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.

Norio Shiba[1,2]
Myoung-Ja Park[1]
Tomohiko Taki[3]
Junko Takita[4]
Mitsuteru Hiwatari[4]
Takashi Kanazawa[2]
Manabu Sotomatsu[1]
Eiichi Ishii[5]
Hirokazu Arakawa[2]
Seishi Ogawa[6]
Yasuhide Hayashi[1]

[1]Department of Haematology/Oncology, Gunma Children's Medical Centre, [2]Department of Paediatrics, Gunma University Graduate School of Medicine, Gunma, [3]Department of Molecular Diagnostics and Therapeutics, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kyoto, [4]Department of Paediatrics, Graduate School of Medicine, University of Tokyo, Tokyo, [5]Department of Paediatrics, Ehime University Graduate School of Medicine, Ehime, and [6]Cancer Genomics Project, Graduate School of Medicine, University of Tokyo, Tokyo, Japan.
E-mail: hayashiy-tky@umin.ac.jp

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References

Biondi, A., Cimino, G., Pieters, R. & Pui, C.H. (2000) Biological and therapeutic aspects of infant leukemia. *Blood*, **96**, 24–33.

Caligiuri, M.A., Briesewitz, R., Yu, J., Wang, L., Wei, M., Arnoczky, K.J., Marburger, T.B., Wen, J., Perrotti, D., Bloomfield, C.D. & Whitman, S.P. (2007) Novel c-CBL and CBL-b ubiquitin ligase mutations in human acute myeloid leukemia. *Blood*, **110**, 1022–1024.

Fu, J.F., Hsu, J.J., Tang, T.C. & Shih, L.Y. (2003) Identification of CBL, a protooncogene at 11q23.3, as a novel MLL fusion partner in a patient with de novo acute myeloid leukemia. *Genes Chromosomes Cancer*, **37**, 214–219.

Isoyama, K., Eguchi, M., Hibi, S., Kinukawa, N., Ohkawa, H., Kawasaki, H., Kosaka, Y., Oda, T., Oda, M., Okamura, T., Nishimura, S., Hayashi, Y., Mori, T., Imaizumi, M., Mizutani, S., Tsukimoto, I., Kamada, N. & Ishii, E. (2002) Risk-directed treatment of infant acute lymphoblastic leukaemia based on early assessment of MLL gene status: results of the Japan Infant Leukaemia Study (MLL96). *British Journal of Haematology*, **118**, 999–1010.

Kosaka, Y., Koh, K., Kinukawa, N., Wakazono, Y., Isoyama, K., Oda, T., Hayashi, Y., Ohta, S., Moritake, H., Oda, M., Nagatoshi, Y., Kigasawa, H., Ishida, Y., Ohara, A., Hanada, R., Sako, M., Sato, T., Mizutani, S., Horibe, K. & Ishii, E. (2004) Infant acute lymphoblastic leukemia with MLL gene rearrangements: outcome following intensive chemotherapy and hematopoietic stem cell transplantation. *Blood*, **104**, 3527–3534.

Niemeyer, C.M., Kang, M.W., Shin, D.H., Furlan, I., Erlacher, M., Bunin, N.J., Bunda, S., Finklestein, J.Z., Sakamoto, K.M., Gorr, T.A., Mehta, P., Schmid, I., Kropshofer, G., Corbacioglu, S., Lang, P.J., Klein, C., Schlegel, P.G., Heinzmann, A., Schneider, M., Starý, J., van, den., Heuvel-Eibrink, M.M., Hasle, H., Locatelli, F., Sakai, D., Archambeault, S., Chen, L., Russell, R.C., Sybingco, S.S., Ohh, M., Braun, B.S., Flotho, C. & Loh, M.L. (2010) Germline CBL mutations cause developmental abnormalities and predispose to juvenile myelomonocytic leukemia. *Nature Genetics*, **42**, 794–800.

Sanada, M., Suzuki, T., Shih, L.Y., Otsu, M., Kato, M., Yamazaki, S., Tamura, A., Honda, H., Sakata-Yanagimoto, M., Kumano, K., Oda, H., Yamagata, T., Takita, J., Gotoh, N., Nakazaki, K., Kawamata, N., Onodera, M., Nobuyoshi, M.,

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Hayashi, Y., Harada, H., Kurokawa, M., Chiba, S., Mori, H., Ozawa, K., Omine, M., Hirai, H., Nakauchi, H., Koeffler, H.P. & Ogawa, S. (2009) Gain-of-function of mutated C-CBL tumour suppressor in myeloid neoplasms. *Nature*, **460**, 904–908.

Shiba, N., Kato, M., Park, M.J., Sanada, M., Ito, E., Fukushima, K., Sako, M., Arakawa, H., Ogawa, S. & Hayashi, Y.. (2010) CBL mutations in juvenile myelomonocytic leukemia and pediatric myelodysplastic syndrome. *Leukemia*, **24**, 1090–1092.

Shiba, N., Taki, T., Park, M.J., Nagasawa, M., Kanazawa, T., Takita, J., Ohnishi, H., Sotomatsu, M., Arakawa, H. & Hayashi, Y. (2011) CBL mutation in childhood therapy-related leukemia. *Leukemia*, **25**, 1356–1358.

Taki, T., Ida, K., Bessho, F., Hanada, R., Kikuchi, A., Yamamoto, K., Sako, M., Tsuchida, M., Seto, M., Ueda, R. & Hayashi, Y. (1996) Frequency and clinical significance of the MLL gene rearrangements in infant acute leukemia. *Leukemia*, **10**, 1303–1307.

Association of an increased frequency of $CD14^{+}HLA\text{-}DR^{lo/neg}$ monocytes with decreased time to progression in chronic lymphocytic leukaemia (CLL)

Clinically important immune dysregulation is an early feature of leukaemia/small lymphocytic lymphoma (CLL) that often precedes other clinical manifestations of this disease. Defects in the adaptive immune system in patients with CLL results in quantitative and qualitative abnormalities in antibody production, and profound changes in T and Natural Killer (NK) cell numbers, ratios, and function (Palmer *et al*, 2008; Gonzalez-Rodriguez *et al*, 2010). Monocytes and macrophages, critical for adaptive and innate immune responses, also have an important role in the function of the CLL cell microenvironment, and this relationship continues to be explored (Caligaris-Cappio, 2011). However, little is known about the effects of CLL on monocyte/macrophage physiology and whether alterations in monocytes have any clinical role in CLL. We were especially interested in the possible role of immunosuppressive CD14+ monocytes with reduced HLA-DR expression in CLL as we have observed this phenomena in glioblastoma (Gustafson *et al*, 2010), non-Hodgkin lymphoma (Lin *et al*, 2011) and prostate cancer (Vuk-Pavlovic *et al*, 2010).

To identify potential monocyte alterations by CLL in patients, we performed flow cytometric analysis of peripheral

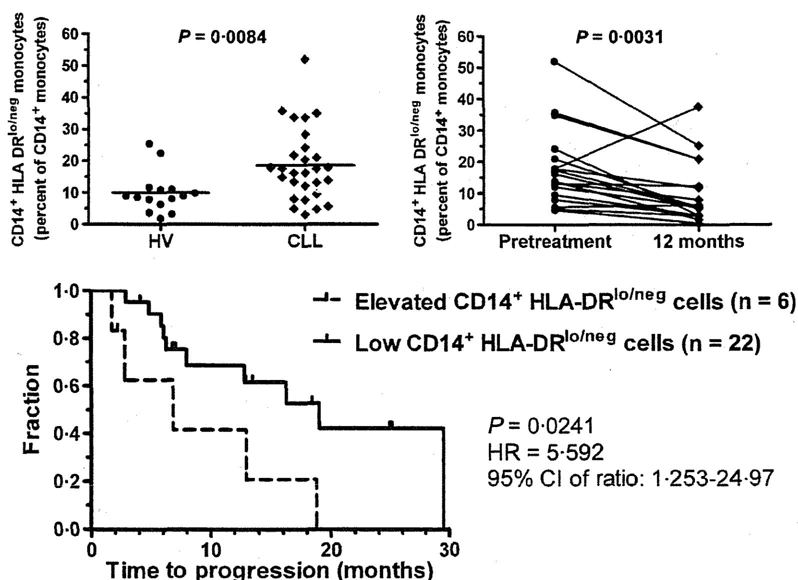

Fig 1. $CD14^{+}HLA\text{-}DR^{lo/neg}$ Monocytes in CLL. Blood from 29 CLL patients and 15 healthy volunteers were used for immune phenotyping. There was no age difference (median of 59 vs. 58 years, respectively; $P = 0{\cdot}2896$). Patients were eligible for the clinical trial (ClinicalTrials.gov NCT00562328) if diagnosed with previously untreated high-risk CLL using standard criteria and did not meet guidelines for conventional treatment. Blood was collected before initiation of treatment and 6, 9, and 12 months after completion of treatment in patients who had a sustained response. The percentage of CD14+ cells with a loss of HLA-DR staining was determined and compared between CLL patients and healthy volunteers (HV; upper left). CLL patients with a sustained response to treatment had a decrease in the frequency of $CD14^{+}HLA\text{-}DR^{lo/neg}$ monocytes 12 months after completion of treatment compared to measurement prior to treatment (upper right). Kaplan–Meyer survival curve comparing CLL patients with elevated ratios (>2·5 standard deviations) of $CD14^{+}HLA\text{-}DR^{lo/neg}$ monocytes when compared to healthy volunteers (dashed line) or with ratios similar to those seen in healthy volunteers (solid line; bottom panel). HR, Hazard Ratio; 95% CI, 95% confidence interval.

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LETTERS TO THE EDITOR

Novel splicing-factor mutations in juvenile myelomonocytic leukemia

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Myelodysplastic syndromes (MDS) and myelodysplastic/myeloproliferative neoplasms (MDS/MPN) are heterogeneous groups of chronic myeloid neoplasms characterized by clonal hematopoiesis, varying degrees of cytopenia or myeloproliferative features with evidence of myelodysplasia and a propensity to acute myeloid leukemia (AML).[1] In recent years, a number of novel gene mutations, involving *TET2*, *ASXL1*, *DNMT3A*, *EZH2*, *IDH1/2*, and *c-CBL*, have been identified in adult cases of chronic myeloid neoplasms, which have contributed to our understanding of disease pathogenesis.[2–7] However, these mutations are rare in pediatric cases, with the exception of germline or somatic *c-CBL* mutations found in 10–15% of chronic myelomonocytic leukemia (CMML) and juvenile myelomonocytic leukemia (JMML),[8] highlighting the distinct pathogenesis of adult and pediatric neoplasms.[9]

Recently, we reported high frequencies of mutations, involving the RNA splicing machinery, that are largely specific to myeloid neoplasms, showing evidence of myeloid dysplasia in adult.[10] Affecting a total of eight components of the RNA splicing machinery (U2AF35, U2AF65, SF3A1, SF3B1, SRSF2, ZRSR2, SF1 and PRPF40B) commonly involved in the 3′ splice-site (3′SS) recognition, these pathway mutations are now implicated in the pathogenesis of myelodysplasia.[10] To investigate the role of the splicing-pathway mutations in the pathogenesis of pediatric myeloid malignancies, we have examined 165 pediatric cases with AML, MDS, chronic myeloid leukemia (CML) and JMML for mutations in the four major splicing factors, *U2AF35*, *ZRSR2*, *SRSF2*, and *SF3B1*, commonly mutated in adult cases.

