Multicolor Analysis of Cell Surface Marker of Human Leukemia Cell Lines Using Flow Cytometry

TOMOKO INOUE[1], ANTHONY SWAIN[1], YOICHI NAKANISHI[2] and DAISUKE SUGIYAMA[1]

[1]*Department of Research and Development of Next-Generation Medicine, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan;*
[2]*Center for Clinical and Translational Research, Kyushu University Hospital, Fukuoka, Japan*

Abstract. *Background: Leukemia cell lines are utilized as tools for molecular analysis. Their implementation in therapy will require standards for quality control, including appropriate selection criteria for functional analysis and efficacy determination. Materials and Methods: Characteristics of six human leukemia cell lines –Kasumi-1, NB-4, MOLM-13, MV-4-11, K562, and Jurkat cells– were investigated using multiple color analysis of surface antigen expression and comparative analysis of gene expression. Results: Differentiation states of Kasumi-1 and MOLM-13 cells are colony-forming units-granulocyte/macrophage equivalent cells to myeloblasts with comparatively high Growth factor independent-1(GFI1) and Transcription factor PU.1 (PU.1) expression, respectively. NB4 and MV-4-11 express high levels of CCAAT/enhancer-binding protein-alpha (CEBPα) and differentiate from myeloblasts to pro-monocytes and myeloblasts, respectively. K562 cells are colony-forming units-erythroid equivalent cells to erythroblasts, with the highest expression of GATA-binding factor 2 (GATA2), GATA1 and Friend of gata-1 (FOG1). Jurkat cells are pro-T to mature T-cells with the highest Neurogenic locus notch-1 homolog protein 1 (NOTCH1) expression. Conclusion: Our study gives a useful guideline of standards for appropriate usage of leukemia cell lines for examining novel targets in vitro.*

Leukemias are a group of disorders characterized by abnormal growth and differentiation of hematopoietic cells due to several factors, including chromosomal abnormalities and transcription factor alterations, which lead to anemia, neutropenia and thrombocytopenia. In almost all cases, leukemia is classified as one of four types; acute myelogenous leukemia (AML), acute lymphocytic leukemia (ALL), chronic myelogenous leukemia (CML), and chronic lymphocytic leukemia (CLL). In acute leukemia, leukocytes proliferate rapidly in the bone marrow at an immature state, and therefore cannot function normally. On the other hand, in chronic leukemia, blastic cells form more slowly than in acute leukemia and consequently cells with abnormal function are transported to the hematopoietic tissues. In AML and CML, myeloid lineage cells such as granulocytes and monocytes, become malignant and lose the capacity to differentiate into mature functional cells and interfere with the production of normal blood cells. In ALL and CLL, lymphoid lineage cells such as T- and B-lymphocytes do likewise. There is considerable research taking place utilizing leukemia cells (both primary cells and immortalized cell lines) to elucidate their pathogenetic mechanisms at cellular and molecular levels, and to develop novel therapies (for example, gene therapy and molecular-target therapy) in addition to existing therapeutic methods, such as chemotherapy, hematopoietic stem cell transplantation, transfusion therapy and induction therapy.

Primary cells from patients with leukemia are obtained from peripheral blood (PB) and bone marrow (BM), which provide much information for diagnosis through morphological observation, cell surface antigen-dependent phenotypical observations and chromosomal tests. However, as primary cells are limited in number and accessibility (especially for BM), it is not easy to extract them for functional analysis in order to examine novel targets to treat patients. To overcome this problem, immortalized leukemia cell lines that can proliferate indefinitely were established and are being utilized as useful tools to analyze molecular mechanisms (transcription factors, signaling pathways, translocations) (1, 2). They are also used for analysis of both gain-of-function, with plasmid transfection and use of recombinant protein, and loss-of-function, with siRNA/shRNA transfection and inhibitors (3, 4).

Correspondence to: Dr. Tomoko Inoue and Dr. Daisuke Sugiyama, Department of Research and Development of Next-Generation Medicine, Faculty of Medical Sciences, Kyushu University, 3-1-1, Maidashi, Higashi-Ku, Fukuoka, 812-8582, Japan. Tel: +81 926426210, Fax: +81 926426146, e-mail: yokotomo@hsc.med.kyushu-u.ac.jp (Tomoko Inoue), ds-mons@yb3.so-net.ne.jp (Daisuke Sugiyama)

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When the cell lines were established, expressions of cell surface markers were studied in terms of single antigens but not multiple antigens together. The transcription factors were examined individually and not compared to other cell lines. It is necessary to characterize leukemia cell lines in terms of protein and gene expression levels, so as to evaluate the state of differentiation and maturation more precisely, which will be very useful in controlling the quality of cell lines, as well as in choosing cell lines to evaluate the effects of molecules and therapies.

Multi-parametric flow cytometry is an adequate method for gaining information on the cell differentiation state, monitoring residual disease and evaluating response to therapy (5, 6). Such application of flow cytometry has become more widespread, and will require the development of standardized approaches with defined specificity and sensitivity, along with suitable quality control schemes.

Herein we examined the multiple color analysis of cell surface markers and comparative analysis of transcription gene expression in using six human leukemia cell lines: four myeloblastic and monoblastic leukemia cell lines: Kasumi-1 (7), NB-4 (8), MOLM-13 (9) and MV-4-11 (10); an erythroleukemia cell line, K562 (11); and the T-cell leukemic cell line Jurkat (12), which have been previously used as *in vitro* leukemia models.

Materials and Methods

Cell culture. The human leukemia cell lines, Kasumi-1, NB4, MOLM-13, K562 and Jurkat were cultured in RPMI-1640 medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum and MV-4-11 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 10 ng/ml human recombinant Granulocyte/macrophage colony-stimulating factor (GM-CSF) (PeproTech, Rochy Hill, NJ, USA) at 37˚C in 5% CO_2.

Cell morphology. Slides of cell suspensions were prepared by CytoSpin 4 (Thermo Fisher scientific, Waltham, MA, USA) at 450 rpm (rcf, 26.06 × *g*) for 7 minutes. Cells were stained with May-Grunwald and Giemsa staining reagents (Muto Pure Chemicals, Tokyo, Japan) and characterized morphologically. One slide containing 2×10^4 cells was prepared for each cell line. Stained cells were observed using an Olympus CKX41 microscope (Olympus, Tokyo, Japan).

Flow cytometry. Kasumi-1 cells and NB4 cells: To analyze hematopoietic stem/progenitor cell (HSPC) markers, cells were stained with a Fluorescein isothiocyanate (FITC)-conjugated anti-human Cluster of differentiation (CD)38 (Biolegend, San Diego, CA, USA), Phycoerythrin (PE)-Cy7-conjugated anti-human Tyrosine-protein kinase (c-KIT, CD117) (Biolegend), Allophycocyanin (APC)-conjugated anti-human CD34 (eBioscience, San Diego, CA, USA), APC-Cy7-conjugated anti-human CD13 (Biolegend) and Pacific Blue-conjugated anti-human leukocyte antigen (HLA)-DR (Biolegend). In addition, PE-conjugated anti-human CD135 (Biolegend) for Kasumi cells and PE-conjugated anti-human CD33 (Biolegend) for NB4 cells were stained to analyze the cells.

