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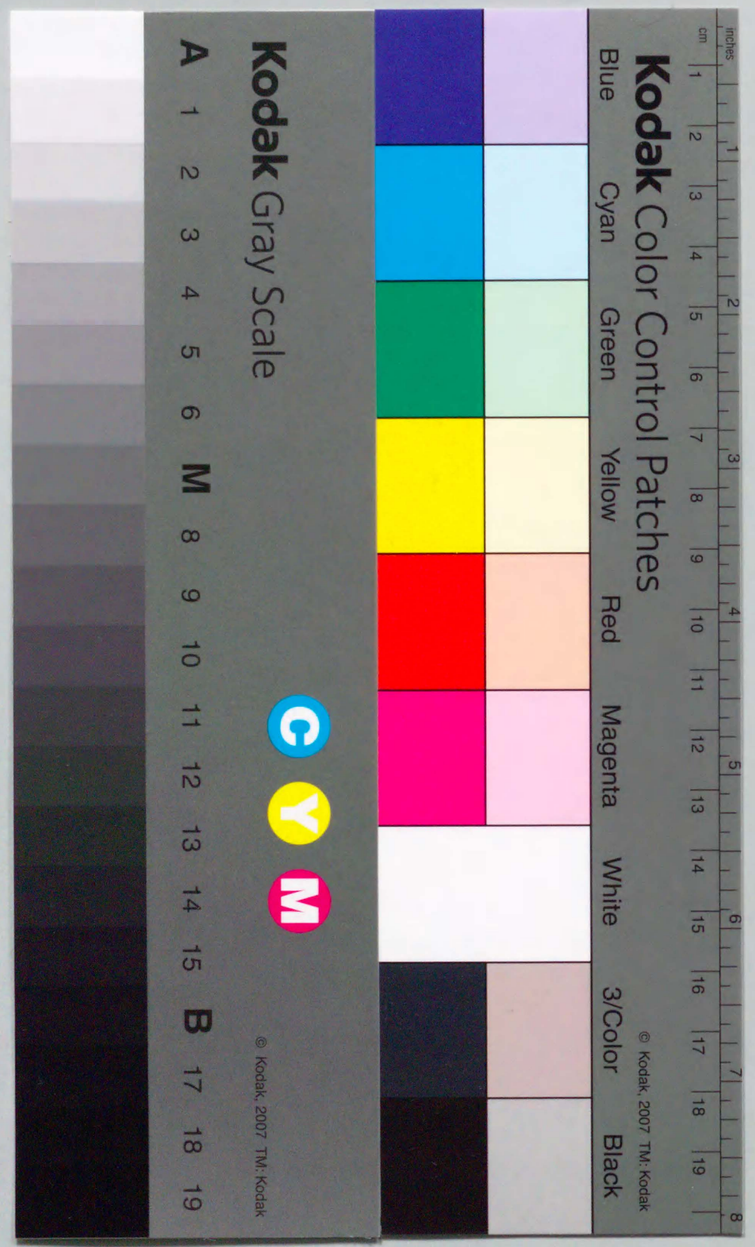

Persistence of Multipotent Progenitors Expressing *AML1/ETO* Transcripts in Long-Term Remission Patients With t(8;21) Acute Myelogenous Leukemia

By Toshihiro Miyamoto, Koji Nagafuji, Koichi Akashi, Mine Harada, Taiichi Kyo, Tomoyuki Akashi, Katsuto Takenaka, Shin-ichi Mizuno, Hisashi Gondo, Takashi Okamura, Hiroo Dohy, and Yoshiyuki Niho

The leukemia-specific *AML1/ETO* fusion gene has been shown to be detected by reverse transcriptase polymerase chain reaction (RT-PCR) analysis in patients with t(8;21) acute myelogenous leukemia (AML) in long-term remission. In the present study, the *AML1/ETO* mRNA could be detected by RT-PCR in bone marrow (BM) and/or peripheral blood (PB) samples from all 18 patients who had been maintaining complete remission for 12 to 150 months (median, 45 months) following chemotherapy or PB stem cell transplantation (PBSCT), whereas it could not be detected in four patients who had been maintaining remission for more than 30 months following allogeneic BM transplantation (BMT). We surveyed the expression of *AML1/ETO* mRNA in clonogenic progenitors from BM in these cases. Notably, 51 of 2,469 colonies from clonogenic progenitors (2.1%) expressed the *AML1/ETO* mRNA in 18 cases who were RT-PCR$^+$ in BM and/or PB samples. Expression was observed in various clonogenic progenitors, including granulocyte-macrophage colonies, mixed colonies, erythroid colonies, and megakaryocyte colonies. Furthermore, we analyzed the clonality of these progenitors by X-chromosome inactivation patterns of the phosphoglycerate kinase (PGK) gene in four female patients. The *AML1/ETO* mRNA$^+$ progenitors showed the PGK allele identical to that detected in the leukemic blasts from the time of initial diagnosis. Normal constitutive hematopoiesis was sustained by polyclonal BM reconstitution in these patients. Accordingly, these committed progenitor cells that express *AML1/ETO* mRNA during remission likely have arisen from common t(8;21)$^+$ pluripotent progenitor cells with at least trilineage differentiation potential. These data strongly suggest that the origin of the clonogenic leukemic progenitors of t(8;21) AML may be multipotent hematopoietic progenitors that acquired the t(8;21) chromosomal abnormality.