Bone marrow or peripheral blood tumor specimens were obtained from 165 pediatric patients with various myeloid malignancies, including *de novo* AML ($n = 93$), MDS ($n = 28$), CML ($n = 17$) and JMML ($n = 27$), and the genomic DNA (gDNA) was subjected to mutation analysis (Supplementary Table 1). The status of the RAS pathway mutations for the current JMML series has been reported previously (Supplementary Table 2).[11,12] Nineteen leukemia cell lines derived from AML (YNH-1, ML-1, KASUMI-3, KG-1, HL60, inv-3, SN-1, NB4 and HEL), acute monocytic leukemia (THP-1, SCC-3, J-111, CTS, P31/FUJ, MOLM-13, IMS/MI and KOCL-48) and acute megakaryoblastic leukemia (CMS and CMY) were also analyzed for mutations. Peripheral blood gDNA from 60 healthy adult volunteers was used as controls. Informed consent was obtained from the patients and/or their parents and from the healthy volunteers. We previously showed that for *U2AF35*, *SRSF2* and *SF3B1*, most of the mutations in adult cases were observed in exons 2 and 7, exon 1, and exons 14 and 15, respectively.[10] Therefore, we confirmed mutation screening to these 'hot-spot' exons. In contrast, all the coding exons were examined for *ZRSR2*, because no mutational hot spots have been detected. Briefly, the relevant exons were amplified using PCR and mutations were examined by Sanger sequencing, as previously described.[10] The Fisher's exact test was used to evaluate the statistical significance of frequencies of mutations for *U2AF35*, *SF3B1*, *ZRSR2* or *SRSF2* in adult cases and pediatric cases. This study was approved by the Ethics Committee of the University of Tokyo (Approval number 948-7).

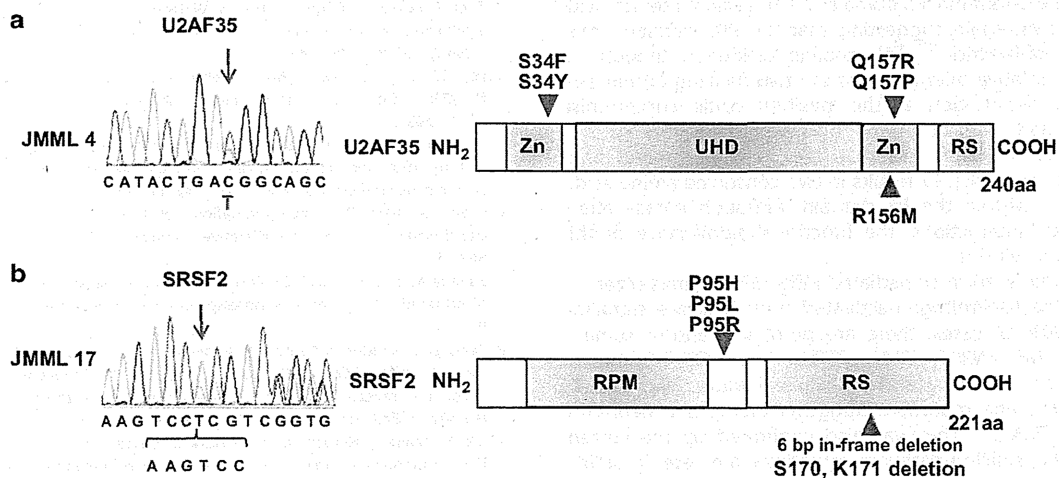

Figure 1. Novel *U2AF35*and *SRSF2* mutations detected in JMML cases. (**a**) Left panel: sequence chromatogram of a heterozygous mutation at R156 in N-terminal zinc-finger motifs of *U2AF35* detected in a JMML case (JMML 4) is shown. Mutated nucleotides are indicated by arrows. Right panel: illustration of functional domains and mutations of U2AF35. Red arrow heads indicate hot-spot mutations at S34 and Q157 detected in the adult cases.[10] Blue arrow head indicates the missense mutation at R156. (**b**) Left panel: sequence chromatogram of a 6-bp in-frame deletion (c.518-523delAAGTCC) in *SRSF2* detected in JMML 17 is shown. Mutated nucleotides are indicated by arrows. Right panel: illustration of functional domains and mutations of SRSF2. Red arrow head indicates hot-spot mutation at P95 frequently detected in the adult cases.[10] Blue arrow head indicates a 6-bp in-frame deletion leading to deletion of S170 and K171.

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No mutations were identified in the 28 cases with pediatric MDS, which included 13 cases with refractory anemia with excess blasts, 5 with refractory cytopenia of childhood, 2 with Down syndrome-related MDS, 2 with Fanconi anemia-related MDS, 2 with secondary MDS and 4 with unclassified MDS. Similarly, no mutations were detected in 93 cases with *de novo* AML or in 17 with CML, as well as 19 leukemia-derived cell lines. Our previous study in adult patients showed the frequency of mutations in *U2AF35, SF3B1, ZRSR2* or *SRSF2* to be 60/155 cases with MDS without increased ring sideroblasts and 8/151 *de novo* AML patients, emphasizing the rarity of these mutations in pediatric MDS ($P<5.0\times10^{-6}$) and AML ($P<0.02$) compared with adult cases. We found mutations in two JMML cases, JMML 4 and JMML 17. JMML 4 carried a heterozygous *U2AF35* mutation (R156M), whereas JMML 17 had a 6-bp in-frame deletion (c.518-523delAAGTCC) in *SRSF2* that resulted in deletion of amino acids S170 and K171 (Figure 1). Both nucleotide changes found in *U2AF35* and *SRSF2* were neither identified in the 60 healthy volunteers nor registered in the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) or in the 1000 genomes project, indicating that they represent novel spliceosome mutations in pediatric cases.

U2AF35 is the small subunit of the U2 auxiliary factor (U2AF), which binds an AG dinucleotide at the 3′SS, and has an essential role in RNA splicing.[13] With the exception of a single A26V mutation found in a case of refractory cytopenia with multilinage dysplasia, all the U2AF35 mutations reported in adult myeloid malignancies involved one of the two hot spots within the two zinc-finger domains, S34 and Q157, which are highly conserved across species, suggesting the gain-of-function mutations.[10] In JMML 4, the R156M U2AF35 mutation affects a conserved amino acid adjacent to Q157, suggesting it may also be a gain-of-function mutation, leading to aberrant pre-mRNA splicing possibly in a dominant fashion.

SRSF2, better known as SC35, is a member of the serine/arginine-rich (SR) family of proteins.[14] SRSF2 binds to a splicing-enhancer element in pre-mRNA and has a crucial role not only in constitutive and alternative pre-mRNA splicing but also in transcription elongation and genomic stability.[14] All mutations thus far identified in adult cases exclusively involved P95 within the intervening sequence between the N-terminal RNA-binding domain and the C-terminal RS domain.[10] This region interacts with other SR proteins, again suggesting that the P95 mutation may result in gain-of-function.[10] This proline residue is thought to determine the relative orientation of the two flanking domains of SRSF2, and a substitution at this position could compromise critical interactions with other splicing factors necessary for RNA splicing to take place. In contrast, the newly identified 6-bp in-frame deletion in JMLL 17 results in two conserved amino acids, S170 and K171, within the RS domain. Although it may affect protein–protein interactions, the functional significance of this deletion remains elusive.

JMML is a unique form of pediatric MDS/MPN characterized by activation of the RAS/mitogen-activated protein kinase signaling pathway; in 90% of cases, there are germ line and/or somatic mutations of *NF1, NRAS, KRAS, PTPN11* and *CBL*.[8] Although JMML shares some clinical and molecular features with CMML, its spectrum of gene mutations suggests that it is a neoplasm distinct from CMML.[15] This was also confirmed by the current results that the splicing-pathway mutations are rare in JMML, whereas they are extremely frequent (~60%) in CMML.[10] Although the two JMML cases carrying the splicing-pathway mutations had no known RAS-pathway mutations, both the pathway mutations frequently coexisted in CMML.[8]

To summarize, no mutations of *SF3B1, U2AF35, ZRSR2* or *SRSF2* are found in pediatric MDS and AML. In our study, except for *ZRSR2*, mutations were examined focusing on the reported hot spots in adult studies, raising a possibility that we may have missed some mutations occurring in other regions. However, these hot spots represent evolutionally conserved amino acids and have functional relevance, it is unlikely that the distribution of hot spots in children significantly differs from adult cases and as such, we could safely conclude that mutations of *SF3B1, U2AF35, ZRSR2* and *SRSF2* are rare in myeloid neoplasms in children. Finally, mutations of *U2AF35* and *SRSF2* may have some role in the pathogenesis of JMML, although further evaluations are required.

CONFLICT OF INTEREST

The authors declare no conflict interest.

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J Takita[1,2], K Yoshida[3], M Sanada[3], R Nishimura[1], J Okubo[1], A Motomura[1], M Hiwatari[1], K Oki[1], T Igarashi[1], Y Hayashi[4] and S Ogawa[3]
[1]Department of Pediatrics, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan;
[2]Department of Cell Therapy and Transplantation Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan;
[3]Cancer Genomics Project, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan and
[4]Gunma Children's Medical Center, Gunma, Japan
E-mail: sogawa-tky@umin.ac.jp

REFERENCES

1 Garcia-Manero G. Myelodysplastic syndromes: 2011 update on diagnosis, risk-stratification, and management. *Am J Hematol* 2011; **86**: 490–498.
2 Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, Masse A *et al.* Mutation in TET2 in myeloid cancers. *N Engl J Med* 2009; **360**: 2289–2301.
3 Thol F, Friesen I, Damm F, Yun H, Weissinger EM, Krauter J *et al.* Prognostic significance of ASXL1 mutations in patients with myelodysplastic syndromes. *J Clin Oncol* 2011; **29**: 2499–2506.
4 Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE *et al.* DNMT3A mutations in acute myeloid leukemia. *N Engl J Med* 2010; **363**: 2424–2433.
5 Nikoloski G, Langemeijer SM, Kuiper RP, Knops R, Massop M, Tonnissen ER *et al.* Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes. *Nature Genet* 2010; **42**: 665–667.
6 Green A, Beer P. Somatic mutations of IDH1 and IDH2 in the leukemic transformation of myeloproliferative neoplasms. *N Engl J Med* 2010; **362**: 369–370.
7 Sanada M, Suzuki T, Shih LY, Otsu M, Kato M, Yamazaki S *et al.* Gain-of-function of mutated C-CBL tumour suppressor in myeloid neoplasms. *Nature* 2009; **460**: 904–908.
8 Perez B, Kosmider O, Cassinat B, Renneville A, Lachenaud J, Kaltenbach S *et al.* Genetic typing of CBL, ASXL1, RUNX1, TET2 and JAK2 in juvenile myelomonocytic leukaemia reveals a genetic profile distinct from chronic myelomonocytic leukaemia. *Br J Haematol* 2010; **151**: 460–468.
9 Oki K, Takita J, Hiwatari M, Nishimura R, Sanada M, Okubo J *et al.* IDH1 and IDH2 mutations are rare in pediatric myeloid malignancies. *Leukemia* 2011; **25**: 382–384.
10 Yoshida K, Sanada M, Shiraishi Y, Nowak D, Nagata Y, Yamamoto R *et al.* Frequent pathway mutations of splicing machinery in myelodysplasia. *Nature* 2011; **478**: 64–69.
11 Chen Y, Takita J, Hiwatari M, Igarashi T, Hanada R, Kikuchi A *et al.* Mutations of the PTPN11 and RAS genes in rhabdomyosarcoma and pediatric hematological malignancies. *Genes Chromosomes Cancer* 2006; **45**: 583–591.
12 Shiba N, Kato M, Park MJ, Sanada M, Ito E, Fukushima K *et al.* CBL mutations in juvenile myelomonocytic leukemia and pediatric myelodysplastic syndrome. *Leukemia* 2010; **24**: 1090–1092.

