-GGAAGACTCCAGTGGTAATC-3′	E fragment	289
E2-AS	5′-GGCATCCTTGAGTTCCAAGG-3′		
F11-S	5′-TTTCACCCTTCAGATCCGTG-3′	F fragment	312
F2-AS	5′-CTGACGCACACCTATTGCAA-3′		

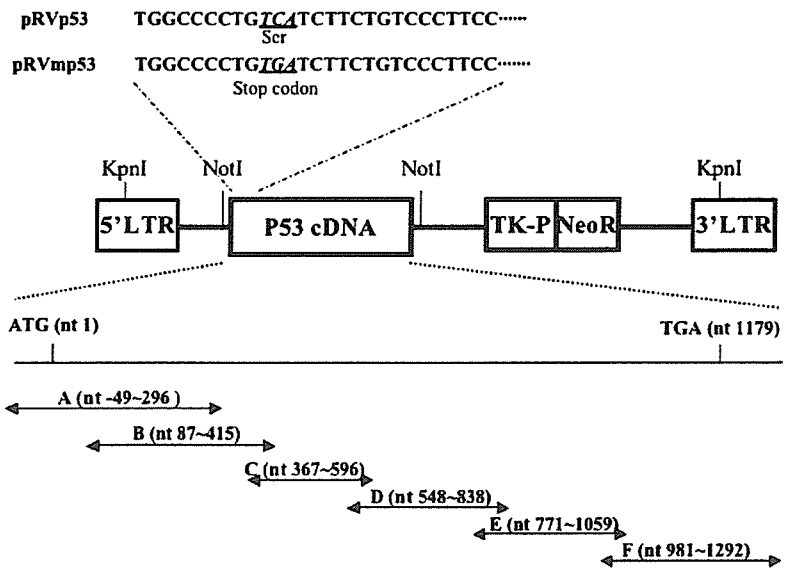

Fig. 1. Schematic representation of the retroviral vector and the primer sets (A–F) for PCR-SSCP analysis. A 1.6-kb fragment containing p53 cDNA was subcloned into the retroviral vector, yielding pRVp53. A mutant p53 cDNA containing a stop codon immediately downstream of the initiation codon was also subcloned into the vector, yielding pRVmp53. The vectors contain the NeoR-selective marker gene (NeoR) driven by the TK promoter (TK-P). The positions of the six PCR fragments are indicated by A, B, C, D, E, and F. As shown in the figure, the PCR fragment overlapped p53 cDNA.

Fig. 1. We constructed a plasmid carrying the functional p53 (pRVp53) and a control plasmid carrying a mutated non-functional p53 (pRVmp53) gene, in which a stop codon was introduced immediately downstream of ATG. The recombinant viral vectors were generated by transfecting the plasmids into GP + env AM12 packaging cells. The titers of retroviral vectors RVp53 and RVmp53, determined with NIH-3T3 cells, were 6.3×10^4 and 5.1×10^4 G418-resistant colony-forming units (CFU)/ml, respectively.

Vectors were incubated with p53-deficient H358 NSCLC cells. TUNEL assay readily detected apoptotic cells among H358 cells transduced with RVp53, but not with RVmp53 (Fig. 2). The apparent titers of RVp53 and RVmp53 in H358 cells were 1.4×10^2 and 1×10^4 CFU/ml, respectively. These results indicate that expression of functional p53 specifically induced apoptosis in cancer cells, as expected. However, a small number of surviving clones remained alive after G418 selection, indicating that some cells are able to escape p53-based gene therapy.

