significantly inhibited the basal binding of α4 integrin to sVCAM-1 (Fig. 2A–D). The results demonstrated that inhibition of FLT3/ITD downregulates the affinity of α4β1 integrin in both cells. Imatinib, an Abl inhibitor, did not deactivate α4β1 integrin suggesting that this event is FLT3-specific (Fig. 2C,D).

Inhibition of FLT3/ITD reduces the α4β1 integrin affinity to sVCAM-1 in leukaemic cells from patients with AML

Next, we analysed the leukaemic cells from patients with AML. Approximately 1×10^6 of leukaemic cells were used for each assay. All primary AML samples were analysed by flowcytometry, indicating that more than 90% of cells expressed FLT3 (data not shown). Preincubation of FLT3/ITD-bearing leukaemic cells with FLT3 selective inhibitor FI-700 (500 nM, 1 hr) significantly inhibited the basal binding of α4 integrin to sVCAM-1 (Fig. 3A). On the other hand, FI-700 had no effects on wtFLT3 leukaemia cells (Fig. 3B).

Inhibition of FLT3/ITD had no effects on Rap1 activity

We then investigated the upstream signal of α4β1 integrin. Rap1 is known to be a critical mediator of inside-out signalling of α4 integrin in Jurkat cells (16) and eosinophils (14). Therefore, we investigated whether FLT3 inhibitor inactivates Rap1. FI-700 treatment of both NAMO-2 and MOLM-13 cells did not alter the GTP loading of Rap1, demonstrating that deactivation of α4β1 integrin by FLT3 inhibitor is not mediated by Rap1 (Fig. 4A).

Inhibition of FLT3/ITD induces dephosphorylation of Akt and Pyk2

A previous study demonstrated that FLT3/ITD expression confers autonomous proliferation capability and induces constitutive activation of downstream signalling molecules such as STAT5, MAP kinase and Akt (3). Consistent with that, FI-700 treatment dephosphorylated Serine 473 of Akt in NAMO-2 cell (Fig. 4B). Pyk2 is a FAK family of non-receptor tyrosine kinase, which is abundantly expressed in haematopoietic cells. The stimulation of cytokine, chemokine and adhesion receptors such as integrin results in Pyk2 phosphorylation, potential recruitment of Src-family kinases and activation of other signalling pathways. A previous study demonstrated that PI3K activation is required for Pyk2 phosphorylation and activation in response to β1 integrin engagement (7). We therefore investigated whether inhibition of FLT3/ITD signalling dephosphorylates Tyr 402 of Pyk2. Addition of FI-700 caused marked dephosphorylation of Pyk2 in NAMO-2 cells (Fig. 4C).

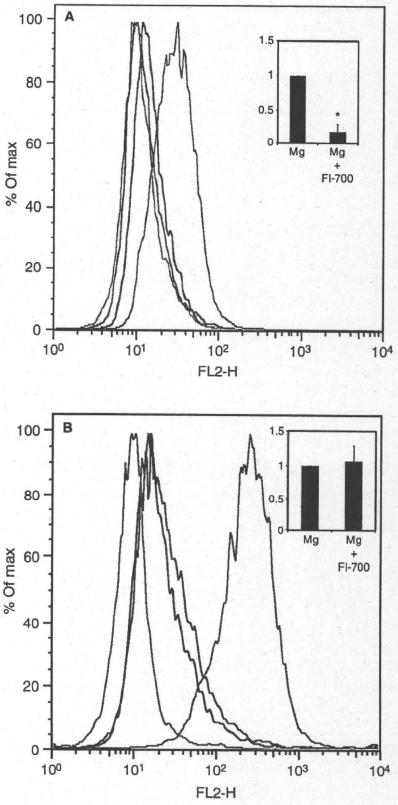

Figure 3 FI-700 reduces sVCAM-1/Fc binding of α4 integrin in the leukaemic blasts with FLT3/ITD but not in the cells with wtFLT3. Leukaemic blasts from the patients with FLT3/ITD (A) and wtFLT3 (B) were pretreated with or without FI-700 (500 nM) for 1 hr, and bound sVCAM-1/Fc was quantified. Cells incubated with 1 mM $MnCl_2$ (orange) and 5 mM EDTA (green) for 60 min at 37°C served as positive and negative controls, respectively. Cells incubated with 1 mM $MgCl_2$ are shown in red and those incubated with FI-700 are shown in blue line. The *y*-axis represents maximal cell numbers (%). Representative flow cytometry profiles of three independent experiments are shown. (Inset) Relative VCAM-1 binding of leukaemic cells with $MgCl_2$ and inhibitors is shown. Bars denote the mean ± SEM of cells of three independent experiments. $*P < 0.02$.