THE 8;21 translocation [t(8;21) (q22;q22)] is one of the most frequent chromosomal abnormalities found in acute myelogenous leukemia (AML), especially in the AML-M2 subtype of the French-American-British (FAB) classification.[1,2] The leukemogenesis of t(8;21) AML has been ascribed to the production of a chimeric protein produced by fusion genes involving *AML1* at 21q22 and *ETO* at 8q22.[3-6] Reverse transcriptase polymerase chain reaction (RT-PCR) analysis has demonstrated the ability to detect *AML1/ETO* fusion transcript in patients with t(8;21) AML.[7,8]

The 8;21 translocation usually predicts a good response to chemotherapy, with a high remission rate and relatively long median survival.[9,10] However, we and other investigators also have demonstrated positive results of minimal residual disease (MRD) from RT-PCR analysis of t(8;21) AML patients who remain in hematologic and cytogenetic long-term remission after chemotherapy alone or autologous bone marrow transplantation (BMT) or peripheral blood stem cell transplantation (PBSCT).[11-16] We have previously reported that the amount of *AML1/ETO* mRNA in BM and PB samples gradually decreased with successive cycles of chemotherapy when evaluated by a quantitative RT-PCR assay.[11] Interestingly, in our experience when the quantity of *AML1/ETO* mRNA has decreased to the lower limit of the quantitative scale, it is still detectable in a qualitative RT-PCR system.[11] This situation is of interest if clinical "cure" represents eradication of the leukemic cell population, including leukemic stem cells. However, results of MRD studies in acute promyelocytic leukemia (APL) with t(15;17) have been reported to be consistent with this concept of leukemia eradication by therapy. A chimeric mRNA of *PML/RARα* produced by this translocation was undetectable in remission by RT-PCR,[17-19] and pluripotent stem cells in t(15;17) APL were reported not to be affected by this translocation.[20]

In the present study, we analyze a larger number of cases and again show that the *AML1/ETO* mRNA can be detected by RT-PCR in all patients who remain in complete remission following chemotherapy or PBSCT. To characterize the residual *AML1/ETO* mRNA-positive cells in remission, we analyzed the presence of this mRNA in individual clonogenic progenitor colonies derived from remission marrow by a two-step nested RT-PCR. We found that the *AML1/ETO* mRNA-positive colonies in remission include various trilineage clonogenic progenitors, such as granulocyte-macrophage colony, mixed colony, erythroid colony, and megakaryocyte colony. These *AML1/ETO* mRNA-positive progenitors and de novo leukemic blasts at initial diagnosis were found to be clonal in origin based on X-linked chromosomal inactivation mosaicism of the phosphoglycerate kinase (PGK) gene in four female patients. This is the first evidence that the t(8;21) chromosomal abnormality involves multipotent hematopoietic progenitors.

MATERIALS AND METHODS

Patients. This study included 10 female and 12 male patients with t(8;21) AML who had been maintaining long-term hematologic remission (remission duration, 12 to 150 months; median, 45 months). In all cases, cytogenetic analysis showed a normal karyo-

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Table 1. Clinical Characteristics of Patients With t(8;21) AML

Patient No.	Age/Sex	FAB	Treatment	Clinical Status	Duration of CR (mo)	Interval From PBSCT/AlloBMT (mo)
1(a)*	47/M	M2	PBSCT	1st CCR	15	11
(b)				1st CCR	27	23
2(a)*	33/F	M2	PBSCT	1st CCR	49	44
(b)				1st CCR	60	55
3	35/F	M2	CHT	1st CCR	45	—
4	31/M	M2	PBSCT	2nd CCR	36	30
5	57/M	M2	CHT	1st CCR	28	—
6	21/F	M2	PBSCT	1st CCR	38	31
7	37/F	M2	CHT	2nd CCR	12	—
8	19/M	M1	CHT	1st CCR	49	—
9	53/F	M2	CHT	1st CCR	50	—
10	52/F	M2	CHT	1st CCR	37	—
11	20/M	M2	CHT	1st CCR	80	—
12	28/F	M2	CHT	1st CCR	30	—
13	25/F	M2	CHT	1st CCR	17	—
14	31/M	M2	CHT	1st CCR	124	—
15	62/M	M2	CHT	2nd CCR	18	—
16	41/F	M1	CHT	1st CCR	34	—
17	54/M	M2	CHT	1st CCR	150	—
18	52/M	M2	CHT	1st CCR	88	—
19	28/M	M4	AlloBMT	1st CCR	74	36
20	29/M	M2	AlloBMT	1st CCR	34	28
21	45/F	M2	AlloBMT	1st CCR	31	20
22	20/M	M2	AlloBMT	1st CCR	30	17

Abbreviations: CHT, chemotherapy; PBSCT, peripheral blood stem cell transplantation; Allo BMT, allogeneic bone marrow transplantation; CCR, continuous complete remission.

