dosage of steroid by a long-term low-dose prednisolone therapy may not be a risk factor for atherosclerosis in SLE, because a prospective study showed that a low-dose prednisolone therapy does not influence atherosclerosis, as determined by carotid IMT in rheumatoid arthritis patients for at least 2 years [33]. Current use of cyclosporine A was significantly associated with decreased carotid IMT (Table 5). Cyclosporine A blocks the phosphatase activity of calcineurin, an essential component of the T cell activation pathway, and is thus considered to be a strong inhibitor of the immune system, most notably of T cells [34]. Tacrolimus was not associated with carotid IMT in our cases, which may not be surprising. Although cyclosporine A and tacrolimus share a common mechanism of calcineurin inhibition, there have been reported differential effects on the molecules other than calcineurin [35]. In fact, clinical effects and adverse events of these two calcineurin inhibitors are not similar, as evidenced in the previous 2 decades of data on organ transplantation [35]. As the number of patients on tacrolimus is small ($n = 8$), further study with a larger sample size is needed before reaching a conclusion. A protective effect of cyclosporine A use against carotid IMT has also been reported by others [36]. Cyclosporine A might be added to the treatment of choice from the standpoint view of reduction of carotid IMT and the resultant prevention of atherosclerosis in SLE.

In conclusion, multivariate-adjusted mean carotid IMT was significantly reduced in SLE patients compared to healthy controls ($P = 0.003$). The current use of cyclosporine A ($P = 0.011$) and a history of steroid pulse therapy ($P = 0.006$) were significantly associated with decreased carotid IMT, while current use of NSAIDs ($P = 0.054$) was marginally associated with increased carotid IMT. Advances in medical therapy and a better understanding of SLE have contributed to a dramatic improvement in the long-term survival of patients.

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Conflict of interest None.

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The ordered acquisition of Class II and Class I mutations directs formation of human t(8;21) acute myelogenous leukemia stem cell

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The cellular properties of leukemia stem cells (LSCs) are achieved at least through Class I and Class II mutations that generate signals for enhanced proliferation and impaired differentiation, respectively. Here we show that in t(8;21) acute myelogenous leukemia (AML), hematopoietic stem cells (HSCs) transform into LSCs via definitively-ordered acquisition of Class II (*AML1/ETO*) and then Class I (*c-KIT* mutant) abnormalities. Six t(8;21) AML patients with *c-KIT* mutants maintaining > 3 years of complete remission were analyzed. At diagnosis, all single LSCs had both *AML1/ETO* and *c-KIT* mutations. However, in remission, 16 out of 1,728 $CD34^+CD38^-$ HSCs and 89 out of 7,187 single HSC-derived myeloerythroid colonies from these patients had *AML1/ETO*, whose breakpoints were identical to those found in LSCs. These cells had wild-type *c-KIT*, which expressed *AML1/ETO* at a low level, and could differentiate into mature blood cells, suggesting that they may be the persistent preleukemic stem cells. Microarray analysis suggested that mutated c-KIT signaling provides LSCs with enhanced survival and proliferation. Thus, in t(8;21) AML, the acquisition of *AML1/ETO* is not sufficient, and the subsequent upregulation of *AML1/ETO* and the additional *c-KIT* mutant signaling are critical steps for transformation into LSCs. © **2014 ISEH - International Society for Experimental Hematology. Published by Elsevier Inc.**

Acute myelogenous leukemia (AML) is characterized by deregulated proliferation and impaired differentiation of immature hematopoietic cells and originates from leukemia stem cells (LSCs). Leukemia stem cells have cellular properties, such as self-renewal activity, impairment of full maturation, and reinforced survival, which may cooperatively play a role in advantageous growth compared with normal hematopoietic stem cells (HSCs). Such cellular properties of LSCs result from multiple genetic abnormalities that are presumably accumulated within the long-surviving, self-renewing HSCs [1,2]. Recent mouse studies have suggested that these genetic abnormalities could be categorized into at least two classes. Class I mutations confer a proliferative and/or survival advantage against hematopoietic progenitors and are exemplified by constitutively activated tyrosine kinases such as BCR-ABL, FLT3 internal tandem duplication (FLT3-ITD), and mutated c-KIT. On the other hand, Class II mutations impair hematopoietic differentiation, which includes the core binding factor (CBF) mutations such as *AML1/ETO* [3,4]. Several mouse models have demonstrated that the combined effects of enhanced proliferation (by Class I abnormalities) and differentiation block (by Class II abnormalities) result in AML development [5–9], but these processes have, to our knowledge, never been documented in de novo human AML.

In AML, *AML1/ETO* achieved by t(8;21) is one of the most common chromosomal abnormalities [10,11]. The enforced *AML1/ETO* expression in hematopoietic cells could block their differentiation [12–14] because AML1/ETO inhibits CBF complexes that can transactivate multiple myeloid-related genes (e.g., *CEBPA*, *MPO*, and *IL3*), in a dominant negative fashion. Frequently, t(8;21) AML patients

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possess constitutively active Class I mutation of *c-KIT* and *FLT3* [7,15,16]. In mouse studies, *AML1/ETO* knock-in or transgenic mice did not develop AML [9,14,17], but these mice developed AML following introduction of Class I genetic abnormalities such as mutations of the *c-KIT* [9,18], *FLT3* [7], and *TEL-PDGFRa* genes [8]. These data strongly suggest that the acquisition of *AML1/ETO* fusion alone is not sufficient, and some additional oncogenic events are needed for the development of t(8;21) AML. However, these studies were based on mouse models, where the expression of Class I and Class II genes was artificially enforced. The critical questions are whether these multistep oncogenic events involve human AML and, if so, whether they occur at random or in a definitive order.

