

Figure 3 CNA analysis of purified ATL cells. (a) Inferred copy number for all SNP sites analysed in MNCs (left) and CD4⁺ cells (right) isolated from an ATL patient in the acute stage (ID6). Copy number is color-coded according to the indicated scheme. Chromosomal regions with an aberrant copy number detected only in CD4⁺ cells are indicated by blue underlines. An inset below demonstrates the raw signal (log₂ ratio) for every SNP locus by the array hybridization (red dots) and corresponding inferred copy number (green lines) on chromosome 1 for the MNC sample, along with the chromosome cytobands. (b) Inferred copy number for all SNP sites (chromosomes 1–22) in all subjects studied ($n = 9$ for chronic stage, $n = 15$ for acute stage) shown according to the color scheme at the bottom. SNP sites are ordered by their physical position from top to bottom (shown on the right), and the borders between chromosomes are indicated by small bars. The most frequently deleted region at 14q11.2 is indicated by the arrowhead. (c) Inferred copy number (yellow columns) for a region of chromosome 6 (nucleotides 16 651 304–16 651 533) compared with the relative amount (blue line) of genomic DNA corresponding to this region (expressed relative to the amount of *GAPDH* genomic DNA). An ATL cell line (KK-1) and CD4⁺ cells isolated from a male (46XY) or female (46XX) volunteer were also analysed. (d) Subject tree based on inferred copy number of chromosomal regions that showed a statistically significant difference in copy number for at least two consecutive SNP sites between the acute and chronic stages of ATL ($P < 0.01$, Student's *t*-test). (e) Comparison of expression intensities of the genes assigned to two chromosomal regions (region #1 and region #2) indicated in (d) between the subjects with or without a gain in chromosome copy number (Chr#) for the corresponding region. Data are means + s.d. The *P*-values were calculated by Student's *t*-test.

silence (expression level of < 10 U) in all normal T cells and chronic ATL specimens and their activation (> 100 U) in at least one of the acute-stage samples. We isolated six probe sets that fulfilled such criteria (Figure 4a and Supplementary Table 4).

Among these acute stage-specific genes, we focused on *MET* (GenBank accession no. NM_000245), given that we recently found, in an independent study, that the amount of *MET* mRNA was specifically increased in ATL cells that manifested liver invasion (Imaizumi *et al.*, 2003). *MET* encodes a transmembrane protein tyrosine kinase that is the receptor for hepatocyte growth factor (HGF) (Bottaro *et al.*, 1991). The expression level of *MET* in the study specimens as determined by microarray analysis was highly correlated with that determined by quantitative RT-PCR analysis (Figure 4b), as revealed by a Pearson's correlation coefficient (r) of 0.851 ($P < 0.001$). (Also see Supplementary Table 5 for verification of microarray data by RT-PCR.) Flow cytometry revealed that the expression of *MET* at the cell surface reflected the abundance of the corresponding mRNA in ATL samples (Figure 4c).

The acute stage-specific expression of *MET* at both the mRNA and protein levels suggested that ATL cells might acquire mitogenic potential as a result of activation of a *MET*-linked signaling pathway. To examine the possible operation of an HGF-*MET* autocrine loop, we quantitated HGF mRNA in ATL cells by both microarray and quantitative RT-PCR analyses. No substantial amounts of HGF mRNA were detected in ATL specimens, however (data not shown).

We therefore next measured the plasma concentration of HGF in the study subjects. High levels of HGF were detected in the plasma of ATL patients, especially in that of individuals in the acute stage (Figure 5a), compared with the previously determined values for healthy adults (0.27 ± 0.08 ng/ml, mean \pm s.d.) and some cancer patients (1–2 ng/ml) (Funakoshi and Nakamura, 2003). To test directly whether activation of the HGF-*MET* signaling pathway is able to promote the proliferation of ATL cells, we examined the *MET*-positive ATL cell line KK-1. HGF induced both the tyrosine phosphorylation of *MET* and proliferation in KK-1 cells (Figure 5b and c). The addition of antibodies to HGF (Montesano *et al.*, 1991) could abolish both effects.

Discussion

We have analysed gene expression and CNA profiles in leukemic cell-enriched fractions of individuals with ATL. We found that both types of profile differ markedly between the chronic and acute stages of ATL, and that the level of gene expression is influenced by the copy number of genomic DNA in ATL cells. Although ATL cells have previously been shown to manifest multiple genomic gains or losses (Ariyama *et al.*, 1999; Tsukasaki *et al.*, 2001), our data have revealed that the ATL genome is more unstable than has been appreciated. Similar complex CNAs have been identified by SNP array-based methods for other types

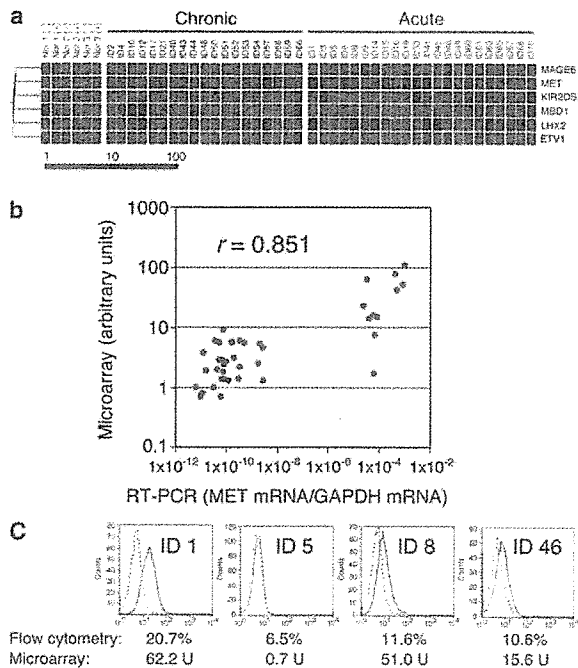


Figure 4 Acute stage-specific expression of *MET* in ATL. (a) Expression profiles of the six acute stage-specific probe sets. The expression level of each probe set is colored according to the indicated scale. Annotations and expression intensities for the probe sets are presented in Supplementary Table 4. (b) Comparison of the abundance of *MET* mRNA in the study specimens as determined by microarray and RT-PCR analyses. For the latter, the amount of *MET* mRNA is expressed relative to that of *GAPDH* mRNA. Pearson's correlation coefficient (r) for the comparison is indicated. (c) Cell surface expression of *MET* was examined by flow cytometry in four ATL samples corresponding to the acute stage. The solid and dashed traces were obtained with antibodies to *MET* and control antibodies, respectively. The proportion of *MET*⁺ cells determined by flow cytometry is indicated together with the corresponding amount of *MET* mRNA determined by microarray analysis.

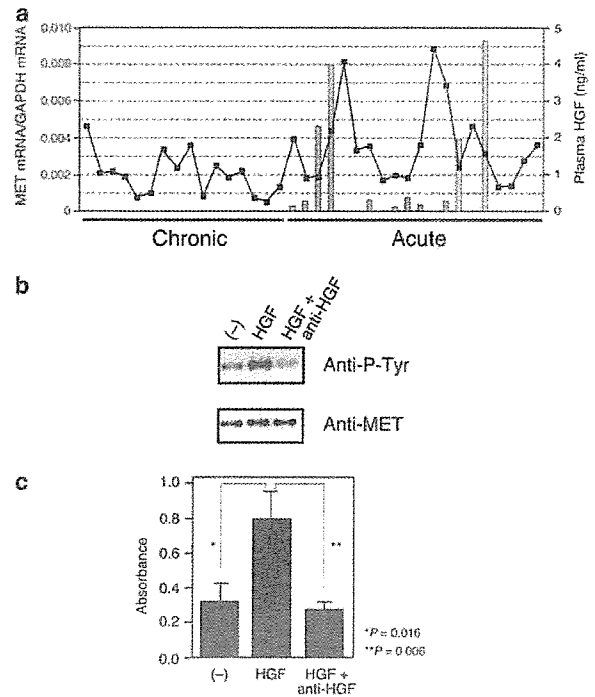


Figure 5 HGF-MET activity induces proliferation in ATL cells. (a) Comparison of the plasma concentration of HGF in ATL patients (as measured by enzyme-linked immunosorbent assay) with the corresponding amount of *MET* mRNA in leukemic cells (as determined by RT-PCR). (b) Tyrosine phosphorylation of *MET* in KK-1 cells incubated for 10 min in the absence (-) or presence of human HGF (50 ng/ml), alone or together with antibodies to HGF (10 μg/ml), was examined by immunoblot analysis of cell lysates with antibodies to phosphotyrosine (Anti-P-Tyr). The blot was also probed with antibodies to *MET*. (c) The proliferation of KK-1 cells (3×10^4) incubated for 1 day in the absence (-) or presence of HGF (50 ng/ml), alone or together with antibodies to HGF (10 μg/ml), was evaluated by the MTS assay. Data are expressed in absorbance units and are means ± s.d. of triplicates from a representative experiment. The P -values for the indicated comparisons were determined by Student's t -test.

of cancer (Zhao *et al.*, 2005). We detected 3386.9 ± 2254.0 (mean ± s.d.) and 4678.5 ± 2874.6 SNP sites showing $\geq 3n$ ploidy as well as 1039.9 ± 2310.0 and 927.1 ± 1137.9 SNP sites with $\leq 1n$ ploidy in samples corresponding to the chronic and acute stages of ATL, respectively. The numbers of probe sets showing gain or loss of DNA content did not differ significantly ($P > 0.05$) between the chronic and acute stages of ATL. Given the large numbers of probe sets with an aberrant DNA content in the ATL genome, a large-scale study will likely be required to pinpoint the *bona fide* disease-dependent or stage-dependent CNAs.

Recently, with the use of BAC array-based CGH (with a mean resolution of 1.3 Mbp), Oshiro *et al.* (2006) have compared CNA of MNCs for 17 patients at the acute stage of ATL to that of lymph nodes for 42 cases with the lymphoma type of ATL. The recurrent gain of chromosomes was found at 3/3p among individuals with the acute stage of ATL, which is in good agreement with our results.

We found that an increased plasma concentration of HGF coexists with an increased expression of *MET* in ATL cells from some individuals at the acute stage of the disease. Together with the demonstrated mitogenic effect of HGF in ATL cells, these data suggest a novel scenario for stage progression in ATL. The mechanism responsible for the increased plasma level of HGF in ATL patients is unclear. Given that ATL is a malignancy of activated mature T cells, the leukemic cells secrete a variety of cytokines, including tumor necrosis factor- α and interleukin-1 β (Wano *et al.*, 1987; Yamada *et al.*, 1996). Both of these cytokines are potent inducers of HGF expression in fibroblasts (Matsumoto *et al.*, 1992; Tamura *et al.*, 1993), suggesting that ATL cells may indirectly increase the plasma HGF level through secretion of these cytokines and consequent activation of fibroblasts.

