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Impact of additional chromosomal abnormalities in patients with acute promyelocytic leukemia: 10-year results of the Japan Adult Leukemia Study Group APL97 study

The t(15;17) chromosome translocation in acute promyelocytic leukemia is classified as a favorable cytogenetic feature among acute myeloid leukemia patients.[1-4] However, the prognostic impact of additional chromosomal abnormalities (ACAs) in acute promyelocytic leukemia has been debated.[5-9] We analyzed the clinical features, biological markers and clinical outcome of Japanese acute promyelocytic leukemia patients with or without ACAs who were treated by all-*trans* retinoic acid (ATRA) and chemotherapy, and tried to determine the role of ACAs on a 10-year follow up.

Adult patients with previously untreated *de novo* acute promyelocytic leukemia were registered consecutively into the JALSG APL97 study.[4] This study was approved by the institutional review boards of each participating institution and registered at *http://www.umin.ac.jp/ctrj/* under C000000206. Informed consent was obtained from patients before registration in the study in accordance with the Declaration of Helsinki.

Chromosomes analyzed by G-banding on bone marrow samples from patients before treatment were classified according to the 1995 International System for Human Cytogenetic Nomenclature (ISCN). Patients were categorized into two groups: those with t(15;17) and ACAs, and those with t(15;17) but without ACAs. Patients with der(17)t(15;17), der(15)t(15;17) or three-way translocation were placed in the group with ACAs.

Details of treatment protocol have been described previously.[4] In brief, remission induction consisted of ATRA and chemotherapy including idarubicin and cytarabine. Dose and duration of chemotherapy were based on initial leukocyte count. After completion of consolidation chemotherapy, patients negative for the *PML-RARA* tran-

Table 1. Clinical features of patients.

Parameters	Total N. (%)	Total Median (range)	t(15;17) N. (%)	t(15;17) Median (range)	t(15;17) with ACAs N. (%)	t(15;17) with ACAs Median (range)	P
N. of patients	225		158		67		
Age, years		48 (15-70)		49 (15-70)		45 (19-70)	0.08
15-29	39 (17%)		21 (13%)		18 (27%)		
30-49	84 (37%)		62 (39%)		22 (33%)		0.06
50-70	102 (46%)		75 (48%)		27 (40%)		
Gender							0.24
Male	122 (54%)		90 (57%)		32 (48%)		
Female	103 (46%)		68 (43%)		35 (52%)		
Leukocyte count, $\times 10^9$/L		1.7 (0.03-256)		1.65 (0.03-256)		1.7 (0.4-70.9)	0.77
Less than 3.0	135 (60%)		93 (59%)		42 (63%)		
3.0-10.0	48 (21%)		31 (20%)		17 (26%)		0.21
10.0 or higher	42 (19%)		34 (21%)		8 (12%)		
Platelet count, $\times 10^9$/L		29 (2-238)		30 (2-238)		29 (3-180)	0.69
Less than 10	31 (14%)		26 (16%)		5 (7.4%)		
10-40	10 (48%)		71 (45%)		38 (57%)		0.12
40 or higher	85 (38%)		61 (39%)		24 (36%)		
DIC score*	n = 213	6 (0-12)	n = 151	6 (0-12)	n = 62	6 (0-11)	0.46
3 or higher	198		139 (92%)		59 (95%)		
10 or higher	12		16 (11%)		5 (8%)		
FAB subtype							0.04
Typical	210 (93%)		144 (91%)		66 (99%)		
Variant	15 (7%)		14 (9%)		1 (1%)		
CD56 expression	n = 192		n = 128		n = 64		0.45
positive	19 (10%)		11 (9%)		8 (13%)		
negative	173 (90%)		117 (91%)		56 (87%)		
Peripheral blood count, $\times 10^9$/L							
leukocyte < 10, platelet > 40	72 (32%)		51 (32%)		21 (31%)		
leukocyte < 10, platelet < 40	112 (50%)		74 (47%)		38 (57%)		0.22
leukocyte > 10	41 (18%)		33 (21%)		8 (12%)		
Incidence of secondary							
MDS/AML	5 (2%)		4 (3%)		1 (1%)		0.63

*FAB: French-American-British; EFS: event free survival; RFS: relapse free survival. NA: not applicable; *DIC score, Score 3 indicates suspected DIC; scores from 4 to 10, definitive DIC; score 10 or more, severe DIC.*

script were randomly allocated either to receive 6 courses of intensified maintenance chemotherapy or to observation. Patients who were positive for the *PML-RARA* fusion transcript received late ATRA therapy followed by maintenance therapy, and received allogeneic hematopoietic stem cell transplantation if they had a human leukocyte antigen-identical donor.

Hematologic response was evaluated by standard criteria according to a previous report.[2] Hematologic and molecular relapse detected by RT-PCR analysis of *PML-RARA* was considered a relapse event.

The primary end point of the JALSG APL97 study was overall survival and disease free survival of patients who achieved complete remission. Overall survival for all patients was calculated from the first day of therapy to death or last visit. Disease free survival was measured from the date of complete remission to relapse, death from any cause or last visit. We also evaluated overall and disease free survival from the time of randomization to maintenance chemotherapy or observation.

Clinical and biological characteristics were compared between patients with or without ACAs by the χ^2 test or Fisher's exact test for categorical data, and Wilcoxon's rank-sum test for continuous data. Overall and disease free survival were estimated by the Kaplan-Meier method and then compared by the log rank test. Clinical outcomes were updated on January 2009 and the median follow-up period is 7.3 years. Statistical analyses were performed using SPSS 11.0 software (SPSS Inc, Chicago, IL, USA).

Among 302 patients enrolled between May 1997 and June 2002, 283 patients were evaluable.[4] Of these, 58 patients were excluded because of insufficient data for ACAs status. Thus, the present analysis was carried out on 225 patients.

Sixty-seven (30%) of 225 patients had ACAs. Trisomy 8 was the most frequently observed ACA and detected in 21 cases (31%). Seven cases (11%) had ACAs in chromosome 15 in addition to t(15;17), 6 (9%) in chromosome 9, 6 (9%) in chromosome 7, 4 (6%) in chromosome 15, and 4 (6%) in chromosome 6. There was no significant difference in clinical or biological characteristics between the two groups, except the frequency of M3v (1% *vs.* 9%, *P*=0.04) (Table 1).

Complete remission rates in patients with or without ACAs were 97% and 95%, respectively (*P*=0.72). There was no difference in cumulative incidence of early death at 50 days, severe hemorrhagic complication or retinoic acid syndrome between the two groups (*P*=0.16, *P*=0.46 and *P*=0.16, respectively). There was also no difference in overall survival, disease free survival or cumulative incidence of relapse between the two groups (91% *vs.* 84%, *P*=0.18; 68% *vs.* 71%, *P*=0.59; 26% *vs.* 22%, *P*=0.51, respectively). Overall and disease free survival are shown in Figure 1A and B. In addition, clinical outcome was analyzed among subgroups of patients with ACAs. However, ACAs including chromosome 8, 7, 9, 15 and 17 did not influence outcomes.

Clinical and biological characteristics have been compared between patients with or without ACAs. ACAs have been detected in 26% to 33% of newly diagnosed acute promyelocytic leukemia patients in whom trisomy 8 was consistently the most frequent ACA.[5-9] In this study, 67 patients (30%) had ACAs, and trisomy 8 was the most frequent (31%). There was no significant difference in overall survival, disease free survival or relapse rate between patients with or without trisomy 8.

The frequency of M3v was significantly lower among our patients with ACAs. This agrees with the report by Schoch *et al.*,[10] although several previous studies showed that the morphology of M3v was not related to the presence of ACAs.[5,6,8] The inconsistency of these results may be caused by a considerably smaller number of M3v cases (16% to 27% of APL). Some authors have reported that the morphology of M3v is related to fms-like tyrosine kinase 3 mutations.[8,11,12] Future analysis of this with ACAs is needed.