Our previous t(8;21) AML patient studies have proven that t(8;21) is acquired in long-term HSCs but it is not sufficient for AML development [19,20]. We found that t(8;21) AML patients maintaining remission long term (>10 years) always possessed a small amount of *AML1/ETO* mRNA in their blood and bone marrow cells. These *AML1/ETO*$^+$ cells may be derived from HSCs having a low level of *AML1/ETO* mRNA, the frequency of which was estimated to be approximately 1% of HSCs [20]. Thus, the acquisition of *AML1/ETO* is not sufficient for leukemic transformation in humans. Therefore, we proposed that such *AML1/ETO*$^+$ HSCs are preleukemic clones that have achieved a precondition for leukemic transformation by additional oncogenic hits [20]. Wiemels et al. have reported that a fraction of t(8;21) AML children had *AML1/ETO*$^+$ clones in their blood samples from neonatal Guthrie blood spots [21], suggesting that t(8;21) translocation can be achieved in utero, and resultant *AML1/ETO*$^+$ HSCs can form a reservoir for the preleukemic clone after birth [21].

Based on these data, we sequentially tracked the involvement of Class I and Class II mutations during clinical courses of t(8;21) AML patients. Here we show that, by single HSC and LSC analyses of *AML1/ETO* in patients with mutated *c-KIT*, all single *AML1/ETO*$^+$ LSCs at diagnosis had *c-KIT* mutations, whereas they were never found within *AML1/ETO*$^+$ HSCs in remission. Our data clearly show that AML1/ETO$^+$ HSCs should belong to the preleukemic clone and are transformed into LSCs by subsequent acquisition of *c-KIT* mutation. This is, to our knowledge, the first clear-cut evidence that normal HSCs transform into LSCs via definitively-ordered acquisition of Class II and then Class I mutations in de novo human AML.

Methods

Patients and samples

Patients' characteristics are shown in Table 1. This study included bone marrow cells from 33 t(8;21) AML cases at diagnosis (Patients 1–33), 13 cases in remission (Patients 1, 3, 7–9, 11, 13, 21–23, 26–27, and 31), and 13 cases at relapse (Patients 2, 5–6, 10, 14, 16–17, 25, 28–30, and 32–33). Remission marrow samples were obtained at least 12 months from first remission, and all the patients remained in remission at the time of this report. Out of 33 cases, 20 obtained complete remission only by chemotherapies. Patients 2 and 10 further received allogeneic bone marrow transplantation, and Patient 5 further received cord blood transplantation. On AML cells, CD19 and CD56 are known as the prognostic markers associated with the possession of *c-KIT* mutation [15,22]. The AML cells of all 13 cases with *c-KIT* mutation at diagnosis were CD19$^-$CD56$^+$. Human marrow was purchased from AllCells (Emeryville, CA). Informed consent was obtained from all patients. The Institutional Review Board of Kyushu University Hospital (Fukuoka, Japan) approved all research.

Flow cytometry analysis and cell sorting

For analysis of CD34$^+$CD38$^-$ cells, bone marrow mononuclear cells were prepared as previously described [19,23]. Cells were stained with APC-anti-CD34, FITC-anti-CD90, PE-anti-CD117 (c-KIT), Cy5-PE-lineage (Lin) mixture (anti-CD3, -CD4, -CD8, -CD10, -CD20, -CD256) (BD Pharmingen, San Jose, CA), and biotin-anti-CD38 (Caltag Laboratories, Buckingham, UK). Streptavidin-Cy7-allophycocyanin (BD Pharmingen) was also used.

Quantitative real-time polymerase chain reaction

We isolated RNA from 5,000 cells using Isogen reagent (*Nippon* gene). We reverse transcribed RNA to cDNA using TaKaRa RNA polymerase chain reaction (PCR) kit (Takara Shuzo, Shiga, Japan). The mRNA levels were quantified by real-time PCR (Applied Biosystems, Carlsbad, CA). β2-microglobulin (B2MG) was used for internal control. The primer and probes for *B2MG, c-KIT, C-X-X chemokine receptor type 4 (CXCR4), B-cell lymphoma 2 (BCL2), myeloid cell leukemia 1 (MCL1)* and *nuclear factor kappa B1 (NFKB1)* were purchased from Applied Biosystems.

Reverse transcription polymerase chain reaction

To examine the *AML1/ETO* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA expression, reverse transcription-PCR (RT-PCR) was performed as previously reported [19,20]. Kasumi-1, a t(8;21) AML cell line, was used as positive control. The nested RT-PCR protocol was previously reported [19].

In vitro assays to evaluate the differentiation potential of myeloid progenitors

The clonogenic colony-forming unit (CFU) assay protocol was previously reported [20]. All of the myeloid colonies were picked up and separated to extract the RNA and genomic DNA.

Identification of the gene mutations

Genomic DNA was extracted by Micro Kit (QIAGEN, Hilden, Germany). The presence of *FLT3-ITD*, *NRAS*, and *c-KIT* mutations was examined as previously descried [7,24]. The primers for the *c-KIT* mutation are shown in Supplementary Table E1 (online only, available at www.exphem.org). The clonal PCR product was purified by QIAquick Spin (QIAGEN) and directly sequenced by ABI 3730 Genetic analyzer (Applied Biosystems).

Identification of breakpoint of AML1/ETO *of genomic DNA*

Patients' breakpoints were determined by sequencing the PCR products of long-distance inverse PCR (LDI-PCR) and conventional long distance PCR (LD-PCR), as previously described [21,25]. The primers are listed in Supplementary Table E2 (online only, available at www.exphem.org).