To analyze myeloid cell lineage markers, cells were stained with a FITC-conjugated anti-human CD15 (Biolegend), PE-conjugated anti-human CD116 (Biolegend), PE-Cy7-conjugated anti-human CD14 (Biolegend), APC-conjugated anti-human CD11c (Biolegend), APC-Cy7-conjugated anti-human CD13 (Biolegend), Pacific Blue conjugated anti-mouse/human CD11b (Biolegend), Biotin-conjugated anti-human CD16 (Biolegend) and V500-conjugated streptavidin (BD Bioscience, San Jose, CA, USA).

To analyze lymphoid cell lineage markers, cells were stained with a FITC-conjugated anti-human CD19 (Biolegend), PE-conjugated anti-human CD2 (Biolegend), APC-conjugated anti-human CD3 (Biolegend) and APC-Cy7-conjugated anti-human CD4 (Biolegend).

MOLM-13 and MV-4-11 cells: To analyze HSPC markers, cells were stained with a FITC-conjugated anti-human CD38, PE-conjugated anti-human CD33, PE-Cy7-conjugated anti-human c-KIT, APC-conjugated anti-human CD34 and Pacific Blue conjugated anti-human HLA-DR.

To analyze myeloid cell lineage markers, cells were stained with a FITC-conjugated anti-human CD15, PE-conjugated anti-mouse/human CD11b (Biolegend), PE-Cy7-conjugated anti-human CD14, APC-conjugated anti-human CD36 (Biolegend), APC-Cy7-conjugated anti-human CD13, Pacific Blue conjugated anti- human HLA-DR, Biotin-conjugated anti-human CD64 (Biolegend) and V500-conjugated streptavidin.

To analyze lymphoid cell lineage markers, cells were stained with a FITC-conjugated anti-human CD10 (Biolegend), PE-conjugated anti-human CD116, PE-Cy7-conjugated anti-human CD14, APC-conjugated anti-human CD11c Ab, APC-Cy7-conjugated anti-human CD4 (Biolegend), Pacific Blue conjugated anti-human HLA-DR, Biotin-conjugated anti-human CD45 (Biolegend) and V500-conjugated streptavidin.

K562 cells: To analyze HSPC markers, cells were stained with a FITC-conjugated anti-human CD38, PE-conjugated anti-human CD33, PE-Cy7-conjugated anti-human c-KIT, APC-conjugated anti-human CD34, APC-Cy7-conjugated anti-human CD13, and Pacific Blue conjugated anti-human HLA-DR.

To analyze erythroid cell lineage markers, cells were stained with a FITC-conjugated anti-human Glycophorin A (GPA, CD235a) (Biolegend), PE-conjugated anti- human CD71 (Biolegend), PE-Cy7-conjugated anti-human c-KIT, APC-conjugated anti-human CD36 (Biolegend), and APC-Cy7-conjugated anti-mouse/human CD44 (Biolegend).

Jurkat cells: To analyze HSPC markers, cells were stained with FITC-conjugated anti-human CD38, PE-conjugated anti-human CD33, PE-Cy7-conjugated anti-human c-KIT, and APC-conjugated anti-human CD34.

To analyze lymphoid cell lineage markers, cells were stained with FITC-conjugated anti-human CD7 (Biolegend), PE-conjugated anti-human CD2 (Biolegend), PE-Cy7-conjugated anti-human CD8 (Biolegend), APC-conjugated anti-human CD3 (Biolegend), APC-Cy7-conjugated anti-mouse/human CD4 (Biolegend), Pacific Blue conjugated anti-human CD5 (Biolegend), Biotin-conjugated anti-human CD38 and V500-conjugated streptavidin.

The cells were analyzed using a FACS Aria cell sorter (BDIS, San Jose, CA, USA), and the data files were analyzed using FlowJo software (Tree Star, Inc., Sac Carlos, CA, USA). Data are presented as means plus standard deviation (SD).

Quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR). Total RNA was extracted from each cell line

Table I. *Human leukemia cell lines*

Cell lines	Cell source	Type	Reference
Kasumi-1	Peripheral blood	Acute myeloblastic leukemia (M2)	7
NB4	Bone marrow	Acute promyelocytic leukemia (M3)	8
MOLM-13	Peripheral blood	Acute monocytic leukemia (M5a)	9
MV-4-11	Bone marrow/peripheral blood	Childhood acute myeloblastic leukemia (M5)	10
K562	Bone marrow	Chronic myelogenous leukemia/erythroleukemia	11
Jurkat	Peripheral blood	Acute T-cell leukemia	12

using an RNAqueous Micro Kit (Life Technologies, Carlsbad, CA, USA). Total RNA was subjected to reverse transcription using a High-Capacity RNA-to-cDNA Kit (Life Technologies) according to established protocols. The mRNA levels of different genes were analyzed by qRT-PCR using SYBR Green and gene-specific primers with the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA). The mRNA level of each target gene was normalized to β-actin (*ACTB*) as an internal control.

Results

Morphological observation of human leukemia cell lines. Leukemia cell lines used in this study are shown in Table I. To examine cell morphology, alive Propidium iodide (PI)-negative cells were sorted-out and stained with May-Grünwald Giemsa solution (Figure 1). Typical cell characteristics were observed under microscopy. Each cell line showed variations in size, nuclear segmentation, nuclear:cytoplasmic (N/C) ratio, and content of cytoplasm, such as granules and vacuoles. Kasumi cells (20.95±2.58 μm) showed nuclear segmentation (22.2%) in nuclei and vacuoles in the cytoplasm (85.7%). Azurophilic granules were contained inside the basophilic cytoplasm (Figure 1, upper left). No nuclear segmentation was observed in NB4 cells (24.49±2.91 μm) whose cytoplasm contained vacuoles (42.9%) with high N/C ratio. Azurophilic granules were contained in the basophilic cytoplasm (Figure 1, lower left). MOLM-13 cells (25.10±3.18 μm) did not have nuclear segmentation or vacuoles in the cytoplasm (42.8%). Azurophilic granules were contained in the basophilic cytoplasm (Figure 1, upper center). Nuclear condensation was observed in MV-4-11 cells (23.57±1.79 μm), but no nuclear segmentation or vacuoles in their cytoplasm were apparent (Figure 1, lower center). K562 cells (25.12±2.23 μm) also exhibited azurophilic granules in the basophilic cytoplasm (Figure 1, upper right). Compared to other cell lines, Jurkat cells were smaller in size (17.08±2.38 μm) and had a lower N/C ratio (Figure 1, lower right).

Surface marker expression of human leukemia cell lines. To further investigate surface marker expression of human leukemia cell lines, we performed flow cytometric analysis using HSPC markers such as c-KIT, CD34, CD33, CD38 for all cell lines; myeloid-specific markers such as CD45, CD13, CD14, CD15 for Kasumi-1, NB4, MOLM-13 and MV-4-11; erythroid-specific markers such as GPA, CD36, CD71 for K562 cells; and T-lymphocyte specific markers such as CD3, CD4, CD8 for Jurkat cells.