Mutations of p53 cDNA in H358 cells

We examined the structure of p53 cDNA in the H358 clones that escaped p53-induced apoptosis. A total of 102

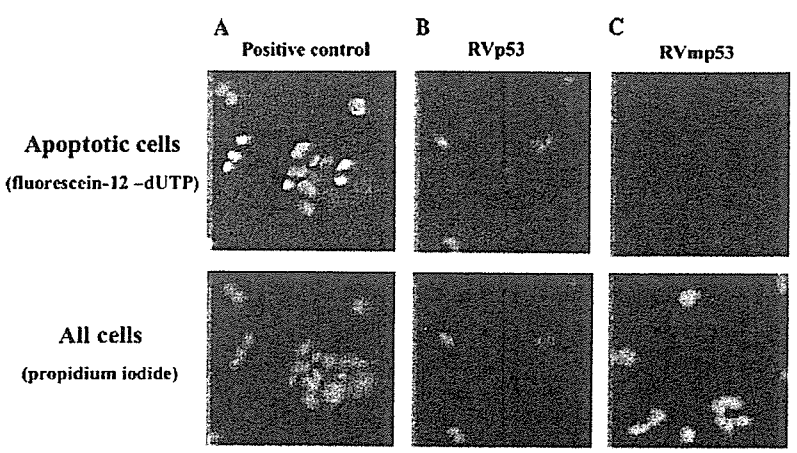

Fig. 2. Analysis of apoptosis among H358 cells using fluorometric TUNEL system. Fluorometric TUNEL labels fragmented DNA in apoptotic cells by catalytically incorporating fluorescein-12-dUTP(a) at 3′-OH DNA ends using the enzyme terminal deoxynucleotidyl transferase (TdT). (A) H358 cells treated with DNase I (positive control). (B) H358 cells transduced with RVp53. Note that the cells are apoptotic. (C) No apoptosis was detected among cells transduced with RVmp53.

clones transduced with RVp53 were isolated for further examination after G418 selection. PCR analysis confirmed that all of the harvested clones carried the NeoR gene from the retroviral vector. Genomic DNA from all clones was subjected to PCR analysis using six sets of primers designed to study whole regions of the p53 cDNA sequence (Fig. 1 and Table 1).

PCR products with the expected size were obtained from most clones. However, some of the clones lacked certain fragments or gave PCR fragments of unexpected sizes. Based on the PCR analysis patterns, 102 clones were classified to several groups (Table 2). In 19 of 102 clones, no p53 specific PCR products were detected (Table 2A). Southern blot analysis of these clones confirmed the lack of p53 sequences (Fig. 3, S5-3), thus suggesting that the entire p53 sequence was deleted in the integrated proviruses. In 53 clones, the selected PCR fragments could not be amplified (Table 2A). Southern blot analysis showed that the proviruses in most of these clones were smaller than the intact RVp53 provirus (Fig. 3; S24-5, S25-1, and

Table 2
Results of PCR-based analysis of the p53 cDNA sequence

(A) Clones with non-amplified PCR fragments (72 clones)

A	B	C	D	E	F	No. of clones
−	−	−	−	−	−	19
−	−	−	−	−	+	6
−	−	−	−	+	+	3
−	−	−	+	+	+	2
−	−	+	+	+	+	10
−	+	+	+	+	+	4
+	−	−	−	−	−	4
+	+	−	−	−	−	4
+	+	+	−	−	−	4
+	+	+	+	−	−	1
+	+	+	+	+	−	2
+	+	−	−	+	+	3
+	+	+	−	+	+	1
+	+	+	−	−	+	4
+	+	−	−	−	+	1
+	+	+	−	+	−	1
−	+	−	−	−	−	1
−	+	+	+	−	−	1
−	−	−	+	+	−	1

(B) Clones with short PCR fragments (1 clone)

A	B	C	D	E	F	Deletion
Short	Short	+	+	+	+	149 bp at 295 nt

(C) Clones with long PCR fragments (3 clones)

A	B	C	D	E	F	Insertion
Long	+	+	+	+	+	98 bp at 278 nt
Long	+	+	+	+	+	104 bp at 278 nt
+	Long	+	+	+	+	127 bp at 522 nt

(D) Clones with aberrant SSCP fragments (12 clones)

Deletion	6 bp at 647 nt
	6 bp at 647 nt
	10 bp at 989 nt
Insertion	C at 597 nt[a]
	A at 1028 nt
Base substitution	C → T at 388 nt
	T → C at 389 nt
	G → A at 517 nt
	C → G at 569 nt
	AA → GT at 595, 596 nt[a]
	A → C at 751 nt
	G → A at 856 nt
	A → G at multiple points

[a] One clone has both an insertion and a base substitution.