Table 1. Patients' characteristics

Patient	Age	Sex	FAB	Karyotype	CD19	CD56	Treatment	c-KIT at diagnosis	c-KIT at relapse	Marrow sampling (month from 1st remission)	Remission duration (month)	NRAS mutation	FLT3-ITD
1	57	M	M2	45,X,-Y,t(8;21)(q22;q22)	(−)	(+)	Ch-Tx	wt	Remission	14	>29	(−)	(−)
2	27	F	M2	45,X,-X,t(8;21)(q22;q22)	(−)	(+)	Allo-BMT	D816V	D816V	2	—	(−)	(−)
3	33	F	M2	46,XX,t(8;21)(q22;q22)	(−)	(+)	Ch-Tx	D816V	Remission	13	>37	(−)	(−)
4	40	F	M2	46,XX,t(8;21)(q22;q22)	(−)	(+)	Ch-Tx	wt	Remission	NA	>41	(−)	(−)
5	24	F	M2	46,XX,t(8;21)(q22:q22)	(−)	(+)	CBT	D816V	D816V	7	—	(−)	(−)
6	65	M	M2	45,X,-Y(AML1/ETO^{+})	(+)	(+)	Ch-Tx	wt	wt	24	—	(−)	(−)
7	41	M	M2	46,XY,t(8;21)(q22;q22),+complex	(−)	(+)	Ch-Tx	D816V	Remission	20	>44	(−)	(−)
8	30	F	M2	45,X,-X,t(8;21)(q22;q22)	(−)	(+)	Ch-Tx	N822K	Remission	60	>90	(−)	(−)
9	84	F	M2	46,XX t(8;21)(q22;q22)	(−)	(−)	Ch-Tx	wt	Remission	14	>23	(−)	(−)
10	32	M	M2	45,X,-Y,t(8;21)(q22;q22)	(−)	(+)	Allo-BMT	wt	wt	7	—	(−)	(−)
11	65	F	M2	46,XX,t(8;21)(q22;q22)	(−)	(+)	Ch-Tx	N822K	Remission	20	>46	(−)	(−)
12	76	F	M2	46,XX,t(8;21) (q22;q22)	(−)	(−)	Ch-Tx	wt	Remission	NA	>39	(−)	(−)
13	56	M	M2	46,XY,t(8;21)(q22;q22)	(−)	(−)	Ch-Tx	wt	Remission	12	>28	(−)	(+)
14	19	M	M2	46,XY t(8;21)(q22;q22)	(−)	(+)	Ch-Tx	N822K	D816Y	10	—	(−)	(−)
15	62	F	M2	46,XX,t(8;21)(q22;q22)	(−)	(−)	Ch-Tx	wt	Remission	NA	>19	(−)	(−)
16	14	F	M2	46,XX t(8;21)(q22;q22)	(−)	(+)	Ch-Tx	N822K	N822K	8	—	(−)	(−)
17	57	F	M2	48,XX,+4,+6,t(8;21)(q22;q22)	(−)	(+)	Ch-Tx	D816Y	D816Y	10	—	(−)	(−)
18	56	M	M2	45,XY,t(8;21)(q22,q22),+complex	(−)	(−)	Ch-Tx	wt	Remission	NA	>88	(−)	(−)
19	21	M	M2	45,X,-Y,t(8;21)(q22;q22)	(−)	(+)	Ch-Tx	wt	Remission	NA	>31	(−)	(−)
20	25	M	M2	46,XY,t(8;21)(q22;q22)	(−)	(+)	Ch-Tx	wt	Remission	NA	>65	(−)	(−)
21	34	M	M2	45,X,-Y,t(8;21)(q22,q22)	(−)	(+)	Ch-Tx	D816Y	Remission	22	>49	(−)	(−)
22	57	M	M2	46,XY,t(8;21)(q22;q22)	(−)	(−)	Ch-Tx	wt	Remission	18	>32	(−)	(−)
23	39	M	M2	46,XY,t(8;21)(q22;q22)	(−)	(+)	Ch-Tx	D816V	Remission	23	>59	(−)	(−)
24	16	M	M2	46,X,Y,t(8;21)(q22;q22)	(−)	(+)	Ch-Tx	wt	Remission	NA	>77	(−)	(−)
25	37	M	M2	45,X,-Y,t(8;21)(q22;q22)	(−)	(+)	Ch-Tx	D816V	D816V	4	—	(−)	(−)
26	48	M	M2	46,XY,t(8;21)(q22:q22)	(+)	(−)	Ch-Tx	wt	Remission	19	>40	(−)	(−)
27	65	M	M2	46,XY,t(8;21)(q22:q22)	(−)	(+)	Ch-Tx	wt	Remission	12	>36	(−)	(−)
28	65	M	M2	45,X,-Y,t(8;21)(q22:q22)	(−)	(+)	Ch-Tx	wt	wt	8	—	(−)	(−)
29	47	M	M2	46,XY,t(8;21)(q22:q22)	(+)	(−)	Ch-Tx	wt	wt	10	—	(−)	(−)
30	65	M	M2	45,X,-Y,t(8;21)(q22:q22)	(−)	(+)	Ch-Tx	wt	wt	11	—	(−)	(−)
31	39	M	M2	46,XY,t(8;21)(q22;q22)	(−)	(−)	Ch-Tx	wt	Remission	29	>69	(−)	(−)
32	39	F	M2	46,XX,t(8;21)(q22:q22)	(−)	(+)	Ch-Tx	N822K	N822K	20	—	(−)	(−)
33	29	M	M2	45,X,-Y,t(8;21)(q22:q22)	(+)	(+)	Ch-Tx	wt	wt	9	—	(−)	(+)

Allo-BMT = allogeneic bone marrow transplantation; CBT = cord blood transplantation; Ch-Tx = chemotherapy; wt = wild type.

Single-cell quantitative polymerase chain reaction and genomic polymerase chain reaction

Single-cell quantitative PCR protocol was previously reported [26]. We used *AML1/ETO* external primers (Supplementary Table E3, online only, available at www.exphem.org). Single-cell genomic PCR was performed by nested PCR utilizing external and internal primers (Supplementary Table E1, online only, available at www.exphem.org). The method of nested PCR for genomic DNA was the same as the RT-PCR method. For single-cell quantitative nested PCR, we performed first round of RT-PCR with external primers for these diluted, pre-amplified cDNA. The protocol of the first round of PCR was same as that for RT-PCR (thermal cycling setting was 16). Nested PCR was performed by using a BioMark 48 × 48 Dynamic Array system with internal primers (Supplementary Table E1, online only, available at www.exphem.org).