Several authors have discussed the clinical importance of ACAs in acute promyelocytic leukemia patients treated with ATRA and chemotherapy. Cervera *et al.*[9] found in the LPA99 trial that ACAs were associated with lower relapse free survival in univariate analysis but not in mul-

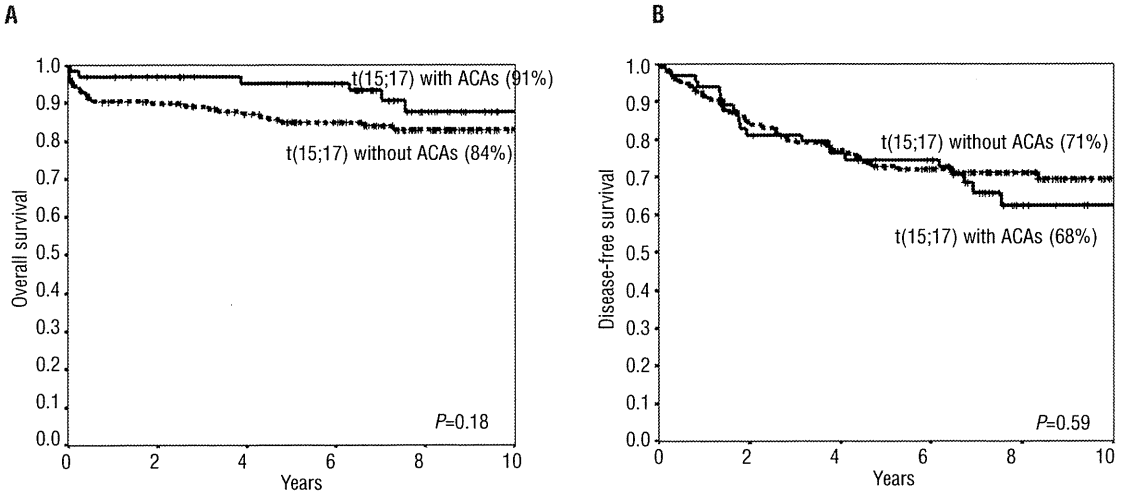

Figure 1. Overall survival and disease free survival of APL patients between with or without additional chromosomal abnormalities in addition to t(15;17). (A) Overall survival (91% *vs.* 84% at 10 years, *P*=0.18), (B) Disease-free survival (68% *versus* 71% at 10 years, *P*=0.59) were similar between two groups.

tivariate analysis. Schlenk *et al.*[8] analyzed 82 patients and reported that ACAs were an unfavorable prognostic marker for overall survival due to early death during the induction therapy. On the contrary, Botton *et al.*[6] and Hernandez *et al.*[7] reported that ACAs had no impact on clinical outcome. In our study, ACAs also did not show any prognostic significance. One of the reasons for this discrepancy would be that the clinical outcome of acute promyelocytic leukemia has recently improved dramatically. The outcome of each subgroup has also been greatly improved, although with some limitations, because patients have been stratified according to risk factors and consequently recent studies have used risk-adapted therapies. Thus, it may become more difficult to identify prognostic factors in acute promyelocytic leukemia.

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ORIGINAL ARTICLE

A decision analysis of allogeneic hematopoietic stem cell transplantation in adult patients with Philadelphia chromosome-negative acute lymphoblastic leukemia in first remission who have an HLA-matched sibling donor

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Clinical studies using genetic randomization cannot accurately answer whether adult patients with Philadelphia chromosome-negative acute lymphoblastic leukemia (ALL) who have a human leukocyte antigen (HLA)-matched sibling should undergo allogeneic hematopoietic stem cell transplantation (HSCT) or chemotherapy in first remission, as, in these studies, patients without a sibling donor undergo alternative donor transplantation or chemotherapy alone after a relapse. Therefore, we performed a decision analysis to identify the optimal strategy in this setting. Transition probabilities and utilities were estimated from prospective studies of the Japan Adult Leukemia Study Group, the database of the Japan Society for Hematopoietic Cell Transplantation and the literature. The primary outcome measure was the 10-year survival probability with or without quality of life (QOL) adjustments. Subgroup analyses were performed according to risk stratification on the basis of white blood cell count and cytogenetics, and according to age stratification. In analyses without QOL adjustments, allogeneic HSCT in first remission was superior in the whole population (48.3 vs 32.6%) and in all subgroups. With QOL adjustments, a similar tendency was conserved (44.9 vs 31.7% in the whole population). To improve the probability of long-term survival, allogeneic HSCT in first remission is recommended for patients who have an HLA-matched sibling.
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Keywords: decision analysis; acute lymphoblastic leukemia; allogeneic hematopoietic stem cell transplantation; HLA-matched sibling donor; first remission

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Introduction

With modern intensive chemotherapy, 74–93% of adult patients with acute lymphoblastic leukemia (ALL) achieve complete remission. However, the overall survival rate is only 27–48% because of the high rate of relapse.[1] Therefore, the establishment of optimal postremission therapy is important. The efficacy of allogeneic hematopoietic stem cell transplantation (HSCT) for adult patients with ALL in first remission has been demonstrated through clinical studies using genetic randomization, in which patients with a human leukocyte antigen (HLA)-matched sibling donor were allocated to the allogeneic HSCT arm, and those without a donor were placed in the chemotherapy or autologous transplantation arm. First, the LALA-87 trial showed that overall survival in patients with a donor was better than that in patients without a donor in a subgroup analysis of patients with high-risk characteristics.[2] A meta-analysis of seven similar studies confirmed that the donor group was superior to the non-donor group in patients with high-risk ALL in first remission.[3] However, such genetic randomization studies cannot accurately answer the question of whether patients with an HLA-matched sibling should undergo allogeneic HSCT or chemotherapy in first remission. In these studies, patients without a sibling donor had to choose transplantation from an alternative donor or chemotherapy alone once they had a relapse. The outcome of these treatments has been reported to be inferior to that of HSCT from an HLA-matched sibling donor in patients with relapsed ALL; therefore, the expected survival after the decision to continue chemotherapy in first remission in patients without a sibling donor is assumed to be originally poorer than that in patients with a sibling donor. However, it is practically difficult to perform a clinical trial in which patients with an HLA-matched sibling in first remission are randomly assigned to receive allogeneic HSCT or chemotherapy alone. Another important problem has been poor compliance with the assigned treatment in some studies. In addition, previous genetic

randomization studies did not consider the quality of life (QOL), especially that associated with graft-versus-host disease (GVHD). Therefore, we performed a decision analysis incorporating QOL adjustments using a decision tree based on the results of Japan Adult Leukemia Study Group (JALSG) prospective studies (ALL93[4] and ALL97[5]), the database of the Japan Society for Hematopoietic Cell Transplantation (JSHCT)[6] and literature. Patients with Philadelphia chromosome (Ph)-positive ALL were not included in our analysis because the outcome of treatment in these patients has improved dramatically since tyrosine kinase inhibitors became available.[7]

Recently, the Medical Research Council/Eastern Cooperative Oncology Group (MRC/ECOG) trial demonstrated the efficacy of allogeneic HSCT in ALL patients and in standard-risk patients, but not in high-risk patients,[8] which was inconsistent with previous studies. This difference might partly depend on the definition of high-risk patients. In the MRC/ECOG study, an age of higher than 35 years was considered to be a high-risk factor. Therefore, we performed separate subgroup analyses according to risk stratification on the basis of white blood cell count and cytogenetics, and according to age stratification with a cutoff of 35 years.

Methods

Model structure

We constructed a decision tree (Figure 1) to identify the optimal treatment strategy for adult patients with Ph-negative ALL in first remission who have an HLA-matched sibling.[9,10] The square at the left represents a decision node. We can decide to either proceed to allogeneic HSCT or continue chemotherapy in first remission. We did not include a decision to perform autologous HSCT, as autologous HSCT has not been shown to be superior to chemotherapy alone in a meta-analysis.[3] Circles, called chance nodes, follow each decision, and each chance node has two or three possible outcomes with a specific probability called the transition probability (TP). Every branch finally ends with triangles, called terminal nodes, and each terminal node has an assigned payoff value, called utility, according to different health states. Calculations were performed backward, from right to left in the decision tree. The sum of the products of TPs and utilities of the branches becomes the expected value for each chance node, and eventually the sum of the expected values in all of the chance nodes following the decision nodes becomes the expected value of each decision. The following analyses were performed using TreeAge Pro 2009 software (Williamstown, MA, USA). This study was approved by the Committee for Nationwide Survey Data Management of JSHCT, and the Institutional Review Board of Jichi Medical University.

Data sources

Outcomes after continuing chemotherapy in first remission were estimated from JASLG studies (ALL93 and ALL97). Patients with Ph-negative ALL, aged 15–54 years, were included, and those who never achieved remission with chemotherapy were excluded. Data from 122 patients in ALL93 and 119 patients from ALL97 were analyzed separately, and then combined by weighting the number of patients. Outcomes after allogeneic HSCT in various disease statuses were estimated from the database of the JSHCT. Patients with Ph-negative ALL, aged 16–54 years, who underwent a first myeloablative allogeneic HSCT from a serologically HLA-A, -B, -DR loci-matched sibling between 1993 and 2007 were included. Of them, 408, 61, 14 and 94 patients were in first remission, second remission, third or later remission and non-remission, respectively, at allogeneic HSCT.