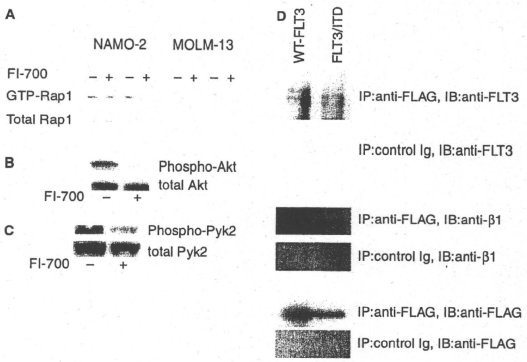

Figure 4 (A) FLT3 inhibitor has no effects on Rap1 activity. NAMO-2 cells or MOLM-13 cells were pretreated with or without FI-700 (500 nM) for 1 hr. Relative Rap1 activity is measured as described in the Materials and methods section. (B and C) Inhibition of FLT3/ITD dephosphorylates Akt and Pyk2. NAMO-2 cells cultured on NamoF stromal cells were pretreated with or without FI-700 (500 nM) for 1 hr, and phosphorylation of either Ser473 of Akt (B) or Tyr402 of Pyk2 (C) is quantified as described in the Materials and methods section. (D) FLT3, $\beta1$ integrin and Pyk2 form a macromolecular complex. pBJ1-α4 integrin, pBJ1-$\beta1$ integrin and pcDNA-FLAG-Pyk2 were cotransfected with either pMKIT-wtFLT3 or pMKIT-FLT3/ITD into COS-7 cells. Cells were lysed and immunoprecipitated with anti-FLAG M2 antibody (Sigma). Notably, both wtFLT3 and FLT3/ITD co-immunoprecipitated with $\beta1$ integrin and Pyk2 (Fig. 4D). These results demonstrated that FLT3 and $\beta1$ integrin form macromolecular complex with Pyk2.

FLT3, $\beta1$ integrin and Pyk2 form a macromolecular complex

We next performed a co-immunoprecipitation assay to evaluate whether Pyk2, $\beta1$ integrin and FLT3 are physically associated. pBJ1-α4 integrin, pBJ1-$\beta1$ integrin and pcDNA-FLAG-Pyk2 were cotransfected with either pMKIT-wtFLT3 or pMKIT-FLT3/ITD into COS-7 cells. Cells were lysed and immunoprecipitated with anti-FLAG M2 antibody (Sigma). Notably, both wtFLT3 and FLT3/ITD co-immunoprecipitated with $\beta1$ integrin and Pyk2 (Fig. 4D). These results demonstrated that FLT3 and $\beta1$ integrin form macromolecular complex with Pyk2.

Discussion

A previous study demonstrated that interactions between $\alpha4\beta1$ integrin and leukaemic cells to fibronectin and bone marrow stromal cells are essential for the increased survival of AML cells, which is at least partially through the PI3K/Akt pathway (5). In the present study, we have demonstrated that basal activity of $\alpha4\beta1$ integrins on FLT3/ITD positive cells is downregulated by FLT3 selective inhibitor FI-700. A previous report demonstrated that stimulation by FL induces increased adhesiveness of haematopoietic progenitor cells related to activation of $\beta1$ integrin (17); however, the precise induction mechanism is still elusive. Rap1 is a major regulator of integrin inside-out signalling; however, FI-700 did not affect Rap1 activation (Fig. 4A). Several reports demonstrated that PI3K is required for the activation of $\alpha v\beta3$ (18) and $\alpha6\beta4$ integrin [15], raising the possibility that FI-700-induced integrin deactivation might be mediated by PI3K. In the anchorage-dependent cells, FAK is a kinase that is critical for initiating the $\beta1$ integrin signalling cascade, including activation of PI3K. Primary AML cells expressing FAK display significantly higher migration and resistance to daunorubicin (19), although FAK is generally expressed at low levels in haematopoietic cells. Pyk2 is expressed abundantly in the central nervous system and haematopoietic cells (20). It is activated as part of a signalling cascade in response to increase in Ca^{2+} levels, activation of protein kinase C or activation of G protein–coupled receptors (21). Based on its ability to respond to these multiple signals, Pyk2 is believed to play a role in the response to many extracellular agonists in several different cell types. Pyk2 activation and Pyk2/c-Src binding are initiated by Pyk2 autophosphorylation on Tyr402, which leads to binding of the c-Src SH2 domain to a motif that includes the Tyr402 residue, with subsequent c-Src activation (22). A previous study demonstrated that activation of PI3K following $\beta1$ integrin engagement on human $CD34^+$ cells results in subsequent phosphorylation of Pyk2 and is

required for the recruitment of the PI3K/Pyk2 complex with $\beta 1$ integrins at the cell surface (7). Therefore, it is possible that FI-700 downregulates Pyk2 through inactivation of PI3K.

The basal activity of $\alpha 4\beta 1$ integrin is downregulated by FI-700 only in FLT3/ITD positive but not in wtFLT3 cells. On the other hand, $\beta 1$ integrin and Pyk2 coprecipitated with both wtFLT3 and FLT3/ITD. A possible explanation of this discrepancy might be owing to the fact that potency of FI-700 against constitutively active FLT3 kinase was > 10 times higher than wt-FLT3 kinase (10).

Recently, siRNA targeting of Pyk2 in both AML cell lines (23) and primary samples from patients with AML (24) yielded reduction in cell growth and viability, suggesting that Pyk2 might be a novel target for therapeutic intervention in AML. In conclusion, our results suggest that inhibition of FLT3 kinase activity by FI-700 dephosphorylates Tyr402 of Pyk2, which is crucial for the signal transduction of multiple pathways. These findings suggest that anti-FLT3 therapy might work as an anti-adhesion therapy of AML.