Our data indicate that the plasma concentration of HGF in ATL patients increases before the onset of

expression of *MET* in the leukemic cells (Figure 5a). The increased concentration of HGF might therefore confer a growth advantage on ATL cells after they upregulate the expression of *MET*. Given that the JAK-STAT signaling pathway is activated in the leukemic cells of patients with advanced ATL (Migone *et al.*, 1995), it may be relevant that binding sites for STAT1 or STAT3 are present in the promoter regions of five (including *MET*) of the six acute stage-specific genes identified in the present study (Figure 4a). We did not detect a significant difference in DNA content (in our data set) for the *MET* locus between chronic and acute stages of ATL. It is thus possible that JAK-STAT signaling contributes to transcriptional activation of *MET*.

Given that our data are derived from purified ATL cells, they can be further used to characterize ATL in various ways. For instance, we attempted to isolate genes whose expression was linked to the presence of hypercalcemia in the study patients (data not shown); such genes included that for parathyroid hormone-like hormone (GenBank accession no. BC005961), which has been shown to be responsible for many instances of humoral hypercalcemia in individuals with cancer including ATL (Broadus *et al.*, 1988; Motokura *et al.*, 1988).

We have demonstrated the existence of an HGF-MET paracrine loop specific to the acute stage of ATL. Given that ligation of MET by HGF promoted the proliferation of ATL cells, activation of the HGF-MET signaling pathway is a candidate molecular mechanism for stage progression in ATL. Furthermore, our observation that this mitogenic effect was blocked by antibodies to HGF provides potential new targets for ATL therapy.

Materials and methods

Expression profiling

All clinical specimens were obtained with written informed consent, and the study was approved by the ethics committees of both Jichi Medical University and Nagasaki University. The diagnosis of ATL in all cases was based on clinical features, immunophenotypes of leukemic cells, and the monoclonal integration of HTLV-1 proviral DNA into the genome of leukemic cells (Shimoyama, 1991). MNCs isolated from PB were labeled with magnetic bead-conjugated mouse monoclonal antibodies to CD4 (CD4 MicroBeads, Miltenyi Biotec, Auburn, CA, USA). For PHA stimulation, purified CD4⁺ cells from healthy individuals were incubated for 48 h in Rosewell's Park Memorial Institute media (RPMI) 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and PHA-P (8 µg/ml) (Sigma, St Louis, MO, USA).

Column fractionation of MNCs, RNA preparation, and hybridization with HGU133A & B microarrays (Affymetrix, Santa Clara, CA, USA) were performed as described previously (Choi *et al.*, 2004). The mean expression intensity of the internal positive control probe sets (http://www.affymetrix.com/support/technical/mask_files.affx) was set to 500 U in each hybridization, and the fluorescence intensity of each test gene was normalized accordingly. All HGU133A & B

microarray data are available at the Gene Expression Omnibus web site (<http://www.ncbi.nlm.nih.gov/geo>) under the accession number GSE1466.

Student's *t*-test with the Benjamini and Hochberg false discovery rate option was performed with GeneSpring 7.0 software (Silicon Genetics). Effect size was defined as an absolute difference in mean expression intensity between a given pair of classes (Dhanasekaran *et al.*, 2001). Education of and prediction by our ANN were performed with NeuralWorks Professional II Plus v.5.3 software (NeuralWare, Carnegie, PA, USA) as described previously (O'Neill and Song, 2003).

Analysis of CNA

Genomic DNA was obtained from purified CD4⁺ ATL cells ($n=24$) and from MNCs of patient ID6 with the use of a QIAmp DNA Mini Kit (Qiagen, Valencia, CA, USA). Each DNA sample (250 ng) was digested with *HindIII*, ligated to Adaptor-Hind (Affymetrix), amplified by PCR, and subjected to hybridization with Mapping 50K Hind 240 arrays (Affymetrix). Chromosome copy number at each SNP site was inferred from hybridization signal intensity on the arrays with the use of CNAG software (<http://www.genome.umin.jp>) (Nannya *et al.*, 2005). For a normal reference, we used array data of PB MNCs isolated from four healthy volunteers. Assessment of copy number for all SNP sites is demonstrated in Supplementary Table 6. The raw data of Mapping 50K Hind 240 arrays is available upon request. Statistical analysis of copy number was performed with GeneSpring 7.0. Alterations in the amount of genomic DNA were confirmed by quantitative real-time PCR with an ABI PRISM 7700 sequence detection system (PE Applied Biosystems, Foster City, CA, USA). The oligonucleotide primers were 5'-AGCATGTCCACAAATGGCCTTTGG-3' and 5'-CAGTTTTCTGTGCATGGGAAAGGG-3' for a region of chromosome 6, and 5'-CTGACCTGCCGTCTA GAAAAACCT-3' and 5'-CAGGAAATGAGCTTGACAA AGTGG-3' for the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene.

MET expression

ATL cells were stained with rabbit polyclonal antibodies to MET (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with mouse monoclonal antibodies to CD3 and to CD4 (both from BD Biosciences, San Diego, CA, USA) and were then subjected to flow cytometry with a FACScan instrument (BD Biosciences). The concentration of HGF in plasma was determined with a Quantikine ELISA kit for human HGF (R&D Systems, Minneapolis, MN, USA).

For quantitative RT-PCR analysis of *MET* expression, portions of nonamplified cDNA were subjected to PCR with a QuantiTect SYBR Green PCR kit (Qiagen). The oligonucleotide primers for PCR were 5'-GTCAGTGGTGGACCTG ACCT-3' and 5'-TGAGCTTGACAAAGTGGTCCG-3' for *GAPDH* cDNA, and 5'-ACTTGTGCAAGGGAGAAGAC TCC-3' and 5'-AGCGTTCACATGGACATAGTGCTC-3' for *MET* cDNA.

KK-1 cell experiments

KK-1 cells were maintained in RPMI 1640 medium supplemented with 10% FBS and recombinant human interleukin-2 (10 ng/ml) (Sigma). For immunoblot analysis, cells were cultured for 48 h without FBS and interleukin-2 and then incubated for 10 min with recombinant human HGF (50 ng/ml) (Sigma) either alone or together with rabbit polyclonal antibodies to HGF (10 µg/ml) (Montesano *et al.*, 1991). The

cells were then lysed and subjected to immunoblot analysis with mouse monoclonal antibodies to phosphotyrosine (4G10, Upstate Biotechnology, Charlottesville, VA, USA) or with rabbit polyclonal antibodies to MET (#05-237, Upstate Biotechnology) as described previously (Yamashita *et al.*, 2001). For assay of cell proliferation, serum-deprived KK-1 cells were cultured for 24 h at a density of 5×10^5 /ml with HGF (50 ng/ml) either alone or together with antibodies to HGF (10 μ g/ml) and were then mixed with MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]. Cell proliferation was measured with a CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA).

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Identification of a constitutively active mutant of JAK3 by retroviral expression screening

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Abstract

To identify transforming genes in acute myeloid leukemia (AML) we here constructed a retroviral cDNA expression library from an AML patient, and then used this library to infect a mouse cell line 32Dcl3-mCAT. cDNA inserts of the cell clones which proliferated in the presence of granulocyte colony-stimulating factor were derived from *JAK3* encoding a JAK3 mutant with a valine-to-alanine substitution at codon 674 and two additional amino acid substitutions. The transforming activity of JAK3(V674A) was confirmed by its introduction into 32Dcl3-mCAT. Sequencing of the original *JAK3* cDNA derived from the patient, however, failed to detect the V674A mutation.
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Keywords: JAK3; Retrovirus; Acute myeloid leukemia; cDNA expression library

1. Introduction

Acute myeloid leukemia (AML) is a clonal disorder of immature progenitor cells in the hematopoietic system. Chromosomal translocations are present in the AML blasts of 20–30% of individuals with this condition, and the fusion genes generated by such translocations have been shown to possess transforming activity [1,2]. Even with current technology, however, chromosomal abnormalities are not detectable in the blasts of almost half of AML patients [3]. Furthermore, although point mutations in a variety of genes implicated in cell growth or differentiation, such as *RAS*, *FLT3*, *KIT*, and *RUNX1*, are detectable in some such blasts, many cases of AML are not associated with a detectable gene anomaly [4]. Clarification of the transforming events in such AML cases would thus be expected to provide a basis for the development of effective treatments.

Functional screening based on transforming activity is one potential approach to the efficient isolation of tumor-promoting genes in AML. Focus formation assays with mouse 3T3 fibroblasts have indeed proved successful for the identification of oncogenes in human cancer [5]. In such screening assays, genomic DNA isolated from cancer specimens is used to transfect 3T3 cells and the formation of transformed cell foci is then evaluated. There are, however, substantial drawbacks to such screening for AML. First, expression of the exogenous genes in conventional 3T3 screening is driven by the associated promoters or enhancers, so that oncogenes are able to exert transforming effects in 3T3 cells only if their regulatory regions are active in fibroblasts, which is not guaranteed. Furthermore, given that the transcriptome and proteome would be expected to differ markedly between fibroblasts and leukemic blood cells, active oncogenes in the latter cells may not function properly in the former cells even if they are adequately expressed.

We reasoned that these concerns may be overcome through expression of test cDNAs under the control of an ectopic

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promoter and with the use of myeloid cells, instead of 3T3 cells, for the assay. We have now constructed a retroviral cDNA expression library from a purified CD133⁺ stem cell fraction isolated from a patient with AML and used this library to infect the mouse myeloid cell line 32Dcl3 [6]. We then screened for transforming genes that override the differentiation program of 32Dcl3 cells triggered by granulocyte colony-stimulating factor (G-CSF). Furthermore, in preparation of the cDNA library, we took advantage of a polymerase chain reaction (PCR)-based system that preferentially amplifies full-length cDNAs [7]. Through this screening, we isolated a cDNA encoding a constitutively active form of the protein kinase JAK3. However, investigation of *JAK3* cDNA sequence in the original RNA failed to detect the activating mutation, indicating that the mutation arose probably from an experimental artifact.

2. Materials and methods

2.1. Cell culture and clinical samples

32Dcl3 cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and mouse interleukin (IL) – 3 (20 U/mL; Sigma, St. Louis, MO). To increase the efficiency of retroviral infection, we generated 32D-mCAT cells, which overexpress a receptor (mCAT) for ecotropic retrovirus [8], through infection of 32Dcl3 cells with a recombinant retrovirus containing both mCAT and blasticidin-S resistance genes. For induction of granulocyte differentiation, 32D-mCAT cells were cultured with mouse G-CSF (0.5 ng/mL, Sigma) instead of IL-3. The BOSC23 packaging cell line [9] was maintained in Dulbecco's modified Eagle's medium – F12 (Invitrogen) supplemented with 10% FBS and 2 mM L-glutamine.

