Molecular Hematology Oncology

research paper

haematologica 2002; 87:569-577
http://www.haematologica.ws/2002_06/569-.htm

Overexpression of translocation-associated fusion genes of *FGFR1*, *MYC*, *NPM1*, and *DEK*, but absence of the translocations in acute myeloid leukemia. A microarray analysis

MARCELO L. LARRAMENDY,*° TARJA NIINI,* ERKKI ELONEN,# BÁLINT NAGY,*@ JUHA OLLILA,^ MAUNO VIHINEN,§‡ SAKARI KNUUTILA*

Correspondence: Sakari Knuutila, PhD, Department of Medical Genetics, POB 21 (Haartmaninkatu 3, 4th flr), FIN-00014 University of Helsinki, Helsinki, Finland. Phone: international +359.191.26527. Fax: international +359.191.26788. E-mail: sakari.knuutila@helsinki.fi

Background and Objectives. Translocation-associated gene fusions are well recognized in acute myeloid leukemia. Other molecular genetic changes are less well known. The novel cDNA technology has opened the avenue to large-scale gene expression analysis. Our aim was to perform cDNA microarray analysis of acute myeloid leukemia (AML).

Design and Methods. We performed cDNA microarray analysis using the Clontech hematology filter (containing 406 genes) on 15 patients to study gene expression profiling in AML. As reference, we used whole bone marrow from 5 healthy donors.

Results. Our results revealed 50 differentially expressed genes in at least 3 out of 15 patients. Twenty-two genes were upregulated (ratio ≥ 4), whereas 28 genes were downregulated (ratio ≤ 0.25). All but one of the 13 genes tested by real-time polymerase chain reaction (PCR) showed the same expression profiles. Among the overexpressed genes, several were those earlier associated with chromosomal translocations and gene fusions. These genes were *FGFR1, MYC, NPM1, DEC,* and *BCL2*. The expression of two upregulated genes, *HOXA4* and *CSF1R*, was significantly higher in patients with a white blood cell count higher than 30×10^9/L cells. In patients whose white blood cell count was higher than 100×10^9/L cells, both *CLC* and *GRN* were significantly underexpressed, whereas *HOXA4* and *DAPK1* were overexpressed. *FGFR1* and *CAMLG* were more frequently significantly overexpressed in patients with CD56 immunophenoytpe.

Interpretation and Conclusions. Clinical and prognostic significance of differential gene expression should be studied with a larger series of patients by using other techniques, such as quantitative real-time PCR.

Key words: cDNA array, acute myeloid leukemia, gene expression, fusion genes.

*Departments of Pathology and Medical Genetics, Haartman Institute and Helsinki University Central Hospital, University of Helsinki, Helsinki, Finland; °Laboratorio de Citogenética y Cátedra de Citología, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, La Plata, Argentina; #Department of Medicine, Helsinki University Central Hospital, Helsinki, Finland; @Genetic Laboratory, 1st Dept. of Obstetrics and Gynecology, Faculty of Medicine, Semmelweis University, Budapest, Hungary; ^Division of Biochemistry, Department of Biosciences, University of Helsinki, Helsinki, Finland; §Institute of Medical Technology, University of Tampere, Tampere, Finland; ‡Tampere University Hospital, Tampere, Finland

It is well-documented that translocation-associated gene fusion plays an essential role in leukemogenesis. Many of the chimeric genes encode protein kinases, transcription factors and/or their receptors and are able to control both cell-cycle progression and apoptosis. *FGFR1-FIM* [t(8;13)(p11;q12)], *FOP-FGFR1* [t(6;8) (q27;p11)], *ZNF198-FGFR1* [t(8;13)(p11;q11-12)], *DEK-CAN* [t(6;9)(p23;q34)], *PML-RARα* [t(15;17) (q21;q22)] and *PLZF-RARα* [t(11;17)(q23;q22)] can be included among them.

However, the biochemical pathway and the target genes activated or suppressed by the chimeric genes are largely unknown.

Since 1995, when the cDNA microarray methodology was developed, it has become possible to study the gene expression pattern of hundreds to thousands of genes relevant for tumor development and progression within a single hybridization experiment.[1,2]

The quantitative information obtained from gene expression profiles with the cDNA microarray methodology provides not only new diagnostic and prognostic parameters for cancer patients but also clues for cancer therapy improvement.[1,3-7]

In the present study we analyzed the gene expression profile from a series of patients with acute myeloid leukemia (AML) at diagnosis using cDNA microarray and clustering analyses to discover the pattern of under- and overexpressed genes. Furthermore, the expression of several up- and downregulated genes was confirmed by quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR).

Design and Methods

Patients and bone marrow samples

Bone marrow cells from fifteen patients (#1-15) were studied using cDNA arrays (Table 1). Real-time PCR of 13 genes was conducted on the samples from these 15 patients to confirm the cDNA array results and on samples from a further 12 AML patients (#16-27, Table 1). Five bone marrow samples from healthy donors obtained during bone marrow transplantation were used as reference for the expression comparison. The patients were diagnosed and treated at the Department of Medicine, Helsinki University Central Hospital. Table 1 shows their clinical and laboratory data.

Aliquots of all bone marrow aspirates were diluted 1:10 in RNA/DNA stabilization reagent for blood/bone marrow (Boehringer Mannheim GmbH, Mannheim, Germany) containing guanidinium thiocyanate and in Triton® X-100 (Boehringer) for simultaneous cell lysis and stabilization of nucleic acids according to the supplier's instructions. Lysates were stored frozen at –70°C for 2 weeks to 8 months until RNA extraction.

