Brief report

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

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Patients with t(8;21) acute myeloid leukemia (AML) are considered to have a good prognosis; however, approximately 50% of them relapse. The genetic alterations associated with a poor outcome in t(8;21) AML remain unknown. Recently, aberrations of receptor tyrosine kinases (RTKs) were frequently found in patients with AML. However, the prevalence and prognostic impact of RTK aberrations in pediatric t(8;21) AML remains undetermined. Here, we found the kinase domain mutations of the *KIT* gene in 8 (17.4%) of 46 patients with t(8;21) AML among newly diagnosed pediatric patients with AML treated on the AML99 protocol in Japan. Significant differences between patients with or without *KIT* mutations were observed in the 4-year overall survival (50.0% versus 97.4%, $P = .001$), disease-free survival (37.5% versus 94.7%, $P < .001$) and relapse rate (47.0% versus 2.7%, $P < .001$). Furthermore, *FLT3* internal tandem duplication was found in only 2 (4.3%) patients. These results suggested that *KIT* mutations are strongly associated with a poor prognosis in pediatric t(8;21) AML. (Blood. 2006; 107:1806-1809)

Introduction

Patients with t(8;21) acute myeloid leukemia (AML) have been reported to have a good prognosis; however, approximately 50% of them relapse.[1,2] A high presenting leukocyte count, CD56 expression, or extramedullary disease has been reported to be associated with a poor prognosis in t(8;21) AML.[1,3,4] However, the genetic alterations associated with a poor outcome in patients with t(8;21) AML remain unknown. Recent studies revealed that internal tandem duplication (ITD) of *FLT3* is considered to be one factor predicting poor prognosis in adult and pediatric patients with AML.[5-9] More recently, *KIT* mutations were found in 12.7% to 48.1% of adult patients with AML with t(8;21)[10-12] and were reported to be associated with a poor prognosis.[13,14] The prevalence and prognostic impact of *KIT* mutations in pediatric t(8;21) AML remain unknown. We performed the mutational analysis of *KIT* and *FLT3* in pediatric patients with t(8;21) AML who were treated on the Japanese Childhood AML Cooperative Study Group Protocol, AML99. We report here that *KIT* mutations are strongly associated with a poor prognosis in pediatric patients with t(8;21) AML.

Study design

Patients and samples

The diagnosis of AML was based on the French-American-British (FAB) classification, and cytogenetic analysis was performed using a routine G-banding method. From January 2000 to December 2002, 318 patients were newly diagnosed as having de novo AML. Of 240 patients, 77 (32.1%), except for 29 AML-M3 and 49 Down syndrome, had t(8;21)(q22; q22) according to cytogenetics or *AML1-MTG8* fusion transcript with the reverse-transcriptase–polymerase chain reaction (RT-PCR) (Figure S1; see the Supplemental Materials link at the top of the online article, at the *Blood* website). Samples were available from 135 (56.3%) of 240 patients with AML, including 46 (59.7%) of 77 patients with t(8;21) AML. Of 46 patients with t(8;21) AML, 3 patients were classified into M1, 39 into M2, and 4 into

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Submitted August 24, 2005; accepted October 20, 2005. Prepublished online as *Blood* First Edition Paper, November 15, 2005; DOI 10.1182/blood-2005-08-3408.

A list of the participating members of the Japanese Childhood AML Cooperative Study Group appears in "Appendix."

Supported in part by a Grant-in-Aid for Cancer Research and a grant for Clinical Cancer Research from the Ministry of Health, Labor, and Welfare of Japan, and by a research grant for Gunma Prefectural Hospitals.

A.S. performed genetic analysis and wrote the paper; T.T. assisted with the genetic analysis; K.T. performed the statistical analysis; A.T., K.H., M.T., and R.H. arranged the clinical data; I.T. designed the AML cooperative study in Japan; and Y.H. designed the study and wrote the paper.

The online version of this article contains a data supplement.

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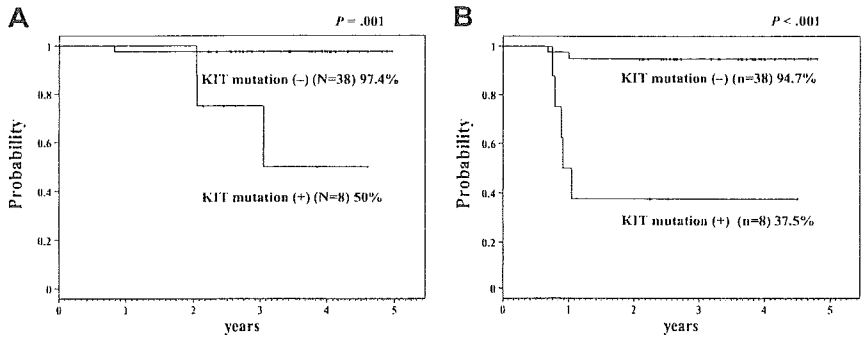

Figure 1. Kaplan-Meier analysis. This analysis shows 4-year overall survival (A) and disease-free survival (B) of the patients with or without *KIT* mutation. The difference is statistically significant (A: $P = .001$; B: $P < .001$).

M4. There were no statistical differences between 46 analyzed patients with t(8;21) AML and the 31 nonanalyzed patients in age (median 7.5 years [range: 2-15 years] versus 9 years [range: 1-15 years]), initial white blood cell (WBC) count (median: 14.4 × 10^9/L; range: 1.65 × 10^9/L-107.7 × 10^9/L; versus 9.1 × 10^9/L; range: 1.4 × 10^9/L-136 × 10^9/L), induction rate (100% versus 93.5%), relapse rate (15.2% versus 19.4%), and 4-year overall survival rate (4y-OS; 87% versus 91%). In the AML99 protocol, patients with t(8;21) with initial WBC count lower than 50 × 10^9/L were categorized into a low-risk group. Thus, after patients with t(8;21) AML obtained complete remission (CR) with induction chemotherapy (cytarabine, etoposide, and mitoxantrone), they were treated with 5 additional courses of intensive chemotherapy (high-dose cytarabine [HDCA], etoposide, idarubicine, and mitoxantron; Figure S2 and Tsukimoto et al[15]). If the initial WBC count was greater than 50 × 10^9/L, patients were categorized into an intermediate-risk group and received allogeneic stem cell transplantation (allo-SCT) in the case of the presence of a donor. Informed consent was obtained from the patients or patients' parents, according to guidelines based on the tenets of the revised Helsinki protocol. The institutional review board of Gunma Children's Medical Center approved this project.

KIT mutation analysis

Mutational analysis of the extracellular (EC) domain (exons 8, 9), transmembrane (TM) domain (exon 10), juxtamembrane (JM) domain (exon 11), and the second intracellular kinase (TK) 2 domain (exons 17 and 18) of *KIT* gene was performed with RT-PCR followed by direct sequencing. Primers used are shown in Table S1.

FLT3 mutation analysis

Mutational analysis of ITD within the JM domain and D835 mutation (D835Mt) within the TK2 domain of the *FLT3* gene was performed as previously reported.[16-18]

Statistical analysis

Estimation of survival distributions was performed using the Kaplan-Meier method and the differences were compared using the log-rank test. Disease-free survival (DFS), event-free survival (EFS), and overall survival (OS) were defined as the times from diagnosis to relapse, from diagnosis to event (relapse or death of any cause), and from diagnosis to death of any cause or the last follow-up. Statistical difference analysis was performed using the χ^2 test.

Results and discussion

KIT and *FLT3* expressions were found in all of the 46 t(8;21) AML samples. Although *KIT* mutations have been reported in a small number of pediatric patients with t(8;21) AML,[8,19] TK2 domain mutations of the *KIT* gene were found in 8 (17.4%) of 46 patients in this study (Table 1). However, we could not find any mutation other than the TK2 domain. The N822K mutation, which has been frequently reported so far,[12] was found in 3 of 8 patients in this study.

The statistical differences between patients with or without *KIT* mutations were not significant in age (median 8 years [range: 1-15 years] versus 7 years [range: 2-15 years]), and the initial WBC count (median: 20.65 × 10^9/L; range: 4.6 × 10^9/L-66.2 × 10^9/L; versus 14.3 × 10^9/L; range: 1.65 × 10^9/L-107.7 × 10^9/L). Interestingly, *KIT* mutations were observed only in M2 patients according to FAB classification. Another report also suggested that *KIT* mutations were frequently found in M2 patients with t(8;21).[19] Significant differences between patients with or without *KIT* mutations were observed in 4-year OS (50.0% versus 97.4%, $P = .001$, Figure 1), DFS (37.5% versus 94.7%, $P < .001$), and relapse rate (47.0% versus 2.7%, $P < .001$). Short CR duration and high relapse rate were more significant than those of the previous report in adults.[14] *KIT* mutations have recently been reported not to influence the clinical outcome in pediatric core-binding factor (CBF) leukemia patients.[20] Although they found *KIT* mutations in 5 of 16 cases of t(8;21) AML, they did not describe the clinical outcome of patients with t(8;21) AML with or without *KIT* mutations. Furthermore, the clinical outcome of the patients

Table 1. Clinical characteristics of patients with t(8;21) AML with *KIT* mutations

Patient no.	Age, y	Sex	WBC count, × 10^9 cells/L	Additional chromosome abnormalities	Time of relapse, mo	Status of allo-SCT	Survival, mo	*KIT* mutation
1	8	F	14.10	None	12	Second CR	37	A814S
2	8	M	27.60	−Y	14	Second CR	47*	N822K
3	8	F	10.77	−X	10	Second CR	25	D816H
4	6	M	34.50	−Y, +4	12	Second CR	26*	N822K
5	3	F	20.50	None	11	—	25	N822K
6	1	F	4.60	−X, t(7;9)	—	—	32*	N822T
7	15	M	20.80	−Y	—	First CR	56*	D816V
8	13	M	66.20	None	—	First CR	30*	V825A

— indicates not applicable.
*Patient still alive.

without *KIT* mutations in their study was poorer than the outcome of those in our study (EFS 63% versus 92.1%). Our result may depend on our good clinical outcome of patients with t(8;21) AML without *KIT* mutations.

Except for 2 patients who received allo-SCT in first CR (patients no. 7 and no. 8 in Table 1), 5 of 6 (83.3%) patients with the mutation relapsed within 14 months after diagnosis. Allo-SCT was performed in 6 of 8 patients with t(8;21) AML with *KIT* mutations (2 in first CR, 4 in second CR) and 4 patients are still alive. In contrast, allo-SCT was also performed in only 1 of 38 patients with t(8;21) AML without *KIT* mutation in second CR, and this patient is still alive.

A high presenting leukocyte count and extramedullary disease were not associated with the poor prognosis in this study. Notably, *KIT* was mapped to chromosome 4 at band q11 and trisomy 4 was reported to be associated with *KIT* mutation.[21] One patient with trisomy 4 in addition to t(8;21) had N822K mutation (patient no. 4). As for additional chromosome abnormality, loss of sex chromosome was observed in 5 (62.5%) of 8 patients with *KIT* mutation and 14 (37%) of 38 patients without mutations, although the difference between them was not statistically significant. Recently, it has been reported that AML blasts with N822K mutation are sensitive to the tyrosine kinase inhibitor Gleevec/STI571/imatinib mesylate.[12] The effectiveness of imatinib mesylate for the patient with AML with *KIT* mutation was also reported.[22] Thus, tyrosine kinase inhibitors may be applicable for these patients in the future.

Two samples examined at relapse showed the same mutations as those at diagnosis (patients no. 3 and no. 5), and these *KIT* mutations disappeared in samples in remission, suggesting that *KIT* mutation was not a constitutional abnormality.

Recently, clonal leukemic cells with *AML1-MTG8* fusion transcript have been reported to arise in utero.[23] Moreover, it was reported that this fusion transcript was not sufficient for full leukemogenesis, and that additional genetic events were required.[24,25] *KIT* mutations may be one of the secondary genetic events of the stepwise leukemogenesis of t(8;21) AML.

FLT3-ITD was found in only 2 (4.6%) of 46 patients with t(8;21). One patient died during chemotherapy, and the other patient was disease free for 42 months from diagnosis. *FLT3*-ITD is considered to be strongly associated with a poor prognosis in AML.[6,7] However, *FLT3*-ITD was rarely reported in patients with t(8;21) AML.[8,9,13,14,20] Our data also confirmed the low incidence of *FLT3*-ITD in patients with t(8;21) AML. As for D835Mt of the *FLT3* gene, we found the mutation in 1 of 46 patients, who was alive for 31 months after diagnosis.

In total, 11 (23.9%) of 46 patients with t(8;21) AML in this study had *KIT* or *FLT3* mutations, suggesting that the pediatric patients with t(8;21) AML had genetic heterogeneity. In conclusion, *KIT* mutations are considered to be strongly associated with poor prognosis in pediatric t(8;21) AML.

Acknowledgment

The authors are grateful to all members of the Japanese Childhood AML Cooperative Study Group.

