

**Masked MLL gene rearrangement was disclosed in the clinical course and sequential development of chromosome abnormality in a patient with therapy related acute myelogenous leukemia.**

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*Abbreviations:* MLL, the mixed lineage leukemia/myeloid-lymphoid leukemia; hDMP1, cyclin D-binding Myb-like protein; IRF-1, interferon regulatory factor-1; AML, acute myelogenous leukemia; TRL, therapy related leukemia; MDS, myelodysplastic syndrome; FAB, French-American-British classification; FISH, fluorescence in-situ hybridization; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

**Abstract** Therapy related acute myelogenous leukemia in a 55-year-old Japanese woman is described. She had been treated for a diagnosis of non-Hodgkin's lymphoma 2 years before the onset of the secondary leukemia. She was diagnosed as AML (FAB: M2) with monosomy 7, and successfully treated by an intensive combination chemotherapy followed by an autologous peripheral blood stem cell transplantation. The disease relapsed shortly after the treatment, and the karyotype analysis revealed a complex abnormality accompanied with t(9;11)(p22;q23), however, monosomy 7 was absent. Southern blotting analysis was performed, and MLL rearrangement was evident in both the bone marrow samples obtained at that time and the cryopreserved marrow cells obtained at the onset of the disease. The bone marrow sample stored in a Carnoy solution at the onset was further analyzed, and 3 karyotype panels showing 45,XX, -7, t(9;11)(p22;q23) were found. Like this situation, a masked MLL rearrangement may have existed in some cases with hematopoietic malignancies, and appear to be disclosed in the clinical course.

## Introduction

TRL has been one of the late complications of chemotherapy for primary malignancies by using alkylating agents and others. Chromosomal abnormalities of entire or partial loss of 7 and 5, i.e., -7, 7q-, -5 and 5q-, have a close relation with alkylating agents, while balanced or unbalanced translocations involving 11q23 or 21q22, such as t(1;21), t(3;21), t(8;21), t(6;11), t(9;11), t(11;17) and t(11;19), do so with DNA-topoisomerase II-inhibitors<sup>1,2</sup>. The tumor suppressor genes responsible for the leukemogenesis, hDMP1 and IRF-1 are located on 7q21<sup>3</sup> and 5q31<sup>4</sup>, respectively. Translocations at 11q23 or 21q22 result in abnormal fusion gene formations and chimeric proteins of MLL<sup>5</sup> or AML1<sup>6</sup> with several partner genes, respectively.

The mechanism of masked Ph chromosome is well known<sup>7</sup>, however, there have been a few studies that focused on the masked abnormality of

other chromosomes. We report here a case with TRL showing monosomy 7 accompanied with masked t(9;11), and the stepwise clonal development of the pathological cells in the clinical course of the case.

## Methods

### *Monoclonal antibodies*

FITC-conjugated anti-LeuM1 (CD15), CALLA (CD10), anti-HLe1 (CD45), Leu4 (CD3), Leu5b (CD2), Leu9 (CD7), Leu3a (CD4), control IgG1, and PE-conjugated anti-HPCA2 (CD34), LeuM9 (CD33), IL2R (CD25), Leu2a (CD8), Leu14 (CD22), Leu12 (CD19), LeuM3 (CD14), Leu16 (CD20), Leu19 (CD56), HLA-DR, control IgG1 were purchased from Becton Dickinson Immunocytometry Systems, Mountain View, CA. FITC-conjugated anti-MCS2 (CD13), c-kit (CD117) and Mik-β1 (CD122) were purchased from Nichirei, Tokyo, Japan. FITC-conjugated

anti-CD36 (FA6.152), CD38 (T16), CD41 (P2), and PE-conjugated anti-Glycophorin A (KC16) were purchased from Immunotech, Marseille, France. FITC-conjugated anti-CD1 was purchased from DAKO, Denmark. PE-conjugated anti-CD21 (B2) was purchased from Coulter Immunology, Hialeah, FL. PE-conjugated anti-AC133 (CD133) was purchased from Miltenyi Biotec, Bergisch Gladbach, Germany.