RNA isolation

Total nucleic acids were isolated from stabilized bone marrow samples using the mRNA isolation kit for blood/bone marrow (Boehringer) based on magnetic glass particle technology following the instructions of the kit's supplier. After elution of nucleic acids from glass beads, the DNA was removed according to Clontech's (Palo Alto, USA) protocol for DNAse treatment of total RNA with the exception that precipitation was carried out at –70°C overnight. The quality and integrity of the RNA were checked using 1% agarose horizontal gel electrophoresis before cDNA arrays so that only non-degradated RNAs were used for gene expression profiling analysis.

cDNA microarray hybridization

The Atlas™ Human Hematology/Immunology cDNA expression array filters from Clontech Laboratories Inc. (Palo Alto, CA, USA) were used. Each filter contains 10 ng/duplicate spots for 406 known and sequence-verified human cDNAs, nine housekeeping control cDNAs, and three negative controls (M 13mp18(+) strand DNA, α-DNA and pUC 18) immobilized on a nylon membrane (for information about genes see *http://www.clontech.com*). The total RNA (3.5-4 μg) from 15 samples (patients 1-15, Table 1) was converted into cDNA and labeled with [α-^{33}P]dATP (Amersham, Buckinghamshire, UK; sp.act. 3,000 Ci/mmol) using the Clontech cDNA array labeling kit (Clontech) following the filter supplier's recommendations and instructions. After overnight hybridization at 68°C in the ExpressHyb solution (Clontech), filters were washed following the manufacturer's instructions. Then the filters were exposed to a high-resolution imaging plate (BAS-MP 2040S; Fuji Photo Film Co., Kanagawa, Japan) for 4-6 days and scanned with a phosphorimager (Bio-Imaging Analyzer, BAS-2500; Fuji) to obtain high-resolution (16 bit) images in TIFF format.

Hybridization controls and cut-off values

Four RNA samples were hybridized twice, each in a different filter and in a different experiment in order to confirm the reproducibility of the results. The two independent hybridization images obtained from the same RNA sample were then compared and plotted to each other. Under the hybridization conditions in our laboratory, the differences in the same sample between any two hybridizations were smaller than 2% with ratios at 1.5 and 0.65 for over- and underexpression, respectively, and smaller than 1% with respective ratio values of 2.0 and 0.5. Accordingly, the threshold ratio values were raised to 4.0 for overexpression and 0.25 for underexpression.

cDNA array and cluster analyses

Filter images were imported into the AtlasImage 1.5 software (Clontech), and hybridization spots were aligned digitally. Local background was subtracted at each gene location. To minimize possible differences either in the amount of RNA hybridized or variations in the hybridization efficiency among samples/filters, the intensities of the hybridization signals were normalized so that the sums of intensities among patients were equal. The intensity ratio value was quantified for each gene, reflecting the relative expression of the gene in each patient compared with its expression in the reference. Ratios were then $\log_2$ transformed and only the genes in which the expression ratio was significantly altered in at least five patients were taken to further analysis. Hierarchical clustering was applied to both axes with software developed by Michael Eisen (Stanford University, CA, USA; *http://rana.lbl.gov*) using uncentered correlation as a similarity metric. Results were visualized with the TreeView software (*Michael Eisen, Stanford University*). For statistical analysis, the Student's t-test was employed using the SPSS statistical software package (SPSS Inc., Chicago, IL, USA) to identify the subsets of genes that may be associated with clinical and chromosomal features in patients with AML. The chosen level of significance was 0.05, unless otherwise noted.

Table 1. Clinical, hematologic and karyotype data of the 27 patients diagnosed with acute myeloid leukemia (AML).