CD133⁺ cells were purified with anti-CD133 MicroBeads and a Mini-MACS magnetic cell separation column (Miltenyi Biotec, Auburn, CA), as described previously [10], from mononuclear cells of bone marrow from a patient with AML, who provided written informed consent.

2.2. Construction of a retroviral library

A retroviral plasmid library was constructed as described previously [7]. In brief, total RNA was extracted from the purified CD133⁺ cells with the use of an RNeasy Mini column and RNase-free DNase (Qiagen, Valencia, CA), and first-strand cDNAs were then synthesized with PowerScript reverse transcriptase, the SMART IIA oligonucleotide, and CDS primer IIA (all from Clontech, Palo Alto, CA). The resulting cDNAs were then amplified for 20 cycles with 5'-PCR primer IIA and LA Taq polymerase (Takara Bio, Shiga, Japan). The PCR products were ligated to a BstXI adapter (Invitrogen) and then incorporated into the pMX retroviral plasmid [11]. The pMX-cDNA plasmids were introduced into ElectroMax DH10B cells (Invitrogen) by electroporation.

2.3. 32D-mCAT transformation assay

BOSC23 cells (1.8×10^6) were seeded in a 6-cm culture plate and transfected with a mixture comprising 2 μ g of retroviral plasmids, 0.5 μ g of pGP plasmid (to express *gag-pol* proteins, Takara Bio), 0.5 μ g of pE-eco plasmid (to express ecotropic *env* protein, Takara Bio), and 18 μ L of Lipofectamine reagent (Invitrogen). Two days after transfection, the culture supernatant was collected and incubated with 32D-mCAT cells (5×10^5) for 24 h in the presence of Retronectin (Takara Bio). The 32D-mCAT cells were then incubated for an additional 24 h with mouse IL-3, washed with cytokine-free medium, and cultured at an initial density of 1×10^4 to 4×10^4 cells/mL in the presence of mouse G-CSF (0.5 ng/mL).

2.4. Recovery of cDNAs from 32D-mCAT cells

Genomic DNA was isolated from 32D-mCAT cells that grew without differentiation (determined by the presence of polymorphonuclear neutrophils as assessed by cytospin preparations stained with the Wright Giemsa solutions) in the presence of G-CSF and was subjected to PCR with 5'-PCR primer IIA and LA Taq polymerase for 50 cycles of 98 °C for 20 s and 68 °C for 6 min. Amplified DNA fragments were purified by gel electrophoresis and ligated into the pT7Blue-2 vector (EMD Biosciences, San Diego, CA) for nucleotide sequencing. To confirm the transforming activity of the isolated cDNAs, they were again isolated by PCR with 5'-PCR primer IIA and PfuUltra High-Fidelity DNA polymerase (Stratagene, La Jolla, CA) for 30 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 4 min. The cDNAs were then individually ligated into the pMX plasmid and used to generate recombinant retroviruses. 32D-mCAT cells were then infected with the resulting viruses and cultured with G-CSF.

2.5. Protein analysis

Wild-type human *JAK3* cDNA (a kind gift of Dr. James N. Ihle) was used to generate mutant cDNAs with the use of a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Oligonucleotides used for mutagenesis were 5'-GGGATGGGGGGCTGTACGTAGATGGGGTGGC-3' and 5'-GCCACCCCATCTACGTACAGCCCCCATCCC-3' for JAK3(H463Y), 5'-GAGTGACCCTGGGGCCAGCCCCGCTGTGTTAAGCC-3' and 5'-GGCTTAACACAGCGGGGCTGGCCCCAGGGTCACTC-3' for JAK3(V674A), and 5'-GATGGGATGTGAGCGGGGTGTCCCCGCCCTCTG-3' and 5'-CAGAGGGCGGGACACCCCGCTCACATCCATC-3' for JAK3(D1043G). The wild-type or mutant cDNAs were individually ligated into the pMX-ires-neo retroviral vector in order to generate recombinant retroviruses encoding both JAK and the neomycin resistance gene. 32D-mCAT cells were infected with the resulting viruses and cultured for >2 weeks in the presence of IL-3 and

G418 (1 mg/mL, Invitrogen). JAK3 was immunoprecipitated from neomycin-resistant mass cultures of 32D-mCAT cells with goat polyclonal antibodies specific for this protein (sc-1078; Santa Cruz Biotechnology, Santa Cruz, CA). Half of the resulting precipitates were subjected to immunoblot analysis with a mouse monoclonal antibody to phosphotyrosine (4G-10; Upstate Biotechnology, Charlottesville, VA) or rabbit polyclonal antibodies to JAK3 (sc-513, Santa Cruz Biotechnology). Antibodies used for the immunoprecipitation of STAT1 were obtained from Upstate Biotechnology, those specific for STAT3 were from Cell Signaling Technology (Danvers, MA), and those specific for STAT5 or STAT6 were from Santa Cruz Biotechnology. Immune complexes on immunoblots were visualized with SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL).

The remaining half of the immunoprecipitates were then washed with kinase buffer [10 mM HEPES–NaOH (pH 7.4), 50 mM NaCl, 5 mM MgCl₂, 5 mM MnCl₂, 0.1 mM Na₃VO₄] and then incubated for 30 min at room temperature in 30 μ L of the kinase buffer containing 0.37 MBq of [γ -³²P]ATP (GE Healthcare Bio-Sciences, Uppsala, Sweden) and 1 μ g of a synthetic peptide (LLPLD-KDYVYVREPGQS) corresponding to amino acids 973–989 of human JAK3 (Operon Biotechnologies, Huntsville, AL).

3. Results

3.1. Screening for transforming genes in 32D-mCAT cells

To isolate transforming genes from a retroviral expression library, we used mouse 32Dcl3 cells, which proliferate in the presence of IL-3 but undergo terminal differentiation to granulocytes in response to G-CSF [6]. Introduction of v-Ha-Ras or v-Abl oncogenes into these cells induces continuous growth even in the presence of G-CSF [12,13], indicating that active oncogenes are able to override the differentiation program of 32Dcl3 cells. We thus reasoned that retroviral transduction of transforming genes present in AML blasts into 32Dcl3 cells might also elicit proliferation in the presence of G-CSF. Given that the efficiency of infection of 32Dcl3 cells with ecotropic retroviruses was low (<2%), we generated 32D-mCAT cells, which overexpress an ecotropic retrovirus receptor and exhibit an infection efficiency of 10–30% (data not shown). We then examined the feasibility of a novel screening system for transforming genes that consists of (1) infection of 32D-mCAT cells with recombinant retroviral cDNA libraries constructed from AML specimens, (2) selection of proliferating 32D-mCAT cells in the presence of G-CSF, and (3) isolation of transforming cDNAs by PCR with a primer targeted to a sequence flanking the cDNA in each retrovirus.

3.2. Screening with a retroviral cDNA expression library from an AML patient

To screen for transforming genes in AML, we prepared a CD133⁺ hematopoietic stem cell-like fraction from an 84-year old Japanese man with AML (M2 subtype according to the FAB classification). The patient had a clinical history of myelodysplastic syndrome prior to the onset of AML, and his malignant blasts had a karyotype of monosomy 7. Purification of a CD133⁺ immature cell fraction likely enriched for

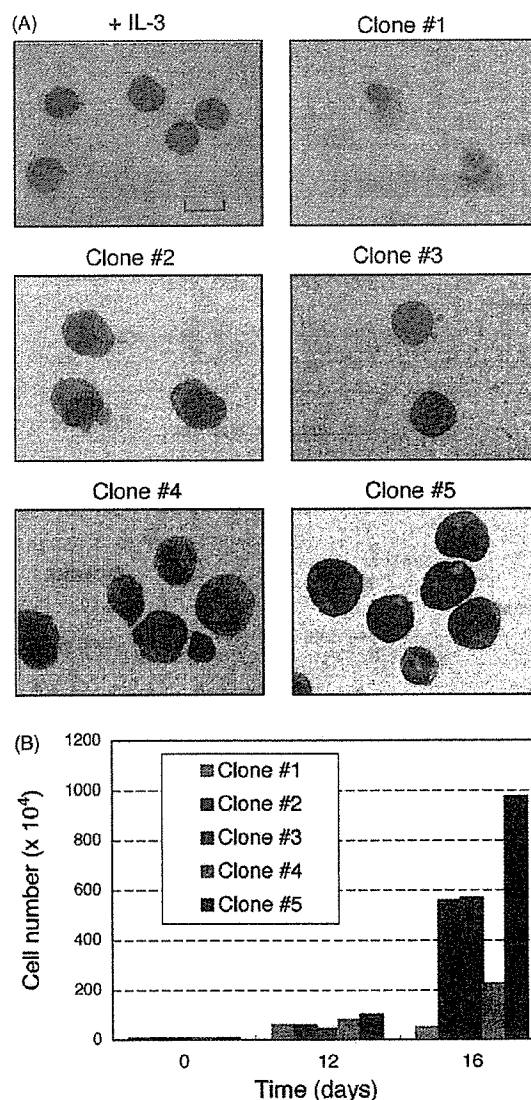


Fig. 1. Isolation of 32D-mCAT clones that proliferate in the presence of G-CSF. (A) 32D-mCAT cells cultured with IL-3 (+IL-3) as well as 32D-mCAT clones infected with the retroviral expression library (clones #1, #2, #3, #4, and #5) and cultured for 16 days with G-CSF were stained with Wright-Giemsa solutions and photographed. Scale bar, 100 μ m. (B) Proliferation of 32D-mCAT cells of clones #1 to #5 was determined by counting the cell number after culture with G-CSF for 12 or 16 days. The experiments were repeated twice, and both data were basically identical.