The percentages of positive cells for each marker are shown in Figure 2. The myelo/monoblastic leukemia cell lines, Kasumi-1, NB4, MOLM-13, and MV-4-11 were mostly positive for CD33 (HSPC marker) and CD45 (pan-leucocyte marker), in 99.8 to 100% and 98.9 to 99.6% of cells, respectively. Among them, Kasumi-1 cells were likely most immature, defined by positivity for c-KIT (99.8%) and CD34 (95.5%), and also expressed CD135 (2.2%). On the other hand, few NB4, MOLM and MV-4-11 cells were positive for CD34 (0.17 to 0.34%) and showed a variety of expression of CD38 (56.5%, 94.3%, 0.68%, respectively) and HLA-DR (19.6%, 23.4%, 85.0%, respectively). Regarding differentiation marker CD13 (pan-myeloid specific marker, widely expressed from Colony formings-granulocyte/macrophage (CFU-GM) equivalent cells to monocytes), NB4 cells were the ones most positive (99.8%), followed by Kasumi-1 cells (44.0%), MV-4-11 (5.38%) and MOLM-13 (1.32%) cells. NB4 cells showed the highest positivity for CD14 (30.4%), CD11b (80.4%) and CD11c (69.2%), and MV-4-11 did for CD15 (88.6%) and CD64 (99.6%) among the four cell lines (Figure 2). Erythroleukemia K562 cells had both immature state defined by c-KIT (12.9%) and CD33 (61.7%) expression and mature state defined by CD36 (75.3%), CD71 (99.8%) and GPA (19.7%). T-cell leukemia Jurkat cells were the most mature among the six cell lines tested, defined by c-KIT (1.22%). They were positive for markers of maturity: CD7 (pan-T-cell-specific marker, widely expressed from pro-T-cell to mature T-cell) in 60.9%, CD2 and CD5 (expressed from early thymocyte to mature thymocyte) as 59.0% and 57.0%, respectively, and CD3 and CD4 (mature T-cell marker) in 45.2% and 28.6%, respectively.

To further characterize the differentiated state of each cell line, we analyzed the surface marker expression under combination (Figure 3). All combination analyses are summarized in Table II. Kasumi-1 cells were double-positive for c-KIT/CD34 at 95.4%, CD33/HLA-DR at 7.4%, and

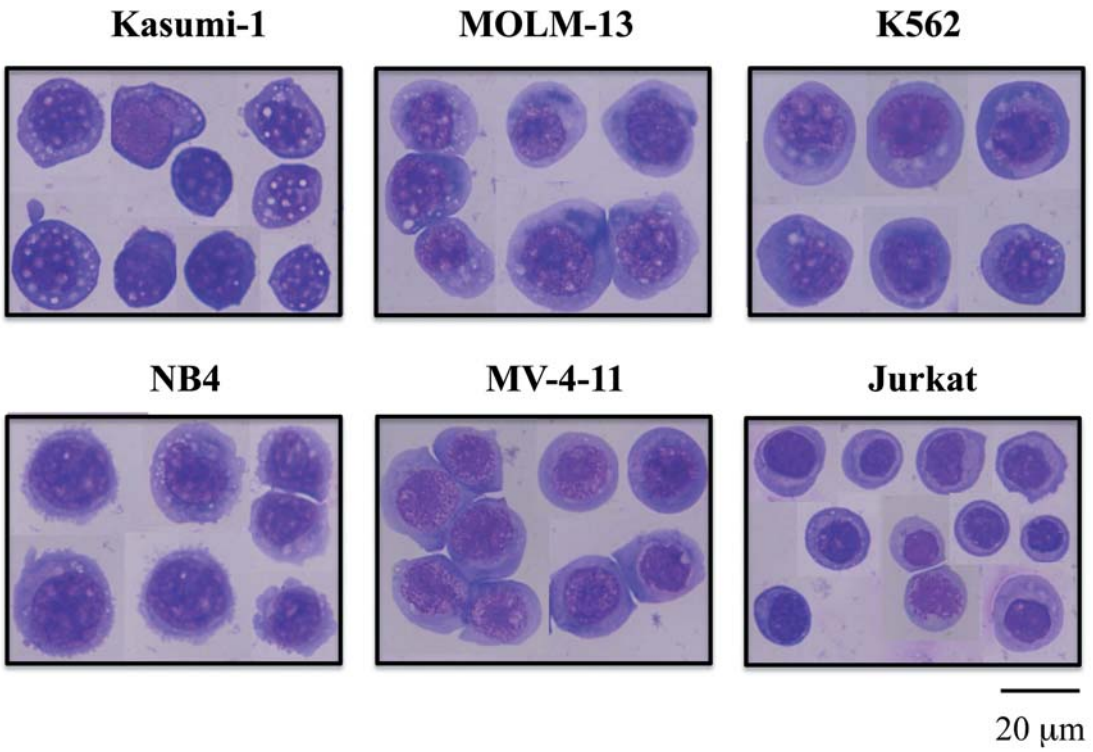


Figure 1. *Morphology of human leukemia cell lines. Morphology of Kasumi-1 (upper left), NB4 (lower left), MOLM-13 (upper center), MV-4-11 (lower center), K562 (upper right) and Jurkat (lower right) cells. All cell lines were stained with the May-Giemsa staining method. Original magnification, ×40 for all panels; scale bar is 20 μm.*

CD13/HLA-DR at 5.2%. Among CD13/CD116 double-positive (both are express from CFU-GM to mature monocytes), CD15-positive cells and CD11b-positive cells were observed at 2.12% and 64.6%, respectively, although we did not see cells highly positive for combinations CD14/CD15, CD15/CD16, CD11b/CD11c cells (Figure 3A). NB4 cells were double-positive for c-KIT/CD34, c-KIT/CD38, CD34/CD38 and c-KIT/HLA-DR cell at 0.06%, 14.4%, 2.6% and 2.7%, respectively. Regarding differentiation markers, positivity for CD15/CD116, CD11b/CD11c and CD14/CD11c cell were observed at 1.96%, 21.2% and 22.4%, respectively (Figure 3B). MOLM-13 cells were double-positive for c-KIT/CD38 and CD33/HLA-DR 9.83%, and 25.2%, but rarely for c-KIT/CD34. They were also positive for HLA-DR/CD13 cells at 2.2%. Compared to other cell lines, they were positive for single-cell markers, such as CD11b, CD14 and CD15, but no double-positivity was observed (Figure 3C). MV-4-11 cells were double-positive for CD33/HLA-DR at 85.8%, but rarely for c-KIT/CD34 and CD34/CD38. They were also positive for HLA-DR/CD13 at 0.9%. Regarding differentiation marker, CD15/CD64 were observed at 27.4%, but were rarely positive for CD14/CD116, CD36/CD63, and CD10/CD13 (Figure 3 D). K562 cells were double positive for c-KIT/CD33 and c-KIT/CD13 at 8.9% and 2.1%, but rarely for CD33/CD38, c-KIT/CD34 or HLA-DR/CD13. Among c-KIT-negative/CD36-positive cells that included Colony forming units-erythroid (CFU-E) equivalent cells, erythroblasts and mature erythrocytes, CD71/GPA double-positive polychromatic and CD44/GPA double-positive orthochromatic erythroblasts at 3.2% and 2.7% (Figure 3E). Jurkat cells were rarely double positive for CD33/CD34, c-KIT/CD34, CD34/CD38 cells and CD38/HLA-DR. Among double positive CD38/CD7 cells that are expected to include pro-T cell and thymocytes, CD2/CD5, CD3/CD4 and CD3/CD8 cells existed at 98.7%, 44.8%, and 1.2%. Among CD38-negative/CD7-positive cells that are expected to be mature, functional T-cells, double positive CD2/CD5 and CD3/CD4 cells were found at 94.6% and 36.0%, but were negative for CD3/CD8 (Figure 3F). Taken together from immunophenotype analysis, the differentiation state of Kasumi-1 and MOLM-13 cells appears to be CFU-GM to myeloblasts, NB4 cells are myeloblasts to pro-monocytes; MV-4-11 cells are myeloblasts; K562 cells are CFU-E to erythroblasts; and Jurkat cells are pro-T cells to mature thymocytes and functional T-cells.