Appendix

Members of the Japanese Childhood AML Cooperative Study Group who contributed data to the study include Akira Morimoto, Department of Pediatrics, Kyoto Prefectural University of Medicine; Ryoji Kobayashi, Department of Pediatrics, Hokkaido University School of Medicine; Hiromasa Yabe, Department of Pediatrics, Tokai University School of Medicine; Kazuko Hamamoto, Department of Pediatrics, Hiroshima Red Cross Hospital; Shigeru Tsuchiya, Department of Pediatric Oncology, Institute of Development, Aging, and Cancer, Tohoku University; Yuichi Akiyama, Department of Pediatrics, National Hospital Organization Kyoto Medical Center; Hisato Kigasawa, Department of Hematology, Kanagawa Children's Medical Center; Akira Ohara, Department of First Pediatrics, Toho University School of Medicine; Hideki Nakayama, Department of Pediatrics, Hamanomachi Hospital; Kazuko Kudo, Department of Pediatrics, Nagoya University Graduate School of Medicine; and Masue Imaizumi, Department of Hematology/Oncology, Miyagi Prefectural Children's Hospital.

References

1. Rubnitz JE, Raimondi SC, Halbert AR, et al. Characteristics and outcome of t(8;21)-positive childhood acute myeloid leukemia: a single institution's experience. Leukemia. 2002;16:2072-2077.
2. Schlenk RF, Benner A, Krauter J, et al. Individual patient data-based meta-analysis of patients aged 16 to 60 years with core binding factor acute myeloid leukemia: a survey of the German Acute Myeloid Leukemia Intergroup. J Clin Oncol. 2004;22:3741-3750.
3. Nguyen S, Leblanc T, Fenaux P, et al. A white blood cell index as the main prognostic factor in t(8;21) acute myeloid leukemia (AML): a survey of 161 cases from the French AML Intergroup. Blood. 2002;99:3517-3523.
4. Baer MR, Stewart CC, Lawrence D, et al. Expression of the neural cell adhesion molecule CD56 is associated with short remission duration and survival in acute myeloid leukemia with t(8;21)(q22;q22). Blood. 1997;90:1643-1648.
5. Yokota S, Kiyoi H, Nakao M, et al. Internal tandem duplication of the FLT3 gene is preferentially seen in acute myeloid leukemia and myelodysplastic syndrome among various hematological malignancies: a study on a large series of patients and cell lines. Leukemia. 1997;11:1605-1609.
6. 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;98:1752-1759.
7. Thiede C, Steudel C, Mohr B, et al. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. Blood. 2002;99:4326-4335.
8. Meshinchi S, Stirewalt DL, Alonzo TA, et al. Activating mutations of RTK/ras signal transduction pathway in pediatric acute myeloid leukemia. Blood. 2003;102:1474-1479.
9. 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;102:2387-2394.
10. Gari M, Goodeve A, Wilson G, et al. c-kit proto-oncogene exon 8 in-frame deletion plus insertion mutations in acute myeloid leukaemia. Br J Haematol. 1999;105:894-900.
11. Beghini A, Peterlongo P, Ripamonti CB, et al. C-kit mutations in core binding factor leukemias. Blood. 2000;95:726-727.
12. Wang YY, Zhou GB, Yin T, 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 U S A. 2005;102:1104-1109.
13. Care RS, Valk PJ, Goodeve AC, 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.
14. Nanri T, Matsuno N, Kawakita T, 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.
15. Tsukimoto I, Tawa A, Hanada R, et al. Excellent outcome of risk stratified treatment for childhood acute myeloid leukemia-AML99 trial. For the Japanese Childhood AML Cooperative Study Group [abstract]. Blood. 2005;106:261a. Abstract 889.
16. 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.

17. 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;103:1085-1088.
18. Yamamoto Y, Kiyoi H, Nakano Y, et al. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. Blood. 2001;97:2434-2439.
19. Beghini A, Ripamonti CB, Cairoli R, et al. KIT activating mutations: incidence in adult and pediatric acute myeloid leukemia, and identification of an internal tandem duplication. Haematologica. 2004;89:920-925.
20. Goemans BF, Zwaan CM, Miller M, et al. Mutations in KIT and RAS are frequent events in pediatric core-binding factor acute myeloid leukemia. Leukemia. 2005;19:1536-1542.
21. Langabeer SE, Beghini A, Larizza L. AML with t(8;21) and trisomy 4: possible involvement of c-kit? Leukemia. 2003;17:1915; author reply 1915-1916.
22. Nanri T, Matsuno N, Kawakita T, Mitsuya H, Asou N. Imatinib mesylate for refractory acute myeloblastic leukemia harboring inv(16) and a C-KIT exon 8 mutation. Leukemia. 2005;19:1673-1675.
23. Wiemels JL, Xiao Z, Buffler PA, et al. In utero origin of t(8;21) AML1-ETO translocations in childhood acute myeloid leukemia. Blood. 2002;99:3801-3805.
24. Yuan Y, Zhou L, Miyamoto T, et al. AML1-ETO expression is directly involved in the development of acute myeloid leukemia in the presence of additional mutations. Proc Natl Acad Sci U S A. 2001;98:10398-10403.
25. Higuchi M, O'Brien D, Kumaravelu P, Lenny N, Yeoh EJ, Downing JR. Expression of a conditional AML1-ETO oncogene bypasses embryonic lethality and establishes a murine model of human t(8;21) acute myeloid leukemia. Cancer Cell. 2002;1:63-74.

bjh short report

Recurrent childhood anaplastic large cell lymphoma: a retrospective analysis of registered cases in Japan

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Received 16 September 2005; accepted for publication 11 November 2005

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Summary

This report presents a retrospective study of 26 Japanese children with recurrent anaplastic large cell lymphoma. The first relapses were documented at a median of 10·5 months after the initial diagnosis. Twenty-four patients achieved a second remission. After a median follow-up period of 47 months, 18 patients are still alive: 15 patients are in second complete remission (CR), three patients are in third CR or later. The 5 year overall and relapse-free survival rates were 61 ± 12% and 51 ± 12% respectively. The patients who received allogeneic haematopoietic stem cell transplantation during second CR showed a superior outcome to other patients.

Keywords: anaplastic large cell lymphoma, relapse, haematopoietic stem cell transplantation, allogenic bone marrow transplantation, autologous stem cell transplantation.

Recently, good results for first-line chemotherapy in children with anaplastic large cell lymphoma (ALCL) have been reported, with event-free survival rates of 60–81% (Brugieres *et al*, 1998; Mora *et al*, 2000; Seidemann *et al*, 2001; Williams *et al*, 2002; Mori *et al*, 2003). However, in most of these series, 20–40% of the patients developed recurrent disease. Brugieres *et al* (2000) reported that most of the patients with relapsed ALCL were highly chemosensitive, but that 40% of patients experienced several relapses. As data concerning ALCL relapses are limited and heterogeneous, the optimal treatment for children with relapsed ALCL has yet to be determined. We report the treatments and outcomes of Japanese children with ALCL relapses.

Patients and methods

We analysed the data on all relapses that occurred in three registered Japanese series of patients treated for ALCL. The Japan Children's Cancer and Leukaemia Study Group (JCCLSG) included 19 patients treated between 1989 and 2003. The Japan Association of Childhood Leukemia Study (JACLS) and the Tokyo Children's Cancer Study Group (TCCSG) included 23 patients treated between 1998 and 2002 and 54 patients treated between 1990 and 2000 respectively. The first-line treatments used in each study differed and were miscellaneous. Of the 96 patients included in these series, 26 patients with relapses were documented (three from JCCLSG,

doi:10.1111/j.1365-2141.2005.05910.x

Table 1. Characteristics of the patients and univariate analysis of possible prognostic factors after first relapse.

Characteristics of the patients							
Age at diagnosis (years)					1–17 (median 10)		
Sex					Male 18, Female 8		
Stage at diagnosis (Murphy's classification)					I, II 5, III, IV 21		
Risk factors of EICNHL*					Low/Standard risk 8, High risk 18		
Time to relapse (months)					1–71 (median 10·5)		
Follow up (months)					8–156 (median 47)		
Possible prognostic factors	Patients	Death	OS	*P*-value	Event	RFS	*P*-value
Time to relapse							
Early relapse (<12 months)	13	6	49 ± 15%	0·089	7	46 ± 14%	0·232
Late relapse (>12 months)	13	2	76 ± 15%		4	61 ± 16%	
During/after the first-line treatment							
Relapse during first-line treatment	7	4	43 ± 19%	0·055	4	43 ± 19%	0·259
Relapse after first-line treatment	19	4	69 ± 14%		7	54 ± 14%	
Year of initial diagnosis							
~1995	8	5	38 ± 17%	0·075	5	38 ± 17%	0·326
1996~	18	3	82 ± 9%		6	64 ± 12%	
Type of first-line treatment							
ALL type	10	5	40 ± 19%	0·121	6	33 ± 17%	0·143
B-NHL type	16	3	77 ± 12%		5	66 ± 13%	
Sites of relapse							
Local site alone	12	5	56 ± 15%	0·538	6	47 ± 15%	0·723
New site	14	3	68 ± 17%		5	58 ± 15%	

Abbreviations: EICNHL: European Inter-Group on Childhood non-Hodgkin Lymphoma, OS: overall survival rate, RFS: relapse-free survival rate, ALL: acute lymphoblastic leukemia, B-NHL: B-cell non-Hodgkin lymphoma
*EICNHL high risk factor: skin and/or mediastinum and/or viscera (lung, liver, spleen) involvement at initial diagnosis

nine from JACLS and 14 from TCCSG). Medical records were retrospectively collected from each treatment group. Twenty of the 26 diagnostic materials were reviewed in each group's reference laboratory. The overall survival (OS) rates and relapse-free survival (RFS) rates were estimated using the Kaplan–Meier method. Statistical differences in the OS and RFS rates were tested using the log-rank test. *P*-values < 0·05 were considered significant.

Results

Patient outcome after first relapse

First relapses were documented 1–71 months (median, 10·5 months) after the initial diagnosis. Characteristics of the patients are summarised in Table 1. Treatments used for first relapse were heterogeneous. Overall, 24 of the 26 patients achieved a second complete remission (CR). The two patients who did not achieve a second CR died of progressive disease 4 months after their first relapse. Among the 24 patients who achieved a second CR, 15 patients are still alive without further relapses after a median follow-up period of 13 months (2–71 months). Three patients died of toxicity after their second CR. The six remaining patients had a second relapse 3–16 months (median, 5·5 months) after their first CR. Of these six relapsed patients, two patients are alive after achieving a third CR and one patient is alive after achieving a fourth CR. One patient died of toxicity after his fourth CR. The two remaining patients died of progressive disease. The 5 year OS and RFS rates were 61 ± 12% and 51 ± 12%, respectively, with a median follow-up of 47 months (8–156 months).

Prognostic factors

In the univariate analysis (Table 1), patients who relapsed during their first-line treatment appeared to have a lower OS (43 ± 19%) than those who relapsed after their first-line treatment (69 ± 14%; $P = 0{\cdot}055$), but the difference was not statistically significant. On the contrary, the RFS rates did not appear to be associated with relapse during first-line treatment or with early relapse (<12 months).

Treatment during second CR and outcome

The treatments used during the second CRs and the outcomes of the patients are shown in Fig 1. Among three treatment groups (chemotherapy alone, autologous haematopoietic stem cell transplantation (auto-HSCT), and allogeneic HSCT (allo-HSCT), there was no significant difference among the possible prognostic factors. Ten of the 24 patients who achieved a second CR were treated using chemotherapy alone. Six of these patients survived without further relapse, one died of treat-

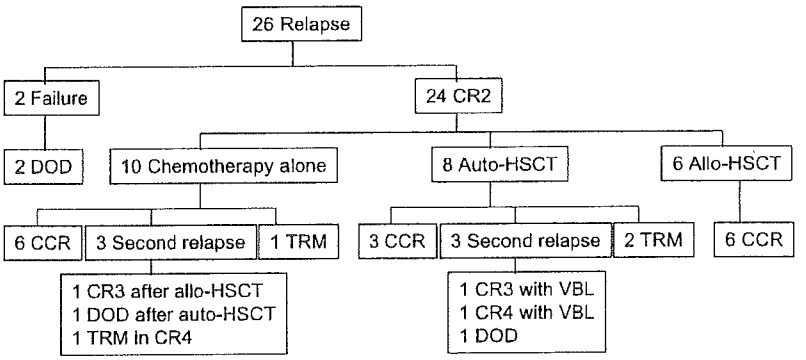

Fig 1. Outcomes of 26 Japanese children with recurrent ALCL. CR, complete remission; DOD, died of disease; Auto-HSCT, autologous haematopoietic stem cell transplantation; Allo-HSCT, allogeneic haematopoietic stem cell transplantation; CCR, continuous complete remission; TRM, treatment-related mortality; VBL, vinblastine.

ment-related toxicity, and three experienced further relapses. Only three of the eight patients who received auto-HSCT during their second CR survived without further relapse. Two patients died of toxicity, and three patients experienced further relapses. Two of the three patients who experienced a second relapse following auto-HSCT during their second CR continued a third CR with the administration of vinblastine. It is noteworthy that all six patients who received allo-HSCT during their second CR survived without further relapse, with a median follow-up period of 42 months (12–124 months). Five of these patients underwent a total body irradiation-containing regimen for allo-HSCT. Three patients experienced chronic graft *versus* host disease. The 3 year RFS rates after the first relapse were 53 ± 17% for the chemotherapy alone group, 33 ± 18% for the auto-HSCT group and 100% for the allo-HSCT group.