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Brief report

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

Takashi Yokoyama,[1] Tsutomu Toki,[2] Yoshihiro Aoki,[2] Rika Kanezaki,[2] Myoung-ja Park,[3] Yohei Kanno,[1] Tomoko Takahara,[1] Yukari Yamazaki,[1] Etsuro Ito,[2] Yasuhide Hayashi,[3] and Takuro Nakamura[1]

[1]Division of Carcinogenesis, Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan; [2]Department of Pediatrics, Hirosaki University Graduate School of Medicine, Hirosaki, Japan; and [3]Department of Hematology/Oncology, Gunma Children's Medical Center, Gunma, Japan

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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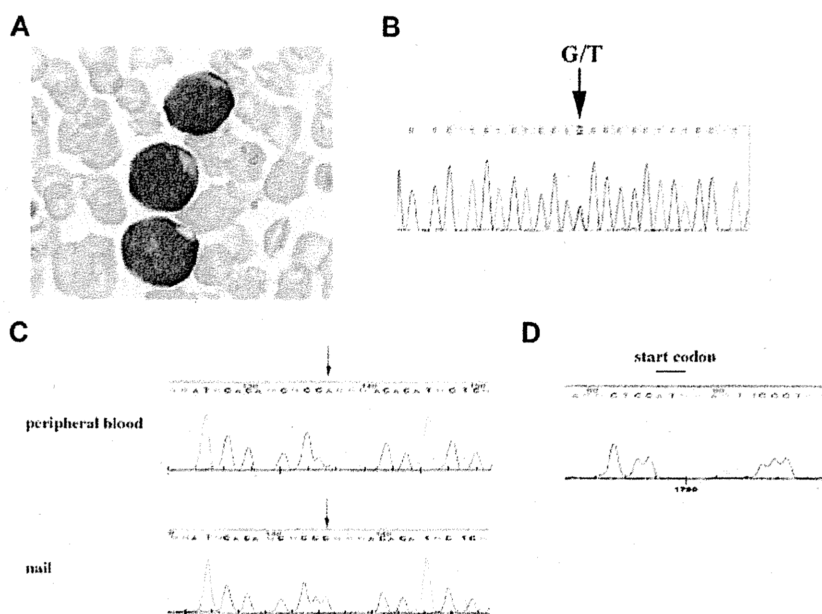

Figure 1. *TRIB1* R107L mutation identified in DS-related leukemias. (A) Giemsa staining of the case 84 peripheral blood smear diagnosed as AMKL. The image was acquired using a BX40 microscope equipped with a 100×/1.30 NA oil objective (Olympus) and a C-4040 digital camera (Olympus). (B) Fluorescent 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

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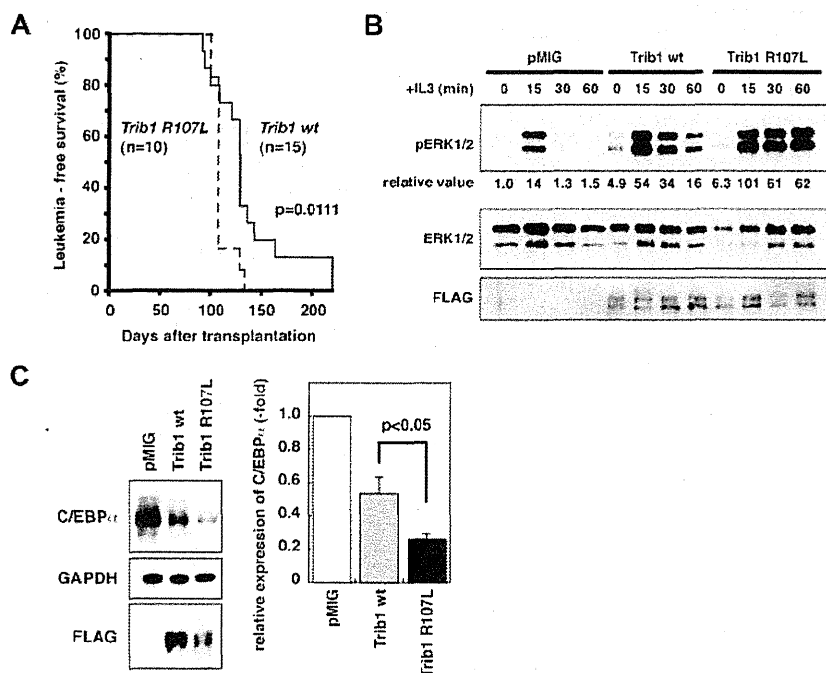

Figure 2. AML development by bone marrow transfer using *Trib1* WT and R107L. (A) Kaplan-Meier survival curves are shown. The *P* value was calculated with the log-rank test. (B) Immunoblot analysis of *Trib1* WT AML (Mac-1 56.2%, Gr-1 52.5%, CD34^{lo}, c-kit^{-}, Sca-1^{-}) and R107L AML (Mac-1 41.4%,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

Correspondence: Takuro Nakamura, Division of Carcinogenesis, Cancer Institute, Japanese Foundation for Cancer Research, 3-8-31 Ariake, Koto-ku, Tokyo 135-8550, Japan; e-mail: takuro-ind@umin.net.

References

1. Shimizu R, Engel JD, Yamamoto M. GATA1-related leukaemias. *Nat Rev Cancer.* 2008;8(4):279-287.
2. 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.
3. 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.
4. Yokoyama T, Kanno Y, Yamazaki Y, et al. Trib1 links the MEK/ERK pathway in myeloid

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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 mye loid 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.

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CASE REPORT

Childhood acute myeloid leukemia with bone marrow eosinophilia caused by t(16;21)(q24;q22)

Nozomu Kawashima · Akira Shimada · Takeshi Taketani · Yasuhide Hayashi · Nao Yoshida · Kimikazu Matsumoto · Yoshiyuki Takahashi · Seiji Kojima · Koji Kato

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Abstract Acute myeloid leukemia with abnormal bone marrow eosinophilia (AML-M4Eo) is often reported in core binding factor (CBF) leukemia, with translocations such as inv(16)(p13q22), t(16;16)(p13;q22) or t(8;21)(q22;q22); however, it is rarely reported with t(16;21)(q24;q22), which produces the *RUNX1-CBFA2T3* (*AML1-MTG16*) chimera. The similarity between this chimera and *RUNX1-RUNXT1* (*AML1-MTG8*) by t(8;21)(q22;q22) remains controversial. Adult leukemia with t(16;21)(q24;q22) was primarily therapy related, and shows poor prognosis; however, pediatric AML with this translocation was quite rare and tended to be de novo AML. We present here a 4-year-old boy with de novo AML-M4Eo and t(16;21)(q24;q22). He received chemotherapy and survived for more than 70 months without transplantation. We speculated that pediatric AML with t(16;21)(q24;q22) showed favorable prognosis, as with t(8;21)(q22;q22).

N. Kawashima (✉) · N. Yoshida · K. Matsumoto · K. Kato (✉)
Division of Hematology/Oncology, Children's Medical Center, Japanese Red Cross Nagoya First Hospital, 3-35, Michishita-cho, Nakamura-ku, Nagoya, Aichi 453-8511, Japan
e-mail: kwnozomu@med.nagoya-u.ac.jp

K. Kato
e-mail: kokato@nagoya-1st.jrc.or.jp

A. Shimada · Y. Takahashi · S. Kojima
Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya, Japan

T. Taketani
Division of Blood Transfusion, Shimane University Hospital, Izumo, Japan

Y. Hayashi
Department of Hematology/Oncology, Gunma Children's Medical Center, Shibukawa, Japan

Keywords AML · Pediatric · Eosinophilia · t(16;21)(q24;q22) · RUNX1-CBFA2T3

Introduction

Chromosomal translocations are frequently associated with hematological malignancies and result in specific gene mutations that affect the clinical outcome. Acute myeloid leukemia with abnormal bone marrow eosinophilia (AML-M4Eo) is highly correlated with chromosomal abnormalities such as inv(16)(p13q22), t(16;16)(p13;q22) or t(8;21) that are involved in the core binding factor (CBF)-AML [1, 2]. Patients with AML-M4Eo which has inv(16)(p13q22) or t(16;16)(p13;q22) generally have a high response rate to chemotherapy and favorable clinical outcome compared to other types of AML [3, 4]. In comparison, t(16;21)(q24;q22) was rarely reported in 22 adult cases and 4 pediatric cases with AML-M1, M2 or M4Eo [5–8].

The t(16;21)(q24;q22) translocation results in *RUNX1-CBFA2T3* (also known as *AML1-MTG16*) fusion gene; however, its contribution to the clinical outcome is not completely established. Adult cases of acute leukemia with t(16;21)(q24;q22) were mostly therapy related [9], whereas pediatric cases with t(16;21)(q24;q22) were mostly de novo AML with better prognosis [8]. Interestingly, the majority of pediatric cases had the characteristic of eosinophilia either in the peripheral blood or in the bone marrow cells [8], although its impact on prognosis remains unknown.

We report here a 4-year-old boy having a diagnosis of AML-M4Eo with t(16;21)(q24;q22) which resulted in the *RUNX1-CBFA2T3* chimera. He achieved complete remission after induction chemotherapy and was disease free for more than 70 months. We will discuss *RUNX1-CBFA2T3* chimera compared with *RUNX1-RUNXT1* chimera in childhood AML.

