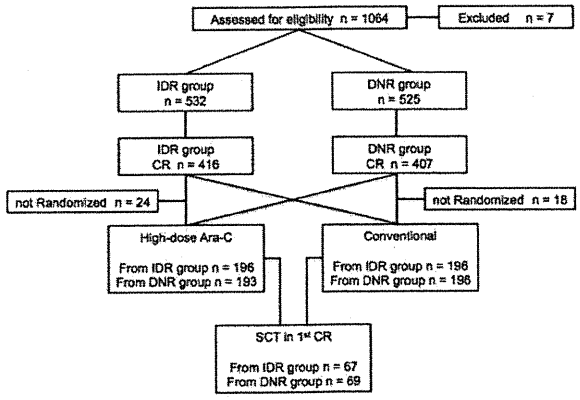

Figure 1. CONSORT flow diagram. IDR indicates idarubicin; DNR, daunorubicin; CR, complete remission; Ara-C, cytarabine; and SCT, stem cell transplantation.

Results

Patient characteristics

Among 1064 registered patients, 7 did not meet the inclusion criteria (misdiagnosis, 1; infectious complication, 1; without therapy, 1; and withdrawal of consent, 4). The study population thus comprised 1057 patients (Figure 1). Patient characteristics are presented in Table 1. Median age was 47 years (range, 15-64 years). Cytogenetics data were available for 1021 patients (96.6%). Among these, 247 (24.2%) were classified in the favorable-risk group, 681 (66.7%) in the intermediate-risk group, and 93 (9.1%) in the adverse group. Five hundred thirty-two patients were assigned to the idarubicin group and 525 to the daunorubicin group. The 2 groups were well balanced with regard to pretreatment characteristics such as age, initial WBC counts, FAB classification, and cytogenetic prognostic grouping.

Table 1. Patient characteristics

	IDR group (n = 532)	DNR group (n = 525)	P
Median age, y (range)	47 (15-64)	47 (15-64)	.781
≤ 50	310	306	
> 50	222	219	.996
Median WBC count, ×10⁹/L (range)	13.7 (0.1-382)	15.3 (0.1-334)	.769
≤ 20 × 10⁹/L	304	297	
20= ≤ 50 × 10⁹/L	95	104	
> 50 × 10⁹/L	125	121	
Unknown	8	3	.427
FAB type			
M0	30	30	
M1	95	94	
M2	232	233	
M4	100	100	
M5	56	51	
M6	17	16	
M7	2	1	.997
Cytogenetic group			
Favorable	128	119	
Intermediate	335	346	
Adverse	49	44	
Unknown	20	16	.561
MPO-positive blasts, %			
< 50	169	187	
≥ 50	307	292	
Unknown	56	46	.330
Performance status			
0, 1, 2	512	509	
3	20	16	.524

Values are number of patients unless otherwise indicated.
IDR indicates idarubicin; DNR, daunorubicin; WBC, white blood cell count; FAB, French-American-British classification; and MPO, myeloperoxidase.

Table 2. Results of induction therapy

	IDR group, n (%)	DNR group, n (%)
Patients	532	525
CR	416 (78.2)	407 (77.5)
CR by 1 course	341 (64.1)	321 (61.1)
CR by 2 courses	75 (14.1)	86 (16.4)
95% CI	74.5-81.5	73.8-80.9

IDR indicates idarubicin; DNR, daunorubicin; and CR, complete remission.

Response to induction therapy

Overall, of 1057 evaluable patients, 823 (77.9%) achieved CR. Of 532 patients in the idarubicin group, 416 (78.2%) achieved CR, and of 525 in the daunorubicin group, 407 (77.5%) obtained CR ($P = .79$). Noninferiority for the primary end point was assessed by determining whether the lower bound of the 95% confidence interval (CI) of the difference between the CR rates for the daunorubicin and idarubicin groups was less than −10%. The CR rate of the daunorubicin group was noninferior to that of the idarubicin group (Table 2). In the idarubicin group, 341 patients (64.1%) achieved CR after the first course, and in the daunorubicin group, 321 (61.1%) did so ($P = .39$). The average period to achieve CR was 33.8 days (95% CI 32.9 to 34.6 days) in the idarubicin group and 32.4 days (95% CI 31.6 to 33.2 days) in the daunorubicin group ($P = .038$). CR rates related to FAB classification, age, and cytogenetics are shown in Table 3. Although they were few, patients with FAB M6 responded better to idarubicin: 78% of 17 patients in the idarubicin group and 38% of 16 in the daunorubicin group achieved CR ($P = .037$). There were no differences in CR rate between the 2 groups in other FAB subtypes, cytogenetic risk groups, age, myeloperoxidase positivity of blasts, initial WBC count, or performance status (Table 3). Overall, logistic regression analysis revealed that induction regimen was not an independent prognostic factor but that cytogenetic group and percentage of myeloperoxidase-positive blasts were significant independent factors for achieving CR (Table 4). A cutoff value of WBCs at 20 or 50 × 10⁹/L did not change the result.

OS and RFS

At a median follow-up of 48 months, 5-year predicted OS rates were 48% for the idarubicin group (95% CI 43% to 53%) and 48% for the daunorubicin group (95% CI 43% to 53%; $P = .54$; Figure 2A), and 5-year predicted RFS rates of CR patients were 41% (95% CI 36% to 46%) and 41% (95% CI 35% to 45%), respectively ($P = .97$; Figure 2B). Significant unfavorable prognostic features for OS by the Cox proportional hazard model were adverse cytogenetic risk group, age greater than 50 years, WBC count more than 20 × 10⁹/L, myeloperoxidase-positive blasts less than 50%, and FAB classification of either M0, M6, or M7; for RFS, the significant unfavorable prognostic features were adverse cytogenetic risk group, WBC count more than 20 × 10⁹/L, myeloperoxidase-positive blasts less than 50%, lactate dehydrogenase of 500 IU/L or more, and age greater than 50 years. Induction regimen was not an independent prognostic factor for either OS or RFS by this multivariate analysis.

Table 3. CR rates by induction therapy

	CR rate, %		
	IDR group (n = 532)	DNR group (n = 525)	*P*
FAB type			
M0	43	63	.195
M1	86	79	.236
M2	80	82	.718
M4	81	79	.86
M5	77	75	.96
M6	76	38	.037
M7	50	100	.999
Cytogenetic group			
Favorable	91	96	.134
Intermediate	79	76	.359
Adverse	51	43	.534
Unknown	50	69	.257
Age, y			
≤ 50	83	77	.108
> 50	73	78	.225
Myeloperoxidase-positive blasts, %			
< 50	68	66	.709
≥ 50	87	88	.699
WBC at diagnosis, ×10⁹/L			
≤ 20	79	76	.767
20= ≤ 50	82	82	.993
> 50	74	77	.824
Performance status			
0, 1, 2	79	78	.762
3	80	75	.999

CR indicates complete remission; IDR, idarubicin; DNR, daunorubicin; FAB, French-American-British classification; and WBC, white blood cell count.

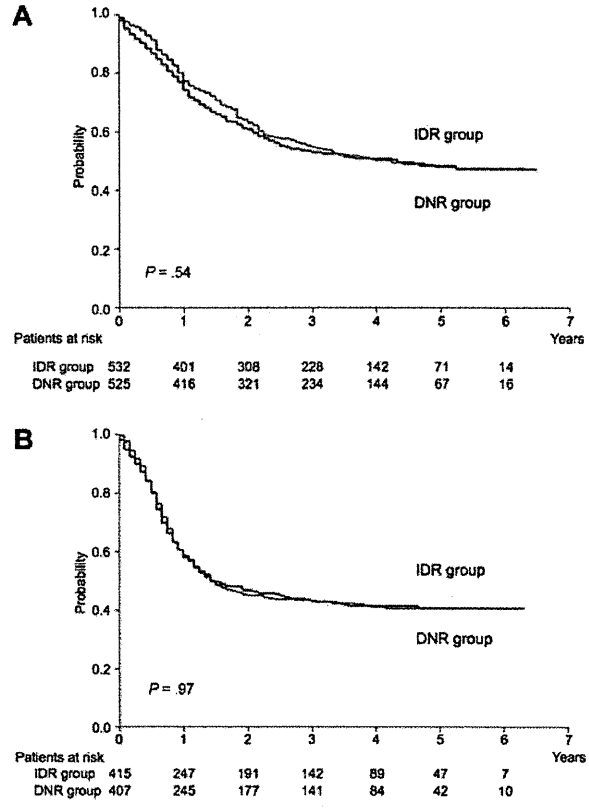

Figure 2. OS and RFS. (A) Predicted 5-year overall survival (OS) was 48% for the idarubicin group (IDR; n = 532; red line) and 48% for the daunorubicin group (DNR; n = 525; blue line; *P* = .54). (B) Predicted 5-year relapse-free survival (RFS) was 41% for the idarubicin group (IDR; n = 416; red line) and 41% for the daunorubicin group (DNR; n = 407; blue line; *P* = .97).

