MATERIALS AND METHODS

Case history. A 46-year-old man was admitted to the hospital of Nippon Medical School because of malaise and a high fever on March 22, 1991. Hematologic examinations showed a hemoglobin level of 5.0 g/dL and a hematocrit value of 17.5%. His white blood cell count was 5.0 x 10^9/L (78% blasts, 1% promyelocytes, 1% myelocytes, 3% segmented neutrophils, and 17% lymphocytes), and the platelet count was 5 x 10^9/L. Bone marrow examination showed a hypercellular marrow containing 58% myeloblasts. He was diagnosed as having acute myeloblastic leukemia (M2) in accordance with the French-American-British (FAB) criteria.11 However, immunophenotypically blasts had characteristics of CD7 + AML. The induction therapy was started on March 25th.13 The marrow was in a state of remission on May 9, 1991. After two cycles of consolidation therapy were administered, his bone marrow relapsed into recurrent disease (35% myeloblasts and 5% promyelocytes). His leukemic cells were refractory to many other chemotherapy regimens.19-21 He died on December 6, 1991 of irreversible septic shock.

Establishment of cell lines. Bone marrow mononuclear cells (MNC) were separated by density gradient centrifugation using Ficoll-Hypaque (specific gravity 1.077; Lymphoprep; Nyegaard, Oslo, Norway) at both diagnosis and relapse of the AML. The MNC were washed three times with Alpha medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 2 x 10^{-3} mol/L glutamine, and 100 μg/mL gentamicin (Schering-Plough Pharmaceutical Corp, Kennilworth, NJ). The cells were cultured in the supplemented Alpha medium with 10% FCS in culture flasks at 37°C in a humidified atmosphere containing 5% CO₂. After 2 weeks, the culture medium was replaced with fresh medium. After initial growth was noted, the cells were propagated in vitro with replacement of the medium containing 10% FCS without addition of growth factors twice weekly.

Immunologic marker studies. The surface markers of leukemic blasts and the cell lines were determined by direct immunofluorescence using flow cytometry on a FACS III (Becton-Dickinson; Sunnyvale, CA) flow cytometer, as previously described.22 The cells were incubated with appropriately diluted fluorescein isothiocyanate (FITC)-conjugated MoAb for 30 minutes at 4°C. The cells were then washed twice, and the percentages of positive cells deter-
mired by flow cytometry. To determine the background fluorescence, control cells were stained with FITC-conjugated normal mouse immunoglobulin. Epstein-Barr virus nuclear antigen (EBNA) was assayed by anticomplement immunofluorescence.23

Cytogenetic studies. Cytogenetic studies were performed by the conventional trypsin-Giemsa banding technique.24 The cells were processed by a direct method. The karyotype was determined by both direct microscopic analysis and photography.24

Polymerase chain reaction (PCR) and direct sequencing of the N-ras gene. The PCR reaction and direct sequencing were performed according to our original protocol.25 The PCR reaction was performed in a DNA Thermal Cycler (Perkin-Elmer/Cetus Corp., Norwalk, CT). Two hundred nanograms of DNA from cells was incubated with 0.5 U of Taq DNA polymerase (Perkin-Elmer/Cetus Corp.) and with 200 ng of each of two synthetic 20-mer oligonucleotides spanning the 5' and 3' ends of the target sequence (5'-5'-ATGACTGAGTACAAACTGGT-3', coding strand; or 3'-5'-ATGGTGGGATCATATTCA-3', antisense strand). A 109-bp amplified fragment was electrophoresed on 2.0% agarose, sliced from the gel, electroeluted, purified with phenol and ethanol precipitated. The fragments were subjected to direct sequencing as described below.