Patient No. (sex/age)	*Patient code*	*Date of diagnosis*	*FAB*	*Immunophenotype*	*Extra-medullary infiltration*	*Blasts in BM (%)*	*WBC (x 10^9/L)*	*Karyotype*	*Duration of first CR (days)*	*Survival (days)*	*Last status*
1 (F, 66)	GA00-9676	04-02-00	M2	CD13$^+$, CD33$^-$, CD34$^-$, CD56$^+$	Gingiva	90	206.0	46,XX	80	180	Dead
2 (M, 53)	GA99-125	29-12-99	M2	CD13$^+$, CD14$^-$, CD33$^+$, CD34$^+$, CD56$^+$, CD64$^+$	-	80	8.6	45,X,-Y,t(8;21)(q22;q22)	147	218	Dead
3 (M, 65)	GA00-10111	09-06-00	M4	CD33$^+$, CD34$^-$, CD56$^+$, CD64$^+$	Gingiva	80	37.5	47,XY,+8	226+	264+	Alive
4 (F, 72)	GA00-10196	29-06-00	M1	CD13$^+$ CD14$^-$, CD33$^+$, CD56$^+$, CD64$^-$, CD117$^+$	Lymph nodes	90	158.0	46,XX	201+	230+	Alive
5 (M, 50)	GA00-9955	19-04-00	M5	CD13$^+$, CD14$^+$, CD20$^+$, CD33$^+$, CD34$^-$, CD56$^+$,CD64$^+$	Lymph nodes, gingiva	95	58.5	46,XY	204+	315+	BMT, Alive
6 (M, 44)	GA00-10515	12-09-00	M2	CD13$^+$, CD33$^+$, CD34$^-$, CD56$^-$, CD64$^-$, CD117$^+$	Skin	45	2.7	46,XY,inv(11)(p15jq23)	140+	169+	Alive
7 (F, 35)	GA00-10463	01-09-00	M3	CD13$^+$, CD33$^+$, CD34$^-$, CD56$^-$, CD117$^+$	-	90	1.2	46,XX,t(15;17)(q22;q11-12)	140+	180+	Alive
8 (F, 26)	GA00-9711	14-02-00	M3	CD13$^+$, CD33$^+$,CD34$^-$	-	75	2.4	46,XX	338+	380+	Alive
9 (F, 51)	GA-10759	03-11-00	M2	CD13$^+$, CD33$^+$, CD34$^-$, CD56-, CD64$^-$, CD117$^+$	-	70	3.4	MFISH:46,XX,t(3;10),t(7;15)	63+	117+	Alive
10 (M, 61)	GA99-51	08-12-99	M4	CD13$^+$, CD14$^+$, CD33$^+$, CD34$^+$, CD56$^+$, CD64$^+$	-	90	80.2	46,XY	315	435+	AML, Alive
11 (F, 68)	GA99-72	13-12-99	M5	CD13$^+$, CD14$^+$, CD33$^+$, CD34$^-$, CD56$^+$,CD64$^+$	-	50	100.6	46,XX	0	24	Dead
12 (M, 23)	GA00-697	20-08-99	M2	CD13$^+$, CD33$^+$, CD34$^+$, CD56$^+$, CD64$^+$	Lymph nodes	65	41.4	45,X,-Y,t(8;21)	527+	558+	Alive
13 (F, 51)	GA00-9655	02-02-00	M4	CD13$^+$,CD14$^+$, CD33$^+$, CD34$^-$, CD56$^-$, CD64$^+$	-	35	10.4	46,XX	329+	392+	BMT, Alive
14 (F, 67)	GA00-9855	24-03-00	M0	CD7$^+$, CD13$^+$, CD33$^+$, CD34$^-$, CD56$^+$, CD64$^-$, CD117$^+$	-	70	1.3	45,XX,-3,del(5)(q821), add(7)cq32),-10, del(12)cq22), add(18)(p11),+2mar	295+	341+	Alive
15 (M, 48)	GA99-133	30-12-99	M2	CD13$^+$, CD33$^+$, CD34$^+$, CD56$^-$	-	35	5.5	46,XY	257+	426+	BMT, Alive
16 (F, 50)	990843	05-06-99	M2	CD13$^+$, CD33$^+$, CD34$^+$, CD56$^-$, CD4$^+$	-	50	105.2	46,XX,t(8;21)(q22;q22)	593+	634+	Alive
17 (M, 21)	981618	02-11-98	M4	CD7$^+$,CD13$^+$, CD14$^-$, CD33$^+$, CD34$^+$, CD56$^-$, CD64$^+$	Lymph nodes, gingiva	90	6.9	46,XY	339	521	BMT, Dead
18 (M, 66)	990423	12-03-99	M4	CD13$^+$,CD14$^+$, CD33$^+$, CD34$^+$, CD56$^-$, CD64$^+$	Gingiva	50	85.8	46,XY,inv(16)(p13q22)	152	179	Dead
19 (F, 30)	981328	18-09-98	M1	CD13$^+$, CD33$^+$, CD34$^+$, CD56$^-$, CD64$^-$	-	91	43.3	46,XX	863+	894+	BMT, Alive
20 (F, 61)	980348	13-03-98	M2	CD13$^-$, CD33$^+$, CD34$^-$, CD56$^+$	-	75	80.9	46,XX	1020+	1083+	Alive
21 (F, 52)	981237	30-01-98	M5	CD13$^+$, CD14$^+$, CD33$^+$, CD34$^-$, CD56$^+$, CD64$^+$	Lymph nodes, gingiva	85	90.4	46,XX	246	286	BMT, Dead
22 (F, 46)	991204	18-08-99	M4	CD7$^+$, CD13$^+$, CD19$^+$, CD33$^+$, CD34$^+$, CD56-, CD64$^+$	-	50	185.7	46,XX	0	64	Dead
23 (M, 51)	991041	16-07-99	M2	CD13$^+$, CD19$^+$, CD33$^+$, CD34$^+$, CD56$^+$, CD64$^+$	-	40	31.0	46,XY,t(8;21)	0	264	Dead
24 (F, 65)	990692	05-05-99	M2	CD13$^+$, CD33$^+$, CD34$^+$, CD56-, CD64$^+$	-	40	2.2	MFISH:45,XX,dup(1)(p?),del(5q),t(14;19),-17,inc	0	131+	AML, Alive
25 (M, 67)	991338	09-09-99	M2	CD13$^+$, CD14-, CD33$^+$, CD34-, CD56-, CD64-	-	60	75.1	46,XY	502+	538+	Alive
26 (M, 52)	991487	11-10-99	M2	CD13$^+$, CD33$^+$, CD34$^+$	-	80	4.6	46,X,-Y,t(8;21)(q22;q22)	425+	506+	BMT, Alive
27 (M, 60)	GA00-9759	03-03-00	M1	CD2$^+$, CD7$^+$, CD13$^+$, CD33$^+$, CD34$^+$, CD117$^+$	Lymph nodes, liver, spleen	90	132.0	46,XY	78	362+	BMT, Alive

BM: Bone marrow; CR: Complete remission; BMT: Bone marrow transplantation from an HLA-identical sibling donor (n=4) or from a matched unrelated donor (n=4); WBC: White blood cell count. + Patients still in complete remission or alive at the last follow-up (28-02-01).

Table 2. Sequence of primers used and the corresponding T_m values.

Gene	*Forward primer*	*T_m (°C)*	*Reverse primer*	*T_m (°C)*
IL-8RA	AgAgggTTTggAAgCCAgT	62.6	TAAgggCTgCTTgTCTCgTT	63.7
FY	AgTgCCCTCTTCAgCATTgT	63.8	gCTgAgCCATACCAgACACA	64.0
MYC	ggCAAAAggTCAgAgTCTgg	56.6	gTgCATTTTCggTTgTTgC	56.5
DEK	gTgggTCAgTTCAgTggC	54.6	AggACATTTggTTCgCTTAg	53.8
LTB	AggAgCCACTTCTCTggtgA	64.0	CTCTggCAgCTTCTgAAACC	64.0
MS4A2	gCAgCAACggAgAAAAACTC	63.7	TgACAAAATgCCCAAgAACA	64.0
CSF1R	CACTAAgCTCgCAATCCCTC	63.7	CTCTACCACCCggAAgAACA	63.9
CBL	ATgTCCCAAAgCCACCTg	56.3	gCAggACCACTACCTTgCT	55.5
DAPK1	CAgTgTTgTTgCTCTAggAAg	57.2	gggACTgCCACAAATgATgAg	58.2
BAX	TgCTTCAgggTTTCATCCAg	56.9	ggCggCAATCATCCTCTg	57.3
BCL2L1	ATggCAgCAgTAAAgCAAgC	57.6	CggAAgAgTTCATTCACTACCTgT	57.3
MCL1	gATgATCCATgTTTTCAgCgAC	56.4	CTCCACAAACCCATCCCAg	57.7
HPRT1	ggCAgTATAATCCAAAGATggTCAA	65.5	gTCTggCTTATATCCAACACTTCgT	65.0