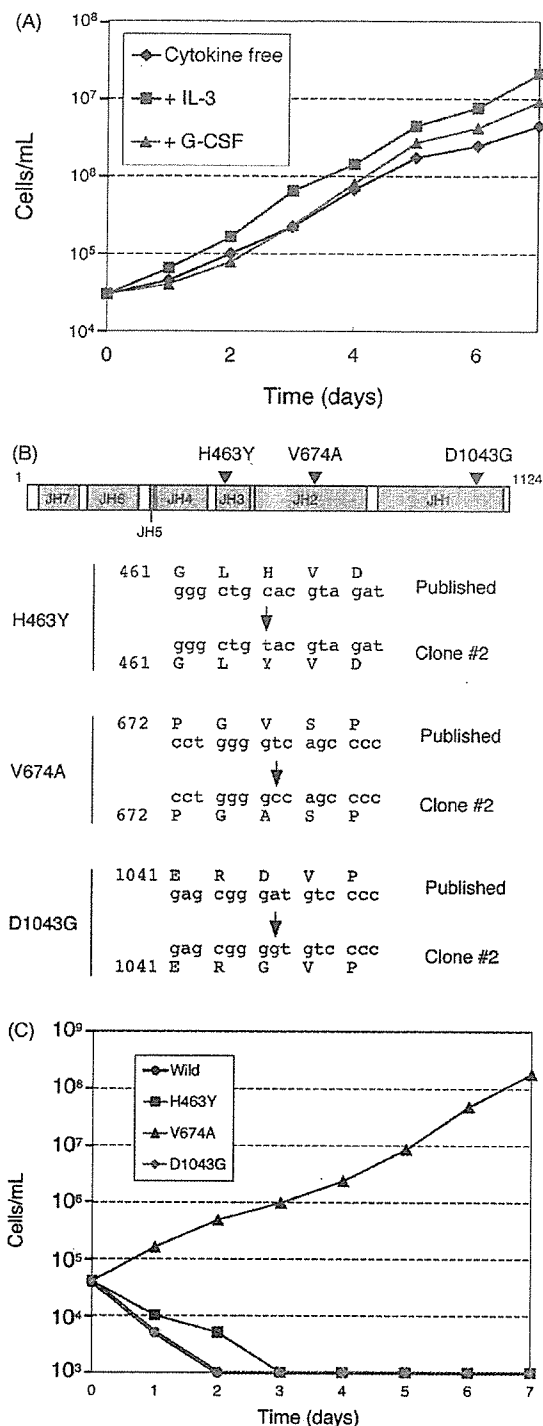


Fig. 2. Expression of JAK3(V674A) abrogates cytokine dependency of 32D-mCAT cell growth. (A) 32D-mCAT cells were infected with recombinant retroviruses containing the *JAK3* cDNA isolated from 32D-mCAT clone #2. The cells were then cultured in the absence or presence of IL-3 or G-CSF, and cell number was counted at the indicated times thereafter. The experiments were repeated twice, and both data were basically identical. (B) Schematic structure of human JAK3 showing the JH domains and the positions of amino

leukemic clones and helped to eliminate mRNA of normal bone marrow cells [10].

We selectively amplified full-length cDNAs from total RNA of the CD133⁺ blasts of the patient and ligated them into the retroviral vector pMX. We obtained a total of 8.7×10^6 colony-forming units of independent plasmid clones. To evaluate the quality of the library, we randomly selected 40 clones and examined the incorporated cDNAs. Thirty-four (85%) of the 40 clones contained inserts with an average size of 1.63 kbp, suggesting that the library was of sufficient complexity for the present study.

A recombinant retroviral library was generated from the plasmids and used to infect 32D-mCAT cells. A total of 2.75×10^6 independent retroviral clones was assayed with a total of 8×10^6 32D-mCAT cells. The cells were subsequently cultured for 16 days in the presence of G-CSF. Whereas proliferating 32D-mCAT cells manifested a high nucleus-to-cytoplasm ratio and basophilic cytoplasm (Fig. 1A, +IL-3), incubation with G-CSF for 16 days induced granulocytic differentiation in most of the cells (Fig. 1A, clone #1). Among the 32D-mCAT cells infected with retroviruses, however, we detected four cell clones (clones #2 to #5) that grew rapidly in the presence of G-CSF (Fig. 1A, data not shown). The cells in each clone were expanded and examined for their growth properties in the presence of G-CSF. The proliferation rate (Fig. 1B) as well as differentiation ability (Fig. 1A, data not shown) varied markedly among the clones. Genomic DNA was isolated from each clone and subjected to PCR in order to amplify the retroviral inserts. Nucleotide sequencing of the inserts revealed that those isolated from clones #2 and #3 were derived from human *JAK3* (GenBank accession number, NM_000215), whereas that from clone #5 was derived from *CSF3R* (accession number, NM_000760), which encodes the G-CSF receptor. PCR failed to detect a cDNA insert in cells of clone #4.

3.3. Identification of constitutively active JAK3(V674A)

JAK3 is a cytoplasmic tyrosine kinase that plays an important role in T cell development and function [14,15]. Given that constitutively active forms of JAK3 have not been detected previously, we focused on the *JAK3* cDNAs identified by retroviral expression screening. To confirm the transforming activity of the cDNAs, we ligated them into the pMX-ires-neo plasmid for generation of recombinant retroviruses. The retroviruses were used to infect 32D-mCAT cells, which were then subjected to selection with G418. The cells expressing the *JAK3* cDNA derived from clone #2 grew exponentially in the presence of G-CSF or IL-3 (Fig. 2A).

acid substitutions encoded by the PCR product obtained from 32D-mCAT clone #2. The corresponding nucleotide changes in the *JAK3* cDNA sequence are shown below. (C) Growth curves for 32D-mCAT cells infected with retroviruses encoding wild-type human JAK3 or the mutants JAK3(H463Y), JAK3(V674A), or JAK3(D1043G) and cultured without cytokine. The experiments were repeated twice, and both data were basically identical.

These cells also grew in the absence of cytokine, showing that the JAK3 derived from clone #2 abrogates cytokine dependency of 32Dc13 cells. Similar results were obtained with the JAK3 cDNA isolated from clone #3, and both of the JAK3 cDNAs (from clones #2 and #3) were identical on both ends (5'- and 3'-termini) (data not shown). We thus chose the JAK3 cDNA from clone #2 for further analysis.

Complete sequencing of the JAK3 cDNA from clone #2 revealed that it spans nucleotide positions 20–3460 of the published human JAK3 cDNA (NM_000215). However, four nucleotides within the isolated cDNA do not match those in the published sequence, and three of these unmatched nucleotides affect the corresponding amino acid sequence of JAK3. A nucleotide change at position 1446 (C to T) results in the replacement of a histidine residue at codon 463 with tyrosine; a nucleotide change at position 2080 (T to C) results in the replacement of valine at codon 674 with alanine; and a nucleotide change at position 3187 (A to G) results in the replacement of aspartic acid at codon 1043 with glycine (Fig. 2B).

To examine the role of these amino acid changes in the transforming activity of the JAK3 cDNA derived from clone #2, we introduced each mutation individually into the wild-type human JAK3 cDNA. Each mutant cDNA was ligated into pMX-ires-neo for the production of recombinant retroviruses, which were then used to infect 32D-mCAT cells. Culture of the infected cells in the absence of cytokine revealed that those expressing JAK3(V674A), but not those expressing JAK3(H463Y) or JAK3(D1043G), grew exponentially (Fig. 2C), demonstrating that the amino acid change from valine to alanine at position 674 is responsible for the transforming activity of JAK3 cDNA from clone #2.

Given that amino acid position 674 is within the JH2 region of JAK3, which negatively regulates the kinase activity of the enzyme [16], the V674A mutation might be expected to affect kinase activity and thereby to confer cytokine independence on 32D-mCAT cell growth. To examine this possibility, we immunoprecipitated JAK3 from 32D-mCAT cells expressing the wild-type or V674A form of the enzyme and subjected the precipitates to immunoblot analysis with an antibody to phosphotyrosine. JAK3(V674A) was constitutively phosphorylated irrespective of cell stimulation with IL-3, and the extent of its phosphorylation was markedly greater than that of wild-type JAK3 (Fig. 3A). Immunoblot analysis of the same samples with antibodies to JAK3 confirmed that the differences in the levels of JAK3 tyrosine phosphorylation were not due to differences in the levels of JAK3 expression. To confirm directly that the kinase activity of JAK3(V674A) is increased, we subjected immunoprecipitates of wild-type JAK3 or JAK3(V674A) to an in vitro kinase assay with a synthetic peptide substrate corresponding to the autophosphorylation site within the kinase domain of JAK3. The kinase activity of JAK3(V674A) was indeed found to be markedly greater than that of wild-type JAK3 and was not influenced by cell stimulation with IL-3 (Fig. 3B).

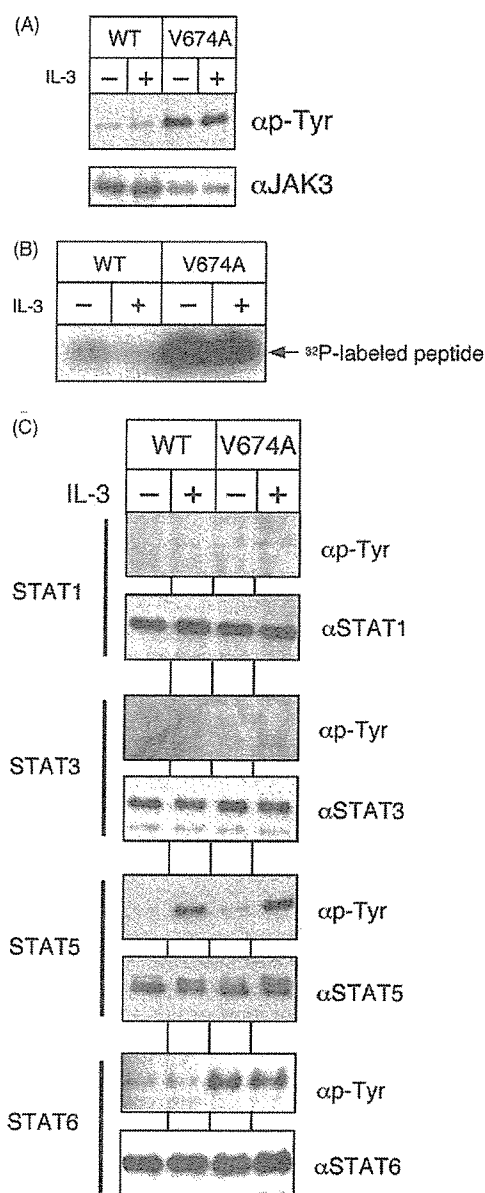


Fig. 3. Constitutive activation of JAK3(V674A). (A) 32D-mCAT cells expressing wild-type JAK3 (WT) or JAK3(V674A) were cultured in the absence of cytokine or FBS for 1 h and then incubated with (+) or without (–) IL-3 for 10 min. Cell lysates were subjected to immunoprecipitation with antibodies to JAK3. Half of the immunoprecipitates were subjected to immunoblot analysis with antibodies to phosphotyrosine (αp-Tyr) or to JAK3 (αJAK3). The experiments were repeated three times, and the data were basically identical. (B) The other half of the precipitates in (A) were subjected to an in vitro kinase assay with a synthetic peptide corresponding to the autophosphorylation site in the kinase domain of JAK3. (C) Cells treated as in (A) were subjected to immunoprecipitation with antibodies to STAT1, STAT3, STAT5, or STAT6, and the resulting precipitates were subjected to immunoblot analysis with antibodies to phosphotyrosine or to the corresponding STAT protein. The experiments were repeated three times, and the data were basically identical.