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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.**

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

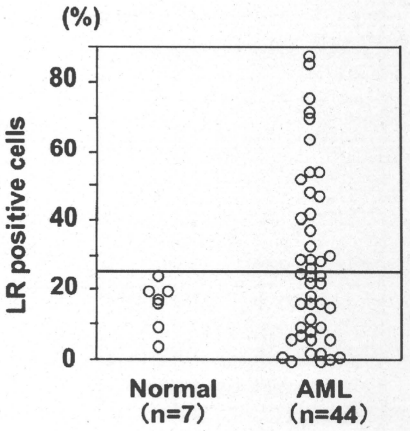

Figure 1. Surface expression of LR on CD34-positive cells. Expression of 67-kD LR was examined in the CD34-positive fraction of AML cells and that of bone marrow cells from healthy volunteer by flow cytometry. AML cases were divided into two groups based on the rate of LR expression: a high-expression 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

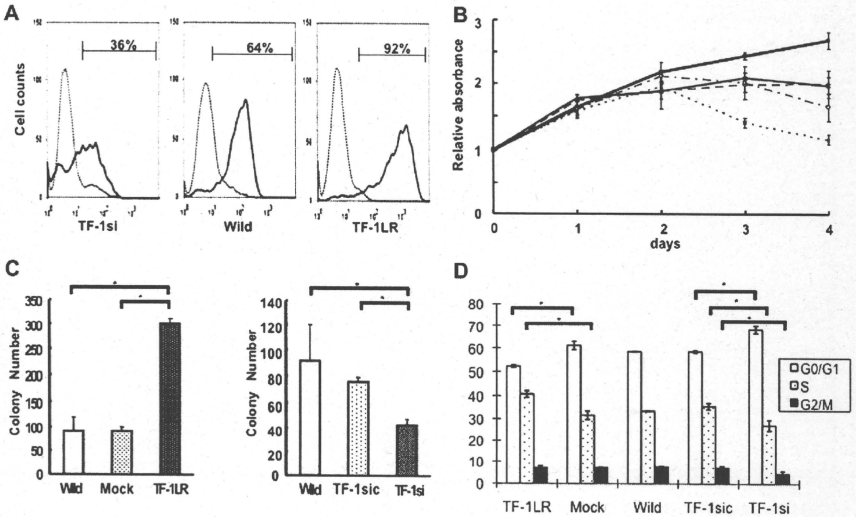

Figure 2. Expression or suppression of LR on leukemia cells modulated the growth characteristics of leukemia cells. (**A**) Surface expression of LR on wild-type TF-1, TF-1LR, and TF-1si was 64%, 92%, and 36%, respectively. Histogram overlays show the data of isotype control (dotted lines) and the anti-LR 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	G_0/G_1	Phase (%) S	G_2/M
TF-1LR	52.2 ± 0.5^{y}	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	$58.6 \pm 0.2^{v,w}$	$33.6 \pm 0.1^{x,y}$	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

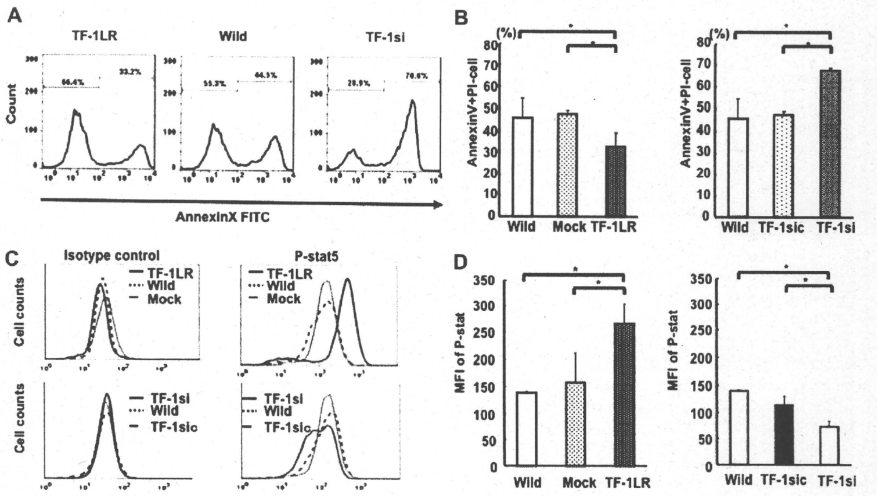

Figure 3. Apoptosis and phosphorylation of STAT5 in TF-1–related cell lines. Cells were cultured in medium with a low concentration of GM-CSF (0.1 ng/mL); then apoptotic cells were assessed using Annexin-V expression and propidium iodide (PI) staining at day 4 of culture. (**A**) One representative experiment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

