# *CBL* mutations in infant acute lymphoblastic leukaemia

Infant acute lymphoblastic leukaemia (ALL) is relatively rare, occurring in approximately 2·5–5% of cases of childhood ALL (Biondi *et al*, 2000). Infant ALLs are much more likely to present with high leucocyte counts, hepatosplenomegaly and overt central nervous system (CNS) diseases (Taki *et al*, 1996). T cell phenotype is much less common in infants, while myeloid antigen co-expression and the absence of CD10 expression are more frequent in infants than in older children with ALL. When molecular techniques [such as fluorescence *in situ* hybridization (FISH) or Southern blot analysis] are used in addition to karyotype, *MLL* gene rearrangements (*MLL*-R) are found in 70–80% of infant ALL compared with only 2–4% of older children with ALL (Taki *et al*, 1996; Biondi *et al*, 2000). Thus, infant ALL appears to be biologically distinct from the disease in older children (more than 1 year old).

In this regard, recent reports of somatic mutations of the *CBL* proto-oncogene in myeloid neoplasms are intriguing, because these *CBL* mutations were shown to result in aberrant tyrosine kinase signalling, which also leads to activation of RAS signalling pathways. So far, we and others have reported that *CBL* mutations occur in a variety of myeloid neoplasms, including *de novo* acute myeloid leukaemia (AML) (Caligiuri *et al*, 2007), myelodysplastic syndrome (MDS), and myeloproliferative neoplasm, especially in chronic myelomonocytic leukaemia (CMML) (Sanada *et al*, 2009), and juvenile myelomonocytic leukaemia (JMML) (Shiba *et al*, 2010). The importance of *CBL* mutations regarding leukaemogenesis is substantially increased. Recently, we found *CBL* mutation in therapy-related AML with *MLL*-R (Shiba *et al*, 2011). Interestingly, the *MLL-CBL* fusion gene has been reported in a *de novo* AML case (Fu *et al*, 2003), and this prompted us to search for possible *CBL* mutations in infant ALL with *MLL-R*.

Because *CBL* mutations thus far reported were almost all clustered within exons 8–9 that encode Linker/RING finger domains (Caligiuri *et al*, 2007; Sanada *et al*, 2009; Shiba *et al*, 2010), we confined our mutation analysis to these exons, in which polymerase chain reaction-amplified exons 8–9 were subjected to direct sequencing using an ABI PRISM 310 Genetic Analyser (Applied Biosystems, Branchburg, NJ, USA). The study adhered to the principles of the Helsinki Declaration, and was conducted under the regulations enacted by the Ethics Board of Gunma Children's Medical Centre.

*CBL* gene analysis was performed in 41 infant ALL patients in which *MLL*-R was found in 33 patients (80·5%), including 15 patients with t(4;11)(q21;q23), 4 with t(9;11)(p22;q23) and 5 with t(11;19)(q23;p13.3). Median age at diagnosis was 4·7 months (range, 0–12 months). We also performed *CBL* gene mutation analysis in 28 B cell precursor (BCP)-ALL patients (age range, 1–14 years).

Heterozygous mutations of the *CBL* gene were identified in 2 (4·9%) of 41 infant ALL patients, but not in older children with BCP-ALL. These were located in exon 8 (Fig 1). One patient was a 3-month-old female with t(4;11)(q21;q23) and the other patient was a 6-month-old male with t(11;19)(q23;p13.3). They were registered and treated on two Japanese infant leukaemia protocols, MLL96 and MLL98 respectively (Isoyama *et al*, 2002; Kosaka *et al*, 2004). Although strong association between *CBL* mutations and 11q-acquired uniparental disomy (aUPD) has been reported (Sanada *et al*, 2009), we did not perform the single nucleotide polymorphism array analysis due to lack of DNA.

*MLL*-R are more frequent in younger infants; up to 90% of infant ALL less than 6 months old at diagnosis have detectable *MLL*-R compared with 30–50% of infant ALL aged 6–12 months (Taki *et al*, 1996). *MLL*-R ALL has a characteristic gene expression profile that significantly differs from that of non-*MLL*-R BCP-ALL and of AML, confirming that *MLL*-R ALL is a biologically unique leukaemia subtype.

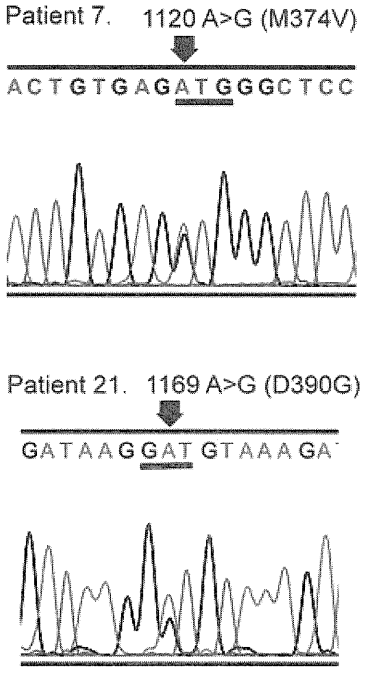


Fig 1. Identification of *CBL* mutations. Heterozygous mutations of the *CBL* gene were identified in Patients 7 and 21.

Thus, the distinctive presenting features and clinical behaviour of infant ALL appear to be primarily due to the high frequency of *MLL*-R in this age group. However, outcome data comparing infant and non-infant patients with *MLL*-R suggest that there may be other factors which impact the prognosis of infant ALL. Both of the patients with *CBL* mutations were diagnosed before 6 months of age. In our previous report, all of three cases with *CBL* mutation developed JMML before 4 months of age (Shiba *et al*, 2010). These data suggested that *CBL* mutation may have a strong association with very early onset disease. *CBL* mutations have been reported as germline mutations in JMML (Niemeyer *et al*, 2010). Unfortunately, we could not investigate whether the mutations in our cases were germline mutations or not, because somatic cells were not available.

*CBL* mutations have been found in approximately 5% of 2000 samples from patients with myeloid neoplasms, including AML transformed from MDS. Gene aberrations in addition to *MLL*-R have rarely been reported in infant ALL. No reports of ALL with *CBL* mutations have so far been reported, suggesting that the pathogenesis of infant ALL is different from paediatric or adult ALL. To our knowledge, this is the first report of infant ALL patients with 11q23 translocation/*MLL*-R and *CBL* mutations. The present study suggests that alterations of *CBL* gene and *MLL*-R may cooperatively play a pathogenic role in the development of infant ALL with *MLL*-R.

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## Author's contributions

TT and YH designed the study. JT, MH, TK, MS and EI provided critical reagents and samples. NS and MP performed the experiments. EI, HA and SO supervised the work. NS and MP analysed the results. NS, TT, and YH wrote the paper and all the authors critically reviewed and revised it.

## Conflict of interest

The authors declare no conflict of interest.

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# Identification of *TRIB1* R107L gain-of-function mutation in human acute megakaryocytic leukemia

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Brief report

# Identification of *TRIB1* R107L gain-of-function mutation in human acute megakaryocytic leukemia

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***Trib1* has been identified as a myeloid oncogene in a murine leukemia model. Here we identified a *TRIB1* somatic mutation in a human case of Down syndrome–related acute megakaryocytic leukemia. The mutation was observed at well-conserved arginine 107 residue in the pseudokinase domain. This R107L mutation remained in leukocytes of the remission stage in which *GATA1* mutation disappeared, suggesting the *TRIB1* mutation is an earlier genetic event in leukemogenesis. The bone marrow transfer experiment showed that acute myeloid leukemia development was accelerated by transducing murine bone marrow cells with the R107L mutant in which enhancement of ERK phosphorylation and C/EBPα degradation by Trib1 expression was even greater than in those expressing wild-type. These results suggest that TRIB1 may be a novel important oncogene for Down syndrome–related acute megakaryocytic leukemia. (*Blood*. 2012; 119(11):2608-2611)**

## Introduction

The Down syndrome (DS) patients are predisposed to developing myeloid leukemia, and those patients frequently exhibit *GATA1* mutations.[1] However, it is proposed that the *GATA1* mutation is important for transient leukemia in DS but not sufficient for full-blown leukemia, suggesting that additional genetic alterations are needed.[1] Therefore, it is important to search the subsequent genetic changes for DS-related leukemia (ML-DS) to predict malignant transformation and prognosis of the patients.

*Trib1* has been identified as a myeloid oncogene that cooperates with *Hoxa9* and *Meis1* in murine acute myeloid leukemia (AML).[2] As a member of the tribbles family of proteins, TRIB1 interacts with MEK1 and enhances ERK phosphorylation.[2,3] Moreover, TRIB1 promotes degradation of C/EBP family transcription factors, including C/EBPα, an important tumor suppressor for AML, and we observed that degradation of C/EBPα by Trib1 is mediated by its interaction with MEK1.[4] Thus, TRIB1 plays an important role in the development of AML by modulating both the RAS/MAPK pathway and C/EBPα function together with Trib2 that has also been identified as a myeloid-transforming gene.[5] Potential involvement of *TRIB1* in human leukemia has been reported in cases of AML with 8q34 amplification in which both *c-MYC* and *TRIB1* are included in the amplicon.[6] The enhancing effect of TRIB1 on the MAPK signaling suggests that TRIB1 alterations may be related to AML cases, which do not show any mutations in the pathway members, such as FLT3, c-Kit, or Ras. In this report, we identified a novel somatic mutation of *TRIB1* in a case of human acute megakaryocytic leukemia developed in DS (DS-AMKL). Retrovirus-mediated gene transfer followed by bone marrow transfer indicated that the mutation enhanced leukemogenic activity and MAPK phosphorylation by TRIB1.

