Table 1 Correlation of imatinib pharmacokinetics with clinical response

Authors	Disease	No. of patients	IM daily dose (mg)	IM-C_{min} (ng/ml)	Correlation with response
Picard *et al.*[8]	CML	50	400	1,058 ± 557	Yes (CCyR, MMR)
		18	600	1,444 ± 710	
Larson *et al.*[7]	CML	351	400	979 ± 530	Yes (CCyR)
Forrest *et al.*[9]	CML	78	400	999 (203–2,910)	No (CCyR, MMR)
Widmer *et al.*[12]	CML	20	400	NA	No (AUC_u vs. HR)
	GIST	38	600	NA	Yes (AUC_u vs. OR)
Kawaguchi *et al.*[10]	CML	13	400	1,400 ± 570	NA
		9	300	1,150 ± 440	
Demetri *et al.*[11]	GIST	36	400	1,530 ± 666	Yes (TTP, OOBR)
		37	600	1,752 ± 794	
Marin *et al.*[6]	CML	84	400	900 (400–1,600)	Yes (MMR)

AUC_u, free area under the curve; CCyR, complete cytogenetic response; CML, chronic myeloid leukemia; GIST, gastrointestinal stromal tumor; HR, hematologic response; IM, imatinib mesylate; MMR, major molecular response; NA, not available; OOBR; overall objective benefit rate; OR, overall response; TTP, time to progression.

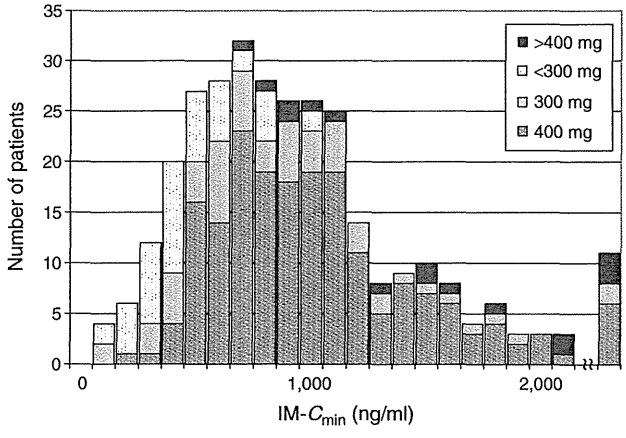

Figure 1 Distribution of steady-state trough plasma IM concentration (IM-C_{min}; $n = 314$, data obtained from all doses). The mean and median values of IM-C_{min} were 1,010.5 ± 564.6 and 900 ng/ml (range, 111–3,620 ng/ml), respectively.

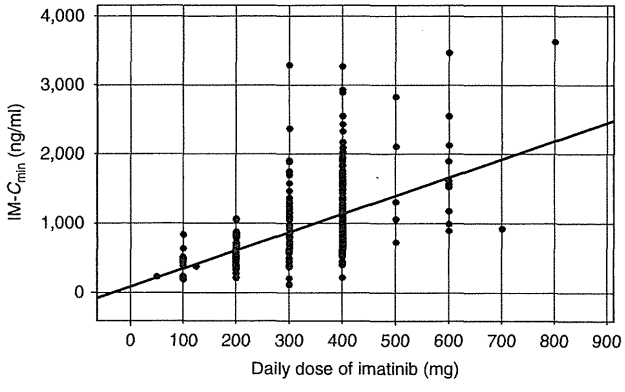

Figure 2 Steady-state trough plasma IM concentration (IM-C_{min}) achieved with the indicated daily dosages ($n = 314$). The IM-C_{min} predicted using linear regression analysis was related to dose as follows: 76.29 + 2.624 × dose ($r^2 = 0.232$; $P < 0.00001$).

may have been influenced by sample size and heterogeneous sampling times.

In Japan, the relationship between IM-C_{min} and clinical response has been independently studied by six groups.[13–18] Although two of these studies identified a significant correlation between IM-C_{min} and clinical response,[13,17] the others did not; however, these latter four studies that detected no correlation may have been insufficiently powered because of their modest sample sizes. In this study, our aim was to investigate the usefulness of monitoring IM-C_{min} in a large cohort of CML patients by integrating the data from these six Japanese studies.

RESULTS

Data for 314 Japanese patients with chronic-phase CML (189 men and 125 women) were integrated in this analysis. The median age of the patients was 60 years (range, 16–91 years), the mean body weight was 61.3 ± 12.1 kg (median, 60.0 kg; range, 37–103 kg), and the mean body surface area was 1.64 ± 0.19 m^2 (median, 1.65 m^2; range, 1.19–2.25 m^2). Among the study participants, 190 (60.5%) received 400 mg of IM daily, 59 (18.8%) received 300 mg, 48 (15.3%) received <300 mg, and 17 (5.4%) received >400 mg. The median duration of IM therapy was 1,435 days (range, 56–2,582 days).

The distribution of IM-C_{min} values across all doses is shown in **Figure 1**. The mean and median IM-C_{min} values were 1,010.5 ± 564.6 and 900 ng/ml (range, 111–3,620 ng/ml), respectively. Although there was substantial interpatient variability among patients treated with the same dose of IM, IM-C_{min} increased significantly and proportionately across doses ranging from 50 to 800 mg (**Figure 2**). Of these 314 patients, 60 patients were excluded from further analysis aimed at correlating IM-C_{min} with clinical response because the duration of IM therapy was <12 months or because a molecular response was not evaluated. The clinical characteristics of the 254 patients included are summarized in **Table 2**. There were no correlations between IM-C_{min} and age, body weight, body surface area, or the duration of IM therapy ($P = 0.343$, $P = 0.073$, $P = 0.075$, and $P = 0.931$, respectively), gender (Student's t-test: $P = 0.648$), or Sokal risk score (analysis of variance: $P = 0.399$).

Table 2 Association between potential predictive factors and imatinib trough concentration

Variable ($n = 254$)	Mean or no. of patients	Correlation with imatinib concentration (r)	P-value
Quantitative features[a]			
Age (years)	59.2	0.060	0.343
Weight (kg)	61.1	−0.113	0.073
Body surface area (m^2)	1.6	−0.112	0.075
Duration of IM therapy (days)	1,454.2	−0.005	0.931
Qualitative features			
Sex (male/female)[b]	151/103		0.648
Sokal risk group (low/intermediate/high)[c]	91/91/52		0.399

IM, imatinib mesylate.

[a]Compared using Pearson's product-moment correlation analysis. Data are presented as correlation coefficient (r) or mean values. [b]Compared using Student's t-test. [c]Compared using analysis of variance.

Table 3 Patient characteristics and clinical response to imatinib therapy

Characteristic (no. of patients)	MMR (166)	No MMR (88)	P-value	CCyR (218)	No CCyR (36)	P-value
Quantitative features						
Imatinib concentration (ng/ml)	1,107.4 ± 594.4	872.7 ± 528.5	0.002	1,057.8 ± 585.0	835.0 ± 524.3	0.033
Age (years)	57.1 ± 15.4	62.8 ± 14.2	0.004	58.3 ± 15.2	64.3 ± 14.4	0.029
Body weight (kg)	61.1 ± 12.1	61.5 ± 12.0	0.808	61.1 ± 12.0	61.6 ± 12.5	0.818
Body surface area (m^2)	1.643 ± 0.195	1.645 ± 0.188	0.926	1.638 ± 0.200	1.643 ± 0.191	0.888
Daily imatinib dose (mg)	367.5 ± 79.6	323.6 ± 135.4	0.006	365.1 ± 94.0	272.0 ± 127.0	0.0002
Duration of imatinib therapy (days)	1,459 ± 623	1,458 ± 697	0.986	1,450 ± 643	1,482 ± 705	0.786
Qualitative features						
Sex (male/female)	95/71	56/32	0.349	128/90	23/13	0.572
Sokal risk group (low/intermediate/high)	63/61/33	28/30/19	0.779	81/81/43	10/11/9	0.529

Data are presented as mean values (±SD) for quantitative features. Quantitative variables were compared using Student's t-test. Qualitative variables were compared using the χ^2 or Fisher's exact test.