Real-time quantitative reverse transcription polymerase chain reaction (QRT-PCR)

The genes for real-time PCR were selected on the basis of the cDNA results to confirm some of the most interesting findings of up- and downregulated genes. The 1st Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostic GmbH, Mannheim, Germany) was used to obtain cDNAs from all 27 AML bone marrow samples and from two healthy donors (references), applying 0.5 μg total RNA from the samples of each patient. Gene-specific PCR primer sequence information corresponding to the PCR targets on the Atlas™ Human Hematology/Immunology cDNA expression array filters (Clontech) were acquired from TIB MOLBIOL Syntheselabor (Berlin, Germany). Primers for the following genes were used: *IL8RA, FY, MYC, DEK, LTB, MS4A2, CSF1R, CBL, DAPK1, BAX, BCL2L1, MCL1*, and *HPRT1* (Table 2). PCRs were performed in the LightCycler thermal cycler (Roche). Standard curves were obtained by using serial dilutions of β-globulin gene (DNA Control kit; Roche) according to the supplier's instructions in each LightCycler run. The 10μL PCR reaction consisted of 1 μL of DNA Master SYBR Green I mix (LightCycler-FastStart DNA Master SYBR Green I kit, Roche; containing Taq DNA polymerase, dNTP, $MgCl_2$, and SYBR Green I dye), 1 μL of cDNA, additional $MgCl_2$ up to 2 mmol and 4-10 pmol of each primer. The amplification program included an initial denaturation at 95°C with 8 min hold, followed by 45 cycles with denaturation at 95°C with 10s hold, annealing at 55-66°C with 5s hold, and elongation at 72°C with 20s hold. Amplifications were followed by melting curve analysis using the program, one cycle at 95°C with 0s hold, 65°C with 15s hold, and 95°C with 0s hold at the acquisition step mode. A negative control without cDNA template was run simultaneously with every assay. The PCR from each cDNA sample was run in duplicate. The concentration of each gene product was determined on the basis of a kinetic approach using the LightCycler software (Roche). Results are expressed as ratio values of expression obtained from comparison of the concentration values of genes from each patient with the average value of concentration from two independent reference samples (normal bone marrow controls). Student's *t*-test was used to compare the mean values of gene expression using the SPSS statistical software package (SPSS Inc.) and the chosen level of significance was 0.05, unless otherwise noted.

Results

Out of the 418 targets present in the microarray filter membrane, the analysis revealed 50 genes differentially expressed in at least three out of the 15 AML samples. Of the differentially expressed genes, 22 were upregulated (ratio ≥ 4) and 28 were downregulated (ratio ≤ 0.25) (Table 3, Figure 1). Table 3 shows the frequencies of the up- and downregulated genes. The gene expression profiles for all the patients are shown in Figure 1, in which hierarchical clustering was used to group the genes on the basis of the similarity of their expression patterns. Several clusters of co-ordinately expressed genes were defined. The same clustering method was used to group the 15 samples on the basis of the similarities in their expression of these genes. The algorithm segregated two main groups of AML patients consisting of 4 (#3, 7, 11, and 14) and 11 samples (#1, 2, 4, 5, 6, 8, 9, 10, 12, 13, and 15) (Figure 1). The clustering results did not correlate with the FAB classification, immunopheno-

Table 3. Differentially expressed genes in cDNA microarray analysis of 418 targets.

