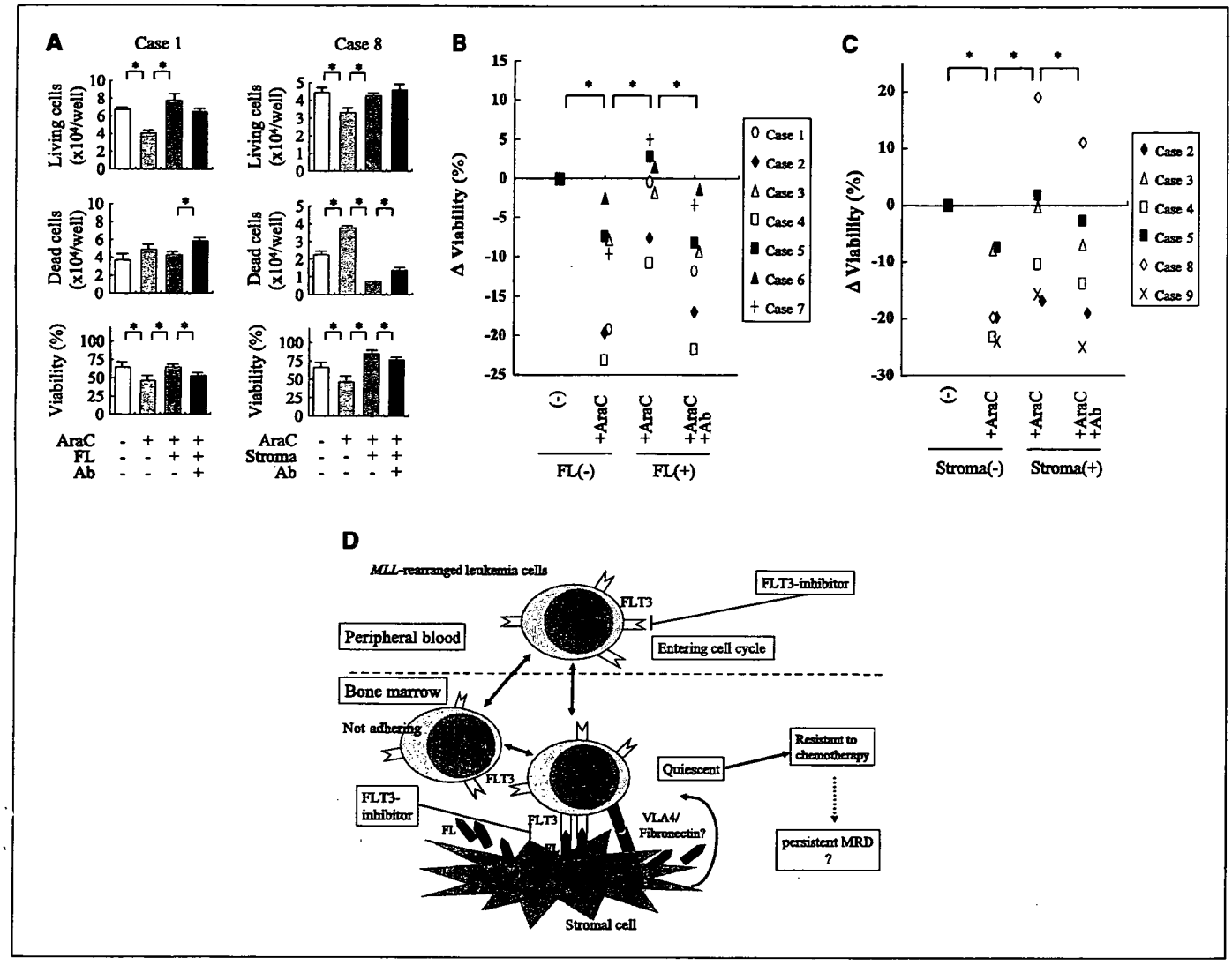

Figure 4. Effect of FL as a soluble or membrane form on chemotherapeutic agent–induced cell death of *MLL*-rearranged primary leukemia cells. *MLL*-rearranged primary leukemia cells (1×10^5/well) were precultured for 24 h in the presence or absence of FL (40 ng/mL) or bone marrow stromal (KM-104) cells with or without the addition of anti-FL antibody (4 μg/mL), and then cultured with or without AraC (100 nmol/L) for 24 h. Numbers of living and dead cells were counted in triplicate by dye-exclusion test and viabilities were calculated. The Δ viability (treated viability − control viability) was also calculated in each culture condition. *A*, representative data in cases 1 and 8. *Columns*, mean; *bars*, SE. *, $P < 0.05$, significant difference by *t* test. *B*, FL-mediating changes in the Δ viabilities in seven primary samples. *, $P < 0.05$, significant difference by the Wilcoxon's test. *C*, bone marrow stromal cell–mediating changes in the Δ viabilities in six primary samples. *, $P < 0.05$, significant difference by the Wilcoxon's test. *D*, schema of the hypothesis illustrating a possible role of the FL/FLT3 interaction for persistent MRD in *MLL*-rearranged leukemia. *MLL*-rearranged leukemia cells not adhering to bone marrow stromal cells are entering cell cycle and considerably sensitive to chemotherapy, but those adhering bone marrow cells are quiescent and resistant to chemotherapy, at least in part via the interaction of FL/FLT3, which results in persistent MRD. The FLT3 inhibitor might be effective by two mechanisms *in vivo*: one directly induces apoptosis of leukemia cells and another awakens quiescent leukemia cells adhering to bone marrow stromal cells to enter cell cycle.

a greater than 90%; the most dismal clinical issue in this disease is an early relapse that frequently occurs during the first 6 months of chemotherapy and before hematopoietic stem cell transplantation (34), resulting in shorter event-free survival and overall survival. Recently, the prognostic value of MRD after induction chemotherapy has been emphasized in childhood ALL (35, 36). Although the importance of MRD is not fully characterized yet in *MLL*-rearranged ALL, persistence of high levels of MRD in bone marrow should be associated with a high and early relapse rate in this disease.

We found that *MLL*-rearranged leukemia cells with wild-type FLT3 showed an inhibitory response to FL. This FL-induced inhibition was due to the induction of cell cycle arrest, in the process of which up-regulation of p27 and dephosphorylation of STAT5 might be implicated profoundly. Importantly, these arrested leukemia cells, not only established lines but also primary samples, showed resistance to apoptosis after exposure to irradiation or chemotherapeutic drugs. Because FL is reported to be expressed at high levels as a soluble or membrane-bound form by bone marrow stromal cells (29), it is postulated that *MLL*-rearranged leukemia cells tightly adhering to bone marrow stromal cells are induced to cell cycle arrest via the FL/FLT3 interaction, which might lead leukemia cells to become "dormant cells" that are resistant to antileukemic agents. In addition, the serum level of FL is reported to increase dramatically in patients who experience chemotherapy-induced bone marrow suppression (37). Therefore, leukemia cells

in patients after intensified chemotherapy are speculated to be exposed to high level of FL not only in bone marrow but also in the periphery. We showed in the *in vitro* model study that *MLL*-rearranged leukemia cells adhering to the stroma cell line partially restored sensitivity to antileukemic agents in the presence of anti-FL antibody. In AML, it has been reported that leukemia cells acquire resistance to AraC and daunorubicin via the interaction of VLA4 expressed on AML cells with fibronectin expressed on bone marrow stromal cells (38). This VLA4/fibronectin interaction was confirmed to play a pivotal role in MRD of AML in both the animal model and the clinical study showing a poor prognosis of VLA4-positive AML compared with a good prognosis of VLA4-negative AML (38). We thus present the hypothesis as illustrated in Fig. 4*D*, postulating that *MLL*-rearranged leukemia cells not adhering to bone marrow stromal cells are sensitive to chemotherapy, but those adhering to bone marrow stromal cells are rendered resistant to chemotherapy, at least in part via the interaction of FL/FLT3, which results in persistent MRD that is closely associated with the high relapse rate of this disease. According to this scenario, the FLT3 kinase inhibitors, such as PKC412, should be effective *in vivo* in the treatment of *MLL*-rearranged leukemia because they can exert their inhibitory action through two mechanisms: one directly induces apoptosis of leukemia cells via blockade of the kinase activity required for their survival as recently reported (21, 22, 39) and another awakens "dormant" leukemia cells and induces them to enter a chemosensitive cell cycling state via blockage of the signal through the FL/FLT3 interaction that occurs on the surfaces of leukemia cells and bone marrow stromal cells. The cell surface adhesion molecule VCAM-1 or asparagine synthetase, expressed on or secreted from bone marrow stromal cells, respectively, have also been reported to be involved in resistance to chemotherapy in ALL cells (40, 41).

The precise molecular mechanism of the FL-induced cell cycle arrest in *MLL*-rearranged leukemia cells remains elusive. We found that p27 is markedly up-regulated after FL stimulation, and this was presumably due to prevention of degradation of this protein in *MLL*-rearranged leukemia. As a key member of the KIP/CIP family of CDKIs, p27 blocks cell cycle progression at G_1 phase, primarily by inhibiting the cyclin E/CDK2 complex (42, 43). It is known that p27 is degraded by the ubiquitin-proteasome pathway and that quiescent cells exhibit a smaller amount of ubiquitinating activity (44), which might account for prolongation of the p27 half-life in FL-treated *MLL*-rearranged leukemia cells. Therefore, it is not clear at present whether up-regulation of p27 is the primary molecular event in FL-induced cell cycle arrest, or it is a secondary event in quiescent ("dormant") cells occurring after cell cycle arrest has been induced by other molecular mechanism(s). We also found that phosphorylation of STAT5, but not p44/42 MAPK and Akt, was almost abolished in arrested *MLL*-rearranged leukemia cells after FL stimulation. Because selective activation of STAT5 is shown to play a pivotal role in the self-renewal of leukemic cells as well as in normal hematopoiesis (45), the specific inactivation of STAT5 after FL stimulation might be critical to the induction of cell cycle arrest. The molecular mechanism of FL-induced STAT5 inactivation is still elusive and will be the subject for the future study.

The most important issue to be addressed is why *MLL*-rearranged ALL cells, unlike other B-precursor ALL cells, show a inhibitory response in proliferation after FL stimulation. Analyses of gene expression in leukemia have provided direct insights into the pathogenesis of leukemias and their responses to therapy. Armstrong et al. (19) reported that *MLL*-rearranged ALL has a distinct gene expression profile, including high FLT3 expression compared with other types of ALL. Thus, specific genes and their products that are uniquely activated in *MLL*-rearranged leukemia might be associated with a unique inhibitory response to FL.

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References

1. Rosnet O, Marchetto S, deLapeyriere O, Birnbaum D. Murine Flt3, a gene encoding a novel tyrosine kinase receptor of the PDGFR/CSF1R family. Oncogene 1991;6:1641–50.
2. Matthews W, Jordan CT, Wiegand GW, Pardoll D, Lemischka IR. A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations. Cell 1991;65:1143–52.
3. Rosnet O, Schiff C, Pebusque MJ, et al. Human FLT3/FLK2 Gene: cDNA cloning and expression in hematopoietic cells. Blood 1993;82:1110–9.
4. Small D, Levenstein M, Kim E, et al. STK-1, the human homolog of Flk-2/Flt-3, is selectively expressed in CD34$^+$ human bone marrow cells and is involved in the proliferation of early progenitor/stem cells. Proc Natl Acad Sci U S A 1994;91:459–63.
5. Broxmeyer HE, Lu L, Cooper S, Ruggieri L, Li ZH, Lyman SD. Flt3 ligand stimulates/costimulates the growth of myeloid stem/progenitor cells. Exp Hematol 1995;23:1121–9.
6. Piacibello W, Fubini L, Sanavio F, et al. Effects of human FLT3 ligand on myeloid leukemia cell growth: heterogeneity in response and synergy with other hematopoietic growth factors. Blood 1995;86:4105–14.
7. Carow CE, Levenstein M, Kaufmann SH, et al. Expression of the hematopoietic growth factor receptor FLT3 (STK-1/Flk2) in human leukemias. Blood 1996;87:1089–96.
8. Drexler HG. Expression of FLT3 receptor and response to FLT3 ligand by leukemic cells. Leukemia 1996;10:588–99.
9. McKenna HJ, Smith FO, Brasel K, et al. Effects of flt3 ligand on acute myeloid and lymphocytic leukemic blast cells from children. Exp Hematol 1996;24:378–85.
10. Nakao M, Yokota S, Iwai T, et al. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. Leukemia 1996;10:1911–8.
11. Abu-Duhier FM, Goodeve AC, Wilson GA, Care RS, Peake IR, Reilly JT. Identification of novel FLT-3 Asp835 mutations in adult acute myeloid leukaemia. Br J Haematol 2001;113:983–8.
12. Yamamoto Y, Kiyoi H, Nakano Y, et al. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. Blood 2001;97:2434–9.
13. Kottaridis PD, Gale RE, Frew ME, et al. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. Blood 2001;98:1752–9.
14. Gilliland DG, Griffin JD. The roles of FLT3 in hematopoiesis and leukemia. Blood 2002;100:1532–42.
15. Xu F, Taki T, Yang HW, 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.
16. Zwaan CM, Meshinchi S, Radich JP, et al. FLT3 internal tandem duplication in 234 children with acute myeloid leukemia: prognostic significance and relation to cellular drug resistance. Blood 2003;102:2387–94.
17. Pui CH, Behm FG, Downing JR, et al. 11q23/MLL rearrangement confers a poor prognosis in infants with acute lymphoblastic leukemia. J Clin Oncol 1994;12:909–15.
18. Taki T, Ida K, Bessho F, et al. Frequency and clinical significance of the MLL gene rearrangements in infant acute leukemia. Leukemia 1996;10:1303–7.
19. Armstrong SA, Staunton JE, Silverman LB, et al. MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. Nat Genet 2002;30:41–7.
20. Taketani T, Taki T, Sugita K, 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.
21. Armstrong SA, Kung AL, Mabon ME, et al. Inhibition of FLT3 in MLL: Validation of a therapeutic target