* Samples were obtained twice from patient no. 1 (a and b) and no. 2 (a and b) at different times.

type after they had achieved complete remission. Clinical characteristics of these patients are listed in Table 1. Fourteen patients were treated by chemotherapy alone, and four patients underwent PBSCT according to the protocol previously reported.[11,21,22] The remaining four patients underwent allogeneic BMT. Five healthy volunteers were included as normal controls. Informed consent was obtained from all patients.

Preparation of CD34⁺ cells for clonogenic progenitor assays. PB mononuclear cells (PBMNCs) and BM mononuclear cells (BMMNCs) were isolated by Ficoll-Hypaque density gradient centrifugation. BMMNCs were suspended in Iscove's modified Dulbecco's medium (IMDM; GIBCO, Grand Island, NY) with 10% fetal calf serum (FCS; ICN Biochemicals, Osaka, Japan) and incubated for 2 hours in a Petri dish to deplete adherent cells. CD34⁺ cells were isolated by positive selection with the use of CD34-conjugated immunomagnetic beads (Dynabeads M-450 CD34; Dynal AS, Oslo, Norway) to focus on more purified hematopoietic colony-forming cells and avoid picking noncolony forming cells.[23] Briefly, these nonadherent BMMNCs were incubated with CD34-conjugated immunomagnetic beads for 1 hour at 4°C with gentle rotation, and CD34⁺ cells were collected with a magnet (MPC-1; Dynal AS). CD34-conjugated immunomagnetic beads were released by incubation with a goat antimouse-Fab polyclonal antibody (DETACHaBEADS; Dynal AS) at room temperature for 1 hour with gentle rotation. More than 90% of these isolated cells expressed CD34 when analyzed by flow cytometry (data not shown).

In vitro assays of hematopoietic progenitors. Clonogenic progenitor assays were performed by using the methylcellulose culture system[24] with minor modifications. Five hundred CD34⁺ cells were cultured in a 1-mL volume of IMDM supplemented with 30% FCS, 50 ng of recombinant human interleukin-3 (rhIL-3, Kirin Brewery Company, Tokyo, Japan), 50 ng of rh-stem cell factor (SCF, Kirin Brewery Co), 10 ng of rhIL-6 (Kirin Brewery Co), 10 ng of rh-granulocyte-macrophage colony-stimulating factor (GM-CSF; Kirin Brewery Co), 10 ng of rh-granulocyte CSF (Kirin Brewery Co), 3 U of rh-erythropoietin (Kirin Brewery Co), 5×10^{-5} mol/L 2-mercaptoethanol (2-ME), and 0.88% methylcellulose. They were incubated at 37°C under a humidified atmosphere with 5% O_2 and 5% CO_2 in 35-mm culture dishes (Nunc 171099, Naperville, IL). At 14 days, colonies were enumerated as colony-forming unit-granulocyte/macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E), and CFU-mixed cells (CFU-Mix) under an inverted microscope.

Megakaryocyte colony assay was performed with a serum-free culture method as described previously.[25,26] Briefly, 500 CD34⁺ cells were cultured in a 1-mL volume of serum-free medium (Ajinomoto serum free medium 104; Ajinomoto Co, Yokohama, Japan) containing 200 μg/mL transferrin (Sigma Chemical Co, St Louis, MO) saturated with $FeCl_3$, 1% deionized bovine serum albumin (BSA; Sigma Chemical Co), 5×10^{-5} mol/L 2-ME, 10 ng of rh-thrombopoietin (Kirin Brewery Co), 10 ng of rhSCF, and 0.88% methylcellulose. After 14 days of culture, colonies containing more than 10 megakaryocytes were enumerated as CFU-megakaryocytes (CFU-Mk) under an inverted microscope.

The mean numbers of CFU-GM, CFU-Mix, BFU-E, and CFU-Mk were 19.8 ± 2.9, 3.5 ± 1.0, 21.8 ± 2.9, and 14.9 ± 2.6. They did not significantly differ between patients and normal controls. These colonies were individually picked up by using fine drawn-out Pasteur pipettes and processed for molecular analysis.

