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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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Abstract

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 1728 $CD34^{+}CD38^{-}$ HSCs and 89 out of 7187 single HSC-derived myelo-erythroid 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 pre-leukemic 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.

Keywords: hematopoietic stem cells, t(8;21) leukemia stem

Introduction

Acute myelogenous leukemia (AML) is characterized by deregulated proliferation and impaired differentiation of immature hematopoietic cells, and originates from leukemia stem cells (LSCs). LSCs have cellular properties such as self-renewal activity, impaired 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 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-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 never been documented in *de novo* human AML.

AML1/ETO achieved by t(8;21) is one of the most common chromosomal abnormalities in

AML[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. t(8;21)AML patients frequently 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 c-KIT[9, 18], FLT3[7], and TEL-PDGFRa gene[8]. These data strongly suggest 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 maintaing remission for a long-term (>10years) 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 AML1/ETO mRNA, the frequency of which were estimated to be ~1% of HSCs[20]. Thus, the

acquisition of AML1/ETO is not sufficient for leukemic transformation in human, therefore, we proposed that such AML1/ETO$^+$ HSCs are “pre-leukemic” clones that have achieved precondition to 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 “pre-leukemic” 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 HSCs and LSCs 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 pre-leukemic clone, and are transformed into LSCs by subsequent acquisition of c-KIT mutation. This is 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, 13 cases in remission (Patient 1, 3, 7-9, 11, 13, 21-23, 26-27 and 31), and 13 cases at relapse (Patient 2, 5-6, 10, 14, 16-17, 25, 28-30, and 32-33). Remission marrow were obtained at least >12 months from 1st remission and all the patients remained remission at the time of this report. 20 out of 33 cases obtained complete remission only by chemotherapies. Patient 2 and 10 were further received allogeneic-bone marrow transplantation and Patient 5 was further received cord blood transplantation. CD19 and CD56 expressions on AML cells 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 marrows were purchased from AllCells Inc. (Emeryville). Informed consent was obtained from all patients. The Institutional Review Board of our hospital 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), and biotin-anti-CD38 (Caltag Laboratories). Streptavidin-Cy7-allophycocyanin (BD Pharmingen) was also used.

Quantitative real-time PCR

RNA was isolated from 5000 cells using Isogen reagent (Nippon gene). RNA was reverse transcribed to cDNA using TaKaRa RNA PCR kit (Takara Shuzo). The mRNA levels were quantified by real-time PCR (Applied Biosystems). β2-microglobulin (B2MG) was used for internal control. The primer and probes for B2MG, c-KIT, CXCR4, BCL2, MCL1 and NFKB1 were purchased from Applied Biosystems.

Reverse transcription-PCR

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, 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

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

Identification of the gene mutations

Genomic DNA was extracted by Micro Kit (QIAGEN). The presence of FLT3-ITD, NRAS and c-KIT mutation was examined as previously descried[7, 24]. The primers for the c-KIT mutation are shown on Table S1. 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 were listed on Table S2.

Single-cell quantitative PCR and genomic PCR

Single-cell quantitative PCR protocol was previously reported[26]. AML1/ETO external primers were used (Table S3). Single-cell genomic PCR was performed by nested PCR with utilizing external and internal primers (Table S1). The method of nested PCR for genomic DNA was same as RT-PCR method. For single-cell quantitative nested PCR, these diluted pre-amplified cDNA was performed first round of RT-PCR with external primers. The protocol of first round of PCR is same as RT-PCR (thermal cycling setting was 16). Nested-PCR was performed by using BioMark 48x48 Dynamic Array system with internal primers (Table S1).

Microarray analysis

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

Cytokine stimulation assays

The c-KIT signaling repercussion for AML1/ETO expression level by addition of stem cell factor was evaluated after 24 hours 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)AML

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 mutation 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 (Figure S1). Out of 13 patients with c-KIT mutations, seven patients relapsed. Six out of seven relapsed patients had a mutation 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 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 pre-leukemic AML1/ETO^{+} HSCs in remission[20].