The characteristics of the patients included in this study are summarized in Table 1. There was no significant difference in their baseline characteristics. To determine the following TPs,

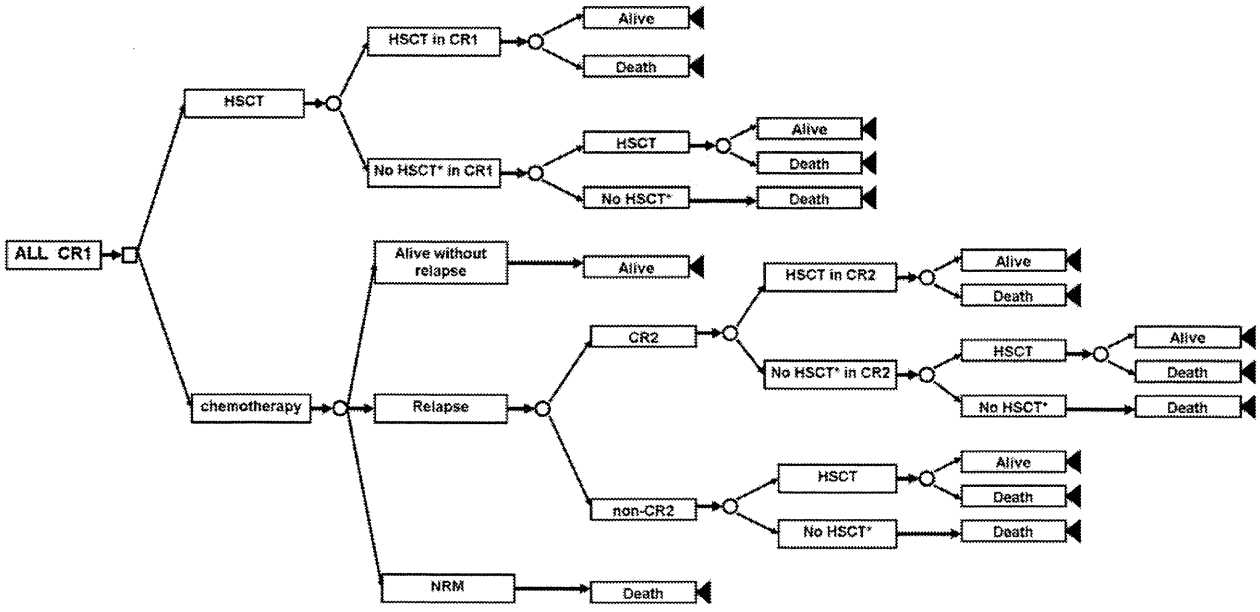

Figure 1 Decision tree used in this study. Decision analysis was performed on the basis of a decision tree. A square indicates a decision node and open circles indicate chance nodes. In analyses with a QOL adjustment, 'Alive' after transplantation was followed by two branches with or without active chronic GVHD. *HSCT was not performed because of early relapse, death and so on. ALL, acute lymphoblastic leukemia; CR, complete remission; NRM, non-relapse mortality.

Table 1 Patient characteristics in the three data sources

	Chemotherapy in CR1		*HSCT in CR1*	P[a]
	JALSG ALL93	*JALSG ALL97*	*JSHCT*	
No. of patients	122	119	408	
Median age (range)	26 (15–54)	26 (15–54)	29 (16–54)	0.72
No. of males/females	72/50	54/65	230/178	0.06
Median WBC count at diagnosis (range) ($\times 10^9$/l)	9.5 (0.6–468.0)	10.2 (0.3–398.0)	10.4 (0.4–801.0)	0.91
Karyotype standard:high[b], ratio	20:1	30:1	15.4:1	0.55

Abbreviations: CR, complete remission; HSCT, hematopoietic stem cell transplantation; JALSG, Japan Adult Leukemia Study Group; JSHCT, Japan Society for Hematopoietic Cell Transplantation; WBC, white blood cell.
[a]Statistical analyses were performed using the Kruskal–Wallis test for continuous variables and the χ^2-test for categorical variables.
[b]t(4;11) and t(1;19) were classified as high-risk karyotypes, and other karyotypes were classified as standard risk.

overall survival and leukemia-free survival (LFS) with a 95% confidence interval (CI) were calculated using the Kaplan–Meier method, whereas the cumulative incidences of non-relapse mortality and relapse with 95% CI were calculated using Gray's method,[11] considering each other as a competing risk. Probabilities that we could not estimate from these data were estimated from the literature.

Transition probabilities (TPs) and utilities

TPs of the whole population were determined as summarized in Table 2. Each TP has a baseline value and a plausible range. Baseline decision analyses were performed on the basis of baseline values.

Patients may have been precluded from undergoing allogeneic HSCT because of early relapse or comorbidities even if they decided to undergo allogeneic HSCT, and therefore the TP of actually undergoing allogeneic HSCT in first remission after the decision branch to undergo allogeneic HSCT was determined as follows: first, the median duration between the achievement of first remission and HSCT without relapse was calculated as 152 days on the basis of JSHCT data. Next, LFS rates at 152 days after achieving first remission were calculated using the data of all patients who achieved remission in the JALSG studies, and the combined LFS was 0.80 (95% CI: 0.76–0.85). We considered this to be the TP for actually receiving HSCT in first remission, and assigned a baseline value of 0.80 and 95% CI to the plausible range. Similarly, patients may be precluded from undergoing allogeneic HSCT even though they have achieved second remission after they had a relapse of leukemia following a decision to continue chemotherapy. This TP of undergoing allogeneic HSCT in second remission could not be calculated from our data. We assigned a plausible range of 0.5–0.80; the former value was the only available rate in a large study[12] and the latter was the TP calculated above. The median of this range was taken as the baseline value. Probabilities regarding the actual rate of receiving HSCT in other disease statuses could not be obtained, even in the literature. Therefore, a baseline value of 0.5 was assigned with a wide plausible range of 0.3–0.7, although these values may not be closely related to the final expected value, as the probability of survival after receiving HSCT in these situations was extremely low. The TPs of 'Alive at 10 years' following HSCT in various disease statuses were determined on the basis of the JSHCT database. We assigned 95% CI to the plausible ranges.

The TPs of 'Alive without relapse at 10 years' and non-relapse mortality following chemotherapy in first remission were determined on the basis of JALSG studies, and the TP of relapse following chemotherapy was determined by subtracting the sum of these TPs from 1. The TP of achieving second remission after relapse in patients who decided not to undergo allogeneic HSCT in first remission was estimated to have a baseline value of 0.4, with a plausible range of 0.3–0.5 based on the literature.[12–14]

The primary outcome measure was the 10-year survival probability as described in the Discussion. The survival curve nearly reaches a plateau after 5 years and therefore 'Alive at 10 years' reflects 'Cure of leukemia', which is the primary goal of allogeneic HSCT. First, we considered only two kinds of health states, 'Alive at 10 years' and 'Dead', and assigned utility values of 100 to the former and 0 to the latter without considering QOL. Next, we performed a decision analysis while adjusting for QOL. 'Alive after chemotherapy without relapse at 10 years', 'Alive with active GVHD at 10 years' and 'Alive without active GVHD at 10 years' were considered as different health states. The proportion of patients with active GVHD among those who

Table 2 Transition probabilities of the whole population

	Baseline value (plausible range)
HSCT in CR1	0.80 (0.76–0.85)
Alive at 10 years following HSCT in CR1	0.57 (0.52–0.63)
HSCT after failure of HSCT in CR1	0.5 (0.3–0.7)
Alive at 10 years following HSCT after failure of HSCT in CR1[a]	0.27 (0.16–0.38)
Alive at 10 years without relapse following CTx	0.21 (0.15–0.28)
NRM at 10 years following CTx	0.07 (0.04–0.10)
Achievement of CR2 after relapse following CTx	0.4 (0.3–0.5)
HSCT in CR2	0.66 (0.5–0.80)
Alive at 10 years following HSCT in CR2	0.38 (0.27–0.53)
HSCT after failure of HSCT in CR2	0.5 (0.3–0.7)
Alive at 10 years following HSCT after failure of HSCT in CR2[b]	0.18 (0.16–0.2)
HSCT in non-CR after relapse following CTx	0.5 (0.3–0.7)
Alive at 10 years following HSCT in non-CR after relapse	0.16 (0.1–0.27)
Rate of active GVHD at 10 years[c]	0.18 (0.1–0.25)

Abbreviations: CR, complete remission; CTx, chemotherapy; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; NRM, non-relapse mortality.
[a]This rate was estimated from the survival rate following HSCT in CR2 and HSCT in non-CR.
[b]This rate was estimated from the survival rate following HSCT in CR3 or more and HSCT in non-CR.
[c]The same baseline value and plausible range were used as the rate of active GVHD at 10 years following HSCT in various disease statuses, but one-way sensitivity analyses were performed separately in each status.

Table 3 Transition probabilities of subgroups

	Baseline value (plausible range)			
	Standard-risk	*High-risk*	*Lower age*	*Higher age*
HSCT in CR1	0.86 (0.81–0.92)	0.65 (0.54–0.77)	0.81 (0.76–0.86)	0.80 (0.72–0.87)
Alive at 10 years following HSCT in CR1	0.6 (0.53–0.68)	0.51 (0.4–0.66)	0.62 (0.55–0.69)	0.48 (0.39–0.58)
Alive at 10 years following HSCT after failure of HSCT in CR1	0.31 (0.24–0.38)	0.28 (0.13–0.43)	0.3 (0.21–0.39)	0.23 (0.11–0.35)
Alive at 10 years without relapse following CTx	0.27 (0.18–0.37)	0.13 (0.03–0.22)	0.19 (0.11–0.27)	0.25 (0.16–0.35)
NRM at 10 years following CTx	0.06 (0.02–0.11)	0.07 (0–0.14)	0.04 (0.01–0.08)	0.11 (0.05–0.18)
HSCT in CR2	0.68 (0.5–0.86)	0.58 (0.5–0.65)	0.66 (0.5–0.81)	0.65 (0.5–0.80)
Alive at 10 years following HSCT in CR2	0.38 (0.23–0.61)	0.43 (0.22–0.84)	0.39 (0.26–0.58)	0.35 (0.19–0.64)
Alive at 10 years following HSCT after failure of HSCT in CR2[a]	0.24 (0.12–0.45)	0.13 (0.05–0.35)	0.21 (0.12–0.36)	0.11 (0.04–0.3)
Alive at 10 years following HSCT in non-CR after relapse	0.24 (0.12–0.45)	0.13 (0.05–0.35)	0.21 (0.12–0.36)	0.11 (0.04–0.3)