## Methods

### Patients

*TRIB1* mutations have been investigated in 12 cases of transient leukemia (TL), 5 of DS-AMKL, and 4 cell lines of DS-AML. Peripheral blood leukocytes of TL and bone marrow cells of DS-AMKL were used as sources for the molecular analysis. This study was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine, and all clinical samples were obtained with informed consent from the parents of all patients, in accordance with the Declaration of Helsinki.

Patient 84 showed trisomy 21 and extensive leukocytosis at birth. Hematologic findings revealed the white blood cell count to be 148 × $10^9$/L, including 87% myeloblasts, a hemoglobin level of 19.4 g/dL, and a platelet count of 259 × $10^9$/L. Patent ductus arteriosus and atrial septal defect have been pointed out. Based on the hematologic data and the chromosomal abnormality, the patient was diagnosed as DS-related TL. The hematologic abnormality was then improved, but 8 months later 3% of 6.9 × $10^9$/L white blood cells became myeloblasts (Figure 1A). A karyotype analysis of bone marrow cells revealed 48, XY,+8,+21 in 3 of 20 cells. In addition, *GATA1* mutation was detected at nt 113 from A to G, resulting in loss of the first methionine.[7] He was diagnosed as AMKL at this time, and his disease was in remission by subsequent chemotherapy.

### PCR and sequencing

The entire coding region of human *TRIB1* cDNA of patients' samples was amplified using Taq polymerase (Promega) and specific primer pairs (the sequences of the primers are available on request). The genomic DNA samples of patient 84 were also analyzed. The sequence analysis of *GATA1* was performed as described previously.[7] After checking the PCR products by agarose gel electrophoresis, the products were purified and directly sequenced.

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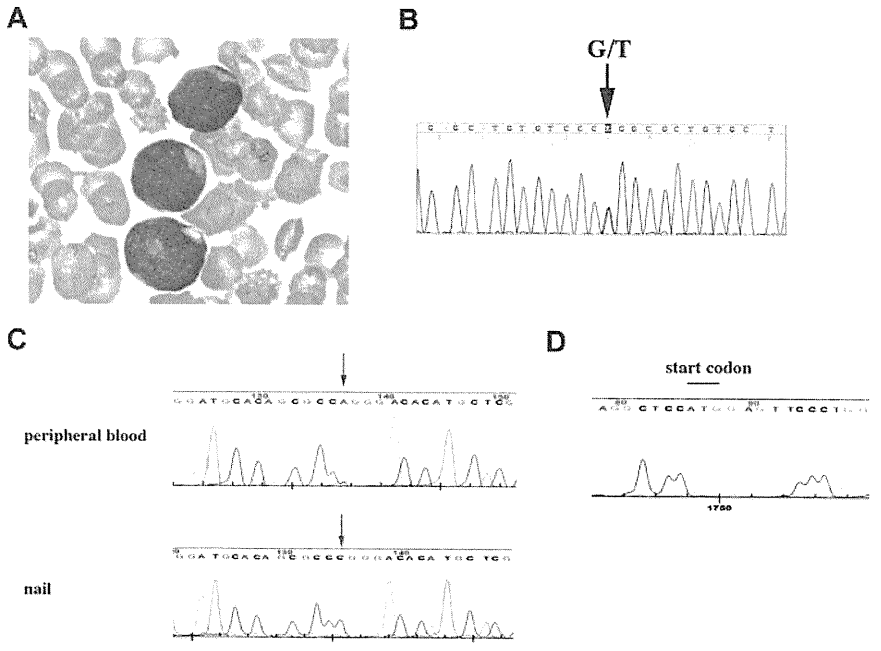


**Figure 1. *TRIB1* R107L mutation identified in DS-related leukemias.** (A) Giemsa staining of the case 84 peripheral blood smear diagnosed as AMKL. The image was acquired using a BX40 microscope equipped with a 100×/1.30 NA oil objective (Olympus) and a C-4040 digital camera (Olympus). (B) Fluorescent dye sequencing chromatographs of *TRIB1* genotyping by direct sequencing of the case 84 using a cDNA sample as a template. The vertical arrow indicates mixed G and T signals at codon 107. (C) Fluorescent dye sequencing chromatographs of *TRIB1* of peripheral blood leukocytes (top) or nail (bottom) in the same case at the complete remission stage. The red arrows indicate that the mutation remains in leukocytes but not in nail. The reverse strand sequences are shown. (D) *GATA1* sequence. The start codon that was mutated in AMKL[7] is normal in the peripheral blood leukocytes at the remission stage.

### Retroviral infection of murine bone marrow cells and bone marrow transfer

Bone marrow cells were prepared from 8-week-old female C57Bl/6J mice 5 days after injection of 150 mg/kg body weight of 5-fluorouracil (Kyowa Hakko Kogyo). Retroviral infection of bone marrow cells and bone marrow transfer experiments were performed as described.[2] Transduction efficiencies evaluated by flow cytometric techniques were comparable between wild-type (WT; 5.3%) and R107L (3.4%). Animals were housed, observed daily, and handled in accordance with the guidelines of the animal care committee at Japanese Foundation for Cancer Research. All the diseased mice were subjected to autopsy and analyzed morphologically, and the blood was examined by flow cytometric techniques. The mice were diagnosed as positive for AML according to the classification of the Bethesda proposal.[8] The survival rate of each group was evaluated using the Kaplan-Meier method, and differences between survival curves were compared using the log-rank test.

### Immunoblotting

Immunoblotting was performed using cell lysates in RIPA buffer as described.[4] Anti-p44/42 ERK (Cell Signaling Technologies), anti–phospho-p44/42 ERK (Cell Signaling Technologies), anti-C/EBPα (Santa Cruz Biotechnology), anti-FLAG (Sigma-Aldrich), and anti-GAPDH (Hy Test Ltd) antibodies were used.

## Results and discussion

The important role of TRIB1 on the MAPK signaling suggests that *TRIB1* alterations may occur in some AML cases, which do not show overlapping mutations in the pathway members, such as FLT3, KIT, or RAS. Therefore, we tried to search mutations of *TRIB1* in cases of ML-DS and TL in which such mutations are infrequent.[9] In a case of DS-AMKL (case 84), a nucleotide change from guanine to thymine has been identified at 902 that results in amino acid alteration from arginine 107 (R107) to leucine (Figure 1B). The sequence changes were confirmed by subcloning the PCR product into the TA-type plasmid vector (data not shown). The nucleotide change was not observed in the DNA sample derived from the nail of the same patient at all (Figure 1C), indicating that this change is a somatic mutation. Interestingly, the mutation was retained in the peripheral blood sample in the complete remission stage in which the GATA1 mutation completely disappeared (Figure 1C-D). These results indicate that the *TRIB1* mutation precedes the onset of TL and the *GATA1* mutation, and suggest that *TRIB1* mutation occurred at the hematopoietic stem cell level and that the clone retaining the *TRIB1* mutation survived after chemotherapy. In case 84, there was no mutation for *FLT3* exons 14, 15, and 20, *PTPN11* exons 3 and 13, *KRAS* exons 2, 3, and 5, and *KIT* exons 8, 11, and 17 by the high-resolution melt analysis (data not shown).

An additional mutation was found in a case of TL (case 109) at the nucleotides 805 and 806 from GC to AT, which results in amino acid conversion from alanine (A75) to isoleucine (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). *TRIB1* expression in DS-related and DS-unrelated leukemias was examined by real-time quantitative RT-PCR (supplemental Figure 2).

R107 is located within a psuedokinase domain of TRIB1 that is considered as a functionally core domain of TRIB family proteins.[10] Sequence comparison among 3 TRIB family proteins as well as tribbles homologs in other organisms revealed that the R107 is well conserved in mammalian TRIB1 and TRIB2,[10] suggesting that this arginine residue is evolutionary conserved and may be related to an important function. On the other hand, A75 is located outside of the pseudokinase domain, not conserved between human and mouse, or other tribbles homologs. Moreover, the N-terminal domain containing A75 is dispensable for the leukemogenic activity of Trib1.[4] Therefore, we tried to investigate whether the R107L mutation could affect the leukemogenic activity of *TRIB1*.