Adverse events

Patients receiving idarubicin required a slightly but significantly longer time to recover from neutropenia and thrombocytopenia. Median duration with a neutrophil count less than 1.0×10^9/L was 28 days for the idarubicin group and 27 days for the daunorubicin group ($P = .0011$; Figure 3A). Median duration with a platelet count less than 100×10^9/L was 25 days for the idarubicin group and 24 days for the daunorubicin group ($P = .0034$; Figure 3B). Sepsis occurred more frequently in the idarubicin group than in the daunorubicin group (8.7% and 4.9%, respectively; $P = .02$). Early death within 60 days occurred more frequently in the idarubicin group than in the daunorubicin group (4.7% and 2.1%, respectively; $P = .03$; Table 5).

Postremission therapy

Of the 823 CR patients, 781 were randomly assigned to receive either 4 courses of conventional standard-dose consolidation therapy (392 patients) or 3 courses of high-dose Ara-C therapy (389 patients), and 136 patients (16% of CR patients) underwent allogeneic SCT in the first CR. There was no significant difference in OS or RFS by postremission therapy between the idarubicin and daunorubicin groups (Table 6). In the idarubicin group, predicted 5-year OS rates were 57% for the conventional standard-dose consolidation arm (95% CI 49% to 65%) and 58% for the high-dose Ara-C arm (95% CI 51% to 66%; $P = .79$; Figure 4A). In the daunorubicin group, predicted 5-year OS rates were 56% (95% CI 48% to 63%) and 58% (95% CI 50% to 65%; $P = .71$; Figure 4B), respectively. If 2 groups were evaluated together, predicted 5-year OS rates were 56% (95% CI 51% to 62%) and 58% (95% CI 53% to 62%; $P = .95$), and predicted 5-year RFS rates were 39% (95% CI 34% to 44%) and 43% (95% CI 38% to 48%), respectively ($P = .72$). The detailed results of this consolidation phase will be reported in a separate paper.[22]

Table 4. Factors that predicted CR in all evaluable patients by multivariate analysis

Variables	Odds ratio	*P*
Cytogenetic group		
Favorable	10.39	< .0001
Intermediate	4.67	< .0001
Myeloperoxidase-positive blast ≥ 50%	2.64	< .0001
Induction therapy: IDR arm	0.97	.854

CR indicates complete remission; and IDR, idarubicin.

Discussion

The present randomized study demonstrates that if the dose intensity is increased appropriately, daunorubicin is as effective as a standard dose of idarubicin for adults less than 65 years of age

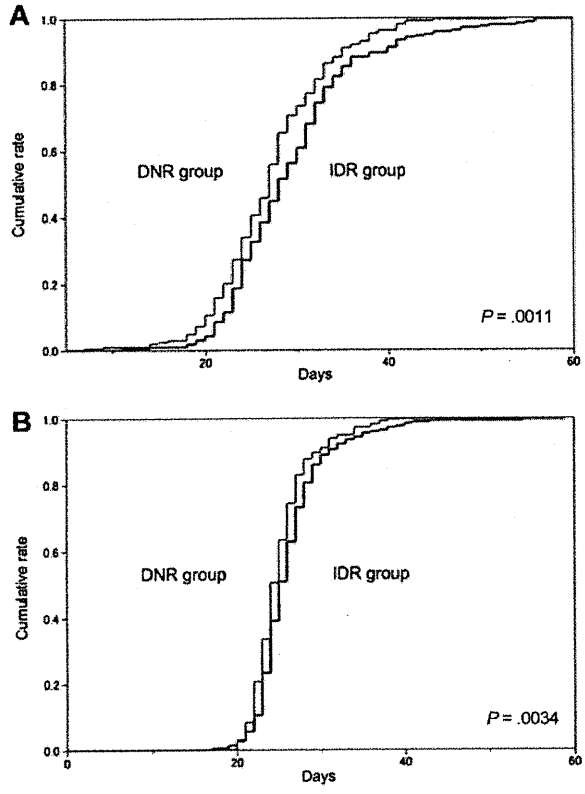

Figure 3. Hematologic recovery. (A) Day of recovery from neutropenia after the first induction course. Neutropenia was defined as neutrophil count $< 1.0 \times 10^9$/L. Median duration until recovery was 28 days for the idarubicin group (IDR; red line) and 27 days for the daunorubicin group (DNR; blue line; $P = .0011$). (B) Day of recovery from thrombocytopenia after the first induction course. Thrombocytopenia was defined as platelet count $< 100 \times 10^9$/L. Median duration until recovery was 25 days for the idarubicin group (IDR; red line) and 24 days for the daunorubicin group (DNR; blue line; $P = .0034$).

who have been newly diagnosed with AML. Remission-induction therapy with 50 mg/m^2 of daunorubicin for 5 days resulted in almost the same CR rate and long-term outcome as seen with 12 mg/m^2 of idarubicin for 3 days in combination with 100 mg/m^2 of Ara-C for 7 days. Generally, daunorubicin is used at a dose of 45 to 50 mg/m^2 for 3 days in combination with 100 to 200 mg/m^2 of Ara-C for 7 days, and 50% to 70% of newly diagnosed adult patients with AML achieve CR. As stated in the "Introduction," JALSG used a response-oriented individualized induction therapy in the AML87, AML89, and AML92 studies for AML, which permitted the additional daunorubicin and other antileukemia drugs

Table 5. Adverse events (World Health Organization grades 3 to 5) after the start of induction therapy

	IDR group, no. of patients (%)	DNR group, no. of patients (%)	*P*
Sepsis	46 (8.7)	26 (4.9)	.021
Early death*	25 (4.7)	11 (2.1)	.026
Bleeding	19 (3.6)	23 (4.4)	.532
Febrile neutropenia	416 (78.2)	406 (77.4)	.761
Acute cardiac toxicity	10 (1.9)	4 (0.8)	.112
Late-onset cardiac failure	2 (0.38)	2 (0.38)	.998

IDR indicates idarubicin; and DNR, daunorubicin.
*Death within 60 days after the start of induction therapy.

Table 6. Effect of induction therapy on outcome by postremission therapies

Consolidation arm	5-year OS IDR group	5-year OS DNR group	5-year RFS IDR group	5-year RFS DNR group
Conventional standard-dose, %	57	56	41	37
P	.759		.332	
High-dose Ara-C, %	58	58	42	44
P	.725		.658	
Allogeneic SCT in first CR, %	59	59	58	64
P	.469		.394	

Number of patients in the conventional standard-dose arm was 196 in the IDR group and 196 in the DNR group; in the high-dose Ara-C arm, the numbers were 196 and 193, respectively; and in the SCT group, the numbers were 67 and 69, respectively, as shown in Figure 1.

OS indicates overall survival; RFS, relapse-free survival; IDR, idarubicin; DNR, daunorubicin; Ara-C, cytarabine; and CR, complete remission.

to be administered according to bone marrow status on day 8 or later.[12-14] The CR rates in these 3 studies ranged from 77% to 80%, and the median total dose of daunorubicin was 240 mg/m^2.

On the basis of these experiences and also because of the regulation of our national medical insurance system, we used a dose and schedule of daunorubicin of 50 mg/m^2 for 5 days, that is, a total dose of 250 mg/m^2. In addition,we avoided higher daily doses, such as 80 mg/m^2 for 3 days, because higher plasma concentration might cause more cardiotoxicity in older patients.[23]

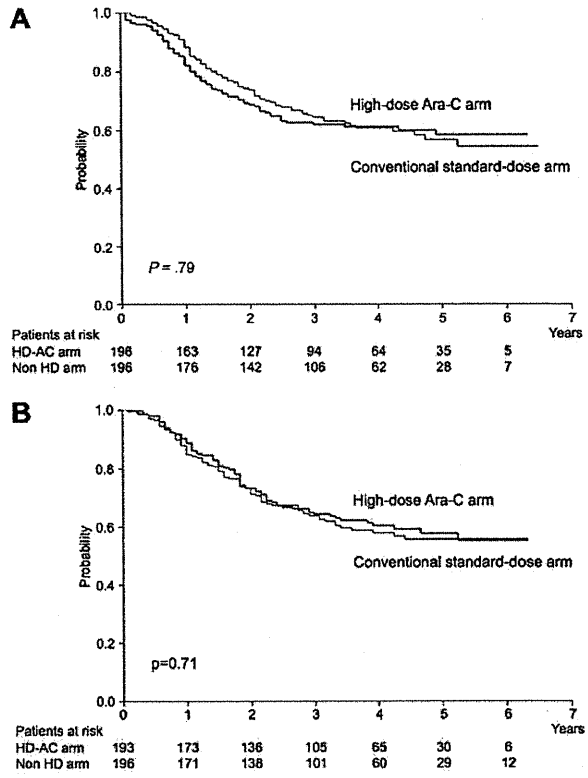

Figure 4. OS of CR patients randomized to receive consolidation therapy. (A) In the idarubicin group, predicted 5-year OS was 58% for the high-dose Ara-C arm (n = 196; red line) and 57% for the conventional standard-dose arm (n = 196; blue line; $P = .79$). (B) In the daunorubicin group, predicted 5-year OS was 58% for the high-dose Ara-C arm (n = 193; red line) and 56% for the conventional standard-dose arm (n = 196; blue line; $P = .71$). Ara-C indicates cytarabine; HD-AC arm, high-dose Ara-C arm; and Non HD arm, conventional standard-dose arm.