Microarray analysis

Eighteen wild-type *c-KIT* LSC and 13 mutated *c-KIT* LSC samples were investigated with Sentrix Bead Chip Assay, Human-6 V2 (Illumina, San Diego, CA) as previously reported [27]. Microarray data were analyzed with Gene Spring GX11.01 (Agilent Technologies, Santa Clara, CA).

Cytokine stimulation assays

The c-KIT signaling repercussion for *AML1/ETO* expression level by addition of stem cell factor was evaluated after 24-hour serum-free liquid culture. The details were previously described [28].

Results

c-KIT *mutation was found in approximately 40% of patients with t(8;21) acute myelogenous leukemia*

Thirty-three t(8;21) AML patients were enrolled in this study. Previous studies have shown that Class I abnormalities, such as *c-KIT*, *NRAS*, and *FLT3* mutations, are frequently found in t(8;21) AML [7,15,16]. As shown in Table 1, 13 out of 33 t(8;21) AML patients had *c-KIT* mutation, one patient had *FLT3-ITD*, and no *NRAS* mutations were observed. In all cases, involvement of Class I mutation was heterozygous.

Of the patients with *c-KIT* mutations, six had D816V mutation, five had N822K, and two had D816Y (Table 1). The expression levels of c-KIT mRNA and protein in the $CD34^+CD38^-$ LSC fraction of t(8;21) AML were equal in all cases, regardless of the involvement of *c-KIT* mutations, and their levels were identical to those of normal $CD34^+CD38^-$ HSCs (Supplementary Figure E1, online only, available at www.exphem.org). Out of 13 patients with *c-KIT* mutations, seven patients relapsed. Six out of seven relapsed patients had mutations identical to those found at diagnosis, whereas Patient 14 acquired an independent de novo *c-KIT* mutation at relapse (N822K at diagnosis and D816Y at relapse) (Table 1). This intriguing case suggests that the acquisition of *c-KIT* mutation is the second event that is independent of t(8;21). These data led us to test whether *c-KIT* mutation is involved in preleukemic $AML1/ETO^+$ HSCs in remission [20].

All single leukemia stem cells possess both AML1/ETO *and* c-KIT *mutations at diagnosis*

Six cases of t(8;21) AML with *c-KIT* mutations, including D816V, N822K, and D816Y, who maintained complete remission for > 3 years (Patients 3, 7, 8, 11, 21, and 23) were investigated to track *AML1/ETO* and *c-KIT* status at both diagnosis and during remission.

We first tested the presence of *AML1/ETO* mRNA and *c-KIT* mutation in LSCs at diagnosis at the single cell level. As shown in Figure 1A, genomic DNA and mRNA were extracted from single $CD34^+CD38^-$ leukemic marrow cells and were subjected to PCR to test for the presence of *AML1/ETO* mRNA. The *c-KIT* gene was amplified from single cell–derived genomic DNA and analyzed by direct sequencing to identify c-KIT mutations.

Figure 1B shows representative results of *AML1/ETO* mRNA analysis of single LSCs (Patient 8). At diagnosis, nearly all LSCs expressed *AML1/ETO* mRNA at a high level, whereas, in remission, only a few percent of HSCs expressed *AML1/ETO* mRNA, whose levels were so low they were only detectable after the second round of PCR (Fig. 1B).

Summarized data are shown in Table 2. In the analysis of six cases at diagnosis, 1,608 (98.9%) out of 1,626 single LSCs that were analyzed had *AML1/ETO* mRNA, and *c-KIT* mutations specific to each patient were observed in all 1,608 *AML1/ETO* $mRNA^+$ cells. In contrast, the remaining 18 $CD34^+CD38^-$ cells that did not express AML1/ETO mRNA had the wild-type *c-KIT*, indicating AML1/ETO mRNA and *c-KIT* mutation always coexist at diagnosis in all single LSCs.

All single preleukemic $AML1/ETO^+$ *hematopoietic stem cells in remission lacked* c-KIT *mutation*

We then tested whether *AML1/ETO*$^+$ HSCs in remission had *c-KIT* mutation. In each patient maintaining remission for >3 years, single $CD34^+CD38^-$ HSCs were sorted from the bone marrow and were subjected to PCR to evaluate the presence of *AML1/ETO* mRNA and *c-KIT* mutations, as shown in Figure 1A. Summarized data are shown in Table 2.

In the six patients analyzed at remission, *AML1/ETO* mRNA was detected in 16 (0.9%) out of 1,728 single cells of $CD34^+CD38^-$ HSC fraction in the remission marrow. The frequency of *AML1/ETO*$^+$ HSCs in remission is consistent with previous estimation based on limit-dilution analysis [20]. All 16 *AML1/ETO* $mRNA^+$ $CD34^+CD38^-$ cells had wild-type *c-KIT*. Furthermore, no *c-KIT* mutations were found in the remaining 1,712 $CD34^+CD38^-$ cells, which did not have *AML1/ETO* mRNA, suggesting that *c-KIT* mutations never precede the acquisition of t(8;21).

