

Signal Transducers and Activators of Transcription 3 Augments the Transcriptional Activity of CCAAT/Enhancer-binding Protein α in Granulocyte Colony-stimulating Factor Signaling Pathway*

Received for publication, July 26, 2004, and in revised form, January 3, 2005
Published, JBC Papers in Press, January 21, 2005, DOI 10.1074/jbc.M408442200

Akihiko Numata[‡], Kazuya Shimoda^{‡§}, Kenjiro Kamezaki[‡], Takashi Haro[‡], Haruko Kakumitsu[‡], Koutarou Shide[‡], Kouji Kato[‡], Toshihiro Miyamoto[‡], Yoshihiro Yamashita[¶], Yasuo Oshima[¶], Hideaki Nakajima^{**}, Atsushi Iwama^{‡‡}, Kenichi Aoki^{‡‡}, Ken Takase[‡], Hisashi Gondo[‡], Hiroyuki Mano[¶], and Mine Harada[‡]

From the [‡]Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-ku, Fukuoka, Fukuoka, 812-8582, the [¶]Division of Functional Genomics, Jichi Medical School, 3311-1 Yakushiji, Kawaguchi-gun, Tochigi, 329-0498, the ^{||}Department of Clinical Pharmacology, Jichi Medical School, 3311-1 Yakushiji, Kawaguchi-gun, Tochigi, 329-0498, the ^{**}Department of Hematopoietic Factors, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo, 108-8639, and the ^{‡‡}Laboratory of Stem Cell Therapy, Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo, 108-8639, Japan

The Janus kinase (Jak)-Stat pathway plays an essential role in cytokine signaling. Granulocyte colony-stimulating factor (G-CSF) promotes granulopoiesis and granulocytic differentiation, and Stat3 is the principle Stat protein activated by G-CSF. Upon treatment with G-CSF, the interleukin-3-dependent cell line 32D clone 3(32Dcl3) differentiates into neutrophils, and 32Dcl3 cells expressing dominant-negative Stat3 (32Dcl3/DNStat3) proliferate in G-CSF without differentiation. Gene expression profile and quantitative PCR analysis of G-CSF-stimulated cell lines revealed that the expression of *C/EBP α* was up-regulated by the activation of Stat3. In addition, activated Stat3 bound to CCAAT/enhancer-binding protein (C/EBP) α , leading to the enhancement of the transcription activity of *C/EBP α* . Conditional expression of *C/EBP α* in 32Dcl3/DNStat3 cells after G-CSF stimulation abolishes the G-CSF-dependent cell proliferation and induces granulocytic differentiation. Although granulocyte-specific genes, such as the G-CSF receptor, lysozyme M, and neutrophil gelatinase-associated lipocalin precursor (*NGAL*) are regulated by Stat3, only *NGAL* was induced by the restoration of *C/EBP α* after stimulation with G-CSF in 32Dcl3/DNStat3 cells. These results show that one of the major roles of Stat3 in the G-CSF signaling pathway is to augment the function of *C/EBP α* , which is essential for myeloid differentiation. Additionally, cooperation of *C/EBP α* with other Stat3-activated proteins are required for the induction of some G-CSF responsive genes including lysozyme M and the G-CSF receptor.

The proliferation and differentiation of hematopoietic progenitor cells are regulated by cytokines (1). Among these, gran-

ulocyte colony-stimulating factor (G-CSF)¹ specifically stimulates cells that are committed to the myeloid lineage (2). The importance of G-CSF to the regulation of granulopoiesis has been confirmed by the observation of severe neutropenia in mice carrying homozygous deletions of their G-CSF or G-CSF receptor genes (3, 4). Cytokines activate several intracellular signaling pathways, and the Janus kinase (Jak) signal transducers and activators of transcription (Stat) pathway is essential for cytokine function (5, 6). The binding of G-CSF to cell surface G-CSF receptors activates Jak1, Jak2, and Tyk2 followed by the activation of Stat1, Stat3, and Stat5 (7–9). Stat3 is the principle protein activated by G-CSF (8, 10). Phosphorylated Stats translocate from the cytoplasm into the nucleus and induce transcription of their target genes within a short period of time. 32Dcl3 cells differentiate to neutrophils following treatment with G-CSF. In contrast to their parental cells, 32Dcl3 cells expressing dominant-negative Stat3 (32Dcl3/DNStat3) proliferate in the presence of G-CSF, but they maintain immature morphologic characteristics without evidence of differentiation (11). Additionally, transgenic mice with a targeted mutation of their G-CSF receptor that abolishes G-CSF-dependent Stat3 activation show severe neutropenia with an accumulation of immature myeloid precursors in their bone marrows (12). To clarify the role of Stat3 in the G-CSF signaling pathway, we wished to identify target genes of Stat3.

We found that the levels of CCAAT/enhancer-binding protein (C/EBP) α mRNA were up-regulated following G-CSF stimulation in 32Dcl3 but were unchanged in 32Dcl3/DNStat3. In addition, the activation of Stat3 augmented the function of *C/EBP α* , which is the essential transcriptional factor for myeloid differentiation. G-CSF-induced granulocytic differentiation was restored in 32Dcl3/DNStat3 cells by the conditional expression of *C/EBP α* . These results show that one of the major

* This work was supported by a grant of the Japan Leukemia Research Foundation (2002) and Grants-in-aid for Scientific Research 11307015 and 15390302 from the Ministry of Education, Science, Sports, and Culture in Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: 81-92-642-5230; Fax: 81-92-642-5247; E-mail: kshimoda@intmed1.med.kyushu-u.ac.jp.

¹ The abbreviations used are: G-CSF, granulocyte colony-stimulating factor; IL, interleukin; C/EBP α , CCAAT/enhancer-binding protein; NGAL, neutrophil gelatinase-associated lipocalin precursor; Jak, Janus kinase; Stat, signal transducers and activators of transcription; DNStat3, dominant-negative Stat3; IRES, internal ribosome entry site; GFP, green fluorescent protein; ER, endoplasmic reticulum; IFN, interferon; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FACS, fluorescence-activated cell sorter; LUC, luciferase; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; TK, thymidine kinase; 4-HT, 4-hydroxytamoxifen.

roles of Stat3 in the G-CSF signaling pathway is to enhance the function of C/EBP α .

MATERIALS AND METHODS

Cell Culture, Expression Plasmid, and Cytokines—32D clone 3 (32Dcl3) and 32Dcl3/DNStat3 cells (DNStat3 deletes the transactivation domain of Stat3) were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (ICN, Osaka, Japan), penicillin/streptomycin (Invitrogen), recombinant murine interleukin-3 (IL-3) (Kirin Brewery, Takasaki, Japan), and recombinant human G-CSF (Chugai Pharmaceutical, Tokyo, Japan). 293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin/streptomycin, and L-glutamine.

For the construction of pTag2A-G-CSF receptor, the human G-CSF receptor cDNA (13) (pHQ3, kindly provided by S. Nagata and R. Fukunaga) was excised from the pBluescript vector and inserted into the FLAG-tagged mammalian expression plasmid pCMV-Tag2A (Clontech). pCDNA3-rat C/EBP α was described before (14). Stat3 cDNA was amplified by PCR and inserted into pCMV-HA vector (Clontech). Stat3c cDNA was elicited from RCMV-Stat3c (15), kindly given from Dr. Darnell, and inserted into pCDNA3.1 (Clontech). For the construction of pMY-IRES-GFP/C/EBP α -ER, full-length human C/EBP α cDNA was fused in-frame with ligand-binding domain (amino acids 281–599) of mouse estrogen receptor harboring a mutation (G525R) that confers selective responsiveness to 4-hydroxytamoxifen (4-HT). A reporter construct of a minimal TK promoter with CEBP-binding sites (pC/EBP2TK) was described previously (14).

Murine recombinant leukemia inhibitory factor, natural IFN- α , and recombinant IFN- γ were purchased from Sigma, HyCult Biotechnology (Uden, The Netherlands), and Peprotech (Rocky Hill, NJ), respectively. For Western blotting, 32Dcl3 cells or 32Dcl3/DNStat3 cells were deprived of IL-3 for 12 h. Then cells were stimulated with G-CSF (10 ng/ml), IL-3 (10 ng/ml), leukemia inhibitory factor (10 ng/ml), IFN- α (1,000 units/ml), or IFN- γ (1,000 units/ml) for 30 min.

Microarray Analysis—32D cl3 and 32Dcl3/DNStat3 cells maintained in IL-3 were washed twice with PBS and starved of cytokine in RPMI 1640 containing 10% fetal bovine serum for 8 h and then stimulated with 10 ng/ml G-CSF. Total RNA was extracted, by the acid guanidinium method, from 32Dcl3 and 32Dcl3/DNStat3 cells before or after the stimulation for 2 h with G-CSF. Double-stranded cDNA synthesized from the total RNA (20 μ g/sample) was then used to prepare biotin-labeled cRNA for the hybridization with GeneChip MGU74Avs2 microarrays (Affymetrix, Santa Clara, CA) harboring oligonucleotides corresponding to ~6000 known genes as well as ~6000 expressed sequence tag sequences. Hybridization, washing, and detection of signals on the arrays were performed with the GeneChip system (Affymetrix).

Quantitative Real-time Reverse Transcription-PCR Assay—32Dcl3 and 32Dcl3/DNStat3 cells maintained in IL-3, were washed twice with PBS and starved of cytokines for 8 h and then stimulated with 10 ng/ml G-CSF. Cells were harvested at the indicated times, and total RNA was isolated using Isogen (Nippon gene, Tokyo, Japan) according to the manufacturer's instructions. One microgram of extracted RNA was transcribed in a 20- μ l cDNA synthesis reaction using an RNA PCR kit (AMV) (Takara, Tokyo, Japan). Real-time PCR for C/EBP α , G-CSF receptor, lysozyme M, neutrophil gelatinase-associated lipocalin precursor (NGAL), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed by a TaqMan assay on an ABI 7000 system. PCR primers and probes were designed as follows: murine C/EBP α , sense, 5'-CCA TGT GGT AGG AGA CAG AGA CCT A-3', and antisense, 5'-CTC TGG GAT GGA TCG ATT GTG-3'; probe FAM-5'-CGG CTG GCG ACA TAC AGT ACA CAC AAG-3'-TAMRA, and sense, 5'-CCA AGA AGT CGG TGG ACA AGA-3', and antisense, 5'-CGG TCA TTG TCA CTG GTC AAC T-3'; probe FAM-5'-AGC ACC TTC TGT TGC GTC TCC ACG TT-3'-TAMRA; murine G-CSF receptor, sense, 5'-CTA AAC ATC TCC CTC CAT GAC TT-3', and antisense, 5'-GGC CAT GAG GTA GAC ATG ATA CAA-3'; probe FAM-5'-CAT CTT CTC TGT CCC CAC CGA CCA A-3'-TAMRA; murine lysozyme M, sense, 5'-TGC CTG TGG GAT CAA TTG C-3', and antisense, 5'-ATG CCA CCC ATG CTC GAA T-3'; probe 5'-FAM-CAG TGA GGT CAT CCT GCA GAC CA-TAMRA-3'; murine NGAL, sense, 5'-GGC CTC AAG GAC GAC AAC A-3', and antisense, 5'-CAC CAC CCA TTC AGT TGT CAA T-3'; probe 5'-FAM-CAT CTT CTC TGT CCC CAC CGA CCA A-TAMRA-3', and murine GAPDH sense, 5'-ACG GCA AAT TCA ACG GCA-3', and antisense, 5'-AGA TGG TGA TGG GCT TCC-3'; probe 5'-FAM-AGG CCG AGA ATG GGA AGC TTG TCA TC-TAMRA-3'. PCR amplifications were performed in a 50- μ l volume, containing 4 μ l of cDNA template, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 10 mM EDTA, 60 nM, 200 μ M dNTPs, 3