Three randomized studies in the early 1990s[4-6] and subsequent studies[24,25] and meta-analyses[7] reported a superior effect of idarubicin (12 to 13 mg/m^2 × 3 days) over that of daunorubicin (45 to 50 mg/m^2 × 3 days), in combination with Ara-C, and AML patients receiving idarubicin obtained 70% to 80% CR without a significant increase in toxic mortality, whereas those receiving daunorubicin achieved 58% to 65% CR.[4-6] However, because the duration of neutropenia and thrombocytopenia was longer in the idarubicin groups, it was questioned whether the doses used in these comparisons were equivalent in terms of levels of toxicity and whether any observed advantage represented an inherent biological advantage of idarubicin rather than biological dose equivalence.[1,2]

In these randomized studies, Wiernik et al reported that patients with initial WBC counts > 50 × 10^9 cells/L obtained only 32% CR by the daunorubicin regimen compared with 68% CR by the idarubicin regimen, whereas patients with WBC counts < 50 × 10^9/L obtained 65% and 69% CR, respectively.[5] Berman et al also reported that patients in the idarubicin group did well regardless of their initial WBC count, whereas patients in the daunorubicin group had a decreased response rate as the WBC count increased.[4] In the present study, however, a total of 250 mg/m^2 of daunorubicin resulted in almost the same CR rate as a total dosage of 36 mg/m^2 of idarubicin regardless of initial WBC counts and other prognostic factors such as cytogenetics, age, and FAB classification except M6. Although among patients with FAB M6, 16 patients in the daunorubicin group had a significantly lower CR rate than 17 patients in the idarubicin group, we have no clear explanation for this observation, because the small number of patients made further analysis difficult. Thus, the increased total dosage of daunorubicin administered in 5 days would be responsible for almost the same satisfactory CR rate and long-term outcome as idarubicin administered in 3 days in the present study. As for adverse events, the recovery from neutropenia and thrombocytopenia was slightly but significantly delayed in the idarubicin group, and sepsis and early mortality occurred more frequently in the idarubicin group, as shown in Figure 3 and Table 5.

Before we initiated the present AML201 study, there was no evidence that a higher dose of daunorubicin was more effective than its standard dose because of the lack of a prospective randomized study. In the sequential studies reported by Southwest Oncology Group, however, the CR rate with daunorubicin at a dose of 70 mg/m^2 was better than that with 45 mg/m^2.[26,27] Very recently, 2 groups reported that a higher dose of daunorubicin improved the CR rate and OS in prospective randomized studies.[28,29] A collaborative group composed of the Dutch-Belgian Cooperative Trial Group for Hemato-Oncology, the German AML Study Group, and the Swiss Group for Clinical Cancer Research compared 3-day daunorubicin at 90 mg/m^2 with 3-day daunorubicin at 45 mg/m^2, in combination with 7-day Ara-C, in elderly patients 60 to 83 years of age who had AML or high-risk refractory anemia and reported a higher CR rate for the escalated-treatment group (52% vs 35%, $P = .002$).[28] Although survival end points did not differ significantly overall, among patients 60 to 65 years of age, the CR rate (73% vs 51%) and OS rate (38% vs 23%) were significantly higher for the 90-mg/m^2 group. The Eastern Cooperative Oncology Group also compared 3-day daunorubicin at 90 mg/m^2 with 3-day daunorubicin at 45 mg/m^2, in combination with 7-day Ara-C, in patients 17 to 60 years of age with AML and reported a higher CR rate (70.6% vs 57.3%, $P < .001$) and longer OS (median 23.7 vs 15.7 months, $P = .003$) for the high-dose group.[29] Given these previous reports and the present report, the optimal total dose of daunorubicin is still to be explored but may rest somewhere between 250 and 270 mg/m^2. Because we used the FAB classification in the present study, we did not include either patients with 20% to 30% of blasts in the bone marrow or those with refractory anemia with excess blasts; therefore, it is unclear whether the present result is applicable to those patients.

Idarubicin is a derivative of daunorubicin and differs from its parent compound by the deletion of a methoxy group at position 4 of the chromophore ring. In vitro and preclinical data have shown that idarubicin is more lipophilic, is faster in cellular uptake, exhibits increased cellular retention, is lower in susceptibility to P-glycoprotein–dependent resistance, and is less cardiotoxic than daunorubicin. Both idarubicin and daunorubicin undergo conversion to their respective alcohol metabolites, idarubicinol and daunorubicinol. Unlike the latter, idarubicinol has a prolonged plasma half-life and is thought to have a pharmacologic advantage.[30-33]

The pediatric Berlin-Frankfurt-Münster group previously compared idarubicin 12 mg/m^2 for 3 days with daunorubicin 30 mg/m^2 twice daily for 3 days, in combination with Ara-C and etoposide, and reported almost the same CR rates (85% vs 86%, respectively) and predicted 5-year event-free survival (55% vs 49%, respectively, $P = .29$) in newly diagnosed childhood AML.[34] Furthermore, daunorubicin at a dose of 60 mg/m^2 for 3 days and idarubicin at a dose of 12 mg/m^2 for 3 days achieved similar CR rates in the studies by Eastern Cooperative Oncology Group that consisted of a large number of adult patients.[35,36]

Recently, the French Acute Leukemia Association reported a randomized study comparing standard doses of idarubicin (12 mg/m^2 for 3 days) with high doses of daunorubicin (80 mg/m^2 for 3 days) or idarubicin (12 mg/m^2 for 4 days) for remission induction in newly diagnosed elderly patients 50 to 70 years of age (median 60 years old) with AML.[37] CR rates were significantly higher for the standard-dose idarubicin group (83%) than for the high-dose daunorubicin group (70%, $P = .007$) but not for the high-dose idarubicin group (78%, $P = .12$). Although OS, relapse incidence, and event-free survival were not different among the 3 arms of the study, daunorubicin (80 mg/m^2 for 3 days) did not improve the CR rate of elderly AML patients to the level of the standard-dose idarubicin regimen.

With regard to adverse events, recovery from myelosuppression was faster and sepsis was less frequent in the daunorubicin group. Both acute and late-onset cardiotoxicity were reported only in a small number of patients in both groups. Given that there was no increase in severe cardiac toxicities in patients receiving high-dose daunorubicin (90 mg/m^2 for 3 days) compared with standard-dose daunorubicin (45 mg/m^2 for 3 days) in the Eastern Cooperative Oncology Group study (7.9% and 7.2%, respectively),[29] daunorubicin may not necessarily be administered for 5 days as in the present study (50 mg/m^2 for 5 days), although further follow-up observation is needed for late-onset cardiotoxicity.

Since the landmark study of the Cancer and Leukemia Group B,[38] it has been believed that high-dose Ara-C is superior to consolidation therapy with intermediate (400 mg/m^2 for 5 days) or conventional (100 mg/m^2 for 5 days) doses of Ara-C. In the present study, we prospectively compared high-dose Ara-C with consolidation therapy that included a conventional dose of Ara-C and non–cross-resistant agents. Our results clearly demonstrate that there is no difference in RFS and OS between the 2 consolidation arms, regardless of whether idarubicin or daunorubicin is used as induction chemotherapy.

In conclusion, the intensified dose of daunorubicin in the present setting, that is, 50 mg/m^2 for 5 days, proved to be biologically equivalent in terms of efficacy and no more toxic in terms of myelosuppression than the standard dose and schedule of idarubicin, that is, 12 mg/m^2 for 3 days, for remission-induction therapy in newly diagnosed younger patients (15 to 64 years old, median 47 years) with AML.

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Authorship

Contribution: S.O. designed and performed research, collected and interpreted data, and wrote the manuscript; S.M. designed and performed research, analyzed data, and participated in writing the manuscript; H.F., H.K., K.S., N.U., H.O., K.M., C.N., Y.M., A.F., T. Nagai, T.Y., M. Taniwaki, M. Takahashi, F.Y., Y.K., N.A., H.S., and H.H. performed research; S.H. analyzed data; K.O. and T. Naoe conducted and performed research; and R.O. conducted research, interpreted data, and participated in writing the manuscript.