All single LSCs possess both AML1/ETO and c-KIT mutation at diagnosis

Six cases of t(8;21)AML with c-KIT mutations including D816V, N822K and D816Y, who maintained complete remission >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, virtually 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 PCR (Figure 1B).

Summarized data are shown in Table 2. In the analysis of six cases at diagnosis, 1608 (98.9%) out of 1626 total 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 pre-leukemic AML1/ETO^+ HSCs in remission did not have c-KIT mutation

We then tested whether AML1/ETO^+ HSCs in remission had c-KIT mutation. In each patient maintaining remission >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 1728 single cells of $CD34^+CD38^-$ HSC fraction in the remission marrow. The frequency of AML1/ETO^+ HSC 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, c-KIT mutations were never found in the remaining 1712 $CD34^+CD38^-$ cells that did not have AML1/ETO mRNA, suggesting that c-KIT mutations never precede the acquisition of t(8;21).

To confirm 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] in three of these patients (Patient 8, 11, and 23), and prepared PCR primers to detect the breakpoint of 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) pre-leukemic HSCs into LSCs

Pre-leukemic AML1/ETO$^+$ HSCs without c-KIT mutation can differentiate into myelo-erythroid 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 (Figure 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 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, each colony was picked up (Figure 2A), and tested for the presence of AML1/ETO and c-KIT mutations (Figure 2B). As summarized in Table 3, within 7187 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 of myelo-erythroid cells and contribute toward maintaining normal hematopoiesis.

Mutated c-KIT signaling endows LSCs with growth advantages through up-regulation of several key molecules

c-KIT mutations in these patients have been shown to constitutively provide active c-KIT signaling and therefore may contribute toward 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, and that from 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 up-regulated or down-regulated by >2-fold in patients with mutant c-KIT are

listed in Table S4. For example, MCL1, BCL2, NFKB1A and CXCR4 were significantly up-regulated in AML LSCs with c-KIT mutations (Figure 3B). These data are consistent with those in previous reports, in which the c-KIT signaling effectively up-regulated these genes to enhance their LSC activity[31-33]. Of note, MCL-1, a survival-promoting protein essential for HSC survival[34], was up-regulated in LSCs with c-KIT mutations (Figure 3B and 3C). This may be reasonable because FLT3-ITD, which is another mutation of the receptor-type tyrosine kinase, is known to up-regulate MCL1 to promote AML LSC survival[35]. These data collectively suggest the acquisition of c-KIT mutation may at least contribute to reinforce proliferation and survival of t(8;21)AML LSCs.

Up-regulation 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 PCR, whereas they are easily detected in LSCs at diagnosis by single PCR (Figure 1B). This suggests 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 >100-fold less than that in LSCs at diagnosis. Taken together, LSCs at diagnosis had ~500-fold higher amount of 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 5000 cells of $CD34^+CD38^-$ LSCs from 10 patients with wild-type c-KIT and in 5000 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 SCF in culture did not affect the AML1/ETO levels in each group (Figure 4B). Thus, c-KIT signaling may not stimulate AML1/ETO transcription, suggesting the acquisition of c-KIT mutation and up-regulation of AML1/ETO transcription are independent events.

Discussion

It has been suggested genetic abnormalities are accumulated in self-renewing, long-surviving HSCs and that these abnormalities cooperatively transform normal HSCs into LSCs[1, 2]. Our intensive analysis of human t(8;21)AML with c-KIT mutation revealed that at diagnosis, all single LSCs had both AML1/ETO and c-KIT mutants (Table 2). During remission, a small fraction (~1%) of HSCs or their myelo-erythloid colonies had AML1/ETO, and such single AML1/ETO$^+$ HSCs always had wild-type c-KIT. HSCs with only c-KIT mutation were never observed (Tables 2 and 3).

Furthermore, the breakpoint of AML1/ETO was identical at diagnosis and in remission in all three patients analyzed (Figure 1C), indicating that AML1/ETO$^+$ cells at diagnosis and those in remission originated from the same "pre-leukemic" clone. In addition, we found a patient who had N822K at diagnosis but newly obtained D816Y at relapse (Patient 14 in Table 1), suggesting acquisition of c-KIT mutation is a subsequent event. Thus, our sequential analysis provides definitive evidence that HSCs first acquire AML/ETO fusion (Class II), and then c-KIT mutation (Class I) for transformation into LSCs. The proposed developmental model for t(8;21)AML is schematized in Figure 5.