Signal transducer and activator of transcription (STAT) proteins are important signal transducers for JAK family members [17], and it is known that JAK3 can activate STAT5 and STAT6 in the context of IL-2- and IL-4-signaling systems, respectively. We thus examined the phosphorylation levels of various STATs in 32D-mCAT cells expressing wild-type JAK3 or JAK3(V674A). STAT1, STAT3, STAT5, and STAT6 were immunoprecipitated from such cells and subjected to immunoblot analysis with the antibody to phosphotyrosine. Only STAT6 manifested a higher level of phosphorylation in 32D-mCAT cells expressing JAK3(V674A) than in those expressing the wild-type kinase (Fig. 3C). In addition, STAT6 phosphorylation in the cells expressing JAK3(V674A) was not affected by IL-3, consistent with the lack of effect of this cytokine on the tyrosine phosphorylation of JAK3(V674A). Although STAT5 underwent tyrosine phosphorylation in response to IL-3 stimulation, the magnitude of this effect did not differ between cells expressing wild-type or V674A forms of JAK3 (Fig. 3C).

Finally, to examine whether the nucleotide substitution responsible for the V674A mutation of JAK3 was detectable in the RNA isolated from the AML patient and used for library construction, we amplified a region of *JAK3* cDNA surrounding codon 674 from the unamplified cDNA of the patient and determined its nucleotide sequence. Extensive sequencing failed to detect this nucleotide change, indicating that the activating mutation was artificially generated in the initial PCR step of library construction.

4. Discussion

We have constructed a retroviral cDNA expression library from a patient with AML. The complexity of the library was sufficient to represent most of the transcriptome of the CD133⁺ cells from which it was prepared. With the use of this retroviral library, we searched for cDNAs whose expression conferred on 32Dcl3 cells the ability to continue proliferating in the presence of G-CSF. Such cDNAs would be expected either (1) to inhibit the differentiation-inducing activity of G-CSF while supporting its mitogenic activity or (2) to possess marked transforming activity. Our screening resulted in the isolation of a cDNA for a constitutively active form of JAK3, which likely conforms to this second possibility.

An activating mutation of *JAK2* was recently identified in blood cells of individuals with myeloproliferative disorders (MPDs) with the exception of chronic myeloid leukemia [18–21]. This *JAK2* mutant, *JAK2*(V617F), possesses an increased kinase activity that is essential for the erythropoietin-independent growth of MPD cell colonies. These observations indicate that some MPD cases are caused by a constitutively active form of *JAK2*, and that reclassification of MPDs on the basis of transforming events may be required. Amino acid position 617 is localized within the JH2 (pseudokinase) domain of *JAK2*, which is thought to function as a negative regulatory region for kinase activity

[22]. Disruption of JH2 function through point mutations (in addition to that associated with MPDs) has thus been shown to activate *JAK2* [23]. The amino acid (valine-674) whose mutation is responsible for the constitutively active form of *JAK3* identified in the present study is also located in the JH2 domain of this kinase, emphasizing the importance of this domain in the negative regulation of *JAK3*. As far as we are aware, oncogenic forms of *JAK3* have not previously been described, and mutation of amino acid residues corresponding to valine-674 of *JAK3* have not been described for the other JAK family members.

Our inability to detect the corresponding mutation at nucleotide position 2080 in the original *JAK3* cDNA prepared from the AML patient indicates that it was introduced into the retroviral library by the initial PCR step of library construction. This mutation thus does not appear to be related to the molecular pathogenesis of AML in the patient. Similarly, we failed to detect the other two mutations (C1446T and A3187G of *JAK3*) in the original RNA of the patient (data not shown). This observation suggests that care is required to minimize the introduction of artificial mutations in the PCR step for this type of screening and that any mutations identified by such screening should be confirmed by analysis of the original sample. Nevertheless, our identification of an active form of *JAK3* demonstrates the potential efficacy of the retroviral library – 32Dcl3 cell screening system for the detection of hematopoietic oncogenes as well as provides insight into the regulation of *JAK3* activity.

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Gene Expression Profiles of CD133-positive Fractions Predict the Survival of Individuals with Acute Myeloid Leukemia

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Abstract. *Background:* The current classification of acute myeloid leukemia (AML) is based predominantly on the cytogenetic abnormalities and morphology of the malignant blasts and is not always helpful for optimization of the treatment strategy. *Gene expression profiles of AML blasts were obtained and a gene expression-based means of predicting the outcome of AML patients was developed. Materials and Methods:* CD133-positive hematopoietic stem cell-like fractions were purified from the bone marrow of 99 individuals with AML-related disorders and the expression profiles of ~33,000 human transcripts in these cells were characterized with the use of DNA microarray analysis. *Results:* The comparison of the expression data between individuals with short- or long-term survival by application of Cox's proportional hazard model led to the identification of four genes, whose expression patterns discriminated between the two groups. The gene expression-based stratification (GES) system, based on a combination of the karyotype approach and the risk index calculated from the expression levels of the four outcome predictor genes, was developed to separate the patients into subgroups with distinct prognoses. *Conclusion:* DNA microarray analysis of purified

fractions provides novel stratification schemes for AML based on the expression profiles of a handful of genes.

Acute myeloid leukemia (AML) is characterized by clonal growth of immature leukemic blasts in the bone marrow (BM). Although current chemotherapeutic regimens induce an initial complete remission in >70% of affected individuals, the long-term survival of such patients remains poor (5-year survival rate of <30%) (1). Given that leukemic blasts of individuals with AML differ in their abilities to differentiate into cells of the granulocyte, monocyte, erythrocyte, or megakaryocyte lineages, the French-American-British Cooperative Group (FAB) established a classification scheme for AML (M0 to M7) based on blast morphology and differentiation commitment (2). Although some FAB subtypes have proved to be related to good or poor prognosis, the clinical relevance of this classification scheme is complicated by other clinical parameters. A preceding history of myelodysplastic syndrome (MDS) or anticancer treatment, for example, is an important indicator of poor prognosis (3).

One of the most robust predictors of AML prognosis is the blast karyotype (1, 4). A good prognosis is thus predicted from the presence in leukemic clones of t(8;21), t(15;17), or inv(16) chromosomal rearrangements ("favorable" karyotype), whereas -7/7q-, 11q23, or more complex (affecting three or more chromosomes) abnormalities are indicative of a poor outcome ("adverse" karyotype). Other karyotypes are classified as "intermediate." The World Health Organization (WHO) has proposed a classification of AML that separates

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individuals with cytogenetic (or molecular) evidence of t(8;21), t(15;17), inv(16)/t(16;16), or 11q23 abnormalities into distinct subcategories (5). However, this classification is of little help for predicting the prognosis of AML patients with a normal karyotype, who constitute ~50% of the AML population. Given that AML patients with a normal karyotype are considered to be at intermediate risk, the corresponding leukemic blasts may harbor heterogeneous minor genomic rearrangements or mutations.

DNA microarray analysis has the potential to provide a stratification scheme for AML based on gene expression profiles and is able to predict the prognosis of each affected individual (6). To facilitate the development of such a genomics approach to the classification of human leukemias, a large-scale cell bank (the Blast Bank) was set up for the storage of CD133 (AC133)-positive hematopoietic stem cell (HSC)-like fractions purified from individuals with a wide range of leukemic disorders (7, 8). CD133 is specifically expressed on HSCs and hemangioblasts that are CD34⁺CD38⁻ (9, 10). There are at least two advantages to the use of such purified immature fractions for the characterization of AML. First, given that the proportion of leukemic blasts within BM varies substantially (20 to almost 100%) among patients and that leukemic blasts possess the ability to differentiate to various extents, a simple comparison of BM mononuclear cells (MNCs) among heterogeneous AML patients is likely to reveal a large number of changes in gene expression that reflect differences either in the percentage of blasts within BM, or in the differentiation ability of the blasts. Analysis of Blast Bank specimens should thus minimize such population-shift effects (7). Second, although leukemic cells in a given patient comprise a mixture of malignant clones at various levels of differentiation, they are thought to be generated from a small number of leukemic stem cells (LSCs), similar to the situation for normal hematopoiesis (11, 12). Characterization of the LSCs should provide insight into the molecular mechanisms of leukemogenesis. Given that such LSCs are exclusively CD34⁺CD38⁻, the Blast Bank may represent a diverse collection of LSC specimens. Cancer stem cells of malignant glioblastoma, but not their progenies, have also been shown to specifically express CD133 (13).

DNA microarray analysis was used here to characterize the expression profiles of ~44,000 probe sets in the CD133⁺ fractions of 99 adults with AML-related disorders. Statistical analyses of the resulting large data set provided the basis for a new classification of AML that facilitates prediction of the long-term prognosis of affected individuals.

Materials and Methods

Purification of CD133⁺ cells. Informed consent was obtained from each of the study subjects and the study was approved by the appropriate institutional review boards. Table I shows the clinical

characteristics of the study subjects. MNCs were isolated by density gradient centrifugation from BM aspirates of each patient and were subjected to chromatography on a miniMACS column (Miltenyi Biotec, Auburn, CA, USA) with magnetic bead-conjugated monoclonal antibodies to CD133 (AC133 MicroBeads; Miltenyi Biotec) as described previously (7). In most instances, the CD34^{hi}CD38^{lo} fraction constituted >90% of the eluate of the affinity column, as judged by flow cytometry.

Microarray analysis. Total RNA was extracted from the CD133⁺ cell preparations with the use of an RNeasy Mini column and RNase-free DNase (Qiagen, Valencia, CA, USA). It was then subjected to two rounds of amplification of mRNA with T7 RNA polymerase (14); the high fidelity of the amplification step has been demonstrated previously (15). The resulting cRNAs were labeled with biotin and subjected to hybridization with GeneChip HGU133 A&B microarrays with the GeneChip system (Affymetrix, Santa Clara, CA, USA). The fidelity of the microarray data was confirmed by quantitative RT-PCR analysis.

Method for real-time RT-PCR. Portions of non-amplified cDNA were subjected to -PCR with a QuantiTect SYBR Green PCR Kit (Qiagen). The amplification protocol comprised incubations at 94°C for 15 sec, 60°C for 30 sec and 72°C for 60 sec. Incorporation of the SYBR Green dye into the PCR products was monitored in real time with an ABI PRISM 7700 sequence detection system (PE Applied Biosystems, Foster City, CA, USA), thereby allowing determination of the threshold cycle (C_T) at which exponential amplification of products begins. The C_T values for cDNAs corresponding to *GAPDH* and the target genes were used to calculate the abundance of target gene mRNA relative to that of *GAPDH* mRNA. The primer sequences used for RT-PCR were as follows:

FGFR1:

Correlation coefficient between RT-PCR and microarray data=0.746.

Sense primer: 5'-CCACCAGAGTGATGTGTG
GTCTTT-3'

Antisense primer: 5'-CATCATCATGTACAGCTC
GTTGGT-3'

NRLN1

Correlation coefficient between RT-PCR and microarray data=0.638.