CCyR, complete cytogenetic response; MMR, major molecular response.

Among all the patients evaluated, 166 (65.3%) achieved an MMR, and 218 (85.8%) achieved a CCyR (**Table 3**). IM-C_{min} values were significantly higher in patients with an MMR than in those without an MMR; the mean values were 1,107.4 ± 594.4 ng/ml (median, 986 ng/ml) and 872.7 ± 528.5 ng/ml (median, 719.5 ng/ml), respectively ($P = 0.002$). In addition, there were significant differences in age and daily dosage between patients with an MMR and those without an MMR ($P = 0.004$ and 0.006, respectively). Importantly, when we subclassified all the patients according to their IM-C_{min} as previously reported,[8] we found that patients with an IM-C_{min} ≥1,002 ng/ml had a higher probability of achieving an MMR than those with an IM-C_{min} <1,002 ng/ml ($P = 0.0120$, **Figure 3**).

In addition, IM-C_{min} was significantly higher in patients with a CCyR ($n = 219$) than in those without a CCyR ($n = 36$); the mean values were 1,057.8 ± 585.0 ng/ml (median, 916 ng/ml) and 835.0 ± 524.3 ng/ml (median, 688 ng/ml), respectively ($P = 0.033$). There were also significant differences in age and daily dosage between the group with a CCyR and those without a CCyR ($P = 0.029$ and 0.0002, respectively, **Table 3**).

In a stepwise forward-selection multiple logistic analysis, MMR was associated with both the age of the patient (odds ratio (OR) = 0.97 (0.958–0.995); $P = 0.0153$) and the IM-C_{min} value (OR = 1.0008 (1.0003–1.0015); $P = 0.0044$), whereas a CCyR was associated with only daily dosage (OR = 1.0073 (1.0036–1.0110); $P = 0.0001$). The association between IM-C_{min} and CCyR was not observed in the stepwise forward-selection multiple logistic analysis.

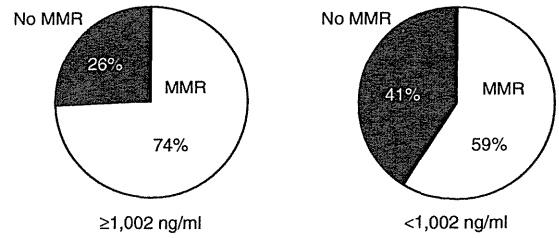

Figure 3 Correlation of trough plasma IM concentration (IM-C_{min}) with MMR ($n = 254$). IM-C_{min} values ≥1,002 ng/ml had a significantly higher probability of achieving an MMR (Fisher's exact test; $P = 0.0120$). MMR, major molecular response.

DISCUSSION

IM has favorable pharmacokinetic characteristics, including complete bioavailability and a proportionate dose–response relationship.[4,19] However, we found that, although there was a

linear relationship between IM-C_{min} and the daily dose of IM, there was also substantial interpatient variability. Factors that could underlie this interpatient variability include body size, age, gender, liver function, renal function, interaction with other medications given concomitantly, adherence to medication regimens, and polymorphisms of enzymes or transporters related to IM pharmacokinetics and/or pharmacodynamics. In this analysis, we did not observe any correlation between IM-C_{min} and body weight, body surface area, or age. Moreover, the eligibility criteria of each of the integrated studies ensured that there were no patients with serious renal or hepatic dysfunction, and no patients who were taking other drugs that might interact with IM. In addition, adherence to medication regimens was monitored by self-report for at least 7 days prior to blood sampling.

In our study of 254 evaluated IM-treated CML patients, steady-state IM-C_{min} correlated significantly with both MMR and CCyR. Among those who achieved an MMR, the mean IM-C_{min} (1,107.4 ng/ml from all doses, 1,154.3 ng/ml from 400 mg) was >1,002 ng/ml, previously shown to be an effective IM-C_{min} threshold.[8] Moreover, we found that patients with an IM-C_{min} <1,002 ng/ml had a lower probability of achieving an MMR ($P = 0.0120$, **Figure 3**), thereby supporting the previously reported 1,002 ng/ml plasma concentration efficacy threshold for Japanese CML patients.

Prior to data integration, two of the six individual studies revisited in this report found a statistically significant difference in plasma IM concentrations between patients with an MMR ($n = 34$) and those without an MMR ($n = 28$) ($P = 0.010$ by Student's t-test)[13] and between patients with an optimal response ($n = 25$) and those with a suboptimal or failed response ($n = 8$) ($P = 0.0087$ by Student's t-test).[17] In the other four Japanese studies, it is possible that the number of patients per study was too small to achieve a statistically significant correlation between IM-C_{min} and outcome, as was previously suggested by Forrest *et al.*[9]

Our results suggest that higher IM-C_{min} is associated with an increased likelihood of achieving MMR and CCyR ($P = 0.002$ and 0.033, respectively, in univariate analysis). Additionally, in a multivariate analysis, both IM-C_{min} and the age of the patient were independently predictive of achieving an MMR. Age is one of the clinical factors that is included in the Sokal risk score as a baseline characteristic. Although we could not find a significant difference in Sokal risk score between patients with an MMR and those without, previous studies have reported that the Sokal risk score predicts outcome for IM-treated CML patients.[9,20] In contrast, by multivariate analysis, daily dosage of IM was the only independent predictive factor for achieving a CCyR. Together, these findings suggest that variability in IM exposure has clinical implications and, probably more so, implications for a molecular response.

Several clinical trials have established that a molecular response is a surrogate marker for predicting the likelihood of disease progression and/or survival in patients with CML.[20] In this study, although we could not directly compare IM-C_{min} with long-term outcomes, higher IM-C_{min} was associated with an increased likelihood of achieving an MMR. Given that an MMR may indicate a decreased risk for progression to accelerated phase or blastic crisis, we speculate that increased IM-C_{min} would be associated with longer survival; however, further study is necessary to test this hypothesis.

Given the importance of the duration of IM treatment in an evaluation of clinical response,[20] 60 of the 314 patients (19%) were excluded from further analysis of the correlation between IM-C_{min} and clinical response, either because the duration of IM therapy was <12 months or because a molecular response was not evaluated. Although there was no significant difference in C_{min} values between the excluded patients ($n = 60$; 994.5 ± 481.9 ng/ml) and the included patients ($n = 254$; 1,026.1 ± 582.2 ng/ml) per Student's t-test ($P = 0.315$), a potential source of bias cannot be entirely ruled out in this retrospective analysis.

In conclusion, on the basis of our data, we propose that, in addition to BCR-ABL mutation analysis for CML patients, it may be useful to assay plasma IM levels when making decisions related to IM therapy. Further study is necessary to prospectively confirm the link between IM-C_{min} and clinical response, including survival, in large multiethnic patient populations.

METHODS

Patients. The 314 patients included in this analysis were those who had previously been enrolled in six independent Japanese clinical studies.[13–18] All the patients had Philadelphia chromosome (Ph)-positive chronic-phase CML, and all were treated orally with IM for >2 months. Informed consent was obtained from each participant in accordance with the Declaration of Helsinki. Each study protocol was reviewed and approved by the ethics committees or institutional review boards of the participating centers.

Clinical parameters including response to the therapy. A CCyR was defined as the absence of Ph^+ metaphase cells among 20 or more bone marrow cells examined. In some cases, fluorescent *in situ* hybridization was also carried out for detection of bcr-abl fusion genes in neutrophils from peripheral blood.[21] An MMR was defined as a threefold log reduction in bcr-abl transcripts measured using real-time reverse transcriptase–mediated quantitative PCR and/or AMP-CML. The samples used to evaluate IM response and those for measurement of IM-C_{min} were collected from patients on the same day.

Measurement of IM concentrations in plasma. Blood samples were collected by venipuncture 24 h (± 2 h) after oral administration of IM. Plasma was isolated by centrifugation at 1,900*g* for 15 min and stored at −40 °C until analysis. IM-C_{min} values were determined using high-performance liquid chromatography coupled to electrospray-ionization tandem mass spectrometry[22] at the TORAY Research Center, (Nihonbashi, Tokyo, Japan), which is the only assay system in Japan authorized by Novartis Global.

