sarcoma, while those were highly expressed in rhabdomyosarcoma cell lines RMS and SJRH-30 (Table VI). Benign tumors examined did not express *ADAMTS4*.

No detection of secreted ADAMTS4 protein in the patient sera. To evaluate the amount of secreted ADAMTS4 protein in the patients, we analyzed ADAMTS4 protein levels in sera by ELISA. In agreement with the results described above, ADAMTS4 was detected in culture supernantants of EWS cell lines SJES-2 and SJES-5 (Fig. 6). The concentration of ADAMTS4 in SJES-2 cells was about twice as high as that in SJES-5 cells. For a positive control, culture supernatant of the NIH3T3/ADAMTS4 cells was also measured (39.9 ng/ml, data not shown). It is noteworthy that ADAMTS4 protein was detected in 2 out of 3 cases of osteosarcoma and in the only case of osteofibrous dysplasia. Consistent with the very high level of *ADAMTS4* transcript shown in the extreme right lane among osteosarcoma samples in Fig. 5, serum from the same patient showed the high level of ADAMTS4 protein in ELISA as shown in Fig. 6 (middle lane among osteosarcoma samples). The other positive samples of osteosarcoma in both figures are not derived from the same patient, because only either serum or RNA was available in these two patients. ADAMTS4 protein was not detected in the 6 EWS patient sera examined. These results indicated that ADAMTS4 is expressed and secreted in EWS cells, but that the ADAMTS4 protein in the serum is not suitable as a marker for EWS.

Discussion

EWS is an aggressive neoplasm with a strong propensity to spread into neighboring tissues. Many patients are diagnosed at advanced stages of EWS. Since EWS has worse prognosis than other soft-tissue sarcomas, it is clinically important to distinguish EWS from other sarcomas. The reason for the poor prognosis in EWS patients is suggested to be that the micro-metastases are formed before clinical symptoms arise and tumors are detected (42). Currently, diagnosis of EWS is determined mainly by CD99 expression or by genetic aberrations that are exemplified by *EWS-FLI1* fusion gene. Since both markers show lack of sensitivity, specificity or feasibility, more useful biomarkers such as surface antigens or secreted proteins are required in clinical areas.

In the present study, we searched for membrane and secreted proteins derived from EWS cell lines using the retrovirus-mediated signal sequence trap method SST-REX, and identified ADAMTS4 as a possible EWS marker. We demonstrated that ADAMTS4 was expressed in EWS cell lines and tissue samples derived from EWS patients. Interestingly, expression of *ADAMTS4* was correlated with expression of *EWS-FLI1*, which is a hallmark of EWS. In addition, we demonstrated that ADAMTS4 was secreted from EWS cells, although we could not detect ADAMTS4 in serum samples derived from EWS patients.

It should be noted that two cases of the osteosarcoma patient samples were found to express high levels of *ADAMTS4*. It is tempting to speculate that a subclass of osteosarcoma with different property may exist.

In conclusion, we identified *ADAMTS4* as a possible marker of EWS by using SST-REX. This is the first report to show the correlation between *ADAMTS4* and EWS. Although ADAMTS4 protein in the serum could not be used as a biomarker for EWS, our study suggested that RNA transcripts of *ADAMTS4* in the tissue sections are useful markers of EWS. Further studies will be required to determine the usefulness of this molecule in differential diagnosis and/or evaluation of the disease activity in clinical settings.

Acknowledgements

We gratefully thank Ms. S. Shoma (The University of Tokyo) for technical support and Dr Y. Murakami (The University of Tokyo) and Dr Y. Fukuchi (Keio University) for helpful comments on this study. This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan.