For a complete list of the members of the JALSG, see the supplemental Appendix (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

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

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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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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,

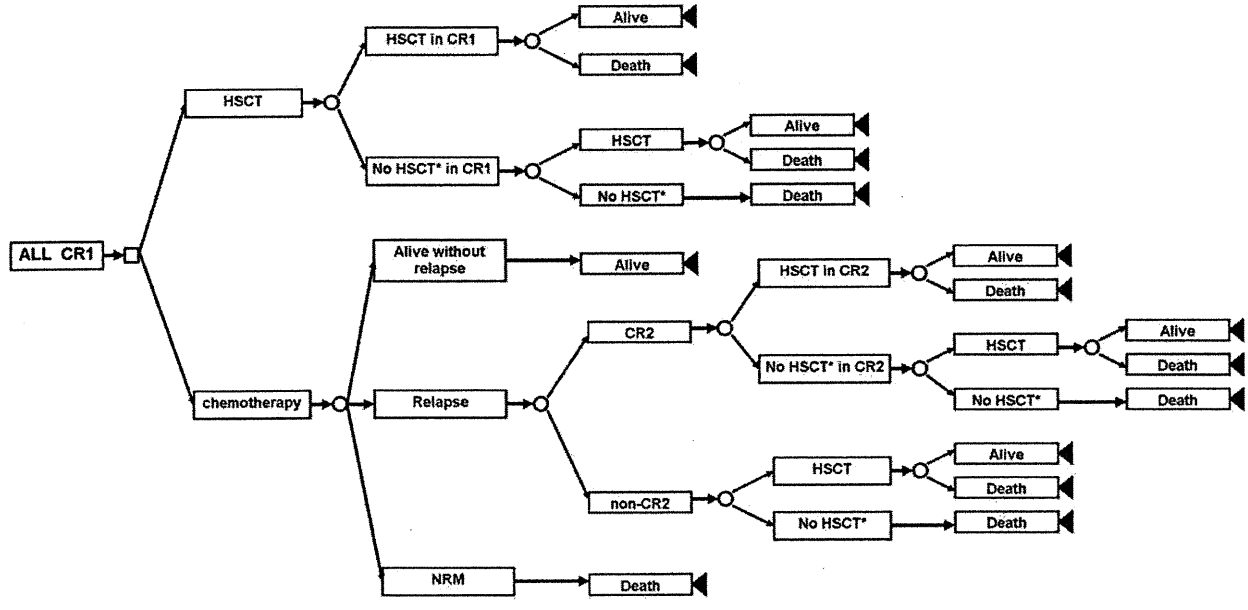

Figure 1 Decision tree used in this study. Decision analysis was performed on the basis of a decision tree. A square indicates a decision node and open circles indicate chance nodes. In analyses with a QOL adjustment, 'Alive' after transplantation was followed by two branches with or without active chronic 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.

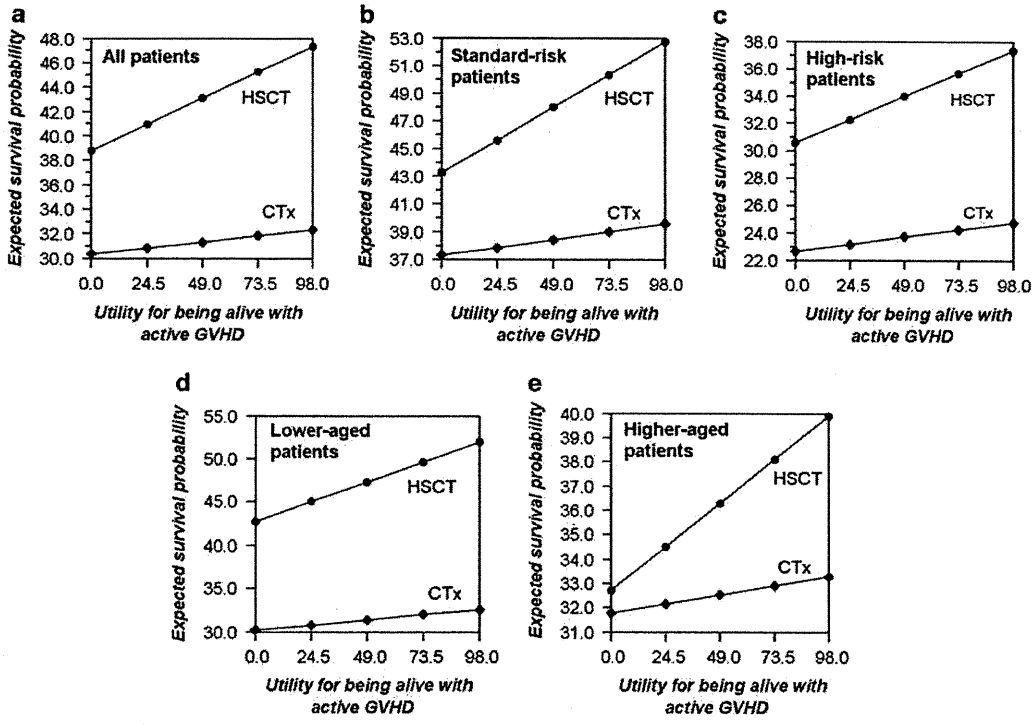

Figure 2 One-way sensitivity analysis for the utility for being alive with active GVHD. We performed one-way sensitivity analyses for the utility for being alive with active GVHD in the model, with adjustment for QOL. The superiority of allogeneic HSCT compared with chemotherapy (CTx) was consistently 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

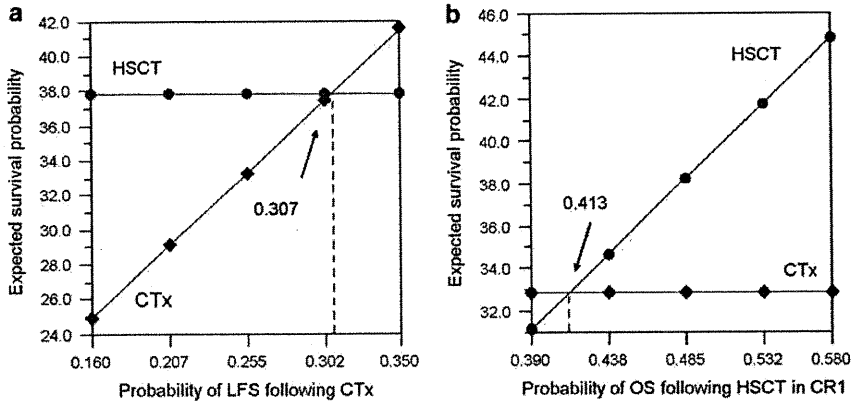

Figure 3 One-way sensitivity analysis in higher-aged patients. We performed one-way sensitivity analyses for all TPs in the decision model both with and without adjustment for QOL. In higher-aged patients, the result reversed if the probability of LFS at 10 years without relapse following chemotherapy (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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Experimental Hematology

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High expression of 67-kDa laminin receptor relates to the proliferation of leukemia cells and increases expression of GM-CSF receptor

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Objective. **The 67-kDa laminin receptor (LR) is a nonintegrin receptor for laminin, a major component of the extracellular matrix. To elucidate the role of LR in leukemia cells, we studied the relationship between the phenotype of leukemia cells and LR expression.**

Materials and Methods. **The relationship between clinical features of acute myeloid leukemia and expression of LR was examined. LR was overexpressed or suppressed by the introduction of complementary DNA or small interfering RNA for LR in a human leukemia cell line to test the effect of LR on the phenotype of leukemia. Expression of granulocyte-macrophage colony-stimulating factor receptors (GM-CSFR) was also tested in leukemia cells, including clinical samples.**

Results. **Expression of LR was significantly related to elevation of white blood cell count, lactate dehydrogenase, and survival among acute myeloid leukemia patients. Forced expression of LR enhanced proliferation, cell-cycle progression, and antiapoptosis of leukemia cells associated with phosphorylation of a transcription factor, signal transducer and activator of transcription 5, in the absence of stimulation by laminin. On the other hand, suppression of LR expression had the opposite effects. The number of GM-CSFR increased in leukemia cells overexpressing LR, and there was a significant relationship between the expression of LR and GM-CSFR in acute myeloid leukemia samples.**

Conclusions. **These results suggest that LR expression influenced the characteristics of leukemia cells toward an aggressive phenotype and increased the number of GM-CSFR. These changes might be partly related to enhanced GM-CSF signaling.** © **2011 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Proliferation and differentiation of hematopoietic cells are strictly regulated via intrinsic and extrinsic signals [1]. Signal from the extracellular matrix (ECM), one of the extrinsic signals, has a significant influence on the control of normal and abnormal hematopoiesis [2,3]. For example, for proliferation and maintenance of leukemia-initiating cell, which is capable of propagating full-blown leukemia, a specific environment called "niche" is required, in which ECM plays a role [4–6]: stimulation from stromal cells through CD44 and its ligand [7,8] and that from the extracellular matrix through very late antigen 4 and fibronectin [9].

Laminin belongs to a family of heterotrimetric glycoproteins composed of α, β, and γ chains, which are major components of ECM [10,11]. There are >12 laminin isoforms that target multiple receptors on the cell surface. The functions of laminin are widely divergent and include the following: structural roles in the basement membrane, adhesion of normal and malignant cells to the matrix, promotion of malignant phenotypes, regulation of growth and metastasis of tumors, and induction of apoptosis through, for example, the Rho and phosphatidylinositol 3 kinase/Akt signaling

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pathways [12–14]. Hereditary abnormalities in laminins result in congenital diseases, such as epidermolysis bullosa, which produces skin fragility and congenital muscular dystrophy [15,16].