Sense primer: 5'-AATAGACGGAAAATGCTGCA
AGGT-3'

Antisense primer: 5'-TGAGGTGGTCTCTCAGTCTCC
AGT-3'

ZNF6

Correlation coefficient between RT-PCR and microarray data=0.779.

Sense primer: 5'-ATCTGGTGCAAAAACAGA
AAGGTG-3'

Antisense primer: 5'-GGCGGGTTTATGCAGTATTT
AACAG-3'

SCGF

Correlation coefficient between RT-PCR and microarray data=0.715.

Sense primer: 5'-TACTACGTCTGCGAGTTC
CCCTTC-3'

Antisense primer: 5'-GCCCTTCAAGGAAAAGA
CACTAAC-3'

HOXA9
Correlation coefficient between RT-PCR and microarray data=0.586.
Sense primer: 5'-CTCAGGTTGTTTATGAGG
GGAAAA-3'
Antisense primer: 5'-ATGAATCTATGCATCCCC
GAGAAC-3'

ANGPT1
Correlation coefficient between RT-PCR and microarray data=0.719.
Sense primer: 5'-GGCTGGGGAATGAGTTT
ATTTTGG-3'
Antisense primer: 5'-AAATCAGCACCGTGTAAG
ATCAGG-3'

241376_at (EST)
Correlation coefficient between RT-PCR and microarray data=0.672.
Sense primer: 5'-CAACTCGAAGCTCAAA
TACCCTCA-3'
Antisense primer: 5'-ACCGTTTATACACCAACGG
TCACA-3'

FLJ13063
Correlation coefficient between RT-PCR and microarray data=0.502.
Sense primer: 5'-AGAGTTCTGCTGTGT
CCTCTG-3'
Antisense primer: 5'-CAGGACAGTGCTGAAC
CAATG-3'

TSPAN-2
Correlation coefficient between RT-PCR and microarray data=0.479.
Sense primer: 5'-GCAGTTGAAAATTGTG
GGAAAGAG-3'
Antisense primer: 5'-CCCACACAACTAGGA
GAAGATG-3'

KIAA0830
Correlation coefficient between RT-PCR and microarray data=0.575.
Sense primer: 5'-TCCAGAGGCATCTGAGG
GAAGTAG-3'
Antisense primer: 5'-ATGGCCATGAAGTATGA
GATGGTG-3'

DJ79P11.1
Correlation coefficient between RT-PCR and microarray data=0.625.
Sense primer: 5'-CCATCCTGCAGTATAG
ATGGGACA-3'
Antisense primer: 5'-GATTCAGGGCATAAGGC
AAAACCTC-3'

POU4F1
Correlation coefficient between RT-PCR and microarray data=0.643.
Sense primer: 5'-ATGAACAAGCCTGAGC
TCTTCAAC-3'
Antisense primer: 5'-GAGAATTTTCATCCGCT
TCTGCTTC-3'

IGHM
Correlation coefficient between RT-PCR and microarray data=0.593.
Sense primer: 5'-CAGAAGAACATCGGAG
ACCAGAGA-3'

Antisense primer: 5'-AACAAGCGTATACACAG
CAAAGCA-3'

GAPDH
Sense primer: 5'-GTCAGTGGTGGACCT
GACCT-3'

Antisense primer: 5'-TGAGCTTGACAAAGTG
GTCG-3'

Statistical analysis. The mean expression intensity of the internal positive control probe sets (http://www.affymetrix.com/support/technical/mask_files.affx) was set to 500 units (U) in each hybridization and the fluorescence intensity of each test probe set was normalized accordingly. All normalized array data are available at the Gene Expression Omnibus web site (<http://www.ncbi.nlm.nih.gov/geo>) under the accession number GSE1427. All expression values were transformed to logarithms prior to statistical analyses. Hierarchical clustering of the data set and Student's *t*-test were performed with GeneSpring 7.0 software (Silicon Genetics, Redwood, CA, USA). Principal component analysis (16) and survival analyses were performed with the SAS software package (ver. 8.0.2).

Results

Purification of CD133⁺ HSC-like fractions. The number of CD133⁺ cells isolated from BM MNCs varied markedly among FAB subtypes of AML. For individuals whose leukemic blasts had a low differentiation capacity (FAB subtypes M0 to M2), for example, the blasts purified by CD133-based affinity chromatography constituted $\geq 30\%$ with MNCs. In contrast, the blasts purified from individuals with FAB subtypes M3 (characterized by prominent differentiation to the promyelocyte level) or M5 (differentiation to the promonocyte level) constituted $< 1\%$ of MNCs. In both the latter instances, large promyelocytes (M3) or large promonocytes (M5) constituted a major proportion of the BM MNCs, whereas the corresponding column eluates contained only medium-sized, agranular blasts with a high nucleus-to-cytoplasm ratio (Figure 1A).

The affinity column thus appeared to select for a minor population of cells at a highly immature level of differentiation. To verify that such cells in the column eluates did indeed represent leukemic clones, fluorescence *in situ* hybridization analysis was performed with the cells purified from several individuals. In the case of FAB subtype M3, described above, a t(15;17) was detected in 75% of the purified blasts (data not shown). Similarly, in an AML case characterized by 5q- or a case of chronic myelogenous leukemia characterized by t(9;22), the corresponding abnormality was detected in > 90 or 70.7% (8) of cells in the column eluates, respectively. These findings indicate that most of the cells in the column eluates were malignant clones, especially given the only moderate sensitivity of fluorescence *in situ* hybridization.

Gene expression profiles of the Blast Bank specimens. All the specimens were collected from the patients before initiation of chemotherapy. The expression profiles of $> 44,000$ probe

Table I. Clinical characteristic of the study subjects.

Blast Bank ID	Age (yr)	Gender	Disease	FAB subtype	Karyotype	Blast Bank ID	Age (yr)	Gender	Disease	FAB subtype	Karyotype
ID020	66	Female	AML evolved from MDS	M2	Normal	ID276	64	Male	de novo AML	M2	-7, >3
ID023	34	Female	de novo AML	M6	Others>=3	ID277	23	Female	de novo AML	M4	inv16
ID026	46	Male	de novo AML	M3	Others≤2	ID278	16	Female	de novo AML	M4	Normal
ID027	49	Male	AML evolved from MDS	M2	Normal	ID279	68	Male	de novo AML	M2	t(8;21)
ID028	62	Male	AML evolved from MDS	M2	Normal	ID280	68	Male	de novo AML	M1	-7
ID032	75	Male	RAEB		Others>=3	ID288	61	Male	de novo AML	M5	Normal
ID035	61	Male	de novo AML	M2	Others≤2	ID292	74	Male	de novo AML	M6	Normal
ID036	74	Male	de novo AML	M0	Normal	ID305	63	Male	de novo AML	M2	Normal
ID042	67	Female	AML evolved from MDS	M2	Others>=3	ID306	43	Male	de novo AML	M1	Normal
ID045	69	Male	RAEB		+8	ID310	75	Male	de novo AML	M0	Normal
ID046	84	Male	de novo AML	M2	-7	ID313	88	Female	AML evolved from MDS	M1	Others>=3
ID051	42	Male	RAEB		Others>=3	ID314	55	Female	de novo AML	M5	+8
ID054	84	Male	AML evolved from MDS	M2	-7	ID315	45	Female	RAEB		Others>=3
ID062	72	Male	de novo AML	M0	Normal	ID316	30	Male	de novo AML	M1	Others>=3
ID063	67	Female	RAEB		Others>=3	ID317	36	Male	de novo AML	M2	t(8;21)
ID066	73	Male	RAEB		t(8;21)	ID318	53	Male	de novo AML	M2	Normal
ID076	37	Male	de novo AML	M2	t(8;21)	ID319	47	Male	de novo AML	M6	Others>=3
ID077	55	Male	RAEB		+8	ID321	68	Female	de novo AML	M2	t(8;21)
ID083	64	Female	de novo AML	M4	Normal	ID325	49	Male	de novo AML	M2	Others>=3
ID087	66	Male	de novo AML	M5	Normal	ID326	68		de novo AML	M1	Normal
ID093	53	Female	de novo AML	M5	Others≤2	ID329	79	Female	de novo AML	M2	Normal
ID098	66	Male	AML evolved from MDS	M0	Normal	ID338	57	Male	RAEB		Normal
ID104	72	Male	de novo AML	M0	Others>=3	ID339	72	Female	de novo AML	M4	inv16
ID107	48	Female	de novo AML	M5	Others≤2	ID347	52	Male	de novo AML	M2	Others≤2
ID109	86	Male	de novo AML	M1	Normal	ID349	70	Male	de novo AML	M6	Others>=3
ID127	41	Male	de novo AML	M2	Normal	ID355	69	Male	de novo AML	M2	t(8;21)
ID139	50	Male	de novo AML	M1	Others≤2	ID362	59	Female	de novo AML	M2	Normal
ID142	38	Male	de novo AML	M2	t(8;21)	ID363	67	Male	de novo AML	M0	Others>=3
ID148	74	Male	AML evolved from MDS	M2	Normal	ID375	32	Male	de novo AML	M2	Normal
ID154	49	Male	RAEB		Normal	ID376	23	Male	de novo AML	M0	Others≤2
ID174	51	Male	de novo AML	M0	Normal	ID378	28	Female	de novo AML	M2	t(8;21)
ID180	47	Male	de novo AML	M4	Normal	ID379	62	Male	de novo AML	M2	Normal
ID183	50	Female	de novo AML	M2	t(8;21)	ID380	51	Female	de novo AML	M6	Others>=3
ID188	59	Male	de novo AML	M2	t(8;21)	ID382	49	Female	AML evolved from MDS	M2	Others>=3
ID195	61	Female	de novo AML	M2	t(8;21)	ID385	79	Male	RAEB		Normal
ID205	39	Male	de novo AML	M1	Normal	ID388	45	Male	de novo AML	M5	inv16
ID215	52	Male	RAEB		Others>=3	ID395	80	Male	AML evolved from MDS	M0	Normal
ID226	52	Male	de novo AML	M2	t(8;21)	ID402	63	Male	de novo AML	M1	Others≤2
ID227	29	Male	de novo AML	M2	Normal	ID409	72	Female	de novo AML	M1	Others≤2
ID234	68	Male	RAEB		Others≤2	ID410	67	Female	RAEB		Normal
ID239	48	Male	de novo AML	M2	t(8;21)	ID413	61	Female	de novo AML	M2	Normal
ID243	54	Female	RAEB		Others>=3	ID414	61	Male	de novo AML	M0	Normal
ID262	70	Female	de novo AML	M2	Others≤2	ID415	85	Female	de novo AML	M2	Others>=3
ID265	65	Male	de novo AML	M7	Normal	ID416	42	Female	de novo AML	M2	Normal
ID266	38	Male	de novo AML	M4	Normal	ID418	57	Female	de novo AML	M2	Normal
ID267	80	Male	de novo AML	M2	Normal	ID421	81	Female	RAEB		Normal
ID269	32	Female	de novo AML	M4	Others≤2	ID427	62	Male	de novo AML	M5	Normal
ID270	46	Female	de novo AML	M1	Others≤2	ID430	66	Female	RAEB		Others≤2
ID272	57	Female	de novo AML	M4	+8						
ID274	67	Male	de novo AML	M1	-7						
ID275	70	Male	AML evolved from MDS	M2	Others>=3						

AML=acute myeloid leukemia; MDS=myelodysplastic syndrome; FAB=French-American-British Cooperative Group; RAEB=refractory anemia with excess of blasts.

sets (corresponding to ~33,000 human genes) were then determined for Blast Bank specimens derived from 83 individuals with AML and 16 individuals in the advanced stage [refractory anemia with excess of blasts (RAEB)] of MDS. The clinical characteristics of the study subjects are provided in Table I.