Gene/Protein°	*Accession number (GenBank)*	*Locus ID*	*Chromosomal location*	*Gene expression profile§* +	–
Chemokine receptors					
High-affinity interleukin-8 receptor A (IL-8R A)	M68932	3577	2q35		13
Chemokine (C-X-C motif), receptor 4 (fusin) (CXCR4)	D10924	7852	2q21		3
Chemokine (C-C motif) receptor 2 (CCR2)	U03905	1231	3p21	5	
Duffy blood group (FY)	U01839	2532	1q21-q22		9
Growth factor and cytokine receptors					
Fibroblast growth factor receptor 1 (FGFR1)	X66945	2260	8p11.2-p11.1	3	
Blood disorder proteins					
v-Myc avian myelocytomatosis viral oncogene homolog (MYC)	V00568	4609	8q24.12-q24.13	8	
Nucleophosmin (nucleolar phosphoprotein B23) (NPM1)	M23613	4869	5q35	11	
ADP-ribosyltransferase (NAD+; poly (ADP-ribose) polymerase) (ADPRT)	M18112	142	1q41-q42	4	
DEK oncogene (DNA binding) (DEK)	X64229	7913	6p23	9	
B-cell CLL/lymphoma 6 (zinc finger protein 51) (BCL6)	U00115	604	3q27		4
Regulator of G-protein signalling 2, 24kD (RGS2)	L13463	5997	1q31		4
Solute carrier family 11, member 1 (SLC11A1)	L32185	6556	2q35		7
Charot-Leyden crystal protein (CLC)	L01664	1178	19q13.1		3
Interleukins and interferons					
Interleukin 1 receptor antagonist (IL1RN)	M63099	3557	2q14.2	3	
Chemokines					
Pro-platelet basic protein (PPBP)	M54995	5473	4q12-q13		4
Small inducible cytokine subfamily B (Cys-X-Cys) (SCYB5)	X78686	6374	4q12-q13		4
Growth factors					
Lymphotoxin beta (TNF superfamily, member 3) (LTB)	L111055	4050	6p21.3		11
Surface antigens					
Fc fragment of IgE, low affinity II, receptor for CD23A (FCER2)	M15059	2208	19p13.3	4	
Decay accelerating factor for complement (CD55) (DAF)	M31516	1604	1q32		7
Membrane-spanning 4-domains, subfamily A, member 2 (CD20) (MS4A2)	X12530	931	11q12-q13.1		5
Integrin, alpha 4 (CD49D) (ITGA4)	L12002	3676	2q31-q32	9	
Membrane cofactor protein (CD46) (MCP)	Y00651	4179	1q32		5
CD44 antigen (homing function and Indian blood group system) (CD44)	M59040	960	11p13		3
Vascular cell adhesion molecule 1 (VCAM1)	M30257	7412	1p32-p31	3	
Tumor necrosis factor receptor superfamily, member 7 (TNFRSF7)	M63928	939	12p13		4
CD34 antigen (CD34)	M81104	947	1q32	5	
Intercellular adhesion molecule 3 (ICAM3)	X69711	3385	19p13.3-p13.2		4
Transcription factors for activation and differentiation of hematopoietic cells					
Homeo box B6 (HOXB6)	X58431	3216	17q21-q22	7	
Homeo box A5 (HOXA5)	M26679	3202	7p15-p14	4	
Myeloid cell nuclear differentiation antigen (MNDA)	M81750	4332	1q22		3
Homeo box A4 (HOXA4)	M74297	3201	7p15-p14	10	
Nuclear factor I/X (CCAAT-binding transcription factor) (NFIX)	L31881	4784	19p13.3	7	
Oncogenes and protein kinases activated in hematopoietic cells					
Neurotrophic tyrosine kinase, receptor, type 1 (NTRK1)	X03663	4914	1q21-q22	3	
Cas-Br-M (murine) ecotropic retroviral transforming sequence (CBL)	X57110	867	11q23.3		3
Calcium modulating ligand (CAMLG)	U18242	819	5q23	3	
Mitogen-activated protein kinase kinase 6 (MAP2K6)	U39657	5608	17		3
Death-associated protein kinase 1 (DAPK1)	X76104	1612	9q34.1	4	
S100 calcium-binding protein A12 (Calgranulin C) (CAGC)	X97859	6283	1q21		6
Apoptosis-associated proteins					
BCL2-associated X protein (BAX)	L22474	581	19q13.3-q13.4	5	
BCL2-like 1 (BCL2L1)	Z23115	598	20pter-20cen		5
Myeloid cell leukemia sequence 1 (BCL2-related) (MCL1)	L08246	4170	1q21		6
Serine (or cysteine) proteinase inhibitor, clade B2 (SERPINB2)	M18082	5055	18q21.3	5	
Eosinophil peroxidase (EPX)	X14346	8288	17q23.1		6
Lymphocyte cytosolic protein 1 (L-plastin) (LCP1)	M22300	3936	13q14.3		3
Bactericidal/permeability-increasing protein (BPI)	J04739	671	20q11.23-q12		6
Proteinase 3 (serine proteinase) (PRTN3)	M29142	5657	19p13.3		3
Granulin (GRN)	M75161	2896	17q21.32		3
Lymphocyte-specific protein 1 (LSP1)	M33552	4046	11p15.5		4
Solute carrier family 20, member 2 (SLC20A2)	L20852	6575	8p12-q21	3	
Housekeeping genes					
Hypoxanthine phosphoribosyltransferase 1 (HPRT1)	V00530	3251	Xq26.1	3	

**Microarrays were performed using the Atlas Human Hematology/Immunology cDNA expression filters (Clontech Laboratories Inc.). Only genes overexpressed (ratio ≥4) or underexpressed (ratio ≤ 0.25) in at least three of the patients studied are shown. °The gene groups are based on Clontech's grouping of the genes in the array. §Number of AML patients with overexpressed (+) and underexpressed (–) genes out of the 15 cases analyzed.*