identified by gene expression based classification. Cancer Cell 2003;3:173–83.
22. Brown P, Levis M, Shurtleff S, Campana D, Downing J, Small D. FLT3 inhibition selectively kills childhood acute lymphoblastic leukemia cells with high levels of FLT3 expression. Blood 2005;105:812–20.
23. Nakamura M, Sugita K, Inukai T, et al. p16/MTS1/INK4A gene is frequently inactivated by hypermethylation in childhood acute lymphoblastic leukemia with 11q23 translocation. Leukemia 1999;13:884–90.
24. Inukai T, Sugita K, Iijima K, et al. Leukemic cells with 11p23 translocations express granulocyte colony-stimulating factor (G-CSF) receptor and their proliferation is stimulated with G-CSF. Leukemia 1998;12:382–9.
25. Harigaya K, Handa H. Generation of functional clonal cell lines from human bone marrow stroma. Proc Natl Acad Sci U S A 1985;82:3477–80.
26. Zhang S, Mantel C, Broxmeyer HE. Flt3 signaling involves tyrosyl-phosphorylation of SHP-2 and SHIP and their association with Grb2 and Shc in Baf3/Flt3 cells. J Leukoc Biol 1999;65:372–80.
27. Zhang S, Fukuda S, Lee Y, et al. Essential role of signal transducer and activator of transcription (Stat)5a but not Stat5b for Flt3-dependent signaling. J Exp Med 2000;192:719–28.
28. Stirewalt DL, Radich JP. The role of FLT3 in haematopoietic malignancies. Nat Rev Cancer 2003;3:650–65.
29. Lisovsky M, Braun SE, Ge Y, et al. Flt3-ligand production by human bone marrow stromal cells. Leukemia 1996;10:1012–8.
30. Pui CH, Gaynon PS, Boyett JM, et al. Outcome of treatment in childhood acute lymphoblastic leukaemia with rearrangements of the 11q23 chromosomal region. Lancet 2002;359:1909–15.
31. Pui CH, Chessells JM, Camitta B, et al. Clinical heterogeneity in childhood acute lymphoblastic leukemia with 11q23 rearrangements. Leukemia 2003;17:700–6.
32. Chessells JM, Harrison CJ, Watson SL, Vora AJ, Richards SM; Medical Research Council Working Party on Childhood Leukaemia. Treatment of infants with lymphoblastic leukaemia: results of the UK Infant Protocols 1987-1999. Br J Haematol 2002;117:306–14.
33. Kosaka Y, Koh K, Kinukawa N, et al. Infant acute lymphoblastic leukemia with MLL gene rearrangements: outcome following intensive chemotherapy and hematopoietic stem cell transplantation. Blood 2004;104:3527–34.
34. Isoyama K, Eguchi M, Hibi S, et al. Risk-directed treatment of infant acute lymphoblastic leukaemia based on early assessment of MLL gene status: results of the Japan Infant Leukaemia Study (MLL96). Br J Haematol 2002;118:999–1010.
35. Cave H, van der Werff ten Bosch J, Suciu S, et al.; European Organization for Research and Treatment of Cancer-Childhood Leukemia Cooperative Group. Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia. N Engl J Med 1998;339:591–8.
36. Coustan-Smith E, Sancho J, Hancock ML, et al. Clinical importance of minimal residual disease in childhood acute lymphoblastic leukemia. Blood 2000;96:2691–6.
37. Wodnar-Filipowicz A, Lyman SD, Gratwohl A, Tichelli A, Speck B, Nissen C. Flt3 ligand level reflects hematopoietic progenitor cell function in aplastic anemia and chemotherapy-induced bone marrow aplasia. Blood 1996;88:4493–9.
38. Matsunaga T, Takemoto N, Sato T, et al. Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia. Nat Med 2003;9:1158–65.
39. Brown P, Small D. FLT3 inhibitors: a paradigm for the development of targeted therapeutics for paediatric cancer. Eur J Cancer 2004;40:707–21.
40. Mudry RE, Fortney JE, York T, Hall BM, Gibson LF. Stromal cells regulate survival of B-lineage leukemic cells during chemotherapy. Blood 2000;96:1926–32.
41. Iwamoto S, Mihara K, Downing JR, Pui CH, Campana D. Mesenchymal cells regulate the response of acute lymphoblastic leukemia cells to asparaginase. J Clin Invest 2007;117:1049–57.
42. Sherr CJ, Roberts JM. Inhibitors of mammalian G_1 cyclin-dependent kinases. Genes Dev 1995;9:1149–63.
43. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G_1-phase progression. Genes Dev 1999;13:1501–12.
44. Pagano M, Tam SW, Theodoras AM, et al. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science 1995;269:682–5.
45. Kato Y, Iwama A, Tadokoro Y, et al. Selective activation of STAT5 unveils its role in stem cell self-renewal in normal and leukemic hematopoiesis. J Exp Med 2005;202:169–79.

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REFERENCES

1. Osato M, Asou N, Abdalla E, et al. Biallelic and heterozygous point mutations in the runt domain of the AML1/PEBP2alphaB gene associated with myeloblastic leukemias. *Blood.* 1999;93:1817–1824.
2. Preudhomme C, Warot-Loze D, Roumier C, et al. High incidence of biallelic point mutations in the runt domain of the AML1/PEBP2 alpha B gene in Mo acute myeloid leukemia and in myeloid malignancies with acquired trisomy 21. *Blood.* 2000;96:2862–2869.
3. Shih LY, Huang CF, Wang PN, et al. Acquisition of FLT3 or N-ras mutations is frequently associated with progression of myelodysplastic syndrome to acute myeloid leukemia. *Leukemia.* 2004;18: 466–475.
4. Gratias EJ, Liu YL, Meleth S, et al. Activating FLT3 mutations are rare in children with juvenile myelomonocytic leukemia. *Pediatr Blood Cancer.* 2005;44: 142–146.
5. Taketani T, Taki T, Takita J, et al. Mutation of the AML1/RUNX1 gene in a transient myeloproliferative disorder patient with Down syndrome. *Leukemia.* 2002;16:1866–1867.
6. Xu F, Taki T, Yang HW, 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;105:155–162.
7. Matsuno N, Osato M, Yamashita N, et al. Dual mutations in the AML1 and FLT3 genes are associated with leukemogenesis in acute myeloblastic leukemia of the M0 subtype. *Leukemia.* 2003;17: 2492–2499.

Routine Use of PET Scans After Completion of Therapy in Pediatric Hodgkin Disease Results in a High False Positive Rate

In Response:

Drs Mardis and Wong raise important points in response to our published report regarding the routine use of positron emission tomography (PET) scans for surveillance of disease recurrence after completion of therapy in children and adolescents with Hodgkin lymphoma.[1] Our study was designed to evaluate PET scans as they are commonly used in the patient population. Hence, our data included all PET scans, as they are used for surveillance even when PET scans were not used in the diagnostic period. Similarly, in practice, multiple physicians read PET scans and variability in interpretation is itself a limitation of the use of PET scans. Drs Mardis and Wong note that perhaps some of false positives in the early stages were due to inexperience, however, we presented information that the false positive rate remained the same throughout the study period suggesting that a learning curve did not account for the findings of the study.

Since our publication Meany et al[2] published a study, evaluating 23 consecutive pediatric patients with Hodgkin disease and compared PET scan results with clinical status and computed tomography (CT) scans. Their results included a strong negative predictive value of 100% and a positive predictive value of 18.2%, findings that are almost identical to our results. They conclude, as we did, that positive PET scans must be interpreted conservatively and that treatment decisions should not be made on the findings of a positive study.

Ultimately, Dr Mardis and Wong's conclusion that CT/PET scanners will solve many of the issues that commonly occur with the use of PET scans supports the conclusion that PET scans are not an ideal imaging modality for off therapy patients. Our concern, and the rationale for publishing our data, is that PET scans are still used in many centers where combined CT/PET scans are not available and also continue to be used in research studies. Thus, the issues that arise with the use of PET scans, including the presence of false positive results, are likely to continue to present management dilemmas to our colleagues and need to be acknowledged. The International Harmonization Project recently evaluated the existing data on the use of PET scans in lymphoma therapy.[3] Consistent with our recommendations, the report concluded that the current data is inadequate to recommend routine surveillance PET scans after completion of therapy.

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REFERENCES

1. Levine JM, Weiner M, Kelly KM. Routine use of PET scans after completion of therapy in pediatric Hodgkin disease results in a high false positive rate. *J Pediatr Hematol Oncol.* 2006;28:711–714.
2. Meany HJ, Gidvani VK, Minniti CP. Utility of PET scans to predict disease relapse in pediatric patients with Hodgkin lymphoma. *Pediatr Blood Cancer.* 2007; 48:399–402.
3. Cheson BD, Pfistner B, Juweid ME, et al. Revised response criteria for malignant lymphoma. *J Clin Oncol.* 2007;25:579–586.

The Accuracy of PET(CT) in Evaluating Pediatric Lymphoma

To the Editor:

We read with great interest the recent article by Levine et al[1] in the November 2006 issue of *Journal of Pediatric Hematology/Oncology*. We have some concerns about the results of this study.

First, only a portion of the cohort had a positron emission tomography (PET) scan at initial diagnosis to serve as a reference for disease response to therapy. Correcting this deficit in surveillance might have minimized false positive results in their patient population. Besides, there was no quantification of metabolic activity, which is important in defining the underlying biologic behavior of the lymphoma. As described by Wong et al,[2] quantitative examination of the glucose metabolic rate by the

AML1 Mutation and *FLT3*-internal Tandem Duplication in Leukemia Transformed From Myelodysplastic Syndrome

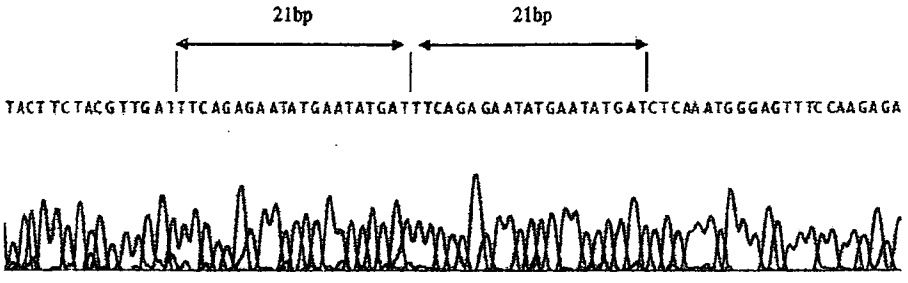

FIGURE 2. Sequence of *FLT3* gene. ITD of 21 bp in exon 11 was observed. The wild type of *FLT3* was not found.

To the Editor:

Myelodysplastic syndrome (MDS) is a clonal disorder of hematopoietic stem cells characterized by ineffective and inadequate hematopoiesis. Recently, gene alterations including *AML1/RUNX1* had been demonstrated to contribute to the development from MDS to secondary acute myeloid leukemia (AML) in adult patients, particular in AML-M0 or AML with acquired trisomy 21.[1,2] Moreover, *FLT3*-internal tandem duplication (ITD) predicts a high risk of progression of MDS to AML in adult patients,[3] but these gene alterations were rarely reported in pediatric MDS.[4]

We reported here a 6-year-old girl with leukocytosis, which consisted of monocytosis and immature myeloid cells. She did not show hepatomegaly, splenomegaly, café-au-lait spots, or other abnormal physical findings. A bone marrow aspirate showed a hypercellular marrow with greatly increased myeloid cells and megakaryocytes, but blasts were less than 1%. Only minimal dysplastic change was seen in her bone marrow cells with normal female karyotype. A provisional diagnosis was adult-type chronic myelomonocytic leukemia. She developed acute mixed-lineage leukemia carrying trisomy 21 after 4 years from the initial diagnosis. The disease progressed rapidly, and she died after allogeneic stem cell transplantation.

We analyzed mutations in the runt domain of *AML1*gene by polymerase chain reaction or reverse-transcription polymerase chain reaction followed direct sequencing using the primers previously described.[5] The patient had a mutation within intron 3 of *AML1* gene (T to A; − 10 from exon 4, Fig. 1). The mutation led to an 8-bp insertion on 1 mRNA allele resulting from change in a splicing acceptor site in intron 3; this induced a frameshift that produced a stop codon. Both normal and mutant *AML1* sequences were found in this patient (Fig. 1).

Moreover, we analyzed the juxtermembrane domain of the *FLT3* gene using primer pairs R5 and R6[6] and found an ITD of 21 bp in exon 11 (Fig. 2), but we could not find the wild-type product of *FLT3* gene. Moreover, mutations of *RAS* and *PTPN11* genes were not found in this patient.

Interestingly, dual mutations in the *AML1* and *FLT3* genes were found in AML-M0 subtype in adult MDS patients.[7] We considered that both *AML1* mutation and *FLT3*-ITD may have a role in disease progression. However, we could not examine the *AML1* mutation and *FLT3*-ITD at the time of chronic phase because the sample was not available. Larger studies regarding gene alterations in pediatric MDS will be needed to clarify these associations.