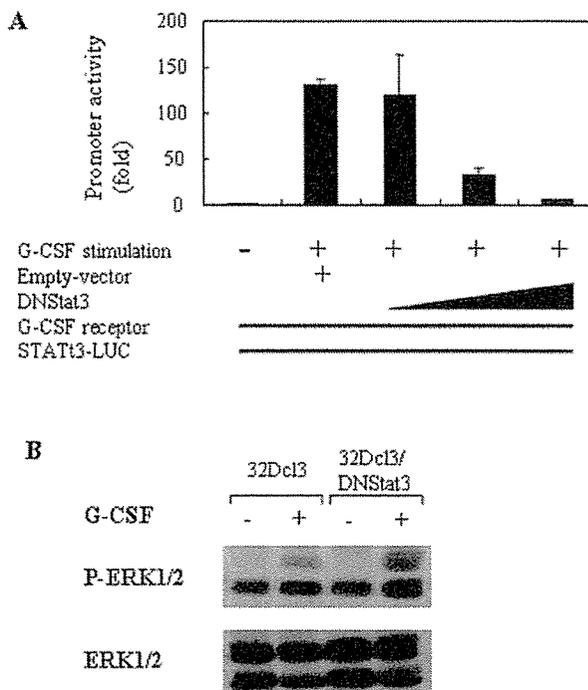


FIG. 1. The effect of dominant-negative Stat3 on G-CSF signaling pathway. **A**, transient transfection in 293T cells with a reporter construct with α 2-macroglobulin promoter (*STAT3-LUC*), dominant-negative Stat3, and G-CSF receptor. Twelve hours after transfection, cells were stimulated with 10 ng/ml G-CSF. Promoter activity was measured as luciferase activity 36 h after transfection. The vertical axis number is the fold induction when compared with control. **B**, 32Dcl3 cells or 32Dcl3/DNStat3 cells were cultured with IL-3 and then deprived of IL-3 for 12 h. Cells were treated with the G-CSF for 30 min and lysed. Post-nuclear supernatants were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed using the indicated antibodies. p-ERK1/2, phosphorylated ERK1/2.

mm MgCl₂, 200 nM each primer, 0.625 units of AmpliTaqGold, and 0.25 units of AmpErase uracil N-glycosylase. Each amplification reaction also contained 100 nM appropriate detection probe. Each PCR amplification was performed in duplicate, using conditions of 50 °C for 2 min preceding 95 °C for 10 min followed by 40 cycles of amplification (95 °C for 15 s, 60 °C for 1 min). In each reaction, GAPDH was amplified as a housekeeping gene to calculate a standard curve and allow for the correction for variations in target sample quantities. Relative copy numbers were calculated for each sample from the standard curve after normalization to GAPDH by the instrument software.

Additional C/EBP α Expression—pMY-IRES-GFP/C/EBP α -ER was transfected into 32Dcl3 and 32Dcl3/DNStat3 cells by electroporation. 5×10^6 cells were transfected with 20 μ g of expression vector, and GFP-positive cells were sorted by FACS Vantage (BD Biosciences). Expression of C/EBP α was determined by Western blotting analysis (see below).

Luciferase Assay—293T cells were transfected by the calcium phosphate precipitation method in 6-well plates, and luciferase activity was assayed using a luminometer Lumat LB9507 (Berthold Technologies, Bad Wildbad, Germany) according to the manufacturer's protocol. Each expression plasmid amount was 50–100 ng/well, and the same amount of empty expression vector was used as control, respectively. Results of reporter assays represent the average values for relative luciferase activity generated from five independent experiments.

Flow Cytometry— 1×10^7 cells were incubated with 5 μ l of recombinant phosphatidylethanolamine-conjugated rabbit anti-murine Gr1 monoclonal antibody (BD Biosciences) for 30 min at 4 °C, washed twice in PBS, and analyzed on a FACS Calibur (BD Biosciences).

Immunoprecipitation and Immunoblotting—Cells were lysed with lysis buffer, and lysates were immunoprecipitated with anti C/EBP β (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (8). Total cell lysates or the immunoprecipitates were resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes

TABLE I
Microarray analysis

32Dcl3 and 32Dcl3/DNStat3 cells were starved of cytokines for 8 h and then stimulated or left unstimulated with 10 ng/ml G-CSF. Total RNA was extracted from each fraction and was subjected to the hybridization with high-density oligonucleotide microarrays (MGU74Av2). Fold induction means a rate of increase in gene expression level by G-CSF stimulation. Candidate genes were identified as transcripts that were up-regulated in 32Dcl3 cells and down-regulated or unchanged in 32Dcl3/DNStat3 cells after G-CSF stimulation.

Gene product name	Abbreviation	Accession number	Fold induction	
			32Dcl3	32Dcl3/DNStat3
B-cell leukemia/lymphoma α	<i>Bcl2</i>	L31532	35.6	0.0629
CyclinE1	<i>Ccne1</i>	NM007633	29.7	0.690
Serotonin-gated ion channel	<i>5HT3</i>	M74425	27.2	0.592
KIF3B protein	<i>kif3b</i>	D26077	21.5	0.921
Protein kinase, serine/arginine-specific 1	<i>Srpk1</i>	AB012290	18.7	0.321
MAP kinase-interacting serine/threonine kinase 1	<i>Mknk1</i>	Y11091	15.7	0.845
Protein tyrosine phosphatase	<i>Ptpn13</i>	D83966	12.4	0.964
Transferrin receptor	<i>Trfr</i>	X57349	10.6	0.964
Lymphocyte antigen 57	<i>Ly57</i>	AF068182	9.62	0.968
Macrophage stimulating 1 receptor	<i>Mst1r</i>	X74736	8.83	0.762
Mitogen-activated protein kinase kinase 7	<i>MKK7</i>	AB005654	8.14	0.980
RAR-related orphan receptor alpha	<i>Rora</i>	U53228	7.94	0.861
Hemoglobin Y, β -like embryonic chain	<i>Hbb-y</i>	V00726	7.38	0.375
Runt related transcription factor 1	<i>Runx1</i>	NM009821	7.01	0.226
Microtubule-associated protein 6	<i>Mtap6</i>	Y14754	5.06	0.885
CCAAT/enhancer binding protein α	<i>C/EBPα</i>	M62362	2.05	0.840
Ecotropic viral integration site 1	<i>Evi1</i>	M21829	1.55	0.239
Integrin alpha L	<i>Itgal</i>	M60778	1.35	0.567
Ninjurin 1	<i>Ninj1</i>	U91513	1.34	0.783
Interleukin 17 receptor	<i>IL17R</i>	U31993	1.24	0.449
Mucosal addressin	<i>MAdCAM</i>	D50434	1.14	0.527
Carbon catabolite repression 4 homolog	<i>Ccr4</i>	X16670	1.06	0.0768
Friend leukemia integration 1	<i>Fli1</i>	X59421	1.01	0.305

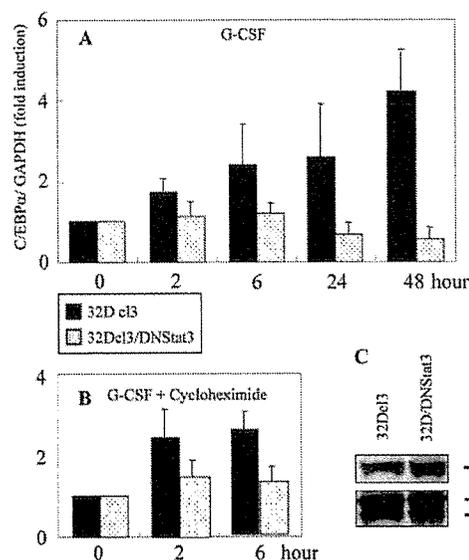


FIG. 2. Expression of C/EBP α mRNA in G-CSF-stimulated 32Dcl3 and 32Dcl3/DNStat3 cells. A and B, 32Dcl3 and 32Dcl3/DNStat3 cells maintained in IL-3 were washed twice with PBS and starved of cytokines for 8 h and stimulated with 10 ng/ml G-CSF (A) or 10 ng/ml G-CSF and 10 μ g/ml cycloheximide (B). Total RNA was isolated from both cell lines at the indicated times and transcribed to cDNA, which was subjected to real-time PCR for murine C/EBP α . The numbers given on the vertical axis represent the fold induction of the ratios of GAPDH-normalized expression values when compared with those before G-CSF stimulation. Results are expressed as mean fold of two independent experiments.

were probed using the indicated antibodies followed by an IgG-horse-radish peroxidase-conjugated secondary antibody (Amersham Biosciences) and visualized with the ECL detection system (Amersham Biosciences). Anti-phospho-ERK1/2 antibodies were purchased from Cell Signaling (Beverly, MA). Anti-phospho-Stat1 and -Stat5 antibodies were obtained from New England Biolabs (Beverly, MA), and anti-Stat1, -Stat3, and -C/EBP α antibodies were purchased from Santa Cruz Biotechnology. Membranes were probed using and visualizes with the

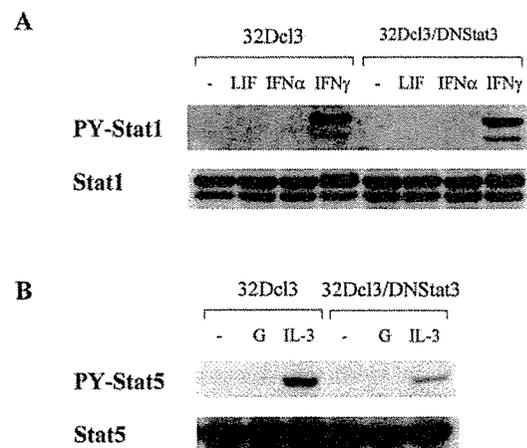


FIG. 3. The effect of the abrogation of Stat3 on other cytokine signaling pathway. 32Dcl3 cells or 32Dcl3/DNStat3 cells were cultured with IL-3 and then deprived of IL-3 for 12 h. Cells were treated with the indicated cytokines for 30 min and lysed. Post-nuclear supernatants were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed using the indicated antibodies. LIF, leukemia inhibitory factor.

ECL detection system (Amersham Biosciences).

Proliferation Assay—32D cl3 and 32Dcl3/DNStat3 cells maintained in IL-3 were washed twice with PBS and starved of cytokine for 8 h and then stimulated with 10 ng/ml G-CSF. The number of viable cells was determined by trypan blue dye exclusion using a hemocytometer. [3 H]Thymidine incorporation assays were also performed. Briefly, cells (1×10^5) in 100 μ l of medium stimulated with murine IL-3 (1.0 ng/ml) or recombinant human G-CSF (10 ng/ml) were cultured for 48 h. During the final 4 h, [3 H]thymidine (1 μ Ci/well) was added. Cells were then harvested by filtration, and radioactivity was counted by scintillation spectrophotometer.

RESULTS

G-CSF-induced Intracellular Signal Response in 32Dcl3/DNStat3 Cells—32Dcl3 cells differentiate into neutrophils following treatment with G-CSF, but 32Dcl3 cells expressing a

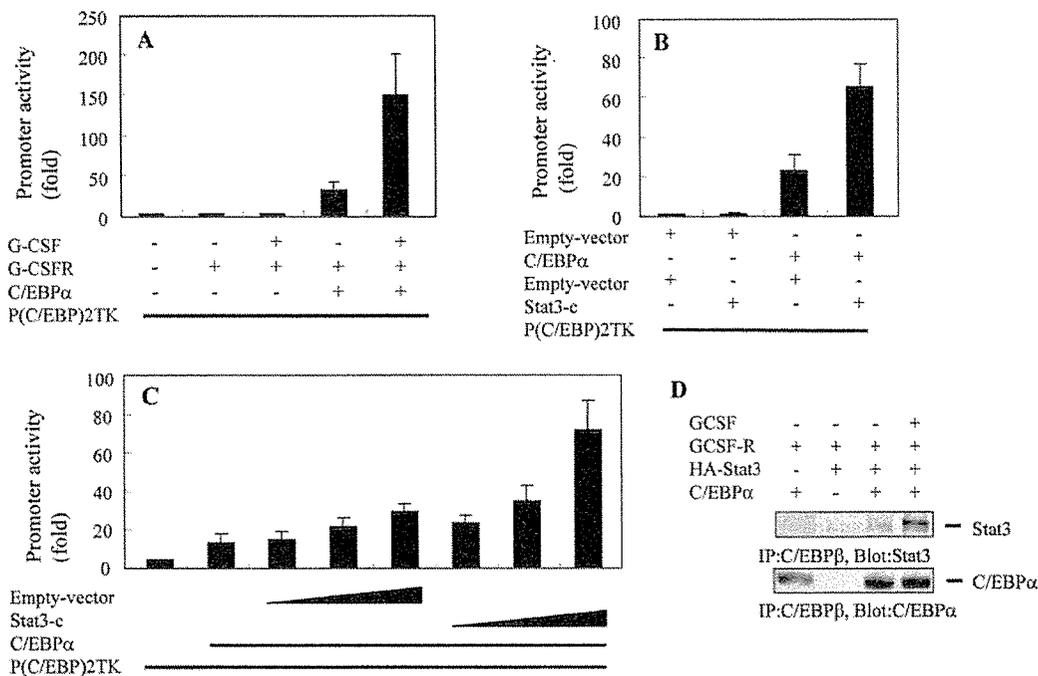


FIG. 4. Activated Stat3 makes complex with C/EBP α , leading to the enhancement of C/EBP α -induced transcription. *A*, transient transfection in 293T cells with a reporter construct of a minimal TK promoter with C/EBP-binding sites (*p(C/EBP)2TK*), C/EBP α , and G-CSF receptor (*G-CSFR*). Twelve hours after transfection, cells were stimulated with 10 ng/ml G-CSF. Promoter activity was measured as luciferase activity 36 h after transfection. The vertical axis number is the fold induction when compared with control. *B* and *C*, transient transfection in 293T cells with a reporter construct of a minimal TK promoter with C/EBP-binding sites (*p(C/EBP)2TK*), C/EBP α , Stat3c, and control vectors. Promoter activity was measured as luciferase activity 24 h after transfection. The vertical axis number is the fold induction when compared with control. *D*, transient transfection in 293T cells with a construct of G-CSF receptor, HA-Stat3, and C/EBP α and control vectors. After 24 h, cells were lysed and immunoprecipitated (IP) with anti C/EBP β . Cells were stimulated with G-CSF during the final 9 h in the culture. The immunoprecipitates were resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Stat3 was detected by immunoblotting.