For analysis of the expression data, the criterion that the expression level of a given probe set should receive the "Present" call (from Microarray Suite 5.0 software) in at least 30% ($n=30$) of the samples was applied in order to exclude transcriptionally-silent genes from the analysis. A total of 11,595 probe sets passed this selection window. Unsupervised two-way hierarchical clustering analysis (17) was then applied to the 99 patients based on the expression profiles of these 11,595 probe sets, generating a dendrogram of the subjects (Figure 1B). Six out of the 16 patients with RAEB (MDS) clustered together in the dendrogram, whereas the remaining ten RAEB patients were intermixed with the AML cases. Given that eleven of our AML subjects had experienced a previous MDS phase, it was not surprising that the gene expression profiles did not clearly separate RAEB from heterogeneous AML.

Patients with a normal karyotype or "other" abnormalities (<3 or ≥ 3) were widely distributed throughout the dendrogram, indicative of the highly heterogeneous character of their blasts. Previous unsupervised clustering analysis of the gene expression profiles of BM MNCs from individuals with AML separated the patients into subgroups that were strongly related to the FAB subtype (18, 19). However, such a relationship was not apparent in our analysis of CD133⁺ cells (data not shown), suggesting that changes in gene expression that accompany the differentiation of leukemic blasts within BM might greatly influence the overall gene expression profiles of MNCs.

Gene expression profiles linked to good prognosis. Among the 83 AML patients studied, 66 individuals were treated with standard chemotherapeutic regimens according to the protocols of the Japan Adult Leukaemia Study Group (JALSG). Kaplan-Meier analysis of these 66 cases revealed that, although the prognosis of individuals with the adverse karyotype was significantly worse than that of the other two karyotype groups (long-rank test, $p<0.001$), the prognosis of patients with the favorable karyotype did not differ from that of those with the intermediate one ($p=0.06$) (Figure 2). The poor prognosis of individuals with the adverse karyotype (5-year survival rate of ~10%) has been confirmed extensively (1, 4, 20), but the intermediate group apparently contains both patients with a curable disorder and those with an intractable one. It is, therefore, of clinical importance to be able to identify individuals with a *bona fide* good prognosis from among those with a favorable or intermediate karyotype.

We next compared the transcriptomes of individuals with these two karyotype designations. Among the 66 AML patients who underwent standard chemotherapy, the blasts of 16 and 39 individuals had the favorable and intermediate karyotypes, respectively. Probe sets that received the "Present" call in $\geq 10\%$ ($n=6$) of these 55 cases were selected first. The selected 17,724 probe sets were then screened for those whose expression differed significantly between the two karyotype groups (Student's *t*-test, $p<0.001$), resulting in the identification of 378 such sets. Application of principal component analysis reduced the number of principal gene expression patterns from 378 to three. On the basis of the calculated coordinates of these three principal expression patterns, the 55 samples were projected into a virtual three-dimensional space (Figure 3A). The samples with favorable and intermediate karyotypes were not clearly separated from each other, however.

We, therefore, examined whether it was possible to identify a gene expression profile, or "molecular signature," that was directly linked to the prognosis of leukemic patients. Among the 55 patients with favorable or intermediate karyotypes who underwent standard chemotherapy, 14 individuals survived for ≥ 755 days (long-term survivors) and eight individuals died within 365 days (short-term survivors) after the first chemotherapy. The application of the Student's *t*-test to the gene expression data for these 22 cases identified five probe sets, the expression level of which contrasted the two groups ($p<0.001$) (Table II). Principal component analysis and three-dimensional projection indicated that the molecular signature was clearly different between the two patient classes (Figure 3B).

Gene expression-based stratification scheme for AML. We next attempted to develop a gene expression-based stratification (GES) system for AML. Given that the adverse karyotype is a reliable indicator of poor prognosis, we tried to construct a GES scheme to isolate those individuals with good prognosis from other patients. The 66 AML patients who underwent standard chemotherapy were divided into training ($n=44$) and test ($n=22$) sets (Figure 4A). The former contained 37 patients with favorable or intermediate karyotypes, seven and six of whom were long-term or short-term survivors, respectively. To identify a prognosis-related molecular signature, the Student's *t*-test was first applied to the expression data for these latter 13 cases to isolate probe sets whose expression differed significantly ($p<0.01$) between the two groups. The expression profiles of the resulting 38 probe sets were then subjected to Cox's proportional-hazard regression analysis (21) ($p<0.01$) for the 13 selected individuals. Four independent probe sets (outcome predictor genes) were finally isolated, the expression profiles of which are shown in a dendrogram in Figure 3B. The risk index (RI)

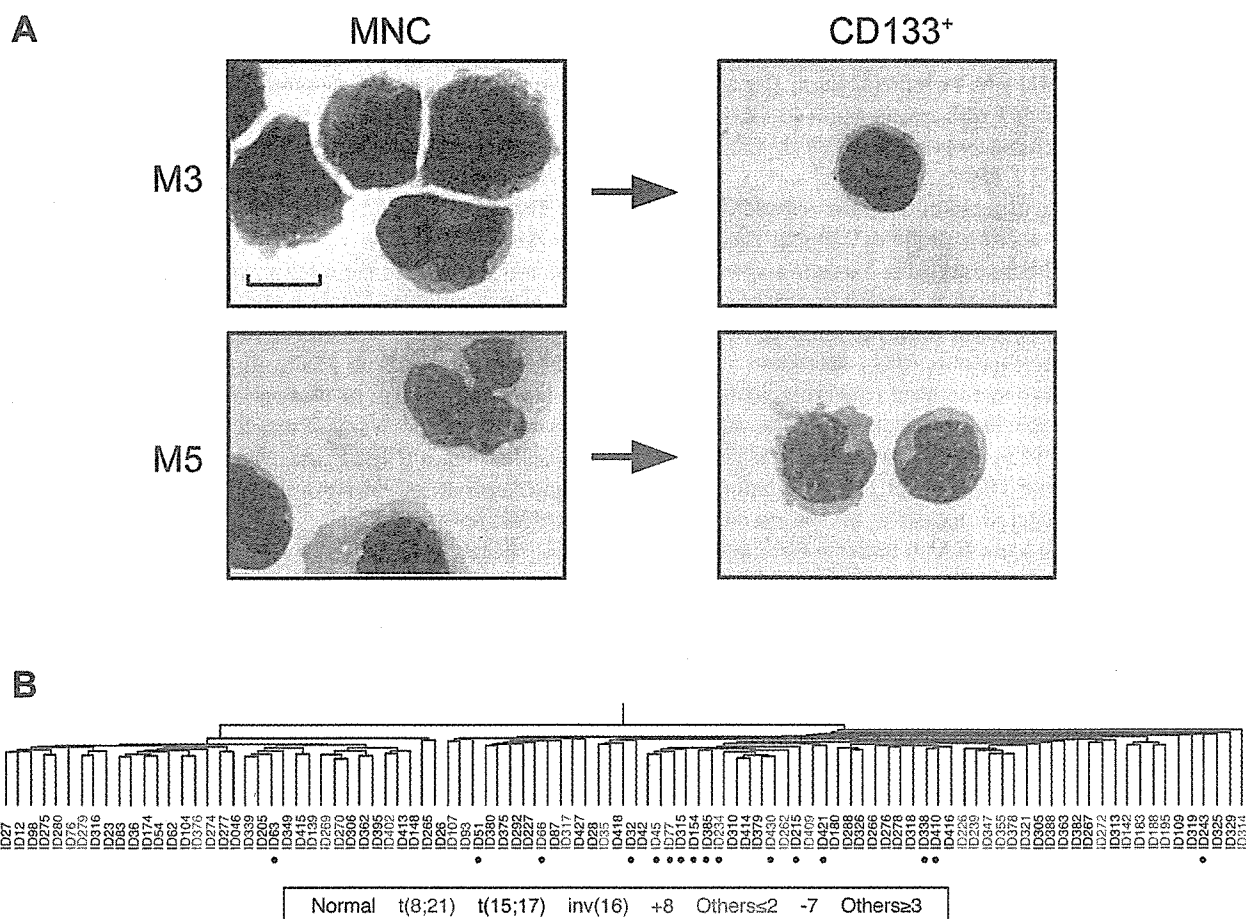


Figure 1. Clustering analysis of purified CD133⁺ fractions from individuals with acute myeloid leukemia (AML)-related disorders. A) The CD133⁺ fractions (right panels) were purified from bone marrow mononuclear cells (MNCs) (left panels) of AML patients of FAB subtypes M3 (upper panels) or M5 (lower panels). The cells were stained with Wright-Giemsa solution. Scale bar, 10 μ m. B) Unsupervised clustering of the study subjects based on the similarity in expression profiles of 11,595 probe sets. The karyotype of each patient is colored differentially. Individuals with refractory anemia with excess blasts are indicated by dots.

(22) was calculated for each patient based on the expression intensity and the parameter estimate (Table III) for each of these four probe sets.