Materials and methods

Case report

A 4-year-old Japanese boy with a diagnosis of acute myeloid leukemia with abnormal bone marrow eosinophilia (AML-M4Eo) according to the French–American–British (FAB) criteria presented with fever and purpura. On admission, his peripheral white blood cell count was 43,100/mm^3 (blast cells 37 %). His bone marrow aspirate revealed a nucleated cell count of 576,000/mm^3 comprised 63 % of myeloid blasts and 32 % of atypical eosinophilic granulocytes (Fig. 1a). The immunophenotype was positive for CD13, 14, 33, 34 and HLA-DR, and negative for CD3, 5, 7, 10, 19, 20 and 56. The karyotype of bone marrow cells (20 metaphases) by G-banding was 46,XY,inv(3)(p23q26),del(7)(q?),t(16;21)(q24;q22) [20] (Fig. 1b). He had no known type I mutations of the blast and FLT3-internal tandem duplication was not detected. *RUNX1-CBFA2T3* fusion gene was detected by reverse transcription polymerase chain reaction (RT-PCR) as described later. He underwent standard chemotherapy according to AML99 protocol [10]. After the induction therapy, he achieved hematological and molecular complete remission (CR). He underwent 5 courses of consolidation therapy during which he stayed in CR. He did not receive transplantation, considering his good response to chemotherapy and negative minimal residual disease (MRD). He lived without relapse of AML-M4Eo for more than 70 months.

Molecular studies

RT-PCR was performed after RNA extraction from bone marrow mononuclear cells. RT-PCR of *RUNX1-CBFA2T3* fusion gene was performed using a *RUNX-1* exon 5 specific primer, AMLex5f1 (CCACCTACCACAGAGCCATCAA AA) and a *CBFA2T3* specific primer and MTG16r2 (GT TCTCGTTGACTTCCAGTAGCAG),as described previously [11]. Briefly, PCR amplification was performed for 35 cycles (94 °C for 30 s, 58 °C for 60 s, and 72 °C for 60 s), followed by denaturation at 94 °C for 3 min and extension at 72 °C for 10 min. The PCR products were electrophoresed by a 1 % Sea Plaque GTG agarose gel (FMC, Rockland, ME, USA). Sequencing was performed using the PRISM Dye Terminator FS cycle sequencing kit and an ABI PRISM 377 DNA Sequencer (Perkin-Elmer Japan).

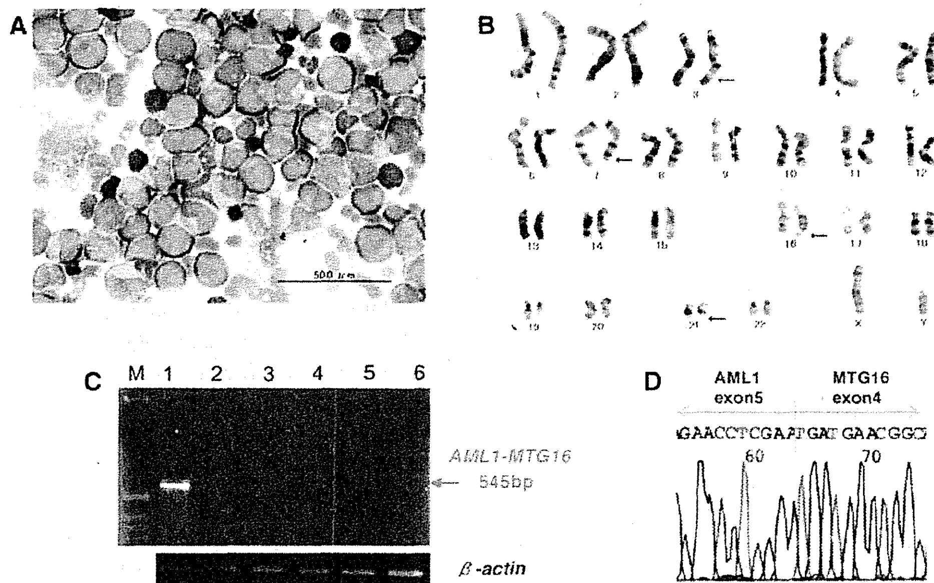

Fig. 1 **a** May-Giemsa stain of bone marrow aspirate at the time of diagnosis. Monocyte-like blasts and atypical eosinophilic granulocytes were observed, consistent with acute myeloid leukemia with abnormal bone marrow eosinophilia (AML-M4Eo) according to the French–American–British (FAB) criteria. **b** G-banding of the blasts: 46,XY,inv(3)(p23q26),del(7)(q?),t(16;21)(q24;q22). **c** Reverse transcription polymerase chain reaction (RT-PCR) for *RUNX1-CBFA2T3* (also known as *AML1-MTG16*) fusion gene. *Lane M* molecular size marker (100-bp ladder); *lane 1* bone marrow (BM) sample from the patient at the diagnosis; *lane 2* BM after induction chemotherapy; *lanes 3, 4, 5, 6* BM after first, second, third, fourth course of consolidation therapy, respectively. *RUNX1-CBFA2T3* was detected only at the time of diagnosis. **d** Sequence analysis of *RUNX1-CBFA2T3* fusion gene. In-frame fusion of *RUNX1* exon 5 and *CBFA2T3* exon 4 was confirmed

Results

RT-PCR revealed the presence of *RUNX1-CBFA2T3* fusion transcript in bone marrow cells at the time of diagnosis (Fig. 1c). The sequence analysis showed in-frame fusion of *RUNX1* exon 5 and *CBFA2T3* exon 4 (Fig. 1d). We monitored the MRD by conducting RT-PCR every time before the beginning of new course of chemotherapy. *RUNX1-CBFA2T3* fusion transcript was not detected at the end of induction chemotherapy and was persistently negative throughout the course of therapy (Fig. 1c).

Discussion

Although the number of reported cases of AML with t(16;21)(q24;q22) is quite limited, the prognosis of pediatric patients seems to be favorable. To date, only 5 pediatric cases including our case have been reported (Table 1) [5–8]. Of interest, 4 of 5 patients had eosinophilia either in peripheral blood or bone marrow at the diagnosis, excluding one patient without detailed clinical information. Three of the 4 patients survived more than 1 year without relapse of leukemia, and the last case of a 4-year-old girl with AML-M4Eo died of septic shock during induction chemotherapy, whose genuine prognosis remains uncertain [8]. We retrospectively reanalyzed 240 pediatric patients with AML treated by AML99 protocol from 2000 to 2003 in Japan, there were no other patients with t(16;21)(q24;q22) except for our patient, which verifies the rarity of this translocation in pediatric population (unpublished data). Adult cases of acute leukemia with t(16;21)(q24;q22) have been reported more frequently; 22 cases were found in English-written literatures [9]. Of note, 17 of 22 adult cases were therapy-related leukemia, whereas 3 of 5 pediatric cases were de novo AML [8, 9], and the prognosis seems to be better in pediatric patients.

The *RUNX1* (also known as *AML1*) gene is located on chromosome 21, and encodes the alpha subunit of core binding factor (CBF) that regulates hematopoietic cell-specific genes [12]. The chromosomal translocations generate fusion genes involving *RUNX1* and are associated with several types of leukemia. The *RUNX1-RUNX1T1* (also known as *MTG8*) fusion gene is frequently observed in patients with AML-M2, and its product is considered to suppress the wild-type *RUNX1* gene product by competitive inhibition leading to leukemogenesis [13]. The sequences of *CBFA2T3* and *RUNX1T1* show nearly 70 % identity with the exception of the N-terminal amino acid sequences, and the common structures between *CBFA2T3* and *RUNX1T1* suggest that *RUNX1-CBFA2T3* is a variant of *RUNX1-RUNX1T1*, sharing similar biological activity

Table 1 Pediatric cases of acute myeloid leukemia with t(16;21)(q24;q22)

Reference	Age (years)	Sex	Diagnosis	Karyotype	Eosinophil % (source)	Primary cancer	HSCT	Survival months	Outcome
Raimondi et al. [5]	<15	F	AML-M1	46,XX,t(16;21)(q24;q22)	10 % (BM)	de novo	N/A	N/A	N/A
Kondoh et al. [6]	12	M	AML-M2	46,XY,add(12)(p13),t(16;21)(q24;q22)	19 % (BM)	AML-M3	UR-BMT	>20	Alive
Frascella et al. [7]	14	M	AML-M5a	47,XY,add(4)(q35),+8,add(16)(q24)[23]/47,XY,+8[1]	N/A	Ewing's sarcoma	No	14	Dead from infection
Park et al. [8]	4	F	AML-M4	47,XX,t8,t(16;21)(q24;q22)[18]/46,XX[2]	11 % (BM) 10 % (PB)	de novo	No	0.75	Dead from infection
Present case	4	M	AML-M4	46,XY,inv(3)(p23q26),del(7)(q?), t(16;21)(q24;q22)	32 % (BM)	de novo	No	>70	Alive

HSCT hematopoietic stem cell transplant, *BM* bone marrow, *PB* peripheral blood, *UR-BMT* bone marrow transplant from unrelated donor, *N/A* not available

[11, 13], thus, we estimate that RUNX1-CBFA2T3 chimera has a prognostic impact similar to *RUNX1-RUNX1T1* chimera.

Acute leukemia with inv(16)(p13q22), t(16;16)(p13; q22) or t(8;21) involves CBF, and presents with concomitant eosinophilia. However, there is no relationship between hypereosinophilic syndrome and chronic eosinophilic leukemia caused by the translocation of PDGFα/β [14]. A recent report suggests that eosinophilia is not a predictor of good outcome in pediatric patients with AML [15], although the molecular mechanism of eosinophilia in AML related to CBF and t(16;21)(q24;q22) still remains unknown.

In conclusion, the rare chromosomal translocation of t(16;21)(q24;q22) may not be a sign of poor prognosis in children with AML. Further studies are needed for the evaluation of prognosis in AML with t(16;21)(q24;q22).

Conflict of interest The authors declare no conflict of interest.

