Jpn. J. Cancer Res. 87, 269–274, March 1996

Establishment of an Undifferentiated Leukemia Cell Line (Kasumi-3) with t(3;7)(q27;q22) and Activation of the EVI1 Gene

Hiroya Asou,[1] Kazumi Suzukawa,[3] Kenkichi Kita,[4] Kazunori Nakase,[4] Haruo Ueda,[2] Kazuhiro Morishita[3] and Nanao Kamada[1,5]

[1]Department of Cancer Cytogenetics, Research Institute for Nuclear Medicine and Biology, [2]Department of Pediatrics, School of Medicine, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734, [3]Biology Division, National Cancer Center Research Institute, Tsukiji 5-1-1, Chuou-ku, Tokyo 104 and [4]Department of Second Internal Medicine, Mie University School of Medicine, Edobashi 2-174, Tsu, Mie 514

A novel human leukemia cell line (Kasumi-3) was established from the blast cells of a 57-year-old man suffering from myeloperoxidase-negative acute leukemia. The cell line had five distinctive features, as follows. 1) Flow cytometric analyses showed cell surface expression of CD7, CD4, CD13, CD33, CD34, HLA-DR and c-Kit. This phenotype is compatible with that of acute myelocytic leukemia cells with the M0 subtype in the French-American-British classification. 2) Kasumi-3 cells carried chromosomal abnormalities of t(3;7)(q27:q22), del(5)(q15), del(9)(q32), and add(12)(p11). The breakpoint of 3q27 was located near the EVI1 gene, and a high level of expression of the EVI1 gene was observed. 4) Kasumi-3 cells treated with TPA showed maturation to monocytic lineage. 5) Treatment with either interleukin (IL)-2, IL-3, IL-4, granulocyte-macrophage colony-stimulating or stem cell factor induced the proliferation of Kasumi-3 cells. Thus, the Kasumi-3 cell line shows the characteristic features of undifferentiated leukemia. It should, therefore, be useful both for studying the biological characteristics of acute myelogenous leukemia M0 subtype and for investigating the role of the EVI1 gene in leukemogenesis.

Key words: Lëukemia — Cell line — AMLM0 — t(3;7) — EVI1

Rearrangement of chromosome 3 involving bands 3q21 and 3q26 has been found in approximately 2–3% of acute myelogenous leukemia (AML) or myelodysplastic syndrome (MDS).[1,2] Clinically, AMLs with 3q26 chromosomal abnormality have been associated with morphologically abnormal megakaryocytes, elevated or normal platelet counts and a poor prognosis.[1,3] Therefore, this group of AMLs was termed the 3q21q26 syndrome. Monosomy 7 is the most frequent additional chromosomal abnormality in AMLs with 3q26 abnormalities.[2,3] Recently, aberrant expression and rearrangements of the ectopic virus integration-1 (EVI1) gene, located at 3q26, has been reported in AMLs with inv(3)(q21q26) and t(3;3)(q21;q26).[4,5] But the role of the EVI1 gene in leukemogenesis is not yet clear. To investigate the function of the EVI1 gene in leukemogenesis, leukemia cell lines with activation of the EVI1 gene and 3q chromosomal abnormalities are essential. We report here the establishment of a unique undifferentiated leukemia cell line (Kasumi-3), which has t(3;7)(q27; q22) and activation of the EVI1 gene.

MATERIALS AND METHODS

Case history The Kasumi-3 cell line was derived from the blast cells in bone marrow of a 57-year-old Japanese man with myeloperoxidase-negative acute leukemia refractory to chemotherapy. He had been admitted to the Yamada Red Cross Hospital (Mie) on July 3, 1990. Cytogenetic analysis showed 46, XY, t(3;7)(q27;q22), del(5)(q15), −8, del(9)(q32), add (12)(p11), +mar. Chemotherapy including doxorubicin, vincristin, cyclophosphamide, and prednisolone was completely ineffective. He died 16 days after admission, due to leukemic cell infiltration into the central nervous system. A detailed case report has already been published.[6]

Cell culture Mononuclear cells were separated by Ficoll-Conray gradient centrifugation from a bone marrow sample obtained on July 18, 1990, when the patient's leukocyte count was 552,000/μl with 100% blasts. Leukemic cells were cultured in RPMI-1640 medium with 20% fetal bovine serum (FBS) at 37°C in humid conditions in a 7.5% CO_2 atmosphere. The cells were suspended in the medium to give a final cell concentration of 1×10^6 cells/ml.