RNA isolation and RT-PCR analysis. Total RNA was extracted from PBMNCs, BMMNCs, and each colony with 4 μg of carrier RNA (MS2 phage RNA, Boehringer Mannheim, Germany) by the acid guanidine/phenol/chloroform method.[27]

Reverse transcription and double-amplification PCR were performed with a commercial RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT).[11] Briefly, total RNA was reverse transcribed to cDNA by incubation at 20°C for 20 minutes, 42°C for 15 minutes, 99°C for 5 minutes, and finally 4°C for 5 minutes in a total volume of 40 μL containing 1 × PCR reaction buffer (10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl), 100 U Moloney murine leukemia virus reverse transcriptase, 5 mmol/L $MgCl_2$, 1 mmol/L each dNTP, 40 U RNase inhibitor, and 2.5 μmol/L random hexamers. The first PCR amplification was performed in a volume of 50 μL using 10 μL of the reverse transcription mixture with additional 1 × PCR reaction buffer containing 2 mmol/L $MgCl_2$, 1.25 U thermostable Taq DNA polymerase (Perkin Elmer Cetus), and 30 pmol/L of each upstream and downstream primer. The PCR amplification was performed on a programmed-temperature system (PC-700; Astec, Fukuoka, Japan) as follows: denaturation at 93°C for 1 minute, annealing at 65°C for 1 minute, and extension at 72°C for 1 minute. Forty cycles were followed by a final round of extension at 72°C for 10 minutes. Four percent of the first PCR product was added to a second PCR reaction as a template. Two-step nested PCR was then performed. The primer sequences were obtained from the sequenced chimeric *AML1/ETO (MTG8)* cDNA[7,8] (Table 2). The second PCR products were electrophoresed on an ethidium bromide-stained 2% agarose gel. The PCR products from PBMNCs and BMMNCs were transferred to nylon membranes and Southern-blot analysis was performed using 5′-^{32}P labelled oligodeoxynucleotide probe (5′-TCTCAGTACGATTTC-GAGGTTCTC-3′) spanning the *AML1/ETO* fusion point. With this method, we could detect a single Kasumi-1 cell, a t(8;21) AML cell line,[28] among 10^7 HL-60 cells (data not shown). The glyceraldehyde-3-phosphate dehydrogenase messenger RNA was amplified as a control.[29]

To confirm the single lineage of individual colonies picked up and exclude the possibility of cross-contamination of different lineages, we examined the expressions of myeloperoxidase, β-globin, and von Willebrand factor genes in colonies from CFU-GM, BFU-E, and CFU-Mk. In each colony the lineage specific gene expression expected to be positive was exclusively observed and other lineage gene expressions were negative. The primers used are shown in Table 2. Rigorous precautions were taken to avoid contamination of PCRs according to the recommendations of Kwok and Higuchi.[30] False positive results were not obtained throughout the entire study.

Clonality analyses. Clonality analysis of leukemic blasts at diagnosis, remission marrow, and individual colonies was performed on the X-linked inactivation pattern of the PGK gene according to the methods of Gilliland et al[31] and Turhan et al.[32] Four of nine female patients (patients nos. 2, 3, 6, and 7) showed heterozygosity for restriction fragment length polymorphism of the PGK gene by methylation-sensitive enzymes.

One half of the DNA sample was digested with *Hpa*II (Takara Shuzo Co, Shiga, Japan) in a total volume of 20 μL for 1 hour at 37°C, and the remaining half was left undigested. PCR-PGK analysis was performed on both fractions using the external and internal PGK primers as described.[31] Following two rounds of PCR, 20 μL of the PCR product was digested with 10 U of *Bst*XI (Takara Shuzo Co) overnight at 45°C. Amplified fragments were electrophoresed on an ethidium bromide-stained 2% agarose gel. In the *Hpa*II-digested fraction, DNA from single progenitor cells should give rise to either a 530-bp or 433-bp PGK allele as each colony arises from a single CD34⁺ cell, and *Hpa*II-digested fragments could not be amplified by PCR.

Statistical analysis. A two-tailed Student's *t*-test was used for comparing numbers of individual colonies between patients and normal controls. Differences in the frequency of *AML1/ETO* mRNA⁺ colonies between four kinds of progenitors were assessed by Mann-Whitney's U test. Spearman rank correlation analysis was employed to evaluate the relationship between the frequency of *AML1/ETO* mRNA⁺ colonies and the remission duration.

Table 2. Oligonucleotides Used for the RT-PCR Analysis

Origin	Oligonucleotide Sequences	Size (bp) of RT-PCR Product
AML1 external sense	5′-GAGGGAAAAGCTTCACTCTG-3′	
ETO external antisense	5′-TCGGGTGAAATGTCATTGCC-3′	448
AML1 internal sense	5′-GCCACCTACCACAGAGCCATCAAA-3′	
ETO internal antisense	5′-GTGCCATTAGTTAACGTTGTCGGT-3′	200
Myeloperoxidase sense	5′-GTATGACGGAGGCTTCTCTC-3′	
Myeloperoxidase antisense	5′-CAGTTGACGCCAGTGACGAA-3′	237
β-globin sense	5′-ACACAACTGTGTTCACTAGC-3′	
β-globin antisense	5′-AGTGATGGGCCAGCACACAG-3′	392
von Willebrand factor sense	5′-CTTGAATCCCAGTGACCCTGAGCAC-3′	
von Willebrand factor antisense	5′-GCACTTCAAACTCAGCCTCGGACAG-3′	248

RESULTS

Detection of AML1/ETO transcripts in PBMNCs and BMMNCs. Table 3 shows results of the *AML1/ETO* mRNA detection by the two-step nested RT-PCR. *AML1/ETO* mRNA could be detected in all PB and BM samples from 18 patients treated with chemotherapy or PBSCT. In contrast, it could not be detected in any of the four patients who underwent allogeneic BMT: representative results are shown in Fig 1. These data strongly suggest that the *AML1/ETO* fusion transcript is still produced in the circulating leukocytes of patients in long-term remission.