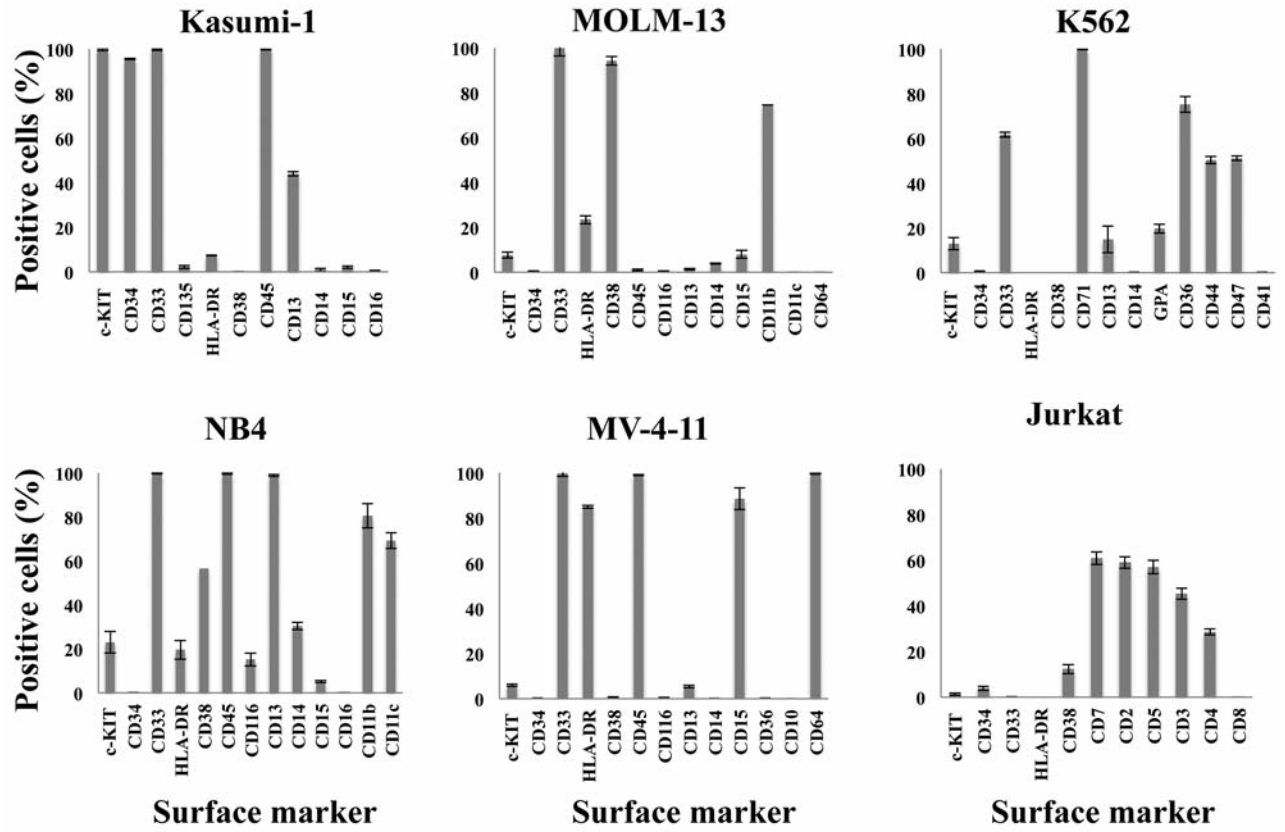


Figure 2. *Surface phenotype analysis with flow cytometry. Cells were stained with surface antigens, such as Tyrosine-protein kinase (c-KIT), Cluster of differentiation (CD) 34, CD33, CD38 and CD45, and analyzed with flow cytometry. Data are shown for each individual positive cell marker.*

Hematopoietic transcription factor expression in human leukemia cell lines. Next, we examined expression of hematopoietic transcription factor genes, such as *PU.1, CEBPα, GFI1, NOTCH1, E2A, GATA2, GATA1* and *FOG1* in six leukemia cell lines (Figure 4). Myeloblastic/ monoblastic cell lines (Kasumi-1, NB4, MOLM-13, and MV-4-11) express myeloid-specific transcription factors *PU.1* and *GFI1*. In contrast, CEBPα expression differed among cell lines, its expression in MV-4-11 was the highest and 9.45-fold higher in NB4 cells. Jurkat T-cell leukemia cells more highly expressed *NOTCH1* and *E2A* than did K562 erythroleukemia cell lines, at 8.09-fold and 6.82-fold higher, respectively. When compared to myeloblastic/monoblastic cell lines, Jurkat had the highest expression of both *NOTCH1* and *E2A*. K562 cells more highly expressed *GATA2* and *GATA1* than Jurkat cells, at 110.5-fold and 839.4-fold higher, respectively. When compared to myeloblastic/monoblastic cell lines, it had the highest expression of both *GATA2* and *GATA1* (Figure 4).

Among cell lines, Kasumi cells expressed *GFI1* (7837.28), *PU.1* (6010.33) and low expression of *CEBPα* (52.69). NB4 cells had higher expression of *CEBPα* (12699.06) than *PU.1* (7230.01) and *GFI1* (5565.11). MOLM-13 had higher expression of *PU.1* (11553.68) than *GFI1* (3848.21) and low expression of *CEBPα* (214.20). MV-4-11 predominantly expressed *CEBPα* (120013.11) and also expressed *PU.1* (11364.33) and GFI1 (4338.27). K562 cells predominantly expressed *GATA1* (1.2E+09) and also expressed *GATA2* (2432.562). Jurkat cells expressed 9.16-fold more *NOTCH1* than *E2A* (Figure 4). Overall, we found that the expression of hematopoietic transcription factors varies among leukemia cell lines at different levels.

Discussion

Herein, we carried-out multiple color analysis of cell surface markers by flow cytometry, and comparative analysis of expression of transcription genes on six human leukemia cell lines that are commonly used for studies on *in vitro* leukemic models. Our results suggest guidelines of standards for (i) quality control of cell lines, (ii) choosing appropriate cell lines for functional analysis, and (iii) determination of efficacy after functional analysis and drug screening.