Statistical analyses. Statistical analyses were carried out using SPSS statistical software (version 17.0; SPSS Japan, Tokyo, Japan). Data are presented as mean values ± SD unless indicated otherwise. Pearson's product moment correlation was applied to assess the relationship between IM-C_{min} and clinical variables (age, body weight, body surface area, and duration of IM therapy). A linear regression analysis was applied to assess the correlation between IM-C_{min} and the daily dose of IM. Differences in IM-C_{min} between two patient groups were evaluated using the Student's t-test. Comparison of IM-C_{min} among three groups was made using one-way analysis of variance with *post hoc* Tukey's multiple-comparison procedure. The χ^2 test or Fisher's exact test was used to compare the proportions of patients with an MMR or a CCyR and to compare groups. Stepwise forward-selection multiple logistic analyses

were performed for MMR and CCyR in order to determine the effects of the factors examined in the univariate analysis. Values of $P < 0.05$ were considered significant.

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CONFLICT OF INTEREST

The authors declared no conflict interest.

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ORIGINAL ARTICLE

Pre-transplant imatinib-based therapy improves the outcome of allogeneic hematopoietic stem cell transplantation for *BCR–ABL*-positive acute lymphoblastic leukemia

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A high complete remission (CR) rate has been reported in newly diagnosed Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph + ALL) following imatinib-based therapy. However, the overall effect of imatinib on the outcomes of allogeneic hematopoietic stem cell transplantation (allo-HSCT) is undetermined. Between 2002 and 2005, 100 newly diagnosed adult patients with Ph + ALL were registered to a phase II study of imatinib-combined chemotherapy (Japan Adult Leukemia Study Group Ph + ALL202 study) and 97 patients achieved CR. We compared clinical outcomes of 51 patients who received allo-HSCT in their first CR (imatinib cohort) with those of 122 historical control patients in the pre-imatinib era (pre-imatinib cohort). The probability of overall survival at 3 years after allo-HSCT was 65% (95% confidence interval (CI), 49–78%) for the imatinib cohort and 44% (95% CI, 35–52%) for the pre-imatinib cohort. Multivariate analysis confirmed that this difference was statistically significant (adjusted hazard ratio, 0.44, $P = 0.005$). Favorable outcomes of the imatinib cohort were also observed for disease-free survival ($P = 0.007$) and relapse ($P = 0.002$), but not for non-relapse mortality ($P = 0.265$). Imatinib-based therapy is a potentially useful strategy for newly diagnosed patients with Ph + ALL, not only providing them more chance to receive allo-HSCT, but also improving the outcome of allo-HSCT.
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Keywords: Philadelphia chromosome-positive acute lymphoblastic leukemia; imatinib; allogeneic hematopoietic stem cell transplantation

Introduction

The Philadelphia chromosome (Ph) presents in 20–25% of adult patients with acute lymphoblastic leukemia (ALL) and is an extremely unfavorable prognostic factor. The outcome of patients with Ph-positive ALL (Ph + ALL) following conventional chemotherapy is dismal, showing <20% long-term survival.[1–4] Although allogeneic hematopoietic stem cell transplantation (allo-HSCT) has offered a curative option in Ph + ALL,[3–5] relatively high rates of relapse and non-relapse mortality (NRM) impair the treatment success even after allo-HSCT. The International Bone Marrow Transplant Registry reported a leukemia-free survival rate of 38% following human leukocyte antigen (HLA)-identical allo-HSCT for Ph + ALL patients transplanted in the first complete remission (CR).[6] Previously, we and others reported that imatinib-based chemotherapy produced very high CR rate, thus allowing a high proportion of patients to prepare for allo-HSCT.[7,8] However, because of the short observation period, the impact of imatinib-based therapy upon the survival outcomes after allo-HSCT remains unclear. To address whether allo-HSCT after imatinib-based therapy is a superior treatment approach to that after conventional chemotherapy, we conducted a retrospective analysis of Ph + ALL patients who underwent allo-HSCT before and after imatinib became available, using data from the Japan Adult Leukemia Study Group (JALSG) Ph + ALL202 study and from the nationwide database of the Japan Society of Hematopoietic Stem-cell Transplantation (JSHCT) and the Japan Marrow Donor Program (JMDP).

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Patients and methods

Data source and patient selection criteria

We compared the transplantation outcome of patients treated by the JALSG Ph + ALL 202 study (imatinib cohort) with those in the historical control data in the pre-imatinib era from the JSHCT and JMDP (pre-imatinib cohort), in which information on patient survival, disease status and long-term complications, including chronic graft-versus-host disease (cGVHD) and second malignancies, is renewed annually using follow-up forms.[9,10] To

attain an adequate level of comparability in terms of allo-HSCT, patients were selected according to the following criteria: (1) patients with *de novo* Ph + ALL; (2) age range of 15–65 years and (3) allo-HSCT during their first CR. A total of 122 patients who received allo-HSCT between January 1995 and December 2001 (before the approval of imatinib by the Japanese government) were selected. This study period of the pre-imatinib cohort included the pioneering period of cord blood transplantation (CBT) when the relevance of cell dose and HLA matching had not yet been recognized. Thus, the subjects were limited to those who received bone marrow (BM) or peripheral blood (PB) as a treatment graft.

Patients

Between September 2002 and May 2005, 100 newly diagnosed patients with Ph + ALL were registered to the JALSG Ph + ALL202 study, and received a phase 2 imatinib-combined chemotherapy as described previously.[7] Ph + ALL was diagnosed by the presence of Ph through chromosome and/or FISH analysis, and positivity for *BCR–ABL* fusion transcripts detection by real-time quantitative polymerase chain reaction (RQ–PCR) analysis.

Of 97 patients who achieved CR, 60 patients received allo-HSCT in their first CR. Of these 60 patients, 9 patients who received unrelated CBT were excluded in this analysis because of the reason as described at the selection criteria for control patients in the pre-imatinib era. Thus, 51 patients transplanted between February 2003 and December 2005 were analyzed. In the JALSG Ph + ALL202 study, allo-HSCT was recommended after achieving CR if an HLA-identical donor was available. The stem cell source for allo-HSCT was chosen in the following order: (1) matched-related allo-HSCT; (2) HLA-A, B and DRB1 allele matched (6/6) or DRB1 one-allele mismatched-unrelated allo-BMT, if patients had no HLA-matched-related donor and (3) unrelated CBT or HLA-mismatched-related allo-HSCT, if they had no donors described in (1) and (2). A prophylaxis for GVHD was determined by each institute, but did not include T-cell depletion. The study was approved by the institutional review board of each participating center and conducted in accordance with the Declaration of Helsinki.

Definition of engraftment and GVHD

Engraftment day was defined as the first day of three consecutive days when the absolute neutrophil count was $\geqslant 0.5 \times 10^9$/l. Graft failure was defined as the lack of any sign of neutrophil recovery. Engraftment that occurred after day 60 was also considered to be a graft failure. Patients who died early (<day 29) were excluded from the analysis of engraftment. Acute GVHD (aGVHD) and chronic GVHD (cGVHD) were defined according to previously described standard criteria.[11]

Quantitation of BCR–ABL transcripts

The copy number of *BCR–ABL* transcripts in BM was determined at a central laboratory using the RQ–PCR as described previously.[7] To minimize the variability in the results because of differences in the efficiency of cDNA synthesis and RNA integrity among the patient samples, the copy number of the *BCR–ABL* transcripts was converted to molecules per microgram RNA after being normalized by means of *GAPDH*. The normalized values of the *BCR–ABL* copies in each sample were reported as *BCR–ABL* number of copies. At least 5.7×10^5 copies/μg RNA *GAPDH* levels were required in a sample to consider a negative PCR result valid; otherwise, the sample was not useful for minimal residual studies. The threshold for quantification was 50 copies/μg RNA. The levels below this threshold were designated as 'not detected' or '<50 copies/μg'. In this study, the former was categorized as PCR negativity.

Minimal residual disease (MRD) at the time of HSCT was evaluated by the result of RQ–PCR within 30 days prior to transplantation.