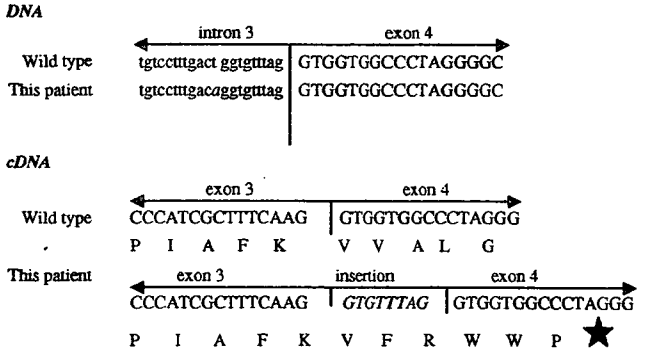

FIGURE 1. The schema of mutation in the runt domain of the *AML1/RUNX1* gene. A point mutation at intron 3 (t→a; italic letter -10 from exon 4), led to an 8-bp insertion (italic letters) on 1 cDNA allele because of the change in the splicing acceptor site in intron 3, inducing a frameshift resulting in a stop codon (pentagram). The wild type of *AML1* was also found.

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ORIGINAL ARTICLE

Outcome of risk-based therapy for infant acute lymphoblastic leukemia with or without an *MLL* gene rearrangement, with emphasis on late effects: a final report of two consecutive studies, MLL96 and MLL98, of the Japan Infant Leukemia Study Group

D Tomizawa[1], K Koh[2], T Sato[3], N Kinukawa[4], A Morimoto[5], K Isoyama[6], Y Kosaka[7], T Oda[8], M Oda[9], Y Hayashi[10], M Eguchi[11], K Horibe[12], T Nakahata[13], S Mizutani[1] and E Ishii[14]

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We evaluated the efficacy of a treatment strategy in which infants with acute lymphoblastic leukemia (ALL) were stratified by their *MLL* gene status and then assigned to different risk-based therapies. A total of 102 patients were registered on two consecutive multicenter trials, designated MLL96 and MLL98, between 1995 and 2001. Those with a rearranged *MLL* gene (MLL-R, $n=80$) were assigned to receive intensive chemotherapy followed by hematopoietic stem cell transplantation (HSCT), while those with germline *MLL* (MLL-G, $n=22$) were treated with chemotherapy alone. The 5-year event-free survival (EFS) rate for all 102 infants was 50.9% (95% confidence interval, 41.0–60.8%). The most prominent late effect was growth impairment, observed in 58.9% of all evaluable patients in the MLL-R group. This plan of risk-based therapy appears to have improved the overall prognosis for infants with ALL, compared with previously reported results. However, over half the events in patients with *MLL* rearrangement occurred before the instigation of HSCT, and that HSCT-related toxic events comprised 36.3% (8/22) of post-transplantation events, suggesting that further stratification within the MLL-R group and the development of more effective early-phase intensification chemotherapy will be needed before the full potential of this strategy is realized.

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Keywords: infant acute lymphoblastic leukemia; *MLL* gene; hematopoietic stem cell transplantation; late effects

Introduction

The outcome of therapy for children with acute lymphoblastic leukemia (ALL) has markedly improved over the last four decades, to the extent that approximately 80% of affected patients are now cured.[1] However, infants with ALL, who represent 2.5–5% of all childhood ALL cases, continue to have high relapse rates and a dismal prognosis, as illustrated by recently published event-free survival (EFS) rates of 22–54%.[2–8] ALL in infancy has several distinctive clinical characteristics compared with common childhood ALL, including hyperleukocytosis, hepatosplenomegaly, central nervous system (CNS) disease and a high frequency of molecularly evident rearrangement of the *MLL* gene at chromosome band 11q23.[9–12] Among these features, *MLL* gene rearrangement, found in 70–80% of infant ALL cases studied with molecular techniques, is an independent risk factor most predictive of recurrent leukemia.[13–15]

The high failure rate in infants with ALL, especially those with *MLL* gene rearrangement, can be attributed to early relapse after remission rather than toxicity, and warrants consideration of intensified therapy. We therefore segregated infants with ALL into two subgroups according to their *MLL* gene status in two consecutive nationwide multicenter studies, designated MLL96 and MLL98. Infants with a rearranged *MLL* (MLL-R) gene received intensive chemotherapy followed by hematopoietic stem cell transplantation (HSCT), while those with a germline *MLL* gene were treated with standard intensive chemotherapy for ALL alone. This report updates findings published earlier[16–18] and extends the analysis to long-term side effects. By combining data from two studies with similar treatment strategies, we were able to analyze results for a relatively large cohort with this rare subtype of ALL. The additional detail on prognostic features should facilitate further risk stratification among *MLL*-rearranged cases, while outcome data on the increased number of patients undergoing HSCT should stimulate critical discussion of the role of this procedure in future treatment strategies for infants with ALL.

Materials and methods

Patients

Between December 1995 and December 2001, 102 consecutive infants with ALL, younger than 12 months, were registered and treated on two protocols, designated MLL96 (55 patients) and MLL98 (47 patients). These studies accrued more than 80% of all Japanese infants with ALL over the 6-year enrollment period, based on results of a nationwide surveillance study.[17] The

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diagnosis of ALL was based on bone marrow morphology and cytochemical staining results. Each patient was evaluated with respect to the characteristics of the leukemic cells, including immunophenotype, cytogenetics and *MLL* gene status. Written informed consent, provided according to the Declaration of Helsinki, was obtained from the guardians of the patients, with institutional review board approval obtained for all aspects of this investigation.

Detection of MLL *gene rearrangement and cytogenetic analysis*

The *MLL* gene status of each patient was determined by Southern blot analysis and/or split-signal fluorescence *in situ* hybridization (FISH) as previously described.[16] Leukemic cell karyotypes were determined by cytogenetic analysis with a G-banding technique.[16]

Treatment

The details of the therapeutic regimens used in the MLL96 and MLL98 studies are described in Supplementary Tables.[16–18] Briefly, patients with rearranged *MLL* (MLL-R) received induction therapy and three courses of postremission intensification therapy followed by HSCT if a suitable donor was available. Patients with germline *MLL* (MLL-G) were assigned to a chemotherapy arm consisting of induction, consolidation and CNS prophylaxis, intensification, reinduction and maintenance phases, administered over 83–85 weeks. Except for vincristine, drug dosages on the MLL98 protocol were calculated on the basis of body surface area, while dosages on the MLL96 protocol were based on body weight. This modification increased the dosages of all antileukemic drugs used in the MLL98 study by 1.2- to 2-fold over those in the MLL96 study.

Patients in the MLL-R group underwent HSCT in first remission (CR1), if a 5 to 6/6 human leukocyte antigen-matched related donor, 6/6-matched unrelated donor or 4 to 6/6-matched unrelated cord blood donor was available. The protocol-specified conditioning regimen comprised either a total-body irradiation (TBI; 12 Gy in six fractions, twice a day on days −7 to −5) or busulfan (BU; 35 mg/m^2/dose orally, 4 times a day on days −8 to −5) with a combination of etoposide (60 mg/kg intravenously on day −4) and cyclophosphamide (60 mg/kg intravenously on days −3 and −2). Prophylaxis for graft-vs-host disease (GVHD) consisted of either cyclosporine or tacrolimus combined with short-term methotrexate.

Evaluation of the late effects

Late effects studied included cardiac, pulmonary, renal, endocrine, dental, orthopedic, dermatologic, ophthalmologic, auditory, psychological, growth and development and occurrence of secondary malignancies. Medical records regarding these issues were reviewed by each physician of the participating centers, and these data were collected by questionnaire which was sent to each participating center.

Statistical considerations

The analysis of treatment outcome was updated on 30 September 2006, combining data from both the MLL96 and MLL98 studies because of their similar 5-year survival estimates (see Results). EFS and overall survival (OS) rates were estimated by the method of Kaplan–Meier and standard errors (s.e.) with the Greenwood formula, and then were compared with the log-rank test. Confidence intervals (CIs) were computed with a 95% confidence level. The clinical, demographic and biologic features of patients were compared with χ^2 tests for homogeneity. A Cox regression model was used for the multivariate analysis. *P*-values, when cited, are two-sided, with a value of 0.05 or less taken to indicate statistical significance.

Table 1 Characteristics of infants with ALL treated in the MLL96 and MLL98 studies

	Overall, n *(%)*	*MLL-R,* n *(%)*	*MLL-G,* n *(%)*	P-*value**
Total no. of patients	102	80	22	
Age (months)				<0.001
<3	19 (18.6)	19 (23.8)	0 (0.0)	
3 to <6	31 (30.4)	27 (33.7)	4 (18.2)	
⩾6	52 (51.0)	34 (42.5)	18 (81.8)	
Gender				<0.001
Male	52 (51.0)	32 (40.0)	20 (90.9)	
Female	50 (49.0)	48 (60.0)	2 (9.1)	
WBC count (× 10^9/l)				<0.001
<100	44 (43.2)	25 (31.3)	19 (86.4)	
100 to <300	34 (33.3)	32 (40.0)	2 (9.1)	
⩾300	24 (23.5)	23 (28.7)	1 (4.5)	
CNS disease[a]				0.05
Positive	15 (14.7)	15 (18.8)	0 (0.0)	
Negative	81 (79.4)	59 (73.8)	22 (100.0)	
Unknown	6 (5.9)	6 (7.4)	0 (0.0)	
CD10				<0.001
Positive	24 (23.5)	3 (3.8)	21 (95.5)	
Negative	78 (76.5)	77 (96.2)	1 (4.5)	
Karyotype[b]				<0.001
t(4;11)(q21;q23)	41 (40.1)	41 (51.2)	0 (0.0)	
t(11;19)(q23;p13)	7 (6.9)	7 (8.8)	0 (0.0)	
t(9;11)(p22;q23)	6 (5.9)	6 (7.5)	0 (0.0)	
Other 11q23	6 (5.9)	6 (7.5)	0 (0.0)	
No 11q23 rearrangement	35 (34.1)	13 (16.2)	22 (100.0)	
Unknown	7 (6.9)	7 (8.8)	0 (0.0)	

Abbreviations: ALL, acute lymphoblastic leukemia; CNS, central nervous system; FISH, fluorescence *in situ* hybridization; MLL-G, patients with germline *MLL*; MLL-R, patients with MLL gene rearrangement; WBC, white blood cell count.
*Comparison between the MLL-G and MLL-R subgroups.
[a]CNS disease was diagnosed if more than 5 cells/mm^3 with recognizable blasts were found in cerebrospinal fluid.
[b]All 80 patients in the MLL-R group, including the 13 cases with "No 11q23 rearrangement" and the 7 "Unknown" cases by normal karyotypic analysis, were confirmed as "*MLL* rearranged" by Southern blotting and/or split-signal FISH.

Results

Patient characteristics

The characteristics of the patients at diagnosis are reported in Table 1. We identified 80 patients with MLL-R and 22 with MLL-G by Southern blot analysis and/or FISH. Patients in the MLL-R group were significantly younger (median age, 4 vs 9 months, $P<0.001$), and had higher leukocyte counts (median, 168.4×10^9/l vs 21.8×10^9/l, $P<0.001$). The frequency of CNS disease (defined as more than 5 cells/mm^3 with recognizable blasts in cerebrospinal fluid) was also significantly higher in the MLL-R group. Expression of the CD10 antigen was closely

Table 2 Distribution of events by *MLL* gene status and treatment phase

	No. of patients	No. of events	No. of events during induction		No. of events before HSCT		No. of events after HSCT	
			No CR	Death	Relapse	Death in CR	Relapse	Death in CR
Overall	102	50	5	1	21	1	14	8
MLL-G	22	1	0	0	1	0	—	—
MLL-R	80[a]	49	5	1	20	1	14	8

Abbreviations: CR, complete remission; HSCT, hematopoietic stem cell transplantation; MLL-G, patients with germline *MLL*; MLL-R, patients with MLL gene rearrangement.
[a]No events were observed among all four patients in the MLL-R group who did not receive HSCT for lack of a suitable donor.

associated with MLL-R. By karyotyping analysis, 11q23 abnormalities were found in 60 of 73 evaluable cases (82.2%) in the MLL-R group, half of whom had the t(4;11)(q21;q23) abnormality. The clinical characteristics of patients enrolled in the MLL96 study were comparable to those in the MLL98 study (data not shown).

Treatment outcome

Remission induction and subsequent events. The numbers and types of events are summarized in Table 2. The overall remission induction rate was 94.1% (96/102): Remission induction rates were high in both the MLL-R and MLL-G groups: 92.5% (74/80 patients) in the MLL-R group and 100% (22/22 patients) in the MLL-G group. There was one induction death due to a fatal adenoviral infection and five induction failures in the MLL-R group. Two of the latter patients survived for 4.9 and 6.0 years, respectively, without evidence of disease after alternative therapies (either acute myeloid leukemia-directed chemotherapy or second HSCT).