#### *Flow cytometry*

For phenotype analysis, a single cell suspension was obtained from a sample, and aliquots of the sample were stained simultaneously with FITC- and PE-conjugated monoclonal antibodies. Cell fluorescence was analyzed with a FACScan<sup>TM</sup> Flow Cytometer using CELLQuest<sup>TM</sup> software (Becton Dickinson).

#### *Southern blotting analysis*

The rearrangement of the MLL gene was evaluated according to the standard method. In brief, 5 µg of DNA from the sample was extracted according to the standard methods, digested with *Bam*HI and *Ech*RV, electrophoresed through a 0.6% agarose gel and transferred onto a nylon membrane filter. The filter was hybridized with a <sup>32</sup>P-labeled MLL/ALL1 probes, and washed under appropriate stringency conditions, then bands were visualized by autoradiography.

#### *FISH*

The samples were also analyzed by means of FISH designed for detection of 7-chromosome. The Translocation DNA Probe kits were purchased from Vysis, Downers Grove, IL. Cells were placed on the slide in the same manner as that for chromosomal analysis. The denatured slide was hybridized with the probe labeled with SpectrumGreen<sup>TM</sup> (D7Z1 sequence of 7-chromosome) in the Translocation DNA Probe kits. Following hybridization, the slide was washed and counter stained with DAPI. Signal numbers were counted in 1,000 cells.

#### **Case report**

A 53-year-old Japanese female was admitted to our hospital with a right neck tumor in June, 1996. Informed consent was obtained from the patient for the following examinations and analyses to be performed. The histology of the tumor and computed tomography allowed a diagnosis of non-Hodgkin's lymphoma (diffuse large B cell

type, clinical stage IA). The patient was treated with weekly alternated combination chemotherapy for 8 weeks including pirarubicine, cyclophosphamide, methotrexate, etoposide, vincristine, and prednisolone. Complete remission was achieved, and the patient was discharged. The cumulative dosages of pirarubicine, cyclophosphamide and etoposide were 120 mg, 1,000 mg and 600 mg in the course, respectively.

Thrombocytopenia appeared in May, 1998, and the patient was admitted in July, 1998. A bone marrow aspiration showed increased numbers of peroxidase-positive myeloblasts with the typical morphology and phenotype of AML (M2) as shown in Figure 1. The myeloblasts were CD4/CD13/CD15/CD33/CD38/HLA-DR-positive and CD1/CD2/CD3/CD7/CD8/CD10/CD14/CD19/CD20/CD21/CD22/CD25/CD34/CD36/CD41/CD56/CD117/CD122/CD133/Glycophorin A (GPA)-negative. Trilineage myelodysplasia was minimal at that time. Reverse transcriptase-polymerase chain reaction analysis for AML1-MTG8 was negative (data not shown). Chromosome analysis of the bone marrow specimen showed a simple abnormal karyotype of 45,XX, -7 in 14/20 metaphases studied (Table 1). She was treated with daily intensive chemotherapy including daunorubicine, cytosine arabinoside, 6-mercaptopurine and prednisolone, and complete remission was achieved. The karyotype analysis was normal, and FISH analysis for monosomy 7 was negative in bone marrow cells at that time. The prognosis of TRL is usually poor, so the patient was further treated with autologous peripheral blood stem cell transplantation after high dose cytosine arabinoside and idarubicine in February, 1999. Monosomy 7 was not also detected in the harvested cells by FISH.

The disease relapsed shortly after the transplantation in May, 1999. The bone marrow aspirate showed increased number of myeloblasts with morphology of AML (M1) and a more immature phenotype as shown in Figure 1 and Table 1. The blasts were CD13/CD15/CD33/CD34/CD117/HLA-DR-positive and CD1/CD2/CD3/CD4/CD7/CD8/CD10/CD14/CD19/CD20/CD21/CD22/CD25/CD36/CD38/CD41/CD56/CD122/CD133/GPA-negative. Marked myelodysplasia was observed in erythroblasts, neutrophils and megakaryocytes.