References

1. Schnittger S, Bacher U, Haferlach C, Kern W, Haferlach T. Rare CBFB-MYH11 fusion transcripts in AML with inv(16)/t(16;16) are associated with therapy-related AML M4eo, atypical cytomorphology, atypical immunophenotype, atypical additional chromosomal rearrangements and low white blood cell count: a study on 162 patients. Leukemia. 2007;21(4):725–31.
2. Tobal K, Johnson PR, Saunders MJ, Harrison CJ, Liu Yin JA. Detection of CBFB/MYH11 transcripts in patients with inversion and other abnormalities of chromosome 16 at presentation and remission. Br J Haematol. 1995;91(1):104–8.
3. Delaunay J, Vey N, Leblanc T, Fenaux P, Rigal-Huguet F, Witz F, et al. Prognosis of inv(16)/t(16;16) acute myeloid leukemia (AML): a survey of 110 cases from the French AML Intergroup. Blood. 2003;102(2):462–9.
4. Balgobind BV, Hollink IH, Arentsen-Peters ST, Zimmermann M, Harbott J, Beverloo HB, et al. Integrative analysis of type-I and type-II aberrations underscores the genetic heterogeneity of pediatric acute myeloid leukemia. Haematologica. 2011;96(10): 1478–87.
5. Raimondi SC, Kalwinsky DK, Hayashi Y, Behm FG, Mirro J, Williams DL. Cytogenetics of childhood acute nonlymphocytic leukemia. Cancer Genet Cytogenet. 1989;40(1):13–27.
6. Kondoh K, Nakata Y, Furuta T, Hosoda F, Gamou T, Kurosawa Y, et al. A pediatric case of secondary leukemia associated with t(16;21)(q24;q22) exhibiting the chimeric AML1-MTG16 gene. Leuk Lymphoma. 2002;43(2):415–20.
7. Frascella E, Zampieron C, Sainati L, Casula L, Pasquali F, Mura R, et al. AML1-MTG16 gene rearrangement in a pediatric therapy related AML after Ewing sarcoma: a case discussion and review of literature. Cancer Therapy. 2005;3(A):285–92.
8. Park IJ, Park JE, Kim HJ, Jung HJ, Lee WG, Cho SR. Acute myeloid leukemia with t(16;21)(q24;q22) and eosinophilia: case report and review of the literature. Cancer Genet Cytogenet. 2010;196(1):105–8.
9. De Braekeleer E, Douet-Guilbert N, Le Bris MJ, Morel F, Férec C, De Braekeleer M. RUNX1-MTG16 fusion gene in acute myeloblastic leukemia with t(16;21)(q24;q22): case report and review of the literature. Cancer Genet Cytogenet. 2008;185(1): 47–50.
10. Tsukimoto I, Tawa A, Horibe K, Tabuchi K, Kigasawa H, Tsuchida M, et al. Risk-stratified therapy and the intensive use of cytarabine improves the outcome in childhood acute myeloid leukemia: the AML99 trial from the Japanese Childhood AML Cooperative Study Group. J Clin Oncol. 2009;27(24):4007–13.
11. Gamou T, Kitamura E, Hosoda F, Shimizu K, Shinohara K, Hayashi Y, et al. The partner gene of AML1 in t(16;21) myeloid malignancies is a novel member of the MTG8(ETO) family. Blood. 1998;91(11):4028–37.
12. Ito Y. Oncogenic potential of the RUNX gene family: 'overview'. Oncogene. 2004;23(24):4198–208.
13. Kitabayashi I, Ida K, Morohoshi F, Yokoyama A, Mitsuhashi N, Shimizu K, et al. The AML1-MTG8 leukemic fusion protein forms a complex with a novel member of the MTG8(ETO/CDR) family, MTGR1. Mol Cell Biol. 1998;18(2):846–58.
14. Monma F, Nishii K, Lorenzo F, Usui E, Ueda Y, Watanabe Y, et al. Molecular analysis of PDGFRalpha/beta genes in core binding factor leukemia with eosinophilia. Eur J Haematol. 2006; 76(1):18–22.
15. Agarwal R, Vishnubhatla S, Gupta R, Bakhshi S. Diagnostic and follow-up eosinophilia is not predictive of outcome in childhood acute myeloid leukemia. J Pediatr Hematol Oncol. 2011;33(2): e51–3.

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ORIGINAL ARTICLE

RAS mutations are frequent in FAB type M4 and M5 of acute myeloid leukemia, and related to late relapse: a study of the Japanese Childhood AML Cooperative Study Group

Hirozumi Sano · Akira Shimada · Tomohiko Taki · Chisato Murata · Myoung-ja Park · Manabu Sotomatsu · Ken Tabuchi · Akio Tawa · Ryoji Kobayashi · Keizo Horibe · Masahiro Tsuchida · Ryoji Hanada · Ichiro Tsukimoto · Yasuhide Hayashi

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Abstract Mutations in *RAS* are frequent in acute myeloid leukemia (AML), and are thought to contribute to leukemogenesis in a subset of patients; however, their prognostic significance has not been firmly established. One hundred and fifty-seven pediatric patients with AML were analyzed for *NRAS* and *KRAS* mutations around hot spots at codons 12, 13, and 61. Twenty-nine patients (18.5%) had an activating mutation of *RAS*. We found *KRAS* mutations to be more frequent than *NRAS* mutations (18/29, 62.1% of patients with *RAS* mutation), in contrast to previous reports (18–40%). The frequency of *RAS* mutation was higher in French-American-British types M4 and M5 than other types ($P = 0.02$). There were no significant differences in other clinical manifestations or distribution in cytogenetic subgroups, or aberrations of other genes, including *KIT* mutation, *FLT3*-ITD, and *MLL*-PTD, between patients with and without *RAS* mutations. No significant differences were observed in the 3-year overall survival and disease-free survival; however, the presence of *RAS* mutation was related to late relapse. The occurrence of clinical events at relatively late period should be monitored for in AML patients with mutations in *RAS*.

Keywords Acute myeloid leukemia · *RAS* · Late relapse · Prognosis · FAB

H. Sano · A. Shimada · C. Murata · M. Park · M. Sotomatsu · Y. Hayashi (✉)
Department of Hematology/Oncology, Gunma Children's Medical Center, 779 Shimohakoda, Hokkitsu, Shibukawa, Gunma 377-8577, Japan
e-mail: hayashiy-tky@umin.ac.jp

T. Taki
Department of Molecular Diagnostics and Therapeutics, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, 465 Kajii-cho, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan

K. Tabuchi
Department of Hematology, Kanagawa Children's Medical Center, 2-138-4 Mutsugawa, Minami-ku, Yokohama, Kanagawa 232-8555, Japan

A. Tawa
Department of Pediatrics, National Hospital Organization Osaka National Hospital, 2-1-14 Hoenzaka, Chuo-ku, Osaka 540-0006, Japan

R. Kobayashi
Department of Pediatrics, Sapporo Hokuyu Hospital, 6-6-5-1 Higashi-Sapporo, Shiroishi-ku, Sapporo 003-0006, Japan

K. Horibe
Clinical Research Center, National Hospital Organization Nagoya Medical Center, 4-1-1 Sannomaru, Nakaku, Nagoya, Aichi 460-0001, Japan

M. Tsuchida
Department of Pediatrics, Ibaraki Children's Hospital, 3-3-1 Futabadai, Mito, Ibaraki 311-4145, Japan

R. Hanada
Division of Hematology/Oncology, Saitama Children's Medical Center, 2100 Magome, Iwatsuki-ku, Saitama 339-8551, Japan

I. Tsukimoto
First Department of Pediatrics, Toho University School of Medicine, 6-11-1, Omori-nishi, Ota-ku, Tokyo 143-8541, Japan

Introduction

The prognosis of pediatric acute myeloid leukemia (AML) has improved markedly over the past decade, with an overall survival rate of about 60–70% according to the results of various clinical trials; however, relapse remains the major cause of treatment failure, occurring in 30–40% of patients in their first complete remission (CR) [1–6]. Several study groups have shown that chromosome abnormalities are independent and strong predictors of the outcome, both in childhood and adult AML [1, 4, 7].

It is hypothesized that AML results from two classes of cooperating mutations [8–10]. Type II mutations affect hematopoietic stem cell differentiation, typically by mutations or translocations (e.g., *AML1-ETO*, *PML-RARα*, or *CBFβ-MYH11*), resulting in the aberrant function of transcription factor complexes; however, these mutations alone are insufficient to induce leukemia. Type I mutations provide a proliferative and survival advantage to hematopoietic progenitors [11]. This group includes activating mutations in receptor tyrosine kinases (RTKs) such as *FLT3* and GTPase *RAS* [11–14].

The *RAS* family of genes consists of three G-proteins, *NRAS*, *KRAS*, and *HRAS* [15]. The RAS proteins are important in relaying proliferation and survival signals from cell membrane receptors (including KIT and FLT3) to intracellular signal transduction pathways. Certain mutations in *RAS* induce the permanent activation of RAS. Mutations in *NRAS* or *KRAS* have been identified in numerous malignancies [15], including hematologic malignancies such as AML [15–17].

Although mutations in *RAS* are frequent in AML and considered to contribute to leukemogenesis in a subset of patients, their prognostic significance has not been firmly established. Some reports have suggested that patients with AML showing *RAS* mutation have poorer [18–20] or similar clinical outcomes to patients carrying the wild-type *RAS* gene [16, 21–26], whereas others have found that mutations in *RAS* are associated with a more favorable prognosis [17, 27]. Although these conflicting results may stem from variations in the pretreatment features of patient populations analyzed in different series, they may also be related to differences in the treatment regimens used.

We performed mutational analysis of *RAS* in pediatric AML patients who were treated on the Japanese Childhood Cooperative Study Group Protocol, AML99, and demonstrated that *RAS* mutations were frequently found in French-American-British (FAB) types M4 and M5, and related to late relapse. Furthermore, we analyzed the association between *RAS* mutations and other gene aberrations, including *KIT* mutation, *FLT3*-ITD, and *MLL*-PTD.

Materials and methods

Patients

The diagnosis of AML was based on the FAB classification, and cytogenetic analysis was performed using a routine G-banding method. From January 2000 to December 2002, 318 patients were newly diagnosed as having de novo AML. Of these, samples from 157 patients were available for molecular analysis, including 13 with FAB-M3 and 10 with Down syndrome (DS), who were treated on different treatment protocols [5, 28–30]. There were no significant differences between the 134 patients without FAB-M3 or DS and the 106 non-analyzed patients in terms of the age [median 6 years (range 0–15 years) vs. 6 years (range 0–15 years)] and initial WBC count [median 24.8×10^9/L (range $1.65–621.0 \times 10^9$/L) vs. 13.8×10^9/L (range $1.0–489.0 \times 10^9$/L, $P = 0.0764$)]. Patients who were younger than 2 years old or had an initial WBC count <100,000/μL were treated using the induction A regimen [etoposide (VP-16), cytarabine (CA), and mitoxantrone (MIT), (ECM)]. Patients who were older than 2 years and had an initial WBC count >100,000/μL were treated using the induction B regimen [VP-16, CA, and idarubicin (IDA), (ECI)]. If patients achieved a complete remission (CR), they were classified into three risk groups (62 low, 57 intermediate, and 10 high) according to the results of cytogenetic analyses or the achievement of CR after the 2 initial courses of chemotherapy [5, 28–30]. AML patients with t(8;21) (except for those with WBC counts >50,000/μL) or inv(16)(p11q22) were classified into the low-risk (LR) group. Patients with monosomy 7, 5q-, t(16;21), or Philadelphia chromosome (Ph1) were classified into the high-risk (HR) group. Patients were treated with additional chemotherapy or allogeneic stem cell transplantation (allo-SCT) in each risk group.

Informed consent was obtained from the patients or their parents, according to guidelines based on the tenets of the revised Helsinki protocol. The institutional review board of Gunma Children's Medical Center approved this project.

Detection of *RAS* mutations

Total RNA (4 μg) extracted from the bone marrow or peripheral blood samples at diagnosis was reverse transcribed to cDNA with a cDNA Synthesis Kit (Amersham Bioscience, Tokyo, Japan). Mutations of codons 12, 13, and 61 of *NRAS* and *KRAS* genes were directly sequenced using the following primers: NRAS EX1S 5′-TACAAACT GGTGGTGGTTGG-3′; NRAS EX2R 5′-CAAATGACTT GCTATTATTGATG-3′; KRAS EX1S 5′-GGCCTGCTGA AAATGACTGAATAT-3′; KRAS EX2R 5′-ACTGGTCC CTCATTGCACT-3′.