Abbreviations: CR, complete remission; CTx, chemotherapy; HSCT, hematopoietic stem cell transplantation; NRM, non-relapse mortality.
Transition probabilities that are not in Table 3 are the same as those mentioned in the whole population.
[a]As the number of patients who underwent HSCT in CR3 or more was not enough, the same rate of survival following HSCT in non-CR was used.

were alive at 10 years was determined on the basis of the literature.[15–17] We assigned a value of 100 to the utility for being alive without relapse at 10 years after chemotherapy alone, and a value of 0 to the utility for being dead in all situations. We assigned a fixed value of 98 to the utility for being alive without active GVHD at 10 years following HSCT, and assigned a value of 70 with a wide plausible range of 0–98 to the utility for being alive with active GVHD at 10 years. These utilities were determined on the basis of opinions of 10 doctors who were familiar with HSCT and the literature.[9,18]

Subgroup analyses were also performed according to risk stratification on the basis of white blood cell count and cytogenetics, and according to age stratification with a cutoff of 35 years. Patients with a high white blood cell count (more than 30×10^9/l for B lineage and more than 100×10^9/l for T lineage) and/or with t(4;11) or t(1;19) were classified as a high-risk group, and all other patients were classified as standard-risk group. All TPs, based on the JALSG studies and the JSHCT data, were recalculated using the data of patients in each subgroup (Table 3). Other TPs and utilities were the same as those for the overall patient analyses.

Sensitivity analyses

To evaluate the robustness of the decision model, we performed one-way sensitivity analyses for all TPs, in which the decision tree was recalculated by varying each TP value in its plausible range, and confirmed whether the decision of the baseline analyses changed. In the analyses that included adjustments for QOL, the utility for being alive with active GVHD at 10 years was also subjected to a one-way sensitivity analysis.

We also performed a probabilistic sensitivity analysis using Monte Carlo simulation in which the uncertainties of all TPs were considered simultaneously.[19] The distribution of the random variables for each TP was determined to follow a normal distribution, with 95% of the random variables included in the plausible range. Following 1000 simulations based on the decision tree, the mean and s.d. of the expected value for each decision were calculated.

Results

Baseline analysis

The baseline analysis in the whole population without adjusting for QOL revealed an expected 10-year survival of 48.3% for the decision to perform allogeneic HSCT in first remission, which was better than that of 32.6% for the decision to continue chemotherapy. The decision to perform allogeneic HSCT continued to be superior even after adjusting for QOL (44.9% for HSCT vs 31.7% for chemotherapy, Table 4).

Table 4 Expected 10-year survival probabilities with and without adjusting for QOL

	Expected survival probability without a QOL adjustment		*Expected survival probability with a QOL adjustment*	
	HSCT (%)	*Chemotherapy (%)*	*HSCT (%)*	*Chemotherapy (%)*
All patients	48.3	32.6	44.9	31.7
Standard-risk patients	53.8	39.8	50.0	38.9
High-risk patients	38.0	25.0	35.4	24.1
Lower-aged patients[a]	53.1	32.9	49.3	31.9
Higher-aged patients[a]	40.7	33.4	37.8	32.8

Abbreviation: HSCT, hematopoietic stem cell transplantation; QOL, quality of life
[a]Lower-aged patients include those aged 35 years or younger. Higher-aged patients include those aged older than 35 years.

Sensitivity analysis

First, we performed one-way sensitivity analyses for all TPs in the decision model without adjusting for QOL. A better expected survival for the decision to perform HSCT was consistently demonstrated in all TPs within the plausible ranges. In the probabilistic sensitivity analysis, the mean value and s.d. of the expected survival probability for HSCT were 48.3 and 2.6%, and those for chemotherapy were 32.7 and 3.4%, respectively.

Next, we performed one-way sensitivity analyses for all TPs and for the utility for being alive with active GVHD at 10 years in the decision model adjusted for QOL. Even in these analyses, the result of the baseline analysis did not reverse in all TPs. In addition, a higher expected survival probability for HSCT was retained, assuming that the utility for being alive with active GVHD ranged between 0 and 98 (Figure 2a). In the probabilistic sensitivity analysis, the mean value and s.d. of the expected survival probability for HSCT were 44.8 and 2.6%, and those for chemotherapy were 31.8 and 3.4%, respectively.

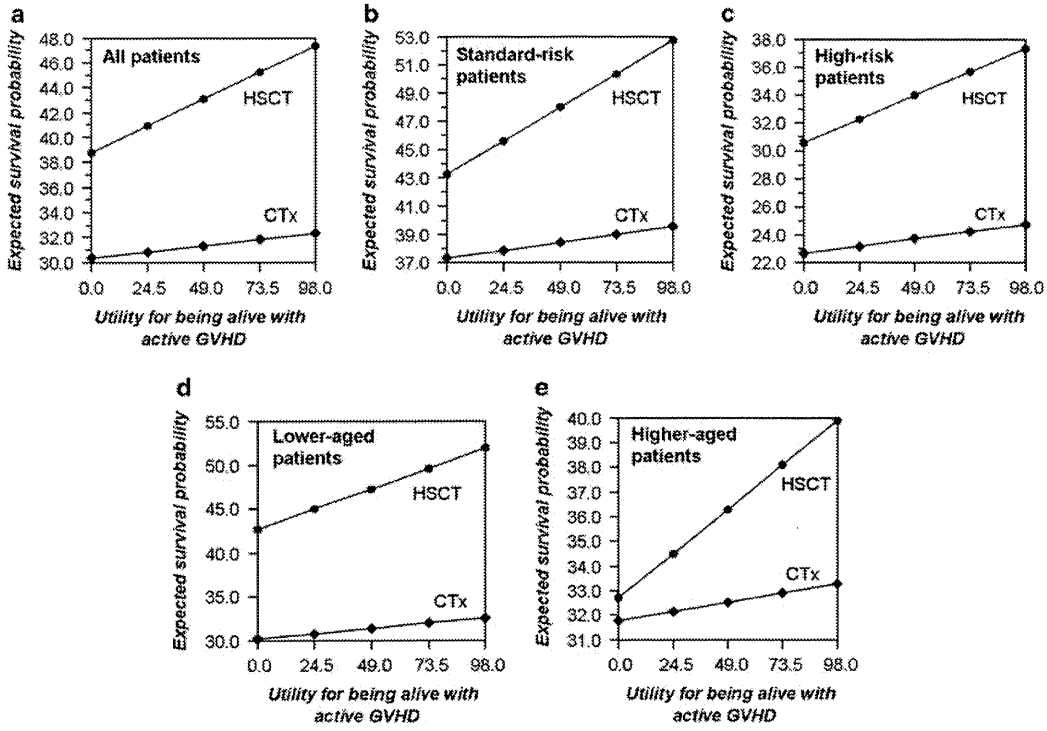

Figure 2 One-way sensitivity analysis for the utility for being alive with active GVHD. We performed one-way sensitivity analyses for the utility for being alive with active GVHD in the model, with adjustment for QOL. The superiority of allogeneic HSCT compared with chemotherapy (CTx) was consistently observed even with a wide plausible range of the utility in the whole population (**a**) and all subgroups (**b–e**).

Subgroup analyses

In subgroup analyses, both with and without adjustment for QOL, a better expected survival probability for HSCT was consistently observed in all subgroups (Table 4).

We also performed one-way sensitivity analyses in all subgroups. In the decision model without adjusting for QOL, varying each TP value in its plausible range did not affect the results of baseline analyses in all subgroups, except for higher-aged patients. In higher-aged patients, the result of the baseline analysis reversed only if the probability of LFS at 10 years following chemotherapy in first remission was more than 0.334. Even in the decision model with adjustment for QOL, varying each TP value did not affect the result of the baseline analyses in all subgroups, except for higher-aged patients. In higher-aged patients, the result reversed in favor of chemotherapy if the probability of LFS at 10 years without relapse following chemotherapy was more than 0.307 (Figure 3a) or the probability of overall survival at 10 years following HSCT in first remission was less than 0.413 (Figure 3b). On the other hand, non-relapse mortality at 10 years following chemotherapy did not affect the result. We also performed one-way sensitivity analyses for the utility of being alive with active GVHD ranging between 0 and 98. A higher expected survival probability for HSCT was retained in all subgroups (Figures 2b–e).