dominant-negative Stat3 (32Dcl3/DNStat3) proliferate following G-CSF treatment. These cells maintain immature morphologic characteristics without evidence of differentiation (11). First, we examined the effect of dominant-negative Stat3, carboxyl-truncated Stat3 that lacked 55 amino acids including the transactivation domain. We transfected reporter construct of STAT3-LUC, in which the α 2-macroglobulin promoter (16) drives expression of the luciferase (LUC) reporter gene and G-CSF receptor, together with empty vector (pcDNA3) or DNStat3 to 293T cells. After 12 h of transfection, cells were stimulated with 10 ng/ml G-CSF. Cells were cultured for more 24 h, and luciferase assay was performed. As shown in Fig. 1A, G-CSF induced the transcriptional activity of Stat3 by 150-fold, and DNStat3 inhibited this G-CSF-induced Stat3 activation in a dose-dependent manner.

G-CSF mainly induces the phosphorylation of Stat3, but it also phosphorylates Stat1 and Stat5 in some cells among the Stats family (8) and induces the activation of MAP kinases. In both 32Dcl3 cells and 32Dcl3/DNStat3 cells, neither Stat1 nor Stat5 was phosphorylated in response to G-CSF (data not shown). As for the MAP kinase activation, the degree of the phosphorylation of ERK1/2 by G-CSF stimulation in 32Dcl3/DNStat3 cells was stronger than that in 32Dcl3 cells (Fig. 1B).

Identification of Genes Regulated by Stat3 in the G-CSF Signaling Pathway by Oligonucleotide Array Analysis—To identify Stat3-regulated genes involved in granulocytic differentiation, we compared gene expression change in both cell lines using microarray analysis. 32D cl3 and 32Dcl3/DNStat3 cells maintained in IL-3 were washed twice with PBS and starved in RPMI 1640 containing 10% fetal bovine serum lacking cytokine for 8 h and then stimulated with 10 ng/ml G-CSF.

Total RNA was isolated from 32Dcl3 cells and 32Dcl3/DNStat3 cells treated with G-CSF after 0 and 2 h, transcribed to biotin-labeled cRNA, and hybridized to GeneChip MGU74Av2 arrays to compare the expression profile of ~12,000 murine genes. The fold induction in the expression level of each gene was calculated as the ratio of GAPDH-normalized fluorescence intensity value of G-CSF-stimulated cells when compared with those before G-CSF stimulation. As shown in Table I, we could identify a set of candidate genes for Stat3 targets, expression of which was up-regulated in 32D cl3 cells but down-regulated or unchanged in 32Dcl3/DNStat3 cells. Such Stat3-dependent expression profiles were confirmed in triplicate experiments.

C/EBP α Is a Target Gene for Stat3 in G-CSF Signaling Pathway—Among the identified genes, it was decided to focus further efforts on C/EBP α . C/EBP α has been shown to be critical for early granulocytic differentiation (17–19), and the factors regulating its activity are unclear. The expression of C/EBP α was examined by real-time quantitative reverse transcription-PCR. C/EBP α mRNA levels are rapidly up-regulated in 32Dcl3 cells, being elevated 2.39-fold after 6 h and 4.20-fold after 48 h (Fig. 2A). In contrast to 32Dcl3 cells, the C/EBP α mRNA levels were not changed in 32Dcl3/DNStat3 cells after G-CSF stimulation (Fig. 2A). A similar expression pattern was seen in separate experiments with independently designed primers and probes (data not shown). Levels of C/EBP α mRNA were unaffected by cycloheximide treatment (Fig. 2B). The expression level of the sum of Stat3 plus dominant-negative Stat3 in 32Dcl3/DNStat3 cells is a little larger than that of Stat3 in 32Dcl3 cells (Fig. 2C).

Activated Stat3 Binds to C/EBP α and Enhances the Transcription Activity of C/EBP α —We next examined the effect of

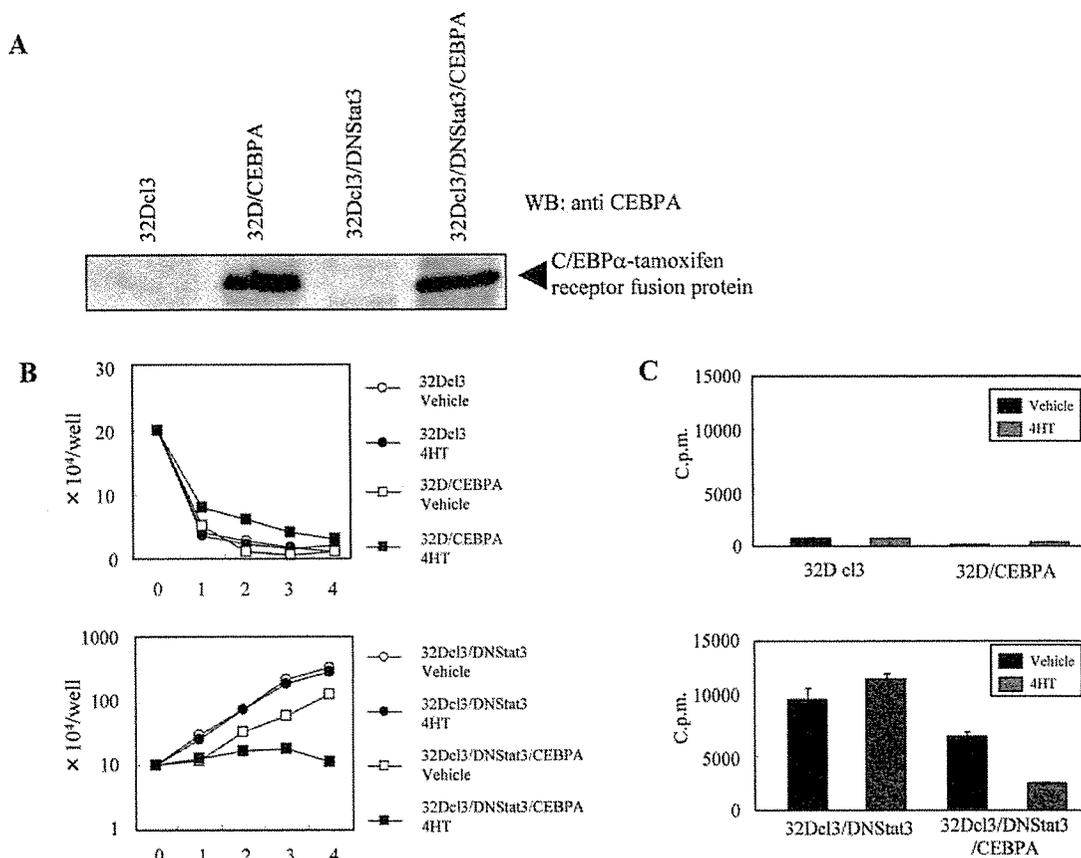


FIG. 5. Proliferation of 32Dcl3 and 32Dcl3/DNStat3 by restoration of C/EBP α . *A*, the expression vector pMY-IRES-GFP/C/EBP α -ER was transfected into 32Dcl3 and 32Dcl3/DNStat3 cells. The expression of C/EBP α -ER was examined by Western blotting (WB) using anti-C/EBP α polyclonal antiserum. *Lane 1*, 32Dcl3; *lane 2*, 32D/CEBPA; *lane 3*, 32Dcl3/DNStat3; *lane 4*, 32Dcl3/DNStat3/CEBPA cells (*upper panel*), and 32Dcl3/DNStat3, 32Dcl3/DNStat3/CEBPA cells (*lower panel*). Cells maintained in IL-3 were washed twice with PBS and starved of cytokines for 8 h and stimulated with 10 ng/ml G-CSF plus 0.5 μ M 4-HT or vehicle. Viable cells were counted daily by trypan blue dye exclusion method at the indicated times. The numbers given on the vertical axis represent the mean cell counts ($\times 10^4$ /well) of triplicate wells. Standard deviations (S.D.) were less than 15% of each mean. Three independent experiments were performed, and similar results were obtained. Data shown are representative of these results. *C*, 3 H incorporation assays in 32Dcl3, 32Dcl3/CEBPA (*upper panel*) and 32Dcl3/DNStat3 and 32Dcl3/DNStat3/CEBPA cells (*lower panel*). Cells maintained in IL-3 were washed twice with PBS and starved of cytokines for 8 h and stimulated with 10 ng/ml G-CSF plus 0.5 μ M 4-HT or vehicle for 48 h. During the final 4 h, 1 μ Ci of [3 H]thymidine was added, cells were harvested by filtration, and radioactivity was counted by scintillation spectrophotometer. Results are expressed as mean cpm of triplicate wells \pm S.D. Three independent experiments were performed, and similar results were obtained. Data shown are representative of these results.

Stat3 abrogation on the balance of intracellular signals in other cytokine pathways. Although Stat1 was not phosphorylated by leukemia inhibitory factor stimulation in neither 32Dcl3 cells nor 32Dcl3/DNStat3 cells, its activation in response to IFN- γ occurred at the same degree in both 32cl3 cells and 32Dcl3/DNStat3 cells (Fig. 3A). As for the Stat5 activation, the phosphorylation of Stat5 by IL-3 stimulation in 32Dcl3 cells was stronger than that in 32Dcl3/DNStat3 cells (Fig. 3B). These data indicated that there was the possibility that abrogation of Stat3 signaling can alter the balance of intracellular signals in other cytokine signaling pathways. The transcription of C/EBP α is regulated by C/EBP α itself (20, 21). Then we examined whether activated Stat3 in G-CSF signaling enhance C/EBP α activity or not.

We transfected a reporter construct of a minimal TK promoter with CEBP-binding sites (p(C/EBP)2TK), C/EBP α , and G-CSF receptor to 293T cells. After 12 h of transfection, cells were stimulated with 10 ng/ml G-CSF. Cells were cultured for more 24 h, and a luciferase assay was performed. C/EBP α up-regulated the C/EBP α -dependent gene expression, and the G-CSF stimulation enhanced this C/EBP α -dependent gene expression (Fig. 4A). Next we examined the effect of constitutive

active Stat3 (Stat3C) on the augmentation of C/EBP α transcriptional activity instead of the G-CSF stimulation. We transfected reporter construct p(C/EBP)2TK, C/EBP α , and Stat3C to 293T cells. After 24 h of transfection, luciferase assay was performed. Stat3C augmented the C/EBP α -dependent gene expression, although Stat3C alone had no influence on the luciferase activity (Fig. 4, B and C).