Discussion

The present study evaluated the treatments and outcomes of Japanese children with recurrent ALCL. Although the second-line therapies used were miscellaneous, responses to chemotherapy after the first relapse were very good, as previously reported (Brugieres *et al*, 2000). Although Brugieres *et al* (2000)reported that an interval of <12 months between the initial diagnosis and relapse were found to be associated with a higher risk of failure after relapse, prognostic factors for recurrent ALCL were not clearly identified in the present study. As this was a retrospective study spanning over 14 years, the treatment modalities and the indications for HSCT treatments have changed during this period. To clarify accurate prognostic factors in recurrent ALCL children, a prospective study involving a large number of patients would be necessary.

It was interesting that all the patients who received an allo-HSCT during their second CR survived without further relapse, whereas only three of the eight patients who received auto-HSCT survived without further relapse. Some previous reports have described the results of high-dose chemotherapy with auto-HSCT for ALCL patients. The outcome of the patients varied according to their remission status prior to the auto-HSCT, being better in the first CR or chemosensitive-relapse patients (Fanin *et al*, 1999; Deconinck *et al*, 2000; Sandlund *et al*, 2002; Attarbaschi *et al*, 2005). Brugieres *et al* (2000) reported that patients treated with auto-HSCT during their second CR did not appear to have a better outcome than other patients. A small number of experiences concerning the use of allo-HSCT for the treatment of high-risk ALCL have been previously reported (Chakravarti *et al*, 1990; Sandlund *et al*, 2002; Attarbaschi *et al*, 2005; Cesaro *et al*, 2005). Theoretically, allo-HSCT offers the advantage of a tumour-free graft and the possible induction of a graft *versus* lymphoma effect. Although there has been no clear evidence showing a graft *versus* lymphoma effect from allo-HSCT in patients with ALCL, the results of the present study suggested that allo-HSCT for recurrent ALCL might have additional therapeutic effects to those of high-dose chemotherapy. Prolonged anti-tumour effects, such as a graft *versus* lymphoma effect or maintenance treatment with vinblastine, might be more crucial than high-dose chemotherapy for some recurrent ALCL patients.

Acknowledgements

This work was supported by the Pfizer Fund for Growth and Development Research, and the Ministry of Education, Culture, Sports, Science and Technology of Japan. This work was also supported in part by a grant for Clinical Cancer Research from the Ministry of Health, Labor and Welfare, Japan.

References

Attarbaschi, A., Dworzak, M., Steiner, M., Urban, C., Fink, F.M., Reiter, A., Gadner, H. & Mann, G. (2005) Outcome of children with primary resistant or relapsed non-Hodgkin lymphoma and mature B-cell leukemia after intensive first-line treatment: a population-based analysis of the Austrian Cooperative Study Group. *Pediatric Blood and Cancer*, **44**, 70–76.

Brugieres, L., Deley, M.C., Pacquement, H., Meguerian-Bedoyan, Z., Terrier-Lacombe, M.J., Robert, A., Pondarre, C., Leverger, G., Devalck, C., Rodary, C., Delsol, G. & Hartmann, O. (1998) CD30(+) anaplastic large-cell lymphoma in children: analysis of 82 patients enrolled in two consecutive studies of the French Society of Pediatric Oncology. *Blood*, **92**, 3591–3598.

Brugieres, L., Quartier, P., Le Deley, M.C., Pacquement, H., Perel, Y., Bergeron, C., Schmitt, C., Landmann, J., Patte, C., Terrier-Lacombe, M.J., Delsol, G. & Hartmann, O. (2000) Relapses of childhood anaplastic large-cell lymphoma: treatment results in a series of 41 children – a report from the French Society of Pediatric Oncology. *Annals of Oncology*, **11**, 53–58.

Cesaro, S., Pillon, M., Visintin, G., Putti, M.C., Gazzola, M.V., D'Amore, E., Scarzello, G., Zanesco, L., Messina, C. & Rosolen, A. (2005) Unrelated bone marrow transplantation for high-risk anaplastic large cell lymphoma in pediatric patients: a single center case series. *European Journal of Haematology*, **75**, 22–26.

Chakravarti, V., Kamani, N.R., Bayever, E., Lange, B., Herzog, P., Sanders, J.E. & August, C.S. (1990) Bone marrow transplantation for childhood Ki-1 lymphoma. *Journal of Clinical Oncology*, **8**, 657–660.

Deconinck, E., Lamy, T., Foussard, C., Gaillard, F., Delwail, V., Colombat, P., Casassus, P., Lemevel, A., Brion, A. & Milpied, N. (2000) Autologous stem cell transplantation for anaplastic large-cell lymphomas: results of a prospective trial. *British Journal of Haematology*, **109**, 736–742.

Fanin, R., Ruiz de Elvira, M.C., Sperotto, A., Baccarani, M. & Goldstone, A. (1999) Autologous stem cell transplantation for T and null cell CD30-positive anaplastic large cell lymphoma: analysis of 64 adult and paediatric cases reported to the European Group for Blood and Marrow Transplantation (EBMT). *Bone Marrow Transplantation*, **23**, 437–442.

Mora, J., Filippa, D.A., Thaler, H.T., Polyak, T., Cranor, M.L. & Wollner, N. (2000) Large cell non-Hodgkin lymphoma of childhood: analysis of 78 consecutive patients enrolled in 2 consecutive protocols at the Memorial Sloan-Kettering Cancer Center. *Cancer*, **88**, 186–197.

Mori, T., Kiyokawa, N., Shimada, H., Miyauchi, J. & Fujimoto, J. (2003) Anaplastic large cell lymphoma in Japanese children: retrospective analysis of 34 patients diagnosed at the National Research Institute for Child Health and Development. *British Journal of Haematology*, **121**, 94–96.

Sandlund, J.T., Bowman, L., Heslop, H.E., Krance, R., Mahmoud, H., Pui, C.H., Hale, G. & Benaim, E. (2002) Intensive chemotherapy with hematopoietic stem-cell support for children with recurrent or refractory NHL. *Cytotherapy*, **4**, 253–258.

Seidemann, K., Tiemann, M., Schrappe, M., Yakisan, E., Simonitsch, I., Janka-Schaub, G., Dorffel, W., Zimmermann, M., Mann, G., Gadner, H., Parwaresch, R., Riehm, H. & Reiter, A. (2001) Short-pulse B-non-Hodgkin lymphoma-type chemotherapy is efficacious treatment for pediatric anaplastic large cell lymphoma: a report of the Berlin-Frankfurt-Munster Group Trial NHL-BFM 90. *Blood*, **97**, 3699–3706.

Williams, D.M., Hobson, R., Imeson, J., Gerrard, M., McCarthy, K. & Pinkerton, C.R. (2002) Anaplastic large cell lymphoma in childhood: analysis of 72 patients treated on the United Kingdom Children's Cancer Study Group chemotherapy regimens. *British Journal of Haematology*, **117**, 812–820.

Appendix

Chairpersons of each Lymphoma Committee and the Study Group

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Leukemia (2005) 19, 1145–1152

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MEETING REPORT

The Seventh International Childhood Acute Lymphoblastic Leukemia Workshop Report: Palermo, Italy, January 29–30, 2005

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Between 1995 and 2004, six International Childhood Acute Lymphoblastic Leukemia (ALL) Workshop have been held, and the completion of several collaborative projects has established the clinical relevance and treatment options for several specific genetic subtypes of ALL. This meeting report summarizes the data presented in the seventh meeting and the discussion.
Leukemia (2005) **19,** 1145–1152. doi:10.1038/sj.leu.2403783
Published online 5 May 2005
Keywords: childhood acute lymphoblastic leukemia; minimal residual disease; glucocorticoids; methotrexate; anthracycline; intrathecal therapy

Introduction

To further improve the clinical outcome of therapy for childhood acute lymphoblastic leukemia (ALL), the 'Ponte di Legno' working group meets regularly to identify issues that require international collaboration to address.[1–4] The group recognizes the need not only to increase the cure rate beyond the 80% achieved with contemporary treatments,[5] but also to improve the quality of life of leukemia survivors by limiting both acute and long-term sequelae. At the start of this workshop, Giuseppe Masera stressed that we are limited in our ability to perform clinical trials for ALL because of the current high cure rate, and that large numbers of patients are now often needed to ensure adequate statistical power in the clinical studies. Moreover, with the exception of very high-risk ALL, events tend to occur late such that answers to randomized questions may only become apparent 5–6 years after a study is opened. There was also recognition that the efficacy of specific treatments varies with the dosages used and with the mix of treatment components. This report summarizes the discussion of these and other issues raised by workshop participants.

Strategies to optimize international collaboration

Is a common treatment backbone or question feasible?

Contemporary clinical protocols for ALL share many of the same treatment elements while differing in the detail; yet, they have all yielded similar end results. This indicates that ALL cells can be killed to the same extent with a variety of therapeutic strategies. This complicates attempts to incorporate data from the randomized clinical trials (RCTs) of one study group into the protocols of other groups. For instance, pulse therapy with vincristine and steroid in continuation therapy may not be beneficial when the backbone of therapy already includes intensive reinduction or consolidation therapy, while it is beneficial when little or no reinduction therapy is given.[6,7] Hence, the results of RCTs appear to be treatment backbone dependent. Meta-analyses do not necessarily give answers that can be extrapolated to every protocol.

Workshop participants discussed whether a common treatment backbone and stratification of patients by uniform criteria would be helpful in addressing key clinical questions. In this regard, the same backbone and the same criteria for risk group stratification are used by the Berlin–Frankfurt Münster group (BFM), Associazione Italiana di Ematologia ed Oncologia Pediatrica (AIEOP), and Dutch Childhood Oncology Group (DCOG). Likewise, similar protocols are used in the Medical Research Council/National Cancer Research Institute/United Kingdom (MRC/NCRI/UK) and the US Children's Oncology Group (COG), and in the European Organization for Research and Treatment of Cancer (EORTC) and the French Acute Lymphoblastic Leukemia Study Group (FRALLE). However, protocols of the COG, despite being based on a BFM treatment model, differ in many respects from those of European groups because of decades of treatment modifications and because the COG has elected to use flow cytometry as the measure of minimal residual disease (MRD) rather than PCR, which is the primary method utilized by most European groups. Thus, worldwide introduction of a common backbone of therapy is not feasible at present. Although this complicates the extrapolation of RCT data from one group to another, certain findings have been useful to all groups, as demonstrated by the wide acceptance of reinduction therapy as a critical addition to first-line protocols. Many participants felt that RCT results frequently are most relevant to the treatment programs used to obtain the data. However, the results of an RCT in one treatment program may provide a hypothesis for a trial in a different context.

As therapy becomes more specific for patients with certain subtypes of ALL, such as *BCR-ABL*-positive and infant leukemias, it should be possible to initiate large international RCTs to ensure that adequate numbers of patients with relatively rare ALL subtypes are studied. Indeed, such RCTs are already under way. For the majority of ALL cases, it is often possible for a very

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Received 8 March 2005; accepted 9 March 2005; Published online 5 May 2005

large group, such as the US COG, to accrue enough patients for a statistically valid trial. In Europe, collaboration between the AIEOP and BFM, and the similarity of treatment backbones among many national study groups have made it feasible to conduct several major RCTs at the same time. There is also developing consensus on a common framework for risk stratification and therapy in relapsed ALL.

New standards in clinical monitoring

The criteria used to define induction failure (an M3 marrow with greater than 25% blasts at the end of induction), hematologic relapse (occurrence of an M3 marrow after complete remission), and central nervous system (CNS) relapse (5 or more white blood cells/μl in the cerebrospinal fluid with blasts identified by morphology) were established decades ago. These adverse events represent the failure of treatment regimens to eradicate leukemia. Given the wide use of technologies that allow investigators to definitively identify leukemic cells at low levels, the COG recently proposed new definitions of induction failure, hematologic relapse, and CNS relapse, which were the subject of considerable discussion.

In the proposed new definitions, hematologic relapse is defined as an M3 marrow at any time after the induction of complete remission, or an M2 marrow (5–25% blasts) at any time after remission induction with definitive confirmation that greater than 1% blasts are derived from the original leukemic clone by multiparameter flow cytometry, conventional or molecular cytogenetics, or PCR amplification of clonotypic rearrangements of antigen receptor gene loci or fusion transcripts produced by chromosome translocations.

CNS relapse is established by the presence of more than 5 white blood cells/μl in cerebrospinal fluid with blasts on cytospin preparations or two consecutive specimens obtained at least 4 weeks apart with $\leqslant 5$ white blood cells/μl in cerebrospinal fluid with definitive confirmation that the blasts are derived from the original leukemic clone by molecular cytogenetics, multiparameter flow cytometry, or immunostaining with at least two antigens.

There was vigorous debate concerning these proposed definitions. The general consensus was that relapse must be defined strictly to minimize any possibility of false positives. No consensus was reached regarding the proposed new definition for hematologic relapse. Although there was general agreement that the proposed new definition of CNS relapse was reasonable and, in fact, the DCOG and St Jude Children's Research Hospital are using the same criteria, discussion on this issue was brief and a decision concerning adoption was tabled. COG investigators will circulate the proposed definitions with a detailed rationale for further discussion. While the COG has already incorporated the modified definition of CNS relapse into its new ALL protocols, they will defer implementation of the proposed new definition of hematologic relapse pending the outcome of additional discussions with international colleagues.