References

1. Jaffe R, Santamaria M, Yunis EJ, Tannery NH, Agostini RM, Medina J Jr and Goodman M: The neuroectodermal tumor of bone. Am J Surg Pathol 8: 885-898, 1984.
2. Hashimoto H, Enjoji M, Nakajima T, Kiryu H and Daimaru Y: Malignant neuroepithelioma (peripheral neuroblastoma). A clinicopathologic study of 15 cases. Am J Surg Pathol 7: 309-318, 1983.
3. Khoury JD: Ewing sarcoma family of tumors. Adv Anat Pathol 12: 212-220, 2005.
4. Turc-Carel C, Philip I, Berger MP, Philip T and Lenoir G: Chromosomal translocation (11; 22) in cell lines of Ewing's sarcoma. C R Seances Acad Sci III 296: 1101-1103, 1983.
5. Zucman J, Delattre O, Desmaze C, *et al*: Cloning and characterization of the Ewing's sarcoma and peripheral neuroepithelioma t(11;22) translocation breakpoints. Genes Chromosomes Cancer 5: 271-277, 1992.
6. Sorensen PH, Lessnick SL, Lopez-Terrada D, Liu XF, Triche TJ and Denny CT: A second Ewing's sarcoma translocation, t(21;22), fuses the EWS gene to another ETS-family transcription factor, ERG. Nat Genet 6: 146-151, 1994.
7. Jeon IS, Davis JN, Braun BS, Sublett JE, Roussel MF, Denny CT and Shapiro DN: A variant Ewing's sarcoma translocation (7;22) fuses the EWS gene to the ETS gene ETV1. Oncogene 10: 1229-1234, 1995.
8. Arvand A and Denny CT: Biology of EWS/ETS fusions in Ewing's family tumors. Oncogene 20: 5747-5754, 2001.
9. May WA, Gishizky ML, Lessnick SL, Lunsford LB, Lewis BC, Delattre O, Zucman J, Thomas G and Denny CT: Ewing sarcoma 11;22 translocation produces a chimeric transcription factor that requires the DNA-binding domain encoded by FLI1 for transformation. Proc Natl Acad Sci USA 90: 5752-5756, 1993.
10. Ohno T, Rao VN and Reddy ES: EWS/Fli-1 chimeric protein is a transcriptional activator. Cancer Res 53: 5859-5863, 1993.
11. Jambhekar NA, Bagwan IN, Ghule P, Shet TM, Chinoy RF, Agarwal S, Joshi R and Amare Kadam PS: Comparative analysis of routine histology, immunohistochemistry, reverse transcriptase polymerase chain reaction, and fluorescence in situ hybridization in diagnosis of Ewing family of tumors. Arch Pathol Lab Med 130: 1813-1818, 2006.
12. Llombart-Bosch A and Navarro S: Immunohistochemical detection of EWS and FLI-1 proteinss in Ewing sarcoma and primitive neuroectodermal tumors: comparative analysis with CD99 (MIC-2) expression. Appl Immunohistochem Mol Morphol 9: 255-260, 2006.
13. Bernstein M, Kovar H, Paulussen M, Randall RL, Schuck A, Teot LA and Juergens H: Ewing's sarcoma family of tumors: current management. Oncologist 11: 503-519, 2006.
14. Folpe AL, Hill CE, Parham DM, O'Shea PA and Weiss SW: Immunohistochemical detection of FLI-1 protein expression: a study of 132 round cell tumors with emphasis on CD99-positive mimics of Ewing's sarcoma/primitive neuroectodermal tumor. Am J Surg Pathol 24: 1657-1662, 2000.
15. Bernard G, Zoccola D, Deckert M, Breittmayer JP, Aussel C and Bernard A: The E2 molecule (CD99) specifically triggers homotypic aggregation of $CD4^+$ $CD8^+$ thymocytes. J Immunol 154: 26-32, 1995.