Discussion

Decision analysis is a statistical technique that aids the clinical decision-making process under uncertainty. This approach has also been used in situations in which a well-designed clinical trial is practically difficult to perform. In the present case, a prospective trial to randomly assign patients with ALL in first remission who have an HLA-matched sibling to undergo allogeneic HSCT or chemotherapy alone is practically difficult. Therefore, we tried to determine the optimal strategy in this clinical situation by using a decision analysis. We chose the 10-year survival probability as the primary outcome measure rather than life expectancy, as the cure rate, rather than how long they can survive, is important for young patients with acute leukemia to make a decision whether they should undergo allogeneic HSCT in first remission. When we performed the decision analysis using the 5-year survival probability as the primary outcome measure, however, the findings in this study did not change, as the survival curve nearly reaches a plateau after 5 years. Further, we adjusted for QOL by considering the presence or absence of persisting symptoms associated with chronic GVHD rather than by calculating quality-adjusted life years, as most patients who choose allogeneic HSCT may tolerate transiently impaired QOL and attach much importance to long-term QOL. Under these conditions, we decided to use a simple decision analysis model rather than a Markov model that allows probabilities and utilities to change with time, as the benefit of using a Markov model is limited in this situation. In addition, a large number of patients are required for the Markov model to define appropriate TPs that change with time. In this study, the number of patients was limited because we used data from the JALSG prospective studies to avoid biases of using retrospective data. We used the database of the JSHCT to calculate TPs in patients who underwent HSCT, because the number of patients who underwent HSCT was further limited in the JALSG prospective studies. However, outcomes after allogeneic HSCT in first remission were not significantly

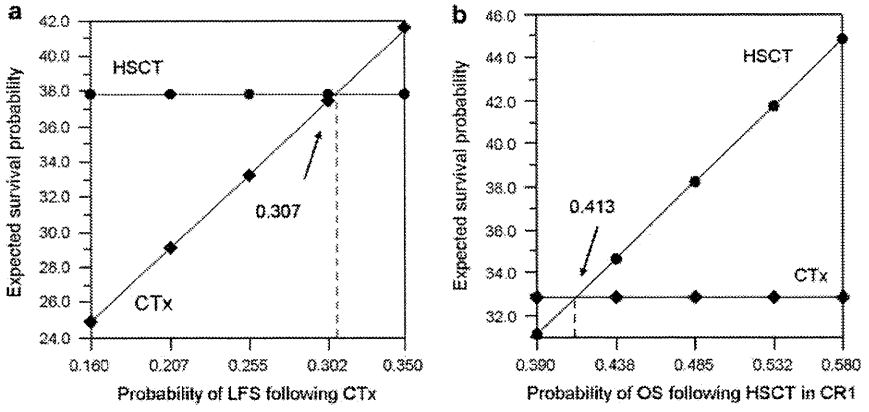

Figure 3 One-way sensitivity analysis in higher-aged patients. We performed one-way sensitivity analyses for all TPs in the decision model both with and without adjustment for QOL. In higher-aged patients, the result reversed if the probability of LFS at 10 years without relapse following chemotherapy (CTx) was more than 0.307 (**a**), or the probability of overall survival at 10 years following allogeneic HSCT in first complete remission (CR1) was less than 0.413 (**b**).

different among the JALSG prospective studies and the JSHCT database (data not shown).

In our baseline analysis both with and without adjustment for QOL, the superiority of HSCT in first remission was demonstrated in the whole population and also in all subgroups. In the whole population, probabilistic sensitivity analysis using a Monte Carlo simulation also supported this result. However, in one-way sensitivity analyses, we should note that the decision model was sensitive to the probability of LFS following chemotherapy in first remission in higher-aged patients (Figure 3a). The adaptation of intensified chemotherapy according to pediatric regimens has led to improved outcomes in adolescents and young adults,[20] and even in older patients in recent trials,[21] and therefore this decision might change in the future.

The risk stratification we used in subgroup analyses was different from that used in the MRC/ECOG study.[8] Therefore, we added subgroup analyses according to the risk stratification used in the MRC/ECOG study. In analyses without QOL adjustments, allogeneic HSCT in first remission was superior both in standard-risk (56.6 vs 36.2%) and high-risk (42.4 vs 33.3%) patients. With QOL adjustments, the similar tendency was observed in both standard-risk (52.6 vs 35.1%) and high-risk (39.4 vs 32.6%) patients. These findings were consistent with those based on our original risk stratification. In addition, we further subdivided patients into four different age categories: 15–25, 26–35, 36–45 and 46–54 years. The superiority of the decision to perform allogeneic HSCT in first remission was conserved in all age categories (data not shown).

A possible concern in this study was the long median duration of 152 days from achieving complete remission to allogeneic HSCT. In the current decision model, this long duration precluded allogeneic HSCT in first remission in about 20% of patients in the allogeneic HSCT branch (mainly because of early relapse), and thereby impaired the expected probability of survival for the decision to undergo allogeneic HSCT. In reality, a meta-regression analysis by Yanada *et al.*[3] revealed that compliance with allogeneic HSCT was significantly and positively correlated with survival.[3] Another fact to be noted is the low incidence of severe GVHD in Japanese patients, which might have favorably affected the decision to perform HSCT.[22] Therefore, the current conclusion should be cautiously applied to Western patients.

The QOL after HSCT is most strongly affected by the status of chronic GVHD, but it is difficult to determine the appropriate utility for each status of GVHD. Therefore, we performed a one-way sensitivity analysis with a wide plausible range of the utility for being alive with active GVHD. In our decision model, the superiority of HSCT was consistently observed regardless of the utility for being alive with active GVHD both in the whole population and in all subgroups (Figure 2).

In conclusion, to improve the long-term probability of survival, allogeneic HSCT in first remission is recommended for all adult patients with Ph-negative ALL who have an HLA-matched sibling. Even when we considered QOL, the superiority of HSCT was confirmed in the whole population and in all subgroups. However, this result might change by the adaptation of intensified chemotherapy, especially in higher-aged patients.

Conflict of interest

The authors declare no conflict of interest.

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Azacitidine induces demethylation of p16INK4a and inhibits growth in adult T-cell leukemia/lymphoma

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Abstract. Adult T-cell leukemia/lymphoma (ATL) is one of the peripheral T-cell malignant neoplasms strongly associated with human T-cell leukemia virus type-I (HTLV-I). Although the viral transactivator protein Tax has been proposed to play a critical role in leukemogeneis, additional cellular events are required for the development of ATL. One of the genetic events of the disease is inactivation of tumor suppressor genes. The CDKN2A locus on chromosome 9p encodes 2 cell cycle regulatory proteins, p14ARF and p16INK4a, which share exon 2 using different reading frames. The p14ARF and p16INK4a genes have been implicated as tumor suppressor genes by their frequent mutation, deletion or promoter hypermethylation in a variety of human tumors. In this report, we describe the expression status of p14ARF and p16INK4a in 9 ATL cell lines (MT1, MT2, OKM3T, F6T, K3T, Oh13T, S1T, Su9T01 and HUT102). By reverse transcription polymerase chain reaction (RT-PCR), expression of p14ARF was not detected in one cell line (OKM3T), while expression of p16INK4a was not detected in 6 cell lines (OKM3T, MT1, MT2, Oh13T, S1T and Su9T01). In the OKM3T cell line, the shared exon 2 of the p14ARF/p16INK4a gene was deleted; however, the p16INK4a gene, was epigenetically inactivated in 5 other cells lines. In primary tumor cells obtained from ATL patients, p14ARF expression was absent in 6 of the 11 samples. We confirmed the methylation of the p16INK4a gene in MT1 and MT2 cells using the methylation-specific PCR (MSP) method. Treatment with 2.0 μM of Azacitidine (AZA), a demethylating agent, for 72 h restored p16INK4a transcript expression and induced growth inhibition in MT2 cells. Our results demonstrate that p16INK4a is epigenetically silenced in ATL. AZA offers a potential new therapeutic approach to improve the poor outcomes associated with ATL.

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Key words: p14ARF, p16INK4a, methylation, Azacitidine

Introduction

Adult T-cell leukemia/lymphoma (ATL) is an aggressive, fatal malignancy of mature $CD4^+$ T lymphocytes caused by human T-cell lymphotropic virus type I (HTLV-I) infection (1). ATL has been classified into four main subtypes (2). In the relatively indolent smoldering and chronic forms, median survival is 2 years or more. In the acute and lymphoma forms, median survival time is about 13 months (3). Although a pivotal oncoprotein of HTLV-I, Tax, is strongly associated with the development of ATL (4), precise mechanisms of tumorigenesis in ATL have not been well-defined. A long period of clinical latency precedes the development of ATL (5) and only a small percentage of HTLV-I-infected individuals develop this malignancy (6), indicating that additional genetic events probably are required to develop ATL after viral infection of the target T cells. Studies to date by others and ourselves have shown deletions or mutations of several tumor suppressor genes in the pathogenesis of ATL (7-10).

p16INK4a, one of the cyclin-dependent kinase inhibitors (CDKIs), was implicated as a tumor suppressor gene. The gene harboring the CDKN2A locus on chromosome 9p21 binds to CDK4 and thereby inhibits its kinase activities (11,12), resulting in arrest of the cell cycle. The CDKN2A locus also generates another cell cycle-regulatory protein designated as alternative reading frame (ARF), which encodes a protein of 14 kDa (p14ARF) (13). The p14ARF gene has a unique exon 1 (exon 1β), located approximately 20 kb centrometric to exon 1 of p16INK4a. Under the control of each promoter, exon 1β and exon 1 splice into exon 2 in the CDKN2A locus, producing p14ARF and p16INK4a proteins, respectively (13,14). Therefore, the p14ARF and p16INK4a genes share the same exon 2. p14ARF interacts *in vivo* with the MDM2 protein, neutralizing MDM2-mediated degradation of p53 (15,16). Inactivation of the p14ARF and p16INK4a results from homozygous deletion or promoter methylation (17-19). We have previously reported the homozygous deletion of p16INK4a in a significant portion of patietns with ATL (7). In addition, inactivation of p14ARF and p16INK4a by hypermethylation has been described in common neoplasms including T-cell lymphoma (20).