Minimal residual disease

In vivo measurements of leukemia cytoreduction in childhood ALL reflect the combined effects of leukemic cell clinical and biological variables as well as treatment and host pharmacogenetics and pharmacokinetics, and provide a more precise assessment of the effectiveness of treatment. Thus, measurement of MRD at the end of remission induction and/or consolidation treatment was considered by the panel to be the most rationale strategy for tailoring and potentially individualizing treatment. There was also agreement that conclusive evidence to support the clinical application of MRD-based risk assessment is still lacking, making it mandatory to apply this method only in the context of clinical trials. Several techniques have been developed over the past 10–15 years to complement morphology in assessing response to treatment, including immunologic assays, molecular analyses, fluorescent *in situ* hybridization, and colony assays.[8–11] These techniques have drastically changed the definition of 'remission,' which now depends on the sensitivity of the methodology used.[8–15] In most published reports, MRD evaluation at the end of induction (days 28–33) and at day 78 (in the AIEOP-BFM experience) identifies a subgroup of patients (20–40%) who have no MRD detectable at either time point and have an outstanding clinical outcome (long-term event-free survival rate of greater than 90%). Whether these patients could benefit from reduction of treatment intensity is still unknown. At least three ongoing randomized clinical studies (AIEOP-BFM-ALL2000, NCRI ALL2003, and DCOG ALL-10) are assessing a slight or moderate treatment reduction. However, remission induction and consolidation treatment of the AIEOP-BFM study is more intensive than those of the COG study for standard-risk patients, making generalization of treatment reduction problematic.

The panel agreed that caution should be taken not to overemphasize the long-term side effects of current standard treatment; accordingly, treatment reduction may not be the most relevant issue for further RCTs. In the intermediate-risk group, the assessment of MRD identifies different profiles of treatment response. Whether these differences correlate with the time and site of relapse is still unknown. The panel discussed the potential relevance of the assessment of later time points (ie, during maintenance or at cessation of therapy). In the St Jude experience, all intermediate-risk patients had no detectable MRD at the end of treatment, either by immunologic or PCR-based methods with a sensitivity of 10^{-4}–10^{-5} level. The results of the ongoing studies will indicate the relevance of early MRD assessment to identify intermediate-risk patients who would relapse either during or after cessation of treatment. Accordingly, the panel agreed that it is too early to plan for further treatment modifications in intermediate-risk patients in the absence of strong data on the predictive value of early MRD assessment and of proper treatment options to be tested in RCTs. High-risk patients, as defined on the basis of biologic features or early response to treatment, are rather heterogeneous with respect to MRD levels. The introduction of MRD evaluation, either by immunologic or molecular methods, allows the identification of a new category of previously unrecognized very high-risk patients, who have MRD level $>10^{-2}$ at the end of remission induction or $>10^{-3}$ at the end of consolidation treatment, and account for about 5% of the patients overall. It is still unknown whether this very high-risk subgroup would benefit from more intensive treatment, and whether their outcome differs from that of very high-risk patients identified on the basis of biologic features at diagnosis, or early response to pre-phase treatment with glucocorticoid and methotrexate (according to the BFM strategy), or morphologic evaluation of bone marrow at day 7 or 15. The design of some current trials such as the AIEOP-BFM ALL2000, in which these patients are prospectively allocated to the high-risk group, has the potential to address this question. MRD assessment would also help in the future to study the efficacy of new drugs immediately or soon after remission induction in patients who fail to attain molecular or immunologic remission. Finally, the panel recommended the

assessment of MRD levels in the bone marrow before the start of conditioning regimen in all very high-risk patients undergoing hematopoietic stem cell transplantation. There is good evidence that patients with high levels of MRD prior to transplantation have an extremely poor prognosis.

Dexamethasone *vs* prednisone

Dexamethasone has long been considered to be more effective than prednisone or prednisolone for the treatment of ALL, especially in terms of CNS control, because it has a longer half-life, binds to protein less avidly, and penetrates into CNS more readily. Three randomized trials comparing the efficacy of dexamethasone *vs* that of prednisone have been completed.[16–18] In the Children's Cancer Group study for patients with standard-risk ALL, the 530 patients who received dexamethasone at 6 mg/m^2 per day throughout all phases of treatment fared significantly better than the other 530 patients who received prednisone at 40 mg/m^2 per day in all phases of therapy except delayed intensification.[16] Similarly, in the Medical Research Council UK ALL 97/99 study, dexamethasone given at 6.5 mg/m^2 per day during the same phases of therapy yielded significantly better overall results as compared with prednisone at 40 mg/m^2.[18] By contrast, in the Tokyo Children's Cancer Study Group trial of 359 patients with standard-risk or intermediate-risk ALL, comparable results were achieved when dexamethasone was given at 8 mg/m^2 per day and prednisone at 60 mg/m^2 per day.[17] Dexamethasone seems to be well tolerated in a three-drug induction schedule (dexamethasone plus vincristine plus L-asparaginase) or in a four-drug induction regimen (with the addition of anthracycline) in patients <10 years of age. Anecdotal experiences suggest that the degree of toxicity associated with dexamethasone treatment, such as osteonecrosis, osteopenia, hyperglycemia, and thrombosis, is related to the dose and duration of treatment. Several ongoing trials will address the optimal dose and duration of glucocorticoid treatment. In the AIEOP-BFM-ALL2000 and EORTC 58951 studies, dexamethasone at 10 mg/m^2 per day is being compared with prednisone at 60 mg/m^2 per day. In a current COG study for children with high-risk B-cell precursor ALL, the induction steroid is randomized between dexamethasone at 10 mg/m^2 per day on days 1–14 and prednisone at 60 mg/m^2 per day for 28 days. Finally, investigators at St Jude Children's Research Hospital are determining if the pharmacokinetics of dexamethasone correlates with treatment toxicity.

Optimal administration of systemic methotrexate

Common modes of methotrexate administration in childhood ALL include (1) intravenous high dose (1–8 g/m^2) followed by leucovorin rescue, (2) intravenous moderate dose (100–500 mg/m^2) without rescue, commonly given in combination with vincristine and asparaginase (so-called 'Capizzi' methotrexate), and (3) oral, intravenous, or intramuscular low dose (20–40 mg/m^2). A meta-analysis conducted by the Oxford group concluded that intravenous high-dose methotrexate with leucovorin rescue had little impact on the incidence of CNS relapse, but seemed to reduce the risk of bone marrow relapse compared to low-dose methotrexate without rescue.[19]

CCG 1882 was the first study to incorporate the Capizzi methotrexate regimen into front-line therapy for ALL.[20] Capizzi methotrexate was one component of the highly intensified chemotherapy regimen (augmented BFM arm) designed to improve the outcome for patients with high-risk ALL with an M3 bone marrow on day 7 of four-drug remission induction. The augmented BFM arm produced a significantly better event-free survival for these patients compared to the standard modified BFM treatment arm that contained no intravenous methotrexate.[20] Subsequently, in CCG 1961 study, 1284 high-risk patients with a rapid response to induction therapy (M1 or M2 day 7 marrow) were randomized to receive augmented or standard intensity therapy and to one or two delayed intensification phases (NL Seibel *et al. Blood* 2003; **102** (11, part 1 of 2 parts): 224a). Augmented intensification produced a significantly better event-free survival than standard intensity therapy, and there was no significant difference in outcome between those who received one or two delayed intensification phases. Thus, augmented therapy, which includes Capizzi methotrexate and only one delayed intensification phase, is now considered the standard therapy in COG for children with high-risk ALL and a rapid early response. This treatment is now being randomized against the use of high-dose methotrexate. Other high-risk cases continue to receive full augmented therapy. However, the contribution of Capizzi methotrexate *per se* to the improved outcome could not be assessed in either of these trials. Successive COG standard-risk trials are studying the merits of intravenous methotrexate without rescue (CCG 1991) and Capizzi methotrexate (COG ALL0331) in children with standard-risk ALL. Very high-risk patients in the MRC/NCRI ALL 2003 trial also receive Capizzi methotrexate after early intensification therapy.

In the new COG high-risk B-cell precursor ALL trial and the COG T-cell ALL trial, high-dose methotrexate with leucovorin rescue will be randomized against Capizzi methotrexate without leucovorin rescue. A current lymphoblastic lymphoma protocol in the COG randomizes high-dose methotrexate (5 g/m^2) with rescue against standard oral methotrexate. Notwithstanding the paucity of randomized studies on the efficacy of high-dose methotrexate, this treatment has been incorporated into many institutional and collaborative group clinical trials. The use of high-dose methotrexate may allow a relatively short course of intrathecal therapy to provide adequate CNS control. In this regard, the current COG and MRC/NCRI trials utilizing Capizzi methotrexate include protracted intrathecal methotrexate therapy. Currently, there is still some debate on the value of high-dose methotrexate with rescue in pediatric ALL protocols. It was mentioned that several randomized trials failed to show an event-free survival advantage for high-dose methotrexate with leucovorin rescue, for example, CCG 139 (0.5 g/m^2), CCG 144 (33.6 g/m^2), UKALL97 (6–8 g/m^2), and FRALLE 93a (1.5 g/m^2).

Role of anthracyclines for standard-risk patients

Historically, very few studies have compared induction therapy with or without anthracyclines, although meta-analysis of the available data showed a 33% reduction in marrow relapse in trials with anthracyclines ($P=0.004$), without significant improvement in event-free survival or extramedullary leukemia control.[21–23] In the era of those studies, treatment-related morbidity and mortality were much higher than in the current trials.[24] The CCG 105 study demonstrated no benefit for a four-drug remission induction including anthracycline compared to standard three-drug induction for patients 1–9 years old and a leukocyte count $<50 \times 10^9$/l.[25] However, the same study showed a significant benefit for the use of a delayed intensification phase, which included anthracycline.

There is little evidence regarding the optimal dose, optimal type, or optimal duration of anthracycline infusion on efficacy or toxicity in standard- or low-risk patients.[21,26,27] The BFM 95 study decreased daunorubicin during remission induction to two doses (30 mg/m^2 per dose) given on days 8 and 15 for standard-risk patients with leukocyte counts $<20 \times 10^9$/l and aged 1–5 years who are prednisone good responders but therapy contains anthracyclines later (M Schrappe, *et al. Blood* 2003; **102** (II): 222a). In the current BFM 2000 study, all patients received four doses of anthracycline during remission induction. Although the value of the addition of anthracyclines during induction for standard- or low-risk patients remains to be proven, very few study groups totally omit these drugs from the overall treatment plan for such patients. The panel acknowledged that in the context of complex multidrug treatment, it is very difficult to dissect out the value of or even attribute toxicity to specific individual agents. Nonetheless, the cumulative anthracycline dose has been reduced in the Cooperative Acute Lymphoblastic Leukemia Study Group (COALL) for good-risk patients selected by *in vitro* drug sensitivity testing, and in the AIEOP-BFM study for good-risk patients identified by MRD measure.

Role of dexrazoxane in childhood ALL

Dexrazoxane (1CRF-187, ADR-159) is a topoisomerase II inhibitor that was originally introduced in the 1970s as an antineoplastic agent, with disappointing results. As an iron chelator, it was later tested as a cardioprotectant, by preventing free radical formation and scavenging of existing free radicals produced by anthracycline–iron complexes. A recent randomized study by the Dana-Farber Consortium showed that dexrazoxane treatment was associated with a significant decrease in the percentage of patients with acute myocardial injury, as indicated by serum troponin levels.[28] However, a critical review must take into account all available parameters of efficacy and toxicity. First, the serum troponin level has not consistently been shown to be elevated in children with ALL after treatment with anthracyclines (L Kremer, FM Fink, and A Baruchel, unpublished data). Second, the correlation of elevation of troponin level and cardiac abnormalities detected by echocardiography was based on a small number of patients,[29] and long-term echocardiographic measurements have yet to be reported. Third, the dosage of anthracycline used in the Dana-Farber Consortium protocol is much higher than that in the majority of other protocols. Finally, any advantage of dexrazoxane must be weighed against the side effects, including bone marrow suppression and a potential leukemogenic effect. In a randomized study of patients with pediatric Hodgkin's disease, dexrazoxane use was associated with a higher rate of severe infection and a higher incidence of secondary malignancies in the context of simultaneous use of etoposide, cyclophosphamide, and G-CSF (CL Schwartz *et al. Blood* 2003; **102** (11, part 1 of 2 parts): 143a). In view of these findings, dexrazoxane was not given a high priority of investigation by the workshop participants. Nevertheless, an evaluation of this agent in high-risk subgroups (ie, those with poor early responses) who received the highest dose of anthracyclines ($\geqslant$300 mg/m^2) could be envisaged.

Continuation treatment

Continuation treatment with weekly methotrexate and daily mercaptopurine is a phase in which treatment intensity may be affected by differences in pharmacokinetics and pharmacogenetics, variability in physician compliance with protocol guidelines, and patient compliance with the prescribed oral medication. Few RCTs have explored the optimal way to administer, monitor, and adjust methotrexate and mercaptopurine dosage. Intrathecal chemotherapy, high-dose intravenous methotrexate, pulse therapy with vincristine plus a steroid, and the simultaneous use of other drugs further complicate the interpretation of data obtained with intensification of methotrexate and mercaptopurine based on blood counts. The current protocols of major institutional and collaborative study groups use different dose and dose adjustment strategies for this drug pair (Tables 1 and 2).