Reverse transcriptase (RT)-PCR and direct sequencing of the p53 gene. The RT-PCR reaction was performed with a slight modific-

| Table 1. Immunophenotypes of K051 and K052 Cell Lines and Original Bone Marrow Cells at Different Stages |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| CD              | Bone Marrow Cells | Cell Lines      | K051            | K052            |
|                 | Diagnosis        | Relapse         |                 |                 |
| CD2 (Leu 5)    | 14.1            | 3.2             | 1.1             | 39.2            |
| CD3 (Leu 4)    | 8.8             | 1.0             | 3.5             | 0.4             |
| CD5 (Leu 1)    | 9.1             | 7.3             | 0.3             | 36.1            |
| CD7 (Leu 9)    | 50.1            | 53.1            | 8.7             | 99.6            |
| CD10 (CALLA)   | 0.3             | 0.1             | 0.4             | 3.0             |
| CD13 (MY 7)    | 70.1            | 99.7            | 98.2            | 100.0           |
| CD14 (MY 4)    | 6.6             | 0.4             | 2.4             | 28.9            |
| CD19 (Leu 12)  | 1.0             | 0.2             | 0.4             | 1.0             |
| CD20 (Leu 16)  | 3.4             | 0.2             | 3.6             | 1.6             |
| CD33 (MY 9)    | 16.6            | 63.6            | 46.1            | 98.6            |
| CD34 (HPCA-2)  | ND              | ND              | 1.8             | 31.4            |
| CD41 (GP IIb/IIIa) | 0.7          | 5.5             | 42.2            | 25.8            |
| Glycophorin A  | 16.0            | 1.1             | 71.2            | 23.8            |
| HLA-DR         | 65.0            | 98.3            | 1.3             | 99.2            |

The immunophenotypes of the blood MNC from bone marrow cells and cell lines K051 and K052 were assayed by direct immunofluorescence assay. The denoted percentage of cell lines is mean number of that of positive cells of three experiments.

Abbreviation: ND, not done.

Fig 1. Cytocentrifuge preparations of K051 cells (A) and K052 cells (B) (Wright-Giemsa stain; original magnification × 1,000.)

tion of the original PCR procedure of Sugimoto et al.26 All primers used were the same as those of Sugimoto et al. Using the nucleotide numbers of the sequence published by Zakut-Houri et al.27 the sense primers were: ST1, TCTGTGACTGTCAACGATCTC (residues 361-380); SN2, AGCTGACCTCCCCGTCCCTC (residues 373-392); and SC3, GCGGTGAGATTTGGGATG (residues 603-622). The antisense primers were: AST1, CACGGATCTGAA-AGTGGTTT (residues 980-961). Complementary DNA was synthesized from 1 μg of total cellular RNA from bone marrow MNC using 100 ng of 3′-primer AST1 and 200 U of Moloney murine leukemia virus (M-MLV) RT (Bethesda Research Laboratories; Gaithersburg, MD) in 25 μL of solution containing 200 μmol/L each of all four dNTPs, 80 U of RNase inhibitor, 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 10 mmol/L dithiothreitol, and 3 mmol/L MgCl2. The reaction was allowed to proceed for 60 minutes at 37°C, and the reaction solution was used as the substrate for the PCR. The PCR reaction was performed in a DNA Thermal Cycler (Perkin-Elmer/Cetus Corp.) with slight modification of our original protocol.28 To the RT reaction solution, 25 μL of a solution containing 250 μmol/L each of all four dNTPs, 100 ng of 5′-primer ST1, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, and 3 U of Taq DNA polymerase (Perkin-Elmer/Cetus Corp) was added. The reaction conditions and cycle number were as previously described. A second PCR was performed for direct sequencing. Primers SN2 and ASC2 were used to amplify the 5′-side fragment, whereas primers SC3 and ASC3 were used for the 3′-side fragment. One microliter of RT-PCR reaction solution was used in a 50-cycle second PCR with a 10-fold reduction of one of the primers. The resulting single-stranded DNA was purified and sequenced by the di-deoxy chain termination method. The sequencing primers were the reduced primers of the second PCR.

Direct nucleotide sequencing was performed using a modification of the method of Radich et al.29 Six microliters of the total annealing mixture containing approximately 20 to 60 ng of PCR DNA product, 20 pmol of oligonucleotide primer, 1 μL of 5× Se-
Fig 2. Karyotype of KO51 cells by G-banding shows 48, XY, +1, +2, 7q-, 17p, 21q-.

Fig 2. Karyotype of KO51 cells by G-banding shows 48, XY, +1, +2, 7q-, 17p, 21q-.
phenotype of cells in each medium was examined as described above, and the morphology of the cells was determined from Wright-Giemsa-stained cytospin slide preparations.