Azacitidine (AZA) has been administered as a new antineoplastic agent for myelodysplastic syndrome (MDS) with

good outcome (21-24). The agent induces the demethylation of gene promoter via inhibition of DNA methyltransferase (DNMT) that methylates cytosine residues in eukaryotic DNA (22,25).

Because prognosis of ATL is extremely poor, new treatment agents need to be identified. In this study, we investigated the inactivation status of p14ARF and p16INK4a in ATL cell lines as well as primary ATL cells obtained from patients with various clinical types of ATL. We demonstrate that AZA is a potential therapeutic candidate for ATL.

Materials and methods

Cells. Nine ATL cell lines, MT1, MT2, OKM3T, F6T, K3T, Oh13T, S1T, Su9T01 and HUT102 were analyzed. OKM3T was originally established by Miyamoto *et al* (26) and obtained from the Dainippon Sumitomo Pharmaceutical Co., Ltd. (Osaka, Japan). F6T, K3T, Oh13T, S1T and Su9T01 (27) were established in our institute. K562 was used as a negative control. Cell lines were maintained by serial passages in RPMI containing 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 U/ml streptomycin, and incubated in standard tissue culture incubators with 5% CO_2-95% air at 37°C. In addition to these cell lines, mononuclear cells were isolated from either peripheral blood samples from 10 leukemia type ATL patients (6 acute, 3 chronic and 1 undetermined) or from a lymph node sample of a lymphoma type ATL patient. All patients provided written informed consent.

DNA extraction and polymerase chain reaction (PCR) analysis. DNA was isolated with the Sepagene kit (Sanko Jun-yaku, Tokyo, Japan) according to the manufacturer's instructions. All samples were screened for exon 2 of p14ARF/p16INK4a with PCR by our method (7). The primers p16-1S1 (5'-CTTCCTGGACACGCTGGTGGTGCTG-3') and p16-2A2 (5'-GTACAAATTCTCAGATCATCAGTCC-3') were used to obtain a 219-bp fragment of exon 2 of p14ARF/p16INK4a. The cycling conditions were one cycle of 15 min at 95°C, 36 cycles of 60 sec at 95°C, 30 sec at 55°C, 120 sec at 72°C, and one cycle of 10 min at 72°C. The PCR was repeated ≥2 times.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) analysis. Total-RNA was extracted with a single-step method using RNAzol reagent (Sawady, Tokyo, Japan). RT reactions were performed using a First-Strand cDNA Synthesis kit (Amersham Pharmacia Biotec, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's protocol. Products were then used as templates in PCR analysis. The mRNA transcription of the p14ARF and p16INK4a genes was evaluated by RT-PCR. The primer pairs specific for these genes were as follows; p14ARF sense, 5'-GGTTTTCGTGGTTCACATCCCGCG-3' and antisense, 5'-CAGGAAGCCCTCCCGGGCAGC-3'; p16INK4a sense, 5'-TTCGGCTGACTGGCTGGCCA-3' and antisense, 5'-AGC TCCTCAGCCAGGTCCAC-3'. The length of the p14ARF and p16INK4a PCR products was 254 and 330 bp, respectively. Efficacy of reverse transcription was controlled in each sample by PCR amplification of β-actin using the following primer pair: sense, 5'-TACATGGGTGGGGTGTTGAA-3' and antisense, 5'-AAGAGAGGCATCCTCACCCT-3'.

Methylation-specific PCR (MSP). To determine the methylation status at the 5'CpG island in the p16INK4a promoter region, we performed MSP. Bisulfite converts unmethylated cytosine residues to uracil, but methylated cytosines remain nonreactive. PCR amplifies uracil as thymine while methylated cytosines are only amplified as cytosines. MSP distinguishes unmethylated from methylated alleles in a given gene based on sequence changes after bisulfite treatment of DNA using primers designed for either methylated or unmethylated DNA (28). The CpGenome™ DNA Modification kit (Chemicon International, Inc., Temecula, CA) was used for MSP, as recommended by the supplier (29). Genomic DNA was treated with sodium bisulfite. PCR amplification was performed using p16INK4a promoter gene fragment-specific primers for either methylated or unmethylated DNA (CpG WIZ™ Amplification kit, Chemicon International, Inc.) (30). The unmethylated p16INK4a was amplified with a primer pair 5'-TTTTTGGT GTTAAAGGGTGGTGTAGT-3' and 5'-CACAAAAAC CCTCACTCACAACAA-3', which yielded a fragment of 132 bp. The primers for methylated p16INK4a were 5'-GTG TTAAAGGGCGGCGTAGC-3' and 5'-AAAACCCTCAC TCGCGACGA-3', which yielded a PCR product of 122 bp. Universal methylation DNA CpGenome™ (Chemicon International, Inc.) was used as a control for methylated DNA. PCR-amplified products were separated by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining under UV wave.

Treatment with AZA. To determine the optimal dose of AZA (Sigma, St. Louis, MO), MT2 cells were treated with increasing doses of AZA. MT2 cells at a concentration of $1x10^5$/ml were treated with 0.5, 1.0 or 2.0 μM of AZA. After cells were cultured for 72 h with AZA, RNA was harvested. The expression of p16INK4a mRNA was detected by RT-PCR.

Growth inhibition with AZA on MT2 cells. MT2 cells at a concentration of $1x10^5$/ml were treated with 2.0 μM of AZA. The number of viable cells was counted on days 2, 3, 4 and 5 using the trypan blue dye exclusion procedure. All experiments were repeated 3 times.

Results

Deletion of the p14ARF and p16INK4a gene. Nine cell lines were examined for deletions of the exon 2 of p14ARF/p16INK4a. The K562 cell line served as a negative control. The gene was deleted in only one cell line, OKM3T (Fig. 1).

Expression of p14ARF. Expression of p14ARF mRNA was examined in 9 cell lines by RT-PCR. Only OKM3T did not express p14ARF mRNA (Fig. 2) due to deletion of the gene. The p14ARF mRNA was also amplified for primary ATL samples. Of the 11 ATL samples analyzed, p14ARF was not expressed in 6 (4 acute, 1 chronic and 1 undetermined), while 5 samples expressed p14ARF (2 acute, 1 lymphoma and 2 chronic) (Fig. 3).

Expression of p16INK4a. Among 9 cell lines, the OKM3T cell line in which exon2 of the p16INK4a gene was deleted did not express p16INK4a. An additional 5 cell lines (MT1, MT2,

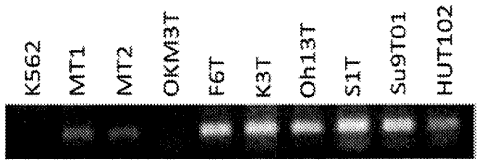

Figure 1. Deletion of the p14ARF and p16INK4a exon 2 gene in ATL cell lines. Among the 9 cell lines, the shared exon 2 of the p14ARF/p16INK4a gene was deleted in the OKM3T cell line. K562 was served as a negative control.

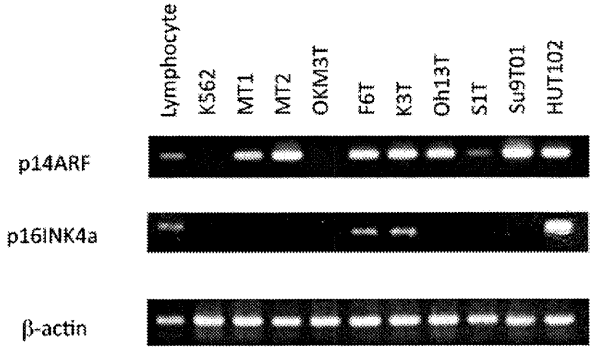

Figure 2. Expression of p14ARF and p16INK4a mRNA in ATL cell lines by RT-PCR. Among the 9 cell lines, the OKM3T cell line in which the shared p14ARF/p16INK4a exon 2 was deleted did not express either p14ARF or p16INK4a. Five other cell lines (MT, MT2, Oh13T, S1T and Su9T01) in which the p14ARF/p16INK4a exon 2 was not deleted, lacked expression of p16INK4a.

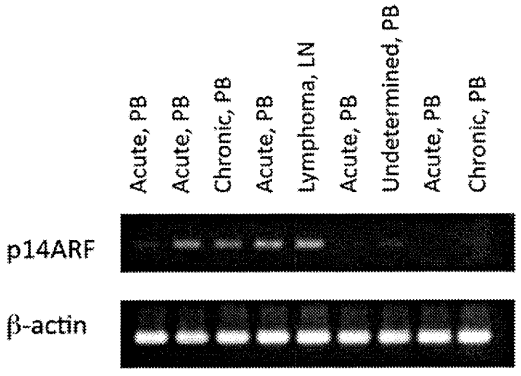

Figure 3. Expression of p14ARF in primary ATL samples. Representative cases are shown. Acute, PB, sample from peripheral blood in acute ATL; chronic, PB, sample from peripheral blood in chronic ATL; lymphoma, LN, sample from lymph node in lymphoma ATL; undetermined, PB, sample from peripheral blood in undetermined type ATL.