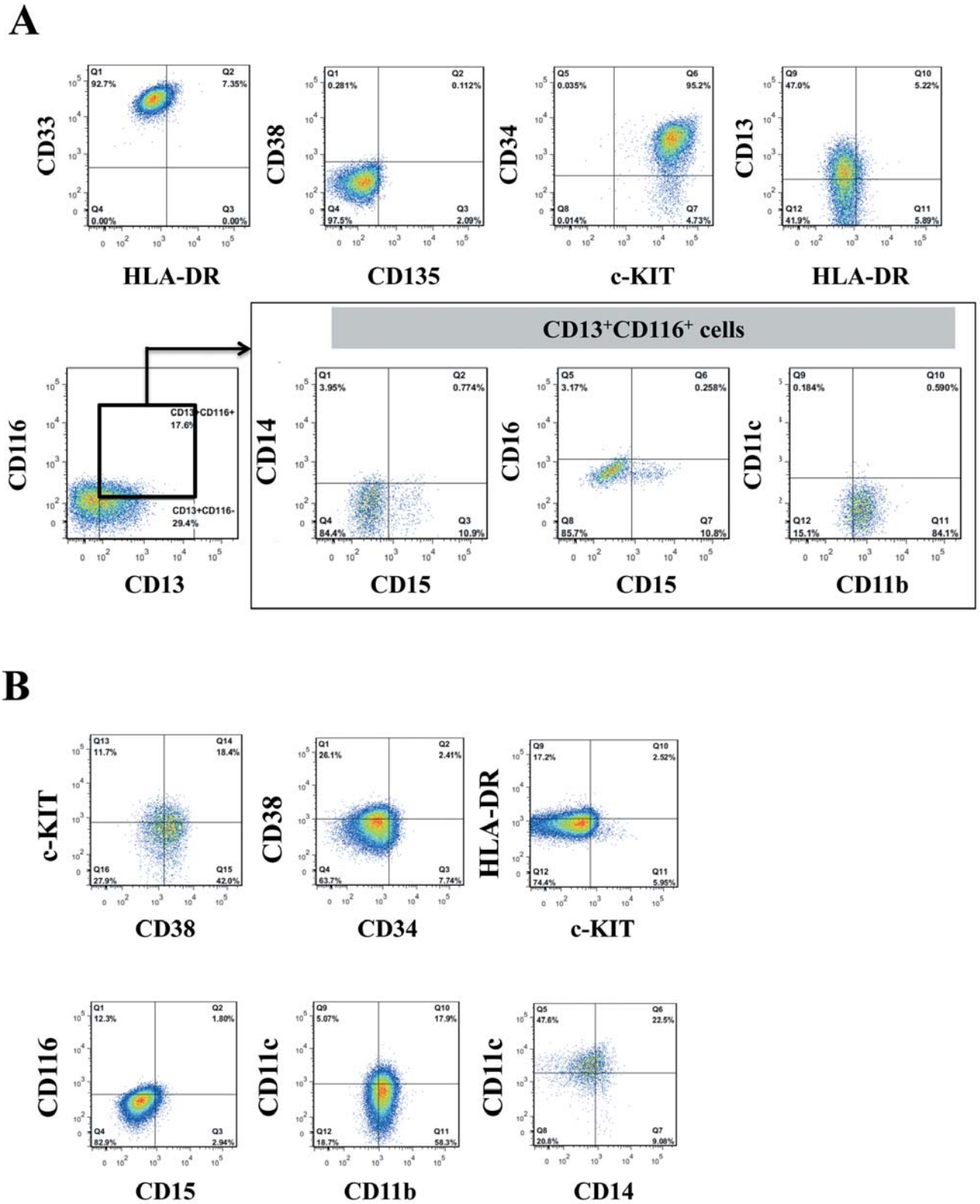


Figure 3. *Multiple color analysis of surface marker phenotype. Multiple color flow cytometric analyses were performed using Kasumi-1 (A), NB4 (B), MOLM-13 (C), MV-4-11 (D), K562 (E) and Jurkat (F) cells. Three independent flow cytometric analyses were carried-out in all cell lines and typical plot patterns are shown here.*

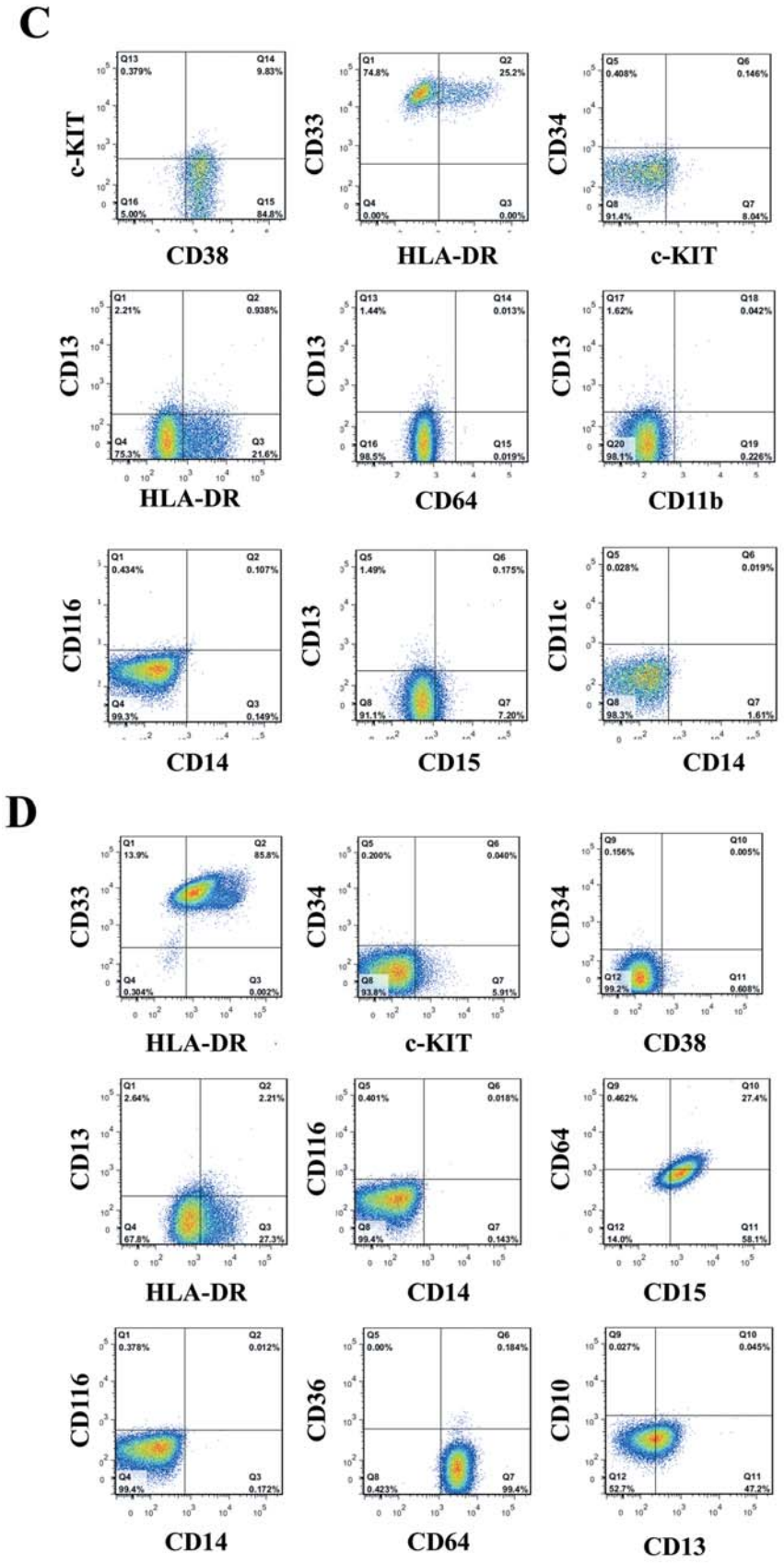
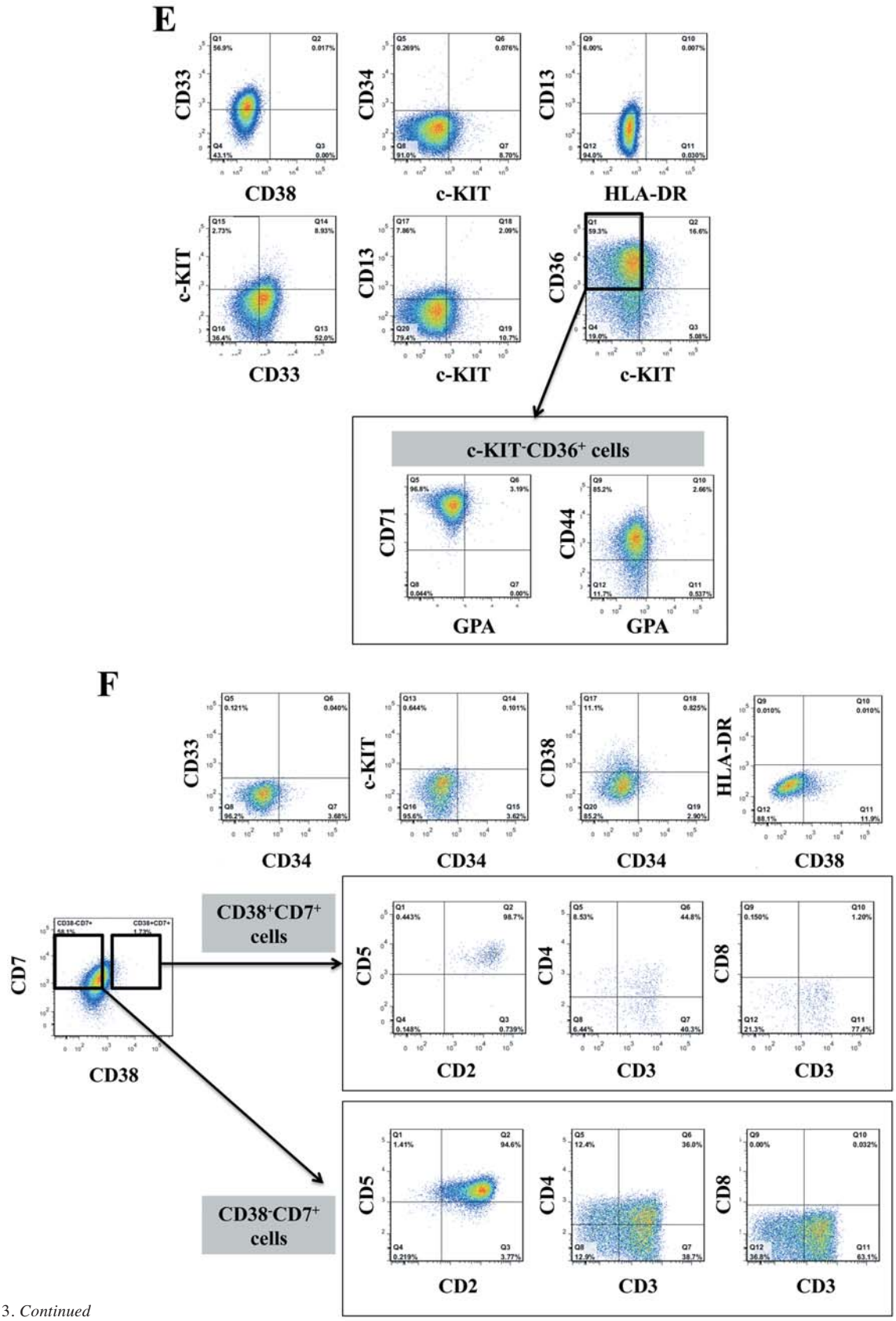