Most of the receptors for laminin are in the integrin family, but the 67-kD laminin receptor (LR) is a nonintegrin receptor [10,11]. LR is widely expressed on cells in different tissues, including hematopoietic cells, under both normal and pathological conditions. LR expression relates to poor prognosis in patients with solid tumors and the metastasis or progression of breast and lung cancer, suggesting the importance of LR expression in malignancy [17–19]. LR is also expressed on various hematopoietic cells such as activated T cells [20], CD34-positive peripheral blood cells mobilized by granulocyte-colony stimulating factor [21], erythroid progenitor cells in bone marrow [22], and AML cells [23]; however, its role in AML is not fully understood.

Granulocyte-macrophage colony-stimulation factor (GM-CSF) is a myeloid hematopoietic cytokine with multiple functions in immature and mature myeloid cells [24]. It has a permissive role in the commitment of myeloid cells [25], promotes growth of granulocytic and monocytic cells, and activates mature myeloid cell functions. The physical and functional relationship between LR and GM-CSF receptors (GM-CSFR) was reported [26]. In that report, it was demonstrated that LR binds to GM-CSFR α- and β-chains, which modulate signaling through the GM-CSFR. Because GM-CSF is an important cytokine for inflammation and host-defense, this provided a new mechanistic basis for the control of host-defense cells via GM-CSF.

These reports prompted us to elucidate the role of LR in AML using clinical samples and leukemia cell lines. Our results demonstrated that increased LR expression on CD34-positive AML cells was related to high white blood cell (WBC) count, elevated lactate dehydrogenase (LDH), and poor prognosis among AML patients. Modulation of the level of LR influenced proliferation and resistance to apoptosis of, and GM-CSFR expression on, leukemia cells. These data suggest important roles for LR in the biology of AML.

Materials and methods

Clinical samples

Bone marrow cells were collected from 44 AML patients before chemotherapy and 7 healthy volunteers, with permission, under a protocol approved by the Internal Review Board of Nagasaki University (approval no. 33-3). CD34-positive AML cells were selected using Ficoll density gradient centrifugation and magnet beads (CD34 Isolation Kit, Auburn, CA, USA) to minimize the confounding effect of LR expression on mature myeloid cells [23].

Flow cytometric analysis

Flow cytometric data (FACScan, Becton Dickinson, San Jose, CA, USA) were analyzed using CellQuest (Becton Dickinson) and FlowJo (Tree Star, Ashland, OR, USA) software. Mean fluorescence intensity (MFI) ratio was calculated by dividing the MFI of the target antigen by that of the respective nonspecific isotype control. Anti-LR antibody (laminin receptor Ab-1, Clone MluC5; Neo Markers, Union City, CA, USA), phycoerythrin-labeled goat anti-mouse IgM Fab fragment (Rockland Immunochemicals, Gilbertsville, PA, USA), phycoerythrin-labeled anti-human GM-CSFR α-antibody (Clone #31916; R & D Systems, Minneapolis, MN, USA), and phycoerythrin-labeled isotype controls were used for the analysis of both LR and GM-CSFR expression.

Modulation of LR expression on leukemia cells

We used the GM-CSF–dependent human leukemia cell lines TF-1 [27] and AML193 [28] to generate leukemia cells with high and low level of LR expression. Wild-type TF-1, AML193, and their related cells were maintained in RPMI-1640 with 10% fetal bovine serum and 2 ng/mL human GM-CSF (R & D Systems). A full-length complementary DNA of the human LR precursor was cloned into a pCI-neo expression plasmid (Promega, Madison, WI, USA), and transfected into TF-1 and AML193 cells by electroporation (Nucleofecta, Amaxa Biosystems, Gaithersburg, MD, USA) to establish LR overexpression models (TF-1LR and AML193LR). Empty pCI-neo plasmids were transfected into TF-1 and AML193 for the controls (TF-1 Mock and AML193 Mock). We used a tetracycline-responsive small interfering RNA (siRNA) method (BLOCK-iT Inducible H1 RNAi Entry Vector kit; Invitrogen, Carlsbad, CA, USA) and established a TF-1si cell line to reduce LR expression. TF-1sic, which expressed mutated LR siRNA, was also obtained as a control. The siRNA sequences for LR and its control were as follows: siRNA (5′-CCA GUCCAGGCAGCCUUC-3′) and mutated siRNA (5′-CCAGUCA AGUCAGCCUUC-3′). After transfection of the plasmids, each cell line was cloned with G418 (Sigma, St Louis, MO, USA).

Cell growth assay

Cell growth was assessed in liquid culture using the Premix WST-1 Cell Proliferation Assay System (Takara Biochem, Tokyo, Japan). For the colony-formation assay, cells (1.5×10^3 cells/well) were cultured in a 24-well plate with RPMI-1640 medium, 3% methyl cellulose, 20% fetal bovine serum, and GM-CSF (2 ng/mL). The number of colonies was counted on day 6 of culture.

Cell-cycle analysis

After 48 hours of culture, the cell-cycle distribution of leukemia cells was measured with a bromodeoxyuridine incorporation assay (BrdU Flow kit, BD Pharmingen, Franklin Lakes, NJ, USA) following manufacturer's instructions.

Detection of Annexin-V/propidium iodide

Cell surface expression of Annexin-V was measured on day 4 of culture under a low concentration of GM-CSF (0.04 ng/mL) using an Annexin-V Fluos staining kit (Roche, Mannheim, Germany).

STAT5 phosphorylation

Phosphorylation of the signal transducer and activator of transcription 5 (STAT5) protein was tested by flow cytometry using BD Phosflow technology (BD Biosciences, Franklin Lakes, NJ, USA). After 16 hours of culture without serum and GM-CSF, cells were stimulated with serum (10%) and GM-CSF (2 ng/mL). Forty-five minutes after stimulation, the cells were stained with antiphospho-STAT5 antibody (Y694, Clone #47; BD Biosciences)

and processed as suggested by the manufacturer. MFI was used for the quantification of phosphorylation.

Immunoprecipitation and immunoblotting

293T cells transfected with expression plasmids for the GM-CSFR α-chain (cloned into p3xFLAG-CMV; Sigma) and for LR (cloned into pcDNA3.1/V5-His; Invitrogen, Carlsbad, CA, USA) were disrupted in lysis buffer (modified phosphate-buffered saline with 135 mM potassium, 5 mM sodium, 0.1% Triton X-100, and protease inhibitor cocktails) with sonication. After clarification by centrifugation, lysates were incubated with antibody against the Flag- (Sigma) or V5- (Invitrogen) tag followed by protein G Sepharose beads (Amersham Bioscience, Buckinghamshire, UK). Immune complexes were washed with lysis buffer, released into sample buffer, then target proteins were detected by immunoblotting using anti-Flag or anti-V5 antibody. For detection of GM-CSFRα, cells were lysed using ProteoExtract Complete Mammalian Proteome Extraction Kit (Calbiochem, San Diego, CA, USA) and identified by immunoblotting using the primary antibody against the GM-CSFR α-chain (Clone #31916; R & D Systems) and β-actin (Abcam, Cambridge, UK) with a peroxidase-labeled secondary antibody (Amersham) and an enhanced chemiluminescence system (ECL Advance Western Blotting Detection Kit, GE Healthcare Bio-Sciences, Buckinghamshire, UK). Quantification of Western blot bands was performed using AE-6982/C/FC and CS Analyzer ver 3.0 Software (ATTO Co., Tokyo, Japan).

Statistical analysis

Results of in vitro experiments are presented as mean ± standard deviation of three independent experiments and were compared using a one-way analysis of variance followed by Scheffe's multiple comparison test. The correlation between the intensity ratio of 67-kDa LR and GM-CSFRα was estimated by the Pearson correlation. Comparisons of patient characteristics between groups were performed using the Wilcoxon test or χ^2 test. Overall survival (OS) for all patients was defined as the interval from the date of diagnosis to that of death. We applied the Kaplan-Meier method to estimate OS and compared the data using the log-rank test. The statistical analyses were performed using the SAS 9.1 software (SAS Japan Institute, Tokyo, Japan). A p value of 0.05 was considered statistically significant for all analyses.