Light microscopy Blood smears were stained with May-Grünwald Giemsa (MGG), myeloperoxidase (MPO), α-naphthyl butyrate esterase (NBE), naphthol AS-D chloroacetate esterase (CAE), acid phosphatase (AP), periodic acid-Schiff (PAS) and neutrophil alkaline phosphatase (NAP) for morphological studies and the determination of cytochemical reactions.

Surface marker analysis Cell surface antigens were detected with a Cytron flow cytometer (Ortho Diagnostic

[5] To whom correspondence should be addressed.

Systems, Tokyo). Acetone-fixed cytocentrifuged smears were used for the detection of cytoplasmic CD3 (cCD3), which was visualized with an alkaline phosphatase substrate kit I (Vector Laboratories, Burlingame, CA) after treatment with biotin-labeled horse antimouse antibody (Vector) and alkaline phosphatase-conjugated avidin (Dakopatts, Glostrup, Denmark).

Cytogenetic studies Chromosome analyses of original leukemic cells in the bone marrow at diagnosis and the Kasumi-3 cell line were done by the Giemsa banding technique as reported previously.[7]

Southern and Northern blot hybridization DNA and RNA extraction and Southern/Northern blot hybridization were performed as described previously.[8] DNA probes for determining the gene structures of the immunoglobulin heavy chain (IgH), TCRβ, TCRγ, TCRδ were; JH for the joining (J) region gene of IgH, Cβ1 for the constant (C) region gene of TCRβ, Jγ1 gene for TCRγ, and Cδ for the C region genes of TCRδ. The cDNA probe for the human c-kit gene was kindly provided by Dr. J. Nakao (Kaketsuken Inc., Kumamoto). H1-la, a cDNA clone of the EVI1 gene, was used as the probe to detect EVI1 gene expression.[9]

Pulsed-field gel electrophoresis (PFGE) Agarose plugs from cultured cells were prepared by adding 1.6% low-melting-point agarose in phosphate-buffered saline (PBS) to an equal volume of cell suspension in PBS ($1.2–2.4 \times 10^7$ cells/ml). The plugs were incubated in 0.5 *M* EDTA, 1% N-laurylsarcosine, and 1 mg of proteinase K per ml for two nights at 50°C, and washed twice at room temperature for 2 h in 10 m*M* Tris HCl (pH 7.5) and 1 m*M* phenylmethylsulfonyl fluoride. DNA in the plugs was digested with the incubated enzymes for one night. After digestion, the plugs were loaded onto a 1% agarose gel. PFGE was carried out on a CHEF Mapper apparatus (Bio-Rad Laboratories, Hercules, CA) at 14°C with ramped pulses from 26.3–115 s for over 25 h at a constant voltage of 200 V. The DNA were transferred onto nylon membranes, and the filters were hybridized as described previously.[10]

^{3}H-Thymidine uptake To determine the proliferation activities of Kasumi-3 cells, ^{3}H-thymidine uptake was measured; various hematopoietic growth factors were also added to the culture, as described previously.[7] Recombinant human interleukin 2, IL-3, IL-4, IL-5, IL-6, IL-7, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), and erythropoietin (EPO) were used as stimulating factors at the following concentrations: 10 ng/ml of interleukin (IL)-2 (Genzyme, Cambridge, MA), 10 ng/ml of IL-3 (Genzyme), 10 ng/ml of IL-4 (Genzyme), 10 U/ml of IL-5 (Genzyme), 10 U/ml of IL-6 (Genzyme), 10 U/ml of IL-7 (Genzyme), 100 ng/ml of G-CSF (Chugai, Tokyo), 10 ng/ml of GM-CSF (Sankyo Co., Tokyo), 1 U/ml of SCF (Genzyme), and 10 U/ml of EPO (Sankyo).