Figure 3. *Continued*



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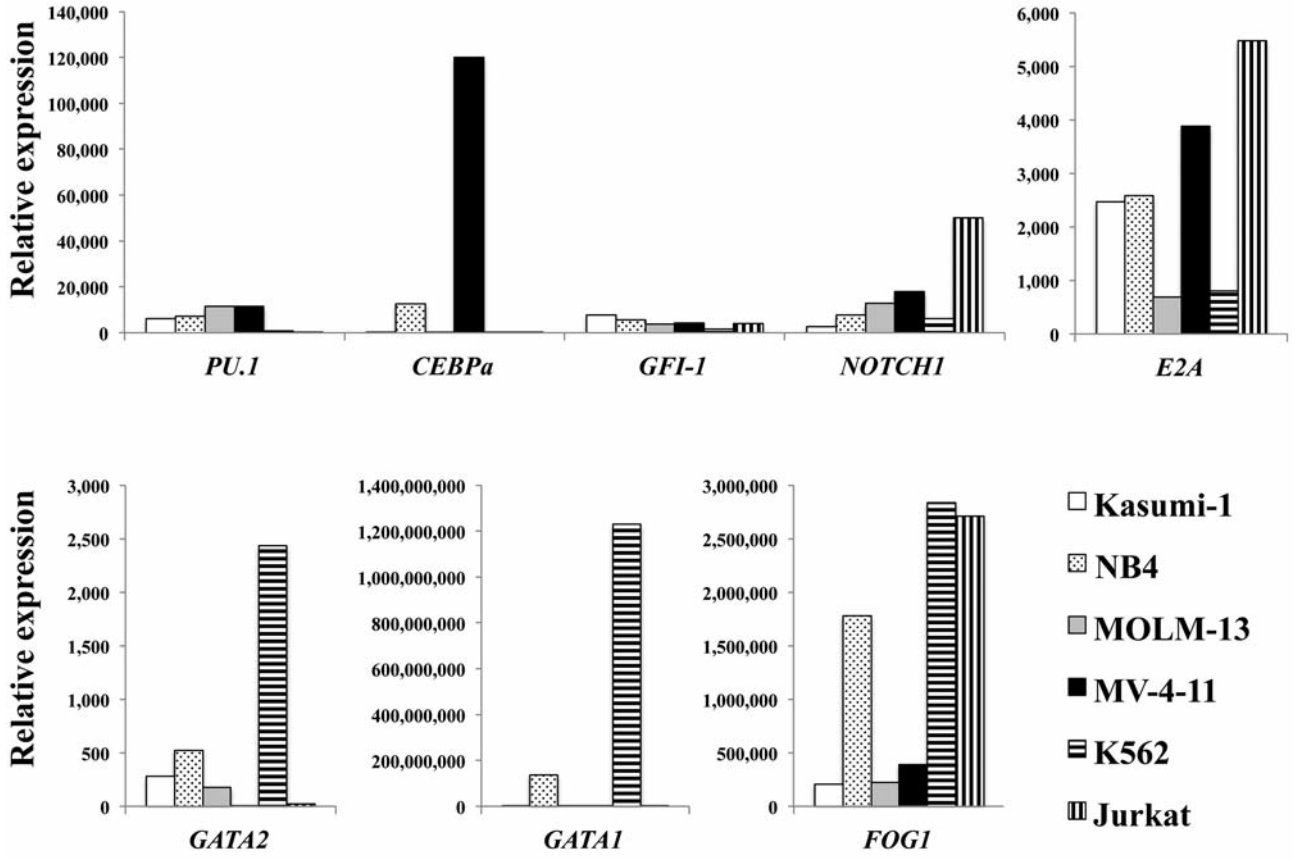

Figure 4. *Comparative expression of genes for hematopoietic transcription factors. RNA was extracted from each cell line and gene expressions of hematopoietic transcription factors, such as Transcription factor PU.1 (PU.1), CCAAT/enhancer-binding protein alpha (CEBPα), Growth factor independent 1(GFI1), Neurogenic locus notch1 homolog protein 1 (NOTCH1), Transcription factor E2-alpha (E2A), GATA-binding factor 2 (GATA2), GATA1, and Friend of gata1 (FOG1) were investigated by real-time reverse transcription–polymerase chain reaction. All data were analyzed with the delta-delta Ct method.*