Detection of *FLT3*-ITD, *FLT3*-D835, *KIT* mutations, and *MLL*-PTD

Mutational analysis of internal tandem duplication (ITD) within the JM domain and D835 mutation (D835Mt) within the TK2 domain of *FLT3* was performed as previously reported [28, 30–32]. Mutation analysis of the kinase domain, extracellular domain, and transmembrane domain of the *KIT* gene was performed with RT-PCR followed by direct sequencing as previously reported [28]. Mutational analysis of partial tandem duplication (PTD) of *MLL* was performed using the primer pair 6.1 (located in exon 9) and E3AS (located in exon 4) as previously reported [30].

Statistical analysis

The χ^2 test was used to compare the frequencies of mutations. Fischer's exact test was used when data were sparse. The survival distribution was assessed using the Kaplan–Meier method, and differences were compared using the log-rank test [33]. Overall survival (OS) was defined as the time from diagnosis until death owing to any cause or the last follow-up. Disease-free survival (DFS) was defined as the time from the date of complete remission until relapse. To compare survival in the relatively late course of treatment, we further measured OS and DFS from the predefined 'landmark' time, but not from the onset, in cases in first complete remission at the landmark time [34]. All patients enrolled in AML99 finished the medical treatment including hematopoietic stem cell transplantations within 1.4 years after complete remission; therefore, the landmark time was defined as 1.4 years after complete remission. In landmark analysis, both OS and DFS were calculated using the survival duration after complete remission. These statistical analyses were based on Dr SPSS II for Windows (release 11.0.1J, SPSS; Japan, Inc.).

Results

RAS mutations

Of 157 pediatric AML patients, 29 (18.5%) had an activating mutation: 10 (6.4%) in *NRAS* exon 1, one (0.6%) in *NRAS* exon 2, 13 (8.3%) in *KRAS* exon 1, and 5 (3.2%) in *KRAS* exon 2. There was no significant difference in age, sex, WBC count at diagnosis, or the frequency of extramedullary infiltration of leukemic cells between patients with and without *RAS* mutations (Table 1).

RAS mutations in cytogenetic subgroups

Fifty-three patients in this study were diagnosed with CBF-AML, including 46 patients with t(8;21) and 7 with inv(16). In this group of patients, 12 (22.6%) had a mutation in *NRAS* or *KRAS*. The incidence of *RAS* mutation in the t(8;21) and inv(16) subgroups was 21.7 and 28.6%, respectively. Thirty-three patients in this cohort had a normal karyotype, with 18.2% of these patients having a mutation in *RAS*. The incidence of mutations in *RAS* was not a significantly frequent event in pediatric CBF-AML among other cytogenetic subgroups (Table 1, in which DS patients were not included in karyotypic abnormalities).

RAS mutations in FAB subgroups

The five most prevalent FAB types in the patient cohort were M2, M1, M5, M4, and M7 in order of descending prevalence. When examining these five subgroups, the frequency of RAS mutation was 31.8% (M4), 28.0% (M5), 19.6% (M2), 12.5% (M1), and 5.3% (M7). The frequency of *RAS* mutation was higher in FAB types M4 and M5 than in the other types (29.8 vs. 13.6%, respectively, $P = 0.02$) (Table 1).

Correlations between RAS mutations and other gene aberrations

In *RAS*-mutated cases, 10 cases (34.5%) had mutations in other genes, including 1 (3.4%) in *FLT3*-ITD, 2 (6.9%) in *FLT3*-D835 mutation, 4 (13.8%) in *KIT* mutation, and 3 in *MLL*-PTD (10.3%). This distribution was not different from those without *RAS* mutation (Table 1).

Clinical outcome and prognostic significance of *RAS* mutations

There were no significant differences in the 3-year OS, DFS, and the induction rate of complete remission (96 vs. 93%, respectively, $P = 0.88$) between those with and without *RAS* mutation in 134 AML patients, excluding those with FAB-M3 and DS (Fig. 1). Between *NRAS*-mutated ($n = 11$) and *KRAS*-mutated patients ($n = 17$) excluding those with FAB-M3 and DS, there were no differences in the 3-year OS (73 vs. 77%, respectively, $P = 0.91$), DFS (73 vs. 50%, respectively, $P = 0.48$), and the induction rate of complete remission (100 vs. 94%, respectively, $P = 0.82$). The frequency of *RAS* mutation did not differ between patients with and without complete remission after induction therapy (22.0 vs. 9.1%, respectively, $P = 0.73$).

On the other hand, by the analysis of OS and DFS from the predefined 'landmark' time of 1.4 years after complete remission, patients with RAS mutation had a poorer 3-year OS ($P = 0.038$) and DFS ($P = 0.026$) than those without RAS mutation in 106 patients (OS) and 84 patients (DFS), excluding those with FAB-M3 and DS (Fig. 2a, b).

Table 1 Clinical characteristics of patients with and without RAS mutations

	All patients	RAS-Wt	RAS-Mt	NRAS-Mt	KRAS-Mt
Age, median (years)	6 (0–15)	6 (0–15)	7 (0–15)	10 (0–15)	6 (0–14)
WBC count, median ($\times 10^9$/L)	20.7 (1.0–620.0)	17.2 (1.0–620.0)	41.6 (1.8–224.4)	47.1 (2.0–198.0)	36.6 (1.8–224.4)
Male/female	89/68	71/57	18/11	9/2	9/9
Patients with Down syndrome	10 (6.4%)	9 (7.0%)	1 (3.4%)	0	1 (5.6%)
FAB classification					
M0	5 (3.2%)	4 (3.1%)	1 (3.4%)	1 (9.1%)	0
M1	24 (15.3%)	21 (16.4%)	3 (10.3%)	1 (9.1%)	2 (11.1%)
M2	46 (29.3%)[a]	37 (28.9%)[a]	9 (31.0%)	4 (36.4%)	5 (27.8%)
M3	13 (8.3%)	13 (10.2%)	0	0	0
M4	22 (14.0%)	15 (11.7%)	7 (24.1%)	3 (27.3%)	4 (22.2%)
M5	25 (15.9%)	18 (14.1%)	7 (24.1%)	2 (18.2%)	5 (27.8%)
M6	1 (0.6%)	0	1 (3.4%)	0	1 (5.6%)
M7	19 (12.1%)[a]	18 (14.1%)[a]	1 (3.4%)[a]	0	1 (5.6%)[a]
Unclassified	2 (1.3%)	2 (1.6%)	0	0	0
Other genomic alterations[b]					
FLT3-ITD	17 (10.8%)	16 (12.5%)	1 (3.4%)	1 (9.1%)	0
FLT3 D835 mutation	8 (5.1%)	6 (4.7%)	2 (6.9%)	0	2 (11.1%)
KIT mutation	12 (7.6%)	8 (6.3%)	4 (13.8%)	1 (9.1%)	3 (16.7%)
MLL-PTD	21 (13.4%)	18 (14.1%)	3 (10.3%)	1 (9.1%)	2 (11.1%)
Total	157	128	29	11	18
Karyotypic abnormalities (excluding Down syndrome)					
Normal	33 (22.4%)	27 (22.7%)	6 (21.4%)	2 (18.2%)	4 (23.5%)
t(8;21)	46 (31.3%)	36 (30.3%)	10 (35.7%)	4 (36.4%)	6 (35.3%)
11q23 abnormality	19 (12.9%)	14 (11.8%)	5 (17.9%)	1 (9.1%)	4 (23.5%)
t(15;17)	13 (8.8%)	13 (10.9%)	0	0	0
inv(16)	7 (4.8%)	5 (4.2%)	2 (7.1%)	2 (18.2%)	0
Others	27 (18.4%)	22 (18.5%)	5 (17.9%)	2 (18.2%)	3 (17.6%)
Unknown	2 (1.4%)	2 (1.7%)	0	0	0
Total	147	119	28	11	17
Risk group (excluding FAB-M3 and Down syndrome)					
Low	62 (46.3%)	49 (46.2%)	13 (46.4%)	4 (36.4%)	9 (52.9%)
Intermediate	57 (42.5%)	44 (41.5%)	13 (46.4%)	6 (54.5%)	7 (41.2%)
High	10 (7.5%)	9 (8.5%)	1 (3.6%)	1 (9.1%)	0
Non-CR	5 (3.7%)	4 (3.8%)	1 (3.6%)	0	1 (5.9%)
Total	134	106	28	11	17

[a] Of 10 cases with Down syndrome, 9 were classified into FAB-M7 and 1 was into FAB-M2. *RAS* (*KRAS*) mutation was observed in one case (FAB-M7) with Down syndrome

[b] One case with Down syndrome harboring *RAS* mutation had no other genomic alteration

Discussion

We analyzed the clinical significance of *RAS* mutation in a cohort of 157 pediatric AML patients. Some studies have reported that *RAS* mutations confer a poor prognosis [18–20]. In this study, the 3-year OS and DFS were not different between patients with and without *RAS* mutation. This discrepancy may be due to the fact that the treatment regimen used in each study was different. In adult cases, AML patients carrying a *RAS* mutation have been reported to benefit from higher cytarabine doses more than those without *RAS* mutation [35]. The current patients were treated on the Japanese Childhood AML Cooperative Study Group Protocol, AML99, in which the intensive use of cytarabine, including high-dose cytarabine, was considered to improve the outcome. Improvement of the clinical outcome of patients with *RAS* mutation in this study might have decreased the differences in the 3-year OS and DFS between patients with and without *RAS* mutation.