Detection of AML1/ETO transcripts in clonogenic progenitors. Individual colonies also underwent RT-PCR analysis (Table 3). The *AML1/ETO* mRNA was detected in progenitor colonies in 18 of 20 bone marrow samples obtained from patients treated with chemotherapy or PBSCT (patients nos. 1 through 18). In total, 51 of 2,469 progenitor cell colonies (2.1%) were PCR⁺. *AML1/ETO* mRNA could be detected in various clonogenic progenitors, such as CFU-GM (16 of 18 cases), CFU-Mix (5 of 18 cases), BFU-E (9 of 18 cases), and CFU-Mk (5 of 15 cases). The frequencies of *AML1/ETO* mRNA-positive colonies were 3.2% (29 of 894 colonies examined) in CFU-GM, 1.9% (5 of 260) in CFU-Mix, 1.3% (11 of 815) in BFU-E, and 1.3% (6 of 457) in CFU-Mk. The positivity of *AML1/ETO* mRNA in CFU-GM was statistically higher than those in other colonies ($P < .05$). RT-PCR analysis of individual colonies in patient nos. 12 and 13 was particularly informative (Fig 2). In these cases, *AML1/ETO* mRNA⁺ colonies were seen in CFU-GM, CFU-Mix, BFU-E, and CFU-Mk. In other cases, there was the

Table 3. RT-PCR Analysis of AML1/ETO mRNA in Individual Progenitor Colonies

Patient No.	Detection of AML1/ETO mRNA		No. of AML1/ETO mRNA-Positive/No. of Colonies Tested				
	PB	BM	CFU-GM	CFU-Mix	BFU-E	CFU-Mk	Total (%)
CHT/PBSCT Group							
1(a)*	+	+	2/38	0/10	1/41	0/25	3/114 (2.6)
(b)	+	+	1/46	0/10	0/42	0/22	1/120 (0.8)
2(a)*	NE	+	2/46	0/10	1/38	0/24	3/118 (2.5)
(b)	+	+	0/55	0/15	0/45	0/28	0/143 (0)
3	NE	+	2/50	0/12	0/40	NE	2/102 (2.0)
4	+	+	2/46	0/10	0/36	NE	2/92 (2.2)
5	+	+	3/42	0/12	2/43	1/31	6/128 (4.7)
6	NE	+	2/52	0/18	0/56	NE	2/126 (1.6)
7	+	+	2/45	0/15	1/47	1/32	4/139 (2.9)
8	+	+	0/42	1/15	0/38	1/29	2/124 (1.6)
9	+	+	1/40	0/12	1/46	0/26	2/124 (1.6)
10	+	+	0/41	0/12	0/40	0/24	0/117 (0)
11	+	+	1/42	1/12	0/40	0/22	2/116 (1.7)
12	+	+	1/49	1/12	1/42	1/35	4/138 (2.9)
13	+	+	3/40	1/13	1/45	2/34	7/132 (5.3)
14	+	+	1/50	0/15	0/44	0/25	1/134 (0.7)
15	NE	+	2/45	0/12	2/40	0/22	4/119 (3.4)
16	+	+	2/45	1/15	1/47	0/28	4/135 (3.0)
17	+	+	1/40	0/18	0/45	0/20	1/123 (0.8)
18	+	+	1/40	0/12	0/43	0/30	1/125 (0.8)
Total			29/894 (3.2)	5/260 (1.9)	11/815 (1.3)	6/457 (1.3)	51/2469 (2.1)
Allo BMT Group							
19	—	—	0/51	0/13	0/55	0/29	0/148 (0)
20	—	—	0/52	0/14	0/48	0/21	0/135 (0)
21	—	—	0/50	0/15	0/50	0/30	0/145 (0)
22	—	—	0/51	0/15	0/50	0/30	0/146 (0)
Total			0/204 (0)	0/57 (0)	0/203 (0)	0/110 (0)	0/574 (0)

Patient nos. 1 to 18 were treated with chemotherapy or PBSCT, while patient nos. 19 to 22 underwent allogeneic BMT (see Table 1).

Abbreviations: CHT, chemotherapy; PBSCT, peripheral blood stem cell transplantation; Allo BMT, allogeneic bone marrow transplantation; NE, not examined.

* Samples were obtained twice from patient no. 1 (a and b) and no. 2 (a and b) at different times. See Table 1.

lack of trilineage positivity of *AML1/ETO* mRNA because of the relatively small number of progenitors analyzed. Although the *AML1/ETO* mRNA never disappeared in PB and BM samples from patients in long-term remission who had been treated with chemotherapy or PBSCT, the frequency of *AML1/ETO* mRNA$^+$ colonies gradually decreased along with the remission duration ($r = -0.692$, $P = .0007$). However, *AML1/ETO* mRNA could not be detected in 574 clonogenic progenitor colonies from all four patients who underwent allogeneic BMT (patient nos. 19 through 22) (Table 3).