16. Zhang PJ, Barcos M, Stewart CC, Block AW, Sait S and Brooks JJ: Immunoreactivity of MIC2 (CD99) in acute myelogenous leukemia and related diseases. Mod Pathol 13: 452-458, 2000.
17. Tashiro K, Tada H, Heilker R, Shirozu M, Nakano T and Honjo T: Signal sequence trap: a cloning strategy for secreted proteins and type I membrane proteins. Science 261: 600-603, 1993.
18. Shirozu M, Nakano T, Inazawa J, Tashiro K, Tada H, Shinohara T and Honjo T: Structure and chromosomal localization of the human stromal cell-derived factor 1 (SDF1) gene. Genomics 28: 495-500, 1995.
19. Kojima T, Morikawa Y, Copeland NG, Gilbert DJ, Jenkins NA, Senba E and Kitamura T: TROY, a newly identified member of the tumor necrosis factor receptor superfamily, exhibits a homology with Edar and is expressed in embryonic skin and hair follicles. J Biol Chem 275: 20742-20747, 2000.
20. Ohta K, Lupo G, Kuriyama S, Keynes R, Holt CE, Harris WA, Tanaka H and Ohnuma S: Tsukushi functions as an organizer inducer by inhibition of BMP activity in cooperation with chordin. Dev Cell 7: 347-358, 2004.
21. Ikeda Y, Imai Y, Kumagai H, Nosaka T, Morikawa Y, Hisaoka T, Manabe I, Maemura K, Nakaoka T, Imamura T, Miyazono K, Komuro I, Nagai R and Kitamura T: Vasorin, a transforming growth factor beta-binding protein expressed in vascular smooth muscle cells, modulates the arterial response to injury in vivo. Proc Natl Acad Sci USA 101: 10732-10737, 2004.
22. Izawa K, Kitaura J, Yamanishi Y, Matsuoka T, Oki T, Shibata F, Kumagai H, Nakajima H, Maeda-Yamamoto M, Hauchins JP, Tybulewicz VL, Takai T and Kitamura T: Functional analysis of activating receptor LMIR4 as a counterpart of inhibitory receptor LMIR3. J Biol Chem 282: 17997-18008, 2007.
23. Ganju RK, Brubaker SA, Meyer J, Dutt P, Yang Y, Qin S, Newman W and Groopman JE: The alpha-chemokine, stromal cell-derived factor-1alpha, binds to the transmembrane G-protein-coupled CXCR-4 receptor and activates multiple signal transduction pathways. J Biol Chem 273: 23169-23175, 1998.
24. Kitamura T, Onishi M, Kinoshita S, Shibuya A, Miyajima A and Nolan GP: Efficient screening of retroviral cDNA expression libraries. Proc Natl Acad Sci USA 92: 9146-9150, 1995.
25. Kojima T and Kitamura T: A signal sequence trap based on a constitutively active cytokine receptor. Nat Biotechnol 17: 487-490, 1999.
26. Kuno K, Kanada N, Nakashima E, Fujiki F, Ichimura F and Matsushima K: Molecular cloning of a gene encoding a new type of metalloproteinase-disintegrin family protein with thrombospondin motifs as an inflammation associated gene. J Biol Chem 272: 556-562, 1997.
27. Porter S, Clark IM, Kevorkian L and Edwards DR: The ADAMTS metalloproteinases. Biochem J 386: 15-27, 2005.
28. Tortorella MD, Burn TC, Pratta MA, *et al*: Purification and cloning of aggrecanase-1: a member of the ADAMTS family of proteins. Science 284: 1664-1666, 1999.