Because all three types of c-KIT mutations found in t(8;21)AML in this study constitutively transduce an active c-KIT signaling[36], the enhanced c-KIT signals may play a critical role in leukemic

transformation. Our patients had D816V, D816Y and N822K c-KIT mutants, and microarray analysis showed that c-KIT mutations at least caused up-regulation of NFKB1A, BCL2, and MCL1, whose signals may promote proliferation/survival of leukemic cells[32, 33, 35]. A variety of c-KIT mutations have been found in several other malignant diseases[36]. Consistently, in a mouse model, the enforced c-KIT mutant signaling induced a myeloproliferative neoplasm-like disease[9]. Notably, D816V and D816Y are frequently found in mast cell leukemia as well as in t(8;21)AML[36]. Thus, it is possible that active c-KIT signaling itself does not decide the phenotype of leukemia, but it may contribute toward development of AML in the presence of CBF mutations such as AML1/ETO.

It is also important to note the significant up-regulation of AML1/ETO transcripts may also be a key event in leukemic transformation. AML1/ETO may not play a role in the inhibition of CBF function because AML1/ETO knock-in mice did not present any leukemia phenotype[17]. In contrast, in LSCs, AML1/ETO mRNA levels were elevated up to >100-fold at diagnosis, and this high level of AML1/ETO may be effective in inhibiting myeloid differentiation. Our data suggest c-KIT signaling is independent of AML1/ETO up-regulation (Figure 4B). Collectively, an unknown, presumably epigenetic mechanism that elevates AML1/ETO mRNA levels may also be critical for the development of t(8;21)AML. This event may precede the acquisition of c-KIT mutations because all AML1/ETO^{+} HSCs

in remission did not have mutated c-KIT (Table 2).

The reason the ordered acquisition of Class II and Class I mutation is consistently observed in t(8;21)AML patients remains unclear. To achieve leukemic hematopoiesis, the single cell with the first oncogenic hit needs to have a clonal advantage for self-renewal compared with normal HSCs. Class I mutations, when enforced to be expressed, are capable of conferring cytokine-independent growth activity into cell lines[9, 37-39]. In mouse models, HSCs with a high level of BCR-ABL (Class I) enforced by retroviruses showed myeloproliferation, whereas in healthy human adults, a low level of BCR-ABL transcripts is sometimes detectable[40-42]. This suggests the acquisition of BCR-ABL in HSCs cannot directly provide clonal advantages against normal HSCs. Previous studies reported that mice having HSCs with FLT3-ITD showed expansion of hematopoietic progenitors resulting in myeloproliferation[7, 43]; however, the HSC compartment declines because FLT3-ITD signals perturb the self-renewal of HSCs[44]. Therefore, in the light of *de novo* development of human AML, if a single HSC achieves FLT3-ITD, the FLT3-ITD$^+$ HSCs may not be able to outgrow normal HSCs. It is possible HSCs with a c-KIT mutation alone cannot exhibit the advantage of self-renewal against normal HSCs to become a dominant clone because c-KIT and FLT3 use similar signal transduction pathways[29, 30].

In contrast, AML1/ETO$^+$ HSCs can persist for >10 years, maintaining their clones at the level

of a few percent of normal HSCs[19, 20]. This evidence indicates HSCs achieved through AML1/ETO do not have advantages in self-renewal but can coexist with normal HSCs for a long period. This is probably because the expression level of AML1/ETO in t(8;21)$^{+}$ HSCs is low, and it does not significantly block hematopoietic differentiation. It is assumed that the long-term coexistence of normal and t(8;21)$^{+}$ HSCs allows the latter to acquire second or third oncogenic hits, including Class I mutations, and some unknown abnormalities that can cause up-regulation of AML1/ETO. It is critical to test whether this hypothesis can be applied to AML with other Class II mutations.