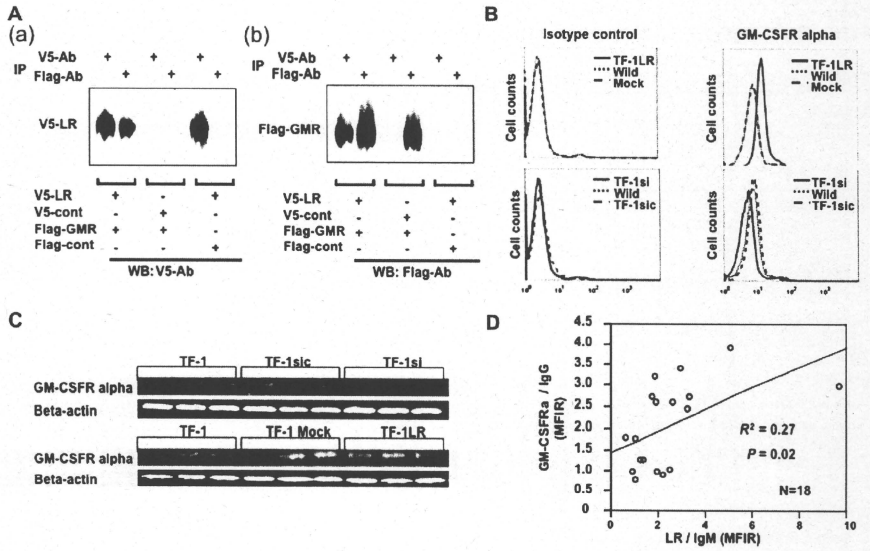

Figure 4. Association of LR and GM-CSFRα, and the relationship of the expression of LR and GM-CSFα in leukemia cell lines and AML samples. (**A**) Expression plasmids for LR (V5-LR) and GM-CSFRα (Flag-GMR) with tags were transfected into 293T cells. Cell lysates were subjected to immunoprecipitation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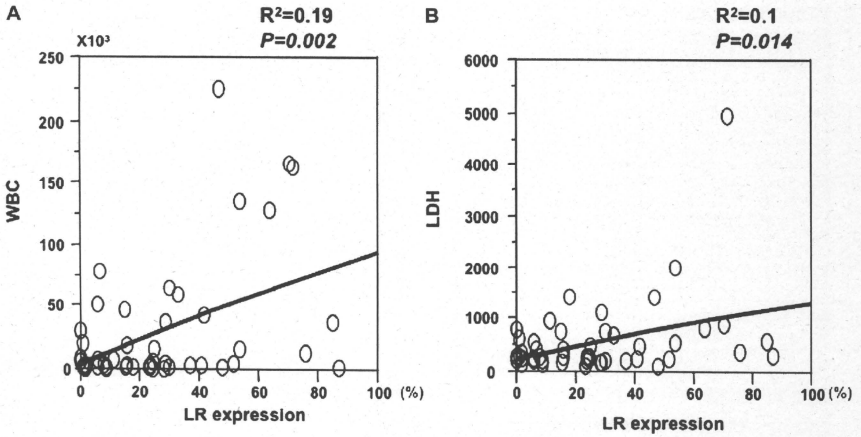
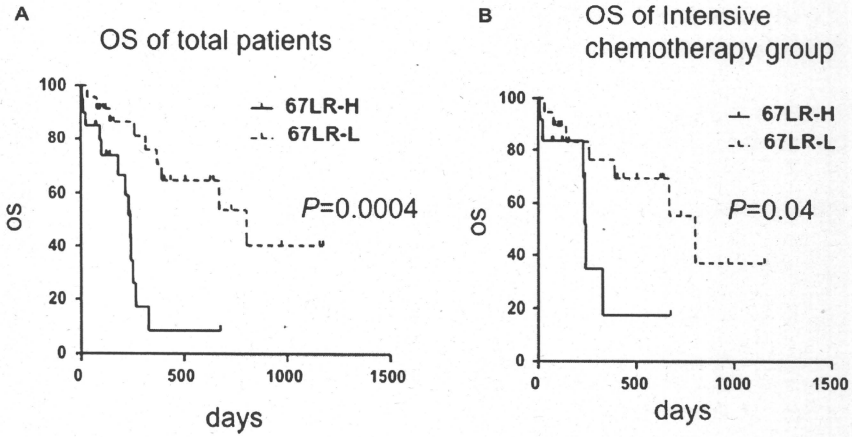
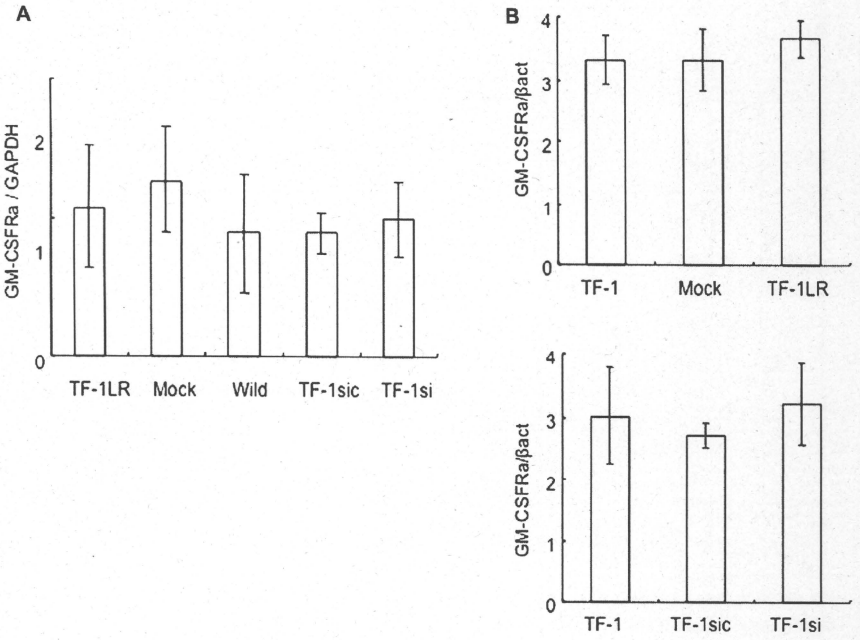