29. Tortorella MD, Malfait AM, Deccico C and Arner E: The role of ADAM-TS4 (aggrecanase-1) and ADAM-TS5 (aggrecanase-2) in a model of cartilage degradation. Osteoarthritis Cartilage 9: 539-552, 2001.
30. Sandy JD, Neame PJ, Boynton RE and Flannery CR: Catabolism of aggrecan in cartilage explants. Identification of a major cleavage site within the interglobular domain. J Biol Chem 266: 8683-8685, 1991.
31. Sandy JD, Flannery CR, Neame PJ and Lohmander LS: The structure of aggrecan fragments in human synovial fluid. Evidence for the involvement in osteoarthritis of a novel proteinase which cleaves the Glu 373-Ala 374 bond of the interglobular domain. J Clin Invest 89: 1512-1516, 1992.
32. Lohmander LS, Neame PJ and Sandy JD: The structure of aggrecan fragments in human synovial fluid. Evidence that aggrecanase mediates cartilage degradation in inflammatory joint disease, joint injury, and osteoarthritis. Arthritis Rheum 36: 1214-1222, 1993.
33. Kashiwagi M, Tortorella M, Nagase H and Brew K: TIMP-3 is a potent inhibitor of aggrecanase 1 (ADAM-TS4) and aggrecanase 2 (ADAM-TS5). J Biol Chem 276: 12501-12504, 2001.
34. Yamanishi Y, Boyle DL, Clark M, Maki RA, Tortorella MD, Arner EC and Firestein GS: Expression and regulation of aggrecanase in arthritis: the role of TGF-beta. J Immunol 168: 1405-1412, 2002.
35. Matthews RT, Gary SC, Zerillo C, Pratta M, Solomon K, Arner EC and Hockfield S: Brain-enriched hyaluronan binding (BEHAB)/brevican cleavage in a glioma cell line is mediated by a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family member. J Biol Chem 275: 22695-22703, 2000.
36. Morita S, Kojima T and Kitamura T: Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Ther 7: 1063-1066, 2000.
37. Kitamura T, Koshino Y, Shibata F, Oki, T, Nakajima H, Nosaka T and Kumagai H: Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. Exp Hematol 11: 1007-1014, 2003.
38. Prieur A, Tirode F, Cohen P and Delattre O: EWS/FLI-1 silencing and gene profiling of Ewing cells reveal downstream oncogenic pathways and a crucial role for repression of insulin-like growth factor binding protein 3. Mol Cell Biol 24: 7275-7283, 2004.
39. Nosaka T, Kawashima T, Misawa K, Ikuta K, Mui AL and Kitamura T: STAT5 as a molecular regulator of proliferation, differentiation and apoptosis in hematopoietic cells. EMBO J 18: 4754-4765, 1999.
40. Tirode F, Laud-Duval K, Prieur A, Delorme B, Charbord P and Delattre O: Mesenchymal stem cell features of Ewing tumors. Cancer Cell 11: 421-429, 2007.
41. Riggi N, Cironi L, Provero P, Suva ML, Kaloulis K, Garcia-Echeverria C, Hoffmann F, Trumpp A and Stamenkovic I: Development of Ewing's sarcoma from primary bone marrow-derived mesenchymal progenitor cells. Cancer Res 65: 11459-11468, 2005.
42. Schleiermacher G, Peter M, Oberlin O, Philip T, Rubie H, Mechinaud F, Sommelet-Olive D, Landman-Parker J, Bours D, Michon J and Delattre O: Increased risk of systemic relapses associated with bone marrow micrometastasis and circulating tumor cells in localized Ewing tumor. J Clin Oncol 21: 85-91, 2003.