Of the 74 patients in the MLL-R group who achieved CR1, 53 remained in continuous complete remission (CCR) during the postremission phase, 1 patient died of infectious pneumonia and 20 relapsed (19 with isolated marrow relapses and 1 with relapse site not specified) before reaching the timepoint of HSCT. Among these 20 relapsed patients, 12 underwent allogeneic HSCT in second remission (CR2) and 3 underwent HSCT without remission; the 5 of 12 who underwent HSCT in CR2 remain in remission for a median duration of 8.4 years (range, 5.7–10.2 years). Forty-nine of the 53 cases in CCR underwent HSCT in CR1: 2 autologous HSCT, 21 HSCT from a related ($n=12$) or unrelated donor ($n=9$) and 26-unrelated cord blood transplantation. The median time from remission to transplantation was 4 months (range, 0–9 months). Twenty-seven of the 49 patients with HSCT remained in CCR at the time of analysis, 8 died in CCR (four of veno-occlusive disease, one of cytomegalovirus infection, one of bacterial sepsis, one of gastrointestinal hemorrhage due to GVHD and thrombotic microangiopathy and one of an unspecified transplant-related complication) and 14 relapsed (nine with an isolated marrow relapses, one with combined marrow/CNS relapse, two with CNS relapse, one with testicular relapse and one with relapse site not specified). Among the 14 relapsed patients, 3 continue to survive for a median duration of 7.2 years (range, 5.0–10.1 years) after subsequent HSCT, while the remaining 11 patients eventually died, mostly of relapsed disease. The four patients who lacked a suitable donor received chemotherapy only as specified by the protocol and remained in CCR for median duration of 8.7 years (range, 3.6–10.8 years).

Analysis of overall outcome. The estimated 5-year OS and EFS rates for all 102 patients were 60.5% (95% CI, 50.7–70.2%) and 50.9% (95% CI, 41.0–60.8%), respectively, after a median follow-up of 7.1 years (range, 1.5–10.8 years). Patients in the MLL-R group had a significantly worse outcome than those in the MLL-G group: 5-year OS, 50.8% (95% CI, 39.6–62.0%) vs 95.5% (95% CI, 86.6–100%) (Figure 1, $P<0.001$) and 5-year EFS, 38.6% (95% CI, 27.7–49.5%) vs 95.5% (95% CI, 86.6–100%) (Figure 1, $P<0.001$).

The only difference between the MLL96 and MLL98 protocols was the higher dosages of antileukemic drugs in the latter study, which was not associated with improved outcome as demonstrated by 5-year EFS rates in the MLL-R group: 35.7% (95% CI, 21.0–50.4%) in MLL96 vs 41.8% (95% CI, 25.8–57.8%) in MLL98 ($P=0.67$). Neither conditioning regimen received nor donor source had a significant impact on post-transplantation EFS rates among the 49 patients with a rearranged *MLL* gene who underwent HSCT after CR1 (Table 3).

Treatment outcome in the MLL-R group according to prognostic factors. The prognostic impact of several potential risk factors (Table 3) was determined in the MLL-R group. Infants younger than 6 or 3 months and those with CNS disease at diagnosis had significantly worse 5-year EFS rates than did infants without these features. Gender, leukocyte count and karyotype lacked prognostic significance in this univariate analysis. Further analysis with a Cox regression model indicated that only age less than 6 months exerted independent predictive strength (data not shown).

Long-term side effects. It was possible to evaluate long-term sequelae among 57 of the all 62 survivors of infant ALL treated on the MLL96 and MLL98 studies: 39 in the MLL-R group and 18 in the MLL-G group (complete follow-up data were not available for the remaining five patients). The median age of the 57 patients at analysis was 7.7 years (range, 1.1–10.4 years). Thirty-six of the 57 patients, all in the MLL-R group, underwent HSCT. Twenty-two received the TBI-based conditioning regimen, while 14 received the non-TBI conditioning regimen. In the TBI group, four patients had undergone allogeneic HSCT twice.

Significant late effects were not observed among patients in the MLL-G group. By contrast, various late complications were observed in the MLL-R group as follows: chronic GVHD in 5; hypothyroidism in 5; short stature (defined as a height standard deviation (s.d.) score below −2.0 or a requirement for growth hormone therapy) in 23; skin abnormalities (alopecia, scleroderma, hyper- or hypo-pigmentation) in 12; fasciitis in 1; ophthalmologic complications (dry eye, corneal opacity, retinal vasculitis) in 5; pulmonary complications (interstitial pneumonia, bronchiolitis obliterans) in 6; chronic diarrhea with malnutrition in 1; dental abnormalities in 6; multiple exostosis in 1; epilepsy in 2 and neurocognitive deficits (learning

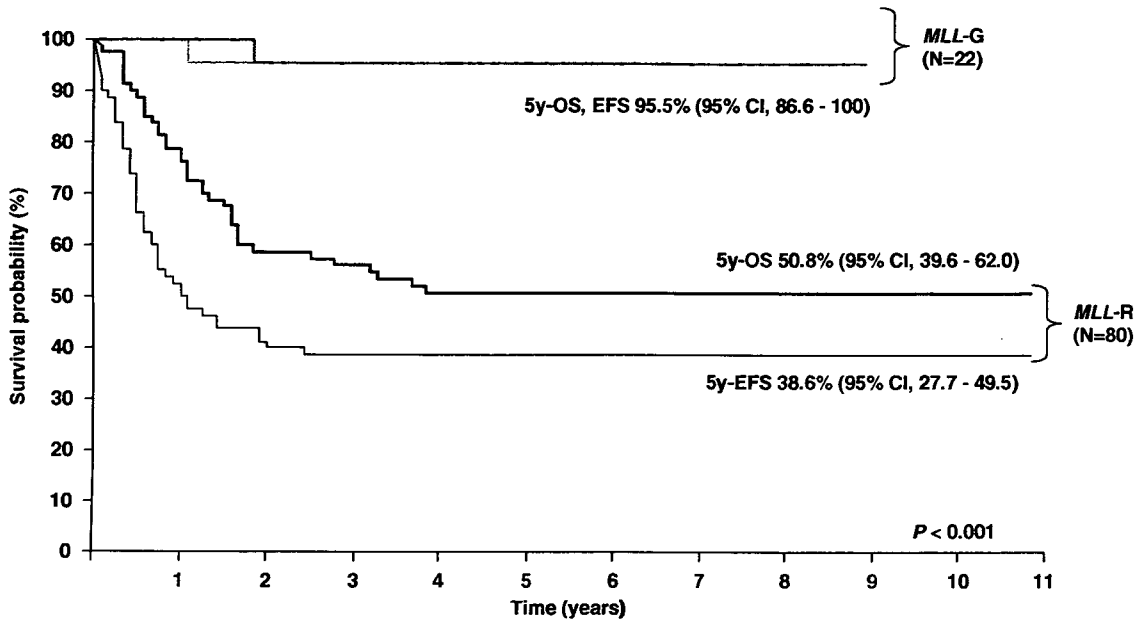

Figure 1 Overall survival (OS) and event-free survival (EFS) rates for infants with ALL treated in the MLL96 or MLL98 study by *MLL* status. Outcome was significantly better in patients with germline *MLL* (MLL-G) than in those with rearranged *MLL* (MLL-R) ($P<0.001$).

disability, intelligence impairment, autism) in 4. There were no cases of secondary malignancy or symptomatic chronic heart failure. Pubertal development could not be evaluated because all study patients were younger than 12 years old and had not entered puberty.

The distribution of height s.d. scores is shown in Figure 2. Median scores for the MLL-R and MLL-G subgroups were −2.49 (range, −8.05 to +2.92) and +0.05 (range, −1.00 to +0.90), respectively (Figure 2a, $P<0.001$). In contrast to the 36 patients who underwent HSCT, height s.d. scores for the three chemotherapy-only MLL-R patients were in the normal range (−1.88, −1.57, −1.48, respectively). The median height s.d. score for patients receiving TBI-based conditioning ($n=22$) was −3.07 (range, −8.05 to −0.08), which was significantly lower than the median value for those given non-TBI conditioning ($n=14$, −1.72 (range, −6.13 to +2.92)) (Figure 2b, $P=0.02$). However, 5 of the 14 patients in the latter group had height s.d. scores below −2.0.

Discussion

The Japanese MLL96 and MLL98 clinical trials are the first published prospective studies to stratify infants with ALL by their *MLL* gene status. The results demonstrated clear differences in clinical features, including treatment outcome, between patients with or without an MLL gene arrangement. In most previous reports of EFS rates for infants with ALL, these two subgroups have been combined,[2–8] when results are listed according to *MLL* gene status or CD10 expression, the EFS rates for those with a rearranged *MLL* gene or negative CD10 expression range from only 21 to 40%, and generally do not exceed 30%. Thus, our overall EFS rate of 50.9% justifies the decision to stratify infants with ALL by *MLL* gene status, so that appropriate risk-based treatments can be applied in each group. The method for determining *MLL* gene status is critical to segregating patients into 'true' *MLL* rearranged and non-rearranged cohorts. Because 16.2% of our *MLL*-rearranged patients lacked abnormalities in band 11q23 by normal karyotypic analysis, we conclude that molecularly based methods such as Southern blotting or split-signal FISH are essential in strategies to determine the accurate *MLL* gene status in infants with newly diagnosed ALL.

Whether allogeneic HSCT has an important role in the treatment of infants with ALL remains controversial because of the limited data on this issue.[19–27] Pui *et al.*[19] retrospectively analyzed cooperative group and individual transplant center data for children with ALL and 11q23 abnormalities, concluding that any type of HSCT was associated with a worse outcome than chemotherapy alone for t(4;11)-positive leukemia. In that study, the EFS rate for the 28 infants who underwent HSCT was only 19±3%, which is extremely low compared to our results and those of Sanders *et al.*,[27] who reported a 3-year disease-free survival rate of 42.2% among 40 infants with ALL following HSCT. However, this apparent improvement in outcome after HSCT must be interpreted with caution, since both the report of Sanders *et al.* and ours lack adequate retrospective or prospective control groups. Moreover, infants are the age group most vulnerable to intensive cytotoxic therapy, especially HSCT with a myeloablative preparative regimen, as illustrated by the high proportion of post-HSCT events in the current study (36.3%, 8/22) that were due to transplant-related toxicity. It is also notable that all four patients in the MLL-R subgroup, who did not receive HSCT for lack of a suitable donor, are alive without any subsequent events.

Allogeneic HSCT in infants always harbors the risk of late effects. In our analysis, 23 of 39 patients (58.9%) in the MLL-R group, especially those receiving TBI, had short stature after a median follow-up of 7.7 years. Sanders *et al.*[27] reported milder growth impairment in their series despite earlier treatment with a TBI-based conditioning regimen. This discrepancy may reflect the hyperfraction method of TBI used by Sanders *et al.*, such that 15.75 Gy was given three times a day over 7 days. Whatever the

Table 3 Five-year EFS by selected prognostic features for infants with a rearranged *MLL* gene

	No. of patients	*5-year EFS, % (s.e.)*	*P-value*
Age (months)			
<3	19	26.3 (10.1)	0.04
⩾3	61	42.4 (6.3)	
<6	46	27.8 (6.6)	0.02
⩾6	34	52.9 (8.5)	
Gender			
Male	32	37.5 (8.5)	0.74
Female	48	39.3 (7.0)	
WBC count ($\times 10^9$/l)			
<100	25	51.2 (10.1)	0.08
⩾100	55	32.7 (6.3)	
<300	57	41.9 (6.5)	0.28
⩾300	23	30.4 (9.6)	
CNS disease			
Positive	15	20.0 (10.3)	0.03
Negative	59	47.3 (6.5)	
Karyotype			
t(4;11)(q21;q23)	41	33.8 (7.4)	0.29
Others	39	46.8 (8.8)	
Conditioning regimen[a]			
TBI-based	26	47.1 (10.1)	
BU-based	23	65.2 (9.9)	0.24
Donor source[b]			
Unrelated cord blood	26	53.8 (9.7)	
Others	23	56.5 (10.3)	0.92

Abbreviations: BU, busulfan; CNS, central nervous system; EFS, event-free survival; HSCT, hematopoietic stem cell transplantation; s.e., standard error; TBI, total-body irradiation; WBC, white blood cell count.
[a]5-year EFS by conditioning regimen was compared among patients who had undergone HSCT in CR1.
[b]5-year EFS by donor source was compared among patients who had undergone HSCT in CR1.

explanation, we would stress that severe growth impairment was also observed among patients in the non-TBI group, which may indicate that any form of conditioning regimen for infants could increase the risk of growth retardation. We observed several other serious long-term side effects, but additional follow-up is needed before the impact of these complications can be fully assessed. Besides, caution is needed to evaluate some of the late effects reported here, because there is a certain methodological limitation in collecting these data, which is questionnaire-based, that may lead to an underestimation of these events.