Analysis of clonality. To determine the clonal origin of these *AML1/ETO* mRNA$^+$ colonies and leukemic blasts at initial diagnosis, we analyzed X-linked chromosomal inactivation mosaicism of the PGK gene. This technique was applied in four female patients treated with chemotherapy or PBSCT (patient nos. 2, 3, 6, and 7). The clonality analysis was performed on BMMNCs, CD34$^+$ cells, individual colonies from remission marrow, and BMMNCs at initial diagnosis, which consisted of more than 90% leukemic blasts. In all four cases examined, the leukemic blasts gave rise to a single band with disappearance of either the 530-bp or 433-bp PGK allele in the *Hpa*II-digested fraction, indicating their clonal origin from leukemic stem cells. In contrast, BMMNCs and CD34$^+$ cells during remission gave rise both to 530-bp and 433-bp bands: representative result (patient no. 3) is shown in Fig 3. This indicates that hematopoiesis during remission was polyclonal. That is, hematopoiesis was sustained by at least two hematopoietic stem cells.

We then tested the clonality of individual *AML1/ETO* mRNA positive and negative colonies (Table 4). The PCR products from the *AML1/ETO* mRNA$^-$ colonies gave rise to 530-bp or 433-bp PGK alleles, and the frequencies of colonies with each genotype were almost equal. This is consistent with the data obtained from BMMNCs and CD34$^+$ cells, which indicated polyclonal hematopoiesis in remission. In contrast, all PCR products from 11 *AML1/ETO* mRNA$^+$ colonies were identical to those of leukemic blasts at initial diagnosis in each patient: representative result (patient no. 7) is shown in Fig 4. These data show the clonal origin of the *AML1/ETO* mRNA$^+$ clonogenic progenitors from remission marrow and also leukemic blasts at initial diagnosis.

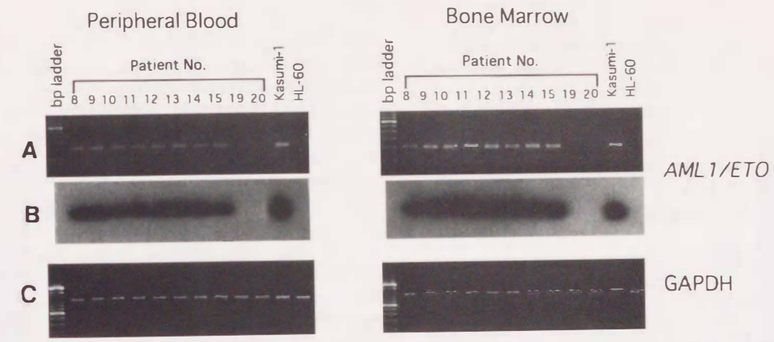

Fig 1. RT-PCR analysis of the *AML1/ETO* fusion gene in PB and BM samples from 10 long-term remission patients. (A) *AML1/ETO*-derived products by RT-PCR; (B) autoradiogram of the PCR products derived from the gel shown in (A); (C) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA-derived products as an internal control. Patient nos. 8 to 15 were treated with chemotherapy alone, while patient nos. 19 and 20 underwent allogeneic BMT. The positive control cells (Kasumi-1) gave rise to a 200-bp product. HL-60 cells were used as the negative control.

DISCUSSION

In the present study, we showed that the positive results of MRD analysis of t(8;21) AML patients in long-term remission could be ascribed to residual t(8;21)$^+$ multipotent hematopoietic progenitors. *AML1/ETO* mRNA expression was not restricted to CFU-GM, but also involved CFU-Mix, BFU-E, and CFU-Mk. Furthermore, clonality analysis indicated that both the *AML1/ETO* mRNA$^+$ progenitors from remission marrow and t(8;21) leukemic blasts at initial diagnosis were clonal in origin. In contrast, *AML1/ETO* mRNA$^-$ progenitors from remission marrow were polyclonal in origin.[16,32] Accordingly, it appears that the leukemic clonogenic progenitor of t(8;21) AML is derived from a t(8;21)$^+$ multipotent progenitor. The persistence of the *AML1/ETO* mRNA$^+$ progenitor does not necessarily indicate impending relapse, as the *AML1/ETO* mRNA was detected in all 12 patients who remained in hematologic and cytogenetic remission for more than 36 months, a group who could be considered clinically "cured" on the basis of historical data.[9,10] The *AML1/ETO* mRNA$^+$ progenitor did not show maturation arrest, but rather retained trilineage differentiation potential, at least, in vitro. These results strongly suggest that expression of the *AML1/ETO* fusion gene does not necessarily mark the leukemic transformation.