Int J Hematol (2010) 91:831–837
DOI 10.1007/s12185-010-0585-x

ORIGINAL ARTICLE

Prognostic significance of the *BAALC* isoform pattern and *CEBPA* mutations in pediatric acute myeloid leukemia with normal karyotype: a study by the Japanese Childhood AML Cooperative Study Group

Yasuhiro Mizushima · Tomohiko Taki · Akira Shimada · Yoshihiro Yui · Yoshimi Hiraumi · Hiroshi Matsubara · Motonobu Watanabe · Ken-ichiro Watanabe · Yuri Kamitsuji · Yasuhide Hayashi · Ichiro Tsukimoto · Ryoji Kobayashi · Keizo Horibe · Akio Tawa · Tatsutoshi Nakahata · Souichi Adachi

Received: 8 December 2009 / Revised: 20 April 2010 / Accepted: 21 April 2010 / Published online: 22 May 2010
© The Japanese Society of Hematology 2010

Abstract High *BAALC* (brain and acute leukemia, cytoplasmic) gene expression may indicate an adverse prognosis for adults who have acute myeloid leukemia (AML) and a normal karyotype, but its prognostic significance for pediatric AML cases is unclear. Whether different *BAALC* isoform patterns are of prognostic significance is also unclear. Newly diagnosed AML patients with normal karyotype who were treated by the Japanese Childhood AML Cooperative Treatment Protocol AML 99 were analyzed in terms of their *BAALC* expression levels ($n = 29$), *BAALC* isoforms ($n = 29$), and *CEBPA* mutations ($n = 49$). Eleven and 18 patients exhibited high and low *BAALC* expression, respectively, but these groups did not differ significantly in terms of overall survival (54.6 vs. 61.1%, $P = 0.55$) or event-free survival (61.4 vs. 50.0%, $P = 0.82$). Three of these 29 patients (10.3%) expressed the exon 1-5-6-8 *BAALC* isoform along with the expected 1-6-8 isoform and had adverse clinical outcomes. Novel

Electronic supplementary material The online version of this article (doi:10.1007/s12185-010-0585-x) contains supplementary material, which is available to authorized users.

Y. Mizushima · Y. Yui · Y. Hiraumi · H. Matsubara · M. Watanabe · K. Watanabe · T. Nakahata · S. Adachi
Department of Pediatrics, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Syogoin, Sakyo-ku, Kyoto 606-8507, Japan

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

A. Shimada · Y. Hayashi
Department of Hematology and Oncology, Gunma Children's Medical Center, 779 Shimohakoda, Hokkitsu, Shibukawa, Gunma 377-8577, Japan

Y. Kamitsuji
Department of Transfusion Medicine and Cell Therapy, Kyoto University Hospital, 54 Kawahara-cho, Syogoin, Sakyo-ku, Kyoto 606-8507, Japan

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

R. Kobayashi
Department of Pediatrics, Graduate School of Medicine, Hokkaido University, North 15, West 7, Kita-ku, Sapporo 060-8638, Japan

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

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

S. Adachi (✉)
Department of Human Health Sciences, Graduate School of Medicine, Kyoto University, 53 Kawahara-cho, Syogoin, Sakyo-ku, Kyoto 606-8507, Japan
e-mail: adachiso@kuhp.kyoto-u.ac.jp

CEBPA mutations were also identified in four of 49 patients (8.2%). All four patients have maintained complete remission for at least 5 years. Thus, 1-5-6-8 isoform expression may be associated with an adverse prognosis in pediatric AML with normal karyotype. *CEBPA* mutations may indicate a favorable prognosis.

Keywords Pediatric AML · Normal karyotype · *BAALC* · *CEBPA*

1 Introduction

Cytogenetic abnormalities in acute myeloid leukemia (AML) that are detected at the time of diagnosis are important prognostic factors that help to determine the clinical outcome. However, 10–20% of pediatric AML cases lack known genetic abnormalities that can be used to predict clinical outcome [1]. For example, while tandem duplications of mixed lineage leukemia gene (*MLL*) and fms-like tyrosine kinase-3 (*FLT3*) correlate with a poor prognosis in pediatric AML [2], internal tandem duplications (ITD) of *FLT3* occur much more rarely in pediatric AML than in adult AML patients; indeed, there is an age-associated increase in this mutation (from 1.5% in infants to nearly 20% in teenage patients) [3]. Thus, most normal karyotype pediatric AML patients lack known markers that are of prognostic significance. To improve the prognostic stratification of this heterogeneous group of patients, novel markers should be identified.

The *BAALC* (brain and acute leukemia, cytoplasmic) gene is believed to participate in the development of AML and chronic myelogenous leukemia in blast crisis [5]. Previous studies have also reported that high *BAALC* expression levels reflect an adverse prognosis for adult AML with a normal karyotype [6–10]. However, how *BAALC* expression levels relate to the clinical outcome of pediatric AML remains unclear.