Induction of cellular differentiation Cells were treated separately with 1.25% dimethyl sulfoxide (DMSO), 10^{-7} mol/liter phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), all-*trans* retinoic acid (ATRA), G-CSF, GM-CSF, IL-2, IL-3, IL-4, IL-5, or SCF. After 5 days of culture in RPMI 1640 medium with 20% FBS, DMSO, TPA, or ATRA at cell concentrations of 2×10^5/ml, smears were prepared for morphologic observations and were analyzed for MGG, MPO, NAP, NBE, and CAE staining. The morphology of cells cultured with cytokines was observed on the 5th and 10th days of culture.

RESULTS

Establishment of Kasumi-3 cell line After 60 days of culture, a cell line was established and designated Kasumi-3. The Kasumi-3 cells have been continuously proliferating in a suspension culture for more than 60 months, with a doubling time of 55 to 60 h. The morphology of the cells is consistent with that of immature lymphoblasts, these cells being uniform in size with abundant basophilic cytoplasm, multiple nucleoli, and no granules (Fig. 1). The original leukemia cells and the Kasumi-3 cell line were positive for AP and negative for MPO, NBE, CAE, PAS, and NAP.

Both the original leukemia cells and the Kasumi-3 cells were positive for CD7, CD4, CD13, CD25, CD33, CD34, and HLA-DR. Surface and cytoplasmic CD3, CD36, CD41 and CD42 were negative (Table I). The Kasumi-3 cells and the original leukemic cells had common chromosomal abnormalities: 46, XY, t(3;7)(q27;q22), del(5)(q15), −8, del(9)(q32), add(12)(p11), +mar (Fig. 2). Additional abnormalities, t(2;5)(p13;q33) and add(16)(q13), appeared 10 months after estab-

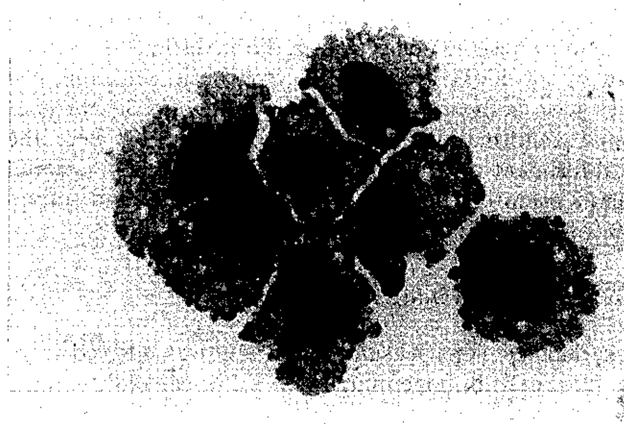

Fig. 1. Morphology of Kasumi-3 cells (MGG staining).

Table I. Immunophenotype of Kasumi-3 Cells and the Patient's Original Leukemic Cells

CD	MoAb	Original leukemia cells (%)	Kasumi-3 cell line (%)
2	T11	30	0
3	Leu4	0	0
cCD3	Leu4		negative
4	Leu3a	81	69
5	Leu1	2	0
7	TP40	74	97
8	Leu8		0
10	J5	0	0
11a	LFA-1		99
11b	OKM1		9
11c	LeuM5		6
13	MCS2	97	99
14	My4		1
15	MCS1		1
19	Leu12	0	0
20	Leu16	0	0
25	Tac		87
33	My9	99	96
34	My10	99	86
36	OKM5		0
41	IIb/IIIa	0	0
42	GPIb		0
c-kit			79
HLA-DR	Nu-la	90	52
HLA-DQ	Leu10		0
	Mac-1		99
	ICAM-1		24

MoAb, monoclonal antibody.