The successful detection of the *AML1/ETO* mRNA in PB collected from long-term remission patients strongly suggests that at least some fraction of the *AML1/ETO* mRNA$^+$ progenitors contribute to the hematopoietic reconstitution in remission. There remains the possibility that the *AML1/ETO* mRNA-positive progenitors can differentiate into mature granulocytes, macrophages, erythrocytes, and megakaryocytes only in vitro, because the concentrations of cytokines used are higher than those experienced under physiologic conditions. These *AML1/ETO* mRNA-positive colonies might not be directly derived from residual t(8;21)$^+$ leukemic cells, because we could not see these colonies from leukemic BMMNCs on leukemic blast colony assay. In this assay, the in vitro conditions are quite similar to those of the progenitor assay employed in this study (data not shown). The frequency of *AML1/ETO* mRNA$^+$ colonies was approximately 2%. This does not directly correlate with the quantitative results because the residual *AML1/ETO* mRNA was

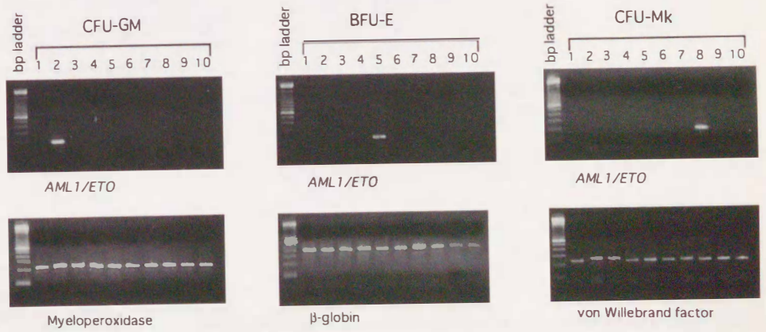

Fig 2. RT-PCR analysis of CFU-GM, BFU-E, and CFU-Mk from patient no. 12. *AML1/ETO* mRNA$^+$ colonies can be seen from CFU-GM (lane 2), BFU-E (lane 5), and CFU-Mk (lane 8). The single lineage commitment of CFU-GM, BFU-E, and CFU-Mk was shown by their exclusive expression of the myeloperoxidase, β-globin, and von Willebrand factor genes, respectively. Other lineage specific gene expressions were negative when applied to other lineages (data not shown).

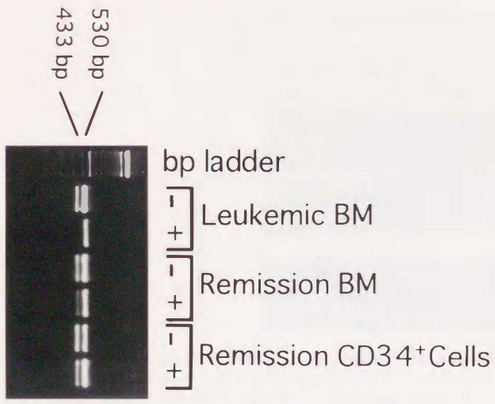

Fig 3. Clonality analysis using the X-linked inactivation assay of the PGK gene in patient no. 3. Lane: −, DNA amplified without *Hpa*II digestion; +, DNA amplified after *Hpa*II digestion. Only one band (530-bp) was present in leukemic BM at initial diagnosis, but both alleles (530- and 433-bp) were evident in remission BM and CD34⁺ cells.

quantified as 50 to 500 molecules/μg of total RNA (unpublished data). The amount of which corresponds to 1×10^{-5}% leukemic contamination when Kasumi-1 cells were used as a positive control. We can, therefore, speculate that the *AML1/ETO* mRNA⁺ progenitors might be quiescent in situ, and that they can proliferate in response to high concentrations of cytokines experienced in vitro. In this context, the gradual decrease in *AML1/ETO* mRNA⁺ progenitors after achieving remission could be ascribed to a growth advantage for normal progenitors in situ. An alternative explanation is that *AML1/ETO*-expressing cells may be responsive to immune-mediated mechanisms that suppress leukemic cell growth.

However, the *AML1/ETO* mRNA⁺ progenitors never disappeared in our series of long-term remission patients. It has been suggested that *AML1/ETO* mRNA may play an essential role in the progression of t(8;21) AML. Sakakura et al[33] have reported that antisense oligomers to the chimeric *AML1/ETO* junction inhibited the proliferation of Kasumi-1 cells, whereas they enhanced the sensitivity to differentiation induction by phorbol 12-myristate 13-estate. Also, Nucifora et al[34] have shown that AML expressing the *AML1/ETO* mRNA has similar morphologic and cytochemical characteristics irrespective of the presence of t(8;21). However, the persistence of multipotent progenitors expressing *AML1/ETO* transcripts in remission raises the possibility that t(8;21)-positive multipotent progenitors are not fully transformed, but rather require another mutational event to achieve full leukemic transformation. This scenario of leukemic transformation has been widely discussed in the stem cell disorder, such as chronic myelogenous leukemia (CML).[35,36] Philadelphia chromosome translocation involves pluripotent progenitors that can differentiate along lymphoid lineages as well as myeloid trilineages.[37,38] The blast crisis is probably because of the acquisition of an additional mutation. It is largely accepted that the cure of patients in CML blast crisis can be achieved only by allogeneic BMT.[36,39] This generally results in a decrease in *BCR/ABL* mRNA to undetectable levels by RT-PCR.[39-41] In our study, *AML1/ETO* mRNA was undetectable in any samples from four patients successfully treated by allogeneic BMT. Allogeneic BMT eradicates the *AML1/ETO*⁺ cells, probably through alloreactive T-cell responses such as the "graft-versus-leukemia" effect. In contrast to *BCR/ABL* in CML,[40-42] the detection of *AML1/ETO* mRNA does not inevitably indicate residual leukemic cells. This is probably because *AML1/ETO* and *BCR/ABL* play different roles in the leukemic transformation process. Because there has been no clear demonstration that the *AML1/ETO* fusion gene is involved in the mechanism of leukemic transformation, it is difficult to address whether detection of the *AML1/ETO* transcript by RT-PCR is because of expression in t(8;21)⁺ multipotent hematopoietic progenitors or t(8;21)⁺ residual leukemic cells.