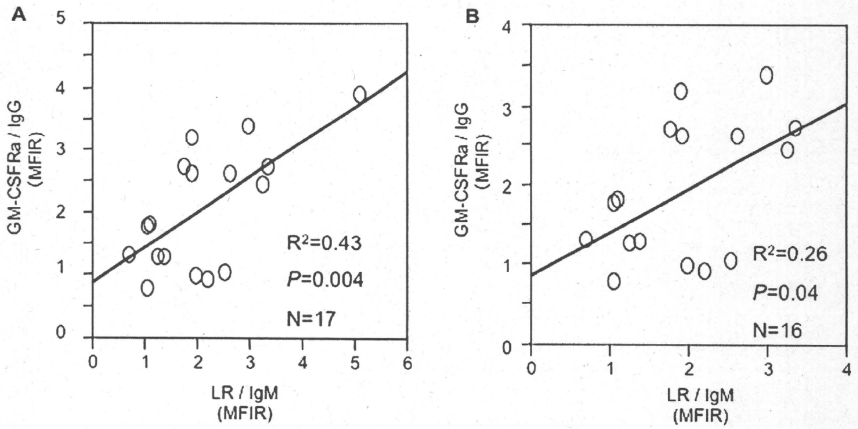
Supplementary Figure E1. Relationship between LR expression with WBC and LDH. There is a statistically significant relationship between expression of LR on CD34-positive AML cells with WBC (**A**) and LDH (**B**), even when these factors are treated as continuum (p = 0.002 and 0.014, respectively).



Supplementary Figure E2. Overall survival of patients treated with any chemotherapy (**A**) and intensive chemotherapy (**B**). Overall survival curve of all patients is shown in (**A**), and that of patients treated with intensive chemotherapy regimens is shown in (**B**). Intensive chemotherapy regimens contained standard dose of arabinofuranosyl cytidine and daunorubicin or idarubicin for induction, and high-dose AraC or induction-like regimens for consolidation therapy. These regimens were used in the protocols of Japan Adult Leukemia Study Group (JALSG-AML97 and -AML201 trials) [29].



Supplementary Figure E3. (**A**) Message level of GM-CSFRα in TF-1–related cell lines. The level of GM-CSFRα message was measured using quantitative PCR. The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference. Average of three independent experiments is shown with standard deviation. (**B**) Densitometric measurement of Western blot analysis for GM-CSFα (data from Fig. 4C). Ratios of GM-CSFRα and β-actin from three independent experiments are shown. Mock, control cell line for TF-1LR.



Supplementary Figure E4. Relationship between MFI ratio (MFIR) of LR and GM-CSFRα on AML samples. (**A**) One sample that was plotted at the far right in Figure 4D was removed, then using 17 samples, their relationship was reanalyzed. There was a significant relationship between these factors ($p = 0.004$). (**B**) Reanalysis of MFIR of LR and GM-CSFRα (n = 16) deleting two samples located at the far right and uppermost of Figure 4D. Significance still remained in the relationship between MFIR of LR and GM-CSFRα.

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ORIGINAL ARTICLE

A single-nucleotide polymorphism of the Fcγ receptor type IIIA gene in the recipient predicts transplant outcomes after HLA fully matched unrelated BMT for myeloid malignancies

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Fcγ receptor type IIIA (FCGR3A) has a functional single-nucleotide polymorphism (rs396991), at which a G-to T-point mutation results in an amino acid substitution at position 158 (valine to phenylalanine; V158F). This study examined the effect of the FCGR3A polymorphism in donors and recipients on the clinical outcomes in unrelated HLA fully matched myeloablative BMT. The FCGR3A-V158F genotype was retrospectively analyzed in a total of 99 recipients with myeloid malignancies, and their unrelated donors. The presence of the 158V genotype in recipients showed a statistically better OS (adjusted hazard ratio (HR) 0.49; 95% confidence interval (CI) 0.26–0.93; $P=0.03$) and TRM (HR 0.30; 95% CI 0.14–0.67; $P=0.003$) without significant influence on the relapse rate. The recipient 158V genotype was also associated with a significantly reduced risk of chronic GVHD (HR 0.45; 95% CI 0.20–0.99; $P=0.049$) and a trend toward a reduced risk of grade II–IV acute GVHD (HR 0.55; 95% CI 0.27–1.10; $P=0.09$), leading to a significantly reduced GVHD-related mortality (HR 0.22; 95% CI 0.06–0.77; $P=0.02$). The donor FCGR3A polymorphism did not have any effect on the transplant outcomes. These results suggest an association between the recipient FCGR3A genotype and the clinical outcomes after BMT.