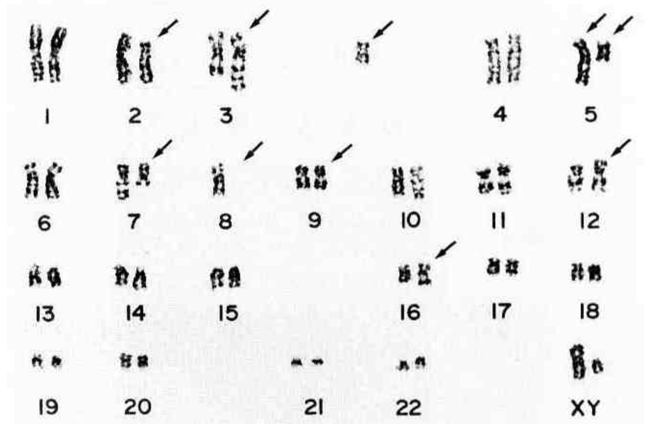

Fig. 2. Representative karyotype of Kasumi-3 cells; 46, XY, t(2;5)(p13;q33), t(3;7)(q27;q22), del(5)(q15), −8, del(9)(q32), add(12)(p11), add(16)(q13), +mar. The t(2;5)(p13;q33) and add(16)(q13) appeared 10 months after the establishment of the cell line.

lishment of the cell line. Southern blot hybridization showed no rearrangement bands with the IgH, TCRβ, TCRγ, or TCRδ gene probes (data not shown).

Detection of EVI1 gene transcripts in the Kasumi-3 cell line To assess EVI1 gene expression, poly(A)RNA was prepared from leukemia cells for Northern hybridization. As shown in Fig. 3, three out of four human cell lines expressed major 6.0 kb transcripts, comparable in size to EVI1 transcripts detected in the ovary.[10] UCSD/AML1 cells[11] showed high levels of expression of EVI1 transcripts, as shown in this figure, but the same sizes of transcripts were detected in UCSD/AML1 RNAs with short-term exposure of the same film (data not shown).

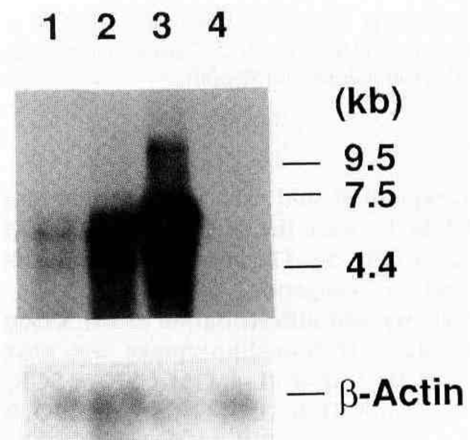

Fig. 3. Detection of EVI1 transcripts by Northern blot analysis in human leukemia cell lines. HEL cell line was used as a positive control.[4] The blot was hybridized with the H1-1 EVI1 cDNA probe (upper panel) and a β-actin probe (lower panel). Lane 1, Kasumi-3; lane 2, HEL; lane 3, UCSD/AML1; and lane 4, SCMC/L1.[18]

Detection of chromosomal breakpoints near the EVI1 gene To identify the breakpoint near the EVI1 gene, we used pulsed-field gel electrophoresis. In previous studies, we had constructed a restriction map of around 1,700 kb surrounding the region of the EVI1 gene and mapped two AML cases with t(3;3)(q21;q26), which are located 15 kb and 300 kb upstream from the 5′ end of the EVI1 gene. Recently we mapped the chromosomal breakpoint of inv(3)(q21q26) to within a 300 kb region downstream from the 5′ end of the EVI1 gene. To identify rearrangement by PFGE, we used an EVI1 cDNA probe (H1-1), which detected a *Sfi* I 1,500 kb fragment and a *Bss*H II 940 kb fragment in control samples. As shown in Fig. 4, the Kasumi-3 DNA contained a novel *Sfi* I 420 kb fragment, but no novel fragment was detected with the H1-1 probe in *Bss*H II-digested DNA. Since the *Sfi* I site is not affected by methylation and we have never detected any DNA polymorphism of the *Sfi* I site, the chromo-