Results

Expression of LR on AML cells

We measured LR expression on CD34-positive AML and normal bone marrow cells using a flow cytometer (Fig. 1). LR expression was also detected in more immature AML cells selected by the expression of CD133 at 10%, 32%, and 80% in three cases tested. However, because of the very limited availability of CD133-positive AML cells from clinical samples, in this study we used a CD34-positive fraction for the analysis. Although normal CD34-positive cells showed relatively low expression of LR, it was widely different in AML cells. Because the normal CD34-positive cells showed up to 25% of positivity of LR expression, we divided AML cases into two groups using 25% as a boundary: a high LR-expression group (LR-H, having LR-positive cells at 25% or more) and a low expression group (LR-L, <25% LR-positive cells). The clinical characteristics of the 44 patients in the LR-H and LR-L groups are shown in Table 1. WBC count and LDH level were significantly higher in the LR-H than in the LH-L group, suggesting that expression of LR on CD34-positive AML cells was associated with a large volume of AML cells at diagnosis. The relationship between LR expression with both WBC and LDH remained significant when these factors were treated as a continuum (Supplementary Figure E1; online only, available at www.exphem.org). Remission rate and OS of LR-H was also significantly worse than LR-L ($p = 0.03$ and 0.0004, respectively; Table 1). The impact of LR expression on OS was still significant for restricted patients who were treated with almost uniform, intensive chemotherapy protocols [29] (Supplementary Figure E2; online only, available at www.exphem.org).

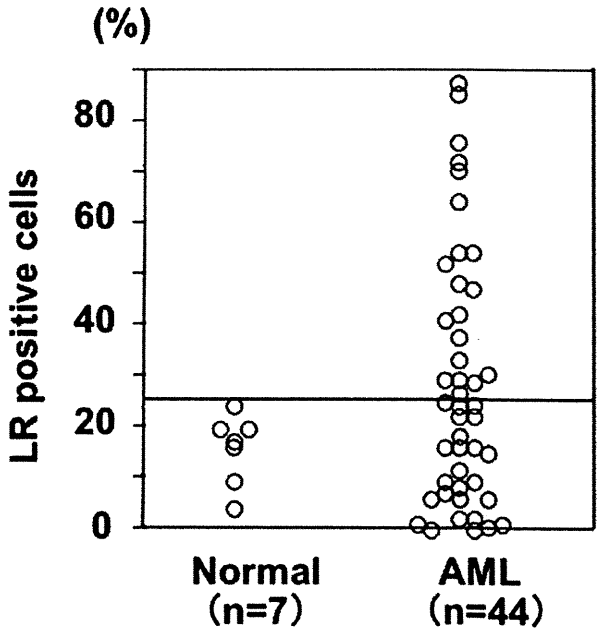

Figure 1. Surface expression of LR on CD34-positive cells. Expression of 67-kD LR was examined in the CD34-positive fraction of AML cells and that of bone marrow cells from healthy volunteer by flow cytometry. AML cases were divided into two groups based on the rate of LR expression: a high-expression group (LR-H, LR-positive cells at ≥25%) and a low-expression group (LR-L, LR-positive cells at <25%).

LR expression level was related to leukemia cell growth

Using a GM-CSF–dependent human leukemia cell line (TF-1), we established cell lines that overexpressed surface LR (TF-1LR), TF-1 with reduced LR expression using siRNA (TF-1si), and their controls (Fig. 2A; TF-1LRc and TF-1sic, respectively) as described in Materials and Methods. These lines were cloned after selection in the presence of G418. There was a clear difference in the

Table 1. Clinical and laboratory features of the patients in LR-H and LR-L groups

No. of patients	LR-L	LR-H	*p* Value
Total	24	20	
Sex			0.37
Male	15	15	
Female	9	5	
Age (y), median (range)	56.5	61.5	0.36
Range	19–89	20–80	
FAB classification			0.48
M0	0	2	
M1	3	1	
M2	11	7	
M4	5	6	
M5	1	2	
M6	2	1	
M7	1	0	
MDS	2	1	
Cytogenetics risk group			0.47
Favorable	3	2	
Intermediate	11	6	
Unfavorable	10	12	
WBC category			0.04
>20,000/μL	5	10	
≤20,000/μL	19	5	
LDH category			0.02
≥2 N	6	12	
<2 N	18	8	
Complete remission rate (%)	83	55	0.03
Median overall survival (d)	803	239	0.0004
Treatment regimen			0.3
Intensive chemotherapy	19	13	
Low-dose chmotherapy	1	3	
Chemotherapy for the elderly	4	5	

Cytogenetic risk group: Favorable, t(8;21), inv(16); Intermediate, normal karyotype, other karyotype than Favorable or Unfavorable; Unfavorable, complex, −7, del(5q), −5.
LDH group was defined as follows: less than or equal to more than twice (2 N) the upper limit of the institutional normal range of LDH.
MDS = myelodysplastic syndromes.

growth of the cell lines as assessed by the WST-1 assay: higher LR expression was correlated with accelerated proliferation (Fig. 2B). To test the effect of signaling from LR on cell proliferation, we next performed the same experiments using dishes coated with laminin for culture. However, there was no difference in growth of these cells with or without stimulation by laminin even in cells expressing high level of LR (TF-1LR) (data not shown). This suggested that it is not the stimulation by laminin but the expression level of LR itself that had a significant effect on the growth of TF-1–related cells. Afterward, we used culture conditions without stimulation by laminin.

Growth of TF-1–related cells in semi-solid media was also affected by expression of LR. The number of colonies increased along with LR expression level (Fig. 2C). Morphology of cells in colonies was that of immature cells with no sign of differentiation (data not shown). In terms of the cell-cycle distribution, TF-1LR showed the higher percentage of cells in the S phase and the lower percentage in the G_0/G_1 phase than its control, TF-1Mock (Table 2, Fig. 2D). In contrast, TF-1si, which had the lowest LR expression level, showed a greater accumulation of cells in the G_0/G_1 phase and a smaller percentage of cells in the S phase compared to its control (TF-1sic) and TF-1LR (Table 2, Fig. 2D). Because these changes in TF-1 and related cells were observed without laminin stimulation, we thought that LR expression and not LR signaling played a role in the change of leukemia cell phenotype.

LR expression conferred resistance to apoptosis in TF-1 cells

Because of the dependence on GM-CSF, a reduction in its concentration leads to apoptosis of TF-1. When TF-1LR was cultured in one-thirtieth the concentration of GM-CSF, it showed resistance against apoptosis as judged by Annexin-V expression. In contrast, the number of apoptotic cells increased in the TF-1si cell line compared with their controls and parental cells (Fig. 3A and B). These data showed that LR expression was not only related to proliferation but also to antiapoptosis of leukemia cells.

STAT5 phosphorylation was modified by LR expression level

Phenotypic change observed in TF-1LR was similar with that under the effect of GM-CSF, such as enhanced proliferation, reduced apoptosis, and acceleration of the cell cycle. This led us to test whether the GM-CSF signaling pathway was modified in TF-1LR and TF-1si cells. STAT5 protein, an important transcription factor for hematopoietic cells, is located downstream in the signaling pathway from GM-CSFR, and STAT5 is phosphorylated after GM-CSF stimulation, which is necessary for its activation [30]. The STAT5 phosphorylation level measured by flow cytometry was higher in TF-1LR and lower in TF-1si than their controls and wild-type cells (Fig. 3C and D), suggesting that modification of STAT5 activity in TF-1–derived cells was related to LR expression level.

Association of GM-CSFR and LR

To address how LR expression modulated STAT5 phosphorylation, we next tested whether LR physically interacts with GM-CSFR α-chain (GM-CSFRα). Using 293T cells transfected with LR and GM-CSFRα expression plasmids, immunoprecipitation and immunoblotting experiments demonstrated that LR and GM-CSFRα were present in the same protein complex (Fig. 4A), confirming previously reported results [26]. Immunoprecipitation experiments using in vitro translated LR and GM-CSFRα also showed the association of these proteins (data not shown).

Surface expression of GM-CSFRα on leukemia cell lines and AML cells

Because GM-CSFRα and LR interacted physically, we examined the surface expression of GM-CSFRα on TF-1 cell lines