To confirm that *AML1/ETO*$^+$ mutant *c-KIT*$^+$ LSCs at diagnosis and *AML1/ETO*$^+$ HSCs in remission belong to a common clone, we tested whether their *AML1/ETO* breakpoints were identical. We amplified specific breakpoints of the *AML1/ETO* fusion gene using a long PCR method [25]

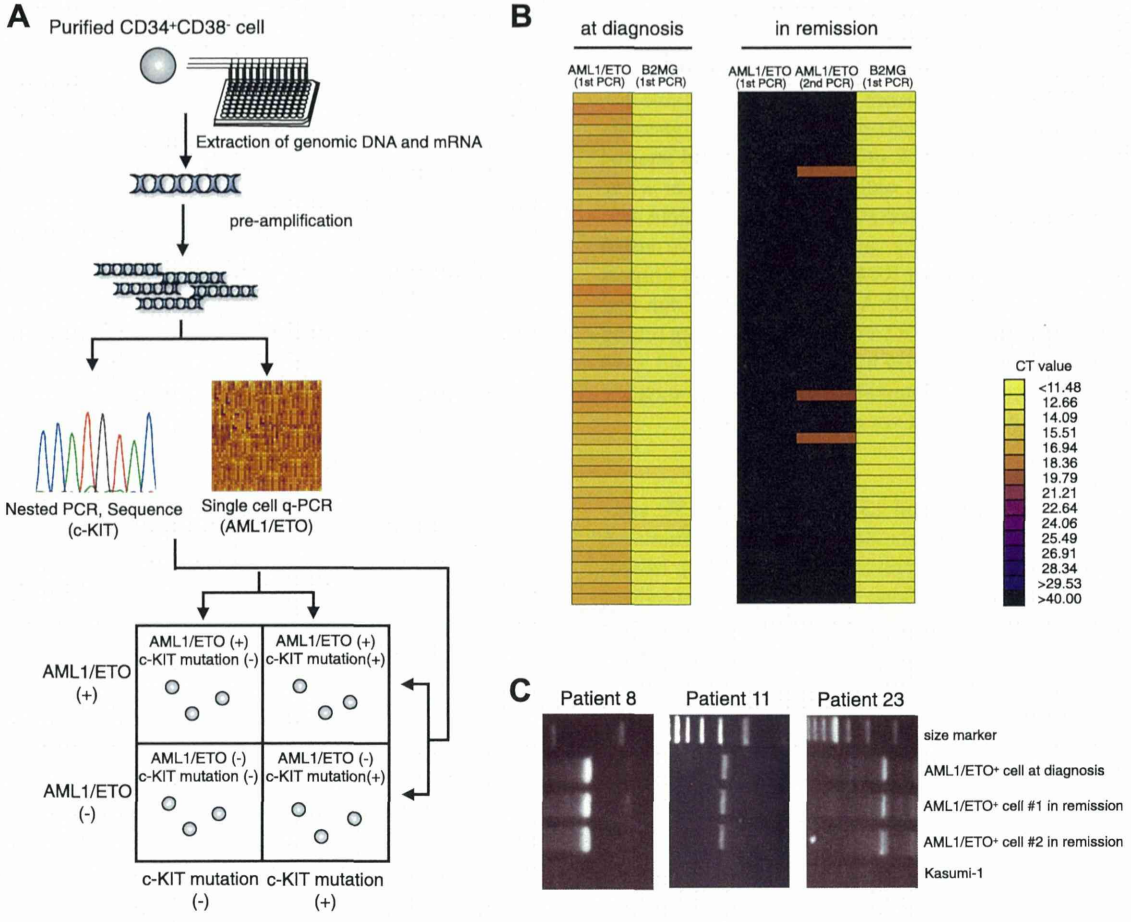

Figure 1. A fraction of single preleukemic HSCs in remission expressed a low level of *AML1/ETO*, whose breakpoints were identical to LSCs at diagnosis. (**A**) The experimental method of single cell analysis. Genomic DNA and mRNA were extracted from fluorescence activated cell sorting-purified single $CD34^+CD38^-$ cells. These extracted genomic DNA and mRNA were preamplified and were then analyzed by nested PCR and direct sequence to detect *c-KIT* mutations, as well as by single-cell quantitative PCR for *AML1/ETO* transcripts. (**B**) The representative single-cell quantitative PCR analysis at diagnosis and in remission (Patient 8). Each lane represents the level of *AML1/ETO* mRNA in single cells. Almost all single $CD34^+CD38^-$ cells in the bone marrow that were detectable at the first round of PCR at diagnosis expressed *AML1/ETO* at a high level. In contrast, a small fraction of single $CD34^+CD38^-$ cells that were detectable only by the second round of PCR in remission expressed *AML1/ETO* at a very low level. The existence of sorted single cells was confirmed by successful detection of *B2MG* mRNA. (**C**) Detection of the breakpoint of the *AML1/ETO* fusion gene specific to each patient. All single *AML1/ETO*$^+$ cells at remission had breakpoints identical to those at diagnosis in all three patients tested. Representative data are shown.

in three of these patients (Patient 8, 11, and 23) and prepared PCR primers to detect the breakpoint of the *AML1/ETO* fusion gene specific to each case. As shown in Figure 1C, in all of these three patients, single *AML1/ETO*$^+$ cells at remission always had breakpoints identical to those at diagnosis, indicating that *AML1/ETO*$^+$ HSCs in remission and the original AML LSCs share their origin. Collectively, these results strongly suggested that acquisition of *c-KIT* mutations in pre-leukemic *AML1/ETO*$^+$ HSCs may be a critical event for the transformation of t(8;21) preleukemic HSCs into LSCs.