CEBPA is a transcription factor that coordinates the granulocytic differentiation of common myeloid progenitors. *CEBPA* mutations have been detected in 7–15% of adult patients with AML and are most frequently found in the AML M1 and M2 subtypes [French–American–British (FAB) classification] [10, 11]. Previous reports indicate that *CEBPA* mutations reflect a favorable prognosis in adult AML with normal karyotype [12, 13]. However, it is unclear whether the same relationship exists between *CEBPA* mutations and pediatric AML.

While previous studies have mainly examined various prognostic factors in terms of gene mutations [9–14] and changes in gene expression [15–20], some recent studies have also reported the prognostic significance of the expression of different isoforms in leukemia [21–25]. Consequently, in the present study, we investigated the prognostic relevance of high *BAALC* expression, *BAALC* isoform patterns, and *CEBPA* mutations in pediatric AML with normal karyotype. This study was performed by the Japanese Childhood AML Cooperative Study Group, which employed the AML 99 protocol [2, 4, 26, 27].

2 Patients and methods

2.1 Patients

This study included 124 of the 241 pediatric patients who were newly diagnosed with de novo AML from January 2000 to December 2002. The 241 patients included 52 patients with a normal karyotype and 49 of those were recruited into the 124-patient group. None of these 49 patients had AML-M3 or Down syndrome. AML was diagnosed according to the FAB classification, and a routine G-banding method was used for cytogenetic analysis. Of the 124 cases, 104 were subjected to *BAALC* expression analysis; of these 104 subjects, 29 had a normal karyotype. These 29 normal karyotype cases were also subjected to *BAALC* isoform analysis. All 49 normal karyotype cases were subjected to *CEBPA* mutation analysis. The characteristics of the patients subjected to *BAALC* isoform analysis and *CEBPA* mutation analysis are shown in Table 1a. The 29 normal karyotype patients who were subjected to *BAALC* isoform and expression analyses did not differ significantly in age, white blood cell (WBC) counts at diagnosis, remission rates or overall survival (OS) from the remaining 20 normal karyotype patients who were not analyzed for *BAALC* isoform and expression (Table 1b). In accordance with the Declaration of Helsinki and upon approval from the Ethics Committees of Kyoto University, informed consent was obtained from each patient or the patient's parents before entering this study.

2.2 *BAALC* expression analysis

Comparative real-time RT-PCR assays were performed and *BAALC* expression levels were measured as previously reported [5, 6]. The *BAALC* expression values of the patient group were divided at the median value (0.57) and patients were said to have a low and high *BAALC* expression if they had expression values within the lower and upper 50% of values, respectively [7].

2.3 *BAALC* isoform analysis

BAALC isoform analysis was performed by RT-PCR followed by direct sequencing. For this, the PCR product was cut out of the gel, purified with a QIAquick gel extraction kit (Qiagen, Chatsworth, CA), and sequenced by the

Table 1 Characteristics of AML patients subjected to *BAALC* expression, *BAALC* isoform, and *CEBPA* mutation analyses

	BAALC		*CEBPA*
	All ($n = 104$)	Normal karyotype ($n = 29$)	Normal karyotype ($n = 49$)
(a)			
FAB			
M0	4	2	4
M1	18	2	6
M2	36	9	12
M4	18	8	12
M5	19	7	12
M7	7	1	3
UN	2	0	0
Age	4 days to 15 years	3 months to 15 years	3 months to 15 years
Sex			
Male	60	16	27
Female	44	13	22
Risk group			
Low	45	0	0
Intermediate	51	29	49
High	8	0	0

	BAALC		*P* value
	Analyzed patients ($n = 29$)	Non-analyzed patients ($n = 20$)	
(b)			
Age			
Median	8 years	8 years	
Range	3 months to 15 years	7 months to 15 years	0.591
WBC at diagnosis ($\times 10^9$/L)			
Median	53.09	15.20	
Range	2.30–343.40	1.20–236.90	0.275
Remission rates	89.7%	100%	0.083
Overall survival	58.6%	65.2%	0.639

UN undifferentiated

dideoxynucleotide termination method with ABI 3100 (Applied Biosystems, Foster City, CA). The primers used are shown in supplementary Table 1. The conditions for the PCR reactions were 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s, and one cycle of 72°C for 7 min.