R107L was introduced into the murine *Trib1* cDNA by site-directed mutagenesis. Both WT and R107L cDNAs were subcloned into the pMYs-IRES-GFP retroviral vector and were used for retrovirus-mediated gene transfer followed by bone marrow transfer according to the method previously described.[1] All the mice

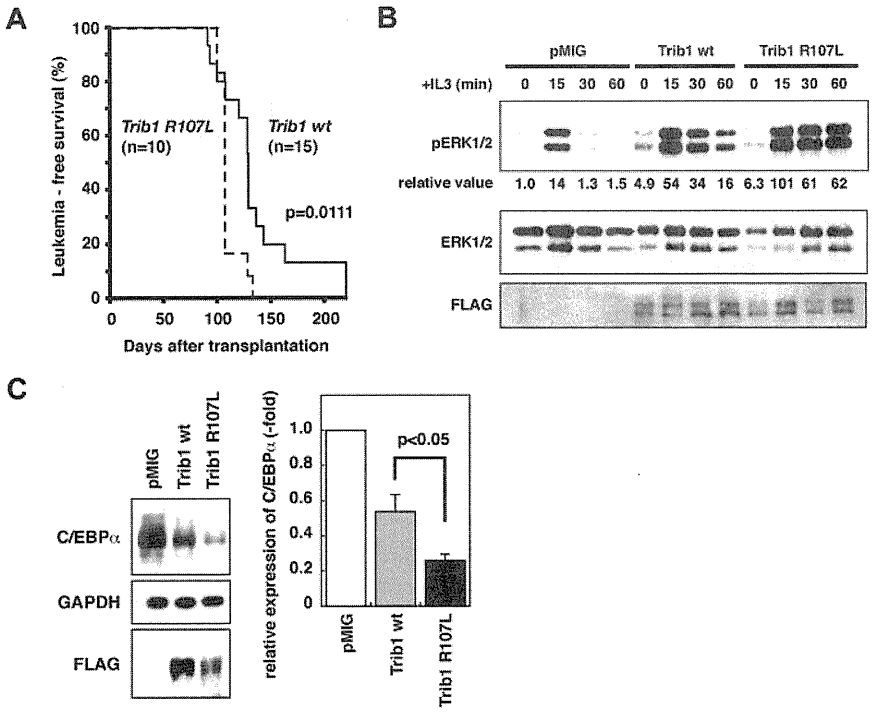


**Figure 2. AML development by bone marrow transfer using *Trib1* WT and R107L.** (A) Kaplan-Meier survival curves are shown. The *P* value was calculated with the log-rank test. (B) Immunoblot analysis of *Trib1* WT AML (Mac-1 56.2%, Gr-1 52.5%, $CD34^{lo}$, c-$kit^{-}$, Sca-$1^{-}$) and R107L AML (Mac-1 41.4%, Gr-1 25.2%, $Cd34^{lo}$, c-$kit^{lo}$, Sca-$1^{-}$) derived from bone marrow of recipient mice (WT #T73 and R107L #T151 in supplemental Table 1). Enhancement of ERK phosphorylation is more significant in R107L. Relative values of ERK phosphorylation were calculated by densitometric analysis. (C) Immunoblot analysis for C/EBPα of the same AML samples as in panel B. Relative expression level of C/EBPα is quantitated (right).

transplanted with bone marrow cells expressing WT (n = 15) or R107L (n = 12) developed AML (Figure 2A). The mean survival time was shorter in the recipients with R107L-expressing bone marrow cells (110 days) than those with WT (136 days; Figure 2A). The difference was significant ($P = .0111$, log-rank test). The result indicates that the R107L mutation enhances the leukemogenic activity of TRIB1. These results also suggest that *TRIB1* mutation might cooperate with *GATA1* mutation in the genesis of DS-AMKL, and that trisomy 21, *TRIB1*, and *GATA1* mutations occurred consecutively, which contributed to the multistep leukemogenic process.

We have shown that TRIB1 interacts with MEK1 and enhances phosphorylation of ERK.[2] The R107L mutant enhanced ERK phosphorylation more extensively than WT (Figure 2B) in AML cells derived from bone marrow of recipient mice, and more significant degradation of C/EBPα was induced by the R107L mutant (Figure 2C). These findings might be correlated to the enhanced leukemogenic activity of the mutant. Both R107L and WT proteins could interact with MEK1, having the binding motif in their C-termini. The residue 107 is located at subdomain II of the pseudokinase domain.[11] The mutation may affect conformation of the domain and may promote the MEK1 function on ERK, although additional studies are required to address the possibility. A recent study demonstrates that Trib1 and Trib2 failed to show ERK phosphorylation in 32D cells.[12] The different response to Trib1 between primary leukemic cells and the cell line might depend on the cellular context and/or combination of additional mutations. The AML phenotypes were somewhat varied in each case and Mac-1–positive/Gr-1–negative AMLs were more remarkable in WT than in R107L, although the difference was not statistically significant (supplemental Figures 3-4; supplemental Table 1). The current study underscores the role of TRIB1 in human leukemogenesis and the significance of the R107L mutation in its function. Further sequence analysis of tribbles family genes in a larger cohort will emphasize the importance of R107L and/or additional mutations of *TRIB1* in leukemic patients.

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## Authorship

Contribution: T.Y., E.I., Y.H., and T.N. designed and performed the research and wrote the manuscript; T. Toki, Y.A., R.K., and M.-j.P. performed the research; and Y.K., T. Takahara, and Y.Y. contributed to the bone marrow transplantation analysis.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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# *CBL* mutation in chronic myelomonocytic leukemia secondary to familial platelet disorder with propensity to develop acute myeloid leukemia (FPD/AML)

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Brief report

# *CBL* mutation in chronic myelomonocytic leukemia secondary to familial platelet disorder with propensity to develop acute myeloid leukemia (FPD/AML)

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**Familial platelet disorder with a propensity to develop acute myeloid leukemia (FPD/AML) is a rare autosomal dominant disease characterized by thrombocytopenia, abnormal platelet function, and a propensity to develop myelodysplastic syndrome (MDS) and AML. So far, > 20 affected families have been reported. Recently, a second *RUNX1* alteration has been reported; however, no additional molecular abnormalities have been found so far. We identified an acquired *CBL* mutation and 11q-acquired uniparental disomy (11q-aUPD) in a patient with chronic myelomonocytic leukemia (CMML) secondary to FPD with *RUNX1* mutation but not in the same patient during refractory cytopenia. This finding suggests that alterations of the *CBL* gene and *RUNX1* gene may cooperate in the pathogenesis of CMML in patients with FPD/AML. The presence of *CBL* mutations and 11q-aUPD was an important "second hit" that could be an indicator of leukemic transformation of MDS or AML in patients with FPD/AML. (*Blood.* 2012; 119(11):2612-2614)**

## Introduction

Familial platelet disorder with a propensity to develop acute myeloid leukemia (FPD/AML) is a rare autosomal dominant disease characterized by thrombocytopenia, abnormal platelet function, and a propensity to develop myelodysplastic syndrome (MDS) and AML.[1,2] Since Song et al reported haploinsufficiency of the *RUNX1/CBFA2* gene,[3] more than 20 affected families have been reported.[4-8] Notably, various types of mono-allelic mutations of the *RUNX1* gene have been found in patients with AML secondary to FPD.[3,7-9] *RUNX1*, which is a key regulator of definitive hematopoiesis and myeloid differentiation, is also commonly involved in sporadic cases of MDS and AML, by translocations in AML[10] and by point mutations in AML[11,12] and MDS.[13] Recently, a second *RUNX1* alteration has been reported[8]; however, no additional molecular abnormalities have been found so far.

In this regard, recent reports of somatic mutations of the *CBL* proto-oncogene in myeloid neoplasms are intriguing because these *CBL* mutations have been shown to result in aberrant tyrosine kinase signaling, which would also lead to the activation of RAS signaling pathways. So far, we and others have reported that *CBL* mutations occurred in a variety of myeloid neoplasms, including de novo AML,[14,15] MDS,[16,17] and myeloproliferative neoplasm,[16,17] especially in chronic myelomonocytic leukemia (CMML)[16,17] and juvenile myelomonocytic leukemia.[18] The importance of *CBL* mutations for leukemogenesis has substantially increased, which prompted us to search for possible *CBL* mutations in this pedigree.

Here, we reported that *CBL* mutation developed at the time of diagnosis of CMML, but not during refractory cytopenia, in a Japanese patient with FPD/AML harboring a *RUNX1* mutation.

## Methods

### *RUNX1* mutation analysis

DNA and RNA were extracted from peripheral blood (PB) of the proband, her sister, and their mother after obtaining informed consent. We performed mutation analysis of the *RUNX1* gene by PCR followed by direct sequencing with the use of an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). For further confirmation of deletion mutations, the PCR products were subcloned with the use of a TOPO TA Cloning Kit (Invitrogen) and then sequenced. Mutations were screened from exons 1-8 of the *RUNX1* gene.

### *CBL* mutation analysis

Because *CBL* mutations thus far reported almost exclusively involved exons 8-9 that encode Linker/RING finger domains, we confined our mutation analysis to these exons, which were subjected to direct sequencing. Because the frequency of 11q-acquired uniparental disomy (11q-aUPD) was reported as ~ 85%-90% in *CBL* mutations, we also analyzed the sample with Affymetrix GeneChip 250K *Nsp*I.[17-19] Genome-wide detection of copy number abnormalities or allelic imbalances was performed with CNAG/AsCNAR Version 3.0 software (http://www.genome.umin.jp), which enabled sensitive detection of copy number neutral loss of heterozygosity (or aUPD).[19] In addition, we examined mutations of the following genes in the proband as previously reported: *FLT3, KIT, RAS, JAK2, PTPN11, ASXL1, IDH1/2,* and *MPL*.[20-22] The study adhered to the principles of the Helsinki Declaration and was conducted under the regulations enacted by the Ethics Board of Gunma Children's Medical Center.