Collectively, results of our intensive analysis of *de novo* t(8;21) human AML suggest there are at least three independent leukemogenic steps in this type of leukemia (Figure 5). First, the normal HSC acquires t(8;21) that generates a low level of AML1/ETO. Second, the long-term existence of such AML1/ETO^{+} HSCs allows them to obtain additional epigenetic or genetic abnormalities that up-regulates AML1/ETO. Finally, the acquisition of Class I mutations, such as c-KIT mutants, transforms AML1/ETO^{+} pre-leukemic HSCs into AML LSCs. Thus, the original description of Class I and Class II mutations[4, 45, 46] is very useful in the understanding of the leukemogenesis of AML. Intensive tracking of mutational processes during clinical courses is critical to understand the step-wise leukemogenesis involved in *de novo* human AML in future studies.

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Figure legends

Figure 1: A fraction of single pre-leukemic 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 FACS-purified single $CD34^{+}CD38^{-}$ cells. These extracted genomic DNA and mRNA were pre-amplified, and then were 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 at diagnosis expressed AML1/ETO at a high level that were detectable at the first round of PCR. In contrast, a small fraction of single $CD34^{+}CD38^{-}$ cells in remission expressed AML1/ETO at a very low level that were detectable only by the second round of PCR. The existences of sorted single cells were confirmed by successful detection of beta-2-microglobulin (B2MG) mRNA. (C) Detection of the breakpoint of AML1/ETO fusion gene specific to each patient. All single $AML1/ETO^{+}$ cells at remission had breakpoints identical to those at diagnosis in all 3 patients tested. Representative data are shown.

Figure 2: Single pre-leukemic 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 were 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 c-KIT gene to evaluate the presence of c-KIT mutation by direct sequencing. Results were summarized in Table 3.

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 up-regulated >2-fold in AML LSCs with mutated c-KIT, as compared to those with the wild-type c-KIT. (C) The quantitative PCR analysis of MCL-1 in $CD34^{+}CD38^{-}$ normal HSC (N), LSCs with wild-type c-KIT (WT) and LSCs with c-KIT mutation (M).

Figure 4: The level of AML1/ETO transcripts in single $CD34^{+}CD38^{-}$ pre-leukemic HSCs and LSCs at diagnosis

(A) Results of quantitative PCR analysis of AML1/ETO mRNA in single pre-leukemic HSCs and LSCs at diagnosis. In all cases, LSCs expressed >100-fold higher level of AML1/ETO than pre-leukemic 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.

Figure 5: The proposed multi-step developmental scheme of human t(8;21) AML with c-KIT mutations

A normal HSC acquires t(8;21), and forms a reservoir of pre-leukemic $AML1/ETO^{+}$ HSCs as the first step. These pre-leukemic $AML1/ETO^{+}$ HSCs up-regulate AML1/ETO transcripts (2nd step), and the acquisition of a c-KIT mutation (3rd step) finally transform pre-leukemic $AML1/ETO^{high}$ HSCs into LSCs. The detailed mechanism of AML1/ETO up-regulation at the 2nd step remains unknown. The time course of 2nd and 3rd steps was not confirmed in this study.