Several steps will need to be taken to further improve the prognosis of infant ALL with *MLL* gene rearrangements. First, additional risk stratification may identify important subsets of patients who would benefit from alternative therapy. Our analysis indicated that age at diagnosis can be used to segregate patients into two subgroups with different 5-year EFS rates: 27.8% for infants younger than 6 months and 52.9% for infants 6 months or older. This approach would not only contribute to better control of the leukemic clone, but might also reduce treatment-related toxicity. Second, we would emphasize that nearly half of the events (21/49) in our MLL-R group occurred before the use of HSCT. Thus, despite an initial remission rate of more than 90%, effective strategies to prevent early relapse are urgently needed. Pieters *et al.*[28] described the *in vitro* drug-resistance data, which demonstrate greater sensitivity to cytarabine (Ara-C) and higher resistance to glucocorticoids and L-asparaginase by leukemic cells from infants. In fact, we did introduce intensive use of Ara-C and dexamethasone in our treatment, which may in part have contributed to improved outcome compared to the historical data. However, one might improve results by further intensifying the early phase of postremission intensification therapy with Ara-C and with intensive use of L-asparaginase, which was not used in the current therapy, to overcome the leukemic cell drug-resistance to this agent. Third, the toxic events and late effects related to HSCT are hardly acceptable for treating infants with ALL. Without clear evidence for benefit of HSCT, it will be necessary to devise an effective chemotherapy regimen without HSCT, at least for the 'lower-risk' MLL-R subgroup. Finally, two large

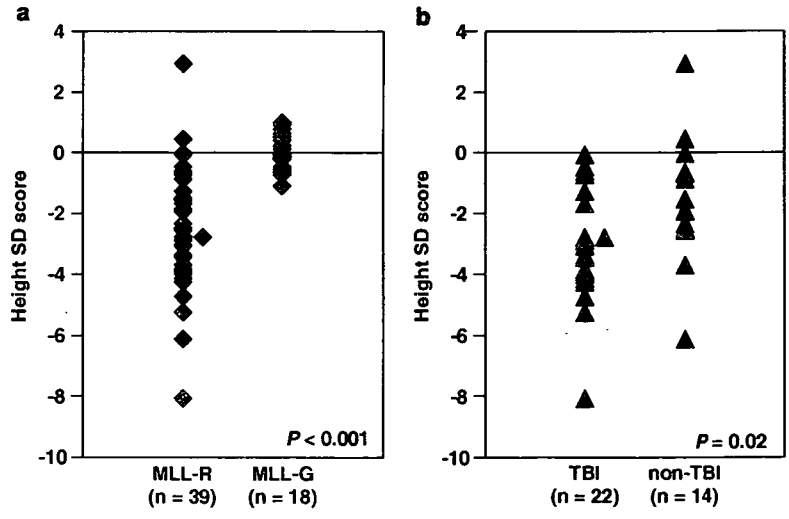

Figure 2 Distribution of height standard deviation (s.d.) scores among survivors of infant ALL in the MLL96 and MLL98 studies. (**a**) Comparison of scores for the *MLL*-rearranged (MLL-R) vs germline *MLL* (MLL-G) patients. (**b**) Comparison of scores between patients who received total-body irradiation (TBI)- or non-TBI-based conditioning regimens.

multicenter studies investigating effective chemotherapy regimens for infant ALL (Interfant 99 and POG/COG9407) have been completed in Europe and in the United States, and these results will be important in designing future protocols for this age group, as will studies to develop innovative targeted therapies for infants with *MLL*-rearranged ALL, now underway.[29,30]

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References

1 Pui C-H, Evans WE. Treatment of acute lymphoblastic leukemia. *N Engl J Med* 2006; **354**: 166–178.
2 Dördelmann M, Reiter A, Borkhardt A, Ludwig W-D, Götz N, Viehmann S *et al.* Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia. *Blood* 1999; **94**: 1209–1217.
3 Ferster A, Benoit Y, Francotte N, Dresse M-F, Uyttebroeck A, Plouvier E *et al.* Treatment outcome in infant acute lymphoblastic leukemia. *Blood* 2000; **95**: 2729–2731.
4 Reaman GH, Sposto R, Sensel MG, Lange BJ, Feusner JH, Heerema NA *et al.* Treatment outcome and prognostic factors for infants with acute lymphoblastic leukemia treated on two consecutive trials of the Children's Cancer Group. *J Clin Oncol* 1999; **17**: 445–455.
5 Hilden JM, Dinndorf PA, Meerbaum SO, Sather H, Villaluna D, Heerema NA *et al.* Analysis of prognostic factors of acute lymphoblastic leukemia in infants: report on CCG 1953 from the Children's Oncology Group. *Blood* 2006; **108**: 441–451.
6 Chessels JM, Harrison CJ, Watson SL, Vora AJ, Richards SM. Treatment of infants with lymphoblastic leukaemia: results of the UK Infant Protocols 1987–1999. *Br J Haematol* 2002; **117**: 306–314.
7 Frankel LS, Ochs J, Shuster JJ, Dubowy R, Bowman WP, Hockenberry-Eaton M *et al.* Therapeutic trial for infant acute lymphoblastic leukemia: the Pediatric Oncology Group experience (POG 8493). *J Pediatr Hematol Oncol* 1997; **19**: 35–42.
8 Silverman LB, Mclean TW, Gelber RD, Donnelly MJ, Gilliland DG, Tarbell NJ *et al.* Intensified therapy for infants with acute lymphoblastic leukemia: results from the Dana-Farber Cancer Institute Consortium. *Cancer* 1997; **80**: 2285–2295.
9 Crist W, Pullen J, Boyett J, Falletta J, van Eys J, Borowitz M *et al.* Clinical and biologic features predict a poor prognosis in acute lymphoblastic leukemias in infants: Pediatric Oncology Group study. *Blood* 1986; **67**: 135–140.
10 Dinndorf PA, Reaman GH. Acute lymphoblastic leukemia in infants: evidence of B cell origin of disease by use of monoclonal antibody phenotyping. *Blood* 1986; **68**: 975–978.
11 Pui C-H, Kane JR, Crist WM. Biology and treatment of infant leukemias. *Leukemia* 1995; **9**: 762–769.
12 Heerema NA, Arthur DC, Sather H, Albo V, Feusner J, Lange BJ *et al.* Cytogenetic features of infants less than 12 months of age at diagnosis of acute lymphoblastic leukemia: impact of the 11q23 breakpoint on outcome: a report of the Children's Cancer Group. *Blood* 1994; **83**: 2274–2284.
13 Chen CS, Sorensen PH, Domer PH, Reaman GH, Korsmeyer SJ, Heerema NA *et al.* Molecular rearrangements on chromosome 11q23 predominate in infant acute lymphoblastic leukemia and are associated with specific biologic variables and poor outcome. *Blood* 1993; **81**: 2386–2393.
14 Pui C-H, Behm FG, Downing JR, Hancock ML, Shurtleff SA, Ribeiro RC *et al.* 11q23/MLL rearrangement confers a poor prognosis in infants with acute lymphoblastic leukemia. *J Clin Oncol* 1994; **12**: 909–915.
15 Taki T, Ida K, Bessho F, Hanada R, Kikuchi A, Yamamoto K *et al.* Frequency and clinical significance of the MLL gene rearrangements in infant acute leukemia. *Leukemia* 1996; **10**: 1303–1307.
16 Isoyama K, Eguchi M, Hibi S, Kinukawa N, Ohkawa H, Kawasaki H *et al.* Risk-directed treatment of infant acute lymphoblastic leukaemia based on early assessment of MLL gene status: results of the Japan Infant Leukaemia Study (MLL96). *Br J Haematol* 2002; **118**: 999–1010.
17 Kosaka Y, Koh K, Kinukawa N, Wakazono Y, Isoyama K, Oda T *et al.* Infant acute lymphoblastic leukemia with MLL gene rearrangements: outcome following intensive chemotherapy and hematopoietic stem cell transplantation. *Blood* 2004; **104**: 3527–3534.
18 Nagayama J, Tomizawa D, Koh K, Nagatoshi Y, Hotta N, Kishimoto T *et al.* Infants with acute lymphoblastic leukemia and a germline MLL gene are highly curable with use of chemotherapy alone: results from the Japan Infant Leukemia Study Group. *Blood* 2006; **107**: 4663–4665.
19 Pui C-H, Gaynon PS, Boyett JM, Chessells JM, Baruchel A, Kamps W *et al.* Outcome of treatment in childhood acute lymphoblastic leukemia with rearrangement of the 11q23 chromosomal region. *Lancet* 2002; **359**: 1909–1915.
20 Marco F, Bureo E, Ortega JJ, Badell I, Verdaguer A, Martinez A *et al.* High survival rate in infant acute leukemia treated with early high-dose chemotherapy and stem-cell support. Grupo Espanol de Transplante de Medula Osea en Ninos. *J Clin Oncol* 2000; **18**: 3256–3261.
21 Copelan EA, Kapoor N, Murcek M, Theil K, Tutschka PJ. Marrow transplantation following busulfan and cyclophosphamide as treatment for translocation (4;11) acute leukemia. *Br J Haematol* 1988; **70**: 127–128.
22 Bordigoni P, Benz-Lemoine E, Vannier JP, Fischer A. Bone marrow transplantation following busulfan, cyclophosphamide and high dose cytosine-arabinoside as treatment for infants with translocation (4;11) acute leukaemia. *Br J Haematol* 1989; **72**: 293–294.
23 Emminger W, Emminger-Schmidmeier W, Haas OA, Urban C, Ambros P, Peters C *et al.* Treatment of infant leukemia with busulfan, cyclophosphamide±etoposide and bone marrow transplantation. *Bone Marrow Transplant* 1992; **9**: 313–318.
24 von Bueltzingsloewen A, Esperou-Bourdeau H, Souillet G, Demeocq F, Mechinaud-Lacroix F, Michel G *et al.* Allogeneic bone marrow transplantation following a busulfan-based conditioning regimen in young children with acute lymphoblastic leukemia: a Cooperative Study of the Société Française de Greffe de Moelle. *Bone Marrow Transplant* 1995; **16**: 521–527.
25 Pirich L, Haut P, Morgan E, Marymount M, Kletzel M. Total body irradiation, cyclophosphamide, and etoposide with stem cell transplant as treatment for infants with acute lymphocytic leukemia. *Med Pediatr Oncol* 1999; **32**: 1–6.
26 Leung W, Pitts N, Burnette K, Cunningham JM, Horwitz EM, Benaim E *et al.* Allogeneic bone marrow transplantation for infants with acute leukemia or myelodysplastic syndrome. *Bone Marrow Transplant* 2001; **27**: 717–722.
27 Sanders JE, Im H-J, Hoffmeister PA, Gooley TA, Woolfrey AE, Carpenter PA *et al.* Allogeneic hematopoietic cell transplantation for infants with acute lymphoblastic leukemia. *Blood* 2005; **105**: 3749–3756.
28 Pieters R, den Boer ML, Durian M, Janka G, Schmiegelow K, Kaspers GJ *et al.* Relation between age, immunophenotype and *in vitro* drug resistance in 395 children with acute lymphoblastic leukemia—implications for treatment of infants. *Leukemia* 1998; **12**: 1344–1348.
29 Stam RW, den Boer ML, Schneider P, Nollau P, Horstmann M, Beverloo HB *et al.* Targeting FLT3 in primary *MLL*-gene-rearranged infant acute lymphoblastic leukemia. *Blood* 2005; **106**: 2484–2490.
30 Brown P, Levis M, Shurtleff S, Campana D, Downing J, Small D. FLT3 inhibition selectively kills childhood acute lymphoblastic leukemia cells with high levels of FLT3 expression. *Blood* 2005; **105**: 812–820.

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Identification of a Novel Fusion Gene *MLL-MAML2* in Secondary Acute Myelogenous Leukemia and Myelodysplastic Syndrome with inv(11)(q21q23)

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We have identified a novel fusion partner of *MLL*, namely the mastermind like 2 (*MAML2* gene), in secondary acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) with inv(11)(q21q23). RT-PCR and sequencing revealed that exon 7 of *MLL* was fused to exon 2 of *MAML2* in the AML and MDS cells. The inv(11)(q21q23) results in the creation of a chimeric RNA encoding a putative fusion protein containing 1,408 amino acids from the NH2-terminal part of MLL and 952 amino acids from the COOH-terminal part of MAML2. The NH2-terminal part of MAML2, a basic domain including a binding site of the intracellular domain of NOTCH, was deleted in MLL-MAML2. MLL-MAML2 in secondary AML/MDS and MECT1-MAML2 in mucoepithelioid carcinoma, benign Wartin's tumor, and clear cell hidradenoma consist of the same COOH-terminal part of MAML2. A luciferase assay revealed that MLL-MAML2 suppressed HES1 promoter activation by the NOTCH1 intracellular domain. MAML2 involving a chimeric gene might contribute to carcinogenesis in multiple neoplasms by the disruption of NOTCH signaling.

INTRODUCTION

11q23 translocations are frequent in hematologic malignancies, occurring in 5–6% of acute myeloid leukemia (AML), 7–10% of acute lymphoblastic leukemia, 60–70% of acute leukemias in infants, and in most patients with therapy-related leukemias induced by inhibitors of topoisomerase II (Rowley, 1998). The *MLL* gene is rearranged as a consequence of 11q23 translocations, and at least 50 partner genes for *MLL* have so far been identified (Meyer et al., 2006).

Although the leukemogenic effect of MLL fusion proteins has been well established in a number of instances lack of functional information about the MLL partners has made it difficult to address their contribution to the oncogenic potential of MLL fusion proteins. A few observations indicated that fusion partners play essential roles in determining the oncogenic capacity of the MLL fusion proteins (So and Cleary, 2003; Liu et al., 2004). In the present study, we have identified the mastermind like 2 (*MAML2*) gene as a novel fusion partner of *MLL* in therapy-related AML and myelodysplastic syndrome (MDS) with inv(11)(q21q23), and provide evidence that the MLL-MAML2 fusion suppresses a promoter activation of the NOTCH target gene, *HES1*.