## Results and discussion

The proband (III-2), who was the second child of nonconsanguineous parents, underwent an 8-year follow-up of mild to moderate

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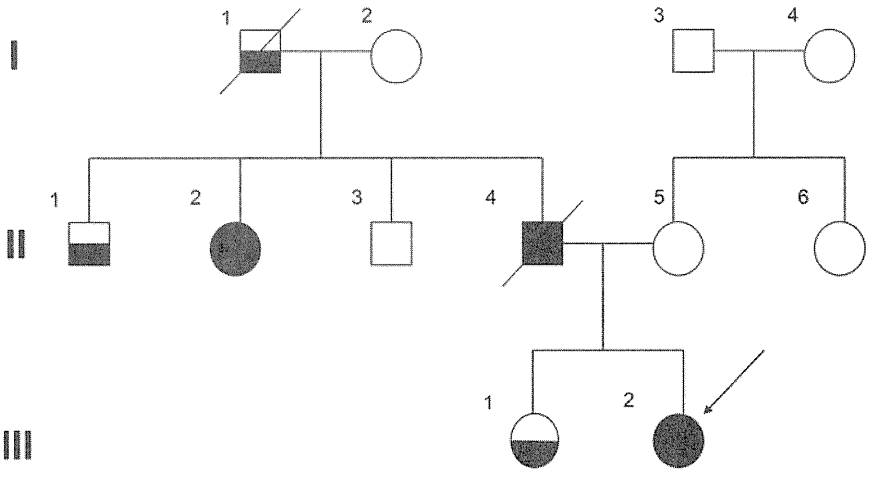


**Figure 1. The family pedigree.** Squares indicate males and circles indicate females. Open symbols represent unaffected persons, half-filled symbols represent persons affected by thrombocytopenia, and closed symbols represent persons affected by FPD who developed MDS/AML. The proband (III-2) is indicated by an arrow.

thrombocytopenia (50-80 $\times$ $10^3/\mu L$), and at that age of 10 years, her condition was diagnosed as refractory cytopenia. Cytogenetic analysis found a normal karyotype, and FISH showed neither monosomy 7 nor trisomy 8. The proband had been closely observed without any therapy for 2 years and 9 months because she did not require transfusion and her disease remained stable; however, at the age of 12 years, leukocytosis and monocytosis developed and she became dependent on platelet transfusions. Finally, the disease evolved to CMML, and allogeneic bone marrow (BM) transplantation from an unrelated donor was performed. During the entire course, the number of blast cells in PB was constantly $< 2\%$, and no additional symptoms were observed, such as hepatosplenomegaly. Her elder sister (III-1) was also followed for 10 years with mild thrombocytopenia; however, the morphologic findings of PB or BM were not compatible with myeloproliferative neoplasms.[17] Because her platelet count has been gradually decreasing, allogeneic BM transplantation is being considered. Although her father (II-4) developed MDS at the age of 41 and died 2 years later, her paternal aunt (II-2) developed MDS at the age of 49 and has remained in complete remission for 11 years after successful allogeneic cord blood transplantation. Her paternal grandfather (I-1) and uncle (II-1) also had a history of thrombocytopenia (Figure 1). Direct sequencing analysis of *RUNX1* found a one-base deletion of adenine at position 2364 within exon 7, resulting in a frameshift mutation that corresponded to AML1b transcript in the proband and her sister (Figure 2A). This resulted in a frameshift after amino acid change G262GfsX21. This mutation was not detected in their mother. All these data suggested that her paternal grandfather (I-1), uncle (II-1), aunt (II-2), and her father (II-4) were considered to have FPD/AML, carrying the same *RUNX1* mutation.

Although no *CBL* mutations were found in the proband sample of refractory cytopenia before development of CMML, homozygous mutation of the *CBL*, which was located in the splice acceptor site of intron 8 (Figure 2B), was identified in the proband sample in the CMML. We also found 11q-aUPD (Figure 2C) in the proband sample, confirming a strong association of *CBL* mutations with 11q-aUPD, as previously described[16-18]; however, no mutations of any other genes, including *FLT3*, *KIT*, *RAS*, *JAK2*, *PTPN11*, *ASXL1*, *IDH1/2,* and *MPL*, were found and no additional somatic *RUNX1* alterations. No *CBL* mutations were found in her sister's sample at this time.

Inherited *RUNX1* mutations were clustered in the N-terminal region in exons 3-5, which affect the runt homology domain. Mutations in the C-terminal region, detected in the present pedigree, have been reported less frequently so far and are considered to affect the transactivation domain (Figure 2D).

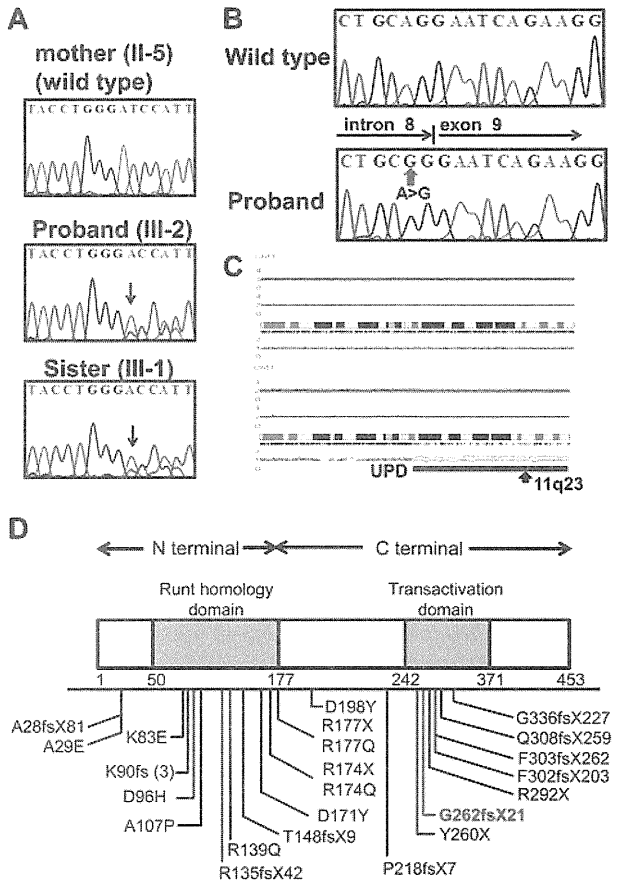


**Figure 2. Mutation analysis of *RUNX1* and *CBL* genes in the pedigree.** (A) Direct sequencing analysis of affected patients (III-1, III-2) and an unaffected family member (II-5) is shown. Arrow indicates a one-base deletion of adenine. (B) Mutated *CBL* is shown in the proband. (C) Identification of acquired uniparental disomy of 11q in the proband. Total copy number (tCN; red plot) is shown above the cytoband, and the results of allele-specific copy number analysis with anonymous references (AsCNAR) plots are shown below the cytoband. Larger allele is presented by a red line, and the smaller allele is presented by a blue line. Allele-specific analysis showed 11q-aUPD (blue line), which contained the *CBL* region (arrow). (D) Schematic representation of wild-type and mutated *RUNX1*. The affected *RUNX1* is truncated at the C terminus of the transactivation domain (TAD). Part of TAD is lacking in this proband (red line).

It has been postulated that disruption of the *RUNX1* gene is not sufficient to cause AML, as previously reported with monoallelic and biallelic inactivation of *Runx1* in mice[23,24] and in mice carrying the knocked-in *Runx1-Eto* chimeric gene. These data indicate that a second-hit mutation in addition to the dysfunction of *RUNX1* is required for the development of AML. Minelli et al postulated that the mutations seen in FPD cases have a mutation effect that induces additional genetic abnormalities and promotes progression to hematologic malignancies.[25]

Marked associations between chromosome translocation and gene mutations have been reported: *KIT* mutation in core binding leukemia, t(8;21)/*AML1-ETO* and inv(16)(p13q22)/*CBFβ-MYH11*, *FLT3*-ITD in leukemia with t(15;17)/*PML-RARα*, or with t(6;9)/*DEK-CAN*. We consider that it is important to find an association to administer clinically relevant treatment. In addition to the germline *RUNX1* mutation, we identified an acquired *CBL* mutation in the proband and assumed it to be a second hit mutation by which FPD evolved into CMML. To our knowledge, this is the first patient with FPD/AML in whom *CBL* mutation has developed. This finding suggests that alterations of the *CBL* gene and *RUNX1* could cooperate in the pathogenesis of CMML or AML in patients with FPD/AML. The presence of 11q-aUPD provided evidence that loss of the wild-type copy of *CBL* with duplication of the mutant copy was an important second hit that could be an indicator of leukemic transformation in patients with FPD/AML.

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## Authorship

Contribution: Y.H. and C.O. designed the study; A.M., C.O., and D.H. provided critical reagents and samples; N.S., M.P., A.S.-O., and C.M. performed the experiments; H.A. and S.O. supervised the work; N.S. and M.P. analyzed the results; N.S. and D.H. constructed the figures; N.S. and Y.H. wrote the paper; and all the authors critically reviewed and revised the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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# Establishment of a Novel Human Myeloid Leukemia Cell Line, AMU-AML1, Carrying t(12;22)(p13;q11) without Chimeric *MN1-TEL* and with High Expression of *MN1*

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In this study, we established and analyzed a novel human myeloid leukemia cell line, AMU-AML1, from a patient with acute myeloid leukemia with multilineage dysplasia before the initiation of chemotherapy. AMU-AML1 cells were positive for CD13, CD33, CD117, and HLA-DR by flow cytometry analysis and showed a single chromosomal abnormality, 46, XY, t(12;22)(p13;q11.2), by G-banding and spectral karyotyping. Fluorescent in situ hybridization analysis indicated that the chromosomal breakpoint in band 12p13 was in the sequence from the 5′ untranslated region to intron 1 of *TEL* and that the chromosomal breakpoint in band 22q11 was in the 3′ untranslated region of *MN1*. The chimeric transcript and protein of *MN1-TEL* could not be detected by reverse-transcriptase polymerase chain reaction or Western blot analysis. However, the *MN1* gene was amplified to three copies detected by array comparative genomic hybridization analysis, and the expression levels of the *MN1* transcript and protein were high in AMU-AML1 cells when compared with other cell lines with t(12;22)(p13;q11-12). Our data showed that AMU-AML1 cells contain t(12;22)(p13;q11.2) without chimeric fusion of *MN1* and *TEL*. The AMU-AML1 cells gained *MN1* copies and had high expression levels of MN1. Thus, the AMU-AML1 cell line is useful for studying the biological consequences of t(12;22)(p13;q11.2) lacking chimeric *MN1-TEL*. 