Oh13T, S1T and Su9T01) which had the p14ARF/p16INK4a exon 2 lacked expression of p16INK4a (Fig. 2).

Analysis of methylation of the p16INK4a gene. Because MT1 and MT2 did not express p16INK4a, the presence of methylation in the p16INK4a promoter region gene was assessed in the MT1 and MT2 cell lines using MSP. DNA obtained from lymphocytes from healthy volunteers was used as an unmethylated control. The K562 cell line served as a negative control. Both MT1 and MT2 cell lines displayed a band only when amplified with primers for methylated and not for unmethylated DNA (Fig. 4).

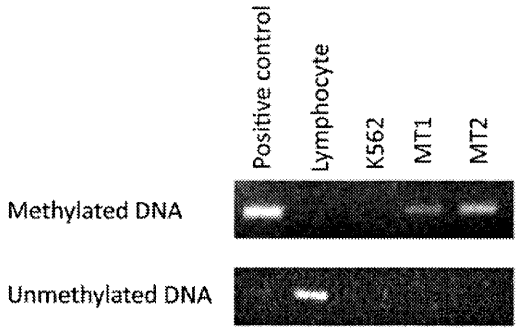

Figure 4. Methylation of the p16INK4a gene in MT1 and MT2 by methylation specific PCR (MSP). Both MT1 and MT2 cell lines displayed a band only when amplified with primers for methlyated DNA but not unmethylated DNA. DNA obtained from lymphocytes from healthy volunteers was used as an unmethylated control. The K562 cell line served as a negative control.

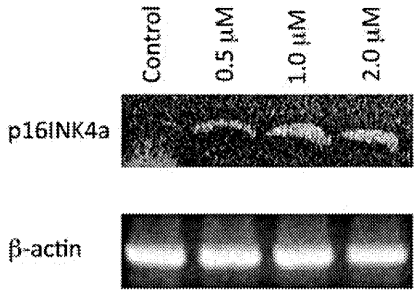

Figure 5. Restoration of p16INK4a gene expression by Azacitidine (AZA). AZA induced the demethylation of the p16INK4a gene promoter. Substantial demethylation of p16INK4a was observed after 72 h of treatment with 0.5 μM AZA. With higher doses of AZA (1.0 or 2.0 μM), the expression of p16INK4a mRNA was clearly up-regulated.

Restoration of p16INK4a gene expression by AZA. AZA-induced the demethylation of the p16INK4a gene promoter. Substantial demethylation of p16INK4a was observed after 72 h of treatment with 0.5 μM AZA. With higher doses of AZA (1.0 or 2.0 μM), the expression of p16INK4a mRNA was clearly up-regulated (Fig. 5).

AZA induces growth inhibition of MT2 cells. MT2 cells at a concentration of $1x10^5$/ml were treated with 2.0 μM of AZA. Growth of 2.0 μM-treated cells was inhibited compared with the control, although the difference was marginal. On Day 3, viable cell numbers in the control and 2.0 μM AZA treated MT2 cells were 4.8 and $4.1x10^6$/ml, respectively. After obtaining maximum cell growth, viable cells decreased in both groups (Fig. 6).

Discussion

ATL is triggered by infection of the human T-cell leukemia virus type I (HTLV-I) (1), with its pivotal oncoprotein Tax being stongly associated with the development of ATL (4). However, expression of Tax disappears in most ATL cells, suggesting that alternative mechanisms may be involved in the development of ATL. A statistical analysis revealed that five independent genetic events are probably required to develop ATL after viral infection of the target T cells (31).

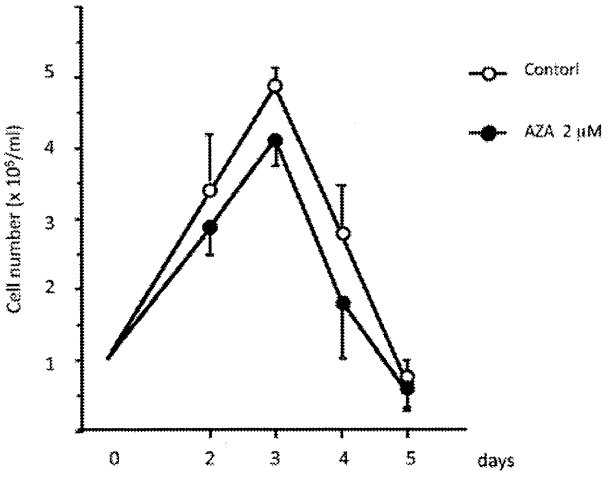

Figure 6. Azacitidine (AZA) induces growth inhibition of MT2 cells. MT2 cells at a concentration of $1x10^5$/ml were treated with 2.0 μM of AZA. Growth of 2.0 μM treated cells was inhibited compared with the control, although the difference was marginal. On Day 3, viable cell numbers in control and 2.0 μM AZA-treated MT2 cells were $4.8x10^6$/ml, and $4.1x10^6$/ml, respectively. After obtaining maximum cell growth, viable cells decreased in both groups.

p16INK4a, one of the CDKIs, resides in the CDKN2A locus on chromosome 9p. p16INK4a inhibits the catalytic activity of the CDK4/cyclin D complexes and blocks G1 to S transition in the cells (11,12). The CDKN2A locus also generates another cell cycle-regulatory protein, p14ARF (13,14). The human p14ARF protein causes arrest in the cell-cycle progression with an accumulation of cells in both G1 and G2/M, through binding to MDM2, which interferes with p53-MDM2 complex formation and proteasome degradation (32,33). Data suggest that both p14ARF and p16INK4A act as tumor suppressors whose inactivation contributes to the development of human tumors.

The homozygous deletion of the p16INK4a gene has been detected at a very high rate in many types of solid tumors as well as leukemias (34,35) especially in T-ALL (36,37). In ATL, we have shown that several tumor suppressor genes including p16INK4a, p18INK4c, and Rb were altered (7,10,36,37). The p16INK4a gene was frequently deleted in ATL (7). Furthermore, methylation of the 5' CpG island in the p16INK4a gene is associated with transcriptional silencing of the gene in many neoplasms including leukemias and lymphomas (38).

We screened the expression of p14ARF and p16INK4a in ATL cell lines. One (OKM3T) and 6 (MT1, MT2, OKM3T, Oh13T, S1T and Su9T01) of the 9 cell lines did not express p14ARF and p16INK4a mRNA, respectively. Absence of p16INK4a mRNA in the OKM3T cell line was due to deletion of the shared exon 2 gene of p14ARF/p16INK4a, while in the remaining 5 cell lines, it was due to gene silencing. Although we did not examine the genetic status of exon 1 of p16INK4a, deletion of the gene is unlikely, because, in our previous study, exons 1 and 2 of p16INK4a were homozygously deleted simultaneously in all ATL samples (7).

In our study, most of ATL cell lines expressed p14ARF mRNA, while 6 of 11 primary samples lacked expression of p14ARF. Esteller *et al* reported that p14ARF was epigenetically inactivated by hypermethylation in colorectal cancer cell lines as well as primary colorectal cancer samples (30). p14ARF genomic alterations are found in T-cell acute lymphocytic leukemias (39). Inactivation, either by deletion or methylation, of p14ARF may account for developing ATL.

DNA hypermethylation is the main epigenetic modification in tumorigenesis (40). Cytosine analogues such as 5-Aza-2'-deoxycytidine and AZA can induce expression of several tumor suppressor genes with unmethylated CpGs in malignant cells by inhibiting DNMTs (41,42). In recent reports, treatment with low-dose AZA was very encouraging in older patients with MDS and AML (24). Although the target genes of AZA in MDS have not been well-defined, one of the candidates is the p15INK4b gene that shares a great deal of nucleotide sequence homology with p16INK4a and is localized 25 kb upstream of the p16 INK4a locus on chromosome 9p21.

Nosaka *et al* reported the epigenetic inactivation of p16INK4a in ATL and suggested that methylation was a critical factor in disease progression (43). We demonstrate the demethylation in p16INK4a and induction of growth inhibition of the ATL cell line, MT2, with AZA. The expression of p16INK4a mRNA was up-regulated by treatment with 0.5 μM of AZA for 72 h. AZA showed inhibition of cell growth of ATL cell lines. Because no obvious toxicity of AZA was observed in phase 3 clinical trials for patients with high-risk MDS (21), our studies suggest a therapeutic approach for ATL. Although single administration of AZA is not sufficient to eradicate ATL cells, concomitant use with other anti-tumor agents such as thalidomide may be more effective as has been reported in MDS (23). This is particularly interesting because ATL cells are highly resistant to the induction of apoptosis and there is still no beneficial treatment other than allogeneic hematopoietic stem cell transplantation for this acute and fatal disease.