As p(C/EBP)2TK contains only a C/EBP α -binding site and does not contain a Stat3-binding sequence, the possibility that Stat3C makes a complex with C/EBP α and augments the function of C/EBP α is raised. Then we transfected C/EBP α , Stat3, and G-CSF receptor to 293T cells and stimulated cells with G-CSF for 6 h. There is no detectable level of endogenous C/EBP α or C/EBP β protein in 293T cells. Cells were lysed and immunoprecipitated with C/EBP β antibody (this antibody cross-reacts with C/EBP α). As shown in Fig. 4D, immunoprecipitants with anti-C/EBP β contain Stat3. In addition, the complex formation between C/EBP α and Stat3 is augmented by G-CSF stimulation, indicating that activated Stat3 makes the complex with C/EBP α .

C/EBP α Restores G-CSF-induced Granulocytic Differentiation in 32Dcl3/DNStat3 Cells—To analyze the role of Stat3-

FIG. 6. Morphologic features of 32Dcl3/DNStat3 and 32Dcl3/DNStat3/CEBPA cells. Granulocytic differentiation of 32Dcl3/DNStat3 cells after induction of C/EBP α is shown. Cells were maintained in IL-3 and washed twice with PBS and then starved of cytokines for 8 h and stimulated with 10 ng/ml G-CSF plus 0.5 μ M 4-HT or vehicle for 5 or 8 days. The cells were cytospun and stained with May-Grunwald and Giemsa stain (original magnification, \times 400).

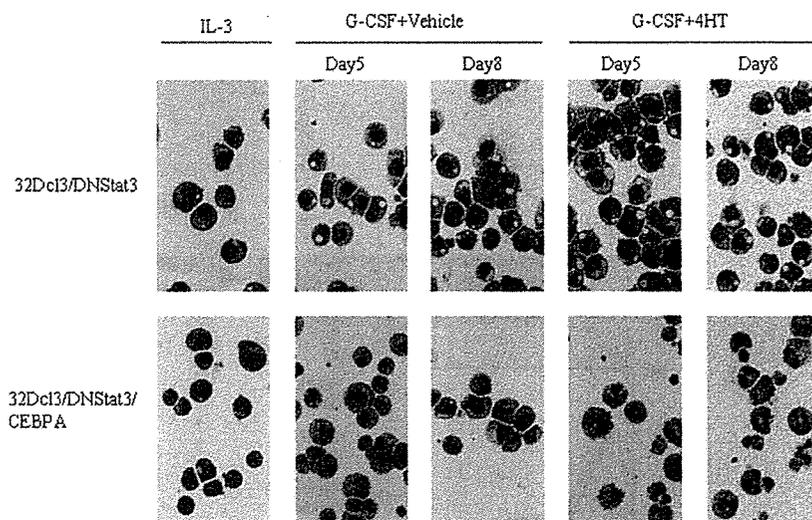


TABLE II
Differential count of 32Dcl3/DNStat3 and 32Dcl3/DNStat3/CEBPA cells

32Dcl3/DNStat3 and 32Dcl3/DNStat3/CEBPA cells were maintained in IL-3 and starved of cytokines for 8 h and stimulated with 10 ng/ml G-CSF plus 0.5 μ M 4-HT or vehicle for 5 days. Differential count was performed by May-Grunwald and Giemsa stain. Values are the mean \pm S.D. percent of cells from three independent experiments. Myelocyte includes promyelocytes, myelocytes, and metamyelocytes. Band(seg) includes band and segmented neutrophils.

Cells	G-CSF+Vehicle	G-CSF+4HT
32Dcl3/DNStat3		
Myeloblasts	98.0 \pm 0	99.3 \pm 0.47
Myelocytes	1.3 \pm 0.94	0.67 \pm 0.47
Band(seg)	0.67 \pm 0.94	0 \pm 0
32Dcl3/DNStat3/CEBPA		
Myeloblasts	90.7 \pm 3.3	3.0 \pm 2.8
Myelocytes	5.0 \pm 0.82	54.3 \pm 2.4
Band(seg)	4.3 \pm 2.6	42.7 \pm 0.47

regulated C/EBP α function in the G-CSF signaling pathway, we transfected a C/EBP α -tamoxifen receptor fusion protein (C/EBP α -ER) into 32Dcl3 and 32Dcl3/DNStat3 cells (32Dcl3/CEBPA cells, 32Dcl3/DNStat3/CEBPA cells, respectively). The expression of C/EBP α -ER in these cells was verified by Western blotting (Fig. 5A). C/EBP α -ER localizes to the cytoplasm and is in an inactive form in the absence of tamoxifen. Upon treatment with tamoxifen, it translocates from cytoplasm to nucleus and becomes active. 32Dcl3, 32Dcl3/CEBPA, 32Dcl3/DNStat3, and 32Dcl3/DNStat3/CEBPA cells were cultured with G-CSF in the presence or absence of tamoxifen, and cell proliferation was examined by both counting viable cells and [3 H]thymidine incorporation. 32Dcl3/DNStat3 proliferated in response to G-CSF, and proliferation was not affected by the presence of tamoxifen. Conversely, G-CSF-induced proliferation of 32Dcl3/DNStat3/CEBPA cells in the presence of tamoxifen was dramatically reduced (Fig. 5, B and C).

32Dcl3/DNStat3 cells maintain morphologically immature characteristics and proliferate without granulocytic differentiation after G-CSF stimulation. We examined the morphological changes in 32Dcl3 and 32Dcl3/DNStat3 cells induced by G-CSF after translocation of C/EBP α from the cytoplasm to the nucleus. When tamoxifen was added to medium containing G-CSF, 32Dcl3/DNStat3/CEBPA cells rapidly began to differentiate into granulocytes, and 5 days later, about 40% of the cells were morphologically similar to mature neutrophils. In contrast, 32Dcl3/DNStat3/CEBPA cells cultured in G-CSF-con-

taining medium without tamoxifen appeared immature with blast-like morphologic features (Fig. 6, Table II). To quantitatively analyze the difference in granulocyte maturation in 32Dcl3/DNStat3/CEBPA cells stimulated by G-CSF in the presence of tamoxifen, the mature granulocyte marker Gr-1 was monitored by FACS analysis. 32Dcl3 cells differentiate into Gr-1-positive neutrophils in response to G-CSF (Fig. 7A). As shown in Fig. 7D, Gr-1-positive cells were increased by the addition of tamoxifen in 32Dcl3/DNStat3/CEBPA cells treated with G-CSF, although low levels were detected in the absence of tamoxifen.

C/EBP α Up-regulates Genes That Are Related to Granulocytic Differentiation—In a conditional expression system, induction of C/EBP α leads to expression of granulocyte-specific genes, such as neutrophil primary granule genes (lysozyme M, NGAL) and the G-CSF receptor gene (17). In 32Dcl3/DNStat3 cells, the expression of these genes following G-CSF stimulation was inhibited (Fig. 8, A, C, and E). Interestingly, only NGAL was up-regulated by G-CSF in 32Dcl3/DNStat3/CEBPA cells following the restoration of C/EBP α (Fig. 8B). Conversely, the expression of lysozyme M and the G-CSF receptor were not changed by the restoration of C/EBP α (Fig. 8, D and F). These data suggest that regulatory factors in addition to C/EBP α may be involved in the induction of expression of granulocyte-specific genes by G-CSF.

DISCUSSION

G-CSF plays a pivotal role in granulopoiesis and granulocytic differentiation. The binding of G-CSF to its receptor leads to the activation of the Jak-Stat pathway, phosphatidylinositol-3 kinase pathway, and Ras-MAP kinase cascade (22). In the Jak-Stat pathway, G-CSF activates Jak1, Jak2, and Tyk2 followed by the activation of Stat1, Stat3, and Stat5 (7, 8).

Dominant-negative Stat3 inhibits G-CSF-induced transcriptional activity of Stat3 (Fig. 1A), as does G-CSF-induced granulocytic differentiation *in vitro* (11). Also, more transgenic mice with a targeted mutation of their G-CSF receptor that abolishes G-CSF-dependent Stat3 activation show severe neutropenia with an accumulation of immature myeloid precursors in their bone marrows (12). Consequently, Stat3 is thought to play an essential role in G-CSF-induced granulocytic differentiation.

32Dcl3 cells differentiate into neutrophils after treatment with G-CSF, and 32Dcl3/DNStat3 cells (32Dcl3 cells expressing dominant-negative Stat3) proliferate in G-CSF without differentiation. The degree of the phosphorylation of ERK1/2 by

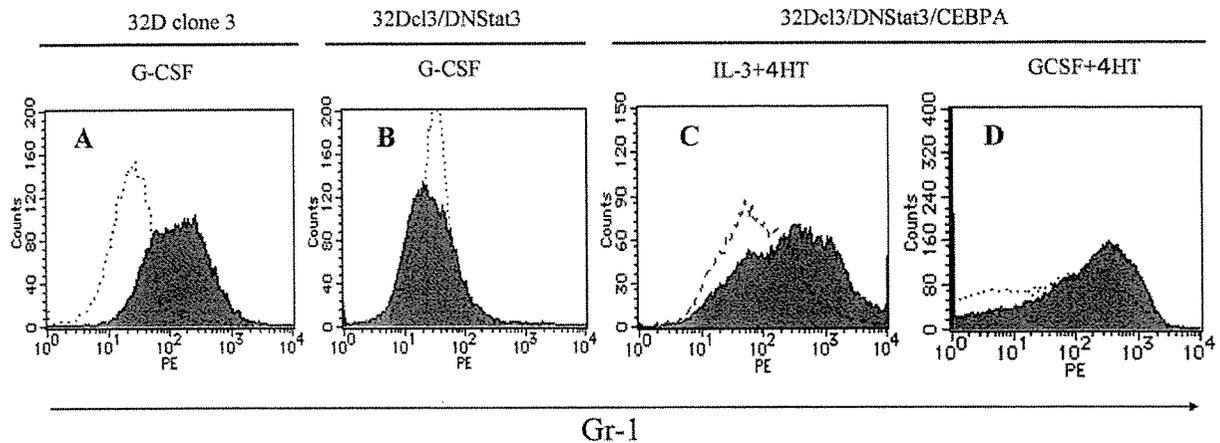


FIG. 7. The expression of Gr-1 on 32Dcl3, 32Dcl3/DNStat3, and 32Dcl3/DNStat3/CEBPA cells. 32Dcl3 (A) and 32Dcl3/DNStat3 cells (B) maintained in IL-3 (broken line) were starved of cytokines for 8 h and stimulated with 10 ng/ml G-CSF for 5 days (solid line). 32Dcl3/DNStat3/CEBPA (C) cells maintained in IL-3 were starved of cytokine for 8 h and stimulated with 1.0 ng/ml IL-3 (C) or 10 ng/ml G-CSF (D) plus 0.5 μ M 4-HT (solid line) or vehicle (broken line) for 5 days.

G-CSF stimulation in 32Dcl3/DNStat3 cells was stronger than that in 32Dcl3 cells (Fig. 1B). We reported that Stat3 null bone marrow cells displayed a significant activation of ERK1/2 after G-CSF stimulation than wild-type bone marrow cells did using Stat3 conditional deficient mice (23). Then the augmented phosphorylation of ERK1/2 in response to G-CSF in 32Dcl3/DNStat3 cells might be caused by the functional abrogation of Stat3 in 32Dcl3/DNStat3 cells.

We compared gene profiles between two cell lines, 32Dcl3 and 32Dcl3/DNStat3 cells, to identify target genes of Stat3 in G-CSF signaling. We found that C/EBP α mRNA levels are rapidly up-regulated in 32Dcl3 cells following G-CSF treatment; these levels are increased 2.39-fold after 6 h and 4.20-fold after 48 h of treatment. In contrast to 32Dcl3 cells, C/EBP α mRNA levels are not changed in 32Dcl3/DNStat3 cells after G-CSF stimulation (Fig. 2A). The observation that cycloheximide does not inhibit G-CSF-induced increases in C/EBP α transcript levels (Fig. 2B) suggests that C/EBP α is induced by G-CSF directly downstream of Stat3. Dahl *et al.* (24) also reported that G-CSF induced the expression of C/EBP α in IL-3-dependent progenitors. SOCS3 is one of the major target genes of Stat3. We previously reported that the expression level of SOCS3 protein in Stat3-deficient bone marrow cells is a trace, and it is not augmented by G-CSF stimulation (23). Contrary to this suppression of SOCS3 in Stat3-deficient cells, the induction of SOCS3 by G-CSF is not abolished in 32Dcl3/DNStat3 cells (data not shown).