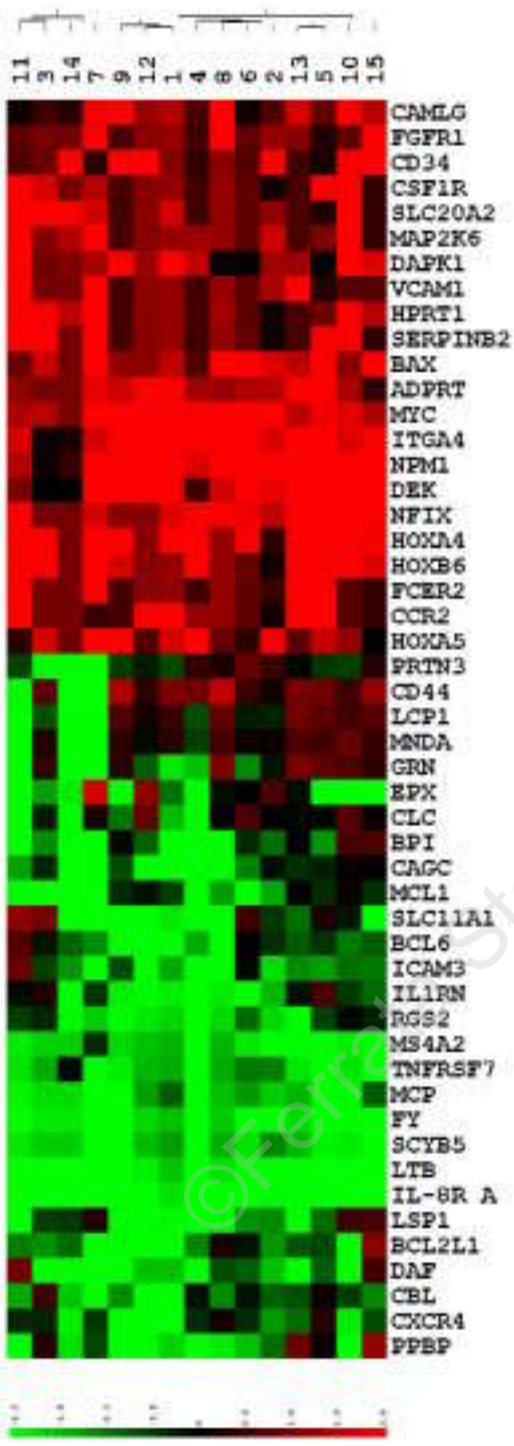

Figure 1. Summary of the gene expression profiles at diagnosis of 15 AML patients. Each row shows the expression of a particular gene in the series of 15 AML patients and each column denotes the gene expression profile for one patient. Patients' codes and gene names are listed on the top and on the right hand side of the figure, respectively. Upregulated genes are red, downregulated green. The intensity of the color correlates with the level of expression as shown by the color scale at the bottom of the figure. Scale ranges from –2.0 to +2.0 in $\log_2$ values (real ratio values from 0.25 to 4.0 for down- and upregulation, respectively).

type, chromosomal features or any other hematologic or survival parameters depicted in Table 1. However, we found one subcluster of 4 patients with normal karyotype (#5, 10, 13, and 15) within the major cluster of 11 AML samples (Figure 1).

A standard *t*-test was used to compare the gene expression levels in different groups of patients to identify gene(s) that may be associated with different clinical, hematologic and chromosomal features. *NFIK* was overexpressed more frequently in patients with abnormal karyotype than in those AML patients with a normal chromosomal complement (p=0.005). Similarly, the expression of two upregulated genes, *HOXA4* and *CSF1R*, was significantly higher in patients with a white blood cell count higher than 30×10^9/L than in the other patients (p=0.02 and p=0.03, respectively). In patients whose white blood cell count was higher than 100×10^9/L, both *CLC* and *GRN* were significantly underexpressed (p=0.007 and p=0.04, respectively), whereas *HOXA4* and *DAPK1* were overexpressed in relation to the values in the other patients (p=0.02). *FGFR1* and *CAMLG* were significantly overexpressed more frequently in patients with CD56 immunophenotype (p=0.04). No differences were observed in gene expression profiling of patients with and without extramedullary infiltrations or t(8;21)(q22;q22) (p>0.05) (Figure 1).

To confirm the gene expression differences obtained using cDNA microarray, we selected 13 genes for real-time quantitative fluorescence PCR, namely *IL8RA, FY, MYC, DEK, LTB, MS4A2, CSF1R, DAPK1, CBL, BAX, BCL2L1, MCL1*, and *HPRT1* (housekeeping gene) (Table 4). Microarray results were confirmed with all genes, except the BCL2-related gene, in which the microarray showed a tendency to underexpression and QRT-PCR showed a tendency to overexpression. There were discrepancies in the results in a few cases involving the expression of *DEK* (1 patient), *CSF1R* (2 patients), *DAPK1* (3 patients), BAX (3 patients), and *HPRT1* (4 patients).

Discussion

For gene expression profiling in AML, we used total RNAs isolated from bone marrow aspirates. For reference, we used total bone marrow cells from healthy donors. Thus the differentially expressed genes, especially those that were downregulated, may be partly related to a decrease in the proportional or absolute amount of cells from non-myeloid bone marrow cells in AML patients rather than to the malignancy *per se*. This notion rests on

Table 4. Validation of array-based gene expression profile by quantitative real-time RT-PCR of selected genes from 27 patients diagnosed with acute myeloid leukemia.

Genes	*Relative gene expression (average ±SD)*		*Validation*
	Microarray	*RT-PCR*	
High-affinity interleukin-8 receptor A (*IL-8R A*)	0.18 ±0.01	0.53 ±0.12	Y
Duffy blood group (*FY*)	0.25 ±0.01	0.29 ±0.05	Y
v-Myc avian myeocytomatosys viral oncogene homolog (*MYC*)	4.06 ±0.34	4.86 ±0.57	Y
DEK oncogene (DNA) binding (*DEK*)	4.92 ±0.60	1.8 ±0.30	Y
Lymphotoxin beta (*TNF* superfamily) (*LTB*)	0.23 ±0.01	0.80 ±0.17	Y
Membrane-spanning 4-domains, superfamily A, member 2 (*CD20*)	0.32 ±0.02	0.16 ±0.02	Y
Macrophage colony-stimulating factor I receptor precursor (*CSF-1-R*)(*c-fms*)	2.83 ±0.39	6.21 ±1.21	Y
Cas-Br-M (murine) ecotropic retroviral transforming sequence (*CBL*)	0.60 ±0.07	0.75 ±0.07	Y
Death-associated protein kinase 1 (*DAPK1*)	3.24 ±0.35	1.46 ±0.18	Y
BCL2-associated X protein (*BAX*)	3.40 ±0.35	2.32 ±0.20	Y
BCL2-like 1 (*BCL2L1*)	0.59 ±0.09	0.41 ±0.18	Y
Myeloid cell leukemia sequence 1 (*BCL2-related*)	0.52 ±0.08	1.44 ±0.18	N
Hypoxanthine phosphoribosyltransferase 1 (*HPRT1*)	5.45 ±1.41	2.38 ±0.35	Y