Chromosomal analysis of the bone marrow specimen showed complex abnormalities: 46,XX,t(9;11)(p22;q23), 47,XX,t(9;11)(p22;q23),+21, 48,XX,+9,t(9;11)(p22;q23),+21, and 48,XX,t(9;11)(p22;q23),+19,+21 (Fig. 2). Monosomy 7 was not detected in the cells by FISH. Southern blotting analysis for the MLL gene rearrangement was performed in the sample as well as the cryopreserved bone marrow sample stored in July, 1998. Rearranged bands of MLL were observed in both of the samples as shown in Figure 3. Then, the bone marrow sample in the Carnoy solution stored in July, 1998 was further examined for karyotyping, and three panels of karyotype excluding previously observed panels, 46,XX and 45,XX,-7, were found, and all three panels showed 45,XX,-7,t(9;11)(p22;q23) as shown in Figure 2. The patient was treated with low dose cytosine arabinoside and aclarubicin combined with G-CSF (CAG regimen). Complete remission was not achieved, and the patient died of sepsis and pulmonary hemorrhage.

### Discussion

There were several questions to be discussed. It was unclear why the chromosome panels of t(9;11) were so few despite the distinctly documented rearranged bands of the MLL gene in July, 1998. The relation between the sequence of clonal evolution and the change of the chromosomal abnormalities was unresolved. The reason was unknown why the abnormality of monosomy 7 disappeared in the sequential clonal evolution despite the finding that the clone, 45,XX,-7, appeared to be the very first origin of all the following subclones.

A detailed cell cycle analysis of normal human bone marrow subpopulations was studied previously<sup>8</sup>. Growing cells were mostly glycophorin A-positive erythroid cells and CD33-positive monomyeloid cells, and about the 90% and 10% of total G2M stage cells in bone marrow were erythroid and monomyeloid, respectively. The G2M % of de novo AML cells distributed from 0.5 to 5%. Therefore, it is easy to understand that the majority of karyotypes arose from erythroid cells in such cases when erythroid hematopoiesis was well preserved as the bone marrow sample studied in July, 1998. Hence, it was possible that the karyotype panels that arose from the pathological myeloblasts were the minority in the study at that time. The one-step-advanced clone by the acquisition of

MLL involvement, 45,XX,-7,t(9;11), might have produced 46,XX,t(9;11) by the second nondisjunction of chromosome 7 during mitosis.

Monosomy 7 is usually found as a simple abnormality in childhood MDS and AML, and is frequently accompanied by other abnormalities in adults<sup>9</sup>. This abnormality is more frequently observed in MDS than AML<sup>2</sup>. In such cases, -7 is suggested to be the first hit of the preleukemia stage, and other 2<sup>nd</sup> and 3<sup>rd</sup> hits on oncogenes may have key roles for leukemic transformation. Balanced and unbalanced translocation involving MLL are frequently observed in overt leukemia rather than MDS<sup>2</sup>. The MLL gene is fused to various translocation partner genes such as AF4/FEL, LTG/AF9 and LTG19/ENL<sup>10</sup>, and such MLL-involved TRL cases show poor prognosis<sup>11,12</sup>. Tandem partial duplication of the MLL gene shows the normal karyotype in patients with AML, and these patients also have poor prognosis<sup>13</sup>.

A patient with monosomy 7 MDS was reported whose clonal cells acquired t(9;11)(p22;q23) at its leukemic transformation<sup>14</sup>. In that case, the MLL gene rearrangement was absent in its MDS stage, present at the leukemic transformation, and -7 was preserved. The authors suggested that MLL gene rearrangement corresponded to the final step of leukemogenesis. In the present case, the MLL-rearranged clone existed at the onset of leukemia as a subclone of -7, and it expanded to be disclosed in the clinical course simultaneously acquiring other complex abnormalities as +21, +9 and +19. A routine analysis of the MLL rearrangement in patients with primary and secondary MDS and AML may predict the clinical prognosis.