Figure 1

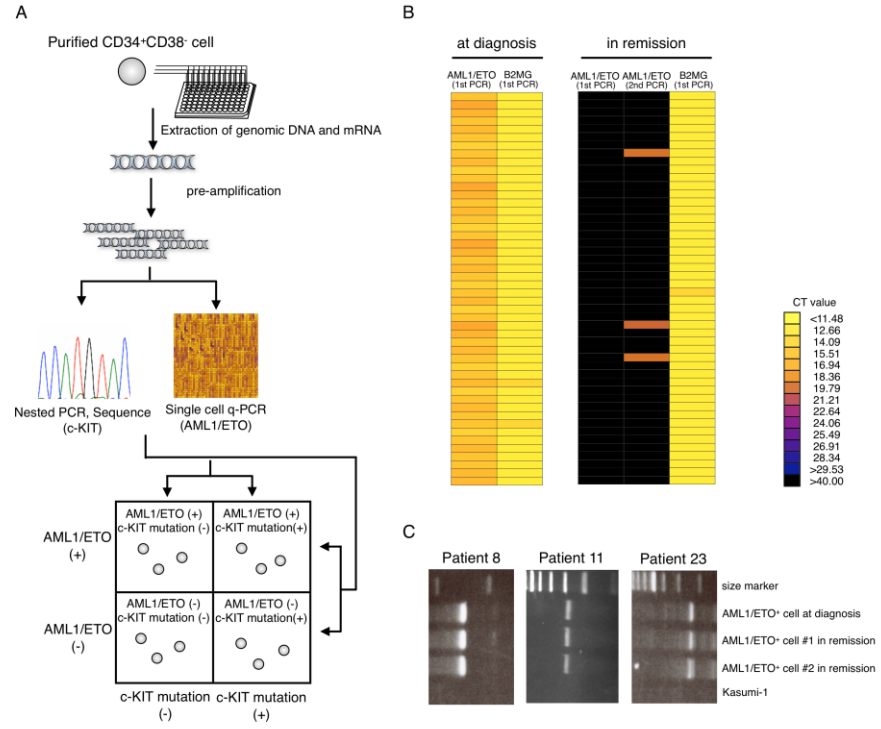


Figure 2

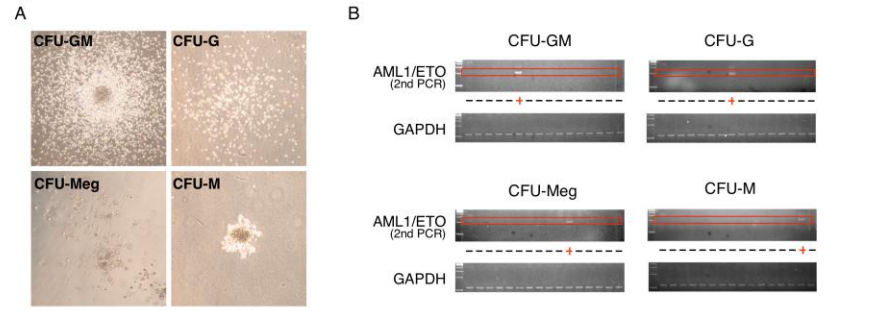


Figure 3

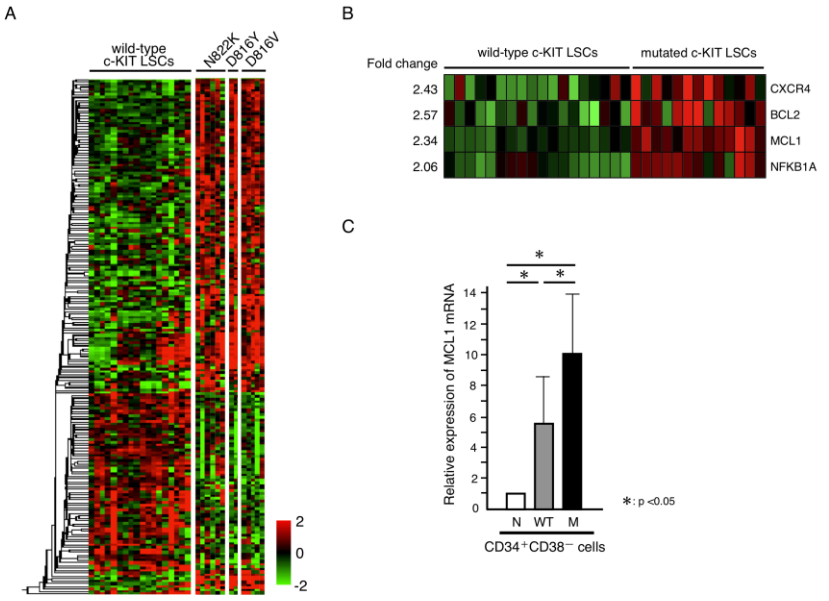


Figure 4

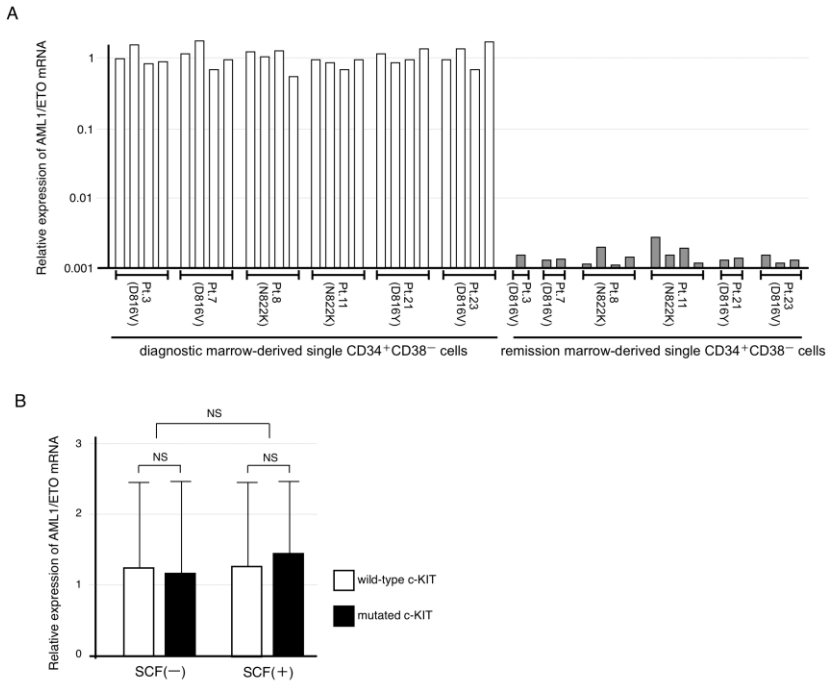
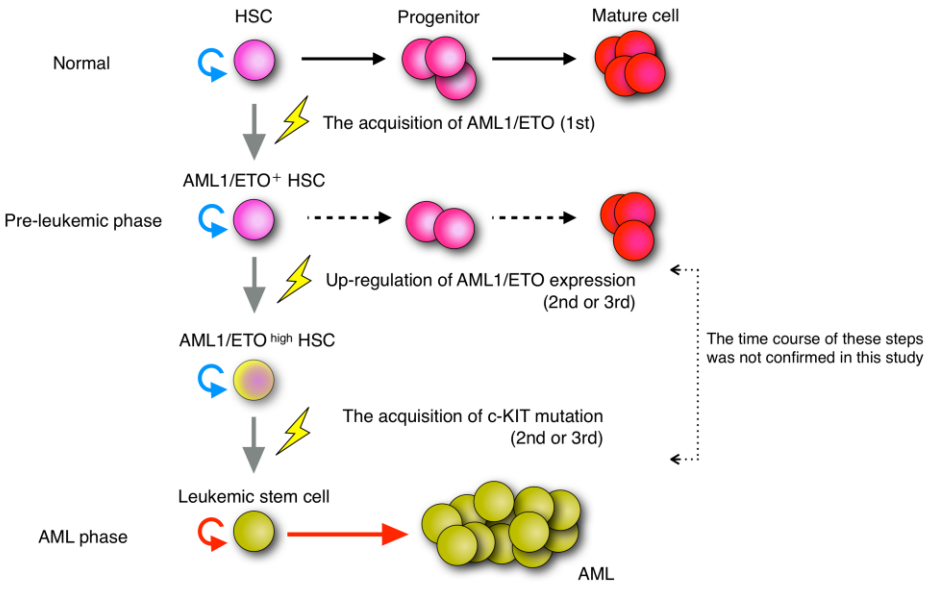


Figure 5


HSC
Progenitor
Mature cell
Normal
The acquisition of AML1/ETO (1st)
AML1/ETO+ HSC
Pre-leukemic phase
Up-regulation of AML1/ETO expression
(2nd or 3rd)
AML1/ETO high HSC
The time course of these steps
was not confirmed in this study
The acquisition of c-KIT mutation
(2nd or 3rd)
Leukemic stem cell
AML phase
AML