## INTRODUCTION

Nonrandom chromosomal translocations usually have an important role in the pathogenesis of leukemia. Establishment of a cell line with a recurrent translocation and analysis of its breakpoints can contribute to the understanding of the molecular biological significance of the translocation and also to the development of new drugs targeting the translocation.

t(12;22)(p13;q11-13) is recurrent in various hematological malignancies and sometimes results in a chimeric fusion of *MN1* and *TEL* (Bohlander, 2005). Cytogenetic abnormalities of chromosome band 12p13 are usually involved with *TEL* (also known as *ETV6*: ets translocation variant gene 6), which encodes a protein of the ETS transcription factor family. Many genes have been reported as fusion partners for *TEL*; these fusion partners include *AML1*, *PDGFβR*, *MN1*, *ABL*, *MDS1/EVI1*, *JAK2*, and others (Bohlander, 2005). The *MN1* gene is located on chromosomal band 22p11, and the product of *MN1* is associated with leukemogenesis as a coactivator of transcription (van Wely et al., 2003; Heuser et al., 2007; Meester-Smoor et al., 2007). A high level of *MN1* mRNA expression is predictive of a poor outcome in patients with acute myeloid leukemia (AML) with normal karyotype (Heuser et al., 2006; Langer et al., 2009).

To the best of our knowledge, t(12;22)(p13;q11-13) has been reported in more than 17 patients with hematological malignancies, and a chimeric fusion gene of *MN1-TEL* was detected in at least seven patients (Johansson et al., 1990; Oval et al., 1990; Callen et al.,1991; Kashimura et al., 1993;

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Geurts van Kessel et al., 1994; Buijs et al., 1995; Vieira et al., 2000; Nakazato et al., 2001; Nakao et al., 2001; Valle et al., 2004). The fusion protein of MN1-TEL has oncogenic activities through the upregulation of *HOXA9* and the inhibition of RAR-RXR-mediated transcription (Kawagoe et al., 2005; van Wely et al., 2007). However, some patients with t(12;22) did not have a chimeric *MN1-TEL*, and the implications of t(12;22) lacking *MN1-TEL* are not understood. In addition, only one cell line of this type, MUTZ-3, has been reported to date (Hu et al., 1996).

Here, we report a novel human myeloid leukemia cell line with t(12;22)(p13;q11) lacking *MN1-TEL*. The cell line was established from a patient with AML with multilineage dysplasia before the initiation of chemotherapy. We examine the genetic and molecular biologic characteristics of this cell line in comparison with other leukemia cell lines with t(12;22).

## MATERIALS AND METHODS

### Case History

A 60-year-old man was admitted to our hospital because of leukocytosis in March 2003. Peripheral blood examination showed a white blood cell level of 37,200 cells/μl with 82% myeloblasts, a hemoglobin level of 10.8 g/dl, and a platelet level of 100,000/μl. Bone marrow examination revealed 56% myeloblasts with Auer bodies and multilineage dysplasia. A surface marker analysis revealed that the blasts from the bone marrow were positive for CD7 (32.1%), CD13 (87.1%), CD33 (76.6%), CD34 (82.2%), and HLA-DR (37.7%). G-banding analysis of cells in the bone marrow showed 46, XY, t(12;22)(p13;q13) [20/20]. Based on these test results, the patient was diagnosed as having AML transformed from myelodysplastic syndrome. Induction therapy was started with CAG therapy consisting of cytosine arabinoside (10 mg/m$^2$) subcutaneously administered every 12 hr for days 1–14; aclarubicin (14 mg/m$^2$) intravenously administered daily for days 1–4; and G-CSF (200 μg/m$^2$) subcutaneously administered daily. However, on the sixth day of therapy, the patient discontinued the treatment for leukemia and was discharged from our hospital because of personal difficulties. He died of leukemia 3 months after diagnosis without reaching complete remission.

### Cell Culture

The patient gave informed consent for bone marrow collection in a procedure approved by the Institutional Review Board of Aichi Medical University. Bone marrow was collected at the time of initial diagnosis and prior to chemotherapy. Mononuclear cells were isolated by Ficoll-Hypaque sedimentation. The cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (Thermo Electron, Melbourne, Australia) and 1% penicillin-streptomycin (GIBCO-BRL, Grand Island, NY, USA). Cultures were maintained at 37°C in 5% $CO_2$, and the cultured cells were fed every 5–7 days by means of partial medium change. After 3 months of culture, the cell proliferation became continuous. Polymerase chain reaction (PCR) and reverse-transcriptase PCR (RT-PCR) were used to test for the presence of the following pathogens: Epstein-Barr virus (EBV), cytomegalovirus (CMV), hepatitis B and C viruses (HBV, HCV), human immunodeficiency virus (HIV)-1, human T cell leukemia virus (HTLV)-I, and mycoplasma.

### Cell Proliferation Assays and Reagents

Cultured cells were observed under an inverted-phase microscope. Growth curves were determined by plating 5 × 10$^5$ cells/ml in 0.2 ml medium in 96-well plates, and viable cell numbers were counted with the aid of trypan blue exclusion. The growth curve was determined from triplicate experiments. To assess the responses to human cytokines, we cultured the cells in 1 ml medium in 12-well plates at a concentration of 5 × 10$^5$ cells/ml in the absence or presence of the following cytokines: rhG-CSF (10 ng/ml), rhGM-CSF (10 ng/ml), rhM-CSF (50 ng/ml), rhIL-3 (10 ng/ml), rhIL-5 (10 ng/ml), rhIL-6 (10 ng/ml), rhSCF (100 ng/ml), and rhEPO (5 U/ml). Viable cells were counted after 96 hr of culture by trypan blue exclusion test.

### Morphology and Immunophenotype

The morphology of cells that were cytocentrifuged and air-dried on a glass slide was determined by May-Giemsa staining. The following cell surface antigens were examined by flow cytometry (SRL Inc., Tokyo, Japan): CD2, CD3, CD4, CD5, CD7,CD8,CD10, CD11b, CD13, CD14, CD15, CD18, CD19, CD20, CD33, CD34, CD41, CD56, CD61, CD64, CD65, CD71, CD117, HLA-DR, and GP-A (glycophorin A).

### Cytogenetics

Chromosome preparations for G-banding, spectral karyotyping (SKY), and fluorescent in situ

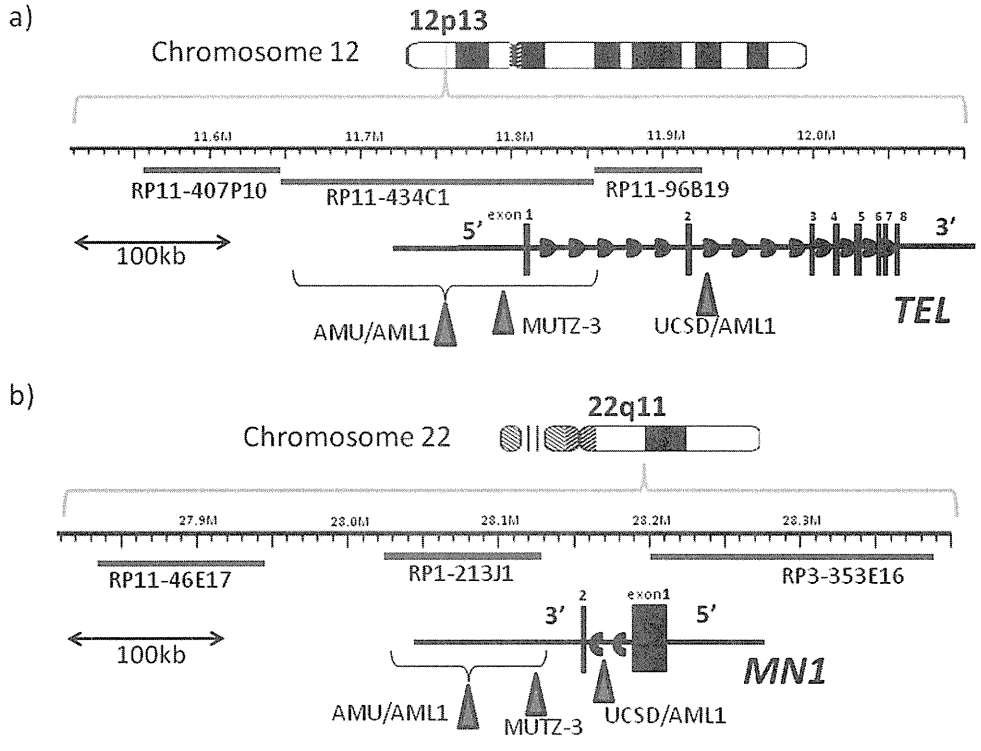


Figure 1. Schema of the relationship among chromosomes, *TEL*, *MN1*, and the contigs of BAC/PAC clones used for FISH analysis and the breakpoints of leukemia cell lines with t(12;22). The positional data for genes, BACs and PACs, were gathered from the NCBI website. The measurements indicate the distance from the telomeric tip on the short arm of each chromosome. The black arrows show the breakpoints of leukemia cell lines with t(12;22). The breakpoints of AMU-AML1 were determined by FISH. The breakpoints of UCSD/AML1 and MUTZ-3 were reported by Buijs and Hu, respectively. The upper panel shows chromosome 12, and the lower panel shows chromosome 22. M; mega bases.