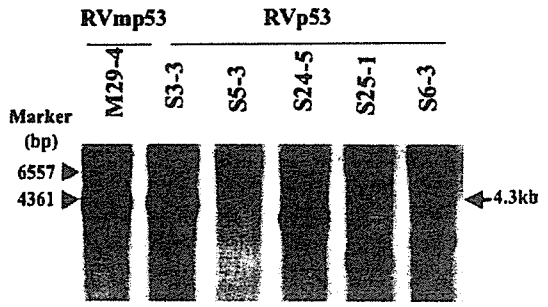

Fig. 3. Southern blot analysis of p53 cDNA in transduced clones. Genomic DNA samples from one or more p53 fragments not amplified by PCR were analyzed by Southern blot. Full-length p53 cDNA as a probe was used. Total absence (S5-3) and large deletions (S24-5, S25-1, and S6-3) of p53 were detected: S, genomic DNA from clones transduced with RVp53; M, genomic DNA from clones transduced with RVmp53. Arrow in the right-hand side of the figure shows the normal position of p53.

S6-3), thus suggesting that deletions of relatively large fragments occurred in these proviruses.

PCR products with aberrant sizes were detected in four clones. Direct sequencing of these DNA fragments revealed that one clone with short PCR fragments had a 149-bp deletion and three clones with longer fragments had insertions of 98–127 bp (Tables 2B and C).

All six sets of PCR products with expected sizes were obtained from 27 of 102 clones and were subjected to SSCP analysis. Aberrant PCR-SSCP patterns were detected in 13 clones. Sequence analyses of DNA fragments from aberrant bands showed that three clones had less then 10-bp deletions, two clones had a 1-bp insertion, and eight clones had substitutions. All of these substitutions were associated with amino acid substitutions in the p53 region (Table 2D).

Fourteen clones showed normal PCR-SSCP patterns, but escaped apoptosis nonetheless. Analysis of p53 expression at the level of the mRNA and protein revealed that these cells did not express p53 (data not shown).

Mutation frequency of p53 sequence

Because functional p53 efficiently induces apoptosis in H358 cells, only clones having inactivated p53 gene were isolated using our strategy. The apparent mutation frequency of 88/102 was overestimated. To measure the mutation frequency of the p53 sequence in all transduced clones, we examined G418-resistant clones transduced with RVmp53, which has a non-functional p53 sequence. PCR-SSCP analysis of 95 clones showed that only 1 clone had a base substitution (from T to C at 210 nt).

Discussion

We examined the structure of p53 cDNA in clones that escaped retroviral-mediated p53 gene therapy. PCR-based analysis showed that 88/102 surviving clones had detectable mutations in the p53 sequence, including gross rearrangements, deletions/insertions and base substitutions. In the remaining 14 clones, no structural changes in the p53 sequence were detected. This may be partially due to limitations in the sensitivity of PCR-SSCP [18]. Alternatively, expression of functional p53 may be inhibited by mutations in the LTR promoter region, which were not studied in this work.

Mutations in retroviral vectors can theoretically arise from any replication/transcription steps, including plasmid replication by the bacterial DNA polymerase, transcription by the cellular RNA polymerase II in packaging cells, or reverse transcription by the viral reverse transcriptase in target cells [19–22]. Because the fidelity of DNA polymerases is much higher than that of RNA polymerases, which lack proofreading activity [23], it is unlikely that DNA replication contributes significantly to the observed mutations. Although it cannot be formally excluded that mutations occur during RNA transcription, it is more likely that the mutations were introduced by a relatively non-processive reverse transcriptase.

