

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 en-

hancement of ERK phosphorylation and C/EBP α 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/EBP α , an important tumor suppressor for AML, and we observed that degradation of C/EBP α 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/EBP α 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 $148 \times 10^9/L$, including 87% myeloblasts, a hemoglobin level of 19.4 g/dL, and a platelet count of $259 \times 10^9/L$. 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 $6.9 \times 10^9/L$ 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.

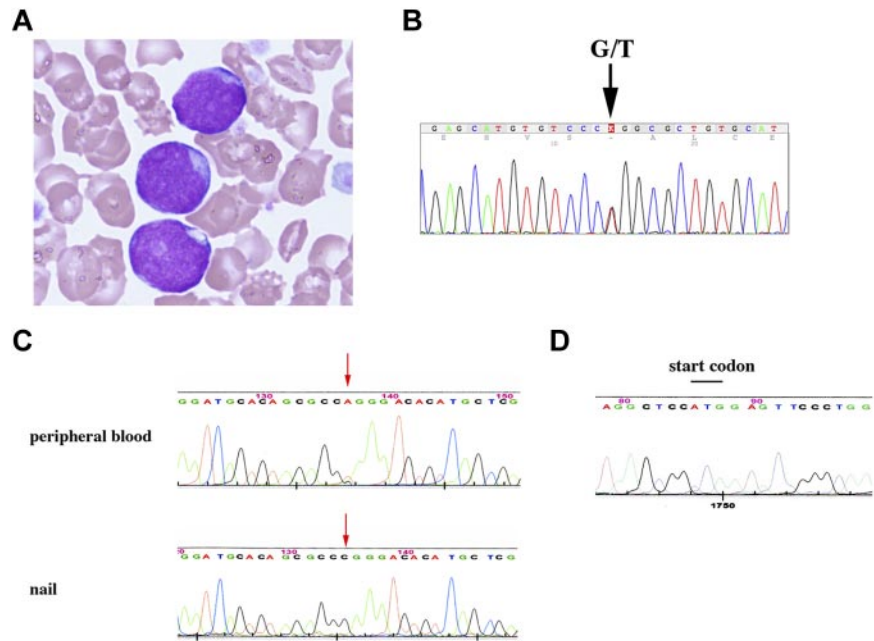
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Figure 1. *TRIB1* R107L mutation identified in DS-related leukemias. (A) Giemsa staining of the case 84 peripheral blood smear diagnosed as AMKL. The image was acquired using a BX40 microscope equipped with a 100×/1.30 NA oil objective (Olympus) and 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 AMKL⁷ 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/EBP α (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

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 pseudokinase 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

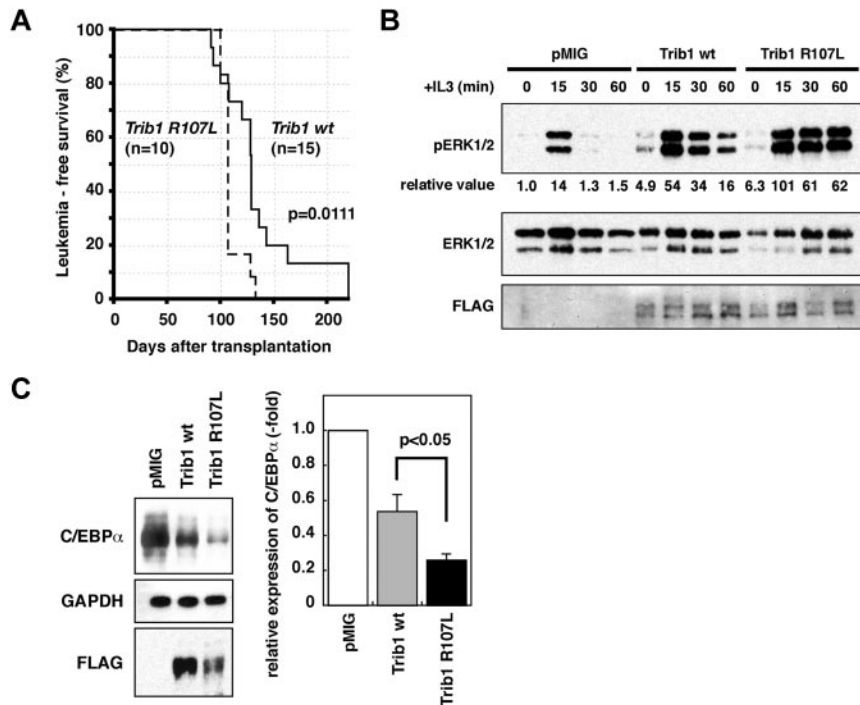


Figure 2. AML development by bone marrow transfer using *Trib1* WT and R107L. (A) Kaplan-Meier survival curves are shown. The *P* value was calculated with the log-rank test. (B) Immunoblot analysis of *Trib1* WT AML (Mac-1 56.2%, Gr-1 52.5%, CD34^{lo}, c-kit⁻, Sca-1⁻) and R107L AML (Mac-1 41.4%, Gr-1 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/EBP α of the same AML samples as in panel B. Relative expression level of C/EBP α 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/EBP α 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.

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References

- Shimizu R, Engel JD, Yamamoto M. GATA1-related leukaemias. *Nat Rev Cancer*. 2008;8(4):279-287.
- Jin G, Yamazaki Y, Takuwa M, et al. Trib1 and Evi1 cooperate with Hoxa and Meis1 in myeloid leukemogenesis. *Blood*. 2007;109(9):3998-4005.
- Kiss-Toth E, Bagstaff SM, Sung HY, et al. Human Tribbles, a protein family controlling mitogen-activated protein kinase cascades. *J Biol Chem*. 2004;279(41):42703-42708.
- Yokoyama T, Kanno Y, Yamazaki Y, et al. Trib1 links the MEK/ERK pathway in myeloid

- leukemogenesis. *Blood*. 2010;116(15):2768-2775.
5. Keeshan K, He Y, Wouters BJ, et al. Tribbles homolog 2 inactivates C/EBPalpha and causes acute myelogenous leukemia. *Cancer Cell*. 2006;10(5):401-411.
 6. Storlazzi CT, Fioretos T, Surace C, et al. MYC-containing double minutes in hematologic malignancies: evidence in favor of the episome model and exclusion of MYC as the target gene. *Hum Mol Genet*. 2006;15(6):933-942.
 7. Kanezaki R, Toki T, Terui K, et al. Down syndrome and GATA1 mutations in transient abnormal myeloproliferative disorder: mutation classes correlate with progression to myeloid leukemia. *Blood*. 2010;116(22):4631-4638.
 8. Kogan SC, Ward JM, Anver MR, et al. Bethesda proposal for classification of nonlymphoid hematopoietic neoplasms in mice. *Blood*. 2002;100(1):238-245.
 9. Toki T, Kanezaki R, Adachi S, et al. The key role of stem cell factor/KIT signaling in the proliferation of blast cells from Down syndrome-related leukemia. *Leukemia*. 2009;23(1):95-103.
 10. Hegedus Z, Czibula A, Kiss-Toth E. Tribbles: a family of kinase-like proteins with potent signaling regulatory function. *Cell Signal*. 2007;19(2):238-250.
 11. Yokoyama T, Nakamura T. Tribbles in disease: signaling pathways important for cellular function and neoplastic transformation. *Cancer Sci*. 2011;102(6):1115-1122.
 12. Dedhia PH, Keeshan K, Uljon S, et al. Differential ability of Tribbles family members to promote degradation of C/EBPalpha and induce acute myelogenous leukemia. *Blood*. 2010;116(8):1321-1328.