MATERIALS AND METHODS

Case Reports

Case 1. Details of the patient, a 48-year-old female, have been previously published (Takei et al., 2006). She initially had AML (M2 in the FAB classification) with t(8;21)(q22;q22). She achieved complete remission by chemotherapy. Consolidation and maintenance chemotherapies containing etoposide (VP-16; total dose 1,150 mg) were administered. Seven months after chemotherapy, a chromosomal abnormality, inv(11)(q21q23), appeared and was constantly detected in bone

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TABLE I. Karyotype Analysis of Bone Marrow Cells (Patient 1)

Date	Number of 46,XX,inv(11) in 20 metaphases	Other abnormality	Status of the bone marrow
Jan 21, 1993	0	+*	AML
Jun 14, 1994	20	–	CR
Apr 3, 1995	19	–	CR
Dec 11, 1995	14	–	CR
Oct 6, 1997	4	–	NHL
Nov 14, 1997	1	–	CR
Feb 23, 1998	19	–	CR
Apr 21, 1998	17	–	CR
Apr 21, 1999	6	+	CR
Mar 13, 2000	19	+	t-AML
Apr 10, 2000	19	+	t-AML
Apr 24, 2000	12	+	t-AML
Jun 27, 2000	19	–	t-AML

*indicates t(8;21), 9q-. AML, acute myeloid leukemia; CR, complete remission; t-AML, therapy-related AML; NHL, non-Hodgkin's lymphoma.

marrow cells during hematologic remission (Table 1). After 6 years, she developed secondary AML with inv(11)(q21q23), and died in 2001.

Case 2. A 69-year-old male was admitted to a regional hospital due to phlegmon of the left thigh in March 2006. He had been diagnosed with rheumatoid arthritis and treated with bucillamine, prednisolone, salazosulfapyridine, and methotrexate (total dose 50 mg) for 8 years. He had no history of malignancy. After recovery of the phlegmon, mild anemia, leukocytosis (peak 136.4 × 10^9/l) and thrombocytopenia (30–40 × 10^9/l) persisted. He was referred to Hitachi General Hospital for further examination. His bone marrow was hypercellular with micromegakaryocytes and hypersegmentation of granulocytes. The karyotype was 46,XY,inv(11)(q21q23)[20]. Fluorescence in situ hybridization (FISH) analysis for *BCR-ABL1* was negative, whereas FISH analysis for *MLL* on peripheral blood revealed that 71% of the white blood cells had deletion of the 3′ part of the *MLL* gene. MDS was diagnosed, and he died of pneumonia in January 2007.

cDNA Panhandle Polymerase Chain Reaction (PCR)

Total RNA was extracted from bone marrow or peripheral blood cells using the acid guanidine thiocyanate-phenol chloroform method (Chomczynski and Sacchi, 1987) and analyzed using a modified cDNA panhandle PCR method (Megonigal et al., 2000; Suzukawa et al., 2005). In brief, first-strand cDNAs were synthesized with *MLL*-random hexamer oligonucleotides, MLL-N. After primer 1 extension with MLL-1, and extension in stem-loop templates, the sample was amplified by first PCR with MLL-1 and MLL-2. Then, 1/25 of the products were used for nested PCR with MLL-3 and MLL-4. The *MLL*-random hexamer oligonucleotides and primers used were as follows: MLL-N, 5′-TCG AGG AAA AGA GTG AAG AAG GGA ATG TCT CNN NNN N-3′; MLL-1, 5′-TGA AGA ACG TGG TGG ACT CT-3′; MLL-2, 5′-GTC CAG AGC AGA GCA A AC AGA-3′; MLL-3, 5′-GTC AGA AAC CTA CCC CAT CA-3′; and MLL-4, 5′-TGT GAA GCA GAA AAT GTG TGG-3′.

Reverse Transcriptase PCR

Five μg of total RNA was reverse transcribed to cDNA in a total volume of 33 μl with random hexamers using the Ready-To-Go You-Prime First-Strand beads (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), after which 1/30 of the cDNA was amplified using PCR in a total volume of 50 μl with 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM TAPS Buffer (pH 9.3 at room temperature), 0.4 μM of each primer, 0.2 mM of each dNTP, and 1 unit of Ex *Taq* polymerase (Takara-Bio, Siga, Japan). After 35 rounds of PCR (30 sec at 94°C, 30 sec at 55°C and 1 min at 72°C), 5 μl of the PCR product was electrophoresed in a 3% agarose gel. The primers used were as follows: MLL7s: 5′-TCC TCA GCA CTC TCT CCA AT-3′; MAML2R: 5′-GTC ATT TGG CCA TCC ATG TG-3′.

Plasmid Construction

The KIAA1816 cDNA clone in pBluescript II SK(+) vector was obtained from the KAZUSA DNA Research Institute in Japan. The GenBank accession number for KIAA1816 is AB058719. Full-length MAML2 and the MAML2 part of the MLL-MAML2 fusion gene (C-MAML2) cDNA were constructed by replacing the 5′ and 3′ untranslated region with PCR-amplified fragments. MLL exon 1–7 cDNA (N-MLL) was provided by Dr. H. Hirai (University of Tokyo, Tokyo, Japan). Both ends of N-MLL were replaced with PCR fragments for subsequent cloning into the mammalian expression vector, pcDNA3.1. FLAG(M2)-tagged MKK6 expression vector in pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) was provided by Dr. T. Sudo (RIKEN, Saitama, Japan). MKK6 cDNA was replaced with MAML2, C-MAML2, and N-MLL for the construction of FLAG tagged genes. The 3′ end of FLAG-tagged N-MLL was replaced with an RT-PCR product containing an

MLL-MAML2 fusion sequence from the leukemia cells and C-MAML2 to construct full-length MLL-MAML2 fusion. All PCR-amplified fragments were confirmed by nucleotide sequence analysis. Further information regarding the primer sequences and cloning is available upon request. Notch1 intracellular domain tagged V5 (N1ICD) expression vector was provided by Dr. F. Ito (Tsukuba University, Japan) (Itoh et al., 2004). The luciferase reporter containing the promoter of the HES1 gene (pHES1-luc) was provided by Dr. R. Kageyama (Kyoto University, Japan) (Takebayashi et al., 1994).

Cell Lines and Antibodies

HEK293, a human embryonic kidney cell line, and KG-1, a leukemic cell line established from an AML patient, were purchased from RIKEN Bio-Resource Center (Tsukuba, Ibaraki, Japan). The HEK293 cells were grown at 37°C in Dulbecco's Modified Eagle's Medium (SIGMA-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml penicillin and 100 μg/ml streptmycin sulfate. The KG-1 cells were grown at 37°C in RPMI 1640 Medium (SIGMA-Aldrich) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml penicillin, and 100 μg/ml streptmycin sulfate.

The anti-FLAG polyclonal antibody (F7425) and the anti-MLL1 (BL1289) polyclonal antibody were purchased from Sigma and Bethyl Laboratory Inc. (Montgomery, TX), respectively.

Transfection and Immunoblotting

1×10^6 cells /well of HEK293 cells were seeded in six well plates. The next day, the cells were transfected with FLAG-tagged MLL-MAML2, MAML2, C-MAML2, or N-MLL expression vector using Lipofectamine 2000 (Invitrogen). The next day, the cells were washed with PBS twice and then lysed with RIPA buffer (1% TritonX-100, 0.1% SDS, 1% Soduim deoxycholate, 158 mM NaCl, 1 mM Na_3VO_4, 5 mM EGTA, 10 mM Tris-HCl, and pH 7.4). The cell lysates were centrifuged at 15,000 rpm for 30 min, and the supernatants were boiled and denatured in Sample buffer containing SDS and DTT (Invitrogen) followed by electrophoresis using SDS-PAGE gradient gel (2–15%) in Tris-Glycin buffer. The proteins were electro-transferred to nitrocellulose membrane (GE Healthcare Bioscience, Tokyo, Japan) using a semi-dry transfer cell (Trans-Blot SD, Bio-Rad Japan, Tokyo, Japan). The resulting protein-bound membrane was blotted with Anti-FLAG (×1,000) or anti-MLL (×1,000) antibody and visualized using ECL reagents (GE Healthcare Bioscience). The cells for all samples were cultured and the protein extracts were also prepared at the same time.

Transfection and Luciferase Assay

The HEK293 cells were seeded (1×10^5 cells per well) in 24-well plates and transfected 48 hr later using Lipofectamine 2000 (Invitrogen) with reporter construct (HES1-luciferase, 100 ng), expression vector (300 ng), ICD expression vector (10 ng) and pRL-TK internal control vector (5 ng) (Promega, Madison, WI, USA). When increasing amounts of the expression vector were transfected, the amount of total DNA was kept constant by adding empty pcDNA3.1 vector to the transfection mixture. Forty-eight hours after transfection, the cells were washed with PBS twice, 100 μl of PLB lysis buffer of the Dual Luciferase Reporter Assay kit (Promega) was added. The samples were gently rocked for 15 min at room temperature. Luciferase activity was determined with 10 μl aliquots of the sample with Dual-Luciferase Reporter assay system (Promega) and Lumat LB9501 luminometer (Berthold Japan, Tokyo, Japan) by following the manufacture's protocol.

2×10^6 of the KG-1 cells were suspended in 100 μl of Nucleofector solution R (Amaxa biosystems, Cologne, Germany). Reporter construct (HES1-luciferase, 500 ng), expression vector (1.5 μg), ICD expression vector (50 ng), and the pRL-TK internal control vector (50 ng) were added to the suspension in the cuvette followed by electrotransfection using Nucleofector II Device (Amaxa biosystems). Transfected cells were incubated in culture medium for 48 hr, after which the cells were washed with PBS and centrifuged at 1,200 rpm for 5 min. PLB lysis buffer (100 μl) was added to the cells; then the samples were rocked for 15 min at room temperature. About 20 μl aliquots of cell lysate were used for quantification of luciferase activity. All experiments were performed in triplicate. Firefly luciferase activity was normalized by reference to the Renilla luciferase activity expressed by the pRL-TK vector.

Exon Nomenclature

The exon nomenclature for *MLL* was taken from a report (Rasio et al., 1996).

RESULTS

Isolation of *MLL-MAML2* Fusion Transcript from AML cells with inv(11)(q21q23)

FISH and Southern blot analyses revealed rearrangement of the *MLL* gene in case 1 (Takei et al.,

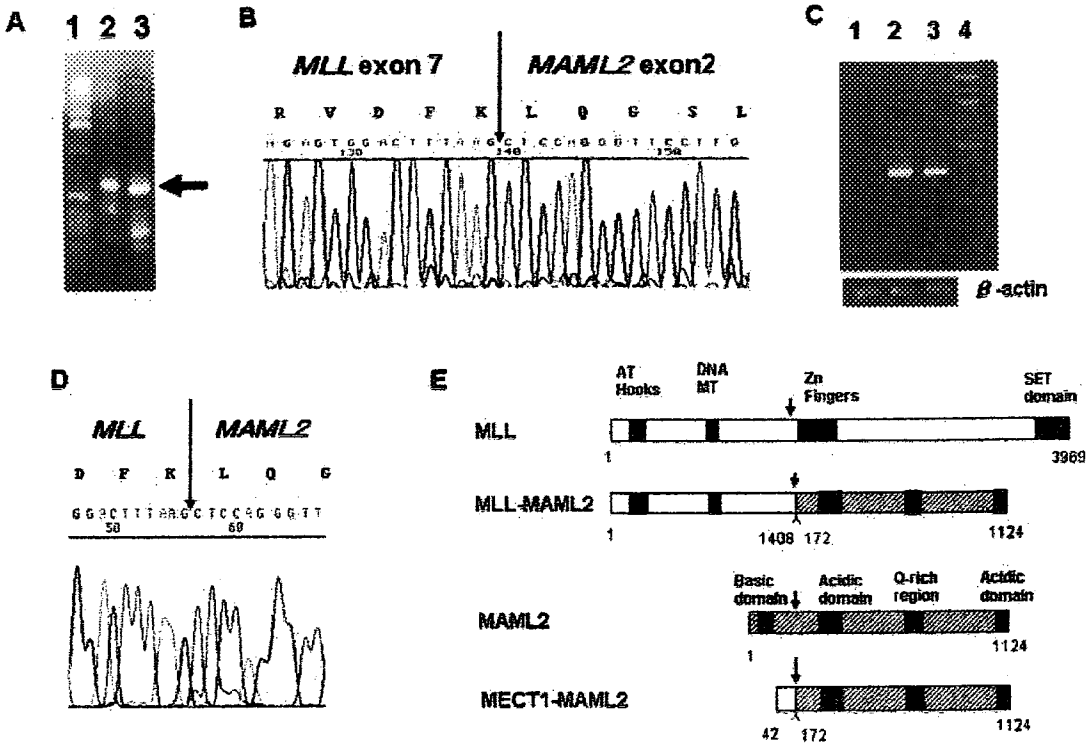

Figure 1. Identification of *MLL-MAML2* fusion transcript. (A) Panhandle PCR for cDNA product. Lane 1: λ *Hind*III digest (marker); lane 2: first PCR product; lane 3: second (nested) PCR product. An arrow indicates a DNA fragment whose sequence is shown in panel B. (B) Sequence analysis of the amplified panhandle PCR product from case 1 revealed an in-frame fusion between *MLL* exon 7 and *MAML2* exon 2 (arrow; GenBank accession no. 828759). (C) RT-PCR of two cases with inv(11)(q21q23). Lane 1: cDNA from normal peripheral blood; lane 2: cDNA from leukemic cells in case 1; lane 3: cDNA from peripheral blood in case 2; lane 4: 1 kb ladder marker. (D) Sequence analysis directly performed on the amplified RT-PCR product from case 2 revealed the same fusion between *MLL* and *MAML2*. (E) Schematic structures of wild-type *MLL*, *MLL-MAML2* fusion gene, wild-type *MAML2* and *MECT1-MAML2* fusion gene. *MT*, DNA methyltransferase homology domain; *Q-rich region*, glutamine rich region; Arrow, fusion point. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

2006). To identify the *MLL* partner gene at 11q21, we performed cDNA panhandle PCR for total RNA from the patient's bone marrow cells. First and second PCR gave a specifically amplified DNA fragment (Fig. 1A). Sequence analysis of the fragment revealed an in-frame fusion between *MLL* exon 7 and *MAML2* exon 2 (Fig. 1B).