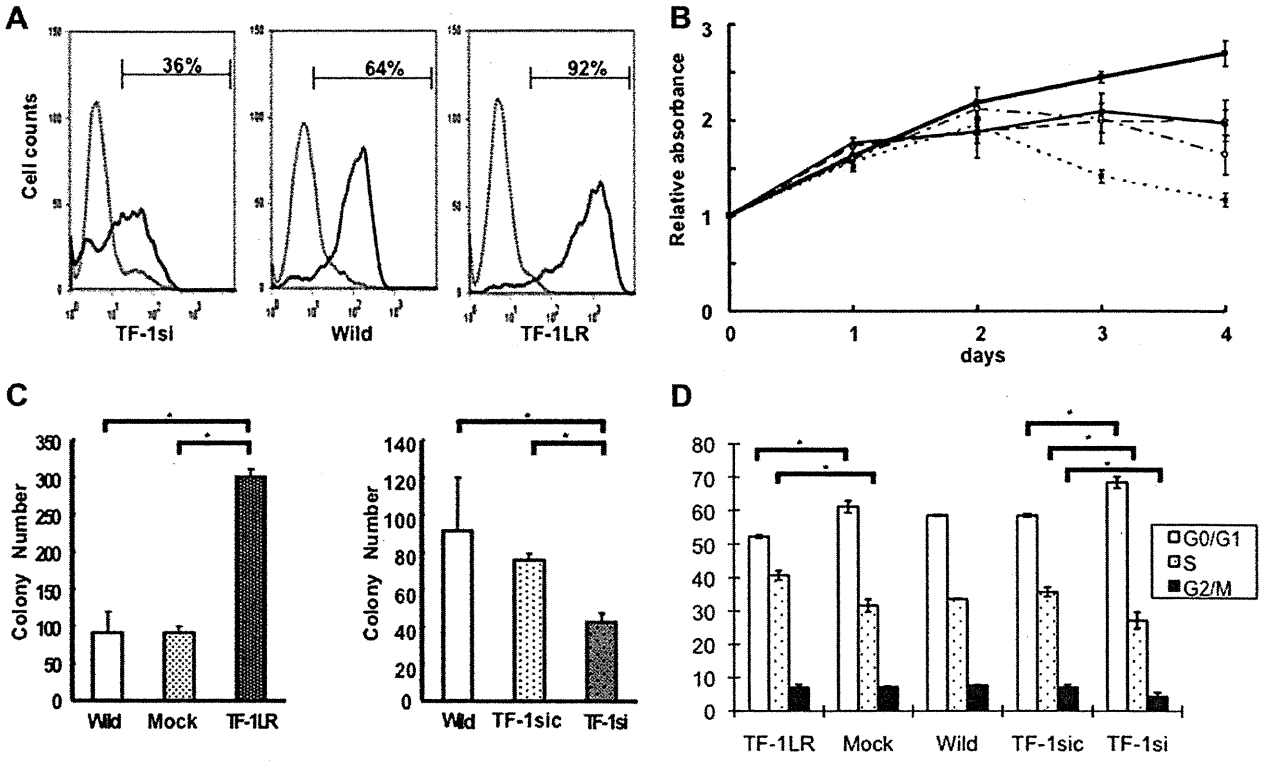

Figure 2. Expression or suppression of LR on leukemia cells modulated the growth characteristics of leukemia cells. (**A**) Surface expression of LR on wild-type TF-1, TF-1LR, and TF-1si was 64%, 92%, and 36%, respectively. Histogram overlays show the data of isotype control (dotted lines) and the anti-LR antibody (bold lines). TF-1LR, TF-1 cells overexpressing cDNA of human LR; TF-1si, TF-1 cells with reduced LR expression using tetracycline-responsive siRNA for LR; TF-1sic, control cells for TF-1si that expressed mutated LR siRNA. (**B**) Growth of wild-type (thin line), TF-1 Mock (broken line), TF-1 LR (bold line), TF-1si (dotted line), and TF-1sic (dot-dash line) were assessed using the WST-1 assay. Data from three independent experiments are shown. (**C**) Cells (1.5×10^3 cells/well) were cultured in semi-solid media with GM-CSF. The number of colonies was counted on day 6 of culture. The number of colonies were: TF-1LR, 300 ± 10 colonies/1500 cells; (**D**) Cell cycle distribution (shown as %) assessed with BrdU assay in TF-1 related cell lines. *statistically significant difference with $p < 0.005$, control cells (TF-1 Mock), 91 ± 8 colonies, TF-1si, 46 ± 3 colonies and TF-1sic, 76 ± 6 colonies ($*p < 0.05$). Wild, wild-type TF-1; Mock; TF-1 Mock.

by flow cytometry. GM-CSFRα expression was upregulated in TF-1LR and downregulated in TF-1si compared with controls (Fig. 4B). In another GM-CSF–dependent human myeloid leukemia cell line, AML193, the forced expression of LR also conferred an increased level of GM-CSFRα on the cell surface (data not shown). However, the message level (detected by quantitative PCR, Supplementary Figure E3A; online only, available at www.exphem.org) and the total amount of GM-CSFRα protein (measured by immunoblotting and densitometer) were quite similar despite the levels of LR among these cells (Fig. 4C and Supplementary Figure E3B; online only, available at www.exphem.org). Flow cytometric analysis revealed a statistically significant relationship between the ratio of MFI of LR and GM-CSFRα in CD34-positive AML cells obtained from clinical samples ($p = 0.02$, Fig. 4D). There was still significant relationship among those parameters even when data of the highest LR/IgH in Figure 4D, or two data of the highest LR/IgH and GM-CSFRa/IgG were removed (Supplementary Figure E4; online only, available at www.exphem.org).

Discussion

In the present study, we demonstrated that LR was expressed on AML cells and its high expression level was significantly related to the elevated WBC and LDH, and to the outcome of treatments such as remission rate and OS. Forced expression of LR increased expression of GM-CSFRα on the surface of both TF-1 and AML193 cells and conferred features of an aggressive nature on leukemia cells, such as enhanced proliferation and resistance against apoptosis. In contrast, the reduction of LR expression resulted in the decrease in GM-CSFRα expression and inversely changed the character of the leukemia cells. It is presumed that clinical features among patients with high LR expression (LR-H group) were in accordance with results obtained using the leukemia cell lines, further suggesting the biological significance of LR expression in AML, which was not apparent in the previous report by Montuori et al. [23]. Because LR is widely expressed in immature to mature hematopoietic cells [12,20,21], it is assumed that the difference in target cells to detect LR

Table 2. Cell-cycle analysis in different cell lines

Cell line	G_0/G_1	Phase (%) S	G_2/M
TF-1LR	52.2 ± 0.5^{v}	40.7 ± 1.4^{x}	7.1 ± 0.9
Mock	61.1 ± 1.8^{v}	31.7 ± 1.9^{x}	7.2 ± 0.2
Wild	58.6 ± 0.2^{v,w}	33.6 ± 0.1^{x,y}	7.7 ± 0.1^{z}
TF-1sic	58.5 ± 0.5^{w}	35.7 ± 1.4^{y}	7.1 ± 0.8^{z}
TF-1si	68.4 ± 1.7^{w}	27.2 ± 2.5^{y}	4.4 ± 1.3^{z}

Values in each column represent mean percentage of cells in each cell-cycle phase ± standard deviation of three independent experiments.
^{v,w,x,y,z}Statistically different in each cell cycle phase among different cell lines ($p < 0.05$).

influenced the results: mononuclear cells in the previous report and CD34-positive AML cells in this study.

To our surprise, for the phenotypic changes in leukemia cells, stimulation with laminin was not necessary. It is interesting that not the signaling from, but the expression of, LR did influence the phenotype of AML cells. Because LR binds the prion protein supporting its internalization [31], LR could bind and act to modulate the metabolism of other proteins, which might contribute to the change of leukemia phenotype. This hypothesis prompted us to study the relationship of LR and GM-CSFR.

Our results suggest that LR expression on leukemia cells enhanced signaling from surface GM-CSFR by increasing its number, which is supported by the in vitro experiments (elevated phosphorylation level of STAT5 in TF-1LR) and the significant relationship between LR expression and MFI of GM-CSFRα on the surface of CD34-positive AML cells obtained from patients. These results seemed to oppose those of a previous report by Chen et al., in which they showed that the physical interaction of LR and GM-CSFR inhibited GM-CSF–induced receptor complex formation, and stimulation of cells with laminin canceled the inhibitory effect of LR on GM-SCFR complex formation in neutrophils [26]. However, the role of LR with GM-CSFR could be different by lineage and stage of differentiation in hematopoietic cells, as the function of GM-CSF on hematopoietic cells is quite divergent in immature progenitors and differentiated myeloid cells, such as neutrophils [28,32].

Although the surface expression of GM-CSFRα was modulated along with the level of LR expression, the total amount of GM-CSFRα protein in whole-cell lysates did not change. Considering the direct association of LR and