Among the 88 mutant clones isolated, 73 had large deletions, 3 had large insertions, 5 had insertions/deletions of less than 10 bp, and 8 clones had base substitutions (Table 2). The mutation spectrum in our study did not reflect the actual frequency of each type of mutation. Because the isolated clones escaped apoptosis induced by p53, only clones carrying inactivate p53 sequences could be analyzed. There may have been many more mutant clones possessing silent mutations.

In the present study, most of the mutant clones had large deletions. Models for generation of large deletions during reverse transcription have been proposed [22,24]. Deletions due to misalignment of the growing point can occur in any region of the proviral sequence. On the other hand, the incorrect plus-strand strong-stop termination models generate deletions of the 5′-regions of the provirus, including the primer binding site, the packaging sequence, and the 5′-region of the structural gene. We found that more than 50% of the inactivated p53 clones lack the 5′-fragment.

The mutation rates of retroviruses have been studied by various experimental systems and the actual numbers can be affected by differences in the viral backbone, the target sequence studied, and the assay method used [9]. The rates of base substitution are estimated to be 10^{-6}–10^{-4} per base per cycle [8,9]. We recently studied the mutation rates and spectra of the herpes simplex virus thymidine kinase (HSV-TK) gene in retroviral vectors among HeLa cells using the same PCR-based assay method as described in this paper. Expression of HSV-TK does not affect the growth of HeLa cells. We found that approximately 20% of clones had mutations in the HSV-TK sequence, among which 13% were base substitutions (manuscript in preparation). In contrast, when the non-functional p53 gene (mp53) was introduced into H358 cells by retroviral vectors, only one in 95 clones had base substitutions. Other mutations, such as large rearrangements, were not detected. These data suggest that the p53 sequence is relatively stable when

compared with the HSV-TK sequence and that the mutation rate is strongly influenced by the sequence of inserted genes. Nevertheless, there remains a significant chance that cancer cells can escape p53 gene therapy. Unwanted mutations in the introduced p53 gene may render the therapy ineffective or even cause the generation of a new cancer [25].

In summary, we demonstrated that mutations in retroviral vectors can influence the consequences of gene therapy. Although the actual mutation rate seems highly variable among different sequences, it is relatively high and should be considered carefully before clinical application of retroviral vectors.

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ORIGINAL ARTICLE

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Corrective effect on Fabry mice of yeast recombinant human α-galactosidase with *N*-linked sugar chains suitable for lysosomal delivery

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Abstract We have previously reported the production of a recombinant α-galactosidase with engineered *N*-linked sugar chains facilitating uptake and transport to lysosomes in a *Saccharomyces cerevisiae* mutant. In this study, we improved the purification procedure, allowing us to obtain a large amount of highly purified enzyme protein with mannose-6-phosphate residues at the non-reducing ends of sugar chains. The products were incorporated into cultured fibroblasts derived from a patient with Fabry disease via mannose-6-phosphate receptors. The ceramide trihexoside (CTH) accumulated in lysosomes was cleaved dose-dependently, and the disappearance of deposited CTH was maintained for at least 7 days after administration. We next examined the effect of the recombinant α-galactosidase on Fabry mice. Repeated intravascular administration of the enzyme led to successful degradation of CTH accumulated in the liver, kidneys, heart, and spleen. However, cleavage of the accumulated CTH in the dorsal root ganglia was insufficient. As the culture of yeast cells is easy and economical, and does not require fetal calf serum, the recombinant α-galactosidase produced in yeast cells is highly promising as an enzyme source for enzyme replacement therapy in Fabry disease.

Keywords Fabry disease · α-Galactosidase · Ceramide trihexoside · Yeast · Enzyme replacement therapy · Fabry mouse

Introduction

Lysosomal α-galactosidase (EC 3.2.1.22) is a critical enzyme for the cleavage of glycolipids with terminal α-D-galactosyl residues, primarily ceramide trihexoside (CTH; also called globotriaosylceramide, GL-3, and

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