Statistical considerations

The primary end point of this study was overall survival (OS) after allo-HSCT. Secondary end points included disease-free survival (DFS) and the incidence of aGVHD, cGVHD, NRM and relapse. We defined DFS events as relapse or death, whichever occurred earlier. The observation periods for OS were calculated from the date of transplantation until the date of the event or last known date of follow-up. The probabilities of OS and DFS were estimated using the Kaplan–Meier product limit method. The cumulative incidences of NRM, relapse, aGVHD and cGVHD were estimated as described elsewhere, taking the competing risk into account.[12] In each estimation of the cumulative incidence of an event, death without an event was defined as a competing risk. Risk factors for OS and DFS were evaluated by a combination of uni- and multivariate analyses. The following variables were evaluated for each analysis: imatinib-based therapy prior to HSCT, age group (under 40 versus 40 to 54 versus 55 and older), stem cell source (BM versus PB), HLA disparity (matched (HLA-identical siblings or 6/6 allele matched unrelated) versus mismatched), duration from diagnosis to HSCT and cGVHD as time-varying covariate (yes versus no). Univariate analysis was performed using Cox regression models or log-rank test. Multivariate analysis was performed using Cox proportional hazards regression model or competing risk regression model[13] as appropriate. For the evaluation of time-varying events, such as aGVHD or cGVHD, upon clinical outcomes, we treated these as time-varying covariates. Differences among groups in terms of demographic characteristics were tested using the χ^2 or Mann–Whitney tests as appropriate. All statistical analyses were conducted using STATA 11 (STATA Corp., College Station, TX, USA).

Results

Patient characteristics

In the imatinib cohort, there were 29 males and 22 females, with a median age of 38 years (range, 15–64 years). Regarding transcript types, 36 patients had minor *BCR–ABL* and 15 had major *BCR–ABL*. In 5 patients, pre-treatment cytogenetic data were not available, and of the remaining 46 patients, 8 showed t(9;22) only, 36 had additional chromosome aberrations and 2 showed normal karyotype. Of 48 patients who were evaluable for MRD analysis, 36 patients achieved PCR negativity at the time of HSCT.

Some of the clinical and biological features (such as presence of additional chromosome aberrations, *BCR–ABL* subtype, MRD status at HSCT and performance status at HSCT) were not available in the pre-imatinib cohort and not included in the present analysis.

Table 1 lists the characteristics of patients included in this comparative analysis. Some of the clinical features were significantly different between two cohorts: age distribution at HSCT ($P=0.048$), conditioning regimens ($P<0.001$), GVHD prophylaxis ($P<0.001$) and duration from diagnosis to HSCT ($P=0.041$). The majority of patients received the preparatory

Table 1 Patient characteristics ($N=173$)

Characteristic	*Imatinib cohort*	*Pre-imatinib cohort*	P
No. of transplantations	51	122	
Age, n (%)			0.048
–39	27 (53)	71 (58)	
40–54	17 (33)	49 (40)	
55–	7 (14)	2 (2)	
Median (range)	38 (15–64)	38 (15–57)	
Gender (male/female)	29/22	73/49	0.717
HSCT donor, n *(%)*			0.460
Related	24 (47)	73 (60)	
Unrelated	21 (41)	43 (35)	
HLA-mismatched related	6 (12)	6 (5)	
Hematopoietic cell source, n *(%)*			0.246
Bone marrow	35 (69)	94 (77)	
Peripheral blood	16 (31)	28 (23)	
Conditioning regimen, n *(%)*			<0.001
CY+TBI	24 (47)	26 (22)	
CY+CA+TBI	14 (27)	37 (31)	
CY+VP+TBI	2 (4)	21 (17)	
CY+TESPA+TBI	—	7 (6)	
CY+BU+TBI	—	6 (5)	
Flu+BU	3 (6)	—	
Flu+ LPAM ± TBI	2 (4)	—	
Others	6 (12)	25 (20)	
GVHD prophylaxis, n *(%)*			<0.001
Cyclosporine + sMTX	24 (47)	95 (80)	
Cyclosporine ± other	3 (6)	3 (2)	
Tacrolimus + sMTX	22 (43)	17 (14)	
Tacrolimus + other	—	4 (3)	
Median days from diagnosis to HSCT (range)	162 (67–512)	182 (66–834)	0.041

Abbreviations: BU, oral busulfan; CA, cytarabine; CY, cyclophosphamide; Flu, fuludarabine; GVHD, graft-versus-host disease; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplantation; LPAM, melphalan; sMTX, short-term methotrexate; TBI, total body irradiation; TESPA, tespamine; VP, etoposide.

regimen of total body irradiation followed by cyclophosphamide and/or cytarabine. Five patients aged >55 in the imatinib cohort were given a reduced intensity regimen consisting of fludarabine and melphalan or busulfan. In the pre-imatinib cohort, a combination of cyclosporine (CsA) and short-term methotrexate (sMTX) was mostly used in the prophylaxis of GVHD. On the other hand, both CsA + sMTX and tacrolimus (FK506) + sMTX combinations were commonly used in the imatinib cohort. In both cohorts, none of the patients received imatinib therapy after HSCT in their first CR. In the imatinib cohort, all patients who showed hematologic relapse after HSCT received salvage treatment comprising of imatinib and/or chemotherapy. As for the pre-imatinib cohort, 13 patients relapsed after the approval of imatinib by the Japanese government (beyond December 2001). However, we have no information on how many patients received imatinib-based therapy after their relapse. The median follow-up period for survivors was 2.6 years (range, 1.0–4.6 years) for the imatinib cohort and 6.9 years (range, 0.1–11.4 years) for the pre-imatinib cohort.

Outcome

OS and DFS. In the pre-imatinib cohort, 80 patients died after HSCT: 46 of disease recurrence and 34 of causes other than leukemia. In the imatinib cohort, 35 patients were alive, 32 of them were free of leukemia and 16 patients died after HSCT: 4 of disease recurrence and 12 of causes other than leukemia. The 3-year OS was 65% (95% confidence interval (CI), 49–78%) for the imatinib cohort and significantly higher than 44% (95% CI, 35–52%) for the pre-imatinib cohort ($P=0.0148$; Figure 1a). The 3-year DFS was 58% (95% CI, 41.8–70.9%) for the imatinib cohort and significantly higher than 37% (95% CI, 28.5–45.6%) for the pre-imatinib cohort ($P=0.039$; Figure 1b).

Table 2 shows the result of risk factor analysis for OS and DFS among all 173 patients. In the multivariate analysis, the only variable found to influence OS and DFS was the pre-transplant imatinib-based therapy (hazard ratio (HR) = 0.44 (95% CI, 0.25–0.77); $P=0.004$ and HR = 0.51 (95% CI, 0.31–0.86); $P=0.011$, respectively). The presence of cGVHD showed a tendency of favorable OS and DFS, but did not reach the statistical significance (HR = 0.66 (95% CI, 0.42–1.06); $P=0.085$ and HR = 0.75 (95% CI, 0.47–1.19); $P=0.217$, respectively).

Other outcomes of transplantation

Relapses. In the pre-imatinib cohort, 48 patients relapsed after HSCT with a median of 240 days (range, 42–2302 days).

In the imatinib cohort, 7 patients (3 of 36 with PCR negative and 4 of 12 with PCR positive at HSCT) relapsed after HSCT with a median of 137 days (range, 68–728 days). The estimated cumulative incidence of relapse at 3 years was 15.0% (95% CI, 6.6–26.7%), and significantly lower than that of the pre-imatinib cohort (50.4% at 3 years (95% CI, 39.6–60.2%); $P=0.002$; Figure 1c). Among patients in the imatinib cohort, patients with PCR negative showed significantly lower relapse rate compared with that of PCR positive (10.0% (95% CI, 2.5–23.6%) versus 41.3% (95% CI, 16.9–64.4%) at 3 years, respectively, $P=0.025$).

Non-relapse mortality. In the pre-imatinib cohort, 34 patients died of non-relapse causes at a median of 159 days (range, 5–2094 days) after HSCT. The estimated cumulative incidence of NRM in the pre-imatinib cohort was 28% (95% CI, 20–36) at 3 years (Figure 2a). In the imatinib cohort, 12 patients died of non-relapse causes at a median of 329 days (range, 41–850 days) after HSCT. The 3-year cumulative incidences of NRM were 21% (95% CI, 11–33%; Figure 2a). There were no significant differences between two cohorts ($P=0.265$).