RESULTS

Establishment of cell lines. The cultured cells began to proliferate 2 weeks after the start of the culture. After subcloning by the limiting dilution technique, the subcloned cells grew well in suspension, and the cell lines were subsequently established. The cells established from bone marrow obtained at diagnosis and at relapse were named KO51 cells and KO52 cells, respectively. The cells have now been maintained in continuous culture for 1.5 years. The doubling time was 48 hours for both cell lines.

Morphology and cytochemistry. The KO51 cells had a folded nucleus with several nucleoli and vacuoles in basophilic cytoplasm (Fig 1A). The KO52 cells had a round nucleus with several nucleoli and occasional azurophilic granules in basophilic cytoplasm (Fig 1B). Both cell lines were positive for peroxidase stain and Sudan black B stain, but negative for nonspecific esterase stain and periodic acid-Schiff stain. Electron microscopic analysis showed that most of the KO51 and the KO52 cells measured 15 to 20 μm and 12 to 18 μm, respectively, in diameter, and that perinuclear cytoplasmic fibrils were seen in many KO51 cells. The patient’s blast cells at diagnosis showed normal chromosomes. On the other hand, the patient’s blasts at relapse and the two cell lines showed a 17p- chromosome aberration, as shown in Fig 2 and Table 2.

Detection of N-ras and p53 gene mutations. Figure 3 depicts the antisense of N-ras exon 1 segments from the DNA and the antisense of p53 cDNA of both the KO51 and KO52 cell lines. We observed neither involvement of codon 12 or 13 of the N-ras gene nor a point mutation of the p53 gene in the leukemic cells at diagnosis. However, the KO51...
cells, which were established from leukemic cells obtained at diagnosis, showed a p53 mutation at codon 248 (C → T) (Table 2). On the other hand, both the leukemic cells at relapse and the K052 cells showed the same mutation (C → T) at codon 248 of the p53 gene and a G → C substitution in codon 13 of the N-ras gene.

Semiquantitative analysis of MDR1 gene expression. As Figure 4 shows, we were able to detect MDR1-mRNA when at least 0.2% of the total cells expressed the MDR1 gene. Only a 243-bp product spanning exons 16-18 was seen in photographs of both ethidium-bromide staining and autoradiography (Figs 4 and 5). β2m was chosen as an internal control for the MDR1 gene expression. Using this assay, the level of MDR1-mRNA in the K052 cell was at least as high as that in the HEL cell (Fig 5). MDR1-mRNA was expressed in the K051 cell, but the level was relatively low compared with that in the K052 cell (Fig 5).

VCR resistance and reversal of resistance by VER or CSA. Table 3 shows the IC50 values of VCR in the four cell lines in the presence or absence of 5.0 μg/mL of VER or CSA. The IC50 values of VCR in the K051, K052, and HEL cells but not the K562 cell were decreased dramatically in the presence of 5 μg/mL of VER or CSA. On the other hand, no significant potentiation of the growth-inhibitory activity of VCR by VER or CSA was observed in the K562 cell. Reversal of VCR resistance by VER or CSA at 0.5 μg/mL in K052 was also observed and greater than that in K051 (data not shown).

Phenotypic and morphologic changes in the K051 and K052 cells induced by various inducers. RA induced phenotypic change of the K051 cell to erythroid-predominant antigen (Table 4). The K052 cells were capable of phenotypically or morphologically differentiating in the medium containing various inducers. In the absence of any inducer, the K052 cells have predominantly a myeloblastic morphology, and show high levels of expression of T-cell and myeloid-associated antigens. As shown in Table 4, IL-2 induced striking changes in the phenotype of the K052 cell characteristic of lympho-monocytic differentiation. PMA induced the pheno-
Table 4. Comparative Study of Cell Surface Markers of K051 and K052 Cells After Incubation With Different Inducers

<table>
<thead>
<tr>
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<th>K051</th>
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<tr>
<td>CD2 (Leu 5)</td>
<td>1.1</td>
<td>39.2</td>
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<tr>
<td>CD3 (Leu 4)</td>
<td>3.5</td>
<td>0.4</td>
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<tr>
<td>CD5 (Leu 1)</td>
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<td>CD7 (Leu 9)</td>
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<tr>
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<tr>
<td>CD33 (MY 9)</td>
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<tr>
<td>CD34 (HPCA-2)</td>
<td>1.8</td>
<td>31.4</td>
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<tr>
<td>CD41 (Ilb/IIla)</td>
<td>42.2</td>
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<tr>
<td>Glycophorin A</td>
<td>71.2</td>
<td>23.8</td>
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<td>HLA-DR</td>
<td>1.3</td>
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K051 or K052 cells were cultured in the presence of one inducer for 7 days. Cell differentiation and immunophenotypic analysis were performed as described in Materials and Methods. The results are expressed as the percentage of positive cells in three experiments.