hybridization (FISH) were performed according to standard procedures as previously described (Kanda-Akano et al., 2004; Hanamura et al., 2006; Kobayashi et al., 2011). Briefly, cells were cultured in the log phase, treated with colchicine (final concentration, 0.02 μg/ml) for 1–2 hr, harvested with hypotonic potassium chloride (0.075 M KCl), fixed with methanol/acetic acid (3:1; Carnoy's solution), and stored at −20°C. BAC and PAC clones at surrounding *TEL* (RP11-434C1, RP11-96B19, RP11-407P10) and *MN1* (RP11-46E17, RP3-353E16, RP1-213J1) were obtained from Invitrogen (Carlsbad, CA, USA) and used for FISH probes (Fig. 1). The BAC/PAC clones were labeled according to the manufacturer's instructions (Abbott, Abbott Park, IL, USA). BAC/PAC information was obtained from the National Center for Biotechnology Information (NCBI) website, and the probes were confirmed to map to the precise chromosome bands by using metaphase spreads from the peripheral-blood lymphocytes of healthy donors.

### High-Density SNP Array Analysis

Genomic DNA was isolated from AMU-AML1 cells (this cell line is available for researchers upon reasonable request) and subjected to GeneChip Human Mapping 250K Sty array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. The chromosome copy number was calculated by using CNAG 3.0 software (Nannya et al., 2005). For data normalization, six normal reference samples were used. The genomic location of probes on the array was determined from the genomic mapping information on NCBI Build 35.1 (National Center for Biotechnology Information).

### RT-PCR and Quantitative Real-Time RT-PCR Analysis

We performed RT-PCR with the primer sets listed in Table 1 to detect *MN1-TEL* and *MN1* transcripts. Total RNA was extracted from 5 × $10^6$ cells by using an RNeasy Mini Kit (Qiagen,

TABLE 1. The Sequences of Primers Used in This Study

| Primer | Sequence (5′→3′) | Source |
|---|---|---|
| *MN1*-1 | GCCATGAGCACCATTGA | Valle et al. (2004) |
| *TEL*-4B | TAGAATTCCAGGGTGGAAGAATG | Valle et al. (2004) |
| *MN1* sense 5 | CGCAGATCCAGCTACAGAGG | Not applicable |
| *MN1* antisense 1 | AGCCACGAATGTCCCAAATC | Not applicable |
| *TEL* sense 2 | CGGGAGAGATGCTGGAAGA | Not applicable |
| *TEL* sense 4 | GAGAGCCCAGTGCCGAGTTA | Not applicable |
| β-*actin* sense | GTGGGGCGCCCCAGGCACCA | Not applicable |
| β-*actin* antisense | GTCCTTAATGTCACGCACGATTTC | Not applicable |

Hilden, Germany), and cDNA was synthesized from total RNA by using the SuperScript 3 First-strand Synthesis System (Invitrogen). RT-PCR was performed with an Advantage2 PCR Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. PCR products were analyzed by agarose gel electrophoresis. The expression levels of *MN1* transcript were quantified by real-time RT-PCR with a specific primer probe set from Applied Biosystems. We used the TaqMan® Gene Expression Assays (Hs 00159202_m1, Applied Biosystems, Branchburg, NJ, USA) with the Applied Biosystems StepOnePlus™ system according to the manufacturer's instructions. The transcript levels were normalized to those of *GAPDH* (TaqMan, Hs 99999905_m1), and the relative expression level was determined by the $2^{-\Delta\Delta CT}$ method.

### 3′RACE and DNA Sequencing

3′ RACE was performed by using a 5′/3′ RACE Kit (second Generation) according to the manufacturer's protocol (Roche, Mannheim, Germany). First-strand cDNA was reverse-transcribed from 1 μg of total RNA from AMU-AML1 cells. Amplicons were extracted from the gels with a QIA Quick Gel Extraction Kit (Qiagen), and sequencing analysis was performed by using Sequence Scanner Software ver.1.0 (Applied Biosystems) with the aid of the NCBI BLAST server and the GenBank database.

### Western Blot Analysis

Cells ($1 \times 10^6$) were washed once in PBS and lysed in 20 μl RIPA buffer. After 5 min of boiling, 20 μl of sample was subjected to 7.5% polyacrylamide gel electrophoresis. The separated proteins were blotted onto a polyvinylidene difluoride membrane (GE Healthcare, Tokyo, Japan). The membrane was blocked with a solution of 5% nonfat dried milk dissolved in Tris-buffered saline (TBS) containing 0.3% Tween 20 for 1–2 hr at room temperature. Then, the membrane was incubated with a 1:200 dilution of primary antibody at 4°C overnight. The primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and consisted of anti-TEL (H-214), anti-n-MN1 (N-20), anti-c-MN1 (T-16), and anti-β-actin (N-21) antibody. The membrane was washed three times with TBS-0.3% Tween 20 and incubated for 1–2 hr with anti-rabbit or anti-goat IgG-HRP antibody (1:2000 dilution, Santa Cruz). After washing in TBS-0.3% Tween 20, the protein was visualized by ECL plus Western Blotting Detection System (GE Healthcare).

### Human Myeloid Leukemia Cell Lines with t(12;22)(p13;q11-12) and Other Cell Lines

We used human myeloid leukemia cell lines UCSD/AML1, MUTZ-3, HL-60, and THP-1 in the present study. The UCSD/AML1 cell line, which was established from a patient with mixed acute leukemia at the time of relapse, had a karyotype of 45, XX, −7, t(3;3)(q21;q26), t(12;22)(p13;q12) (Oval et al., 1990) and a chimeric fusion of *MN1* and *TEL* (Buijs et al., 1995). The MUTZ-3 cell line, which was established from a patient with acute myelomonocytic leukemia at the time of diagnosis, had a karyotype of 46, XY, der(1)t(1;3)(q43;q13.12)inv(3)(q21.2q26.3), der(2)t(2;7)(q35;q35), der(3)t(1;3)(q43;q13.12), der(7)t(2;7)(q35;q35)inv(7)(p14q35), t(12;22)(p13.2;q11.2) but no chimeric *MN1-TEL* (MacLeod et al., 1996; Hu et al., 1996). The UCSD/AML1 and MUTZ-3 cell lines were kindly provided by Drs. VD Valle and OA Bernard (Université Paris Sud 11, Orsay, France), respectively. The HL-60 cell line was established from a patient with AML (FAB M2; Collins et al., 1977), and the THP-1 cell line was established from a patient with acute monocytic

leukemia (Tsuchiya et al., 1980). Both the HL-60 and THP-1 cell lines have complex karyotypes without t(12;22)(p13;q11-12). Detailed references and cultivation protocols for these cell lines have been described previously (Drexler, 2010).

## RESULTS

### Establishment of AMU-AML1 Cell Line

After 3 months of culture, the cells started to grow in suspension. The cell line was designated as AMU-AML1 after confirmation that the cells started growing again after the conventional freeze-thaw procedure. The cells grow in suspension culture as single cells at a density of $10^4$–$10^7$ cells/ml and can grow for a maximum of 21 days in uninterrupted culture. The cells were well maintained more than 100 passages. The cell line was negative for EBV, CMV, HBV, HCV, HIV-1, HTLV-1, and mycoplasma.

### Morphology and Immunophenotype

Light microscopy showed that the AMU-AML1 cells appeared to be myeloblastoid cells (Fig. 2a). Most cells had blue cytoplasm and a round central nucleus with a reticular chromatin pattern. The AMU-AML1 cells expressed CD13, CD15, CD33, CD65, CD117, and HLA-DR, but lacked lymphoid, erythroid, and megakaryocytic markers. This profile is consistent with the profile of the patient's blasts.

### Growth curve and Cytokine Responsiveness

The doubling time of the cells was ~96 hr (Fig. 2b). Proliferation of the cells was significantly stimulated by rhG-CSF, rhIL-3, rhGM-CSF, rhSCF, and rhM-CSF but not by rhIL-6, rhIL-5, and rhEPO (Fig. 2c).

### Karyotype Analysis by G-Banding and SKY

Cytogenetic analysis was performed 8 months after the start of culture (Fig. 3). The representative karyotype of the cells was 46, XY, t(12;22)(p13;q11.2), which was the same as the karyotype of the patient's leukemia cells. AMU-AML1 cells showed only a single structural abnormality.