Cause of death. Recurrence of the primary disease was the leading cause of death in both groups: 55% for the pre-imatinib cohort and 25% for the imatinib cohort. In the pre-imatinib cohort, the causes of NRM were organ failure (11%), infection (9%), GVHD (8%), transplantation-associated thrombotic microangiopathy (TMA) (4%), interstitial pneumonia (3%), graft failure (3%) and other causes (6%). In the imatinib cohort, the causes of NRM included infection (19%), bronchiolitis obliterans with organizing pneumonia (13%), TMA (13%), GVHD (13%), organ failure (6%) and other causes (12%).

Graft-versus-host disease. There was no significant difference in the cumulative incidence of Grades 2–4 aGVHD between two cohorts (31% (95% CI, 19–44%) versus 37% (95% CI, 29–46%), $P=0.391$; Figure 2b). The cumulative incidence of cGVHD at 1 year after HSCT was significantly higher in the imatinib cohort than in the pre-imatinib cohort (49% (95% CI, 31–64%) versus 27% (95% CI, 18–37%), $P=0.0261$; Figure 2c).

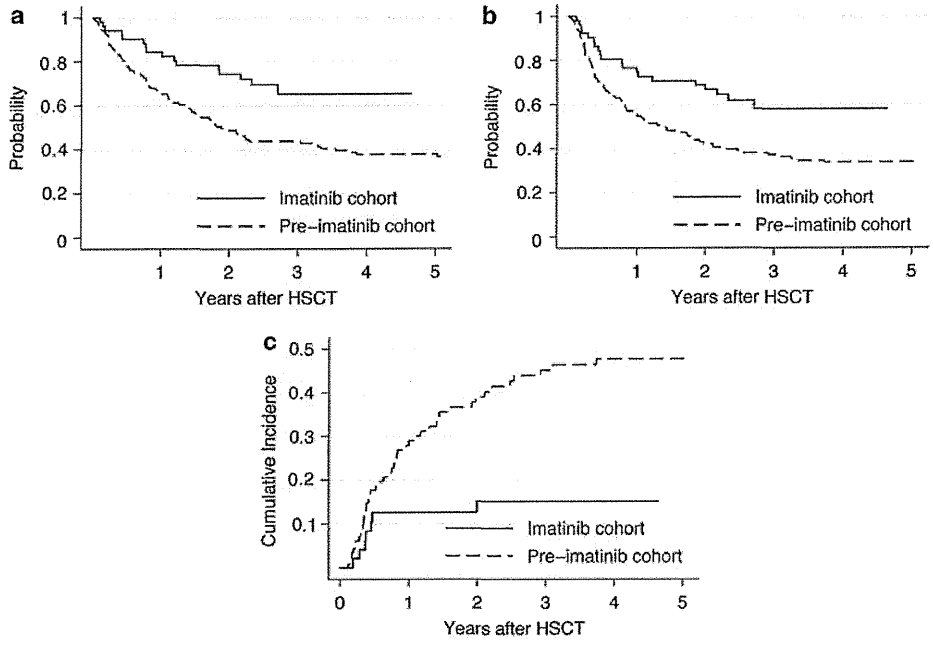

Figure 1 Transplantation outcomes of 51 patients who received imatinib-based therapy and 122 historical patients. (**a**) Overall survival, (**b**) disease-free survival and (**c**) cumulative incidence of relapse.

Table 2 Results of uni- and multivariate analysis of overall survival and disease-free survival among 173 patients with Ph+ALL

Characteristic	*Overall survival*				*Disease-free survival*			
	Univariate analysis		*Multivariate analysis*		*Univariate analysis*		*Multivariate analysis*	
	RR (95% CI)	P	*RR (95% CI)*	P	*RR (95% CI)*	P	*RR (95% CI)*	P
Imatinib-imterim therapy before HSCT	0.45 (0.26–0.77)	0.004	0.44 (0.25–0.77)	0.004	0.51 (0.31–0.83)	0.007	0.51 (0.31–0.86)	0.011
Donor status (RE versus UR)	0.87 (0.57–1.32)	0.521	0.72 (0.40–1.30)	0.275	0.77 (0.51–1.16)	0.211	0.65 (0.37–1.16)	0.147
Age at HSCT (–39 versus 40–55 versus 55–)	1.03 (0.74–1.44)	0.852	1.12 (0.78–1.62)	0.536	0.98 (0.71–1.36)	0.914	1.03 (0.73–1.47)	0.862
HLA-disparity (matched versus mismatched)	0.90 (0.39–2.06)	0.800	0.76 (0.32–1.81)	0.531	1.11 (0.49–2.54)	0.800	1.06 (0.45–2.50)	0.895
Stem-cell source (BM versus PB)	1.15 (0.72–1.82)	0.565	1.23 (0.72–2.10)	0.451	1.30 (0.85–2.00)	0.228	1.34 (0.81–2.20)	0.254
Days from diagnosis to HSCT	1.00 (0.99–1.00)	0.217	1.00 (0.99–1.00)	0.141	1.00 (0.99–1.00)	0.415	1.00 (0.99–1.00)	0.125
cGVHD as time-varying covariate (yes versus no)	0.68 (0.43–1.08)	0.101	0.66 (0.42–1.06)	0.085	0.78 (0.50–1.23)	0.292	0.75 (0.47–1.19)	0.217

Abbreviations: ALL, acute lymphoblastic leukemia; BM, bone marrow; CI, confidence interval; cGVHD, chronic graft-versus-host disease; HLA, human leukocyte antigen; HSCT, hemtopoetic stem cell transplantation; PB, peripheral blood; Ph, Philadelphia chromosome; RE, related; RR, relative risk; UR, unrelated.

However, regarding the cumulative incidence of extensive-type cGVHD, there was no significant difference between two cohorts (22% (95% CI, 10–36%) versus 12% (95% CI, 6–20%), $P=0.119$; Figure 2d).

Association between cGVHD and OS/DFS/relapse. To examine the difference of impacts of cGVHD upon clinical outcome in the pre- and imatinib cohorts, we conducted stratified analysis by cohort, treating cGVHD as a time-varying covariate (Table 3). Multivariate analysis revealed that, in the imatinib cohort, there were no significant associations between cGVHD and OS/DFS/relapse ($P=0.707$, 0.332 and 0.713, respectively). On the other hand, in the pre-imatinib cohort, there was a significant association between cGVHD and OS (HR=0.59 (95% CI, 0.35–1.00), $P=0.048$), but not between cGVHD and DFS/relapse ($P=0.234$ and 0.338, respectively).

Engraftment. In the pre-imatinib cohort, three patients experienced graft failure. The median periods to reach the neutrophil count of $>0.5\times10^6$/l and platelet count of 50×10^6/l were 15 days (range, 8–49 days) and 25 days (range, 9–120 days), respectively, for evaluable patients. In the imatinib cohort, all 51 patients were engrafted. The median period to reach a neutrophil count of $>0.5\times10^6$/l and platelet count of 50×10^9/l was 15 days (range, 5–41 days) and 25 days (range, 11–504 days), respectively, for evaluable patients. There was no

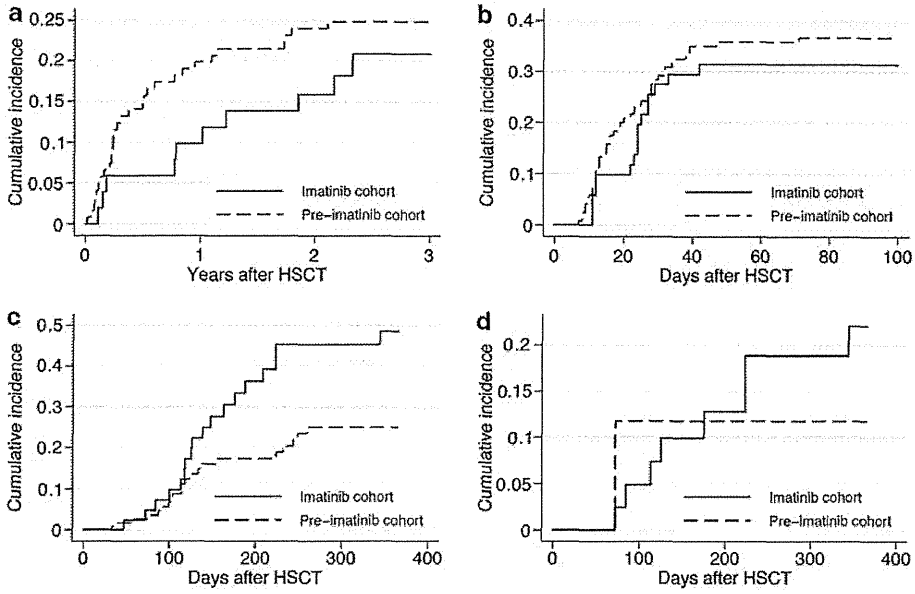

Figure 2 Cumulative incidence of GVHD or NRM. (**a**) Non-relapse mortality, (**b**) Grade 2–4 acute GVHD, (**c**) chronic GVHD and (**d**) extensive-type chronic GVHD.