Based on the published clinical and pharmacological data, the participants agreed to the following guidelines: starting doses should be in the range of 50–75 mg/m^2 for oral mercaptopurine and 20–40 mg/m^2 for oral or parenteral methotrexate. The lower doses may be chosen for patients known or expected to have reduced bone marrow tolerance due to recent intensive chemotherapy. The dose of mercaptopurine should be 10–20% of the conventional dose for patients with homozygous deficiency of thiopurine methyltransferase. Oral mercaptopurine and methotrexate should be administered in the evening due to a significantly increased risk of relapse with morning dosage[30] and should be given without food, especially milk products, due to a lower bioavailability when coadministered with food and possible breakdown of mercaptopurine by xanthine oxidase in milk products. The doses of both drugs should be adjusted upward (with no upper dose limit) to obtain a total white blood cell count below 3.0×10^9/l, a level that appears to be related to a reduced risk of relapse. The drugs should be reduced in dosage or withdrawn if the white blood cell count falls below 1.5×10^9/l, the absolute neutrophil count below $0.3–0.5 \times 10^9$/l, or the platelet count below 50×10^9/l; some participants would only use absolute neutrophil count and platelet count to modify therapy. Routine measurements of liver function are not necessary in patients without symptoms. However, due to the highly significant correlation between red blood cell levels of methylated mercaptopurine metabolites and a rise in aminotransferases,[31] measurements of aminotransferases can be useful in assessing treatment compliance by patients. The 10% of patients who are thiopurine methyltransferase heterozygotes may also have low levels of aminotransferases, but they will respond with leukopenia to dose increments. Most patients with fatigue, nausea, or gastrointestinal complaints during maintenance therapy have normal liver function tests, and dose reductions should be based on a rise in bilirubin to more than three times the upper normal limit, or aminotransferase levels more than 10 times the upper normal limit and rising. In such cases, other causes such as viral hepatitis or Gilbert's syndrome should also be considered.

A survey among cooperative ALL study groups revealed that the majority of them consider 24 months of total treatment duration to be adequate (Table 2). Some groups use longer continuation therapy, particularly for boys. Two randomized studies have demonstrated that abbreviation of continuation therapy can compromise overall disease-free survival.[32,33] The Tokyo Children's Cancer Study Group (TCCSG) analyzed the feasibility and efficacy of their study L92-13 featuring only 12 months of therapy for children 1–15 years of age.[32] Event-free survival and overall survival rates at 8.5 years were 59.2$\pm$2.9 and 78.8$\pm$22.2%, respectively (M Tsuchida *et al. Blood* 2001; **98**: 223b) Results of this study are being updated (K Horibe, personal communication). In the German BFM trials 81 and 83 (performed from 1981 to 1986), the efficacy of 24-month

Table 1 Administration of continuation treatment according to study group

Group	Methotrexate		Mercaptopurine[a]		Duration (months)
	Dose (mg/week)	Route	Dose (mg/day)	Food	
AIEOP	20	Oral	50	Avoid	24
BFM	20	Oral	50	Avoid/no milk	24
COALL	20	Oral	50	Avoid	24
DCOG	20/30[b]	Oral/i.v.[b]	50	Avoid	24
DFCI	30	i.v./i.m.	50	Avoid/no milk	25
EORTC	20	Oral	50	Avoid	24
JACLS	25/150[c]	Oral/i.v.[c]	50	Avoid	24
MRC/NCRI	20	Oral	75	Avoid/no milk	24/36[d]
NOPHO	20	Oral	75	No recommendation	30
SJCRH	40	i.v.	75	Avoid/no milk	30/36[d]

AIEOP, Associazione Italiana di Ematologia ed Oncologia Pediatrica; BFM, Berlin–Frankfurt–Münster; DCOG, Dutch Childhood Oncology Group; DFCI, Dana-Farber Cancer Institute Consortium; EORTC, European Organization for Research and Treatment of Cancer; JACLS, Japan Association of Childhood Leukemia Study; MRC/NCRI, Medical Research Council, National Cancer Research Institute, United Kingdom Acute Lymphoblastic Leukemia Working Group; NOPHO, Nordic Society of Pediatric Haematology and Oncology; SJCRH, St Jude Children's Research Hospital; i.v., intravenous; i.m., intramuscular.
[a]All groups recommend administering mercaptopurine in the evening.
[b]Higher dose (30 mg/m^2 per week) given intravenously for high-risk cases.
[c]Higher dose (150 mg/m^2 every 2 weeks) given intravenously to high-risk cases.
[d]Longer duration (36 months) for boys.

Table 2 Laboratory measures maintained during continuation treatment or used to modify continuation treatment, according to study group

Group	WBC ($\times 10^9$/l)	ANC ($\times 10^9$/l)	Lymphocyte count ($\times 10^9$/l)	Platelet count ($\times 10^9$/l)	ALT (U/l)	Bilirubin (mg/dl)	TPMT[a]
AIEOP	2–3	>1.0	>0.3	>50	500	None	No
BFM	2–3	>0.2	>0.3	>50	5 × UNL	3 × UNL	Yes
COALL	2–3	None	None	>50	500	3 × UNL	No
DCOG	2–4	None	>0.3	None	5 × UNL	UNL	Yes
DFCI	None	0.5–1.0	None	>75	8 × UNL	1.4 × UNL	No
EORTC	2–3	>0.5	None	>50	10 × UNL	None	Yes
JACLS	2–3	>0.5	None	>50	500	None	No
MRC/NCRI	None	0.75–1.0	None	>50	None	3 × UNL	75/75/7.5
NOPHO	1.5–3.5	None	>0.3	None	None	3 × UNL	75/50/7.5
St Jude	1.5–3.5	0.3–1.0	None	>50	500[b]	2 × UNL[c]	75/60/7.5

See Table 1 for study group names. WBC, white blood cell; ANC, absolute neutrophil count; ALT, alanine aminotransferase; TPMT, thiopurine methyltransferase; UNL, upper limit of normal.
[a]Enzyme activity measured in patients with intolerance to treatment in BFM, DCOG, and EORTC groups. TPMT activity is routinely measured in MRC/UK, NOPHO, and SJCRH trials, with the mercaptopurine dose adjusted according to wild type (75 mg/m^2), heterozygous deficiency (50–75 mg/m^2), and homozygous deficiency of TPMT (7.5 mg/m^2), respectively; erythrocyte 6-thioguanine nucleotides are measured in some European centers and at SJCRH.
[b]Withholding high-dose methotrexate only.
[c]Based on direct (conjugated) bilirubin.

treatment was compared to that of 18-month treatment in all study patients.[33] At 8 years, disease-free survival was 77.3% for patients treated for 24 months, compared to 71.2% for those treated for 18 months only. The benefit of longer continuation therapy appeared to apply more to B-cell precursor ALL than to T-cell ALL. It is still not clear why extended therapy in some trials appears to be more beneficial for boys than for girls.

That one-half of the patients in the TCCSG study who were treated for only 1 year remained free of relapse indicates that reduced treatment duration may be an option for a substantial proportion of patients.[32] Until now, there have been no clinical or biologic indicators to identify which patients can be cured with limited therapy. Ongoing MRD studies may provide some new insights into this question. In addition to the duration of continuation therapy, all components of treatment phases have to be analyzed in detail. Several study groups use pulses of prednisone and vincristine in addition to the regular mercaptopurine and methotrexate therapy. With the increasing efficacy of induction and consolidation therapy, the impact of pulse therapy may have to be re-evaluated. Finally, all participants of the workshop felt that treatment duration is not the key issue in terms of treatment burden, as compared to the side effects associated with intensive treatment phases.

Intensification with vincristine plus steroid pulses during continuation treatment

There was a brief discussion on the meta-analysis by the Childhood ALL Collaborative Group, which analyzed four trials conducted in the 1970s showing a better 5-year event-free survival among patients randomized to receive vincristine plus prednisolone pulses during continuation treatment (68.9 *vs* 58.5%).[34] It was emphasized that definitive conclusions cannot be drawn from this analysis, since the event-free survival advantage conferred by pulse therapy did not translate into better long-term survival, and overall the results were clearly inferior to those being obtained with contemporary therapy.

Moreover, in the CCG 1891 study, vincristine plus prednisone pulses failed to improve outcome among intermediate-risk patients who had received a single delayed intensification treatment.[7] International BFM Consortium initiated an international cooperative study in 1995 to evaluate the impact of pulses of vincristine (given weekly twice) plus dexamethasone (6 mg/m^2 daily for 7 days) given every 10 weeks to a total of six pulses during the continuation treatment in patients treated with a BFM backbone strategy. The result of this study, which enrolled approximately 3000 intermediate-risk ALL patients, will provide information relevant for the BFM oriented therapies. It would still be worthwhile to investigate the value of vincristine plus steroid pulses, using a different steroid drug or a different schedule of administration, in the context of different frontline protocols.

Protracted intrathecal chemotherapy during maintenance

The role of protracted intrathecal chemotherapy during continuation therapy for the prevention of CNS relapses was discussed.[35–37] It was agreed that this treatment modality is effective and allows one to omit cranial radiotherapy in the vast majority of patients. However, protocols such as the BFM-ALL 95 and 2000, which include only 11 doses of intrathecal methotrexate during the intensive phases and during the consolidation phase with high-dose methotrexate (5 g/m^2 × 4), and no protracted intrathecal chemotherapy during the continuation treatment, appear equally effective as other protocols. At the moment, it is not possible to extrapolate from these data whether protracted intrathecal chemotherapy can be omitted in the absence or with lower doses of high-dose methotrexate. Considering that intrathecal chemotherapy is associated with some relevant toxicity (paraplegia, seizures, thrombosis), it seems justified to conduct randomized studies to resolve this issue.

Triple intrathecal therapy *vs* intrathecal methotrexate

Several groups have advocated the use of triple intrathecal therapy with methotrexate, hydrocortisone, and cytarabine, as CNS-directed treatment.[38–40] Laboratory data are mixed with regard to likely interactions (synergistic, additive, or antagonistic) between methotrexate and cytarabine.[41–44] Systemic corticosteroids may decrease the concentration of methotrexate in brain tumors after systemic administration of methotrexate.[45] Hence, the COG undertook a randomized study (CCG 1952) of triple intrathecal therapy *vs* intrathecal methotrexate in standard-risk patients between 1996 and 2000 (LC Stork *et al. Blood* 2002; **100**: 36a). All patients received prednisone in a three-drug remission induction and double-delayed intensification. High-dose methotrexate was not used in any of the patients. A total of 2027 patients were randomized to receive either triple intrathecal therapy or intrathecal methotrexate. Triple intrathecal therapy halved the rate of CNS relapse! However, a corresponding increase in bone marrow and testicular relapse in the triple intrathecal arm resulted in no net improvement in event-free survival and a statistically significant decrease in overall survival due to poor salvage rate after bone marrow relapse (LC Stork *et al. Blood* 2002; **100**: 36a). Triple intrathecal therapy provided greater CNS effect but lesser systemic effect, a finding that highlights the link between so-called isolated CNS relapse and occult marrow involvement. One might speculate that hydrocortisone diminished methotrexate-induced meningeal irritation and decreased permeability.

This trial suggests (i) that the addition of hydrocortisone and cytarabine intrathecally may improve CNS control and (ii) an important impact of intrathecal methotrexate on systemic relapse in the context of this therapy, which includes no other parenteral methotrexate. Others have reported measurable amounts of methotrexate and its polyglutamates in erythrocytes,[46] an increased serum concentration in patients with renal impairment[47] after intrathecal methotrexate, and the impact of intrathecal methotrexate on response after a 'prednisone' prophase.[48] Other interventions, such as replacement of prednisone with dexamethasone[16] and replacement of mercaptopurine with 6-thioguanine (60 mg/m^2),[46] decreased the CNS relapse rate and improved event-free survival. The value of additional methotrexate with no leucovorin rescue is under study in the CCG 1991 study, which has just completed accrual. Currently, most groups utilize intrathecal methotrexate alone for CNS-directed therapy, with or without high-dose methotrexate followed by leucovorin rescue.

Acknowledgements

This work was supported in part by the Osterreichische Kinderkrebshilfe and private donations to the Children's Cancer Research Institute; the Associazione Italiana Ricerca sul Cancro, Fondazione Tettamanti, and Consiglio Nazionale Ricerche-Ministero Istruzione Universitá Ricerca; the Deutsche Krebshilfe, Bonn, and Madeleine Schickedanz Foundation, Fürth, Germany; Cancer Research UKJ; grants from the US National Institutes of Health (CA-21765, CA-51001, CA-31566, CA-78824, CA-29139, CA-37379, GM-61393, and GM61374), a Center of Excellence grant from the State of Tennessee, and the American Lebanese Syrian Associated Charities (ALSAC). C-H Pui is the American Cancer Society-FM Kirby Clinical Research Professor and Tim Eden the Cancer Research (UK) Professor of Paediatric Oncology.