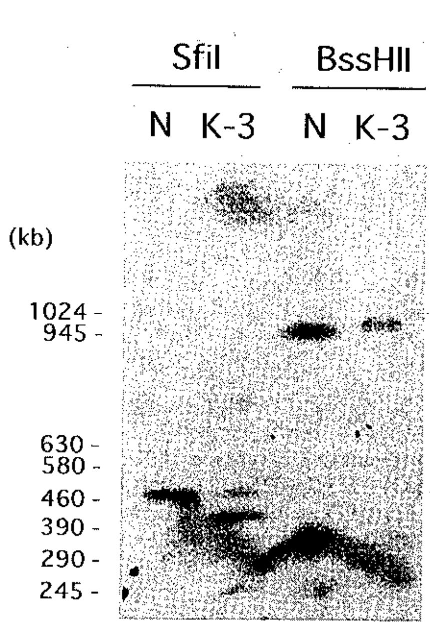

Fig. 4. Identification of chromosomal rearrangements by PFGE analysis. The H1-1 EVI1 cDNA probe was hybridized to *Sfi* I- and *Bss*H II-digested DNA from normal peripheral blood leukocytes (lane N), and Kasumi-3 cells (lane K-3). DNA markers are shown on the left.

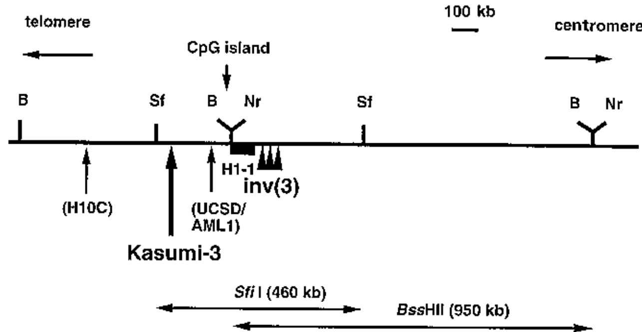

Fig. 5. Mapping of the chromosomal breakpoint at 3q26 in the Kasumi-3 cells with t(3;7). The breakpoint in the Kasumi-3 cells is indicated by a long arrow. The restriction map of the human EVI1 locus was constructed and the breakpoints of two AMLs with t(3;3)(H10C and UCSD/AML1 cells) were determined previously. Recently, we mapped the chromosomal breakpoints at 3q26 in three cases of AMLs with inv(3), as indicated by arrowheads.[5] H1-1, the cDNA clone of the EVI1 gene, was used as a probe for PFGE analysis. The restriction enzymes indicated are: *Nru* I (Nr), *Bss*H II(B), and *Sfi* I (Sf).

somal breakpoint should exist in the region of approximately 200 kb between the *Sfi* I and *Bass*H II sites of the 5′EVI-1 gene (Fig. 5). The precise location of the breakpoint is under investigation.

Growth activity and differentiation of the Kasumi-3 cell line Increased ^{3}H-thymidine uptake was observed in cultures with IL-2, IL-3, IL-4, GM-CSF, or SCF, but not in those with IL-5, IL-6, IL-7, G-CSF, or EPO. SCF and IL-3 had a synergistic effect on the proliferation of the Kasumi-3 cells (Table II).

Table II. ^{3}H-Thymidine Uptake of Kasumi-3 Cells Cultured with Various Cytokines

Cytokine	cpm
Control	494±113
G-CSF	515±119
GM-CSF	1,082±123
IL-2	3,096±779
IL-3	1,777±370
IL-4	2,970±369
IL-5	550±70
IL-6	734±72
IL-7	259±38
SCF	1,601±247
SCF+IL-3	2,561±681

TPA induced the Kasumi-3 cells to differentiate into adherent and monocytoid cells, but the megakaryocytic markers, CD41a and CD42, were not expressed on the cells upon the addition of TPA. DMSO, ATRA, and cytokines did not induce CD2, CD3, and CD8 markers on the Kasumi-3 cells (data not shown).

DISCUSSION

The Kasumi-3 cell line has a unique phenotype which meets the FAB criteria for AML-M0[12]: MPO$^-$ CD13$^+$ CD33$^+$ and B, T lineage markers. Recently, a cytogenetic profile of AML-M0 was reported by Cuneo *et al.*[13] They reported that 5q−, 7q−, and rearrangements involving 12p and 2p were frequently observed among 26 cases with AML-M0. Kasumi-3 cell line contains structural abnormalities of 5q, 7q, 12p and 2p. So, Kasumi-3 cell line could be a representative leukemia cell line with FAB M0 subtype.