Table 3 Impact of overall cGVHD on OS, DFS and relapse in multivariate analysis using cGVHD as a time-varying covariate

Cohort	*OS*			*DFS*			*Relapse*		
	Relative risk	*95% CI*	P	*Relative risk*	*95% CI*	P	*Relative risk*	*95% CI*	P
Imatinib cohort	0.80	(0.26–2.51)	0.707	0.59	(0.21–1.71)	0.332	0.74	(0.15–3.67)	0.713
Pre-imatinib cohort	0.59	(0.35–1.00)	0.048	0.73	(0.43–1.23)	0.234	0.75	(0.39–1.44)	0.388

Abbreviations: CI, confidence interval; cGVHD, chronic graft-versus-host disease; DFS, disease-free survival; HLA, human leukocyte antigen; OS, overall survival; PBSC, peripheral blood stem cell.
Data were adjusted for age categories, donors from unrelated subjects, HLA-matching status, PBSC graft and days to transplantation. Cox proportional hazard models were applied to OS and DFS, and a competing risk regression model was applied to relapse.

significant difference in neutrophil and platelet recovery between two cohorts ($P=0.201$ and 0.783, respectively).

Discussion

This study showed that patients with Ph + ALL who achieved CR by imatinib-based therapy and subsequently received allo-HSCT in their first CR showed significantly superior survival outcome to those in the pre-imatinib era. To our knowledge, our current report is the first to describe the superiority of imatinib-based therapy for this disease by analyzing a substantial number of patients with sufficient follow-up period. The treatment of Ph + ALL has changed dramatically since the introduction of imatinib and >90% of patients have achieved CR,[7,14,15] and allows SCT to be performed in a substantial proportion of patients in major or complete molecular remission.[8,16–18] Actually, in the imatinib cohort, 97 of 100 patients (97%) achieved CR and 60 (60%) could receive allo-HSCT in their first CR. Several studies reported improved OS rates compared with that in the pre-imatinib era by incorporation of imatinib-based therapy.[14,15,19,20] However, there had been few reports focusing on the clinical impact of pre-transplant imatinib administration on the outcome of HSCT. Lee *et al.*[8] reported superior outcome of HSCT by imatinib-based therapy compared with the historical control data, in which 29 patients with prior imatinib treatment showed better outcomes in terms of relapse, DFS and OS than the historical control patients. However, their comparative analysis included patients who received HSCT for refractory disease or beyond their first CR (4 of 29 patients in the imatinib group and 16 of 33 patients in the historical group). Several studies showed that remission status at the time of HSCT was one of the most important prognostic factors for outcome.[21,22] Therefore, we contend that it would be better to assess a greater number of patients and exclude patients with advanced stage at HSCT to accurately compare the clinical impact of imatinib-based therapy on the outcome of HSCT. To our knowledge, this study has the largest number of Ph + ALL patients receiving allo-HSCT in their first CR with the longest follow-up duration yet reported.

It is noteworthy from our findings that a lower rate of relapse was found in the imatinib cohort. Our results thus suggest that an imatinib-based therapy provides a survival benefit for newly diagnosed Ph + ALL patients by lowering the rate of subsequent relapse after HSCT. Despite the lack of comparative data of MRD in the pre-imatinib cohort, 75% of patients in the imatinib cohort achieved RQ–PCR negativity for *BCR/ABL* at the time of HSCT. Moreover, the relapse rate was significantly lower among

patients with PCR negative. From these, we believe that a powerful anti-leukemia activity of the imatinib-based therapy mostly contributed to the prevention of subsequent relapse after HSCT in the present analysis. Thinking of the reduced relapse rate after HSCT, impact of cGVHD should also be considered. Several studies in the pre-imatinib era reported beneficial impact of cGVHD on relapse incidence and survival.[23–25] In this study, the incidence of cGVHD was significantly higher in the imatinib cohort compared with that in the pre-imatinib cohort. In the imatinib cohort, more patients received PB as a stem cell source, which might have contributed to the high frequency of cGVHD. Besides, longer leukemia-free survival period in the imatinib cohort might have contributed to the increased frequency of cGVHD, which is a late complication often observed in the recipients of allo-HSCT who had survived without disease for at least 3 months after transplantation. One could argue that this observation could be related to a stronger graft versus leukemia effect and contribute to the lower relapse rate. However, the presence of cGVHD had no significant impact on the OS/DFS/relapse rate in our imatinib cohort by multivariate analysis.

To assist the proper interpretation of our current results, the strengths and limitations need to be considered. As discussed earlier, one of the strengths of this study is the large sample size for the imatinib cohort, which gives us a better estimation of the end points and also adds statistical power to the analyses. In addition, adjustments for potential confounders in the comparisons with the pre-imatinib cohort from a nationwide registry allow unbiased estimates to be made, at least in Japan. Given the evidence for a substantial impact of imatinib in Ph + ALL patients,[7,14–16] it is unrealistic to conduct a prospective study comparing treatments with or without imatinib. Hence, a retrospective cohort design could be suboptimal to address the key questions.

One of the possible limitations of our current analysis could be the presence of residual confounding factors both of known and unknown. Among the known factors, a difference in the conditioning regimens could be noted. The City of Hope National Medical Center reported a favorable result from the use of a fractionated TBI–etoposide regimen in the treatment of Ph + ALL.[26] However, in the comparative analysis, the clinical advantage of this approach seemed to be established mostly among patients transplanted in their second CR.[27] Moreover, this approach was commonly applied in our pre-imatinib cohort rather than in the imatinib cohort (22 and 4%, respectively). Differences in GVHD prophylaxes should also be considered. Tacrolimus was more frequently used in the imatinib cohort than in the pre-imatinib cohort, which reflects the change in practice within the field of allo-HCT in Japan as tacrolimus was widely used for unrelated allo-HSCT since 2000. Nevertheless, the lack of any differences in the incidence of aGVHD between two cohorts indicates that this factor had minimal impact in our analysis.

It may be argued that the improved outcome of the imatinib cohort have been influenced by the pre-transplant chemotherapy in the JALSG Ph + ALL 202 study. Although detailed information on the pre-transplant chemotherapy in the pre-imatinib cohort was not available, it was clear that the majority of patients were most likely treated by the JALSG ALL93 or JALSG ALL97 protocols as pre-transplant chemotherapy,[2] as these were widely used regimens in Japan at the time. The chemotherapeutic regimen in the JALSG Ph + ALL202 study was similar to those used in these protocols. Thus, the effectiveness on Ph + ALL would have been similar between the two cohorts. At least in JALSG, there had been neither remarkable progress in the chemotherapy of Ph + ALL until the clinical introduction of imatinib, nor in other groups including the MD Anderson Cancer Center.[28] Thus, in the present analysis, the influence of pre-transplant chemotherapy appears to be quite limited.