The phosphorylation of ERK1/2 by G-CSF is stronger and the phosphorylation of Stat5 by IL-3 is weaker in 32Dcl3/DNStat3 cells when compared with those in 32D/C13 cells, although Stat1 phosphorylation by IFN- γ was not changed between these two cells (Figs. 1B and 3). Then there is the possibility that the transfection of dominant-negative Stat3 affects other signaling pathways in 32Dcl3/DNStat3 cells, resulting in the change of C/EBP α regulation. To clarify whether Stat3 directly up-regulates C/EBP α in the G-CSF signaling pathway in 32Dcl3 cells or not, we examined the effect of Stat3C on the transcription of C/EBP α . C/EBP α up-regulated the C/EBP α -dependent gene expression, and the G-CSF stimulation enhanced this C/EBP α -dependent gene expression (Fig. 4A). Strikingly, Stat3C augmented the C/EBP α -dependent gene expression as G-CSF stimulation did (Fig. 4, B and C). This means that G-CSF-induced up-regulation of C/EBP α -dependent gene expression is, at least partly, due to the activation of Stat3.

Two possibilities arise for the mechanism of the induction of C/EBP α transcription by activated Stat3 in the G-CSF signaling pathway. One is that activated Stat3 binds to the promoter region of C/EBP α and induces the transcription of C/EBP α . Analysis of the reported murine C/EBP α promoter sequence (20) identified no Stat-responsive elements (TTN5AA) (25, 26), but we found six Stat-responsive elements between 6 and 4 kb upstream of the C/EBP α transcription initiation site. Activated Stat3 might bind these Stat-responsive elements between 6 and 4 kb upstream of the C/EBP α transcription initiation site. The other possibility is that activated Stat3 might form the complex with C/EBP α and augment the transcriptional activity of C/EBP α because C/EBP α itself is the only protein reported to activate the murine C/EBP α promoter (20, 21). When a minimal TK promoter with CEBP-binding sites (p(C/EBP)2TK) together with C/EBP α was transfected to 293T cells, C/EBP α up-regulated C/EBP α -dependent gene expression. Activated Stat3 (Stat3C) enhanced this C/EBP α -dependent gene expression in collaboration with C/EBP α , although only Stat3C has no transcriptional activity on p(C/EBP)2TK (Fig. 4, B and C). In addition, the stimulation of G-CSF allows Stat3 to make the complex with C/EBP α (Fig. 4D). Then activated Stat3 by G-CSF makes the complex with C/EBP α and augments the transcriptional activity of C/EBP α . This is one of the reasons why induction of C/EBP α transcript through Stat3 activation by G-CSF occurred in 32Dcl3 cells. Several reports have described factors that repress C/EBP α promoter activity, such as SP1 (27), AP2A (28), or MYC (29). We show here for the first time that Stat3 augments the C/EBP α promoter activity.

Intracellular transcript levels of several genes were changed following G-CSF treatment downstream of Stat3 activation (Table I). To better identify the role of C/EBP α in Stat3-mediated signaling in G-CSF-induced granulocyte differentiation, C/EBP α -ER (C/EBP α -tamoxifen receptor fusion protein) was stably expressed in 32Dcl3 and 32Dcl3/DNStat3 cells. C/EBP α -ER translocates from the cytoplasm to the nucleus and becomes activated upon treatment with tamoxifen. Strikingly, transfection of C/EBP α -ER into 32Dcl3/DNStat3 cells abolished proliferation and induced myeloid differentiation by G-CSF without Stat3 activation (Figs. 5, B and C, and 6). These data indicate that C/EBP α activation induced by G-CSF through Stat3 plays an essential role in stopping the cell proliferation and inducing the differentiation to the myeloid lineage.

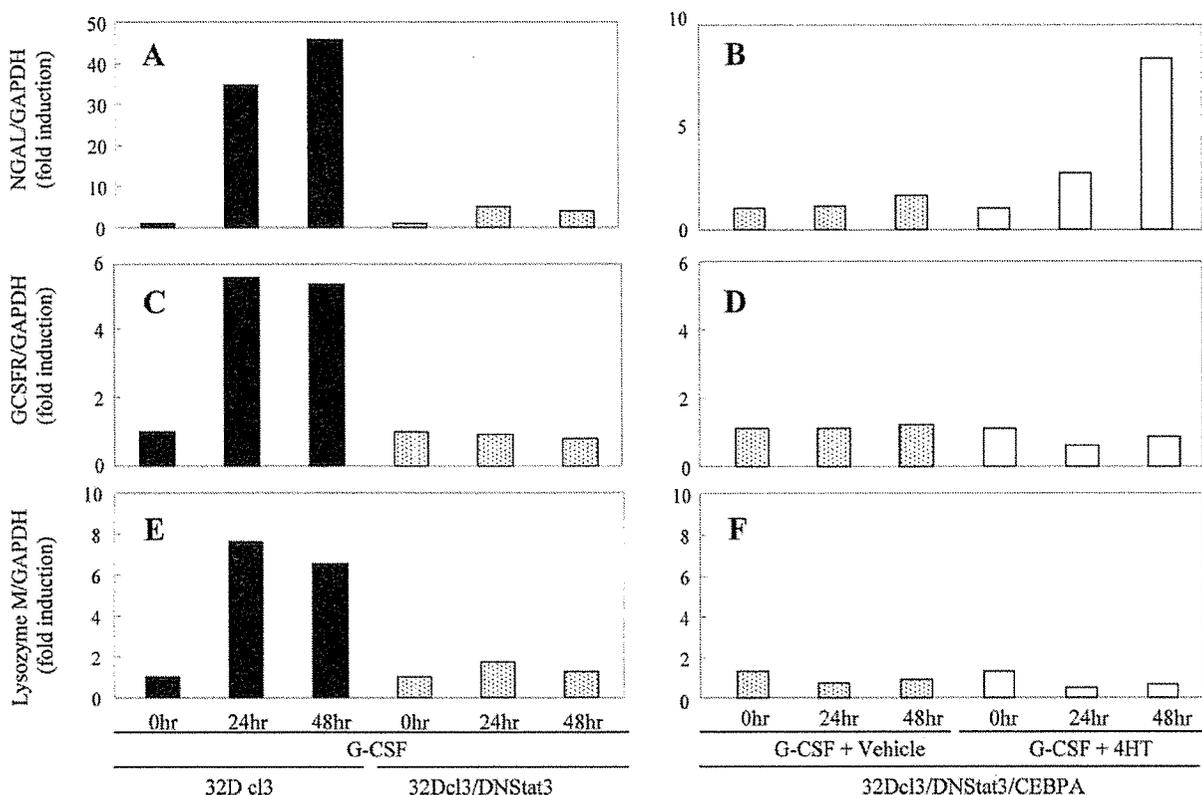


FIG. 8. Granulocyte-specific gene expressions after C/EBP α induction. The time course of NGAL (A and B), G-CSF receptor (*G-CSFR*) (C and D), and lysozyme M (E and F) mRNA expression following G-CSF stimulation in 32Dcl3 and 32Dcl3/DNStat3 cells (A, C, and E) or by G-CSF stimulation with 4-HT or vehicle in 32Dcl3/DNStat3/CEBPA cells (B, D, and F) is shown. Cells maintained in IL-3 were starved of cytokines for 8 h and stimulated with G-CSF, G-CSF, plus 4-HT and G-CSF plus vehicle. Total RNA was isolated at the indicated times after the stimulation and transcribed to cDNA, which was subjected to real-time PCR. The numbers given on the vertical axis represent the fold induction of ratios of average GAPDH-normalized expression values when compared with those before stimulation. Three independent experiments were performed, and similar results were obtained and shown data are the representative of them.

The C/EBP family of transcription factor is expressed in multiple cell types, including hepatocytes, adipocytes, keratinocytes, enterocytes, and cells of the lung (30, 31). C/EBP α transactivates the promoters of hepatocyte- and adipocyte-specific genes, which are important for energy homeostasis (32, 33), and C/EBP α -deficient mice lack hepatic glycogen stores and die from hypoglycemia within 8 h of birth (34). In the hematopoietic system, C/EBP α is exclusively expressed in myelomonocytic cells (35, 36). C/EBP α expression is prominent in mature myeloid cells, and previous investigations found that C/EBP α is critical for early granulocytic differentiation. Mice with a targeted disruption of the C/EBP α gene demonstrate an early block in granulocytic differentiation, but they develop normal monocytes (19). Conditional expression of C/EBP α is sufficient to induce granulocytic differentiation (17). In contrast to the essential role of C/EBP α in granulocytic differentiation, the role of Stat in granulopoiesis is controversial. Stat3 is the principle Stat protein activated by G-CSF, with Stat5 and Stat1 also activated to a lesser degree (8, 10). In mice lacking *Stat5a* and *Stat5b*, the number of colonies produced in response to G-CSF was reduced 2-fold despite normal circulating numbers of neutrophils (9). Myeloid cell lines expressing dominant-negative forms of Stat3 (11, 37, 38) and transgenic mice with a targeted mutation of the G-CSF receptor that abolishes G-CSF-dependent Stat3 activation (12) demonstrate that Stat3-activation is required for G-CSF-dependent granulocytic proliferation and differentiation.

In the present study, we clearly demonstrate that the expression of C/EBP α mRNA is up-regulated through the activation of

Stat3 in response to G-CSF, and the Stat3-C/EBP α signaling cascade plays an important role in G-CSF-induced differentiation. Contrary to these data, however, we and others showed that mice conditionally lacking Stat3 in their hematopoietic progenitors developed neutrophilia, and bone marrow cells were hyper-responsive to G-CSF stimulation (23, 39). Additionally, mice with tissue-specific disruption of *Stat3* in bone marrow cells die within 4–6 weeks after birth with Crohn's disease-like pathogenesis (40). These mice exhibit phenotypes with dramatic expansion of myeloid cells, leading to massive infiltration of the intestine with neutrophils, macrophages, and eosinophils. Cells of the myeloid lineage also demonstrate autonomous proliferation. These apparently disparate results may be explained by the need for molecules in addition to Stat3 to regulate C/EBP α expression *in vivo*, the *in vivo* functional redundancy among C/EBP α regulators, or the absence of the abrogation of SOCS3 induction by G-CSF in 32Dcl3/DNStat3 cells. In 32Dcl3 cells, the Stat3-C/EBP α pathway might be favored, and other pathways may contribute little to granulocytic differentiation in response to G-CSF.

Among C/EBP family, C/EBP ϵ is important for late phase of granulocytic differentiation, and its expression is up-regulated by G-CSF independent of Stat3 (11). A previous report showed that C/EBP ϵ is a transcriptional target of C/EBP α in 32Dcl3 cells (41). From these reports and our results, we speculated that a small amount of C/EBP α is enough for the induction of the transcription of C/EBP ϵ by G-CSF or that there are multiple signaling steps except for Stat3-C/EBP α to induce the transcription of C/EBP ϵ by G-CSF.

Induction of C/EBP α led to not only morphologic differentiation but also expression of granulocyte-specific genes (17). In 32Dcl3/DNStat3 cells, the induction of the G-CSF receptor, lysozyme M, and NGAL in response to G-CSF was abrogated (Fig. 8). Restoration of C/EBP α in these cells led to expression of only the NGAL gene, and thus, 32Dcl3/DNStat3 cells differentiated by the induction of C/EBP α may not be functional as mature neutrophils. In these cells, therefore, activation of C/EBP α is not sufficient for the induction of lysozyme M or G-CSF receptor genes, and the presence of other molecules appears to be required for their expression.

Acknowledgments—We thank M. Sato, R. Hasegawa, and M. Ito for excellent technical assistance.