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Keywords: FCGR3A; unrelated donor; single-nucleotide polymorphism

Introduction

Hematopoietic SCT is a potentially curative therapy in a range of malignant and nonmalignant diseases. However, its utility is limited because of transplant-related life-threatening complications, including GVHD, infections and disease relapse.[1] Although HLA matching represents the major genetic determinant in clinical outcome after allo-SCT, recent evidence suggests that non-HLA immune-associated genes are also implicated.[2] Previous investigations have revealed that several single-nucleotide polymorphisms (SNPs) that effect individual immune response to infections and inflammatory reactions are associated with transplant outcomes.[3–9]

Fcγ receptor type IIIA (FCGR3A), a low-affinity receptor capable of interaction with complexed or monomeric IgG, is expressed on neutrophils, eosinophils, natural killer cells, macrophages, monocytes, DC, γδ-positive T cells and keratinocytes.[10–13] FCGR3A mediates Ab-dependent cell-mediated cytotoxicity, phagocytosis, cytokine production and regulation of Ig production. FCGR3A has a functional SNP (rs396991), at which a G-to-T point mutation results in an amino acid substitution at position 158 (valine to phenylalanine; V158F) in the second Ig-like domain.[14] The cells bearing the FCGR3A-158V genotype show a higher affinity for IgG1 and IgG3 than those without 158V, and are capable of binding IgG4,[15] and thus

can exert Ab-dependent cell-mediated cytotoxicity more efficiently.[16] The 158V genotype is associated with susceptibility to rheumatoid arthritis[17] and immune-mediated thrombocytopenic purpura,[18] better clinical response to rituximab in B-cell lymphomas[19,20] and a lower risk of recurrent periodontitis.[21] In contrast, systemic lupus erythematosus and better clinical outcome of cetuximab against metastatic colorectal cancer correlates with the 158F genotype.[22,23] This study analyzed the effect of donor and recipient SNP (rs396991) in the FCGR3A gene on the clinical outcomes in patients after allogeneic myeloablative BMT using an HLA allele-matched unrelated donor. The data show that the presence of the FCGR3A-158V genotype in the recipient was associated with significantly better transplant outcomes on the OS, TRM and GVHD.

Patients and methods

Patients

FCGR3A genotyping was performed on a total of 99 recipients with myeloid malignancies and their unrelated donors who underwent transplantation after myeloablative conditioning through the JMDP (Japan Marrow Donor Program) with T-cell-replete marrow from an HLA-A, -B, -C and -DRB1 allele-matched donor between November 1995 and March 2000. HLA genotypes of HLA-A, -B, -C and -DRB1 allele of patient and donor were determined by the Luminex microbead method described previously (Luminex 100 System; Luminex, Austin, TX, USA).[24,25] No patient had a history of any previous transplantation. The final clinical survey of these patients was completed by 1 November 2007. Diagnoses were AML in 47 (47%), CML in 42 (42%) and myelodysplastic syndrome in 10 patients (10%; Table 1). The recipients were defined as having standard risk disease if they had AML in first CR, CML in any chronic phase or myelodysplastic syndrome. All others were designated as high-risk disease. CYA- or tacrolimus-based regimens were used in all patients for GVHD prophylaxis, and anti-T-cell therapy, such as antithymocyte globulin and *ex vivo* T-cell depletion, was not. All patients and donors gave their written informed consent to participate in molecular studies of this nature according to the declaration of Helsinki at the time of transplantation. The project was approved by the institutional review board of Kanazawa University Graduate School of Medicine and JMDP.

FCGR3A rs3969913 genotyping

Genotyping of FCGR3A was performed using the TaqMan-Allelic discrimination method[26] with a 7900-HT Real Time PCR system (Applied Biosystems, Foster City, CA, USA), and results were analyzed using the Allelic Discrimination software program (Applied Biosystems). The genotyping assay was conducted in 96-well PCR plates. The amplification reaction contained template DNA, the TaqMan universal master mix and the specific probe rs396991 designed for SNP of FCGR3A (product No C_25815666_10; Applied Biosystems).

Table 1 Donor and recipient characteristics

Variable	*Myeloid malignancies (n = 99)*				
	Recipient FCGR3A genotype				P-value
	158V positive n = 46, 46%		*158V negative n = 53, 54%*		
	No.	*Ratio*	*No.*	*Ratio*	
Age, years					
Recipient					0.89
Median	31		36		
Range	8–49		1–50		
Donor					0.45
Median	33		31		
Range	21–50		22–48		
Donor FCGR3A genotype					0.15
With 158V (V/F or V/V)	24	86%	20	83%	
Without 158V (F/F)	22	79%	33	138%	
Sex, male					0.68
Recipient	30	65%	35	66%	
Donor	29	63%	39	74%	
Recipient/donor sex					0.69
Sex matched	25	54%	31	58%	
Male/female	11	24%	9	17%	
Female/male	10	22%	13	25%	
Disease					0.48
AML	23	50%	24	45%	
MDS	6	13%	4	8%	
CML	17	37%	25	47%	
Disease risk					0.26
Low	30	65%	40	75%	
High	16	35%	13	25%	
ABO matching					0.69
Match	26	57%	32	60%	
Major mismatch	10	22%	13	25%	
Minor mismatch	10	22%	8	15%	
Conditioning regimen					0.25
With TBI	39	85%	40	75%	
Without TBI	7	15%	13	25%	
Pre-transplant CMV serostatus					0.28
CMV-negative recipient	9	20%	13	25%	
Missing	7	15%	3	6%	
GVHD prophylaxis					0.20
With CYA	40	87%	50	94%	
With tacrolimus	6	13%	3	6%	
TNC, $\times 10^8$/kg					0.45
Median	5.4		5.6		
Range	2.3–16.2		2.4–52.6		
Engraftment	1	2%	2	4%	0.64

Abbreviations: FCGR3A = Fcγ receptor type IIIA; MDS = myelodysplastic syndrome; TNC = total nucleated cell count harvested.