the knowledge that the bulk of the cells in AML patients are myeloid blasts, whereas the proportion of them in a normal reference is significantly smaller in relation to other cells. Our results demonstrated downregulated expression of several genes committed mostly to lymphoid lineages and/or mature blood cells. These downregulated genes include surface antigens, such as *CD4, CD8, CD20, CD27*, *CD44*, *CD46*, and *CD55*, several chemokines (*PPBP* and *SCYB5*) as well as chemokine receptors (*IL8RA* and *CXCR4*). *CD34* encodes a transmembrane glycoprotein of unknown function expressed in humans in hematopoietic stem cells, vascular endothelium, and blasts from 30% of patients with AML and ALL.[8] In our sample material, *CD34* was found to be overexpressed in five out of the 15 patients studied (33%) by cDNA microarray analysis. The possibility that this overexpression may be a pseudo-positive result cannot be ruled out, nor that it only reflects the expansion of immature cells (AML blasts) in our patients, as recently demonstrated by Miyazato *et al.* in myelodysplastic syndrome, another myeloproliferative disease.[9] Furthermore, the findings of overexpression of the death promoter *BAX*, apoptosis-associated *BCL2L1*, and *CSF1R* as well as the surface antigen *CD4* could be related to possible expression differences between different cell types. Our decision to use total bone marrow cells as reference material instead of stem cells isolated from a patient or from mononuclear cells separated by density gradient centrifugation was first based on the lack of definite knowledge of the normal counterpart of a malignant cell in AML, and second, it would have been necessary to enrich the stem cells and amplify the RNA derived from them. The use of enrichment methods does not yield a highly purified cell population and the amplification of RNA may render it biased for gene expression profiling. Third, certain leukemias, such as 5;14 translocation-positive ALL,[10] are known to cause changes in gene expression of non-malignant normal myeloid cells. The only way to discover these kinds of changes is to use total bone marrow which best reflects the *in vivo* situation. The detailed discussion below deals only with the overexpressed genes that are least likely to be results from our reference selection.

In the present study, several genes that are known to be involved in chromosomal translocations and fusions were differentially expressed. These genes were *FGFR1* (overexpressed 3/15) involved in 8p11-12 translocations, *MYC* (overexpressed 6/15) in 8q24 translocations, *NPM1* (overexpressed 11/15) in 5q35 translocations, and *DEC* (overexpressed 9/15) in 6p23 translocations.[11] As none of these translocations was observed in our cases, the findings suggest that these genes are also activated by mechanisms other than translocations. Only *MYC* has been previously reported to be overexpressed in numerous human malignancies, including AML and several hematologic disorders with T- and B-cell lineage involvement.[12-14]

In our study, overexpression of *CCR2* (5/15) and *ADPRT* (4/15) is a novel finding. The *CCR2* gene encodes chemokine (C-C motif) receptor 2, which has been reported to be upregulated in human ath-

erosclerotic plaques, in arteries in conditions of hypercholesterolemia, and in vascular smooth muscle cells exposed to minimally modified lipids.[15] Tomoda and collaborators[16] demonstrated that in contrast to reactive proliferative diseases, malignant lymphomas showed increased expression of *ADPTR*. Furthermore, amplification of 1q41-q44 and increased *ADPTR* RNA expression have been correlated with low genetic instability in human breast carcinomas.[17] Enhanced expression of *ADPTR* has not been observed in any other hematologic disease but in malignant lymphomas.[16]

DAPK1, *HOXA4*, and *CSF1R* were significantly more often overexpressed and the *CLC* and *GRN* genes were significantly more often underexpressed in patients with high white blood cell count (>30×10^9 cells/L). Although very little is known about the expression of these genes in leukemias, the following aspects have been reported. *DAPK1* is a positive mediator of the apoptosis by γ-interferon.[18] *HOX4A* has been shown to be expressed in erythroleukemia *HEL* and *K562* cell lines but not in a promyelocytic *HL60* cell line.[19] To date the literature does not include any other expression studies of these genes in AML. Some codons of *CSF1R* are reported to be potentially involved in promotion of the transforming activity of the gene product.[20] Among the samples we analyzed, AML of monocytic lineages (M4/5) was found in seven out of 16 patients (44%) with high WBC, but in two out of 11 patients (18%) with low WBC counts. The enhanced expression of *CSF1R*, a cell surface marker of monocytic cells, may be attributable to an increased population of monocytic blasts. *GRN* represents a family of cysteine-rich polypeptides, some of which have growth modulatory activity, but the biological significance of its expression is unclear, although elevated *GRN* expression has been demonstrated to confer a transformed phenotype on epithelial cells, including anchorage independence *in vitro* and growth as tumors in nude mice.[21] Finally, *CLC* shows similarities to members of the superfamily of the β-galactosidase binding S-type animal lectins, and there is evidence that the gene may confer some specificity for expression in the eosinophilic lineage.[22] *CD56* (neural cell adhesion phenotype) immunophenotype in AML has been reported to be present in a proportion of AML and it has been shown to have an adverse clinical significance in patients with t(8;21)(q22;q22).[23] In our study two genes, *FGFR1* and *CRMLG*, were significantly more often overexpressed in the patients with *CD56* immunophenotype. We can not explain why the overexpression of *CRMLG* (calcium signal-modulating cyclophilin ligand/*CAML*) and *FGFR1*, and *CD56* immunophenotype are associated, but the association of *FGFR1* with *CD56* is intriguing. *FGFR1* is implicated in stem-cell myeloproliferative disorders associated with chromosomal translocation involving 8p12.[24] The translocations are a sign of very poor prognosis. Prognostic significance of *FGFR1* overexpression and its association with *CD56* immunophenotype need to be studied.

In conclusion, our microarray analysis showed numerous differentially expressed genes. Many of the genes are associated with chromosomal translocations. For example, *FGFR1*, *MYC*, *NPM1*, and *DEC* were observed to be overexpressed. Five genes (*HOX4A*, *CSF1R*, *CLC*, *GRN*, and *DAPK1*) were differentially expressed in patients with high white blood cell counts. And finally, the patients with *CD56* immunophenotype had more frequently overexpression of the *FGFR1* and *CAMLG* genes. These results should be confirmed and the clinical and prognostic significance of the findings should be studied in a larger series of patients.

Contribution and Acknowledgments

MLL and SK designed the method and study, and drafted the manuscript. TN and BN performed the cDNA array and PCR analyses, respectively. JO and MV were responsible for the bioinformatics and EE for the clinical studies. All authors contributed to the production of the manuscript. SK is the senior author: he supervised the project and was responsible for its funding.