REFERENCES

1. Metcalf, D. (1989) *Nature* **339**, 27–30
2. Demetri, G. D., and Griffin, J. D. (1991) *Blood* **78**, 2791–2808
3. Lieschke, G. J., Grail, D., Hodgson, G., Metcalf, D., Stanley, E., Cheers, C., Fowler, K. J., Basu, S., Zhan, Y. F., and Dunn, A. R. (1994) *Blood* **84**, 1737–1746
4. Liu, F., Wu, H. Y., Wesselschmidt, R., Kornaga, T., and Link, D. C. (1996) *Immunity* **5**, 491–501
5. Ihle, J. N., Nosaka, T., Thierfelder, W., Quelle, F. W., and Shimoda, K. (1997) *Stem Cells* **15**, Suppl. 1, 105–111; discussion 112
6. Ihle, J. N. (1995) *Nature* **377**, 591–594
7. Shimoda, K., Iwasaki, H., Okamura, S., Ohno, Y., Kubota, A., Arima, F., Otsuka, T., and Niho, Y. (1994) *Biochem. Biophys. Res. Commun.* **203**, 922–928
8. Shimoda, K., Feng, J., Murakami, H., Nagata, S., Watling, D., Rogers, N. C., Stark, G. R., Kerr, I. M., and Ihle, J. N. (1997) *Blood* **90**, 597–604
9. Teglund, S., McKay, C., Schuetz, E., van Deursen, J. M., Stravopodis, D., Wang, D., Brown, M., Bodner, S., Grosveld, G., and Ihle, J. N. (1998) *Cell* **93**, 841–850
10. Tian, S. S., Lamb, P., Seidel, H. M., Stein, R. B., and Rosen, J. (1994) *Blood* **84**, 1760–1764
11. Nakajima, H., and Ihle, J. N. (2001) *Blood* **98**, 897–905
12. McLemore, M. L., Grewal, S., Liu, F., Archambault, A., Poursine-Laurent, J., Haug, J., and Link, D. C. (2001) *Immunity* **14**, 193–204
13. Fukunaga, R., Ishizaka-Ikeda, E., Seto, Y., and Nagata, S. (1990) *Cell* **61**, 341–350
14. Reddy, V. A., Iwama, A., Iotzova, G., Schulz, M., Elsasser, A., Vangala, R. K., Tenen, D. G., Hiddemann, W., and Behre, G. (2002) *Blood* **100**, 483–490
15. Bromberg, J. F., Wrzeszczynska, M. H., Devgan, G., Zhao, Y., Pestell, R. G., Albanese, C., and Darnell, J. E., Jr. (1999) *Cell* **98**, 295–303
16. Aoki, N., and Matsuda, T. (2002) *Mol. Endocrinol.* **16**, 58–69
17. Radomska, H. S., Huettnner, C. S., Zhang, P., Cheng, T., Scadden, D. T., and Tenen, D. G. (1998) *Mol. Cell. Biol.* **18**, 4301–4314
18. Wang, X., Scott, E., Sawyers, C. L., and Friedman, A. D. (1999) *Blood* **94**, 560–571
19. Zhang, D. E., Zhang, P., Wang, N. D., Hetherington, C. J., Darlington, G. J., and Tenen, D. G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 569–574
20. Christy, R. J., Kaestner, K. H., Geiman, D. E., and Lane, M. D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 2593–2597
21. Legraverend, C., Antonson, P., Flodby, P., and Xanthopoulos, K. G. (1993) *Nucleic Acids Res.* **21**, 1735–1742
22. Ihle, J. N. (1996) *BioEssays* **18**, 95–98
23. Kamezaki, K., Shimoda, K., Numata, A., Haro, T., Kakumitsu, H., Yosie, M., Yamamoto, M., Takeda, K., Matsuda, T., Akira, S., Ogawa, K., and Harada, M. (2005) *Stem Cells* **23**, 252–263
24. Dahl, R., Walsh, J. C., Lancki, D., Laslo, P., Iyer, S. R., Singh, H., and Simon, M. C. (2003) *Nat. Immunol.* **4**, 1029–1036
25. Horvath, C. M., Wen, Z., and Darnell, J. E., Jr. (1995) *Genes Dev.* **9**, 984–994
26. Xu, X., Sun, Y. L., and Hoey, T. (1996) *Science* **273**, 794–797
27. Tang, Q. Q., Jiang, M. S., and Lane, M. D. (1999) *Mol. Cell. Biol.* **19**, 4855–4865
28. Jiang, M. S., Tang, Q. Q., McLenithan, J., Geiman, D., Shillinglaw, W., Henzel, W. J., and Lane, M. D. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 3467–3471
29. Mink, S., Mutschler, B., Weiskirchen, R., Bister, K., and Klempnauer, K. H. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 6635–6640
30. Johnson, P. F., Landschulz, W. H., Graves, B. J., and McKnight, S. L. (1987) *Genes Dev.* **1**, 133–146
31. Landschulz, W. H., Johnson, P. F., Adashi, E. Y., Graves, B. J., and McKnight, S. L. (1988) *Genes Dev.* **2**, 786–800
32. Costa, R. H., Grayson, D. R., Xanthopoulos, K. G., and Darnell, J. E., Jr. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 3840–3844
33. Lin, F. T., and Lane, M. D. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 8757–8761
34. Wang, N. D., Finegold, M. J., Bradley, A., Ou, C. N., Abdelsayed, S. V., Wilde, M. D., Taylor, L. R., Wilson, D. R., and Darlington, G. J. (1995) *Science* **269**, 1108–1112
35. Scott, L. M., Civin, C. I., Rorth, P., and Friedman, A. D. (1992) *Blood* **80**, 1725–1735
36. Natsuka, S., Akira, S., Nishio, Y., Hashimoto, S., Sugita, T., Isshiki, H., and Kishimoto, T. (1992) *Blood* **79**, 460–466
37. Shimozaki, K., Nakajima, K., Hirano, T., and Nagata, S. (1997) *J. Biol. Chem.* **272**, 25184–25189
38. de Koning, J. P., Soede-Bobok, A. A., Ward, A. C., Schelen, A. M., Antonissen, C., van Leeuwen, D., Lowenberg, B., and Touw, I. P. (2000) *Oncogene* **19**, 3290–3298
39. Lee, C. K., Raz, R., Gimeno, R., Gertner, R., Wistinghausen, B., Takeshita, K., DePinho, R. A., and Levy, D. E. (2002) *Immunity* **17**, 63–72
40. Welte, T., Zhang, S. S., Wang, T., Zhang, Z., Hesslein, D. G., Yin, Z., Kano, A., Iwamoto, Y., Li, E., Craft, J. E., Bothwell, A. L., Fikrig, E., Koni, P. A., Flavell, R. A., and Fu, X. Y. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 1879–1884
41. Wang, Q. F., and Friedman, A. D. (2002) *Blood* **99**, 2776–2785

Annual Review 血液 2006

2006 年 1 月 25 日 発行

中外医学社

4. マイクロアレイ解析による急性骨髄性白血病の 予後因子の同定

自治医科大学ゲノム機能研究部教授 間野博行

key words DNA microarray, genomics, AC133, karyotype

動 向

ヒトゲノムプロジェクトが終了し我々のもつ蛋白質をコードする遺伝子の全体像が明らかになりつつある。DNA マイクロアレイを用いることで、これら数万種類の遺伝子の発現量を任意の細胞・組織間で簡便に比べることが可能になった。現在急性骨髄性白血病患者サンプルをマイクロアレイで解析し、得られた遺伝子発現プロファイルの中から患者の長期予後にリンクするものをスクリーニングする試みが精力的に行われている。また最近では患者骨髄の単核球全体を解析するのではなく、きわめて幼弱な造血幹細胞分画のみをあらかじめ純化した後マイクロアレイ解析を行うことも試みられている。同定された予後関連遺伝子群の発現量を基に各患者の予後を予測することがこれらのプロジェクトの目標であり、今後は遺伝子発現プロファイルに基づいた急性白血病の新しい分類法が提案されると期待される。

A. 予後予測法の開発—Over view

ついに2003年4月にヒトゲノムプロジェクトの終了宣言が行われ、ヒト染色体の euchromatin 領域のほぼ完全な塩基配列が決定された (<http://www.ncbi.nlm.nih.gov/genome/seq/>)。ヒトのもつ蛋白質をコードする総遺伝子数はおそらく2万~2万5千種類前後になると予想されている¹⁾。かつて夢であったヒトの全遺伝子プール

の全貌がいよいよ明らかになるとうとしており、ポストゲノム時代が訪れたといえる。この膨大な遺伝子情報を有効に使う技術であるゲノミクス・プロテオミクスが、白血病の解析においても重要な役割を担うことは間違いないであろう。今後の医学研究においては、「膨大ではあるがあくまで有限なヒト遺伝子プール」の中から特定の特徴を備えた遺伝子を効率よく同定することが重要になる。ゲノミクス技術の中でもDNA マイクロアレイはすでに複数の疾患で有用な解析ツールとなることが示されてきた^{2,3)}。

マイクロアレイを用いることで数千から数万種類の遺伝子の発現量が比較的簡便に解析可能になった。このようにして得られる遺伝子群の発現様式、発現パターンを「遺伝子発現プロファイル (gene expression profile)」とよぶ。このプロファイルを比べることで白血病の特性に迫るのではないかと考えられる⁴⁾。

具体的な予後予測法の開発手段を図1にまとめる。アルゴリズムの開発を行わずにもととの遺伝子全体の発現パターンから患者を分ける方法 (unsupervised method) もあるが、そのような

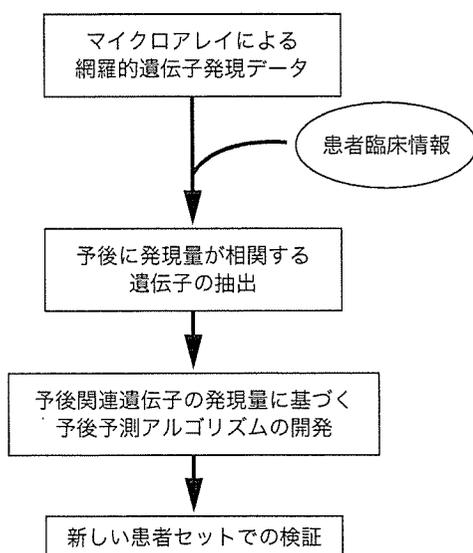


図1 マイクロアレイによる予後予測法開発
DNAマイクロアレイにより得られた膨大な網羅的遺伝子発現データと患者の臨床情報を照合し、予後にリンクした発現量を示す遺伝子をスクリーニングする。次にこれら「予後関連遺伝子」の発現量による予後予測アルゴリズムを開発する。予測法の検証には、最初の解析に用いなかったサンプルを用いることが重要である。

シンプルな解析法で「患者予後」が予測できることはまれであり、図1に示すようなアルゴリズム開発がほぼ必須である。

まず膨大な遺伝子発現データと患者臨床情報（治療反応性、生存期間等）とを照合し、希望する臨床パラメーターに最もよく相関する遺伝子セット、すなわち「予後関連遺伝子」のスクリーニングを行う。具体的には、たとえば患者を「初回寛解導入成功群」と「失敗群」に分け、両群間で統計的有意に発現量が異なる遺伝子を取り出すなどの処理を行う。この場合、一般的なt検定のみのセレクションでは、どちらの群においてもほとんど発現していない遺伝子がしばしば選ばれるため、何らかの付加的なフィルタリングを行う必要がある。また生存期間に直接リンクする遺伝子をCox proportional hazard解析などで取り

出すこともしばしば行われる。さらに後述のアルゴリズム開発の際に、アルゴリズムソフトウェア自身で最適の遺伝子セットを選び出してくれるものもある。

次の段階として選び出された予後関連遺伝子の発現量を用いて患者サンプルの予後を予測するアルゴリズムを開発する必要がある。これには様々なアプローチがあり、代表的なものに weighted-vote 法、k-nearest neighbor 法、Cox proportional hazard 解析による risk index、supported vector machine (SVM) 法、人工知能、などがある。たとえば前2者についてはフリーソフトウェアの GeneCluster2 (<http://www.broad.mit.edu/cancer/software/geneccluster2/gc2.html>) などを用いて解析可能であり、またこのソフトウェアは各アルゴリズムに最適な遺伝子セット自体の抽出も可能である。これらアルゴリズムを用いて各患者の予後関連遺伝子発現量に基づく予後予測を行う。さらにこれら解析法の有用性は、アルゴリズム開発には用いていないサンプルセットによって検証する必要がある。具体的な検証の仕方には、患者全体を「アルゴリズム開発用のトレーニングセット」と「検証用テストセット」に分ける方法と、「全体から1例のみを取りだし残りのサンプルでアルゴリズムを開発して、外しておいた1例を予測する → これを全サンプルについて繰り返す」方法である cross-validation（または drop-one-out 法）とがある。

B. 急性骨髄性白血病 (AML) での応用 —単核球を用いて

AMLは未熟な血液細胞が癌化して生じる疾患であり、これまで様々なパラメーターが治療予後にリンクすることが報告されてきた⁵⁾。現在臨床の場で白血病の診断および予後予測を行う場合、患者骨髄細胞に関して①ペルオキシダーゼ、エス

テラーゼなどの特殊染色, ②FACSを用いた細胞表面マーカーの解析, ③遺伝子異常の有無の解析(免疫グロブリン遺伝子やT細胞表面受容体遺伝子の再構成, BCR-ABL 遺伝子, PML-RAR α 遺伝子などの有無), ④染色体型の解析, などの検査が利用される⁶⁾. これらを総合的に解析することで予後にある程度リンクした診断が行えるが, 上記の解析を全て行うのは煩雑であり多くの専門技術も必要となる. 一方, たとえば1枚のDNAマイクロアレイを用いることで旧来の方法で得られる情報が全て解析可能となれば, 診断はより簡便になりかつ異なった施設間での診断の一致率も上昇するであろう. 骨髄中の血球の多くは単核球(mononuclear cell)とよばれる分画に相当し, これまでAML患者骨髄単核球を用いたDNAマイクロアレイ実験により患者予後を予測する試みが行われてきた.