The difference of transplant year between the two cohorts (1995–2001 and 2002–2005, respectively) could have affected the outcome of HSCT, and the improvement of transplantation procedure might have contributed to the favorable outcome in the imatinib cohort. However, Nishiwaki *et al.*[29] analyzed the clinical outcome of 641 Japanese patients with Ph-negative ALL who had received allo-HSCT in their first CR in 1993–1997, 1998–2002 and 2003–2007, and reported that there was no statistical difference in OS and NRM between three periods. In this study, the incidence of NRM was lower in the imatinib cohort, but did not reach the statistical significance. Therefore, the influence of transplantation year is thought to be limited in this study.

Considering potential benefit by imatinib, the lack of information about post-transplant imatinib use in the pre-imatinib cohort might have led us to underestimate the difference between two cohorts.

In conclusion, we have found that there is a significant improvement in the OS and DFS of Ph + ALL patients who received allo-HSCT following imatinib-based therapy. Although further validation using larger cohorts from different populations is essential to confirm our findings, imatinib-based therapy is likely to be a useful strategy for not only giving patients with Ph + ALL more chance to receive allo-HSCT, but also for improving their outcome after allo-HSCT.

Conflict of interest

Dr Naoe has received research funding and honoraria from Novartis Japan. Dr Ohnishi has received research funding from Novartis Japan. Dr Miyazaki has received honoraria from Novartis Japan. The remaining authors declare no conflict of interest.

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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

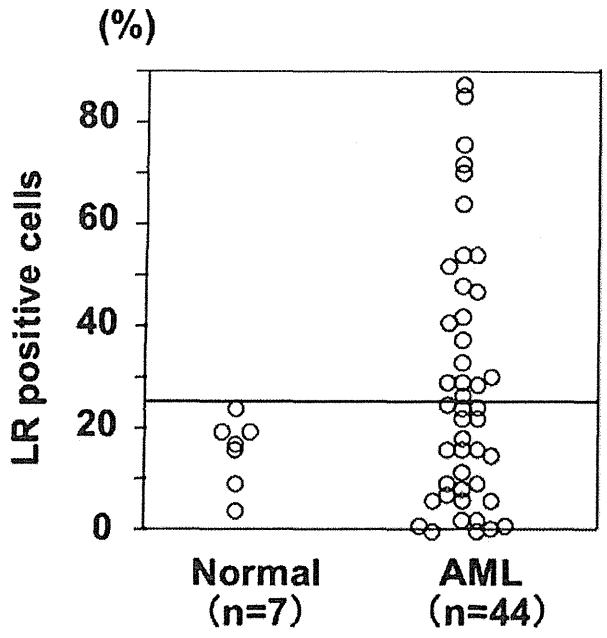

Figure 1. Surface expression of LR on CD34-positive cells. Expression of 67-kD LR was examined in the CD34-positive fraction of AML cells and that of bone marrow cells from healthy volunteer by flow cytometry. AML cases were divided into two groups based on the rate of LR expression: a high-expression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

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).

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

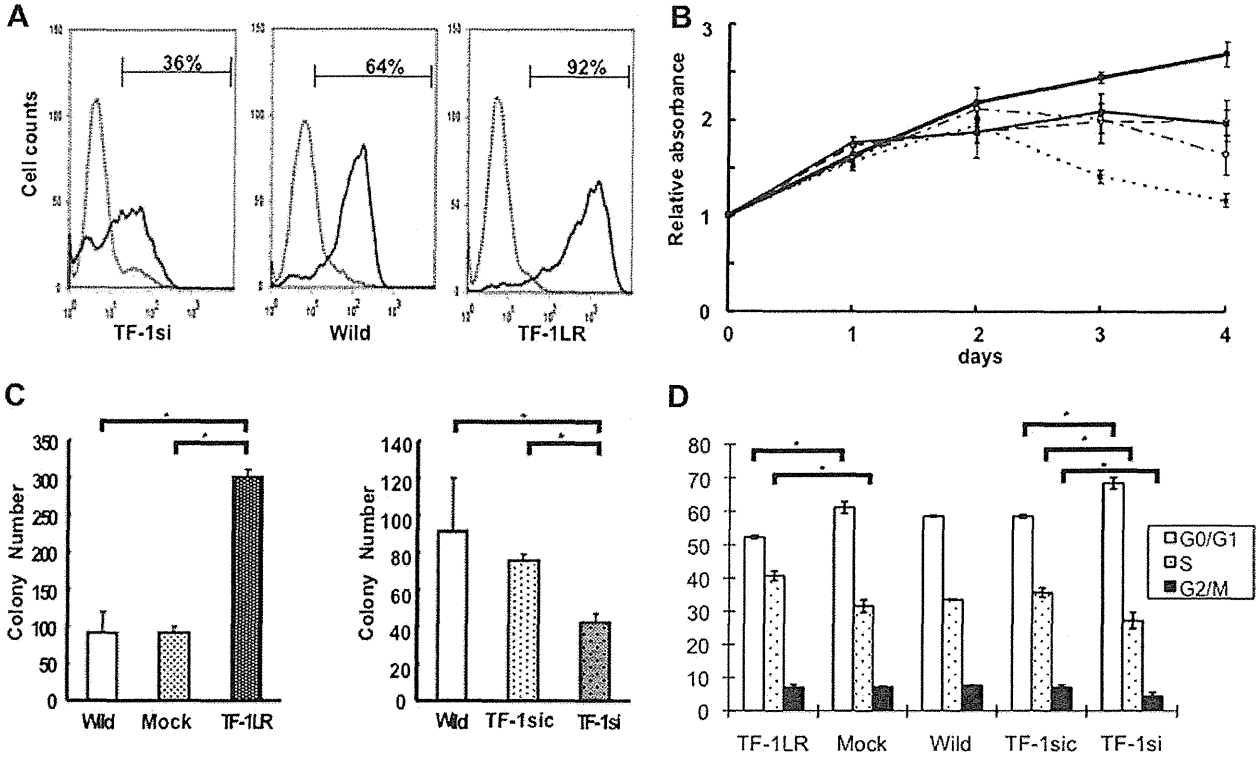

Figure 2. Expression or suppression of LR on leukemia cells modulated the growth characteristics of leukemia cells. (**A**) Surface expression of LR on wild-type TF-1, TF-1LR, and TF-1si was 64%, 92%, and 36%, respectively. Histogram overlays show the data of isotype control (dotted lines) and the anti-LR 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	Phase (%)		
	G_0/G_1	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