References

1 Pui CH, Evans WE, Gilbert JR. Meeting report: International Childhood ALL Workshop: Memphis, TN, 3–4 December 1997. *Leukemia* 1995; **12**: 1313–1318.
2 Pui CH, Sallan S, Relling MV, Masera G, Evans WE. International Childhood Acute Lymphoblastic Leukemia Workshop: Sausalito, CA, 30 November–1 December 2000. *Leukemia* 2001; **15**: 707–715.
3 Gadner H, Haas OH, Masera G, Pui C-H, Schrappe M. 'Ponte di Legno' Working Group – Report on the Fifth International Childhood Acute Lymphoblastic Leukemia Workshop: Vienna, Austria, 29 April–1 May 2002. *Leukemia* 2003; **17**: 798–803.
4 Pui C-H, Schrappe M, Masera G, Nachman J, Gadner H, Eden OB *et al.* Ponte di Legno Working Group: statement on the right of children with leukemia to have full access to essential treatment and report on the Sixth International Childhood Acute Lymphoblastic Leukemia Workshop. *Leukemia* 2004; **18**: 1043–1053.
5 Pui C-H, Relling MV, Downing JR. Acute lymphoblastic leukemia. *N Engl J Med* 2004; **350**: 1535–1548.
6 Childhood ALL Collaborative Group. Duration and intensity of maintenance chemotherapy in acute lymphoblastic leukaemia: overview of 42 trials involving 12 000 randomised children. *Lancet* 1999; **347**: 1783–1788.
7 Lange BJ, Bostrom BC, Cherlow JM, Sensel MG, La MKL, Rackoff W *et al.* Double-delayed intensification improves event-free survival for children with intermediate-risk acute lymphoblastic leukemia: a report from the Children's Cancer Group. *Blood* 2002; **99**: 825–833.
8 Campana D. Determination of minimal residual disease in leukaemia patients. *Br J Haematol* 2003; **121**: 823–838.

9 Szczepanski T, Orfao A, van der Velden VH, San Miguel JF, Van Dongen JJ. Minimal residual disease in leukaemia patients. *Lancet Oncol* 2001; **2**: 409–417.
10 Cazzaniga G, Biondi A. Molecular monitoring of minimal residual disease. In: Pui C-H (ed). *Treatment of Acute Leukemias: New Directions for Clinical Research*. Tototwa, NJ: Humana Press, 2003, pp 537–547.
11 Cazzaniga G, Biondi A. Molecular monitoring of childhood acute lymphoblastic leukemia using antigen receptor gene rearrangements and quantitative polymerase chain reaction technology. *Haematologica* 2005; **90**: 382–390.
12 Pui C-H, Campana D. New definition of remission in childhood acute lymphoblastic leukemia. *Leukemia* 2000; **14**: 783–785.
13 Borowitz MJ, Pullen DJ, Shuster JJ, Viswanatha D, Montgomery K, Willman CL *et al*. Minimal residual disease detection in childhood precursor-B-cell acute lymphoblastic leukemia: relation to other risk factors. A Children's Oncology Group study. *Leukemia* 2003; **17**: 1566–1572.
14 Malec M, van der Velden VHJ, Björklund E, Wijkhuijs JM, Söderhäll S, Mazur J *et al*. Analysis of minimal residual disease in childhood acute lymphoblastic leukemia: comparison between RQ-PCR analysis of Ig/TcR gene rearrangements and multicolor flow cytometric immunophenotyping. *Leukemia* 2004; **18**: 1630–1636.
15 Coustan-Smith E, Gajjar A, Hijiya N, Razzouk BI, Ribeiro RC, Rivera GK *et al*. Clinical significance of minimal residual disease in childhood acute lymphoblatsic leukemia after first relapse. *Leukemia* 2004; **18**: 499–504.
16 Bostrom BC, Sensel MR, Sather HN, Gaynon PS, La MK, Johnston K *et al*. Dexamethasone *vs* prednisone and daily oral *vs* weekly intravenous mercaptopurine for patients with standard-risk acute lymphoblastic leukemia: a report from the Children's Cancer Group. *Blood* 2003; **101**: 3809–3817.
17 Igarashi S, Manabe A, Ohara A, Kumagai M, Saito T, Okimoto Y *et al*. No advantage of dexamethasone over prednisolone for the outcome of standard and intermediate risk childhood acute lymphoblastic leukemia in the Tokyo Children's Cancer Study Group L95-14 protocol. *J Clin Oncol* (in press).
18 Mitchell CD, Richards SM, Kinsey SE, Lilleymann J, Vora AJ, Eden TOB. Benefit of dexamethasone compared with prednisolone for childhood acute lymphoblastic leukaemia: results of the UK Medical research Council ALL 97/99 trial. *Br J Haematol* 2005 (in press).
19 Clarke M, Gaynon P, Hann I, Harrison G, Masera G, Peto R *et al*. CNS-directed therapy for childhood acute lymphoblastic leukemia: childhood ALL collaborative group overview of 43 randomized trials. *J Clin Oncol* 2003; **21**: 1798–1809.
20 Nachman JB, Sather HN, Sensel MG, Trigg ME, Cherlow JM, Lukens JN *et al*. Augmented post-induction therapy for children with high-risk acute lymphoblastic leukemia and a slow response to initial therapy. *N Engl J Med* 1998; **338**: 1663–1671.
21 Sallan SE, Gelber RD, Kimball V, Donnelly M, Cohen HJ. More is better! Update of Dana-Farber Cancer Institute/Children's Hospital acute lymphoblastic leukemia trials. *Haematol Blood Transfus* 1990; **33**: 459–466.
22 van der Does-van den Berg A, van Wering ER, Suciu S, Solbu G, van't Veer MB, Rammeloo JA *et al*. Effectiveness of rubidomycin in induction therapy with vincristine, prednisone, and L-asparaginase for standard risk childhood acute lymphocytic leukemia: results of a Dutch phase III study (ALL V). A report on behalf of the Dutch Childhood Leukemia Study Group (DCLSG). *Am J Pediatr Hematol Oncol* 1989; **11**: 125–133.
23 Eden OB, Lilleyman JS, Richards S, Shaw MP, Peto J. Results of Medical Research Council Childhood Leukaemia Trial UKALL VIII (Report to the Medical Research Council on behalf of the Working Party on Leukaemia in Childhood). *Br J Haematol* 1991; **78**: 187–196.
24 Hargrave DR, Hann IM, Richards SR, Hill FG, Lilleyman JS, Kinsey S *et al*. Progressive reduction in treatment-related deaths in Medical Research Council childhood lymphoblastic leukaemia trials from 1980 to 1997 (UKALL VIII, X and XI). *Br J Haematol* 2001; **112**: 293–299.
25 Tubergen D, Gilchrist G, O'Brien A, Coccia P, Sather H, Waskerwitz M *et al*. Improved outcome with delayed intensification for children with acute lymphoblastic leukemia and intermediate presenting features. *J Clin Oncol* 1993; **11**: 527–537.
26 Lipshultz SE, Giantris AL, Lipsitz SR, Kimball Dalton V, Asselin BL, Barr RD *et al*. Doxorubicin administration by continuous infusion is not cardioprotective: the Dana-Farber 91-01 Acute Lymphoblastic Leukemia protocol. *J Clin Oncol* 2002; **20**: 1677–1682.
27 Harms DO, Janka-Schaub GE. Co-operative study group for childhood acute lymphoblastic leukemia (COALL): long-term follow-up of trials 82, 85, 89 and 92. *Leukemia* 2000; **14**: 2234–2239.
28 Lipshultz SE, Rifai N, Dalton VM, Levy DE, Silverman LB, Lipsitz SR *et al*. The effect of dexrazoxane on myocardial injury in doxorubicin-treated children with acute lymphoblastic leukemia. *N Engl J Med* 2004; **351**: 145–153.
29 Lipshultz SE, Rifai N, Sallan SE, Lipsitz SR, Dalton V, Sacks DB *et al*. Predictive value of cardiac troponin T in pediatric patients at risk for myocardial injury. *Circulation* 1997; **96**: 2641–2648.
30 Schmiegelow K, Glomstein A, Kristinsson J, Salmi T, Schrøder H, Björk O. On behalf of the Nordic Society for Pediatric Hematology and Oncology. Influence of circadian schedule and of food on the pharmacokinetics of methotrexate and 6-mercaptopurine and on relapse risk in childhood acute lymphoblastic leukemia. *J Pediatr Hematol Oncol* 1997; **19**: 102–109.
31 Nygaard U, Toft N, Schmiegelow K. Methylated metabolites of 6-mercaptopurine are associated with hepatotoxicity in childhood leukaemia. *Clin Pharmacol Ther* 2004; **75**: 274–281.
32 Toyoda Y, Manabe A, Tsuchida M, Hanada R, Ikuta K, Okimoto Y *et al*. Six months of maintenance chemotherapy alter intensified treatment for acute lymphoblastic leukemia of childhood. *J Clin Oncol* 2000; **18**: 1508–1516.
33 Schrappe M, Reiter A, Zimmermann M, Harbott J, Ludwig WD, Henze G *et al*. Long-term results of four consecutive trials in childhood ALL performed by the ALL-BFM study group from 1981 to 1995. *Leukemia* 2000; **14**: 2205–2222.
34 Childhood ALL Collaborative Group. Duration and intensity of maintenance chemotherapy in acute lymphoblastic leukaemia: overview of 42 trials involving 12 000 randomised children. *Lancet* 1996; **347**: 1783–1788.
35 Tubergen DG, Gilchrist GS, O'Brien RT, Coccia PF, Sather HN, Waskerwitz MJ *et al*. Prevention of CNS disease in intermediate risk acute lymphoblastic leukemia: comparison of cranial radiation and intrathecal methotrexate and the importance of systemic therapy: a Children Cancer Group report. *J Clin Oncol* 1993; **11**: 520–526.
36 Pullen J, Boyett J, Shuster J, Crist W, Land V, Frankel L *et al*. Extended triple intrathecal chemotherapy trial for prevention CNS relapse in good-risk and poor-risk patients with B-progenitor acute lymphoblastic leukaemia: a Pediatric Oncology Group study. *J Clin Oncol* 1993; **11**: 839–849.
37 Conter V, Aricò M, Valsecchi MG, Rizzari C, Testi AM, Messina C *et al*. Extended intrathecal methotrexate may replace cranial irradiation for prevention of CNS relapse in children with intermediate-risk acute lymphoblastic leukemia treated with BFM intensive chemotherapy. *J Clin Oncol* 1995; **13**: 2497–2502.
38 Hvizdala E, Berry DH, Chen T, Dyment PG, Kim TH, Steuber CP *et al*. Impact of the timing of triple intrathecal therapy on remission induction in childhood acute lymphoblastic leukemia: a Pediatric Oncology Group study. *Med Pediatr Oncol* 1984; **12**: 173–177.
39 Pullen J, Boyett J, Shuster J, Crist W, Land V, Frankel L *et al*. Extended triple intrathecal chemotherapy trial for prevention of CNS relapse in good-risk and poor-risk patients with B-progenitor acute lymphoblastic leukemia: a Pediatric Oncology Group study. *J Clin Oncol* 1993; **11**: 839–849.
40 Pui CH, Sandlund JT, Pei D, Campana D, Rivera GK, Ribeiro RC *et al*. Improved outcome for children with acute lymphoblastic leukemia: results of Total Therapy Study XIIIB at St Jude Children's Research Hospital. *Blood* 2004; **104**: 2690–2696.
41 Grindey GB, Nichol CA. Interaction of drugs inhibiting different steps in the synthesis of DNA. *Cancer Res* 1972; **32**: 527–531.
42 Tattersall MH, Harrap KR. Combination chemotherapy: the antagonism of MTX and Ara-C. *Eur J Cancer* 1973; **9**: 229–232.

43 Edelstein M, Vietti T, Valeriotte F. The enhanced cytotoxicity of combinations of 1-beta-D-arabinofuranosylcytosine and methotrexate. *Cancer Res* 1975; **35**: 1555–1558.
44 Akutsu M, Furukawa Y, Tsunoda S, Izumi T, Ohmine K, Kano Y. Schedule-dependent synergism and antagonism between methotrexate and cytarabine against human cell lines *in vitro*. *Leukemia* 2002; **16**: 1808–1817.
45 Neuwelt EA, Barnett PA, Bigner DD, Frenkel EP. Effects of adrenal cortical steroids and osmotic blood–brain barrier opening on methotrexate delivery to gliomas in the rodent: the factor of the blood brain–brain barrier. *Proc Natl Acad Sci USA* 1982; **79**: 4420–4423.
46 Bostrom B, Erdman GR, Kamen BA. Systemic methotrexate exposure is greater after intrathecal than after oral administration. *J Pediatr Hematol Oncol* 2003; **25**: 114–117.
47 Gregory RE, Pui C-H, Crom WR. Raised plasma methotrexate concentrations following intrathecal administration in children with renal dysfunction. *Leukemia* 1991; **5**: 999–1003.
48 Thyss A, Suciu S, Bertrand Y, Mazingue F, Robert A, Vilmer E *et al*. Systemic effect of intrathecal methotrexate during the initial phase of treatment of childhood acute lymphoblastic leukemia. The European Organization for Research and Treatment of Cancer Children's Leukemia Cooperative Group. *J Clin Oncol* 1997; **15**: 1824–1830.