Yagiらは小児AML患者54例についてAffymetrix社GeneChip HGU95Aチップを用いた遺伝子発現解析を行い, 小児AML内での予後

良好群と不良群とを予測する試みを行っている⁷⁾. 彼らのサンプル内で3年以上完全寛解を維持している症例9例と初回緩解導入に失敗した症例9例の間で発現量が統計的に異なる遺伝子133種類を抽出し(図2A), これら予後関連遺伝子の発現量をもととして, 患者全体を二次元クラスタリング法あるいはSVM法によって層別化した. その結果どちらの方法によってもAML内で生命予後が異なるサブグループを検出することに成功している(図2B). すなわち遺伝子発現プロファイルを基にした新しい疾患分類法の可能性が提示されたことになる.

同様にBullingerらはAML患者116例の骨髄単核球よりmRNAを調整し, 約3万9千種類のcDNAを配置したマイクロアレイで解析を行っている⁸⁾. 彼らは患者をtraining setとtest setに分け, 前者のデータを用いて患者予後に発現量がリンクする遺伝子をスクリーニングした. その結果, 100種類以上の予後予測遺伝子セットを同定することに成功している. さらにこれら遺伝子の発現

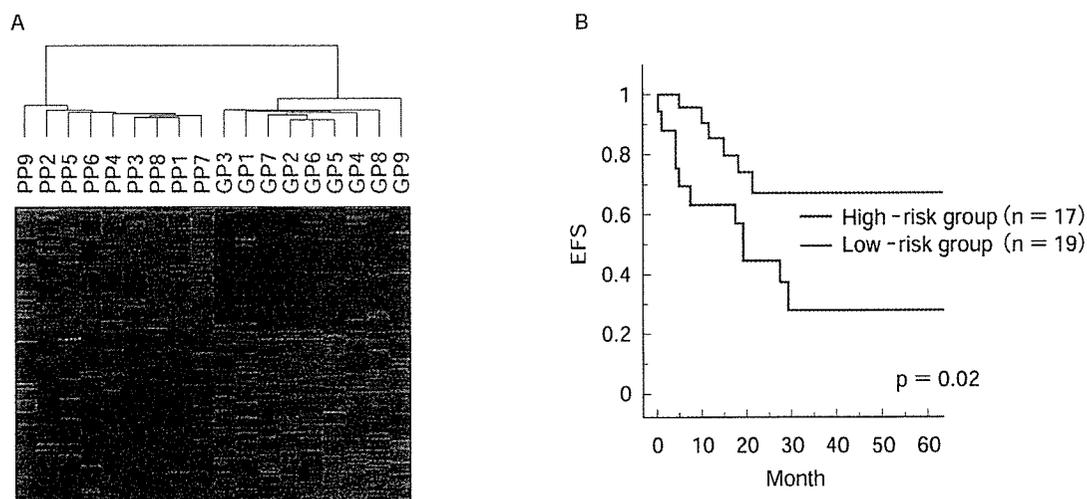


図2 骨髄単核球の発現プロファイルによる予後予測(文献7より改変)

A: 長期生存症例(GP)と予後不良症例(PP)間で発現が異なる遺伝子を取り出したところ133種類同定された. これら遺伝子の発現量で二次元クラスタリングを行った. B: 上記遺伝子の発現量でテストサンプルを二次元クラスタリングし, 大きくわかれた2群間で予後を比較した.

量を基に患者を2群に分け、その予後を比較したところ、2群の生命予後は有意に異なっていた ($p < 0.001$)。またあらかじめ取っておいた test set において検証したところ、やはり2群の予後は大きく違っている ($p = 0.006$) が示された。これらの予後分類法が現行の核型に基づく分類法とは独立したものであることを示すために、彼らは核型分類では中間群に分類される「正常核型」をもった患者のみを選択し、その予後予測も試みている。彼らの分類法を適応したところ、正常核型の中でも予後良好群と不良群とを区別することに成功した ($p = 0.046$)。彼らの解析は旧来の核型分類法とは異なった新しい予後予測法の可能性を示唆しており興味深い。

また Valk らも同様の目的から、285例のAML患者骨髄単核球を用いて約13,000種類の遺伝子に関する発現量を測定した。彼らもやはり予後にリンクした遺伝子発現プロファイルがあることを検出している⁹⁾。

C. 急性骨髄性白血病 (AML) での応用 —純化細胞を用いて

しかし実際にDNAチップを用いた実験を行うと、本法が意外にも偽陽性データが多く効率の悪い手技であることにも気付く。その理由をたとえば健常人と白血病患者の骨髄を比べる場合で考えてみよう (図3)。正常骨髄は様々な系統の細胞群からなる「ヘテロ」な集団である。もし今この骨髄中の多能性幹細胞の1クローンが悪性転化し白血病になったと仮定すると、白血病患者の骨髄は幹細胞由来の白血病細胞が多くを占めるようになり、それ以外の細胞の骨髄における割合は相対的に低下する。したがって両者の骨髄細胞 (あるいは単核球) 全体を単純に比較するようなDNAチップ解析を行うと、造血幹細胞分画以外の細胞 (たとえば図3の青色細胞) 特異的に発現する遺

伝子などは全て減少してみえてしまう。この変化は、それぞれの遺伝子の細胞内における mRNA のコピー数を反映しておらず、誤って「発現減少」と判断されたわけである。一方、幹細胞特異的に発現していた遺伝子は、細胞あたりの発現量の変化の有無にかかわらず骨髄中における割合が増加するために誤って「発現誘導」と判断されることになる¹⁰⁾。これらの様々な偽陽性や偽陰性データのノイズの中から真に「癌化に伴って細胞あたりの発現量が変化する」遺伝子を選択するのはきわめて困難であろう。

ではどのようにしてこれら偽陽性データを回避する効率のよいゲノム解析が可能であろうか？白血病を含む多くの血液悪性疾患が、造血幹細胞あるいはそのごく近傍の幼若細胞における悪性転化に起因することが知られる。そこで患者骨髄より造血幹細胞相当分画のみを純化しこれを用いてマ

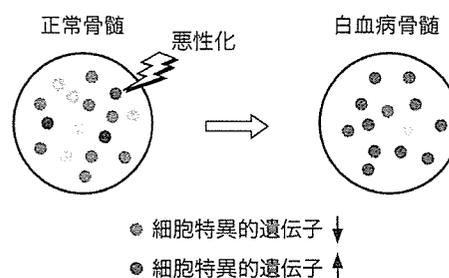


図3 「Population shift 効果」による偽陽性データ
正常骨髄は様々な細胞からなるヘテロな集団である。今その中から緑色細胞が悪性化し白血病が生じたとすると、患者骨髄内は結果的に緑色由来の細胞が大半を占めることになり、それ以外の細胞の割合は相対的に減少する。したがって骨髄全体を正常と白血病患者で比べるような単純なマイクロアレイ解析を行えば、青色細胞特異的な遺伝子は必ず減少しているように見え、逆に緑色細胞特異的な遺伝子の発現は必ず増加しているようにみえてしまう。このような変化は実際の細胞内での発現変化を反映しているわけではなく、単に骨髄内でのポピュレーションの変化を見ているに過ぎない。この「population shift 効果」による見せかけの発現変化が大量の偽陽性データを生み出すもとなる。

マイクロアレイ解析を行えば、患者骨髄中の芽球の割合や芽球の分化傾向の違いに影響されない、精度の高い解析が可能になると期待される。我々は造血幹細胞分画に特異的に発現する膜蛋白 AC133¹¹⁾ に着目し、AC133 に対するアフィニティカラムを用いて広く血液悪性疾患患者より造血幹細胞相当分画を純化保存するバンク事業「Blast Bank」を開始した。2004年12月現在ですでに Blast Bank のサンプル数は600例を超えており、純化細胞のゲノミクスプロジェクトとしては世界最大級といえる^{12,14)}。

では具体的に Blast Bank を用いて白血病の予後予測が可能か否か検討してみよう。我々はヒト全遺伝子が配置された DNA マイクロアレイを用いて標準的化学療法を受けた AML 患者66例の骨髄 AC133 陽性細胞について遺伝子発現プロファイルデータを得た。これら66例の患者中初回化学療法によって完全寛解 (complete remission: CR) に到達した症例は51例あり、残り15例は寛解導入に失敗した。後者15例は1年以内に全員死亡しておりきわめて予後不良なグループといえる。この初回化学療法に成功した患者と失敗した

患者の間では遺伝子発現プロファイルは違うのであろうか？ それを検討するために4万4千種類のプローブセット (3万3千種類の遺伝子に相当) の発現データ中、両グループ間で発現が有意に異なる遺伝子を選び出し、さらに少なくともどちらかのグループでは高い発現量を示す遺伝子をスクリーニングしたところ、最終的に約30種類の遺伝子が得られた。得られた遺伝子の発現プロファイルはそれぞれが互いに独立なわけではなく、よく似たものも多い。そこで correspondence analysis 法¹⁵⁾ によって、これら30種類の発現量パターンから全体を最も代表する仮想発現プロファイルを3種類抽出した (すなわち代表的な仮想発現パターンそれぞれを作る仮想遺伝子3種類を作成したことになる)。これら仮想遺伝子3種類を軸とした仮想空間に各サンプルを投射したのが図4Aである。その結果興味深いことに治療奏効症例と失敗症例とは空間上不連続な位置に独立して存在することがわかった。いい換えると両グループは遺伝子発現プロファイルの面では異なった集団に属するのだ。

このような空間上の位置は患者予後にリンクし

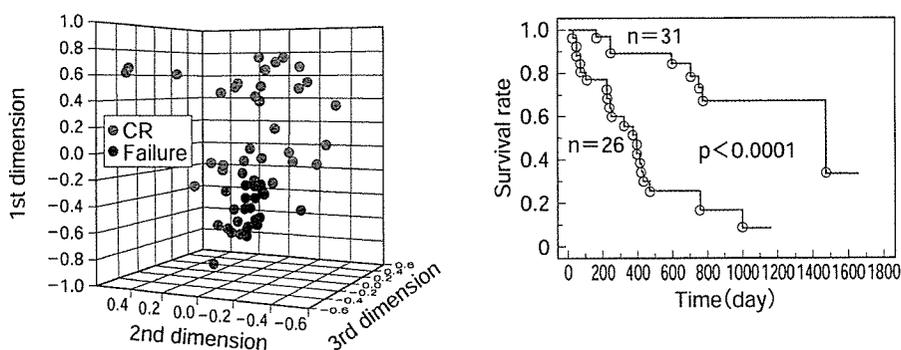


図4 AC133 陽性細胞を用いた予後予測 (文献16より改変)

A: 化学療法による初回寛解導入が成功した群 (CR) と失敗した群 (Failure) との間で有意に発現量が異なる遺伝子を選択し、その発現プロファイルから代表的なものを3種類抽出した。これらプロファイル上の仮想発現量に基づいてサンプルを空間に投射したところ、CR群と Failure群とは異なる位置に配置された。B: 仮想空間のZ軸の値が-0.3未満 (青色) か以上 (赤色) かによって患者を2分し、その予後をKaplan-Meier法で比較した。