### Analysis of Breakpoints by FISH and Array CGH

To confirm the identification of chromosomal breakpoints in AMU-AML1 cells, we performed a series of double-color FISH (DC FISH) with

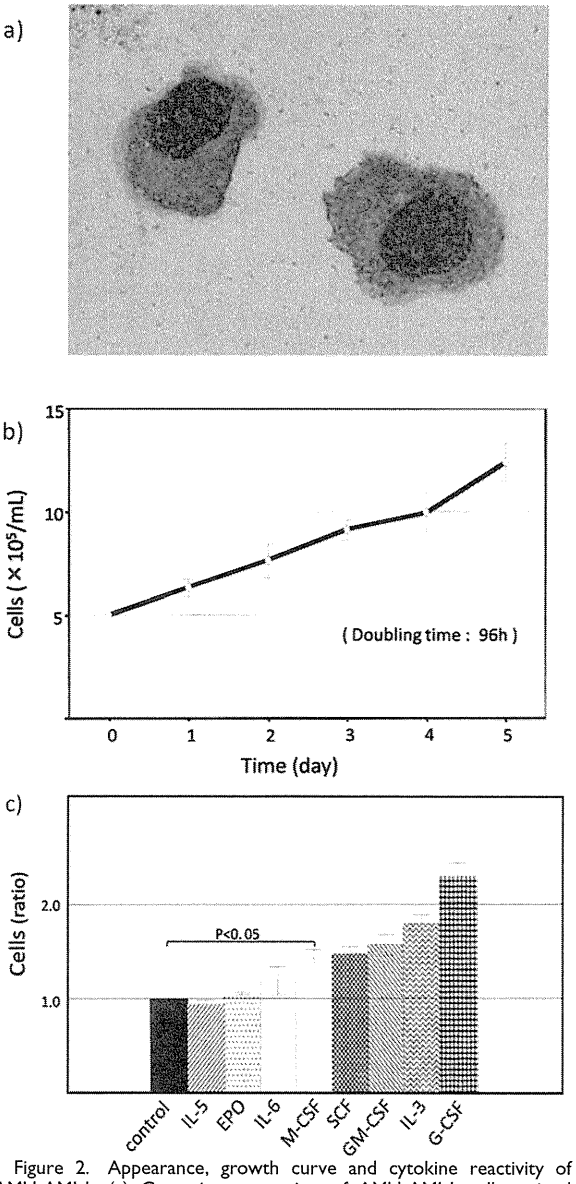


Figure 2. Appearance, growth curve and cytokine reactivity of AMU-AML1. (a) Cytospin preparation of AMU-AML1 cells stained with May-Grünwald-Giemsa (original magnification ×400). AMU-AML1 cells showed a blue cytoplasm and a round central nucleus with a reticular chromatin pattern in morphology. (b) Growth curve for AMU-AML1 cells. The doubling time of the cells was about 96 hr. (c) Cytokine reactivity of AMU-AML1 cells. The cells were counted after 96 hr of culture with the indicated cytokines in triplicate, and the ratio to control cells was calculated for each cytokine. AMU-AML1 cells responded significantly to M-CSF, SCF, GM-CSF, IL-3, and G-CSF but not to IL-5, EPO, and IL-6. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

BAC/PAC clones as FISH probes and array CGH (aCGH) analysis. The relationship between BAC/PAC contigs and genes surrounding chromosomal breakpoints in 12p13 and 22q11 is shown in Figure 1.

To examine the chromosomal breakpoint in band 12p13, we used BAC RP11-407P10, RP11-434C1 and RP11-96B19. RP11-407P10 and RP11-

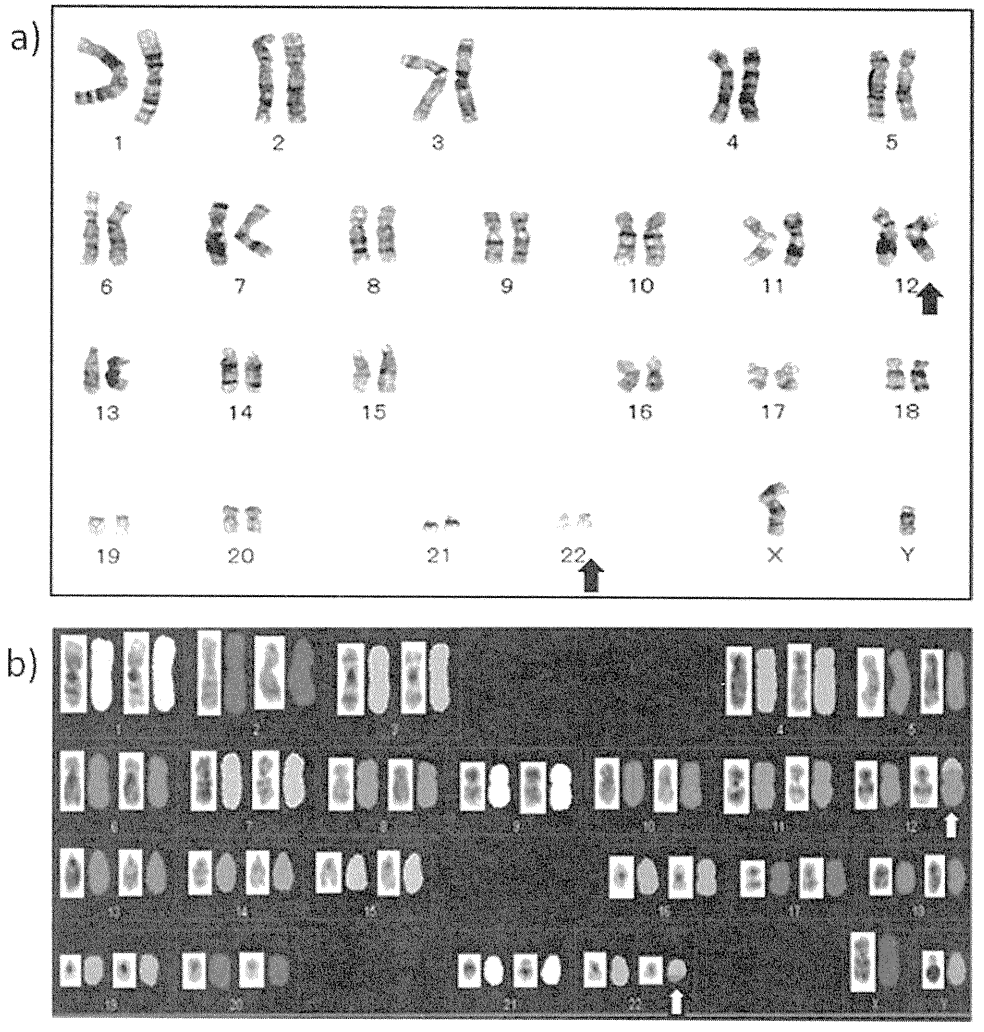


Figure 3. Karyotype analysis of AMU-AML1. The representative karyotype of AMU-AML1 cells is 46, XY, t(12;22)(p13;q11.2) detected by (a) G-banding combined with (b) SKY. The arrows show abnormal chromosomes t(12;22)(p13;q11.2). AMU-AML1 cells have a single structural abnormality, t(12;22)(p13;q11.2), as determined by cytogenetics.

96B19 gave a fusion signal on the normal chromosome 12p13. RP11-407P10 hybridized to der(22), and RP11-96B19 hybridized to der(12) (Fig. 4a). RP11-434C1 gave three signals that were on normal chromosome 12p13, der(12) and der(22) (data not shown). These results suggested that the chromosomal breakpoint in band 12p13 in AMU-AML1 cells existed in the sequences between RP11-407P10 and RP11-96B19 and also covered by RP11-434C1.

To examine the chromosomal breakpoint in band 22q11-12, we performed DC-FISH by using BAC RP11-46E17, PAC RP1-213J1, and PAC RP3-353E16. RP11-46E17 and RP3-353E16 gave a fusion signal on the normal chromosome arm 22q11-12. RP11-46E17 hybridized to der(12) and RP3-353E16 hybridized to der(22) (Fig. 4b). RP1-213J1 gave three signals on der(12), der(22), and normal chromosome 22q11-12 (data not shown). These results indicated that the chromosomal breakpoint in band 22q11-12 in the cell line existed in the sequences covered by RP1-213J1. The t(12;22)(p13;q11) in AMU-AML1 cells was identified by DC-FISH with locus-specific probes for *TEL* (RP11-96B19) and *MN1* (RP3-353E16). Both of these probes gave a fusion signal on der(12) (Figs. 4c and 4d).