Kasumi-1 cells that were established from acute myeloblastic leukemia (M2), reportedly express CD33, CD34, CD38, CD13, and CD15 (7), and the present study confirms their expression. However, we also found expression of CD135, HLA-DR, CD45, CD14 and CD16 with flow cytometric analysis, suggesting that we maintain this cell line as it is and our additional results offer robust evidence for defining these cells. As Kasumi-1 cells express Acute myeloid leukemia 1 (AML1)–Eight twenty one (ETO) fusion gene product (13) and also have mutation in the *c-KIT* gene, they were utilized in research of regulation of the promoter region of the AML1–ETO fusion gene (14), of inhibitors against c-KIT proteins (15) and of mechanisms of apoptosis (16) (17). Zapotocky *et al*. also focused on hematopoietic differentiation and showed that valproic acid triggers differentiation defined by c-Kit, CD33, CD34, CD11b expression in Kasumi-1 cells. They also found the positive effect of valproic acid on up-regulation of *CEBPα* and PU.1 expression. Our results also reveal hematopoietic transcription factors *CEBPα*, *PU.1* and *GFI1* were expressed in Kasumi-1 cell in that order and their expressions are the lowest among the six cell lines we examined (Figure 4), suggesting that Kasumi-1 cells could potentially be a useful tool for examining the effect of increasing the expression of these transcription factors. Using the surface markers that we examined here, we can expect to gain more evidence for differentiation.

NB4 cells that were established from acute promyelocytic leukemia (M3) expressed CD38, CD33, CD13, CD15 and CD11b, but not CD11c, CD34, or CD14 (8). Except for CD11c and CD14, our results shown in Figure 2 and 3B demonstrated the same phenotypic pattern as the previous report. It is likely that this discrepancy was caused by difference in the antibody clones of CD11c and CD14 between our study and previous reports. In addition to the antibodies listed above, we also investigated the expression

Table II. *Summary of flow cytometric data*.

Kasumi-1									
Markers	CD135$^-$	CD135$^+$	CD135$^+$	c-KIT-	c-KIT$^+$	c-KIT$^+$			
	CD38$^+$	CD38$^+$	CD38$^-$	CD34$^+$	CD34$^+$	CD34$^-$			
%	0.31±0.04	0.09±0.03	2.14±0.63	0.07±0.03	95.43±0.25	4.47±0.26			
Markers	HDR$^-$	HDR$^+$	HDR$^+$	HLADR$^-$	HLADR$^+$	HLADR$^+$			
	CD33$^+$	CD33$^+$	CD33$^-$	CD13$^+$	CD13$^+$	CD13$^-$			
%	92.57±0.32	7.28±0.08	0.00±0.00	46.60±0.46	5.74±0.51	6.42±0.53			
Among	CD15$^-$	CD15$^+$	CD15$^+$	CD15$^-$	CD15$^+$	CD15$^+$	CD11b$^-$	CD11$^+$	CD11$^+$
CD13$^+$	CD14$^+$	CD14$^+$	CD14$^-$	CD16$^+$	CD16$^+$	CD16$^-$	CD11c$^+$	CD11c$^+$	CD11c$^-$
CD116$^+$	0.69±0.18	0.13±0.07	1.99±0.43	0.50±0.08	0.05±0.00	1.93±0.50	0.02±0.01	0.12±0.04	14.86±0.61
NB4									
Markers	c-KIT$^-$	c-KIT$^+$	c-KIT$^+$	CD34$^-$	CD34$^+$	CD34$^+$	c-KIT$^-$	c-KIT$^+$	c-KIT$^+$
	CD38$^+$	CD38$^+$	CD38$^-$	CD38$^+$	CD38$^+$	CD38$^-$	HLADR$^+$	HLADR$^+$	HLADR$^-$
%	42.13±0.71	14.37±3.50	10.23±1.28	28.83±2.57	2.61±0.24	7.43±0.37	16.93±3.61	2.66±0.65	6.71±0.88
Markers	CD15$^-$	CD15$^+$	CD15$^+$	CD11b$^-$	CD11b$^+$	CD11b$^+$	CD11c$^-$	CD11c$^+$	CD11c$^+$
	CD116$^+$	CD116$^+$	CD116$^-$	CD11c$^+$	CD11c$^+$	CD11c$^-$	CD14$^+$	CD14$^+$	CD14$^-$
%	13.20±2.86	1.96±0.14	3.15±0.33	5.39±0.71	21.17±2.87	56.63±2.38	9.26±0.33	21.17±1.35	48.03±1.12
MOLM-13									
Markers	HLADR$^-$	HLADR$^+$	HLADR$^+$	c-KIT$^-$	c-KIT$^+$	c-KIT$^+$	c-KIT$^-$	c-KIT$^+$	c-KIT$^+$
	CD33$^+$	CD33$^+$	CD33$^-$	CD34$^+$	CD34$^+$	CD34$^-$	CD38$^+$	CD38$^+$	CD38$^-$
%	76.57±1.86	23.43±1.86	0.00±0.00	0.26±0.17	0.09±0.06	7.63±1.29	85.07±0.64	9.21±1.32	0.38±0.11
Markers	HLADR$^-$	HLADR$^+$	HLADR$^+$	CD64$^-$	CD64$^+$	CD64$^+$	CD11b$^-$	CD11b$^+$	CD11b$^+$
	CD13$^+$	CD13$^+$	CD13$^-$	CD13$^+$	CD13$^+$	CD13$^-$	CD13$^+$	CD13$^+$	CD13$^-$
%	2.16±0.14	0.85±0.10	21.73±0.42	1.37±0.12	0.01±0.01	0.02±0.00	1.56±0.13	0.04±0.01	0.23±0.01
Markers	CD14$^-$	CD14$^+$	CD14$^+$	CD15$^-$	CD15$^+$	CD15$^+$	CD11c$^-$	CD11c$^+$	CD11c$^+$
	CD116$^+$	CD116$^+$	CD116$^-$	CD13$^+$	CD13$^+$	CD13$^-$	CD14$^+$	CD14$^+$	CD14$^-$
%	0.39±0.07	0.10±0.02	0.12±0.03	1.42±0.18	0.18±0.05	7.79±1.63	2.22±0.63	0.01±0.01	0.01±0.01
MV4-11									
Markers	HLADR$^-$	HLADR$^+$	HLADR$^+$	c-KIT$^-$	c-KIT$^+$	c-KIT$^+$	CD38$^-$	CD38$^+$	CD38$^+$
	CD33$^+$	CD33$^+$	CD33$^-$	CD34$^+$	CD34$^+$	CD34$^-$	CD34$^+$	CD34$^+$	CD34$^-$
%	14.80±0.78	85.03±0.67	0.00±0.00	0.14±0.06	0.03±0.01	6.02±0.54	0.14±0.06	0.05±0.01	0.63±0.03
Markers	HLADR$^-$	HLADR$^+$	HLADR$^+$	CD14$^-$	CD14$^+$	CD14$^+$	CD15$^-$	CD15$^+$	CD15$^+$
	CD13$^+$	CD13$^+$	CD13$^-$	CD116$^+$	CD116$^+$	CD116$^-$	CD64$^+$	CD64$^+$	CD64$^-$
%	2.76±0.18	2.62±0.36	30.30±2.67	0.36±0.05	0.02±0.01	0.13±0.06	0.35±0.10	30.70±3.70	57.93±1.16
Markers	CD14$^-$	CD14$^+$	CD14$^+$	CD64$^-$	CD64$^+$	CD64$^+$	CD13$^-$	CD13$^+$	CD13$^+$
	CD116$^+$	CD116$^+$	CD116$^-$	CD36$^+$	CD36$^+$	CD36$^-$	CD10$^+$	CD10$^+$	CD10$^-$
%	0.50±0.16	0.01±0.00	0.17±0.00	0.00±0.00	0.15±0.03	99.43±0.15	0.02±0.01	0.04±0.01	46.13±1.68
K562									
Markers	HDR$^-$	HDR$^+$	HDR$^+$	c-KIT$^-$	c-KIT$^+$	c-KIT$^+$	c-KIT$^-$	c-KIT$^+$	c-KIT$^+$
	CD13$^+$	CD13$^+$	CD13$^-$	CD13$^+$	CD13$^+$	CD13$^-$	CD33$^+$	CD33$^+$	CD33$^-$
%	9.77±6.01	0.01±0.00	0.03±0.02	11.93±6.48	2.99±1.44	9.43±1.34	52.97±1.00	8.77±0.45	2.56±0.15
Markers	c-KIT$^-$	c-KIT$^+$	c-KIT$^+$	CD38$^-$	CD38$^+$	CD38$^+$	c-KIT$^-$	c-KIT$^+$	c-KIT+
	CD34$^+$	CD34$^+$	CD34$^-$	CD33$^+$	CD33$^+$	CD33$^-$	CD36$^+$	CD36$^+$	CD36$^-$
%	0.42±0.18	0.10±0.06	8.51±0.32	57.70±1.14	0.02±0.01	0.00±0.01	57.90±2.09	17.37±1.42	5.43±0.50
Among c-KIT$^-$ CD36$^+$ cells				Markers	CD71$^+$	CD71$^-$	CD44$^+$	CD44$^+$	CD44$^-$
					GPA$^+$	GPA$^+$	GPA$^-$	GPA$^+$	GPA$^+$
				%	1.71±0.17	0.00±0.00	49.03±1.48	1.40±0.15	0.31±0.07
Jurkat									
Markers	CD33$^-$	CD33$^+$	CD33$^+$	c-KIT$^-$	c-KIT$^+$	c-KIT$^+$	CD34$^-$	CD34$^+$	CD34$^+$
	CD34$^+$	CD34$^+$	CD34$^-$	CD34$^+$	CD34$^+$	CD34$^-$	CD38$^+$	CD38$^+$	CD38$^-$
%	3.53±0.54	0.03±0.01	0.13±0.02	3.13±0.74	0.84±0.15	0.78±0.29	11.09±1.11	1.20±0.94	3.37±0.44
Among CD38$^+$ CD7$^+$ (1.50±0.40%)									
Markers	CD2$^-$	CD2$^+$	CD2$^+$	CD3$^-$	CD3$^+$	CD3$^+$	CD3$^-$	CD3$^+$	CD3$^+$
	CD5$^+$	CD5$^+$	CD5$^-$	CD4$^+$	CD4$^+$	CD4$^-$	CD8$^+$	CD8$^+$	CD8$^-$
%	0.00±0.00	1.48±0.40	0.01±0.00	0.12±0.04	0.67±0.18	0.62±0.16	0.00±0.00	0.01±0.01	1.18±0.31
Among CD38$^-$ CD7$^+$ cells (59.43±2.40%)									
Markers	CD2$^-$	CD2$^+$	CD2$^+$	CD3$^-$	CD3$^+$	CD3$^+$	CD3$^-$	CD3$^+$	CD3$^+$
	CD5$^+$	CD5$^+$	CD5$^-$	CD4$^+$	CD4$^+$	CD4$^-$	CD8$^+$	CD8$^+$	CD8$^-$
%	0.36±0.39	56.73±2.55	2.26±0.07	6.84±0.31	21.82±1.12	23.44±1.18	0.01±0.01	0.03±0.01	39.01±2.60