ているのであろうか？ 図4Aをよく見てみると、患者サンプルはZ軸上で最も強く分離されていることがわかる。そこで症例の仮想空間上でのZ軸上の値が -0.3 未満か -0.3 以上かによって大きく2群に分け、その長期生命予後をKaplan-Meier法によって解析してみた。図4Bで明らかのように両群は大きく予後が異なることがわかる。しかも様々な方法で予後関連遺伝子を絞り込んだ結果、数種類の遺伝子の発現量を測定するだけで患者の長期予後を予測可能なことまで明らかになった¹⁶⁾。これらの予後関連遺伝子数は、以前の骨髄単核球による解析で同定された100種類以上の予後関連遺伝子数に比べて明らかに少ない。純化した細胞を標的として解析することで分化傾向などによるノイズが低減し、少数の遺伝子で予後予測可能になったのではないかと考えられる。

むすび

DNA マイクロアレイがその新規性のみで注目・検討された時期は終わりつつあり、比較的高額な実験費用に見合うだけの有用性が本当にあるのかが問われる時代になった。ヒトゲノムプロジェクトの成果は、医学にとってきわめて貴重（かつ膨大）な情報リソースであり、この有効活用なくして21世紀の医療はなり立たないであろう。必ずしもDNA マイクロアレイが理想のシステムとはいえないが、現段階でヒトゲノム情報の網羅的利用に関してDNA マイクロアレイを超える技術は存在しない。実際の実験にあたっては「網羅的発現比較によって何を明らかにしたいのか」を明確に念頭に置き、そのための最も効率よいアプローチを検討することが重要であろう。DNA マイクロアレイシステムは慎重にデザインされた実験においてのみ、そのきわめて高いスクリーニング能力を発揮すると考えられる。

文献

- 1) International Human Genome Sequencing Consortium. Finishing the euchromatic sequence of the human genome. *Nature*. 2004; 431: 931-45.
- 2) Dhanasekaran SM, Barrette TR, Ghosh D, et al. Delineation of prognostic biomarkers in prostate cancer. *Nature*. 2001; 412: 822-6.
- 3) van't Veer LJ, Dai H, van de Vijver MJ, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature*. 2002; 415: 530-6.
- 4) Rhodes DR, Chinnaiyan AM. Integrative analysis of the cancer transcriptome. *Nat Genet*. 2005; 37 Suppl: S31-7.
- 5) Grimwade D, Walker H, Oliver F, et al. The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. *Blood*. 1998; 92: 2322-33.
- 6) Bennett JM, Catovsky D, Daniel MT, et al. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. *Ann Intern Med*. 1985; 103: 620-5.
- 7) Yagi T, Morimoto A, Eguchi M, et al. Identification of a gene expression signature associated with pediatric AML prognosis. *Blood*. 2003; 102: 1849-56.
- 8) Bullinger L, Dohner K, Bair E, et al. Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *N Engl J Med*. 2004; 350: 1605-16.
- 9) Valk PJ, Verhaak RG, Beijten MA, et al. Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med*. 2004; 350: 1617-28.
- 10) Miyazato A, Ueno S, Ohmine K, et al. Identification of myelodysplastic syndrome-specific genes by DNA microarray analysis with purified hematopoietic stem cell fraction. *Blood*. 2001; 98: 422-7.
- 11) Hin AH, Miraglia S, Zanjani ED, et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood*. 1997; 90: 5002-12.
- 12) Tsutsumi C, Ueda M, Miyazaki Y, et al. DNA microarray analysis of dysplastic morphology associated with acute myeloid leukemia. *Exp Hematol*. 2004; 32: 828-35.

- 13) Ota J, Yamashita Y, Okawa K, et al. Proteomic analysis of hematopoietic stem cell-like fractions in leukemic disorders. *Oncogene*. 2003; 22: 5720-8.
- 14) Oshima Y, Ueda M, Yamashita Y, et al. DNA microarray analysis of hematopoietic stem cell-like fractions from individuals with the M2 subtype of acute myeloid leukemia. *Leukemia*. 2003; 17: 1990-7.
- 15) Fellenberg K, Hauser NC, Brors B, et al. Correspondence analysis applied to microarray data. *Proc Natl Acad Sci USA*. 2001; 98: 10781-6.
- 16) Mano H. Stratification of acute myeloid leukemia based on gene expression profiles. *Int J Hematol*. 2004; 80: 389-94.

表 題

著 者 名

醫學のあゆみ 別刷

第 卷・第 号： 年 月 日号

遺伝子発現解析に基づく予後判定

Prognosis prediction based on gene expression profile



間野 博行

Hiroyuki MANO

自治医科大学ゲノム機能研究部

◎すでに病因となる遺伝子が明らかなリンパ腫においては、その遺伝子の発現を RT-PCR 法で定量することにより鑑別診断および患者の予後判定が可能になりつつある。一方、病因が不明なリンパ腫においては、DNA マイクロアレイを用いた患者の層別化が新しいリンパ腫の予後予測法として注目されている。DNA マイクロアレイを用いることで数千~数万種類の遺伝子の発現量を一度の実験で明らかにすることができるが、これら膨大な発現データのなかから患者の治療反応性・長期予後にリンクした遺伝子を同定することが現在研究されている。



Key
word

定量的real-time RT-PCR法, 染色体転座, DNAマイクロアレイ, 遺伝子発現プロファイル

t(15;17)を有する急性前骨髄球性白血病が、その原因遺伝子産物を標的としたオールトランスレチノイン酸によって寛解導入可能なこと、またt(9;22)を有する慢性骨髄性白血病が、やはりその原因遺伝子産物を標的としたSTI571によって高率に細胞遺伝学的寛解に導入可能なことは、いずれも病因単位の疾患分類が治療・予後予測に直結していることを明瞭に示している。したがって、WHOによる新しい白血病分類案も原因遺伝子による区分を優先したものになった¹⁾。

一方、リンパ腫の発生メカニズムはいまだ不明な点が多く、原因遺伝子異常が明らかになったものとして、①マンツル細胞リンパ腫におけるcyclin D1の高発現、②濾胞性リンパ腫におけるBCL2の高発現、③びまん性大細胞型リンパ腫におけるBCL6の高発現、④未分化大細胞型リンパ腫におけるNPM-ALK融合遺伝子の発現、および、⑤MALTリンパ腫におけるAPI2-MALT1融合遺伝子の発現などがあげられるにすぎない。したがって、現行のリンパ腫の疾患分類単位は多くの場合“症候群”と考えるべきであろう。

病因が解明されている場合は、その発症責任遺伝子あるいは遺伝子産物の発現を定量すること

で、治療法の選択および患者の予後予測などに重要な情報を得ることができる。しかし、病因が不明な場合のさらなる層別化には、このようなアプローチは不可能であり、現段階ではDNAマイクロアレイが有効なツールと期待されている。これら遺伝子発現の定量法は、他の免疫組織化学染色、核型解析、フローサイトメータ解析などと組み合わせられることで、各患者の予後予測に重要な役割を果たすと予想される。



単一遺伝子の発現定量

1. マントル細胞リンパ腫

マンツル細胞リンパ腫の多くにt(11;14)(q13;q32)転座が認められる。この染色体異常の結果、免疫グロブリンIgH遺伝子の転写調節領域がサイクリンD1(CCND1)遺伝子と結合し、後者の遺伝子発現を誘導することになる。CCND1は代表的なG1期サイクリンであり、サイクリン依存性キナーゼ(CDK)を活性化させることで細胞周期をG1期からS期に移行させる。マンツル細胞リンパ腫においてはCCND1の発現が正常のコントロールからはずれ、IgHのプロモーターエンハンサーによって強制的に活性化されることで細胞の

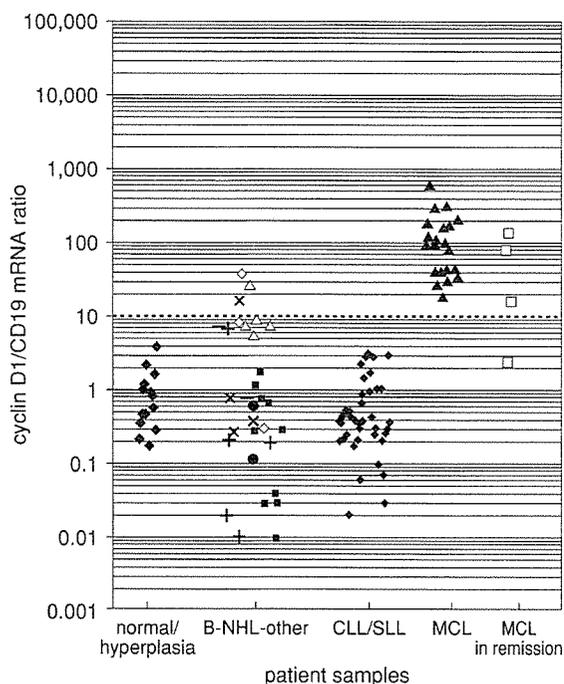


図1 リンパ腫患者におけるcyclin D1 mRNAレベル(文献³⁾より改変)

健常人および良性リンパ節過形成(normal/hyperplasia), 慢性リンパ性白血病および小リンパ球性リンパ腫(CLL/SLL), マントル細胞リンパ腫(MCL)およびその寛解期(in remission), さらにそれ以外のBリンパ球性非Hodgkinリンパ腫(B-NHL-other)の各検体について定量的real-time RT-PCR法を行い, cyclin D1 cDNAとCD19 cDNAの比をプロットした。

異常増殖がもたらされると予想されている。CCND1転写の異常活性化は他のリンパ腫ではほとんど認められず, マントル細胞リンパ腫の鑑別診断上きわめて重要である。とくに同リンパ腫の生命予後は悪いため, CD5⁺リンパ腫の診断上, CCND1の発現確認は必須の検査といえよう。

これまで, CCND1の発現は抗CCND1抗体を用いた免疫組織化学染色によって判定されることが多かった。しかし, CCND1蛋白の染色性は用いる抗体によってまちまちであり, 近年は定量的RT-PCR法によってCCND1 mRNAを直接測定することが試みられるようになった。Huiらは定量的PCR法であるreal-time RT-PCR法によりパラフィン包埋リンパ節検体のCCND1 mRNAの定量を行い, 他の蛋白質定量法との比較検討を行っている。その結果, mRNA定量法は免疫組織化学染色法と同程度以上の検出感度を有することが明らか

かになった²⁾。CCND1 mRNAの高発現が真にマントル細胞リンパ腫に特異的であれば, 本来悪性細胞が微量にしか存在しない末梢血や骨髓血細胞などを検体としても, 同疾患の診断が可能ではないかと予想される。そこでHoweらは, これら腫大リンパ節以外の臨床検体においてRT-PCR法により疾患特異的なCCND1 mRNA増加を検出できないか検討した³⁾。CCND1 mRNAとCD19 mRNAとの量比をプロットしたところ, 図1に示されるとおりマントル細胞リンパ腫特異的な上昇が確認された。本法は, 最適化されることで実際の臨床の場できわめて予後不良のリンパ腫を精度よく診断可能にすると期待される。しかも図1にあるように, その測定値はリンパ腫細胞量の減少に伴い低下しており, 末梢血を用いた検査でリンパ腫の残存量をフォローできる可能性が示唆された。

2. MALTリンパ腫

一部の悪性リンパ腫においては, 染色体転座の結果, 疾患特異的な融合遺伝子が生じる。融合遺伝子は疾患細胞以外には存在しないため, その検出は鋭敏かつ信頼性の高い診断法となる。悪性リンパ腫における融合遺伝子として, これまでMALTリンパ腫におけるAPI2-MALT1と未分化大細胞型リンパ腫のNPM-ALKが知られている。前者はt(11;18)(p21;q21.2)の, 後者はt(2;5)(p23;q35)の染色体転座の結果それぞれ生じたものである。たとえば, API2-MALT1融合遺伝子はAPI2遺伝子の図2上の矢印に示す位置で切断が生じ, 同図下の矢印の箇所でMALT1遺伝子に結合する。したがって, この結合部位を挟む形でAPI2 cDNA上に5'-プライマーを, MALT1 cDNA上に3'-プライマーを設定すれば, 融合遺伝子由来cDNAのみを増幅することができる。実際, Liuらは罹患部位より調整したRNAをもとにcDNAを作製し, 融合部位の5'側, 3'側それぞれに2種類のプライマーを設定したnested PCRをかけて融合cDNAの検出(RT-PCR)を行った⁴⁾。彼らは100万個中に1個しか存在しない悪性細胞もこの方法で検出可能なことを示しており, きわめて鋭敏に診断可能なことを明らかにした(ただしnested PCRのデータは定量的ではなくなるが多い)。