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Table 1

| Table 1 Sequential change of the bone marrow karyotypes. |         |         |         |                 |          |   |      |      |      |
|--|---------|---------|---------|-----------------|----------|---|------|------|------|
|  | Status  | BM (%)  |         | chromosome (BM) |          | FISH (BM)   |      |      |      |
|  |         | Ery (%) | Bla (%) | 46,XX           | 45,XX,-7 |   |      |      |      |
| Jul, '98   | 1st AML | 37.2    | 43.6    | 6/20            | 14/20    | 45,XX,-7,t(9;11), 46,XX,t(9;11), 47,XX,t(9;11), +21, +9, 48,XX,t(9;11),+21,+19,-7 (*) |      |      |      |
| Aug  | CR      | 18.8    | 2.1     | 20/20           |          |   |      |      | 3.8  |
| Oct  | CR      | 23.2    | 6.0     |                 |          |   |      |      | 2.1  |
| Nov  | CR      | 28.8    | 1.6     |                 |          |   |      |      | 2.1  |
| Dec  | CR      | 16.0    | 2.8     |                 |          |   |      |      | 2.4  |
| Jan, '99   | CR      | 12.4    | 6.2     |                 |          |   |      |      | 2.4  |
| Mar  | CR      | 19.2    | 4.8     |                 |          |   |      |      | 1.8  |
| May, 7   | 2nd AML |         |         | 17/20           |          |   |      | 2/20 | 1/20 |
| May, 17  |         | 12.8    | 44.8    | 4/10            |          | 1/10  | 4/10 | 1/10 | 2.5  |
| Jun  |         | 0.8     | 28.0    |                 |          |   | 7/8  | 1/8  |      |

-7 (\*): normal 3.2 + 1.8 %. Ery (%) and Bla (%), erythroid cells and blasts, respectively. <1/20\*\*: observed later by an intensive examination.

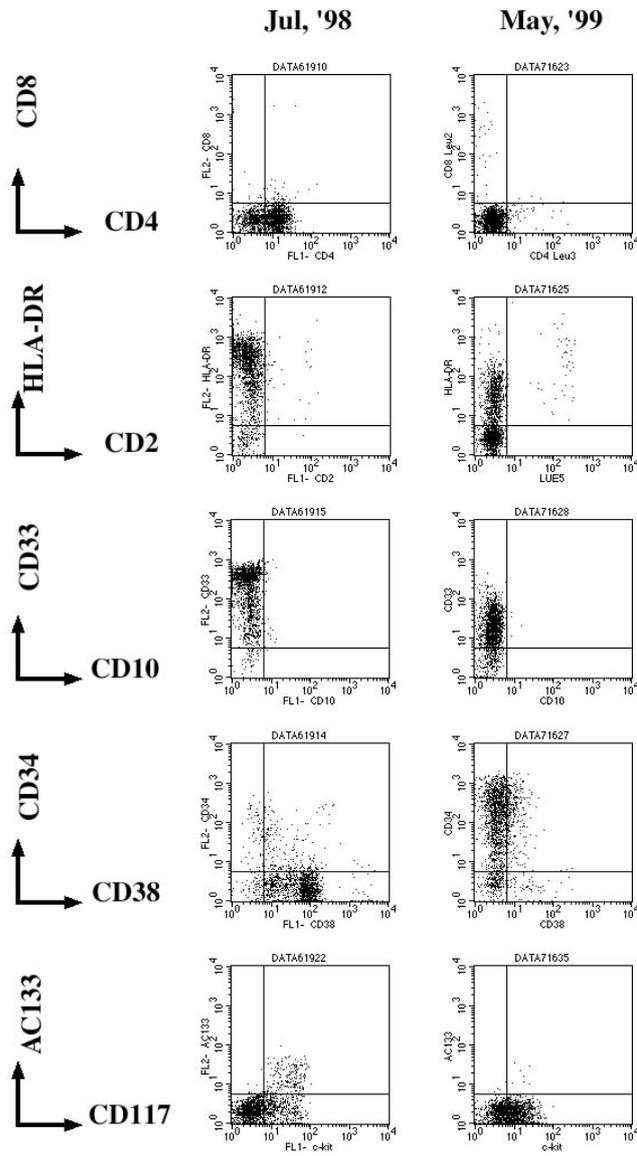


Fig. 1: Phenotype analysis of bone marrow blasts in July, 1998 and May, 1999. Horizontal and vertical axis, FITC and PE fluorescence of the blasts, respectively.