The CD7 expression on the Kasumi-3 cell line is interesting, because CD7 is frequently expressed in undifferentiated leukemias.[14–16] Neither rearrangement of T cell receptor genes nor expression of CD3 antigen was detected in the Kasumi-3 cell line. So, the CD7 positivity in the Kasumi-3 cell line seems to reflect the primitive characteristics of the cell line rather than commitment to T cell lineage.

Kasumi-3 cells have EVI1 gene expression, as detected by Northern blot analysis, and the chromosomal break-

point exists in a 200 kb region on the 5′ side of the EVI1 gene. A point of particular interest is the partner of 3q27; t(3;7)(q27;q22). Loss of a chromosome 7 is frequently accompanied with 3q21 and/or 3q26 abnormalities.[2, 3] Moreover, abnormality of chromosome 7 is commonly observed in secondary leukemia.[17] It is conceivable, therefore, that the rearranged gene located in 7q22 of the Kasumi-3 cell line may play an important role in leukemogenesis, as well as the EVI1 gene at 3q26. Precise analysis of the breakpoint is now being undertaken by chromosomal walking. Gene(s) located in 7q22 will be cloned from the cell line. Furthermore, the mechanism of the EVI1 gene activation will be investigated using the Kasumi-3 cell line.[19] The Kasumi-3 cell line should be a useful model to investigate biological characteristics and oncogenes related to human acute leukemias of FAB M0 type.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science, Sports and Culture of Japan.

(Received September 20, 1995/Accepted December 18, 1995)

REFERENCES

1) Lee, E. J., Schiffer, C. A., Tomiyasu, T. and Testa, J. R. Clinical and cytogenetic correlations of abnormal megakaryopoiesis in patients with acute leukemia and chronic myelogenous leukemia in blast crisis. *Leukemia,* **4,** 350–353 (1990).
2) Fonatsch, C., Gudat, H., Lengfelder, E., Wandt, H., Silling-Engrelhardt, C., Ludwig, W. D., Thiel, E., Freund, M., Bodenstein, H., Schwieder, G., Gruneisen, A., Aul, C., Schnittger, S., Rieder, H., Haase, D. and Hild, F. Correlation of cytogenetic findings with clinical features in 18 patients with inv(3)(q21q26) or t(3;3)(q21;q26). *Leukemia,* **8,** 1318–1326 (1994).
3) Bitter, M. A., Neilly, M. E., Le Beau, M. M., Pearson, M. G. and Rowley, J. D. Rearrangements of chromosome 3 involving bands 3q21 and 3q26 are associated with normal or elevated platelet counts in acute nonlymphocytic leukemia. *Blood,* **66,** 1362–1370 (1985).
4) Morishita, K., Parganas, E., Willman, C. L., Whittaker, M. H., Drabkin, H., Oval, J., Taetle, R. and Ihle, J. N. Activation of EVI1 gene expression in human acute myelogenous leukemias by translocations spanning 300–500 kilobases on chomosome 3q26. *Proc. Natl. Acad. Sci. USA,* **89,** 3937–3941 (1992).
5) Suzukawa, K., Parganas, E., Gajjar, A., Abe, T., Takahashi, S., Tani, K., Asano, S., Asou, H., Kamada, N., Yokota, J., Morishita, K. and Ihle, J. N. Identification of a breakpoint cluster region 3′ of the Ribophorin I associated with the transcriptional activation of the EVI1 gene in acute myelogenous leukemias with inv(3)(q21q26). *Blood,* **84,** 2681–2688 (1994).
6) Nakase, K., Kita, K., Sekine, T., Otsuji, A., Shirakawa, S., Tsuji, K., Miyanishi, E., Asou, H. and Kamada, N. CD7, CD4 and myeloid antigen positive acute lymphoblastic leukemia. *Int. J. Hematol.,* **59,** 41–45 (1993).
7) Asou, H., Tashiro, S., Hamamoto, K., Otsuji, A., Kita, K. and Kamada, N. Establishment of a human acute myeloid leukemia cell line (Kasumi-1) with 8;21 chromosome translocation. *Blood,* **77,** 2031–2136 (1991).
8) Nosaka, T., Kita, K., Miwa, H., Kawakami, K., Ikeda, T., Ohno, T., Matsuoka, N., Arita, Y., Doi, S., Nishikiori, M., Matsuda, F., Lee, K. H., Shirakawa, S., Honjo, T. and Hatanaka, M. Cross-lineage gene rearrangements in human leukemic B precursor cells occur frequently with V-DJ rearrangements IgH genes. *Blood,* **74,** 361–368 (1989).
9) Morishita, K., Parker, D. S., Mucenski, M. L., Copeland, N. G. and Ihle, J. N. Retroviral activation of a novel gene encoding a zinc finger protein in IL-3-dependent myeloid leukemia cell lines. *Cell,* **54,** 831–840 (1988).
10) Morishita, K., Parganas, E., Douglass, E. C. and Ihle, J. N. Unique expression of the human Evi-1 gene in an endometrial carcinoma cell line; sequence of cDNAs and structure of alternatively spliced transcripts. *Oncogene,* **5,** 963–970 (1990).
11) Oval, J., Jones, O. W., Montoya, M. and Teatle, R. Characterization of a factor-dependent acute leukemia cell line with translocation (3;3)(q21;q26). *Blood,* **76,** 1369–1375 (1990).
12) Bennet, J. M., Catovsky, D., Daniel, M.-T., Flandrin, G., Galton, D. A. G., Gralnick, H. R. and Sultan, C. Proposal for the recognition of minimally differentiated acute myeloid leukaemia (AML-M0). *Br. J. Haematol.,* **78,** 325–329 (1991).
13) Cuneo, A., Ferrant, A., Michaux, J. M., Boogaerts, M., Demuynck, H., Van Orshoven, A., Criel, A., Stul, M., Cin, P. D., Hernandez, J., Chatelain, B., Doyen, C., Louwagie, A., Castoldi, G., Cassiman, J. J. and Van Den Berghe, H. Cytogenetic profile of minimally differentiated (FAB M0) acute myeloid leukemia: correlation with clinicobiologic findings. *Blood,* **85,** 3688–3691 (1995).
14) Kurtzberg, J., Waldmann, T. A., Davey, M. P., Binger, S. H., Moore, J. O., Hershfield, M. S. and Haynes, B. F. CD7+,CD4−,CD8− acute leukemia: a syndrome of malignant pluripotent lymphohemopoietic cells. *Blood,* **73,** 381–390 (1989).
15) Bassan, R., Biondi, A., Benvestito, S., Tini, M. L., Abbate, M., Viero, P., Barbui, T. and Rambaldi, A. Acute undifferentiated leukemia with CD7+ and CD13+ immunophenotype. *Cancer,* **69,** 396–404 (1991).
16) Kita, K., Miwa, H., Nakase, K., Kawakami, K.,