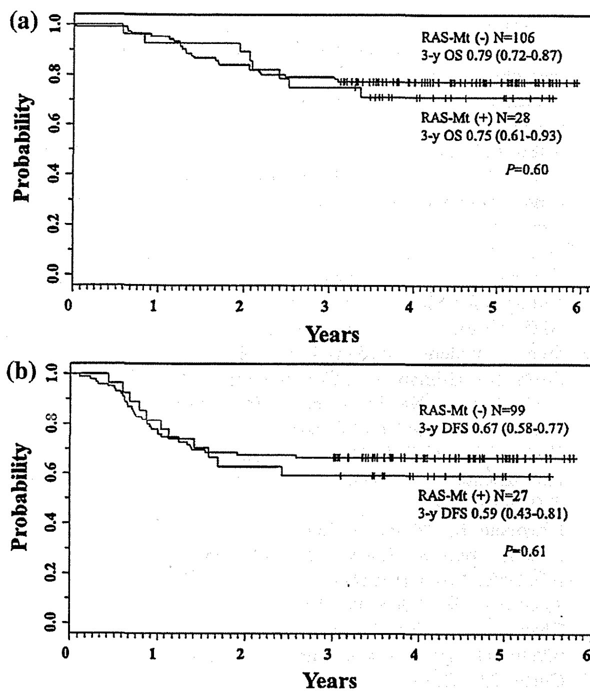

Fig. 1 Probability of 3-year OS (**a**) and DFS (**b**) in 134 AML patients, excluding those with FAB-M3 and Down syndrome. Kaplan–Meier estimates for patients with and without *RAS* mutation are shown

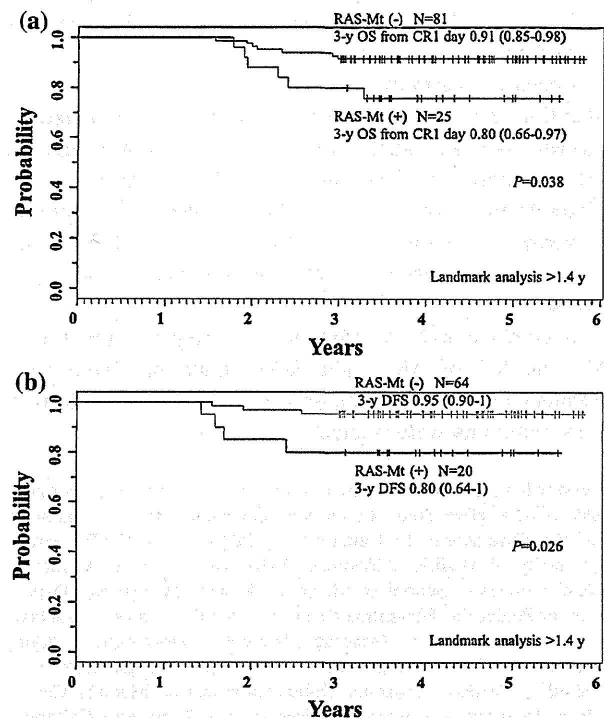

Fig. 2 Probability of 3-year OS in 106 patients (**a**) and DFS in 84 patients (**b**) from the landmark time of 1.4 years from the date of complete remission (CR1 day), excluding those with FAB-M3 and Down syndrome. Kaplan–Meier estimates for patients with and without *RAS* mutation are shown

To compare survival in the relatively late course of treatment, OS and DFS were also calculated from the predefined 'landmark' time of 1.4 years after complete remission, excluding patients with FAB-M3 and DS. As a result of landmark analysis, the 3-year OS and DFS of patients with *RAS* mutation were inferior to those without *RAS* mutation, which was different from the results of conventional analysis. This discrepancy might be due to the influence of cases with *FLT3*-ITD, since all relapses in cases with *FLT3*-ITD had taken place within 9 months [30]. This landmark analysis was meaningful in clarifying the influence of *RAS* mutation on late relapse by excluding the influence of *FLT3*-ITD on early relapse. To the best of our knowledge, no reports have discussed the meaning of *RAS* mutation in such relatively late course of treatment. Although further studies should focus on the prognostic impact of *RAS* mutation, the occurrence of death or relapse at a relatively late period should be paid attention to in AML patients with *RAS* mutation.

The incidence of *RAS* mutation in this study was 18.5%, almost the same as in previous reports [36–40]. In adult cases, *NRAS* mutations were reported to be more frequent than *KRAS* mutations [19, 41]; however, there have been few reports on pediatric AML. In pediatric cases, *KRAS* mutations were reported to be less frequent than *NRAS* mutations (18–40% in pediatric patients with *RAS* mutations) [20, 25, 42]; however, *KRAS* mutations were more frequent than *NRAS* mutations in this study (18/29, 62.1% in patients with *RAS* mutation). Although the reason for this discrepancy is not clear, this might be due to racial differences. As another possibility, the frequency of *NRAS* or *KRAS* mutation may differ between adult and pediatric patients.

The frequency of *RAS* mutation was higher in FAB types M4 and M5 than other types in this study ($P = 0.02$). Mutant *RAS* was suggested to promote a myeloid maturation defect, with relative sparing of the monocyte-macrophage lineage in vitro [43]. This may be consistent with the high incidence of *RAS* mutation in FAB types M4 and M5. In adult cases, *KRAS* mutation was reported to frequently demonstrate significant heterogeneity among FAB subtypes, being more common in M4 [24]; however, the frequency of FAB type M4 was not significantly high in patients with *KRAS* mutation in our study (22.2% of *RAS*-mutated vs. 13.3% of wild-type *RAS* samples, $P = 0.38$). In pediatric cases, the frequencies of FAB types M2 and M4 were reported to be high in patients with *RAS* mutation [25]; however, no significant differences were observed in this study (55.2% of *RAS*-mutated vs. 40.6% of wild-type *RAS* samples, $P = 0.15$). Further study is needed to clarify

the distribution of *RAS* mutation in FAB types, especially in pediatric cases.

Recently, germline mutation of *NRAS* has attracted attention as a cause of juvenile myelomonocytic leukemia (JMML) [44] and autoimmune lymphoproliferative disease [45]. Notably, germline mutation of the *RAS* gene may be linked to the development of AML; however, the presence of germline mutation of the *RAS* was not identified in this study because normal somatic cells of patients were not available.

In conclusion, *RAS* mutations were frequent in FAB type M4 and M5 of AML, and *KRAS* mutations were more frequent than *NRAS* mutations in this study. In addition, *RAS* mutations were related to late relapse.

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Conflict of interest There is no conflict of interest.

References

1. Gibson BE, Wheatley K, Hann IM, Stevens RF, Webb D, Hills RK, et al. Treatment strategy and long-term results in paediatric patients treated in consecutive UK AML trials. Leukemia. 2005;19:2130–8.
2. Lie SO, Abrahamsson J, Clausen N, Forestier E, Hasle H, Hovi L, et al. Long term results in children with AML: NOPHO-AML Study Group—report of three consecutive trials. Leukemia. 2005;19:2090–100.
3. Creutzig U, Zimmermann M, Lehrnbecher T, Graf N, Hermann J, Niemeyer CM, et al. Less toxicity by optimizing chemotherapy, but not by addition of granulocyte colony-stimulating factor in children and adolescents with acute myeloid leukemia: results of AML-BFM 98. J Clin Oncol. 2006;24:4499–506.
4. Lange BJ, Smith FO, Feusner J, Barnard DR, Dinndorf P, Feig S, et al. Outcomes in CCG-2961, a children's oncology group phase 3 trial for untreated pediatric acute myeloid leukemia: a report from the children's oncology group. Blood. 2008;111:1044–53.
5. Tsukimoto I, Tawa A, Horibe K, Tabuchi K, Kigasawa H, Tsuchida M, et al. Risk-stratified therapy and the intensive use of cytarabine improves the outcome in childhood acute myeloid leukemia: The AML99 trial from the Japanese Childhood AML Cooperative Study Group. J Clin Oncol. 2009;27:4007–13.
6. Rubnitz JE, Inaba H, Dahl G, Ribeiro RC, Bowman WP, Taub J, et al. Minimal residual disease-directed therapy for childhood acute myeloid leukaemia: results of the AML 02 multicentre trial. Lancet Oncol. 2010;11:543–52.
7. Grimwade D, Walker H, Oliver F, Wheatley K, Harrison C, Harrison G, et al. The importance of diagnostic cytogenetics on outcome of AML: analysis of 1612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. Blood. 1998;92:2322–33.
8. Speck NA, Gilliland DG. Core binding factors in haematopoiesis and leukaemia. Nat Rev Cancer. 2002;2:502–13.
9. Kelly LM, Kutok JL, Williams IR, Boulton CL, Amaral SM, Curley DP, et al. PML/RARalpha and FLT3-ITD induce an APL-like disease in a mouse model. Proc Natl Acad Sci USA. 2002;99:8283–8.
10. Grisolano JL, O'Neal J, Cain J, Tomasson MH. An activated receptor tyrosine kinase, TEL/PDGFbetaR, cooperates with AML1/ETO to induce acute myeloid leukemia in mice. Proc Natl Acad Sci USA. 2003;100:9506–11.
11. Chan IT, Gilliland DG. Oncogenic K-ras in mouse model of myeloproliferative disease and acute myeloid leukemia. Cell Cycle. 2004;3:536–7.
12. Janssen JW, Steenvoorden AC, Collard JG, Nusse R. Oncogene activation in human myeloid leukemia. Cancer Res. 1985;45:3262–7.
13. Kitayama H, Tsujimura T, Matsumura I, Oritani K, Ikeda H, Ishikawa J, et al. Neoplastic transformation of normal hematopoietic cells by constitutively activating mutations of c-kit receptor tyrosine kinase. Blood. 1996;88:995–1004.
14. Hayakawa F, Towatari M, Kiyoi H, Tanimoto M, Kitamura T, Saito H, et al. Tandem-duplicated Flt3 constitutively activates STAT5 and MAP kinase and introduces autonomous cell growth in IL-3-dependent cell lines. Oncogene. 2000;19:624–31.
15. Bos JL. ras oncogenes in human cancer: a review. Cancer Res. 1989;49:4682–9.
16. Radich JP, Kopecky KJ, Willman CL, Weick J, Head D, Appelbaum F, et al. N-ras mutations in adult de novo acute myelogenous leukemia: prevalence and clinical significance. Blood. 1990;76:801–7.
17. Neubauer A, Dodge RK, George SL, Davey FR, Silver RT, Schiffer CA, et al. Prognostic importance of mutations in the ras proto-oncogenes in de novo acute myeloid leukemia. Blood. 1994;83:1603–11.
18. De Melo MB, Lorand-Metze I, Lima CS, Saad ST, Costa FF. N-RAS gene point mutations in Brazilian acute myelogenous leukemia patients correlate with a poor prognosis. Leuk Lymphoma. 1997;24:309–17.
19. Kiyoi H, Naoe T, Nakano Y, Yokota S, Minami S, Miyawaki S, et al. Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. Blood. 1999;93:3074–80.
20. Meshinchi S, Stirewalt DL, Alonzo TA, Zhang Q, Sweetser DA, Woods WG, et al. Activating mutations of RTK/ras signal transduction pathway in pediatric acute myeloid leukemia. Blood. 2003;102:1474–9.
21. Stirewalt DL, Kopecky KJ, Meshinchi S, Appelbaum FR, Slovak ML, Willman CL, et al. FLT3, RAS, and TP53 mutations in elderly patients with acute myeloid leukemia. Blood. 2001;97:3589–95.
22. Ritter M, Kim TD, Lisske P, Thiede C, Schaich M, Neubauer A. Prognostic significance of N-RAS and K-RAS mutations in 232 patients with acute myeloid leukemia. Haematologica. 2004;89:1397–9.