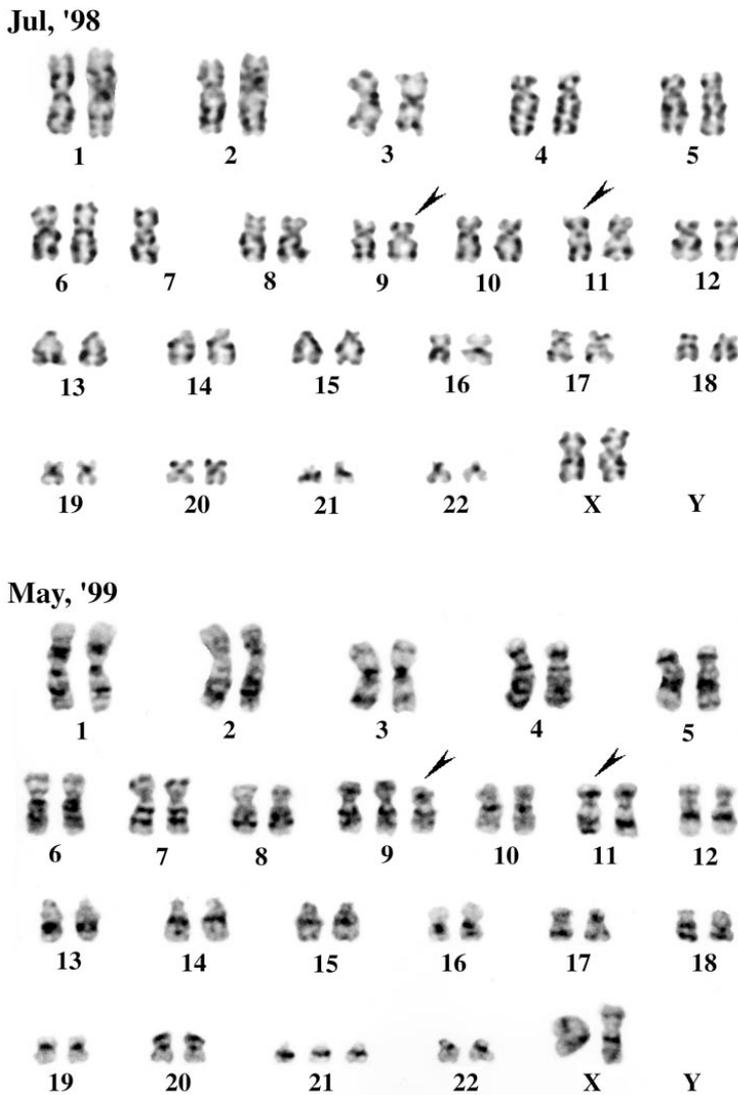


Fig. 2: Karyotypes of the bone marrow cells. Some of the karyotypes showed 45,XX,-7,t(9;11)(p22;q23) in July, 1998, and 48,XX,+9,t(9;11)(p22;q23),+21 in May, 1999. The arrowheads indicate abnormal chromosomes.

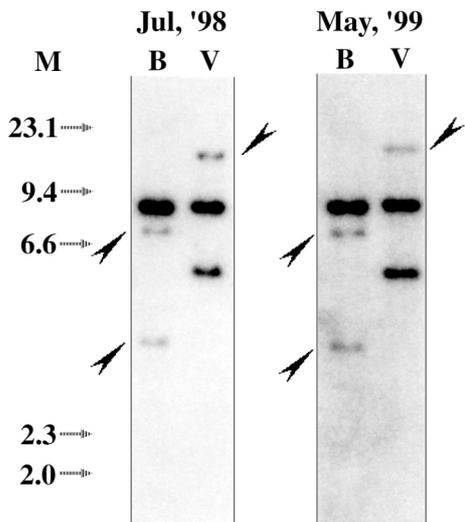


Fig. 3: Southern blot analysis for the MLL gene. B and V, digestion using Bam HI and Eco RV, respectively. The arrowheads indicate rearranged bands, otherwise germline bands.