Preleukemic AML1/ETO$^+$ *hematopoietic stem cells without* c-KIT *mutation can differentiate into myeloerythroid cells in vitro*

The main leukemogenic function of AML1/ETO may be to block differentiation by abrogating the CBF function through dominant-negative inhibition of AML1 [12–14]. However, because the expression of *AML1/ETO* is very low in remission (Fig. 1B), such a low level of *AML1/ETO* may not be able to inhibit differentiation of *AML1/ETO*$^+$ HSCs. In fact, *AML1/ETO*$^+$ mRNA is detectable in a small fraction of mature granulocytes and lymphoid cells in remission [20]. Thus, we wished to confirm that *AML1/ETO*$^+$ HSCs with the wild-type *c-KIT* in remission differentiate into mature blood cells. Single $CD34^+CD38^-$ HSCs purified from remission marrow were cultured in methylcellulose, and each colony was picked up (Fig. 2A) and tested for the presence of *AML1/ETO* and *c-KIT* mutations (Fig. 2B). As summarized in Table 3, of 7,187 total myeloid colonies from six patients, 89 (1.2%) were positive for *AML1/ETO* mRNA, and all of these colonies had wild-type *c-KIT*. These data confirm that AML1/ETO$^+$ HSCs in remission with wild-type *c-KIT* are capable of differentiating into a variety

Table 2. Summary of detection of *AML1/ETO* mRNA$^+$ cells in single CD34$^+$CD38$^-$ cells in diagnostic and remission marrow

Patient	(*c-KIT* mutation)	At diagnosis	In remission
Patient 3	(D816V)		
	No. of cells	262	288
	AML1/ETO$^+$ cells/No. of cells	262/262 (100%)	1/288 (0.3%)
	mutated *c-KIT* cells/*AML1/ETO*$^+$ cells	262/262 (100%)	0/1 (0%)
Patient 7	(D816V)		
	No. of cells	285	288
	AML1/ETO$^+$ cells/No. of cells	279/285 (97.9%)	2/288 (0.7%)
	mutated *c-KIT* cells/*AML1/ETO*$^+$ cells	279/279 (100%)	0/2 (0%)
Patient 8	(N822K)		
	No. of cells	274	288
	AML1/ETO$^+$ cells/No. of cells	270/274 (98.5%)	4/288 (1.4%)
	mutated *c-KIT* cells/*AML1/ETO*$^+$ cells	270/270 (100%)	0/4 (0%)
Patient 11	(N822K)		
	No. of cells	235	288
	AML1/ETO$^+$ cells/No. of cells	235/235 (100%)	4/288 (1.4%)
	mutated *c-KIT* cells/*AML1/ETO*$^+$ cells	235/235 (100%)	0/4 (0%)
Patient 21	(D816Y)		
	No. of cells	285	288
	AML1/ETO$^+$ cells/No. of cells	284/285 (99.6%)	2/288 (0.7%)
	mutated *c-KIT* cells/*AML1/ETO*$^+$ cells	284/284 (100%)	0/2 (0%)
Patient 23	(D816V)		
	No. of cells	285	288
	AML1/ETO$^+$ cells/No. of cells	278/285 (97.5%)	3/288 (1.0%)
	mutated *c-KIT* cells/*AML1/ETO*$^+$ cells	278/278 (100%)	0/3 (0%)
Total	*AML1/ETO*$^+$ cells/No. of cells	1,608/1,626 (98.9%)	16/1,728 (0.9%)
	mutated *c-KIT* cells/*AML1/ETO*$^+$ cells	1,608/1,608 (100%)	0/16 (0%)

of myeloerythroid cells and contribute toward maintaining normal hematopoiesis.

Mutated c-KIT *signaling endows leukemic stem cells with growth advantages through upregulation of several key molecules*

In these patients, *c-KIT* mutations have been shown to constitutively provide active c-KIT signaling and may therefore contribute to proliferation and survival of leukemic cells [29,30]. To understand the function of mutant c-KIT signaling, we compared the gene expression profile of the CD34$^+$CD38$^-$ LSC fraction purified from 18 t(8;21) patients with wild-type *c-KIT* with that of 13 patients with *c-KIT* mutants using microarray analysis. As shown in Figure 3A, the clustering analysis showed t(8;21) AML LSCs with *c-KIT* mutation had a distinct expression pattern, regardless of their type of *c-KIT* mutation. Genes upregulated or downregulated by > twofold in patients with mutant *c-KIT* are listed in Supplementary Table E4 (online only, available at www.exphem.org). For example, *MCL1*, *BCL2*, *NFKB1A* and *CXCR4* were significantly upregulated in AML LSCs with *c-KIT* mutations (Fig. 3B). These data are consistent

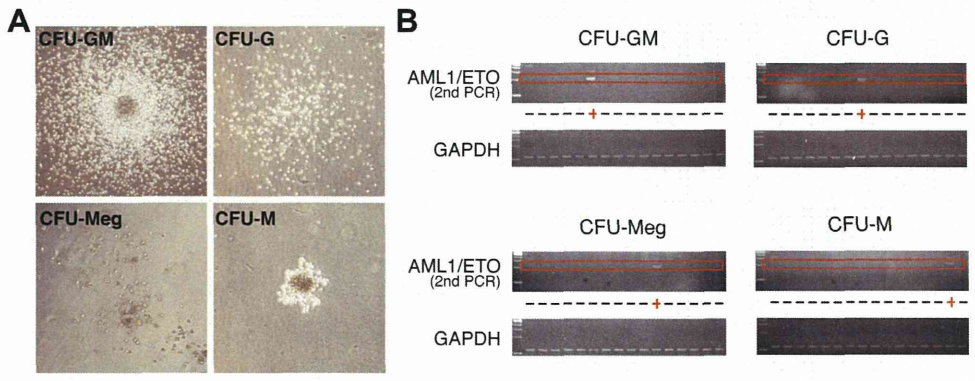

Figure 2. Single preleukemic HSCs in remission do not have *c-KIT* mutation and can differentiate into mature myeloid cells. (**A**) Morphology of *AML1/ETO*-positive myeloid colonies derived from single CD34$^+$CD38$^-$ cells in remission. The representative results of Patient 11 are shown. (**B**) PCR analyses for *AML1/ETO* mRNA and *c-KIT* genes of cells picked from single HSC-derived myeloid colonies. *AML1/ETO* mRNA can be detected in a fraction of myeloid colonies only by nested PCR. Simultaneously, genomic DNA from these colonies was subjected to PCR amplification for the *c-KIT* gene to evaluate the presence of *c-KIT* mutation by direct sequencing. Results were summarized in Table 3.