In aCGH analysis of the region surrounding t(12;22), two regions were amplified to three copies in 12p13 (~700-kb long) and 22q11 (~200-kb long) (Fig. 5a). The amplicon in 12p13 contained all or part of *TEL*, and one end of the amplicon was within the sequence from the 5′ untranslated region to intron1 of *TEL* (Fig. 5b). The only

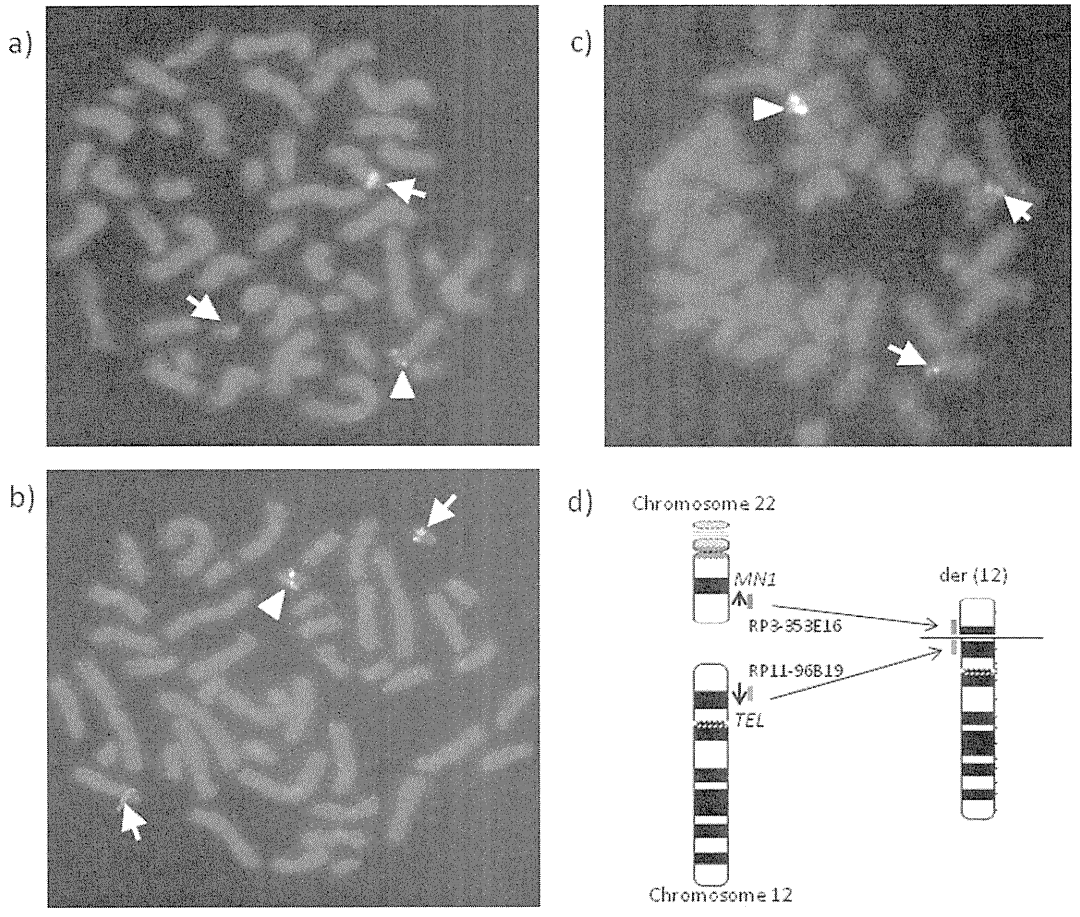


Figure 4. FISH pinpointed the breakpoints of t(12;22) (p13;q11) in AMU-AML1 cells. (a) RP11-407P10 (red) and RP11-96B19 (green) hybridized to der (22) and der (12), respectively (white arrows), while RP11-407P10 (red) and RP11-96B19 (green) gave a fusion signal on the normal chromosome 12 (white arrowhead). The chromosomal breakpoint in arm 12p13 was between RP11-407P10 (red) and RP11-96B19 (green). (b) RP3-353E16 (red) and RP11-46E17 (green) gave a fusion signal on the normal chromosome 22 (white arrowhead). RP3-353E16 (red) and RP11-46E17 (green) hybridized to der (12) and der (22), respectively (white arrows). The breakpoint in arm 22q11 was between RP3-353E16 (red) and RP11-46E17 (green). (c) RP3-353E16 (red) and RP11-96B19 (green) gave a fusion signal on der (12) (white arrowhead). White arrows show RP3-353E16 (red) on chromosome 22 and RP11-96B19 (green) on chromosome 12. (d) Schema of chromosome 12, 22 and der (22) in AMU-AML1 cells with FISH probes used in Figure 4c.

gene contained in the amplicon in 22q11 was *MN1* (Fig. 5c). Both ends of the amplicon in 22q11 were outside *MN1*. Our aCGH data suggested that an 800-kb region across the junction of t(12;22)(p13;q11) was amplified to three copies. Moreover, the breakpoint in 12p13 was in the 5′ untranslated region or within *TEL*, and the breakpoint in 22q11 was in the 3′ untranslated region of *MN1*. The t(12;22)(p13;q11) in AMU-AML1 cells was not completely reciprocal as determined by aCGH analysis.

To confirm the detection of the identification of *MN1* as the fusion partner gene of *TEL*, we attempted to perform 3′ RACE by using specific primers for *TEL* (TEL sense 2 and TEL sense 4, Table 1), but we could not obtain specific products containing the *TEL* sequence (data not shown).

Taken together, our results indicate that the breakpoint in 12p13 was within *TEL* or telomeric to 5′ *TEL* and that the breakpoint in 22q11 was in the centromeric 3′ untranslated region of *MN1* in the AMU-AML1 cell line, which is similar to the MUTZ-3 cell line (Table 2).

### Expression Analysis of Transcripts of *MN1-TEL* and *MN1* by RT-PCR

We performed RT-PCR analysis for the mRNA expression of *MN1-TEL* and *MN1* in AMU-AML1, UCSD/AML1, MUTZ-3, HL-60, and THP-1 cell lines. As previously reported

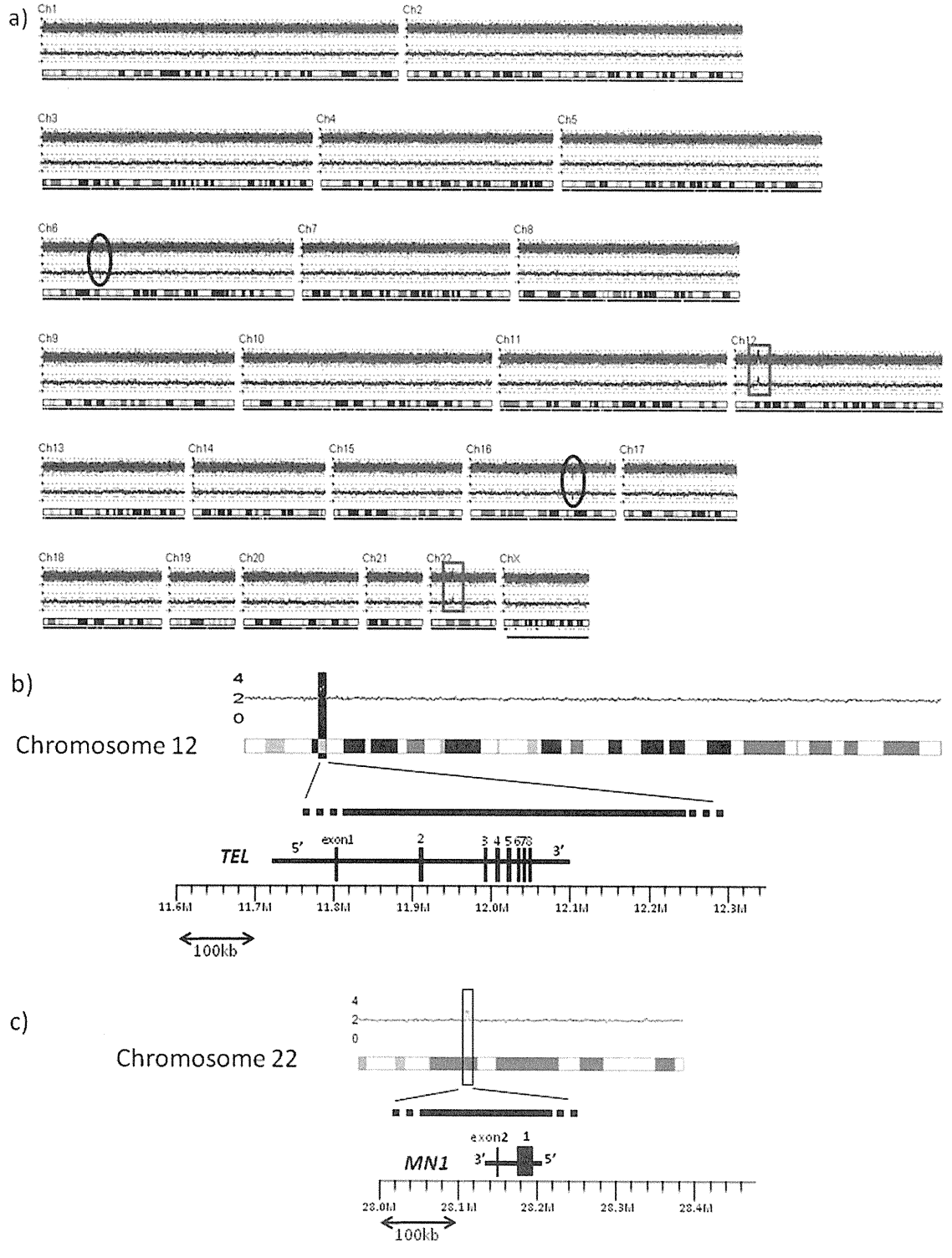


Figure 5. Array CGH analysis of AMU-AML1 cells. (a) Genome-wide copy number aberrations in AMU-AML1 cells determined by Affymetrix SNP array. Two regions surrounding the breakpoint at t(12;22) were amplified to three copies (red rectangles), and two regions at chromosome band 6p21 and 16q22 were deleted to one copy (black ovals). These regions correspond to copy number variation loci. (b,c) Copy number aberrations on chromosomes 12 and 22 and the genomic structure of *TEL* and *MN1*. (b) The amplicon at chromosome 12p13 was the region from ~11,700 kb to 12,400 kb containing all or part of *TEL*. The telomeric end of the amplicon was in the sequence from the 5′ untranslated region to intron 1 of *TEL*. (c) The amplicon at chromosome 22 was in the region from ~26,350 kb to 26,600 kb containing the entire *MN1*. The measurements indicate the distance from the telomeric tip on the short arm of each chromosome. M; mega bases.