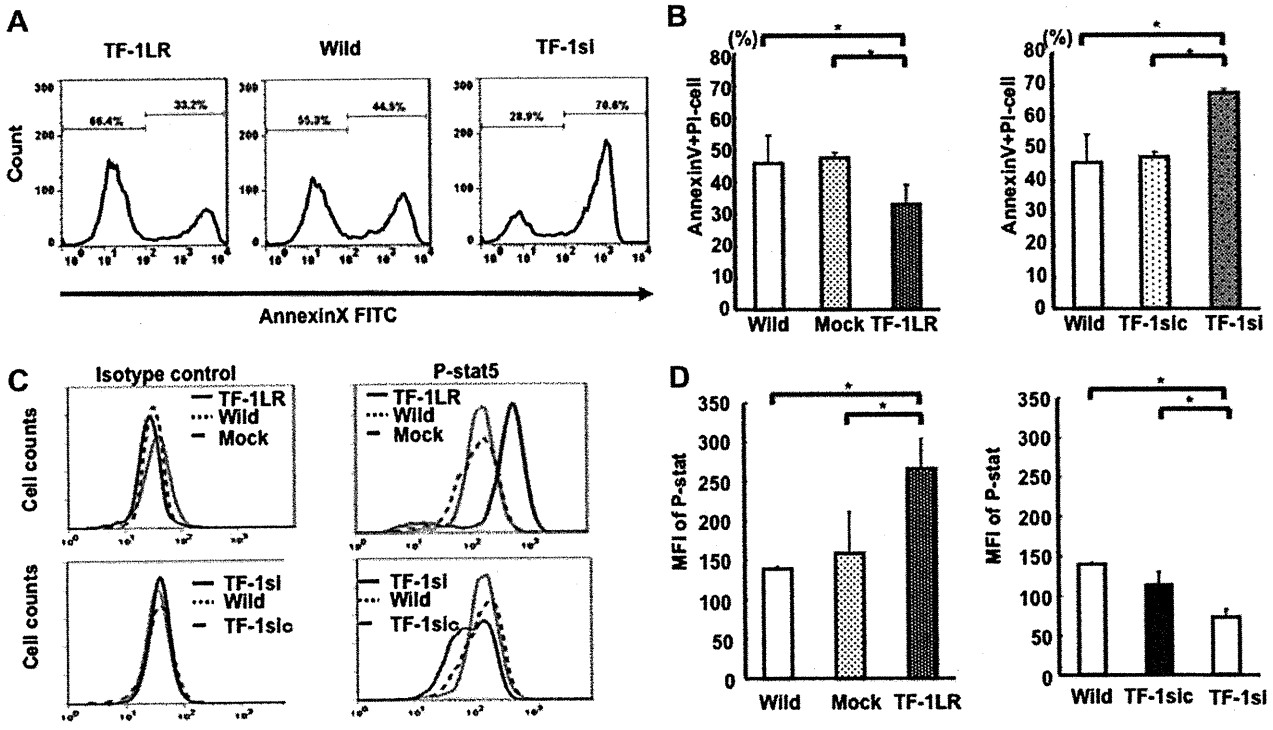

Figure 3. Apoptosis and phosphorylation of STAT5 in TF-1–related cell lines. Cells were cultured in medium with a low concentration of GM-CSF (0.1 ng/mL); then apoptotic cells were assessed using Annexin-V expression and propidium iodide (PI) staining at day 4 of culture. (**A**) One representative experiment is shown. (**B**) Data from three independent experiments (±standard deviation) are shown as a bar graph. There were statistically significant differences in apoptosis among cell lines (Annexin-V^{+} PI^{-}, Annexin-V–positive and PI-negative cells; *$p < 0.05$). (**C**) Flow cytometric detection of STAT5 phosphorylation (P-stat5). After 16 hours of culture without serum, cells were incubated with anti–phospho-STAT5 antibody, and then stimulated with serum and GM-CSF. Data of representative experiments are shown. (**D**) Results of P-stat5 in TF-1 related cells are presented as mean ± standard deviation of three independent experiments. MFI of STAT5 was higher in TF-1LR than that in the control (265 ± 39 and 158 ± 54, respectively), and lower in TF-1si than that in its control (72 ± 9.6 and 138.9 ± 2.5, respectively) (*$p < 0.05$).

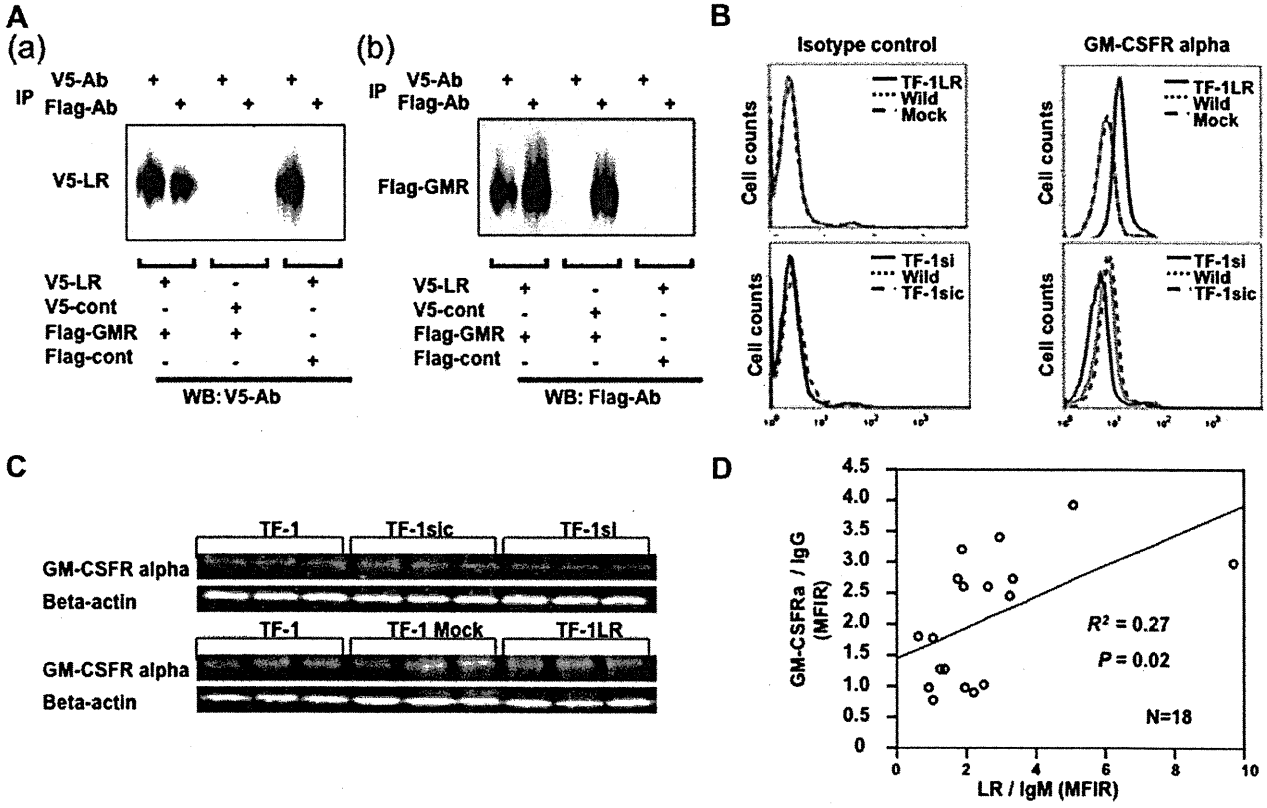

Figure 4. Association of LR and GM-CSFRα, and the relationship of the expression of LR and GM-CSFα in leukemia cell lines and AML samples. (**A**) Expression plasmids for LR (V5-LR) and GM-CSFRα (Flag-GMR) with tags were transfected into 293T cells. Cell lysates were subjected to immunoprecipitation with anti-V5 or anti-Flag antibody. (**a**) Western blot (WB) analysis was performed using anti-V5 and (**b**) anti-Flag antibodies. IP, immunoprecipitation; V5/Flag-Ab, antibody against V5 or Flag tag; V5-LR, LR tagged with V5; V5-cont, control for V5-LR; Flag-GMR, GM-CSFRα tagged with Flag; Flag-cont, control for Flag-GMR. (**B**) Representative results were shown regarding the surface expression of GM-CSFRα on TF-1–related cell lines. (**C**) The total amount of GM-CSFRα protein of each cell line as assessed by Western blot analysis using anti–GM-CSFRα or anti–β-actin antibody. (**D**) Using CD34-positive AML cells, expression of LR and GM-CSFRα were measured by flow cytometry. There was a significant relationship between the values of MFI ratio of LR and GM-CSFRα, and their controls (isotype IgM and isotype IgG, respectively). MIFR, mean fluorescent intensity ratio.

GM-CSFRα that was shown previously [26] and confirmed in this study, and our experimental condition lacking stimulation by laminin through LR, it is suggested that LR itself and not signaling through LR influenced GM-CSFRα surface expression. The multifunctional properties of LR as shown by its crystal structure [33] might contribute to the dodecamer complex formation of GM-CSFR that has been recently demonstrated to be important for active signaling [34].

The importance of GM-CSF signaling in myeloid leukemia is clearly emphasized by the analysis of juvenile myelomonocytic leukemia [35,36]. Because mutually exclusive abnormalities in the signaling pathway from GM-CSFR have been found in >50% of juvenile myelomonocytic leukemia cases, it would be interesting to examine the role of LR in juvenile myelomonocytic leukemia [35,36]. A recent report on the phosphoprotein network in AML [37] also demonstrated the significant involvement of GM-CSF signaling in AML: enhanced STAT5 phosphorylation was found after treatment with GM-CSF in some AML cases. In the same report, a relationship between GM-CSF–induced STAT5 phosphorylation and FLT3 mutation was also shown. Given that AML cases with FLT3 mutation show leukocytosis [38,39], there might be complex interactions in the signals from GM-CSFR and FLT3 for the increased WBC count in the LR-H group, but this awaits further analysis. Considering the distribution of LR in bone marrow cells, our results suggested a new role for LR in leukemia cells that might be a future target of AML treatment.

It will be necessary to further analyze the mechanism of how LR contributes to expression of GM-CSFR on AML cells, and the role of GM-CSF signaling in leukemia, particularly in the immature fraction of AML cells.

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