Dimerization of MLL fusion proteins and FLT3 activation synergize to induce multiple-lineage leukemogenesis

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The mechanisms by which mixed-lineage leukemia (MLL) fusion products resulting from in utero translocations in 11q23 contribute to leukemogenesis and infant acute leukemia remain elusive. It is still controversial whether the MLL fusion protein is sufficient to induce acute leukemia without additional genetic alterations, although carcinogenesis in general is known to result from more than 1 genetic disorder accumulating during a lifetime. Here we demonstrate that the fusion partner–mediated homo-oligomerization of MLL-SEPT6 is essential to immortalize hematopoietic progenitors in vitro. *MLL-SEPT6* induced myeloproliferative disease with long latency in mice, but not acute leukemia, implying that secondary genotoxic events are required to develop leukemia. We developed in vitro and in vivo model systems of leukemogenesis by MLL fusion proteins, where activated FMS-like receptor tyrosine kinase 3 (FLT3) together with MLL-SEPT6 not only transformed hematopoietic progenitors in vitro but also induced acute biphenotypic or myeloid leukemia with short latency in vivo. In these systems, MLL-ENL, another type of the fusion product that seems to act as a monomer, also induced the transformation in vitro and leukemogenesis in vivo in concert with activated FLT3. These findings show direct evidence for a multistep leukemogenesis mediated by MLL fusion proteins and may be applicable to development of direct *MLL* fusion–targeted therapy.

Introduction

Recurrent translocations involving chromosome 11 band q23 (11q23) are frequent cytogenetic abnormalities observed in hematological malignancies, occurring in approximately 80% of infant and 10% of adult acute leukemias (1, 2). The *mixed-lineage leukemia* (*MLL*) gene (also called *ALL1* or *HRX*) has been cloned in 11q23 translocations, such as t(4;11), t(9;11), and t(11;19) (3, 4), and more than 30 partner genes fused with *MLL* have been identified in various types of 11q23 translocations (5).

MLL encodes a nuclear protein characteristic of several domains with assigned activities including an N terminus with 3 AT-hook motifs, a CXXC domain, 4 cysteine-rich zinc fingers, a transactivation domain, and a highly conserved C-terminal Su(var)3-9, Enhancer of zeste, and Trithorax (SET) domain with histone methyltransferase activity (6, 7). Recent studies demonstrated that MLL is cleaved by Taspase1, generating 2 fragments that heterodimerize to stabilize the complex (8) and assemble in a chromatin-modifying supercomplex (7).

Some important insights into the leukemogenesis by MLL fusion proteins have been obtained (9, 10). *MLL* fusion genes express in-frame chimeric proteins that harbor the N-terminal amino acids of MLL and C-terminal amino acids of each partner protein and reside in the nucleus. Fusion of MLL with a translocation partner, not simple truncation of MLL, leads to development of leukemias in experimental models. Despite sharing little sequence homology to each other among various MLL fusion partners, the oncogenic activity of MLL fusion proteins has been demonstrated to arise from at least 2 distinct common mechanisms (10). One mechanism leads to the aberrant activation of target genes including homeobox genes (*HOX*s) by fusion of MLL with transcriptional activation domains within translocation partners such as *eleven nineteen leukemia* (*ENL*) (9). The other leads to the similar aberrant activation by dimerization of MLL fusion proteins through oligomerization domains within various translocation partners (11, 12). However, it remains unclear whether fusion with all MLL translocation partners leads to development of leukemia through either mechanism. Furthermore, various experimental models in vivo using biotechnological methods such as knock-in (13) and an inducible Cre-lox translocation system (14) as well as retroviral transduction did not reproduce acute leukemia with as short a latency as expected from clinical features of infant leukemia. Therefore, some secondary genotoxic events acquired by the disruptive effects of MLL fusion genes on DNA repair and/or cell-cycle regulation might play an essential role in leukemogenesis by MLL fusion proteins (15); however, this hypothesis has not been thoroughly investigated.

Nonstandard abbreviations used: AML, acute myeloid leukemia; ENL, eleven nineteen leukemia; FLT3, FMS-like receptor tyrosine kinase 3; 5-FU, 5-fluorouracil; hER, human estrogen receptor; HOX, homeobox gene; IRES-EGFP, internal ribosomal entry site-enhanced GFP; ITD, internal tandem duplication; LBD, ligand-binding domain; Meis1, myeloid ecotropic viral integration site 1; MEs, *MLL-ENLs*; MLL, mixed-lineage leukemia; 5′-MLL, the portion of MLL within MLL-SEPT6; MLL-ENLs, MLL-ENL short form; MPD, myeloproliferative disease; MS6, *MLL-SEPT6*; 4-OHT, 4-hydroxy-tamoxifen; SET, Su(var)3-9, Enhancer of zeste, and Trithorax.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* 115:919–929 (2005). doi:10.1172/JCI200522725.

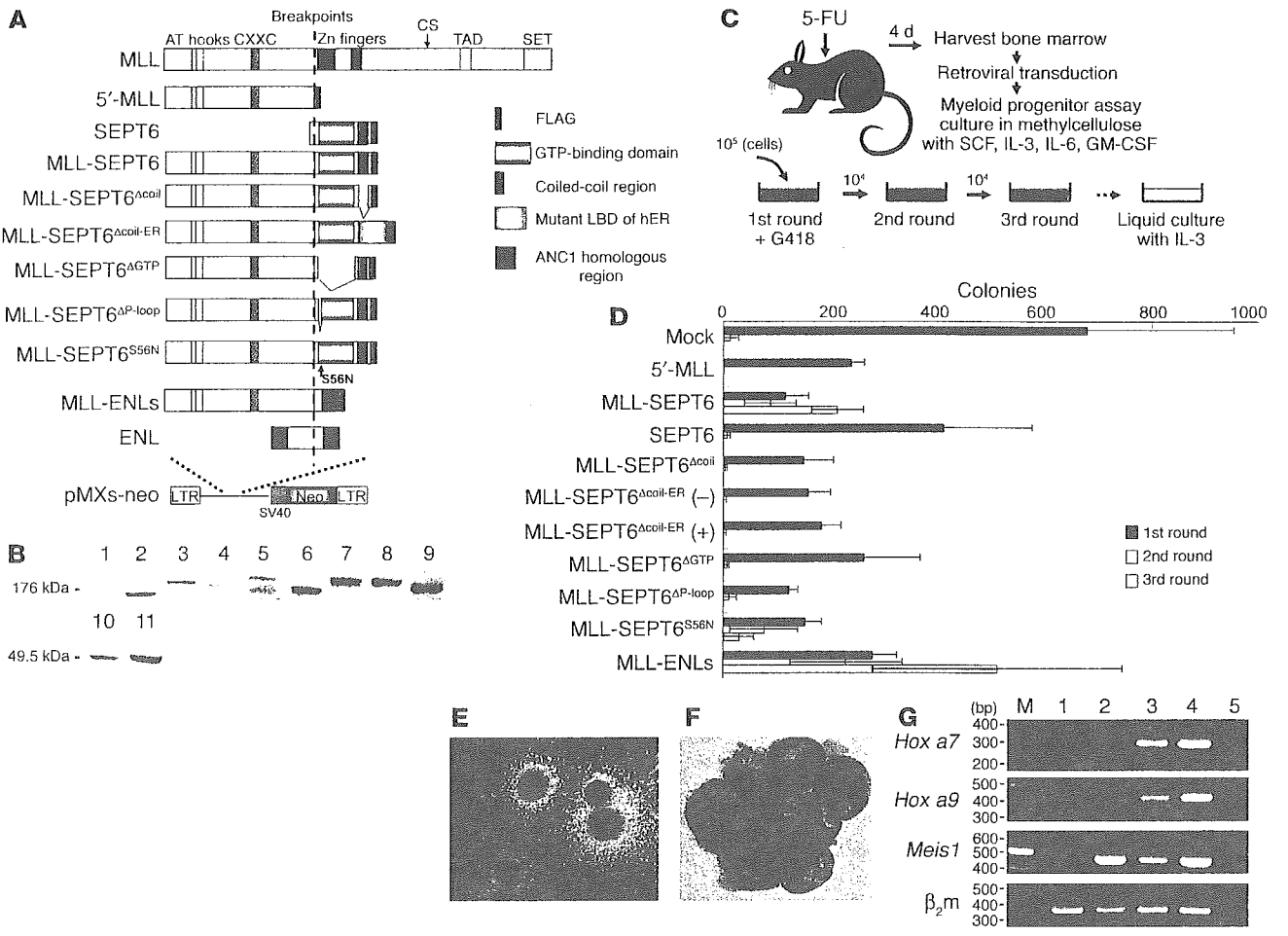

Figure 1

Immortalization of murine hematopoietic progenitors by MLL-SEPT6 fusion protein via aberrant expression of *Hox* genes. (**A**) Schematic representation of the retroviral constructions used. CXXC, CXXC domain; Zn fingers, zinc fingers; CS, cleavage sites; TAD, transactivation domain; SET, SET domain. (**B**) Western blot analysis of proteins extracted from PlatE cells transfected with the constructs shown in **A**, after immunoprecipitation using the anti-MLL Ab (lanes 1–9). Each lysate was blotted with the anti-FLAG Ab (lanes 1–9) or anti-SEPT6 Ab (lanes 10 and 11). Endogenous expression of SEPT6 was detected in lane 10. Lane 1, mock; lane 2, 5′-MLL; lane 3, MLL-SEPT6; lane 4, MLL-SEPT6$^{\Delta coil}$; lane 5, MLL-SEPT6$^{\Delta coil\text{-}ER}$; lane 6, MLL-SEPT6$^{\Delta GTP}$; lane 7, MLL-SEPT6$^{\Delta P\text{-}loop}$; lane 8, MLL-SEPT6^{S56N}; lane 9, MLL-ENLs; lane 10, pMXs-neo alone (endogenous SEPT6); lane 11, SEPT6. (**C**) Experimental strategy for myeloid immortalization assay. (**D**) Myeloid immortalization assay using the constructs shown in **A**. Lanes for MLL-SEPT6$^{\Delta coil\text{-}ER}$ indicate the presence (+) or absence (–) of 4-OHT. The bar graph shows numbers of colonies obtained after each round of replating in methylcellulose (average ± SD). (**E** and **F**) Typical morphology of the colonies generated by *MLL-SEPT6* (**E**), and the cells constituting these colonies (**F**). Original magnification, ×40 (**E**), ×400 (**F**). (**G**) Expression of *Hox a7*, *Hox a9*, and *Meis1* by RT-PCR in the cells from third-round cultures. β_2m was used as an internal standard. M, 100-bp DNA ladder (New England Biolabs Inc.); lane 1, control (Ba/F3 with IL-3) cells; lane 2, mock; lane 3, MLL-SEPT6; lane 4, MLL-ENLs; lane 5, negative control.

We identified the *SEPT6/SEPTIN6* gene on chromosome Xq24 as a novel fusion partner of *MLL* in infant acute myeloid leukemia (AML) (16). SEPT6 contains a short coiled-coil region at the C terminus, and a GTP-binding domain characteristic of the septin family proteins that are important for cytokinesis through polymerization (17, 18). Besides *SEPT6*, 3 of 12 human *SEPTINs* identified to date, *SEPT5/CDCREL1*, *SEPT9/AF17q25/MSF*, and *FLJ10849*, have been shown to be fusion partners of *MLL* (19–22). MLL-SEPT6 contains almost the entire SEPT6, similar to other MLL-SEPTINs. Interestingly, according to previous case reports of 8 *MLL-SEPT6* patients (23–26), *MLL-SEPT6* is involved preferentially in infant AML, while the other *MLL-SEPTINs* remain unclear.

In the present study, we first analyzed the oncogenic potential of *MLL-SEPT6* in vitro by colony-formation assay after retroviral transduction and found that *MLL-SEPT6* immortalized murine hematopoietic progenitors with aberrant expression of *Hox a7* and *Hox a9* as it did in a previously reported case of *MLL-ENL* (9). Mutation analysis of the portion of SEPT6 demonstrated that the fusion partner–mediated homo-oligomerization of MLL-SEPT6 is essential for leukemogenesis. We next analyzed the leukemogenic potential of *MLL-SEPT6* in vivo using retroviral transduction followed by bone marrow transplantation. *MLL-SEPT6* induced no acute leukemia but, rather, lethal myeloproliferative disease (MPD) with prolonged latency in vivo, far from clinical features. To examine the effect of a putative secondary genotoxic stress on

the *MLL-SEPT6*–transduced cells that proliferated with IL-3, the effect of additional expression of *FMS-like receptor tyrosine kinase 3* (*FLT3*) internal tandem duplication (ITD) was tested in vitro. Transduction of *FLT3*-ITD enabled the *MLL-SEPT6*–transduced cells to grow and expand in the absence of IL-3, and simultaneous expression of *MLL-SEPT6* and *FLT3*-ITD led to AML with short latency in vivo. Furthermore, *MLL-ENL*, which requires no dimerization, also induces the same transformation in vitro and AML in vivo in concert with *FLT3*-ITD. This in vitro transformation system and in vivo leukemic development system using murine hematopoietic progenitors through the MLL fusion protein together with the secondary genotoxic stress are useful complementary models to elucidate the molecular mechanism of leukemogenesis by MLL fusion proteins.