In conclusion, p16INK4a is epigenetically inactivated in ATL. AZA should be further investigated as a novel therapeutic agent for the management of ATL.

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ORIGINAL ARTICLE

Efficacy and safety of nilotinib in Japanese patients with imatinib-resistant or -intolerant Ph+ CML or relapsed/refractory Ph+ ALL: a 36-month analysis of a phase I and II study

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Abstract Although the tyrosine kinase inhibitor (TKI) imatinib is often used as first-line therapy for newly diagnosed chronic myelogenous leukemia (CML), some patients fail to respond, or become intolerant to imatinib. Nilotinib is a potent and selective second-generation TKI, with confirmed efficacy and tolerability in patients with imatinib-resistant or -intolerant CML. A phase I/II study was conducted in Japanese patients with imatinib-resistant or -intolerant CML or relapsed/refractory Ph+ acute lymphoblastic leukemia. Thirty-four patients were treated with nilotinib for up to 36 months. Major cytogenetic response was achieved in 15/16 patients (93.8%) with chronic-phase CML within a median of approximately 3 months. Major molecular response was achieved in 13/16 patients (81.3%). These responses were sustained at the time of the most recent evaluation in 13 patients and 11 patients, respectively. Hematologic and cytogenetic responses were also observed in patients with advanced CML. The BCR-ABL mutation associated with the most resistance to available TKIs, T315I, was observed in three patients. Common adverse events included rash, nasopharyngitis, leukopenia, neutropenia, thrombocytopenia, nausea, headache and vomiting. Most adverse events resolved following nilotinib dose interruptions/reductions. These results support the favorable long-term efficacy and tolerability of nilotinib in Japanese patients with imatinib-resistant or -intolerant chronic-phase chronic myeloid leukemia.

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Introduction

The tyrosine kinase inhibitor (TKI) imatinib (ST1571, Glivec™; Novartis) has been shown to induce durable responses in a high proportion of patients with chronic-phase chronic myeloid leukemia (CML-CP) [1–5]. However, disease progression caused by resistance to imatinib occurs in some CML patients treated with this drug [6].

CML patients in the accelerated phase (CML-AP) or in blast crisis (CML-BC) also show a complete cytogenetic response (CCyR) following treatment with imatinib, but the proportion of such patients achieving CCyR is considerably lower than that of CML-CP patients [7, 8]. Moreover, imatinib resistance and relapse are also common in CML-AP and -BC patients [6, 9]. Imatinib is also used to treat patients with Philadelphia chromosome-positive (Ph+) acute lymphoblastic leukemia (ALL), and many of these patients also achieve CCyR. However, the CCyRs in these patients are not sustained for as long as they are in CML-CP patients, both in Japan [10] and in other countries [11].

Approximately half of the cases of imatinib resistance are now known to result from mutations in *BCR-ABL* [12–16], which make particular leukemic cells resistant to BCR-ABL tyrosine kinase inhibition by imatinib.

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Nilotinib (AMN107, Tasigna®; Novartis) is a second-generation TKI that inhibits BCR-ABL-dependent cell proliferation and induces cell death in BCR-ABL phenotypic cells [17, 18]. Nilotinib was originally approved as second-line treatment for imatinib-resistant or -intolerant CML-CP and -AP patients [19–22]. More recently, it was approved as first-line therapy for CML-CP and -AP patients [23, 24] in Japan. Several studies have reported hematologic response (HR) and cytogenetic response (CyR) with nilotinib in patients with imatinib-resistant or -intolerant CML-BC and those with relapsed/refractory Ph+ ALL [25, 26].

We recently reported the results of a phase I and II study of nilotinib in which Japanese patients with imatinib-resistant or -intolerant Ph+ CML, or relapsed/refractory Ph+ ALL were treated for up to 12 months [22]. Here, we report the effects of treatment with nilotinib for up to 36 months in these patients, as well as the results of mutation analysis and the response by BCR-ABL mutation status.

Materials and methods

Study design and objectives

This was an open-label, multicenter, continuous-dose, 36-month extension of a phase I and II clinical study. The study protocol and documentation were approved by the institutional review boards of each participating center. The observation period was defined to be 36 months, including the entire 3 months of the Ph I/II clinical study. The study was conducted in accordance with the ethical principles established by the Declaration of Helsinki and in compliance with institutional guidelines.

The primary objective of this extension study was to evaluate the long-term safety of nilotinib, including chronic toxicity. Secondary objectives included the long-term efficacy of nilotinib, the relationship between BCR-ABL mutations or BCR-ABL transcript levels determined by quantitative RT-PCR, and the clinical efficacy of nilotinib. The time of last evaluation in this study was the time at which patients had received treatment for more than 3 years or the time at which the drug became commercially available at each of the study institutions, whichever was the later.

Patients

The inclusion and exclusion criteria are described in the original study report [22]. Briefly, Japanese patients were eligible if they had imatinib-resistant or -intolerant CML-CP, CML-AP, CML-BC or relapsed/refractory Ph+ ALL, were at least 20 years of age, had a World Health Organization (WHO) performance status (PS) ≤ 2, and had normal hepatic, renal and cardiac function.

Treatments

Nilotinib 400 mg was administered orally twice daily. Patients were required to fast for 2 h before and after each dose. One treatment course (1 cycle) was defined as 28 consecutive days of twice-daily nilotinib. If administration was delayed for more than 21 days (42 days for hematologic toxicity) after the previous dose, the patient was withdrawn from the study. Dose reductions to 400 mg once daily (one level lower than the standard dose) or 200 mg once daily (two levels lower than the standard dose) were permitted. The nilotinib dose at re-introduction was one level lower than that at cessation. The mean dose in each patient was calculated by assuming the dose during the cessation period to be 0 mg.

Treatment with nilotinib was continued until disease progression or unacceptable toxicity was observed, or at the investigator's discretion that treatment be discontinued. After the regulatory approval date for nilotinib in Japan (January 29, 2009), its administration was continued for longer than 3 years or until the drug became commercially available, whichever was later.

Measurements

Response rates

Criteria for HR and CyR were similar to those reported elsewhere [19, 21, 27] and are described in more detail in Tojo et al. [22]. Briefly, CyR was determined as the percentage of Ph+ cells of ≥20 cells in the metaphase in each bone marrow sample, and was classified as complete (0% Ph+ cells), partial (1–35% Ph+ cells), minor (36–65% Ph + cells) or minimal (66–95% Ph+ cells). Major CyR (MCyR) included complete and partial CyR. Fluorescent in situ hybridization was used if <20 cells were examined or if the bone marrow sample was not adequate for assessment.

The proportion of patients who experienced major molecular response (MMR) was also determined for each disease phase and subtype. BCR-ABL transcript levels were measured by quantitative RT-PCR and reported in the international scale using a conversion factor of 1.25 established by the Institute of Medical and Veterinary Science, Australia. MMR was defined as a BCR-ABL/BCR ratio ≤0.1%. Loss of MMR was defined as a BCR-ABL/BCR ratio >0.1%. Patients with MMR at baseline were considered "not evaluable" and were excluded from the analysis. Only evaluable patients in the intention-to-treat (ITT) population were included in the analyses of overall response rates.

Patients whose BCR-ABL transcript levels were not evaluated at baseline were considered "not assessable", and were not included in the denominator when calculating the proportion of patients who achieved MMR.

Mutation analysis

Efficacy was also examined based on the subtype of BCR-ABL mutation at baseline and after nilotinib administration. Mutation analysis was performed by the direct sequence identification method. The number and proportion of patients with HR, CyR or MMR were calculated for the following categories of mutation [22]: no mutation, any mutation, multiple mutations, P-loop mutations (amino acids 248–255), non-P-loop mutations, and protocol-specified subgroup mutations associated with imatinib resistance mutations (L248, Q252, T315, F317, H396, M237, M244, G250, D325, S348, M351, E355, A380, L387, M388, F486, Y253, E255, and F359). The impact of baseline mutations or development of new mutations on patient outcomes was assessed.

Safety analyses

Safety assessment included an evaluation of the frequency and severity of adverse events, which included hematologic and biochemical laboratory tests, vital signs, physical examinations (including body weight), WHO PS, cardiac function tests (12-lead ECG, cardiac enzyme test, echocardiography), and chest X-rays, as needed. Adverse events were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (version 3.0). The monitoring was continued for at least 28 days after the last dose of nilotinib.

Statistical analyses

The ITT population was used for the efficacy analysis and was pre-specified as all patients enrolled in either the phase I or phase II studies, and who were treated with nilotinib 400 mg twice daily, irrespective of when they withdrew from the study. The safety (SAF) population comprised all patients in the ITT population who underwent safety assessments. HR, CyR and MMR were summarized by disease phase and subtype (CML-CP, CML-AP, CML-BC, and Ph+ ALL). The time to first response and duration of response were assessed by descriptive statistics or Kaplan–Meier analysis, as appropriate. No statistical comparisons were made.

Results

Patients and treatment administration

This 36-month study included 34 Japanese patients with imatinib-resistant or -intolerant CML (CML-CP, $N = 16$; CML-AP, $N = 7$; CML-BC, $N = 4$) or Ph+ ALL ($N = 7$).