Funding

This study was supported by the Finnish Foundation for Cancer Research, the Sigrid Jusélius Foundation and Helsinki University Central Hospital Research Funds, the Academy of Finland, the Medical Research Fund of Tampere University Hospital in Finland, and by the National Council of Scientific and Technological Research (CONICET) and the National University of La Plata in Argentina.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

References

1. DeRisi J, Penland L, Brown PO, Bittner ML, Meltzer PS, Ray M, et al. Use of a cDNA microarray to analyse gene expression patterns in human cancer. Nat Genet 1996; 14:457-60.
2. Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complimentary DNA microarray. Science 1995; 270:467-70.

3. Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP, et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 1999; 286:531-7.
4. Aalto Y, El-Rifai W, Vilpo L, Ollila J, Nagy B, Vihinen M, et al. Distinct gene expression profiling in chronic lymphocytic leukemia with 11q23 deletion. Leukemia 2001; 15:1721-8.
5. Zhu Y, Hollmén J, Oinonen R, Aalto Y, Elonen E, Kere J, et al. Gene expression profiling of mantle cell lymphoma and its blastoid variant. Unpublished data.
6. Kettunen E, Nissèn A-M, Ollikainen T, Taavitsainen M, Tapper J, Mattson K, et al. Gene expression profiling of malignant mesothelioma cell lines on cDNA microarray study [abstract]. Cancer Genet Cytogenet 2001; 128:72.
7. Wolf M, Tarkkanen M, El-Rifai W, Kononen J, Serra M, Eriksen EF, et al. Novel findings in gene expression detected in human osteosarcoma by cDNA microarray. Cancer Genet Cytogenet 2000; 123:128-32.
8. Satterthwaite AB, Burn TC, Le Beau MM, Tenen DG. Structure of the gene encoding CD34, a human hematopoietic stem cell antigen. Genomics 1992; 12:788-94.
9. Miyazato A, Ueno S, Ohmine K, Ueda M, Yoshida K, Yamashita Y, et al. Identification of myelodysplastic syndrome-specific genes by DNA microarray analysis with purified hematopoietic stem cell fraction. Blood 2001; 98:422-7.
10. Knuutila S, Alitalo R, Ruutu T. Power of the MAC (Morphology Antibody Chromosomes) method in distinguishing reactive and clonal cells: report of a patient with acute lymphatic leukemia, eosinophilia and t(5;14). Genes Chromosom Cancer 1993; 8:219-23.
11. Heim S, Mitelman F. Cancer Cytogenetics. 2nd ed. New York: Wiley-Liss, 1995.
12. Marcu KB, Bossone SA, Patel AJ. MYC function and regulation. Ann Rev Biochem 1992; 61:809-60.
13. Preisler HD, Kinniburgh AJ, Wei-Dong G, Khan S. Expression of the protooncogenes c-myc, c-fos, and c-fms in acute myelocytic leukemia at diagnosis and in remission. Cancer Res 1987; 47:874-80.
14. Preisler HD, Raza A, Larson R, LeBeau M, Browman G, Goldberg J, et al. Protooncogene expression and the clinical characteristics of acute nonlymphocytic leukemia: a Leukemia Intergroup pilot study. Blood 1989; 73:255-62.
15. Boring L, Gosling J, Cleary M, Charo IF. Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. Nature 1998; 394:894-7.
16. Tomoda T, Kurishige T, Moriki T, Yamamoto H, Fujimoto S, Taniguchi T. Enhanced expression of poly(ADP-ribose) synthetase gene in malignant lymphoma. Am J Hematol 1991; 37:223-7.
17. Bieche I, de Murcia G, Lidereau R. Poly(ADP-ribose) polymerase gene expression status and genomic instability in human breast cancer. Clin Cancer Res 1996; 2:1163-7.
18. Deiss LP, Feinstein E, Berissi H, Cohen O, Kimchi A. Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the gamma interferon-induced cell death. Genes Dev 1995; 9:15-30.
19. Taniguchi Y, Komatsu N, Moriuchi T. Overexpression of the HOX4A (HOXD3) homeobox gene in human erythroleukemia HEL cells results in altered adhesive properties. Blood 1995; 85:2786-94.
20. Ridge SA, Worwood M, Oscier D, Jacobs A, Padua RA. FMS mutations in myelodysplastic, leukemic, and normal subjects. Proc Natl Acad Sci (USA) 1990; 87:1377-80.
21. He Z, Bateman A. Progranulin gene expression regulates epithelial cell growth and promotes tumor growth in vivo. Cancer Res 1999; 59:3222-9.
22. Gomolin HI, Yamaguchi Y, Paulpillai AV, Dvorak LA, Ackerman SJ, Tenen DG. Human eosinophil Charcot-Leyden crystal protein: cloning and characterization of a lysophospholipase gene promoter. Blood 1993; 82:1868-74.
23. Daniels JT, Davis BJ, Houde-McGrail L, Byrd JC. Clonal selection of CD56+ t(8;21) AML blasts: further suggestion of the adverse clinical significance of this biological marker? Br J Haematol 1999; 107:381-3.
24. Guasch G, Mack GJ, Popovici C, Dastugue N, Birnbaum D, Rattner JB, et al. FGFR1 is fused to the centrosome-associated protein CEP110 in the 8p12 stem cell myeloproliferative disorder with t(8;9)(p12;q33). Blood 2000; 95:1788-96.

PEER REVIEW OUTCOMES

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor. Francesco Lo Coco, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Prof. Lo Coco and the Editors. Manuscript received January 21, 2002; accepted April 11, 2002.

What is already known on this topic

Gene array technology has been employed recently as a promising approach to classify human cancer and leukemia based on the identification of differentially expressed genes.

What this study adds

Some genes frequently involved in chromosome translocations have been found to be overexpressed through the above technology in AML cases lacking translocations.

Potential implications for clinical practice

The study has no relevant clinical implication in the short term. However, it may foster further investigation in larger patient series aimed at better classification of human leukemias.

Francesco Lo Coco, Deputy Editor