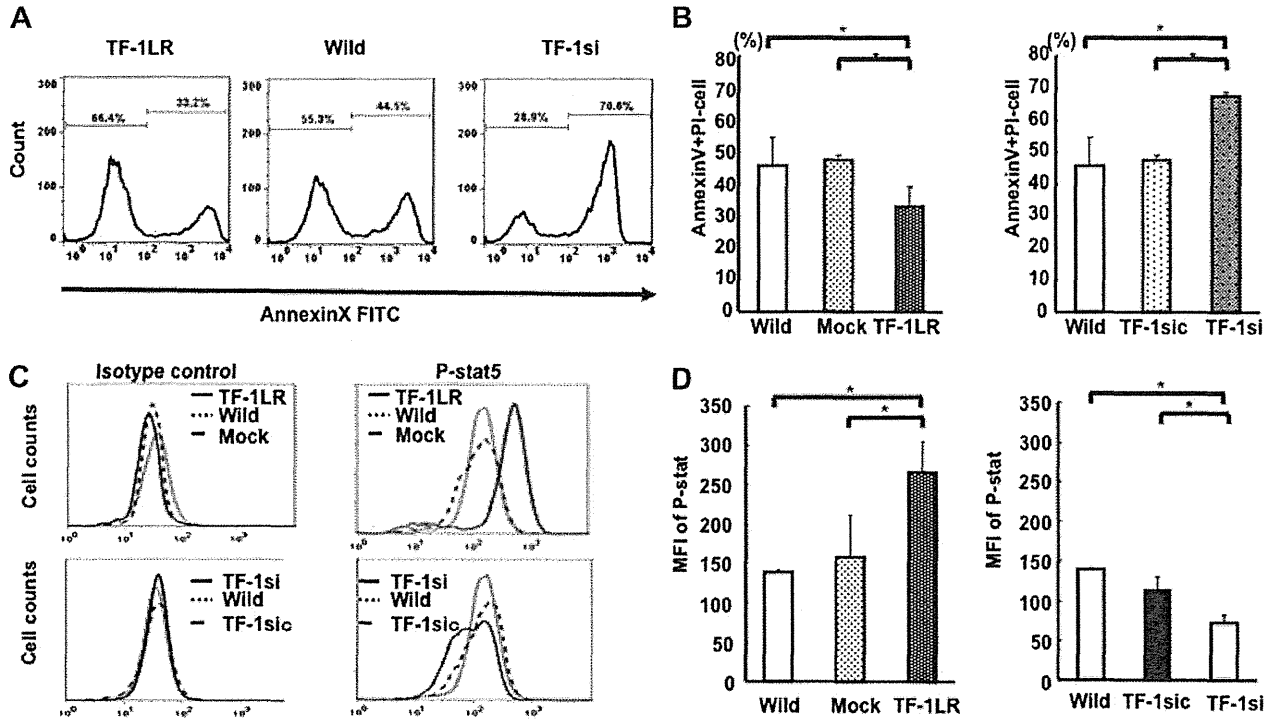

Figure 3. Apoptosis and phosphorylation of STAT5 in TF-1–related cell lines. Cells were cultured in medium with a low concentration of GM-CSF (0.1 ng/mL); then apoptotic cells were assessed using Annexin-V expression and propidium iodide (PI) staining at day 4 of culture. (**A**) One representative experiment 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) (*$p < 0.05$).

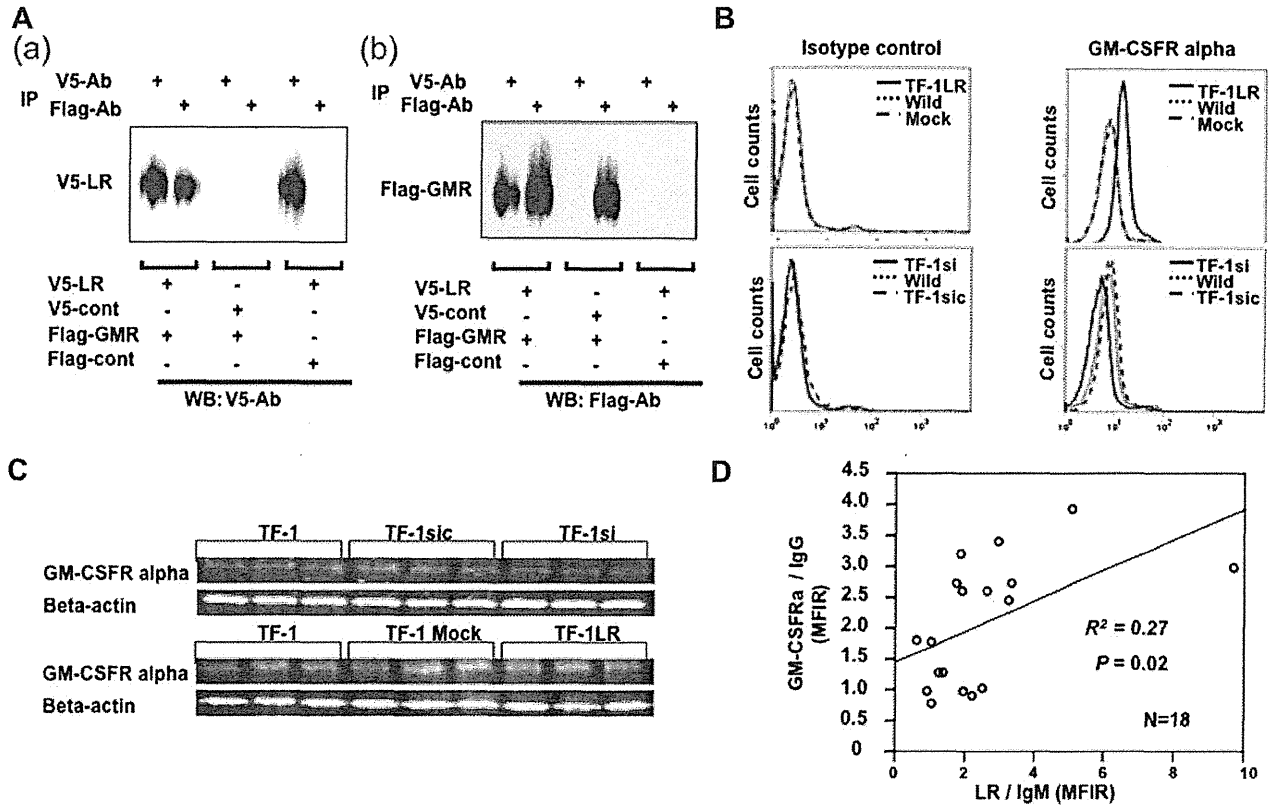

Figure 4. Association of LR and GM-CSFRα, and the relationship of the expression of LR and GM-CSFα in leukemia cell lines and AML samples. (**A**) Expression plasmids for LR (V5-LR) and GM-CSFRα (Flag-GMR) with tags were transfected into 293T cells. Cell lysates were subjected to immunoprecipitation 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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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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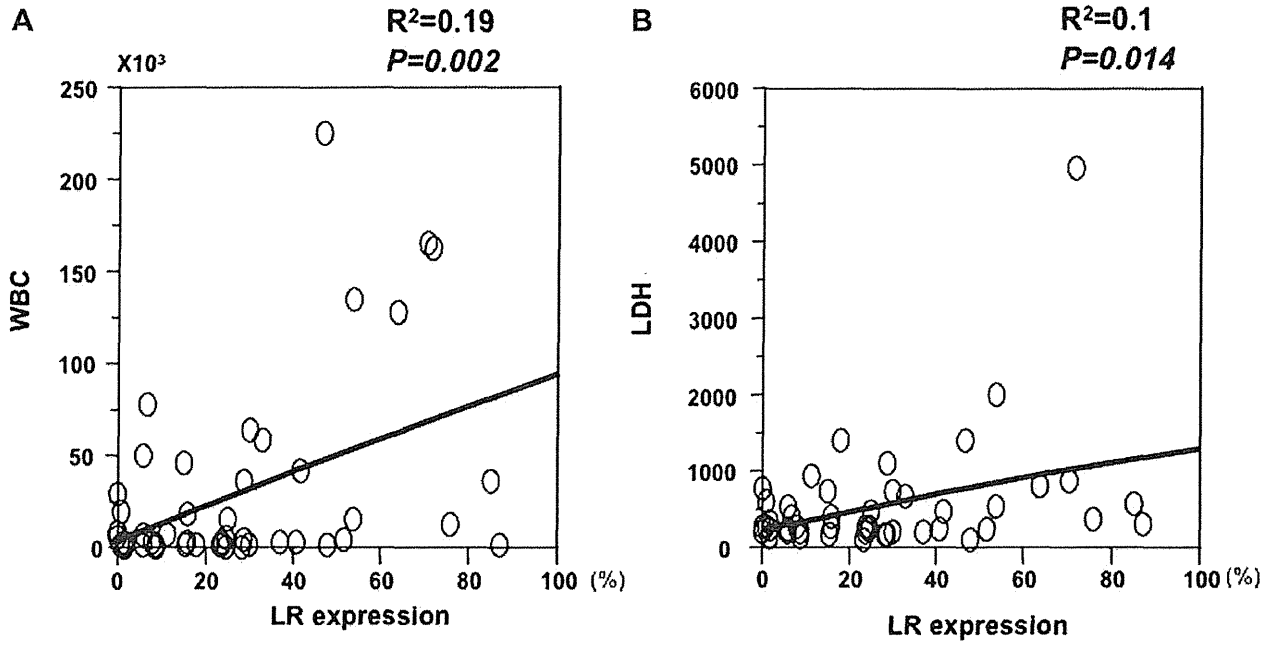

Supplementary Figure E1. Relationship between LR expression with WBC and LDH. There is a statistically significant relationship between expression of LR on CD34-positive AML cells with WBC (**A**) and LDH (**B**), even when these factors are treated as continuum (p = 0.002 and 0.014, respectively).