DISCUSSION

The K051 cell line was established from leukemic cells at diagnosis. The K051 cell had a p53 mutation, although the leukemic cells at diagnosis had no mutations of either the N-ras, p53 gene, or deletion of 17p. The PCR-direct sequencing technique is capable of detecting an N-ras point mutation only if this mutation is present in 10% of the cell samples. Taking the cytogenetic and RT-PCR data into consideration, we speculate that a new leukemic clone carrying both the p53 mutation and 17p deletion represented a minor clone of less than 1% to 10% in the presentation sample. This quite minor clone was established as the K051 cell line. As the disease progressed, this minor clone expanded possibly because of acquisition of the N-ras mutation. This clone with two mutations acquired strong drug-resistance and the capacity for leukemic progression. The new, progressed clone was established as the K052 cell line.

The presence of the same p53 and N-ras mutations in the relapse sample and the K052 cell line indicates that these changes arose in the patient and were not an artifact of culture.

Coordinated loss of one copy of the p53 gene and mutation of the remaining copy have been reported in many human malignancies. Both mutations and allelic loss generally occur near the time of transition from a benign to malignant state in colorectal tumorigenesis, and from the chronic phase to the acute phase in CML. In the present case, leukemic cells with both mutations and allelic loss already existed at the time of presentation.

One of the mechanisms of drug-resistance in cancer is said to be associated with high expression of the P-glycoprotein encoded by the MDR1 gene normally found at substantial levels in many organs. Expression of the MDR1 gene in cancers is increased following relapse after chemotherapy, suggesting that MDR1 expression might be selected in tumor populations exposed to chemotherapy. As shown in Fig 5, the K051 (having p53 mutation) and K052 (having p53 and N-ras mutations) cells expressed a small and large amount of mRNA of the MDR1 gene, respectively. Expression of a functionally active MDR1 protein by
the KO51 and KO52 cells was demonstrated using assay of VCR-resistance reversal by VER or CSA. The MDR1 gene might be regulated positively as a result of activation of ras and inactivation of the p53 tumor suppressor gene. Thus, we speculated that the emergence of multidrug resistance might be closely associated with p53 and N-ras mutations.

The original blasts of the FAB-M2 patient had characteristics of multilineage CD7 + AML phenotypically. A high level of CD7 expression, concurrent CD2 expression in the original blasts, and high levels of expression of T-cell, platelet, and erythroid antigens on both cell lines are evidence of the multilineage nature of the cell of origin. The unusual multiple antigen phenotype of the KO52 cell supports the concept that these cells originated from multilineage cells. As shown in Table 1, the immunophenotypes of the KO51 and the KO52 cells are quite different. There are also marked differences between the phenotypes of the cell lines and the respective samples from which they were obtained. Although we are currently unable to explain these marked differences, the possibility is that CD7 + AML may have multilineage characteristics and change phenotype easily.

The KO51 and KO52 cells may be useful for investigating the cellular and molecular events in leukemogenesis and drug resistance. Moreover, these cell lines may be productive models for investigating the process of differentiation.

ACKNOWLEDGMENT

We thank Dr Hisamaru Hirai and Dr Koichi Sugimoto for kindly providing the primers of RT-PCR analysis, Dr Tamiko Shinohara for performing the chromosome analysis, Dr Sachiko Inokuchi for performing the ultrastructural study, Dr Koichi Miyake for helpful discussions, and the Japanese Cancer Research Resources Bank for providing cell lines.

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p53 and N-ras mutations in two new leukemia cell lines established from a patient with multilineage CD7-positive acute leukemia

J Abo, K Inokuchi, K Dan and T Nomura