23. Illmer T, Thiede C, Fredersdorf A, Stadler S, Neubauer A, Ehninger G, et al. Activation of the RAS pathway is predictive for a chemosensitive phenotype of acute myelogenous leukemia blasts. Clin Cancer Res. 2005;11:3217–24.
24. Bowen DT, Frew ME, Hills R, Gale RE, Wheatley K, Groves MJ, et al. RAS mutation in acute myeloid leukemia is associated with distinct cytogenetic subgroups but does not influence outcome in patients younger than 60 years. Blood. 2005;106:2113–9.
25. Goemans BF, Zwaan CM, Miller M, Zimmermann M, Harlow A, Meshinchi S, et al. Mutations in KIT and RAS are frequent events in pediatric core-binding factor acute myeloid leukemia. Leukemia. 2005;19:1536–42.
26. Bacher U, Haferlach T, Schoch C, Kern W, Schnittger S. Implications of NRAS mutations in AML: a study of 2502 patients. Blood. 2006;107:3847–53.
27. Coghlan DW, Morley AA, Matthews JP, Bishop JF. The incidence and prognostic significance of mutations in codon 13 of the N-RAS gene in acute myeloid leukemia. Leukemia. 1994;8: 1682–7.
28. Shimada A, Taki T, Tabuchi K, Tawa A, Horibe K, Tsuchida M, et al. KIT mutations, and not FLT3 internal tandem duplication, are strongly associated with a poor prognosis in pediatric acute myeloid leukemia with t(8;21): a study of the Japanese Childhood AML Cooperative Study Group. Blood. 2006;107:1806–9.
29. Kobayashi R, Tawa A, Hanada R, Horibe K, Tsuchida M, Tsukimoto I. Extramedullary infiltration at diagnosis and prognosis in children with acute myeloid leukemia. Pediatr Blood Cancer. 2007;48:393–8.
30. Shimada A, Taki T, Tabuchi K, Taketani T, Hanada R, Tawa A, et al. Tandem duplications of MLL and FLT3 are correlated with poor prognoses in pediatric acute myeloid leukemia: a study of the Japanese Childhood AML Cooperative Study Group. Pediatr Blood Cancer. 2008;50:264–9.
31. Xu F, Taki T, Yang HW, Hanada R, Hongo T, Ohnishi H, et al. Tandem duplication of the FLT3 gene is found in acute lymphoblastic leukaemia as well as acute myeloid leukaemia but not in myelodysplastic syndrome or juvenile chronic myelogenous leukaemia in children. Br J Haematol. 1999;105:155–62.
32. Taketani T, Taki T, Sugita K, Furuichi Y, Ishii E, Hanada R, et al. FLT3 mutations in the activation loop of tyrosine kinase domain are frequently found in infant ALL with MLL rearrangements and pediatric ALL with hyperdiploidy. Blood. 2004;103:1085–8.
33. Kaplan E, Meier P. Nonparametric estimation from incomplete observations. J Am Stat Assoc. 1957;53:457–81.
34. Anderson JR, Cain KC, Gelber RD. Analysis of survival by tumor response. J Clin Oncol. 1983;1:710–9.
35. Neubauer A, Maharry K, Mrózek K, Thiede C, Marcucci G, Paschka P, et al. Patients with acute myeloid leukemia and RAS mutations benefit most from postremission high-dose cytarabine: a Cancer and Leukemia Group B Study. J Clin Oncol. 2008;26:4603–9.
36. Vogelstein B, Civin CI, Preisinger AC, Krischer JP, Steuber P, Ravindranath Y, et al. RAS gene mutations in childhood acute myeloid leukemia: a Pediatric Oncology Group study. Genes Chromosomes Cancer. 1990;2:159–62.
37. Farr C, Gill R, Katz F, Gibbons B, Marshall CJ. Analysis of ras gene mutations in childhood myeloid leukemia. Br J Haematol. 1991;77:323–7.
38. Lübbert M, Mirro J Jr, Kitchingman G, McCormick F, Mertelsmann R, Herrmann F, et al. Prevalence of N-ras mutations in children with myelodysplastic syndrome and acute myeloid leukemia. Oncogene. 1992;7:263–8.
39. Sheng XM, Kawamura M, Ohnishi H, Ida K, Hanada R, Kojima S, et al. Mutations of the RAS genes in childhood acute myeloid leukemia, myelodysplastic syndrome and juvenile myelocytic leukemia. Leuk Res. 1997;21:697–701.
40. Mahgoub N, Parker RI, Hosler MR, Close P, Winick NJ, Masterson M, et al. RAS mutations in pediatric leukemias with MLL gene rearrangements. Genes Chromosomes Cancer. 1998;21:270–5.
41. Nakagawa T, Saitoh S, Imoto S, Itoh M, Tsutsumi M, Hikiji K, et al. Multiple point mutation of N-ras and K-ras oncogenes in myelodysplastic syndrome and acute myelogenous leukemia. Oncology. 1992;49:114–22.
42. Shih LY, Liang DC, Huang CF, Chang YT, Lai CL, Lin TH, et al. Cooperating mutations of receptor tyrosine kinases and Ras genes in childhood core-binding factor acute myeloid leukemia and a comparative analysis on paired diagnosis and relapse samples. Leukemia. 2008;22:303–7.
43. Darley RL, Burnett AK. Mutant RAS inhibits neutrophil but not macrophage differentiation and allows continued growth of neutrophil precursors. Exp Hematol. 1999;27:1599–608.
44. De Filippi P, Zecca M, Lisini D, Rosti V, Cagioni C, Carlo-Stella C, et al. Germline mutation of the NRAS gene maybe responsible for the development of juvenile myelomonocytic leukaemia. Br J Haematol. 2009;147:706–9.
45. Oliveira JB, Bidère N, Niemela JE, Zheng L, Sakai K, Nix CP, et al. NRAS mutation causes a human autoimmune lymphoproliferative disease. Proc Natl Acad Sci USA. 2007;104:8953–8.

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ORIGINAL ARTICLE

Aberrant activation of ALK kinase by a novel truncated form ALK protein in neuroblastoma

J Okubo[1], J Takita[1,2], Y Chen[1], K Oki[1], R Nishimura[1], M Kato[1], M Sanada[3], M Hiwatari[1], Y Hayashi[4], T Igarashi[1] and S Ogawa[3]

Anaplastic lymphoma kinase (ALK) was originally identified from a rare subtype of non-Hodgkin's lymphomas carrying t(2;5)(p23;q35) translocation, where ALK was constitutively activated as a result of a fusion with nucleophosmin (NPM). Aberrant ALK fusion proteins were also generated in inflammatory fibrosarcoma and a subset of non-small-cell lung cancers, and these proteins are implicated in their pathogenesis. Recently, ALK has been demonstrated to be constitutively activated by gene mutations and/or amplifications in sporadic as well as familial cases of neuroblastoma. Here we describe another mechanism of aberrant ALK activation observed in a neuroblastoma-derived cell line (NB-1), in which a short-form ALK protein (ALK^{del2-3}) having a truncated extracellular domain is overexpressed because of amplification of an abnormal *ALK* gene that lacks exons 2 and 3. ALK^{del2-3} was autophosphorylated in NB-1 cells as well as in ALK^{del2-3}-transduced cells and exhibited enhanced *in vitro* kinase activity compared with the wild-type kinase. ALK^{del2-3}-transduced NIH3T3 cells exhibited increased colony-forming capacity in soft agar and tumorigenicity in nude mice. RNAi-mediated ALK knockdown resulted in the growth suppression of ALK^{del2-3}-expressing cells, arguing for the oncogenic role of this mutant. Our findings provide a novel insight into the mechanism of deregulation of the ALK kinase and its roles in neuroblastoma pathogenesis.

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INTRODUCTION

Anaplastic lymphoma kinase (ALK) (OMIM: 105590) is an orphan receptor tyrosine kinase (RTK) that was initially characterized as a fusion partner of the nucleophosmin (NPM)-ALK chimeric protein associated with the t(2;5)(p23;q35) translocation in anaplastic large-cell lymphoma.[1,2] Subsequent studies have revealed that various ALK-containing fusion proteins with different fusion partners are generated in various solid tumors, such as inflammatory myofibroblastic tumors, non-small-cell lung cancer and squamous cell carcinoma of the esophagus.[3-6] Furthermore, recent genome-wide studies have revealed that *ALK* is activated by gene amplification and nucleotide mutations and is involved in the pathogenesis of both familial and sporadic neuroblastoma.[7-10]

Neuroblastoma is an intractable, solid tumor of childhood arising from the neural crest and can arise anywhere along the sympathetic nervous system.[11] The overall 5-year survival rate for neuroblastoma is ⩽40%, despite current intensive multimodality treatments.[12-14] Considering that *ALK* mutations preferentially involve advanced neuroblastoma with a poor outcome, the more relevant implication of these findings is that ALK inhibitors may improve the clinical outcome of children suffering from intractable neuroblastoma.

In this study, we demonstrated another mechanism of aberrant ALK activation in neuroblastoma, in which an abnormal *ALK* gene with a deletion of exons 2 and 3 was amplified in a neuroblastoma-derived cell line (NB-1), leading to high-level expression of an ALK protein variant with a truncated extracellular domain (ALK^{del2-3}). Furthermore, we demonstrated that ALK^{del2-3} had constitutive kinase activity and showed a transforming capacity in NIH3T3 cells. Moreover, ALK inhibition experiments using small interfering RNA (siRNA)-mediated gene knockdown and the low-molecular-weight compound, TAE684, also supported the oncogenic role of ALK^{del2-3}. Our results will help elucidate the mechanism of aberrant activation of ALK kinase and the role of activated ALK in the pathogenesis of neuroblastoma.

RESULTS

Detection of a short-form ALK protein in NB-1 cells

To examine the status of ALK in neuroblastoma, western blotting analysis was performed with a panel of 24 neuroblastoma-derived cell lines (Table 1). Among the 24 samples examined, the NB-1 cell line showed high-level expression of an ALK protein having a low molecular weight of 208 kDa compared with the molecular weight of 220 kDa for the wild-type protein (Figure 1a). Subsequent sequencing and reverse transcription–polymerase chain reaction (RT–PCR) analysis of *ALK* messages from NB-1 cells revealed the presence of an aberrant *ALK* transcript with a 285-bp in-frame deletion in the 5′ region corresponding to exons 2 and 3 (Figures 1b and c), which should result in the production of an abnormal ALK protein with a truncated N-terminal extracellular domain. Using a primer set for exons 2 and 3, a 166-bp product was also detected in NB-1 cells, indicating the presence of the wild-type *ALK* allele in NB-1 cells (Figures 1b and c). The deletion spanned 224–318 amino acids (aa), including the N-terminal end of the first meprin A5 protein and receptor protein tyrosine phosphatase mu (MAM) domain (aa 264–427) (Figure 1d).[15,16] We analyzed full-length *ALK* cDNAs isolated from 71 primary neuroblastoma samples for possible nucleotide deletions using RT–PCR (Table 2), but no deletions were detected.

[1]Department of Pediatrics, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; [2]Department of Cell Therapy and Transplantation Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; [3]Cancer Genomics Project, Graduate School of Medicine, University of Tokyo, Tokyo, Japan and [4]Gunma Children's Medical Center, Maebashi, Japan. Correspondence: Dr J Takita, Cell Therapy and Transplantation Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: jtakita-tky@umin.ac.jp
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