Table 3. Summary of detection *c-KIT* mutation and *AML1/ETO* mRNA in single HSC-derived myeloid colonies in remission

Patient	*c-KIT* mutation	CFU-GM	BFU-E	CFU-Meg	CFU-Mix	CFU-G	CFU-M	Total
Patient 3	(D816V)							
	No. of colonies	411	101	98	85	65	197	957
	AML1/ETO$^+$ colonies/No. of colonies	3/411	0/101	0/98	0/85	1/65	1/197	5/957 (0.5%)
	mutated *c-KIT* colonies/*AML1/ETO*$^+$ colonies	0/3	0/0	0/0	0/0	0/1	0/1	0/5 (0%)
Patient 7	(D816V)							
	No. of colonies	706	181	199	90	45	140	1,361
	AML1/ETO + colonies/No. of colonies	6/706	4/181	0/199	0/90	0/45	1/140	11/1,361 (0.8%)
	mutated *c-KIT* colonies/*AML1/ETO*$^+$ colonies	0/6	0/4	0/0	0/0	0/0	0/1	0/11 (0%)
Patient 8	(N822K)							
	No. of colonies	662	110	132	55	53	259	1,271
	AML1/ETO$^+$ colonies/No. of colonies	7/662	1/110	0/132	1/55	0/53	3/259	12/1,271 (0.9%)
	mutated *c-KIT* colonies/*AML1/ETO*$^+$ colonies	0/7	0/1	0/0	0/1	0/0	0/3	0/12 (0%)
Patient 11	(N822K)							
	No. of colonies	765	100	112	98	71	219	1,365
	AML1/ETO$^+$ colonies/No. of colonies	12/765	6/100	1/112	1/98	3/71	5/209	28/1,355 (2.1%)
	mutated *c-KIT* colonies/*AML1/ETO*$^+$ colonies	0/12	0/6	0/1	0/1	0/3	0/5	1/28 (0%)
Patient 21	(D816Y)							
	No. of colonies	597	143	88	59	58	202	1,147
	AML1/ETO$^+$ colonies/No. of colonies	9/597	5/143	0/88	0/59	1/58	7/202	22/1,147 (1.9%)
	mutated *c-KIT* colonies/*AML1/ETO*$^+$ colonies	0/9	0/5	0/0	0/0	0/1	0/7	0/22 (0%)
Patient 23	(D816V)							
	No. of colonies	504	69	142	66	23	282	1,086
	AML1/ETO$^+$ colonies/No. of colonies	5/504	1/69	0/142	0/66	1/23	4/282	11/1,086 (1.0%)
	mutated *c-KIT* colonies/*AML1/ETO*$^+$ colonies	0/5	0/1	0/0	0/0	0/1	0/4	0/11 (0%)
Total	mutated *c-KIT* colonies/*AML1/ETO*$^+$ colonies/No. of colonies	0/42/3,645	0/17/704	0/1/771	0/2/453	0/6/315	0/21/1,299	0/89/7,187
		(0/1.2%)	(0/2.4%)	(0/0.1%)	(0/0.4%)	(0/1.9%)	(0/1.6%)	(0/1.2%)

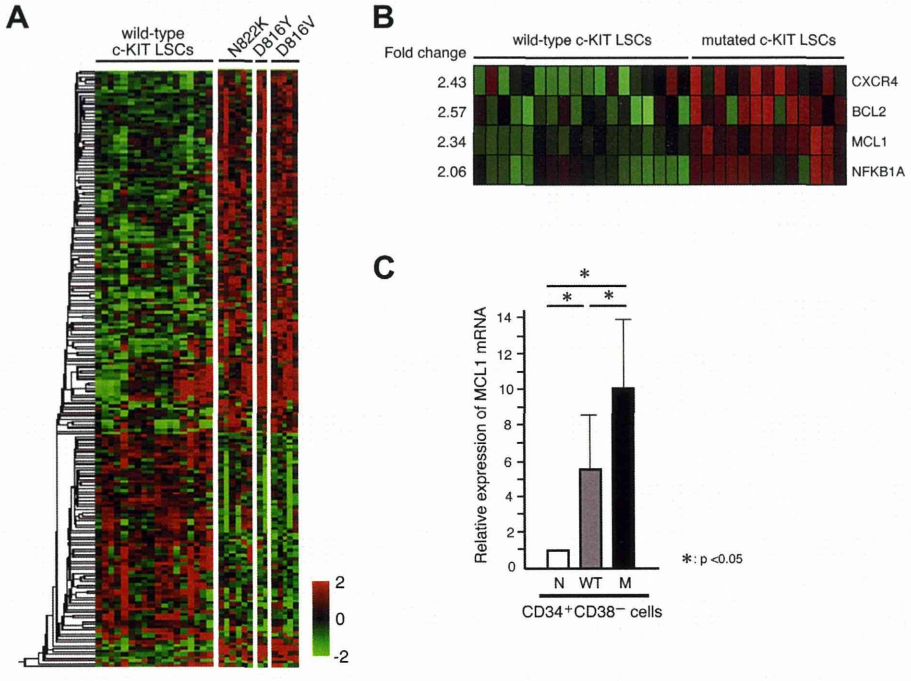

Figure 3. The expression of molecules that enforce the survival of LSCs. (**A**) Results of microarray analysis of t(8;21) LSCs with wild-type *c-KIT* and mutated *c-KIT*. t(8;21) AML LSCs with *c-KIT* mutation had a distinct expression pattern, irrespective of their types of *c-KIT* mutation. (**B**) Representative molecules that upregulated greater than twofold in AML LSCs with mutated *c-KIT*, as compared with those with the wild-type *c-KIT*. (**C**) The quantitative PCR analysis of MCL-1 in N, WT and M cell types. M = LSCs with *c-KIT* mutation; N = $CD34^+CD38^-$ normal HSCs; WT = LSCs with wild-type *c-KIT*.

with those in previous reports, in which the c-KIT signaling effectively upregulated these genes to enhance their LSC activity [31–33]. Of note, *MCL1*, a survival-promoting protein essential for HSC survival [34], was upregulated in LSCs with *c-KIT* mutations (Fig. 3B and C). This may be reasonable because *FLT3-ITD*, which is another mutation of the receptor-type tyrosine kinase, is known to upregulate MCL1 to promote AML LSC survival [35]. These data collectively suggest that the acquisition of *c-KIT* mutation may at least contribute to reinforce proliferation and survival of t(8;21) AML LSCs.