Kobayashi, T., Shirakawa, S., Tanaka, I., Ohta, C., Tsutani, H., Oguma, S., Kyo, T., Dohy, H., Kamada, N., Nasu, K. and Uchino, H. Clinical importance of CD7 expression in acute myelocytic leukemia. *Blood,* **81,** 2399–2405 (1993).

17) Le Beau, L. L., Albain, K. S., Larson, R. A., Virdiman, J. W., Davis, E. M., Blough, R. R., Colomb, H. M. and Rowley, J. D. Clinical and cytogenetic correlation in 63 patients with therapy related myelodysplastic syndrome and acute non-lymphocytic leukemia: further evidence for characteristic abnormalities of chromosome no. 5 and no. 7. *J. Clin. Oncol.,* **4,** 325–345 (1986).

18) Hayashi, Y., Kawamura, M., Kobayashi, S., Moriwaki, K., Kobayashi, M., Besho, H., Hamada, R., Yamamoto, K., Mori, Y., Nakazawa, S., Yaginuma, S., Honma, Y. and Hozumi, M. Establishment and characterization of cell lines derived from childhood leukemia. *Int. J. Hematol.,* **57** (Suppl. 1), 124 (1993).

19) Tanaka, T., Nishida, J., Mitani, K., Ogawa, S., Yazaki, Y. and Hirai, H. Evi-1 raises AP-1 activity and stimulates *c-fos* promoter transactivation with dependence on the second zinc finger domain. *J. Biol. Chem.,* **269,** 24020–24026 (1994).