Detection of *MLL-MAML2* in Two Hematologic Malignancies with inv(11)(q21q23)

To confirm the presence of the *MLL-MAML2* fusion mRNA in the leukemic cells of the case 1 and peripheral blood of case 2, we performed RT-PCR analysis with MLL7s and MAML2R, which successfully yielded specific DNA fragment (Fig. 1C). We confirmed that the RT-PCR product from case 2 was an *MLL-MAML2* fusion transcript by sequencing (Fig. 1D). The putative MLL-MAML2 fusion protein of 2,389 amino acids (aa) contained 1,408 aa, from the NH2-terminal part of MLL and 981 aa from the COOH-terminal part of MAML2 (Fig. 1E).

MLL-MAML2 Suppresses HES1 Promoter Activation by N1ICD

To investigate the function of the MLL-MAML2 fusion gene, we constructed FLAG-tagged MLL-MAML2 (M-M2), MLL part of MLL-MAML2 (N-MLL), MAML part of the MLL-MAML2 (C-M2), and full-length MAML2 gene (M2) (Fig. 2A). The expression of recombinant proteins was confirmed by Western blot analysis (Fig. 2B). Immunohistchemical experiment using anti-FLAG antibody revealed that both M2 and M-M2 localized in the nucleus (data not shown). We evaluated the ability of MLL-MAML2 to participate in NOTCH signaling by examining the activation of a NOTCH target gene, *HES1*, the best characterized member of the HES gene family (Leong and Karsan, 2006). M-

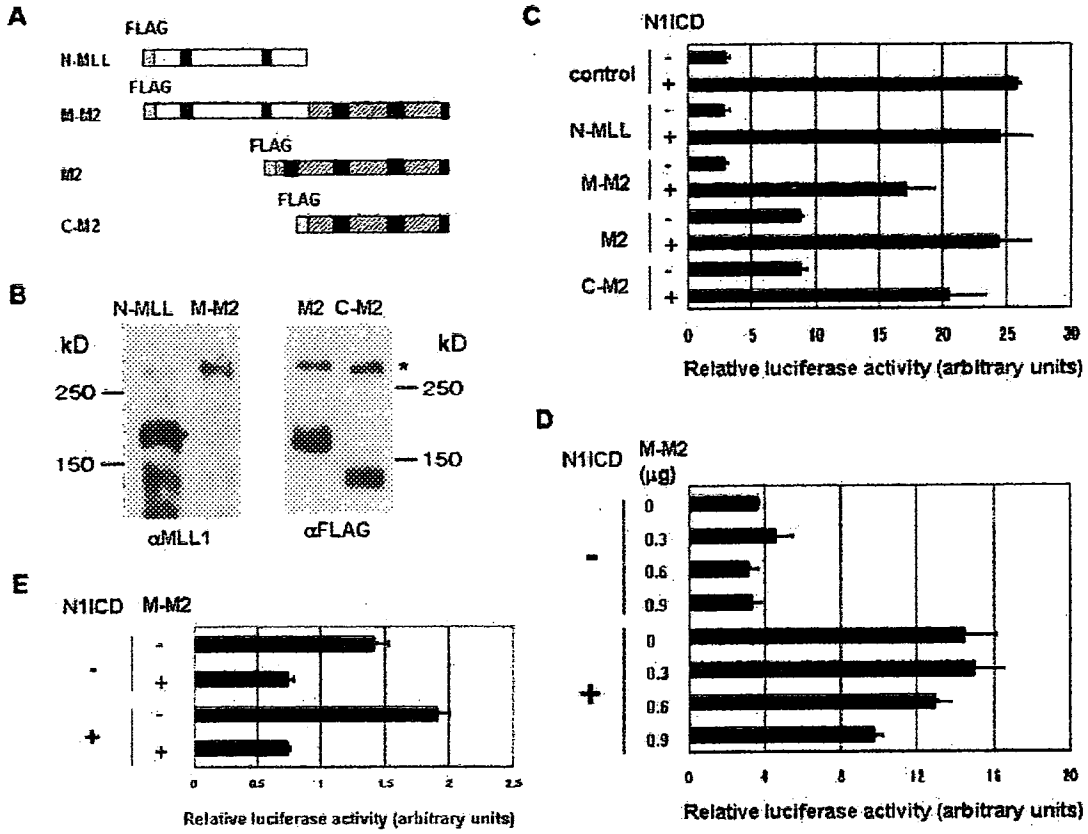

Figure 2. Functional analysis of the fusion gene. (A) a schematic presentation of recombinant genes. FLAG tag was fused to the NH2 terminal end of each recombinant construct. N-MLL; NH2 terminal part of MLL-MAML2: M-M2; MLL-MAML2 fusion gene: M2; full length MAML2: C-M2; COOH terminal part of MLL-MAML2. (B) Detection of recombinant proteins by Western blot analysis. Each protein was detected either anti MLL (left panel) or anti FLAG antibody (right panel). Asterisk in the right panel indicates a nonspecific band. (C) Comparison of basal (N1ICD-) and N1ICD-induced activation (N1ICD+) of HES1 promoter activity. Each recombinant gene was transfected as indicated. Empty pcDNA3.1 vector was used as control. (D) Dose escalation of MLL-MAML2 fusion gene. (E) Luciferase assay using KG-1 leukemia cell line. The error bars indicate the standard deviations ($n = 3$).

M2 did not increase the basal HES1 promoter activity, but suppressed N1ICD-induced HES1 promoter activation in a dose dependent manner (Figs. 2C and D). In contrast, MECT1-MAML2 was reported to activate the HES1 promoter, and this was independent of NOTCH stimulation (Tonon et al., 2003). Thus, the MAML2 containing fusion gene might disrupt NOTCH signaling by both activation and inhibition. This is not surprising because NOTCH activation can be both oncogenic and tumor suppressive in different tumors (Leong and Karsan, 2006), although there is a possibility that the difference is due to the different cell lines used for the experiments. N-MLL changed neither the basal nor N1ICD-induced HES1 promoter activity, suggesting that this part is not involved in the NOTCH signaling pathway. M2 and C-M2 enhanced the basal HES1 promoter activity. C-M2 suppressed the N1ICD-induced activation of the HES1 promoter, whereas M2 did not. Next, we used a leukemia cell line, KG-1, for luciferase assay. The N1ICD-induced activation of the HES1 promoter in KG-1 was less compared to the one in HEK293. M-M2 suppressed both basal and N1ICD-induced HES1 promoter activity to the same level (Fig. 2E).

DISCUSSION

The mammalian *MAML1, MAML2,* and *MAML3* genes are widely expressed in adult tissues and localize to nuclear bodies. They share a conserved basic domain in their N termini that binds to the ankyrin repeat domain of NOTCH, and contain a transcriptional activation domain in their C termini. They function as transcriptional co-activators for NOTCH, forming a complex in the nucleus

with the intracellular domain of an activated NOTCH receptor (ICN) and the CSL family of transcription factors, resulting in the activation of NOTCH downstream targets, such as *HES1* and *HES5* (Lin et al., 2002; Wu et al., 2002).

The acquisition of novel properties by the combination of MLL with the fusion partners, rather than the loss of wild-type MLL function leads to the generation of an active oncoprotein. Recent studies suggest that different mechanisms might be involved in the leukemogenesis by MLL fusion proteins (Li et al., 2005). The MLL fusion partners can be divided into nuclear and cytoplasmic factors, according to their compartment of protein expression/function. There is increasing evidence that the nuclear factors have transcription activity themselves and belong to important chromatin remodeling and transcription modulating complexes. A subgroup of fusion partners have oligomerization/dimerization domains, such as leucine zippers and a-helical coiled-coil domains. Recent reports indicate that the dimerization of the N-terminal portion of MLL fusion protein is oncogenic (Martin et al., 2003; So et al., 2003). In the case of MLL-MAML2, the N-terminal portion of MLL may acquire oncogenic activity by transcriptional activation, since the transcriptional activation domain of MAML2 is retained (Wu et al., 2005) and no self-association domain has been identified.

MLL is the second fusion partner of *MAML2* in human neoplasm. It has been reported that the same part of MAML2 as in MLL-MAML2 is fused to the NH2-terminal part of MECT1 in mucoepidermoid carcinoma, Warthin's tumor, and clear cell hidradenoma (Tonon et al., 2003; Enlund et al., 2004; Behboudi et al., 2005). A recent report demonstrated that not only MECT1, but also the MAML2 component is required for the transformation of RK3E cells (Wu et al., 2005). A common structural alteration of MAML2 in solid tumors and leukemia suggests that common functional aberration(s) of MAML2 contribute to carcinogenesis in multiple tissues. There are a few genes that are found as part of fusion genes in both leukemia and solid tumors. For example, the *ETS* family gene *ERG* is found as part of a fusion gene with different partner genes in myeloid leukemia, Ewing sarcoma, and prostate cancer (Ichikawa et al., 1994; Tomlins et al., 2005). The common alteration of *ERG* in these tumors is the overexpression of the COOH-terminal part, including the ETS domain. Especially, the same part of ERG is fused to either TLS/FUS or EWSR1 in myeloid leukemia or Ewing sarcoma, respectively. Thus, overexpression of the COOH-terminal portion of MAML2 may be required for common carcinogenesis. Functional alteration of partner gene might be required in tissue-specific carcinogenesis. In accordance with this, MLL-MAML2 did not transform RK3E cells (unpublished data), while transformation by MECT1-MAML2 was been reported (Wu et al., 2005). Intriguingly, both M-M2 and C-M2 lack the ICN binding site in the basic domain (Wu and Griffin, 2004).

Detection of chromosomal translocations involving MAML2 in not only benign tumors, Warthin's tumor (Enlund et al., 2004) and clear cell hidradenoma (Behboudi et al., 2005), but also in bone marrow cells before overt leukemia (Takei et al., 2006) and MDS suggests that the disruption of NOTCH signaling by the emergence of a MAML2 containing fusion gene is an early event of carcinogenesis. The inv(11)(q21q23) is rare in hematologic malignancies. There is another case report of secondary AML with inv(11)(q21q23) and rearrangement of the *MLL* (Obama et al., 1998). It is likely that the leukemia cells of the patient have MLL-MAML2 fusion gene. Although the clinical effect of this infrequent fusion hence may be limited, their characterization will certainly provide additional mechanistic insights into both MLL- and MAML2-mediated carcinogenesis.

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REFERENCES

Behboudi A, Winnes M, Gorunova L, van den Oord JJ, Mertens F, Enlund F, Stenman G. 2005. Clear cell hidradenoma of the skin-a third tumor type with a t(11;19)–associated TORC1-MAML2 gene fusion. Genes Chromosomes Cancer 43:202–205.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Enlund F, Behboudi A, Andren Y, Oberg C, Lendahl U, Mark J, Stenman G. 2004. Altered Notch signaling resulting from expression of a WAMTP1-MAML2 gene fusion in mucoepidermoid carcinomas and benign Warthin's tumors. Exp Cell Res 292:21–28.

Ichikawa H, Shimizu K, Hayashi Y, Ohki M. 1994. An RNA-binding protein gene, TLS/FUS, is fused to ERG in human myeloid leukemia with t(16;21) chromosomal translocation. Cancer Res 54:2865–2868.

Itoh F, Itoh S, Goumans MJ, Valdimarsdottir G, Iso T, Dotto GP, Hamamori Y, Kedes L, Kato M, ten Dijke Pt P. 2004. Synergy and antagonism between Notch and BMP receptor signaling pathways in endothelial cells. Embo J 23:541–551.

Leong KG, Karsan A. 2006. Recent insights into the role of Notch signaling in tumorigenesis. Blood 107:2223–2233.

Li ZY, Liu DP, Liang CC. 2005. New insight into the molecular mechanisms of MLL-associated leukemia. Leukemia 19:183–190.

Lin SE, Oyama T, Nagase T, Harigaya K, Kitagawa M. 2002. Identification of new human mastermind proteins defines a family that consists of positive regulators for notch signaling. J Biol Chem 277:50612–50620.

Liu H, Chen B, Xiong H, Huang QH, Zhang QH, Wang ZG, Li BL, Chen Z, Chen SJ. 2004. Functional contribution of EEN to leukemogenic transformation by MLL-EEN fusion protein. Oncogene 23:3385–3394.

Martin ME, Milne TA, Bloyer S, Galoian K, Shen W, Gibbs D, Brock HW, Slany R, Hess JL. 2003. Dimerization of MLL fusion proteins immortalizes hematopoietic cells. Cancer Cell 4:197–207.

Megonigal MD, Rappaport EF, Wilson RB, Jones DH, Whitlock JA, Ortega JA, Slater DJ, Nowell PC, Felix CA. 2000. Panhandle PCR for cDNA: A rapid method for isolation of MLL fusion transcripts involving unknown partner genes. Proc Natl Acad Sci USA 97:9597–9602.