of c-KIT, HLA-DR, CD45, CD116 and CD16. NB4 cells have been utilized to examine the effect of differentiation inducers (8, 18) and examined their effect by morphological observation and surface expression utilizing CD11b antibodies. Although NB4 cells have been also used for apoptosis studies (19, 20), no mention about surface marker expression has been made. Combined with studies on surface markers, these studies will provide us with more information on which cell fractions are most effective in apoptosis. Additional surface markers tested here, such as c-KIT, HLA-DR, CD45, CD116 and CD16, support the robust evidence to define the cells.

MOLM-13 cells that were established from acute monocytic leukemia (M5a) expressed CD33, CD34, CD15, but not CD13 or CD14 (9), and our expression patterns for CD34 and CD14 differed compared with those previously reported (Figures 2 and 3C). This discrepancy also seemed likely to be due to differences in antibody clones of CD34 and CD14 between our study and previous reports. There was no previous clarification of surface phenotype for MV-4-11 cells. p53-Dependent apoptosis and cell cycle was investigated in MOLM-13 cells (21) (22) but without differentiation-based analyses. MOLM-13 and MV-4-11 cells are both CD135- internal tandem duplication (ITD)-positive AMLs, were utilized in CD135-dependent drug-screening studies (23, 24) in which outcomes were evaluated with cell proliferation and phosphorylation of internal signal, such as CD135 itself and Signal transducer and activator of transcription 5 (STAT5), but not with surface phenotype and transcription factor expression. Our results shown in Figure 3C and D suggest that MOLM-13 cells and MV-4-11 are CFU-GM to myeloblasts and myeloblasts, respectively, which implies that these cell lines will also be fit for functional analysis to examine differentiation after the myeloblast state to mature monocytes. Regarding gene expression, as *CEBPα* expression is quite high in MV4-11 cells (Figure 4), reduction of expression would seem to be difficult for this cell line, suggesting it is not suitable for studies of loss-of-function of CEBPα and its downstream genes.

K562 cells are chronic myelogenous leukemia-derived erythroleukemia cells and were not defined on surface marker-based differentiation state when established (11); later studies confirmed the surface phenotype of GPA (25). When examining the differentiation stage of erythroid cells, hemoglobinized cells were stained with benzidine and hemoglobinization was quantified, depending on the staining intensity. Recently Hu *et al*. reported that the distinct stages in erythroid differentiation were clearly separated based on dynamic changes in membrane proteins, such as CD36, CD44, CD71 and GPA using human cord blood-derived erythroid cells (26). Herein, we confirm the expression of these proteins in K562 cells. Concerning gene expression, *GATA1* expression and its target gene effect was checked to investigate its effect on erythroid differentiation in K562 cells (27). *GATA2* and *FOG1* expression was also confirmed (Figure 4) although their expression levels were lower than *GATA1*, suggesting that *GATA2* and *FOG1* expression can be used to trace the differentiation of K562 cells so as to evaluate the differentiation status more accurately.

Jurkat cells were established from acute T-cell leukemia and not defined in terms of surface marker-based differentiation state when they were established (12). Our results revealed that Jurkat cells are differentiated into mature functional T-cells with mature T-cell markers (Figures 2 and 3F), and T-cell-specific genes (*NOTCH1* and *E2A*) were highly expressed (Figure 4), which implies that this cell line is fit for studies that focus on transcriptional regulation, rather than differentiation studies.

The information mentioned herein can be used for establishing standards for choosing appropriate cell lines for functional analyses, and for determination of efficacy after functional analysis and drug screening.

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