A GES system for AML, based on a combination of this RI-based classification scheme and the karyotype-based scheme, is thus proposed (Figure 5A). AML patients with an adverse karyotype are classified as GES class III, whereas the other patients are classified as either GES class I (RI < -9.36) or GES class II (RI \geq -9.36) on the basis of the calculated RI for the four outcome predictor genes. To examine whether the GES system is able to select patients with good prognosis, Kaplan-Meier analysis was performed with the 44 individuals in the training set classified according either to the karyotype-based scheme (Figure 5B) or to our GES scheme (Figure 5C). The prognosis of the favorable group of the karyotype-based classification did not

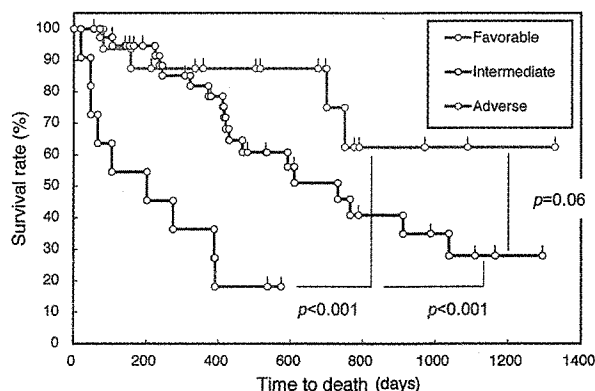


Figure 2. Long-term survival according to the keryotype-based stratification of the 66 acute myeloid leukemia patients treated with standard chemotherapy.

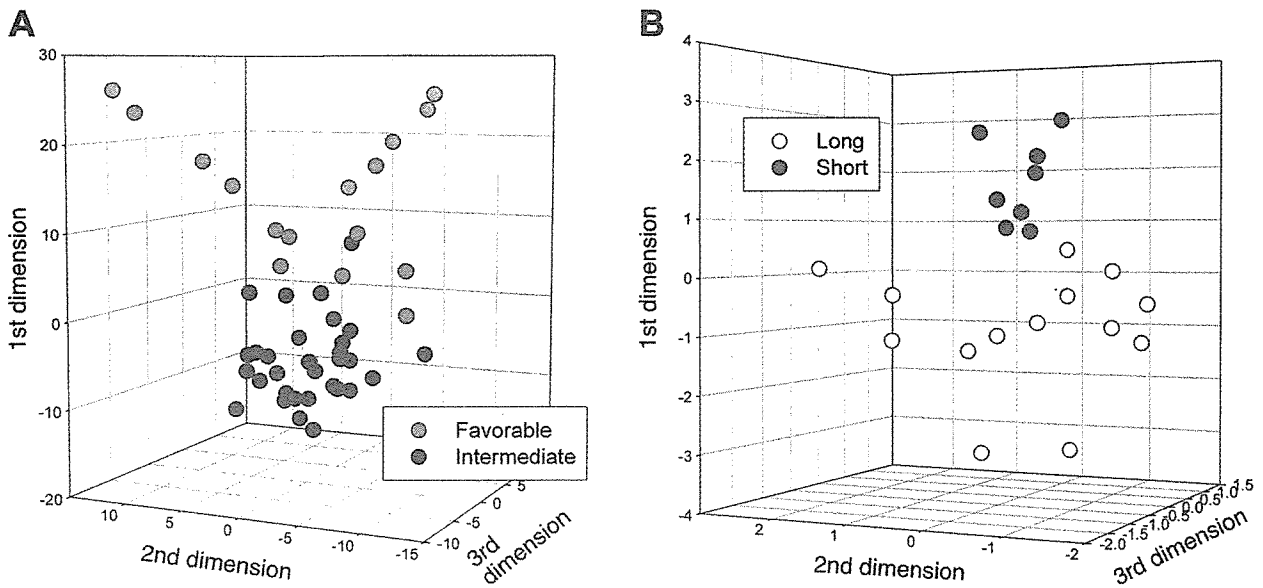


Figure 3. Comparison of gene expression profiles by principal component analysis. A) Three principal components were derived from the gene expression patterns of 378 probe sets that contrasted acute myeloid leukemia (AML) patients with favorable or intermediate karyotypes who underwent standard chemotherapy. Samples were projected into a virtual space based on the coordinates of the three components. B) Sample projection was similarly performed for five probe sets that contrasted the long-term and short-term survivors among the patients in A.

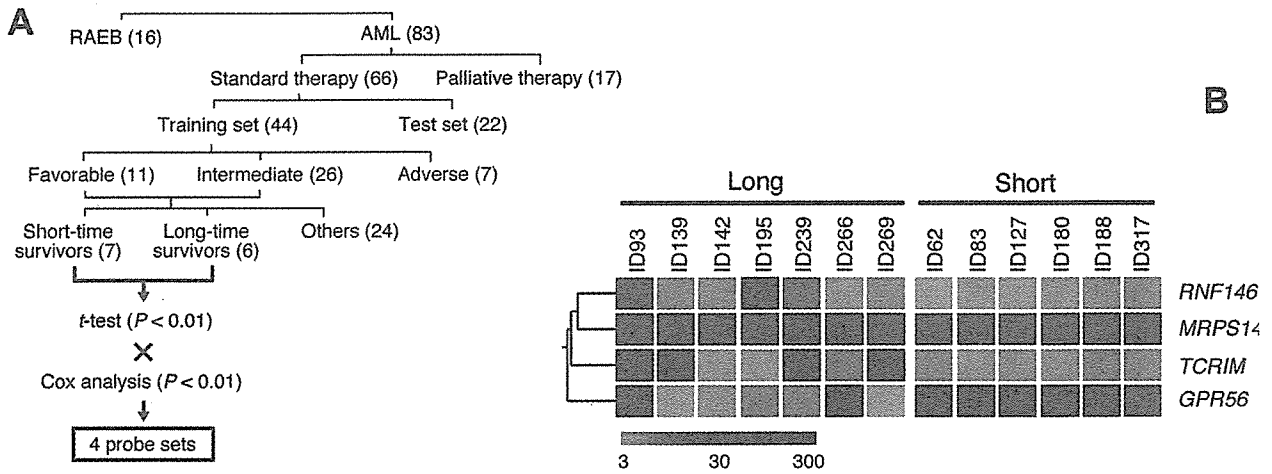


Figure 4. Isolation of probe sets linked to survival time. A) Approach adopted to identify four probe sets whose expression contrasted the long-term and short-term survivors in a training set of acute myeloid leukemia (AML) patients. The numbers in parentheses indicate the corresponding numbers of subjects. RAEB; refractory anemia with excess of blasts. B) Gene tree for the expression levels (color coded as indicated by the scale at the bottom) of the four human genes identified in A for CD133⁺ cells derived from seven long-term and six short-term survivors. Each row corresponds to a single gene (symbol shown at right) and each column to a different patient (Blast Bank ID shown at top).

differ significantly (log-rank test, $p=0.10$) from that of the intermediate group. In contrast, the GES system clearly separated the patients into three groups with distinct prognoses. Importantly, only GES class I contained the long-term survivors.

The efficacy of the GES system was then examined with the 22 individuals in the test set, who were also classified by either the karyotype-based (Figure 5D) or GES (Figure 5E) schemes. Again the former scheme failed to separate the patients into prognosis-related

Table II. Expression intensities of five probe sets linked to survival of 14 long-term and eight short-term survivors of acute myeloid leukemia.

Affymetrix designation	Gene symbol	GenBank accession no.	Long-term survivors													
			ID026	ID035	ID087	ID093	ID139	ID142	ID174	ID195	ID226	ID227	ID239	ID266	ID269	ID270
217147_s_at	TRIM	AJ240085	22.2	22.4	5.3	42.9	26.5	4.9	0.9	3.6	14.2	6.4	43.0	11.0	30.5	27.9
203098_at	CDYL	AL050164	6.9	15.5	8.4	18.8	49.9	10.2	20.5	6.0	42.5	11.7	60.8	20.3	23.5	30.8
226333_at	IL6R	AV700030	16.3	34.0	68.4	39.2	20.2	52.3	60.5	80.3	54.2	60.6	49.4	107.4	50.6	24.4
243023_at		N34402	1.7	10.2	6.9	4.5	31.3	20.8	16.7	7.1	2.0	8.7	2.9	3.0	29.3	2.4
200663_at	CD63	NM_001780	170.0	73.0	132.4	181.5	74.4	97.7	60.4	94.2	112.9	112.8	102.1	68.2	82.0	76.6

Affymetrix designation	Gene symbol	GenBank accession no.	Short-term survivors							
			ID027	ID062	ID083	ID127	ID180	ID188	ID288	ID317
217147_s_at	TRIM	AJ240085	1.3	7.3	1.1	2.9	2.7	5.0	1.5	4.1
203098_at	CDYL	AL050164	48.5	60.1	35.1	30.7	44.3	67.4	33.2	47.0
226333_at	IL6R	AV700030	177.2	146.2	127.5	72.5	120.7	85.7	61.8	70.1
243023_at		N34402	28.8	33.5	33.4	23.4	24.5	10.8	19.7	19.5
200663_at	CD63	NM_001780	57.9	65.9	67.7	52.7	61.8	49.4	62.5	76.6

groups. In contrast, the GES scheme efficiently isolated the long-term survivors.

Discussion

In the present study, we attempted to develop a new classification scheme for AML based on the gene expression profiles of purified highly immature leukemic cells. Microarray analysis has proved effective for the prediction of prognosis in other hematological malignancies including diffuse large B cell lymphoma (DLBCL) (23-25) and acute lymphoblastic leukemia (ALL) (26, 27). The clinical specimens for these latter two disorders, however, appear to be more homogeneous than are those for AML. Lymphoma clones constitute most of the cell population within the affected lymph nodes of individuals with DLBCL and most ALL clones remain at an early precursor stage of the B or T cell lineage. The clinical specimens derived from individuals with DLBCL or ALL are thus probably representative of the corresponding malignant clones.

Caution is warranted, however, in the interpretation of microarray data for certain blood cell disorders. In Hodgkin's lymphoma, for example, the malignant cells constitute only a small proportion of cells within lymph nodes, most of which are inflammatory cells and normal lymphocytes. Hodgkin's lymphoma cells have thus been isolated with the use of laser-capture microdissection before microarray analysis (28, 29). Similar caution is required with AML, given the heterogeneity in both the proportion and differentiation ability of the blasts within BM. Indeed, our data indicated that so-called "leukemic blasts" are highly heterogeneous with regard to their differentiation ability, even within the BM of a single patient (Figure 1A), consistent with the notion of the presence of multiple types of LSC (12).

In the present study, statistical analysis of the gene expression profiles of CD133⁺ HSC-like fractions resulted in the isolation of a very small number of outcome-predictor genes, including those for G protein-coupled receptor 56 (GPR56; GenBank accession number, NM_005682), ring finger protein 146 (RNF146; NM_030963), mitochondrial ribosomal protein S14 (MRPS14; NM_022100) and T cell receptor-interacting protein (TCRIM; NM_016388). Their predicted amino acid sequences indicate that GPR56 belongs to the family of cell surface proteins with seven transmembrane domains and that RNF146 participates in protein ubiquitination. MRPS14 is a component of the 28S subunit of mitochondrial ribosomes and contributes to protein synthesis in this organelle (30). TCRIM is a transmembrane protein that undergoes phosphorylation by Src family kinases (31). TCRIM potentially functions as a scaffold protein that recruits a variety of signaling proteins via its Src homology 2 (SH2) domain.