Meyer C, Schneider B, Jakob S, Strehl S, Attarbaschi A, Schnittger S, Schoch C, Jansen MW, van Dongen JJ, den Boer ML, Pieters R, Ennas MG, Angelucci E, Koehl U, Greil J, Griesinger F, Zur Stadt U, Eckert C, Szczepanski T, Niggli FK, Schafer BW, Kempski H, Brady HJ, Zuna J, Trka J, Nigro LL, Biondi A, Delabesse E, Macintyre E, Stanulla M, Schrappe M, Haas OA, Burmeister T, Dingermann T, Klingebiel T, Marschalek R. 2006. The MLL recombinome of acute leukemias. Leukemia 20:777–784.

Obama K, Furukawa Y, Tara M, Niina K. 1998. Secondary monocytic leukemia with rearrangement of the MLL gene occurring during the course of adult T-cell leukemia. Int J Hematol 68:323–326.

Rasio D, Schichman SA, Negrini M, Canaani E, Croce CM. 1996. Complete exon structure of the ALL1 gene. Cancer Res 56:1766–1769.

Rowley JD. 1998. The critical role of chromosome translocations in human leukemias. Annu Rev Genet 32:495–519.

So CW, Cleary ML. 2003. Common mechanism for oncogenic activation of MLL by forkhead family proteins. Blood 101:633–639.

So CW, Lin M, Ayton PM, Chen EH, Cleary ML. 2003. Dimerization contributes to oncogenic activation of MLL chimeras in acute leukemias. Cancer Cell 4:99–110.

Suzukawa K, Shimizu S, Nemoto N, Takei N, Taki T, Nagasawa T. 2005. Identification of a chromosomal breakpoint and detection of a novel form of an MLL-AF17 fusion transcript in acute monocytic leukemia with t(11;17)(q23;q21). Int J Hematol 82: 38–41.

Takebayashi K, Sasai Y, Sakai Y, Watanabe T, Nakanishi S, Kageyama R. 1994. Structure, chromosomal locus, and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-1. Negative autoregulation through the multiple N box elements. J Biol Chem 269:5150–5156.

Takei N, Suzukawa K, Mukai HY, Itoh T, Okoshi Y, Yoda Y, Nagasawa T. 2006. Therapy-related acute myeloid leukemia 6 years after clonal detection of inv(11)(q21q23) and MLL gene rearrangement. Int J Hematol 83:247–251.

Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R, Lee C, Montie JE, Shah RB, Pienta KJ, Rubin MA, Chinnaiyan AM. 2005. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 310:644–648.

Tonon G, Modi S, Wu L, Kubo A, Coxon AB, Komiya T, O'Neil K, Stover K, El-Naggar A, Griffin JD, Kirsch IR, Kaye FJ. 2003. t(11;19)(q21;p13) translocation in mucoepidermoid carcinoma creates a novel fusion product that disrupts a Notch signaling pathway. Nat Genet 33:208–213.

Wu L, Griffin JD. 2004. Modulation of Notch signaling by mastermind-like (MAML) transcriptional co-activators and their involvement in tumorigenesis. Semin Cancer Biol 14:348–356.

Wu L, Liu J, Gao P, Nakamura M, Cao Y, Shen H, Griffin JD. 2005. Transforming activity of MECT1-MAML2 fusion oncoprotein is mediated by constitutive CREB activation. EMBO J 24:2391–2402.

Wu L, Sun T, Kobayashi K, Gao P, Griffin JD. 2002. Identification of a family of mastermind-like transcriptional coactivators for mammalian notch receptors. Mol Cell Biol 22:7688–7700.

To investigate further the oncogenic function of $BRAF^{L597Q}$, we examined its transforming ability by comparing colony formation of various BRAF transfectants of NIH3T3 cells on soft agar. We found significant colony formation with the two activating mutants, $BRAF^{V600E}$ and $BRAF^{L597Q}$, in a similar manner (Figures 1b and c). No colony formation occurred with vector transfections (control). We also did not see colony formation with the $BRAF^{WT}$ transfectant. This was an expected result as the $BRAF^{WT}$ itself is not oncogenic and $BRAF^{WT}$ was unable to overactivate the MAP kinase pathway (Figure 1a). These data demonstrated the transforming ability of the T1790A *BRAF* mutation and, together with its constitutive kinase activation of BRAF (Figure 1a), demonstrated its oncogenic function.

In this study, we have for the first time explored the functionality of the T1790A *BRAF* mutation and demonstrated that the *BRAF* gene with this mutation is an oncogene. In addition to childhood ALL,[7] this mutation was reported also in cutaneous melanoma with a lower prevalence (3%).[12] The relative common occurrence (10%) of the T1790A mutation in childhood ALL suggests that this mutation plays an important role in a subpopulation of patients with this blood malignancy and may represent a novel therapeutic target for these patients.

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References

1 Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S *et al.* Mutations of the BRAF gene in human cancer. *Nature* 2002; **417**: 949–954.
2 Garnett MJ, Marais R. Guilty as charged: B-RAF is a human oncogene. *Cancer Cell* 2004; **6**: 313–319.
3 Wan PT, Garnett MJ, Roe SM, Lee S, Niculescu-Duvaz D, Good VM *et al.* Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell* 2004; **116**: 855–867.
4 Oler G, Ebina KN, Michaluart Jr P, Kimura ET, Cerutti J. Investigation of BRAF mutation in a series of papillary thyroid carcinoma and matched-lymph node metastasis reveals a new mutation in metastasis. *Clin Endocrinol (Oxf)* 2005; **62**: 509–511.
5 Moretti S, Macchiarulo A, De Falco V, Avenia N, Barbi F, Carta C *et al.* Biochemical and molecular characterization of the novel BRAF (V599Ins) mutation detected in a classic papillary thyroid carcinoma. *Oncogene* 2006; **25**: 4235–4240.
6 Hou P, Liu D, Xing M. Functional characterization of the T1799-1801del and A1799-1816ins BRAF mutations in papillary thyroid cancer. *Cell Cycle* 2007; **6**: 377–379.
7 Gustafsson B, Angelini S, Sander B, Christensson B, Hemminki K, Kumar R. Mutations in the BRAF and N-ras genes in childhood acute lymphoblastic leukaemia. *Leukemia* 2005; **19**: 310–312.
8 Hunter T, Pines J. Cyclins and cancer. II: cyclin D and CDK inhibitors come of age. *Cell* 1994; **79**: 573–582.
9 Lee RJ, Albanese C, Stenger RJ, Watanabe G, Inghirami G, Haines III GK *et al.* pp60 (v-src) induction of cyclin D1 requires collaborative interactions between the extracellular signal-regulated kinase, p38, and Jun kinase pathways. *J Biol Chem* 1999; **274**: 7341–7350.
10 Guo SS, Wu X, Shimoide AT, Wong J, Moatamed F, Sawicki MP. Frequent overexpression of cyclin D1 in sporadic pancreatic endocrine tumours. *J Endocrinol* 2003; **179**: 73–79.
11 Wellbrock C, Karasarides M, Marais R. The RAF proteins take centre stage. *Nat Rev Mol Cell Biol* 2004; **5**: 875–885.
12 Cruz III F, Rubin BP, Wilson D, Town A, Schroeder A, Haley A *et al.* Absence of BRAF and NRAS mutations in uveal melanoma. *Cancer Res* 2003; **63**: 5761–5766.

N822 mutation of *KIT* gene was frequent in pediatric acute myeloid leukemia patients with t(8;21) in Japan: a study of the Japanese childhood AML cooperative study group

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Recently, *KIT* mutations were found in 12.7–48.1% of adult acute myeloid leukemia (AML) patients.[1–6] Our previous analysis revealed that *KIT* mutations were found in 8 (17.4%) of 46 pediatric t(8;21)-AML patients and these patients had the poorer prognosis than those without *KIT* mutations.[7] Recent Berlin-Frankfurt-Münster study and Dutch Childhood Oncology Group study revealed that 5 (31.3%) of 16 t(8;21)-AML patients had *KIT* mutations.[8] On the other hand, Children's Cancer Group study revealed that *KIT* mutations were not found in eight t(8;21)-AML patients.[9] There remains the possibility of the different frequency about *KIT* mutations in ages and races. Thus, we further investigated *KIT* mutations in a larger number of pediatric t(8;21)-AML patients.

We examined for *KIT* mutations in 42 newly diagnosed t(8;21)-AML patients who were treated on the same AML99 protocol from January 2003 to December 2006 after AML99 study closed.[7] We found *KIT* mutations in 6 (14.3%) of 42 t(8;21)-AML patients (D816mutation (Mt) ($n=4$), N822Mt ($n=1$), deletion of D419 ($N=1$)).

Furthermore, we searched for other *KIT* mutations and found novel mutations in transmembrane domain (exon 10) in 3 (3.4%) of total 88 t(8;21)-AML patients, including previous 46 patients[7] (Table 1). Two patients having the same I538V (ATT to GTT) in *KIT* have been in complete remission for 30 and 48 months, respectively. However, the remaining patient having V540L (GTG to CTG) in *KIT* relapsed 4 months after diagnosis. V540L was also found in relapsed bone marrow sample. These mutation sites were in the vicinity of the codon 541, which was reported as single nucleotide polymorphism (SNP) site (ATG to CTG, M541L). Therefore, we examined these mutations (I538V

Table 1 *KIT* gene mutation in 88 pediatric t(8;21)-AML

	No. of patients	%
Kinase domain (exon 17)	13	14.8
D816Mt	6	
D816H	1	
D816V	3	
D816Y	2	
N822Mt	5	
N822K	4	
N822T	1	
A814S	1	
V825A	1	
Extracellular domain (exon 8)	1	1.1
del. D419	1	
Transmembrane domain (exon 10)	3	3.4
I538V	2	
V540L	1	
Total	17	19.3

Abbreviations: AML, acute myeloid leukemia; Mt, mutation.

and V540L) and M541L (SNP) in 42 normal peripheral blood samples, and detected only M541L (SNP) in 4 (9.5%) samples. Internal tandem duplication of *KIT*[10] was not found in this study.

In total, *KIT* mutations were found in 17 (19.3%) of 88 t(8;21)-pediatric AML patients (Table 1). This frequency was lower than that of several adult reports including Japan.[1–3,5] Interestingly, in Europe, D816Mt and N822Mt were found in 5 and 0 of 47 adult patients in Netherlands,[1] 3 and 0 of 56 adult patients in France,[5] 12 and 0 of 42 adult patients in Italy, respectively.[6] On the other hand, in east Asia, D816Mt and N822Mt were found in 7 and 3 of 37 adult patients in Japan,[3] 9 and 10 of 54 adult patients in China,[2] respectively. Our study revealed that D816Mt and N822Mt were found in 6 and 5 of 88 t(8;21)-pediatric AML patients, respectively. The frequency of N822Mt in *KIT* is different between Europe (Caucasian) and east Asia (oriental people). Boissel *et al.*[5] suggested that ethnic and geographic variations may also be responsible for such disparities. N822Mt in *KIT* was frequent in Japanese and Chinese t(8;21)-AML patients. Notably, Wang *et al.*[2] suggested that Imatinib, a tyrosine kinase inhibitor, has the efficacy for the leukemic cells with N822Mt, but not with D816Mt. Further larger study is needed to clarify N822Mt and the efficacy of Imatinib.

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References

1 Care RS, Valk PJ, Goodeve AC, Abu-Duhier FM, Geertsma-Kleinekoort WM, Wilson GA *et al.* Incidence and prognosis of c-KIT and FLT3 mutations in core binding factor (CBF) acute myeloid leukaemias. *Br J Haematol* 2003; **121**: 775–777.
2 Wang YY, Zhou GB, Yin T, Chen B, Shi JY, Liang WX *et al.* AML1-ETO and C-KIT mutation/overexpression in t(8;21) leukemia: implication in stepwise leukemogenesis and response to Gleevec. *Proc Natl Acad Sci USA* 2005; **102**: 1104–1109.
3 Nanri T, Matsuno N, Kawakita T, Suzushima H, Kawano F, Mitsuya H *et al.* Mutations in the receptor tyrosine kinase pathway are associated with clinical outcome in patients with acute myeloblastic leukemia harboring t(8;21)(q22;q22). *Leukemia* 2005; **19**: 1361–1366.
4 Paschka P, Marcucci G, Ruppert AS, Mrozek K, Chen H, Kittles RA, Cancer Leukemia Group B *et al.* Adverse prognostic significance of KIT mutations in adult acute myeloid leukemia with inv(16) and t(8;21): a Cancer and Leukemia Group B Study. *J Clin Oncol* 2006; **24**: 3904–3911.
5 Boissel N, Leroy H, Brethon B, Philippe N, de Botton S, Auvrignon A, Acute Leukemia French Association (ALFA); Leucemies Aigues Myeloblastiques de l'Enfant (LAME) Cooperative Groups *et al.* Incidence and prognostic impact of c-Kit, FLT3, and Ras gene mutations in core binding factor acute myeloid leukemia (CBF-AML). *Leukemia* 2006; **20**: 965–970.
6 Cairoli R, Beghini A, Grillo G, Nadali G, Elice F, Ripamonti CB *et al.* Prognostic impact of c-KIT mutations in core binding factor leukemias: an Italian retrospective study. *Blood* 2006; **107**: 3463–3468.
7 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**: 1806–1809.
8 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**: 1536–1542.
9 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**: 1474–1479.
10 Corbacioglu S, Kilic M, Westhoff MA, Reinhardt D, Fulda S, Debatin KM. Newly identified c-KIT receptor tyrosine kinase ITD in childhood AML induces ligand-independent growth and is responsive to a synergistic effect of imatinib and rapamycin. *Blood* 2006; **108**: 3504–3513.