Data management and statistic analysis

Data were collected by the JMDP using a standardized report form. Follow-up reports were submitted at 100 days, 1 year and annually after transplantation. The pre-transplant CMV serostatus was routinely tested only for

Table 2 Univariate analysis of the association between the FCGR3A genotype and the clinical outcomes after transplantation

	Donor FCGR3A genotype			*Recipient FCGR3A genotype*		
	With 158V	*Without 158V*	P-*value*	*With 158V*	*Without 158V*	P-*value*
No.	44	55		46	53	
5-year OS	50%	60%	0.52	65%	49%	0.15
5-year TRM	36%	27%	0.28	22%	40%	0.07
5-year relapse	16%	16%	0.92	17%	15%	0.79
II–IV aGVHD	39%	40%	0.81	33%	45%	0.19
cGVHD	34%	36%	0.81	26%	43%	0.07
GVHD-related mortality	15%	18%	0.63	7%	25%	0.01
Infection-related mortality	27%	20%	0.46	20%	26%	0.33

Abbreviations: aGVHD = acute GVHD; cGVHD = chronic GVHD; FCGR3A = Fcγ receptor type IIIA.

patients but not for their donors. Engraftment was confirmed by an ANC of $>0.5 \times 10^9$/L for at least 3 consecutive days. Acute and chronic GVHD were diagnosed and graded using established criteria.[27,28] The OS was defined as the number of days from transplantation to death from any cause. Disease relapse was defined as the number of days from transplantation to disease relapse. TRM was defined as death without relapse. Any patients who were alive at the last-follow-up date were censored. The data on the causative microbes of infections, postmortem changes in the cause of death and staging of acute GVHD, as well as the data on supportive care including infection prophylaxis and therapy of GVHD, which were given on institution basis, were not available in this cohort. The analysis was performed using the Excel 2007 (Microsoft Corp., Redmond, WA, USA), OriginPro version 8.0J (Lightstone Inc., Tokyo, Japan) and R (The R Foundation for Statistical Computing, Perugia, Italy) software programs.[29] The probability of OS was calculated using the Kaplan–Meier method and compared using the log-rank test. The probabilities of TRM, disease relapse, acute GVHD, chronic GVHD and each cause of death were compared using the Grey test[30] and analyzed using the cumulative incidence analysis,[29] considering relapse, death without disease relapse, death without acute GVHD, death without chronic GVHD and death without each cause as respective competing risks. The variables included the recipient age at the time of transplantation, sex, CMV serostatus before transplantation, disease characteristic (disease type and disease risk at transplantation), donor characteristics (age, sex, sex compatibility and ABO compatibility), transplant characteristics (TBI-containing regimen, tacrolimus vs CYA and total nucleated cell count harvested per recipient weight). The median values were used as the cutoff point for continuous variables. The χ^2 test and Mann–Whitney test were used to compare two groups. The Hardy–Weinberg equilibrium for the FCGR3A gene polymorphism was tested using the Haploview program.[5] Multivariate Cox models were used to evaluate the hazard ratio (HR) associated with the FCGR3A polymorphism. Covariates found to be significant in univariate analyses ($P \leqslant 0.10$) were included in the models. The *P*-values were two sided and outcomes were considered to be significant with $P \leqslant 0.05$ in both the univariate and multivariate analyses.

Results

Frequencies of the FCGR3A genotyping

The FCGR3A gene polymorphism (rs396991) was analyzed in 99 unrelated BM donor-myeloablative transplant recipient pairs (Table 1). The genotype frequencies of 158V/V, 158V/F and 158F/F were 4, 42 and 54% in donors, and 3, 41 and 56% in recipients. These were similar to a previous report[14,31] in Japanese populations and were in accord with the Hardy–Weinberg equilibrium ($P = 0.91$).

Transplant outcome according to the FCGR3A genotype

The median follow-up duration in the cohort was 109 months among the survivors (range 43–134 months), and 16 recipients (16%) had relapsed or progressed and 47 (47%) had died. Three patients (3%) died before undergoing engraftment.

The transplant outcomes according to the FCGR3A genotype are summarized in Table 2. The recipient 158V genotype was associated with a significantly reduced incidence of GVHD-related mortality (7 vs 25%, $P = 0.01$; Figure 1b), and a trend toward a reduced incidence of chronic GVHD (33 vs 45%, $P = 0.07$) and reduced 5-year TRM (22 vs 40%, $P = 0.07$; Figure 1a). The donor genotype had no significant effects on the transplant outcomes.

Multivariate analysis

All factors that were found to be significant in univariate analyses were included in the model. The presence of the 158V genotype in recipients were statistically significant in the multivariate analyses for better OS (HR 0.49; 95% confidence interval (CI) 0.26–0.93; $P = 0.03$; Table 3) and TRM (HR 0.30; 95% CI 0.14–0.67; $P = 0.003$). In addition, the recipient 158V genotype was associated with a significantly reduced incidence of chronic GVHD (HR 0.45; 95% CI 0.20–0.99; $P = 0.049$) and a trend toward a lower incidence of grade II–IV acute GVHD (HR 0.55; 95% CI 0.27–1.10; $P = 0.09$), resulting in a significantly reduced GVHD-related mortality (HR 0.22; 95% CI 0.06–0.77; $P = 0.02$). A correlation between the recipient 158V genotype and low infection-related death was also observed (HR 0.42; 95% CI 0.17–1.01; $P = 0.05$). The donor 158V genotype did not significantly influence the transplant outcomes.