Results

MLL-SEPT6 immortalizes murine hematopoietic progenitors with aberrant expression of Hox a7 and Hox a9 in vitro as MLL-ENL does. To examine the oncogenic potential of MLL-SEPT6 in vitro, MLL-SEPT6, the portion of MLL within MLL-SEPT6 (5′-MLL), SEPT6, and MLL-ENL short form (MLL-ENLs) (9) were subcloned into pMXs-neo (27) (Figure 1A). After confirmation, by Western blot analysis, of expression of these genes in packaging cells, the enriched murine hematopoietic progenitors were analyzed by the myeloid immortalization assay using retroviruses harboring these genes or harboring none of them (named mock) (Figure 1, B and C). Initial plating showed similar morphologies and reflected transduction efficiencies. In serial replating, MLL-SEPT6–transduced cells yielded and maintained increasing numbers of compact colonies similar to those generated by MLL-ENLs, although the cells transduced with mock, 5′-MLL, or SEPT6 rapidly failed to form colonies (Figure 1E). Wright-Giemsa–stained cytospin preparations of the cells constituting these compact colonies showed features consistent with myelomonocytic blasts (Figure 1F). Furthermore, RT-PCR of total RNA extracted from the colonies transduced with mock, MLL-SEPT6, and MLL-ENLs at the third round using primers Hoxa7S and Hoxa7AS, or Hoxa9S and Hoxa9AS, revealed that expression of both Hox a7 and Hox a9 was upregulated in MLL-SEPT6–transduced cells, as in MLL-ENLs (9), but not in mock-transduced cells (Figure 1G). The RT-PCR using primers M1S and M1AS also generated 468-bp PCR products as expected, and sequence analysis confirmed that these products represented transcripts of the Hox cofactor myeloid ecotropic viral integration site 1 (Meis1), which was reported to be upregulated with aberrant expression profiles of Hox (11). Unexpectedly, we could not detect any significant difference of expression levels of Meis1 among these colonies.

Single-cell suspensions obtained from the third-round colony could grow and expand in liquid culture supplemented with IL-3 for more than 10 months but failed to survive without IL-3. These immortalized cells expressed Gr-1, CD11b, and c-kit but were negative for Sca-1, B220, CD3, and Ter119 as determined by fluorescence-activated cell sorting (FACS; BD Biosciences) analysis (Figure 2B and data not shown) and contained oligoclonal bands of proviral integration sites, as well as a single band of the full-length proviral DNA, by Southern blot analysis using the Neo probe (Figure 2, A and C). Expression of MLL-SEPT6 chimeric transcripts could be detected by RT-PCR of total RNA extracted from the immortalized cells (Figure 2D), while MLL-SEPT6 fusion proteins were hardly detectable in the lysate of the immortalized cells (data not shown). This showed the toxicity of high expression levels, as previously reported in the literature on leukemogenesis by MLL fusion proteins using retroviral transduction (9). Taken together, these results demonstrated that *MLL-SEPT6* directly immortalizes murine hematopoietic cells via both the block of differentiation and the enhancement of self-renewal in vitro, with aberrant upregulation of *Hox a7* and *Hox a9*, as *MLL-ENL* does.

Myeloid immortalization in vitro by MLL-SEPT6 requires dimerization of MLL fusion proteins through both its GTP-binding domain and its coiled-coil region. Because of common structural characteristics in 4 kinds of MLL-SEPTIN fusion proteins, we examined whether both a GTP-

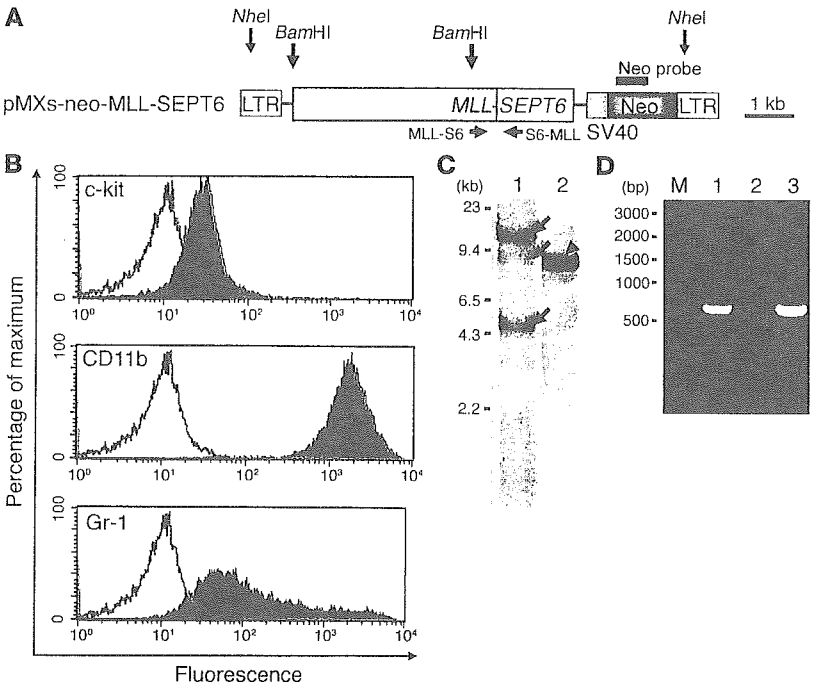

Figure 2
Characterization of the cells immortalized by *MLL-SEPT6*. (**A**) Schematic representation of pMXs-neo-MLL-SEPT6. The restriction endonuclease sites and the Neo probe used in the Southern blot analysis are indicated by vertical arrows and a thick horizontal line, respectively. The primers used are indicated by horizontal arrows. LTR, long-terminal repeat. (**B**) Immunophenotype of the cells immortalized by *MLL-SEPT6*. Shadow profiles represent FACS staining obtained with Abs specific for the indicated cell surface antigens. Green lines represent staining obtained with isotype control Abs. (**C**) Southern blot analysis to detect clonality (lane 1) and integration (lane 2). Genomic DNA extracted from the cells immortalized by *MLL-SEPT6* was digested with *Bam*HI (lane 1) or *Nhe*I (lane 2), and hybridized with the Neo probe. Oligoclonal bands of proviral integration sites and a single band of the intact proviral DNA are indicated by arrows and an arrowhead, respectively. (**D**) Expression of *MLL-SEPT6* fusion transcripts by RT-PCR. M, 1-kb DNA ladder (New England Biolabs Inc.); lane 1, cells immortalized by *MLL-SEPT6*; lane 2, negative control; lane 3, positive control (pMXs-neo-MLL-SEPT6).

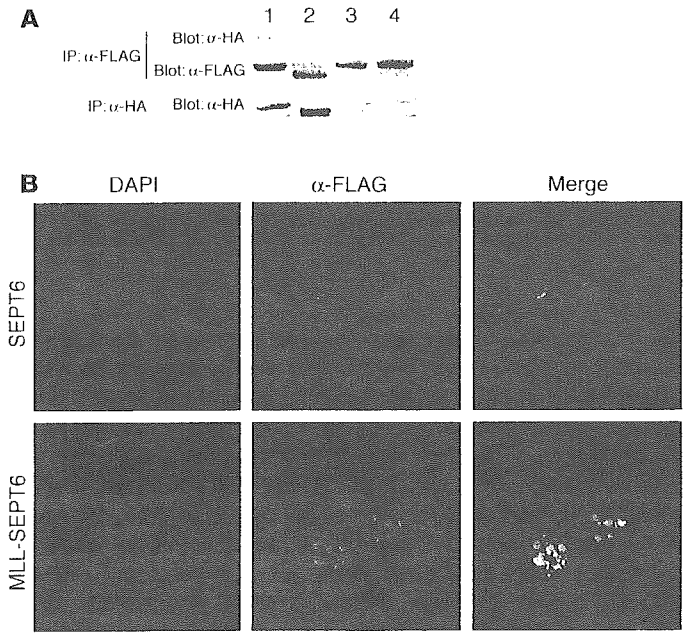

Figure 3
Oligomerization of MLL-SEPT6 fusion protein through both its GTP-binding domain and its coiled-coil region in the nucleus. 293T cells were cotransfected with equal amounts of FLAG-tagged and HA-tagged constructs (**A**), and transfected with pMXs-neo-SEPT6 or pMXs-neo-MLL-SEPT6 (**B**). (**A**) Self-interaction among MLL-SEPT6 fusion proteins or MLL-SEPT6 mutants, analyzed by Western blot analysis after immunoprecipitation. Lysates of 293T cells coexpressing FLAG-tagged and HA-tagged MLL-SEPT6 or its mutants (top and middle) were immunoprecipitated by the anti-FLAG Ab, and lysates of the cells expressing HA-tagged MLL-SEPT6 or its mutants (bottom) were immunoprecipitated by the anti-HA Ab. Anti-FLAG immunoprecipitates were blotted with the anti-HA Ab (top) or the anti-FLAG Ab (middle), while anti-HA immunoprecipitates were blotted with the anti-HA Ab (bottom). Lane 1, MLL-SEPT6; lane 2, MLL-SEPT6$^{\Delta coil}$; lane 3, MLL-SEPT6$^{\Delta P\text{-}loop}$; lane 4, MLL-SEPT6^{S56N}. (**B**) Localization of SEPT6 and MLL-SEPT6, analyzed by immunofluorescent confocal microscopy. FITC-conjugated secondary Abs reacting with the anti-FLAG Ab in 293T cells expressing SEPT6 or MLL-SEPT6 visualized their cellular localizations (middle). Nuclei were visualized with DAPI (left), and merged images are displayed (right). Original magnification, ×400.

binding domain and a coiled-coil region were required for myeloid immortalization by *MLL-SEPT6* in vitro, using various mutants shown in Figure 1A. Our myeloid immortalization assay demonstrated that lack of the GTP-binding domain (MLL-SEPT6$^{\Delta GTP}$), a highly conserved P-loop motif within the GTP-binding domain (MLL-SEPT6$^{\Delta P\text{-}loop}$), or the coiled-coil region (MLL-SEPT6$^{\Delta coil}$) led to failure in colony formation (Figure 1, B and D). Substitution of the critical single amino acid within the P-loop with reduction of GTP-binding activity (MLL-SEPT6^{S56N}), based on previous findings of the well-characterized septin (17), was also found to lead to failure in maintenance of colonies (Figure 1D), and to result in no growth in the liquid culture, demonstrating that MLL-SEPT6^{S56N} also failed to immortalize murine hematopoietic progenitors. These data indicated that the P-loop motif within the GTP-binding domain of SEPT6 was required for MLL-SEPT6–mediated immortalization, suggesting that GTP-binding activity was associated with MLL-SEPT6–mediated immortalization.

To examine whether MLL-SEPT6 homo-oligomerizes through its GTP-binding domain and/or coiled-coil region in the same way as other septins (18, 28), mutual-interaction assay of MLL-SEPT6 and its mutants using immunoprecipitation/Western blot analysis was first performed as shown in Figure 3. The intact MLL-SEPT6, not any mutants, was coprecipitated (Figure 3A), consistent with the activity of the immortalization in vitro. Furthermore, to investigate whether homo-oligomerization was sufficient for the immortalization, the coiled-coil region of pMXs-neo-MLL-SEPT6 was replaced with a mutant ligand-binding domain (LBD) of human estrogen receptor (hER) that induces dimerization specifically in response to 4-hydroxy-tamoxifen (4-OHT) (MLL-SEPT6$^{\Delta coil\text{-}ER}$) (29) (Figure 1A). Unexpectedly, MLL-SEPT6$^{\Delta coil\text{-}ER}$ (Figure 1B) failed to induce the immortalization in the presence of 4-OHT (Figure 1D), suggesting that homo-oligomerization with proper whole structure is essential for MLL-SEPT6–mediated immortalization. On the other hand, immunofluorescent confocal microscopy of 293T cells transiently expressing MLL-SEPT6 or SEPT6 revealed that MLL-SEPT6 was distributed with a pattern of small speckles or dots in the nucleus (Figure 3B, bottom), while overexpression of SEPT6

Table 1
Characteristics of morbid mice transplanted with cells immortalized by *MLL-SEPT6* in vitro and mice transplanted with hematopoietic progenitors, directly after transduction with *MLL-SEPT6*, *MLL-ENL*s, and/or *FLT3*-ITD

Mouse	Latency (d)	Liver wt (g)	Spleen (g)	WBC (cells/μl)	Hb (g/dl)	Plt (× 10^4/μl)
Immortal. cells (n = 5)	210 ± 24	3.94 ± 2.24	0.72 ± 0.23	85,000 ± 75,000	8.3 ± 3.1	17 ± 2.6
PBS (n = 5)	NA	1.45 ± 0.13	0.09 ± 0.01	10,000 ± 2,100	14.5 ± 1.1	93 ± 5.3
MS6/FLT3 (n = 9)	63 ± 18	1.88 ± 0.29	0.38 ± 0.07	95,000 ± 70,000	9.0 ± 3.6	6.0 ± 2.5
MS6/GFP (n = 6)	120 ± 32	2.00 ± 0.87	0.40 ± 0.15	51,000 ± 18,000	7.3 ± 4.0	9.6 ± 6.4
MEs/FLT3 (n = 5)[A]	17.6 ± 1.8	2.37 ± 0.36	0.39 ± 0.09	226,200	12.6	12
MEs/GFP (n = 4)	92 ± 9.1	2.84 ± 0.48	0.71 ± 0.17	71,000 ± 22,000	5.7 ± 0.4	7.5 ± 2.1
Mock/FLT3 (n = 5)[B]	NA	NA	NA	13,000 ± 4,000	13.8 ± 0.9	80 ± 12
Mock/GFP (n = 4)	NA	1.27 ± 0.11	0.07 ± 0.01	13,000 ± 5,000	14.1 ± 1.2	59 ± 28

Averages with SDs are shown. [A]Blood cell counts of only 1 morbid mouse were performed. [B]Analyzed 160 days after the transplantation. Hb, hemoglobin; Immortal. cells, cells immortalized by *MLL-SEPT6* in vitro; MEs, *MLL-ENL*s; MS6, *MLL-SEPT6*; Plt, platelets; WBC, white blood cells.