The t(8;21) AML has several biological characteristics of immature stem cells, although there is also a tendency to granulocytic differentiation in vivo; the leukemic blasts of t(8;21) AML frequently express CD34 and CD19, either of which marks primitive progenitor cells and early stage B cells, respectively.[43] We have shown that leukemic progenitors in CD34⁺ AML simulate the proliferative response to various cytokines in normal blast colony-forming cells.[24] The cross lineage expression of surface markers in leukemic blasts strongly suggests that they preserve surface molecules of immature leukemic progenitors transformed at the differentiation stage of bipotent (ie, myeloid/lymphoid) progenitors (mixed lineage leukemias).[44,45] Accordingly, it is of significant interest to determine whether the *AML1/ETO* fusion gene involves progenitors that can differentiate along the B-cell lineage, in addition to the myeloid lineage.

Table 4. Clonal Analysis of Individual Colonies From Four Female Patients

Patient No.	PGK Allele of Leukemic BM	No. of Colonies Examined (AML 1/ETO mRNA)	PGK Allele in AML1/ETO mRNA-Positive or Negative Colonies							
			CFU-GM		CFU-Mix		BFU-E		CFU-Mk	
			433 bp	530 bp	433 bp	530 bp	433 bp	530 bp	433 bp	530 bp
2(a)	530 bp	115 (Neg.)	22	22	5	5	18	19	11	13
		3 (Pos.)*	—	2	—	—	—	1	—	—
3	530 bp	100 (Neg.)	22	26	6	6	19	21	NE	NE
		2 (Pos.)*	—	2	—	—	—	—	NE	NE
6	433 bp	124 (Neg.)	22	28	10	8	27	29	NE	NE
		2 (Pos.)*	2	—	—	—	—	—	NE	NE
7	530 bp	135 (Neg.)	22	21	7	8	23	23	17	14
		4 (Pos.)*	—	2	—	—	—	1	—	1

Abbreviation: NE, not examined.
* All 11 AML1/ETO mRNA⁺ colonies show the PGK allele identical to that of leukemic blasts at diagnosis.

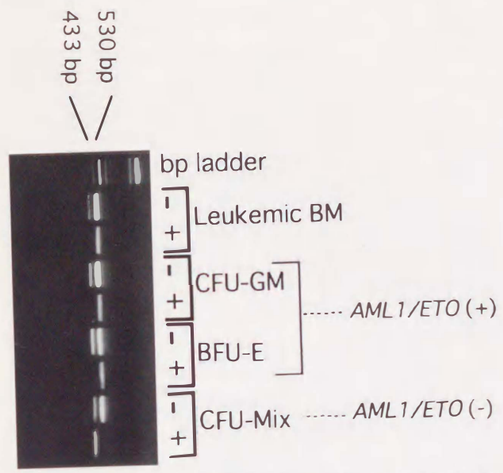

Fig 4. Clonality analysis of *AML1/ETO* mRNA⁺ progenitor colonies from patient no. 7. Lane: −, DNA amplified without *Hpa*II digestion; +, DNA amplified after *Hpa*II digestion. The PGK alleles of the *AML1/ETO* mRNA⁺ CFU-GM and BFU-E are identical to those of leukemic BM at initial diagnosis, showing their clonal origin. In CFU-Mix that do not express *AML1/ETO* mRNA, the genotype is different from the pattern observed in leukemic BM.

In summary, we showed that the *AML1/ETO* mRNA was expressed in multipotent hematopoietic progenitors in long-term remission patients with t(8;21) AML. These *AML1/ETO* mRNA⁺ progenitors were of the same clonal origin as leukemic blasts from the time of diagnosis. However, this finding did not necessarily indicate impending relapse because the *AML1/ETO* mRNA⁺ progenitors could persist for more than 10 years after achieving remission. These progenitors can differentiate into mature trilineage myeloid cells in vitro, suggesting that an additional transformation event would be required for them to become a malignant leukemic clone. Thus, leukemic stem cells in de novo t(8;21) AML might be derived from t(8;21)⁺ multipotent hematopoietic progenitors.

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