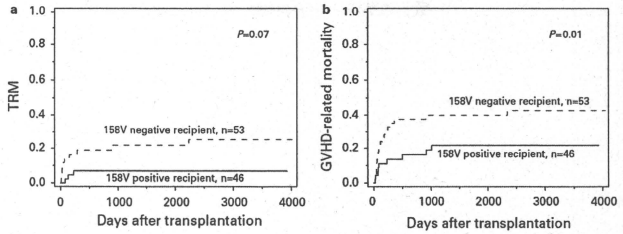

Figure 1 Estimated cumulative incidence curves of TRM (**a**) and GVHD-related mortality (**b**) according to the recipient FCGR3A genotype. Solid lines and dashed lines represent the 158V-positive recipient and the 158V-negative recipient, respectively.

Table 3 A multivariate analysis of the association of the FCGR3A genotype with the clinical outcomes after transplantation

Variable	*Donor 158V positivity*			*Recipient 158V positivity*			*Minor ABO incompatibility*		
	HR	*95% CI*	P-*value*	*HR*	*95% CI*	P-*value*	*HR*	*95% CI*	P-*value*
OS	1.29	0.70–2.38	0.42	0.49	0.26–0.93	0.03	—	—	—
TRM	1.73	0.84–3.54	0.14	0.30	0.14–0.67	0.003	—	—	—
Relapse	0.82	0.27–2.55	0.74	0.67	0.22–2.02	0.47	—	—	—
II–IV aGVHD	1.24	0.63–2.46	0.53	0.55	0.27–1.10	0.09	—	—	—
cGVHD	0.55	0.25–1.21	0.14	0.45	0.20–0.99	0.049	0.26	0.03–1.88	0.18
GVHD-related mortality	1.65	0.61–4.45	0.32	0.22	0.06–0.77	0.02	—	—	—
Infection-related mortality	1.36	0.58–3.20	0.48	0.42	0.17–1.01	0.05	—	—	—
	High-risk disease			*Age (>27 years)*			*Donor age (>32 years)*		
	HR	*95% CI*	P-*value*	*HR*	*95% CI*	P-*value*	*HR*	*95% CI*	P-*value*
OS	2.17	1.17–4.04	0.01	—	—	—	—	—	—
TRM	—	—	—	—	—	—	2.56	1.22–5.37	0.01
Relapse	6.44	1.92–21.59	0.003	0.43	0.13–1.43	0.17	—	—	—
II–IV aGVHD	—	—	—	0.38	0.19–0.74	0.005	2.84	1.42–5.69	0.003
cGVHD	—	—	—	—	—	—	—	—	—
GVHD-related mortality	0.43	0.10–1.90	0.27	—	—	—	—	—	—
Infection-related mortality	2.78	1.19–6.46	0.02	—	—	—	3.27	1.31–8.12	0.01

Abbreviations: aGVHD = acute GVHD; CI = confidence interval; cGVHD = chronic GVHD; FCGR3A = Fcγ receptor type IIIA; HR = hazard ratio.

Discussion

This study showed a considerable effect of the recipient FCGR3A-158V genotype on GVHD development and GVHD-related mortality, thus positively contributing to a significantly better TRM and OS for patients with myeloid malignancies receiving HLA-matched myeloablative BMT. The presence of 158V in recipients did not influence disease relapse. Therefore, recipients with the 158V genotype may be capable of avoiding GVHD without compromising a GVL effect. The recipient 158V genotype also showed a trend toward reduced infection-related mortality, which might result from a reduced need for immunosuppressive therapy due to a low incidence of GVHD, although the data regarding treatment for GVHD were unavailable in this cohort. This is the first report to show that the FCGR3A-V158F polymorphism influences transplant outcomes.

Little is known about the involvement of FCGR3A in the pathogenesis of GVHD. Indirect evidence in animal and human studies showed that B cells have an important role in the immunopathophysiology of acute and chronic GVHD,[12,32–35] in which FCGR3A is involved through Ab-mediated immune responses, including Ab-dependent cell-mediated cytotoxicity and FCGR3A-mediated endocytosis. Previous reports showing the effectiveness of B-cell depletion in the treatment of acute[33] and chronic GVHD[35] prompted a working hypothesis that the presence of the FCGR3A-158V genotype, which potentially mediates Ab-dependent cell-mediated cytotoxicity more efficiently in comparison to its absence,[16] may be a risk factor contributing to the development of acute and chronic GVHD. However, the current results contradict this hypothesis. One explanation of this conflict may be observed in reports that B-cell-deficient mice experience more exacerbated acute GVHD than wild-type mice,[36] and that a high number of B-cell progenitors in the stem cell graft or in patients after allo-SCT is associated with a significantly lower rate of acute and chronic GVHD.[37,38] A study in HIV-infected men showed the presence of the FCGR3A-158V genotype to be associated with susceptibility to Kaposi's sarcoma and human herpesvirus-8