Upregulation of AML1/ETO *may also constitute a critical step for transformation into leukemia stem cells*

In LSCs in remission, *AML1/ETO* transcripts become detectable only after the second round of PCR, whereas they are easily detected in LSCs at diagnosis by single PCR (Fig. 1B). This suggests that the increase in *AML1/ETO* expression may also be important in LSC development. Therefore, we quantified *AML1/ETO* transcripts in $CD34^+CD38^-$ cells at diagnosis and remission using a single cell quantitative PCR method.

Figure 4A shows the amount of *AML1/ETO* transcripts in 16 single *AML1/ETO*$^+$ $CD34^+CD38^-$ cells in remission relative to those in single *AML1/ETO*$^+$ LSCs at diagnosis in the six patients listed in Table 1. In every case, regardless of the *c-KIT* mutant type, the amount of *AML1/ETO* transcripts per cell in remission was more than one hundredfold less than that in LSCs at diagnosis. Taken together, LSCs at diagnosis had approximately five hundred times more *AML1/ETO* transcripts compared with *AML1/ETO*$^+$ HSCs in remission at the single cell level. We could not conduct a similar analysis for *AML1/ETO*$^+$ *c-KIT* wild-type patients because of the lack of sufficient samples.

We hypothesized that, in *AML1/ETO*$^+$ LSCs with *c-KIT* mutations, constitutively active c-KIT signaling may stimulate the expression of *AML1/ETO* transcripts. Therefore, we quantified the levels of *AML1/ETO* transcripts in 5,000 cells of $CD34^+CD38^-$ LSCs from 10 patients with wild-type *c-KIT* and in 5,000 cells from patients with *c-KIT* mutations. However, as shown in Figure 4B, the *AML1/ETO* transcript level was not significantly different, regardless of the presence of *c-KIT* mutation. Furthermore, the ligation of c-KIT by addition of stem cell factor (SCF) in culture did not affect the *AML1/ETO* levels in each group (Fig. 4B). Thus, c-KIT signaling may not stimulate *AML1/ETO* transcription, suggesting that the acquisition of *c-KIT* mutation and the upregulation of *AML1/ETO* transcription are independent events.

Discussion

It has been suggested that genetic abnormalities are accumulated in self-renewing, long-surviving HSCs and that these

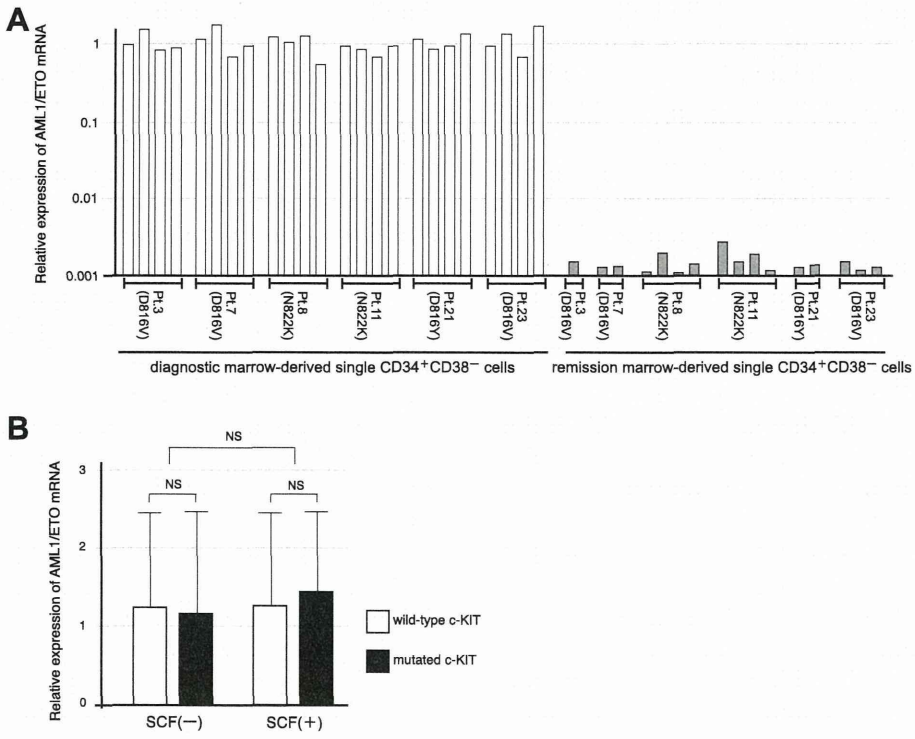

Figure 4. The level of *AML1/ETO* transcripts in single $CD34^+CD38^-$ preleukemic HSCs and LSCs at diagnosis. (**A**) Results of quantitative PCR analysis of *AML1/ETO* mRNA in single preleukemic HSCs and LSCs at diagnosis. In all cases, LSCs expressed over one hundredfold higher levels of *AML1/ETO* than preleukemic HSCs in remission, irrespective of their *c-KIT* mutant types. (**B**) *AML1/ETO* mRNA expression in human t(8;21) LSCs with wild type *c-KIT* and those with mutated *c-KIT* in the presence or absence of SCF. The amounts of *AML1/ETO* transcripts were not affected by c-KIT signaling.