2.4 *CEBPA* mutation analysis

The entire coding region of the gene was amplified using overlapping PCR primer pairs followed by direct sequencing as previously described [28].

2.5 Statistical analysis

Survival distributions were estimated using the Kaplan–Meier method and the differences were compared using the log-rank test. OS and event-free survival (EFS) were defined as the time from diagnosis to death from any cause or the last follow-up and the time from diagnosis to event (relapse or death from any cause), respectively.

3 Results

3.1 *BAALC* expression levels

High *BAALC* expression was associated with M0, M1, and M2 FAB subtypes, while M4 and M5 FAB subtypes correlated with low *BAALC* expression (Fig. 1). Healthy volunteers (I) have remarkably small range of *BAALC* expression levels compared to AML patients (II, III), as the previous study was reported [15]. We did not observe significant differences between normal karyotype patients

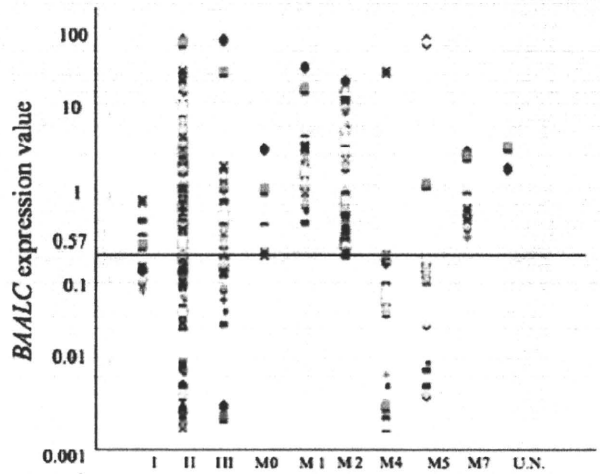

Fig. 1 *BAALC* expression levels in pediatric AML with normal karyotype and FAB subtype patients. The *dot plot* indicates the individual *BAALC* expression levels of healthy volunteers (I, $n = 9$), all AML patients (II, $n = 104$), AML patients with normal karyotype (III, $n = 29$), and the M0 ($n = 4$), M1 ($n = 18$), M2 ($n = 36$), M4 ($n = 18$), M5 ($n = 19$), M7 ($n = 7$), and undifferentiated (U.N., $n = 2$) FAB subtype patients

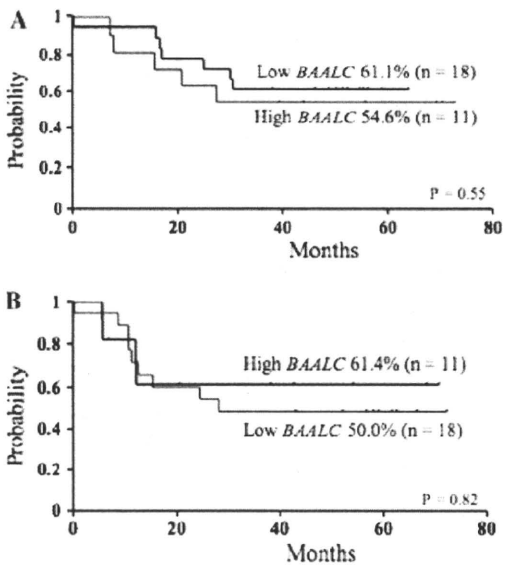

Fig. 2 Kaplan–Meier analysis of the overall survival (OS) and event-free survival (EFS) of pediatric AML patients with normal karyotype who express *BAALC* at high and low levels. The OS (**a**) and EFS (**b**) of the high and low *BAALC*-expressing pediatric AML with normal karyotype patients did not differ significantly

with high ($n = 11$) and low ($n = 18$) *BAALC* expression with regard to their OS (54.6 vs. 61.1%, $P = 0.55$, Fig. 2a) or EFS (61.4 vs. 50.0%, $P = 0.82$, Fig. 2b).

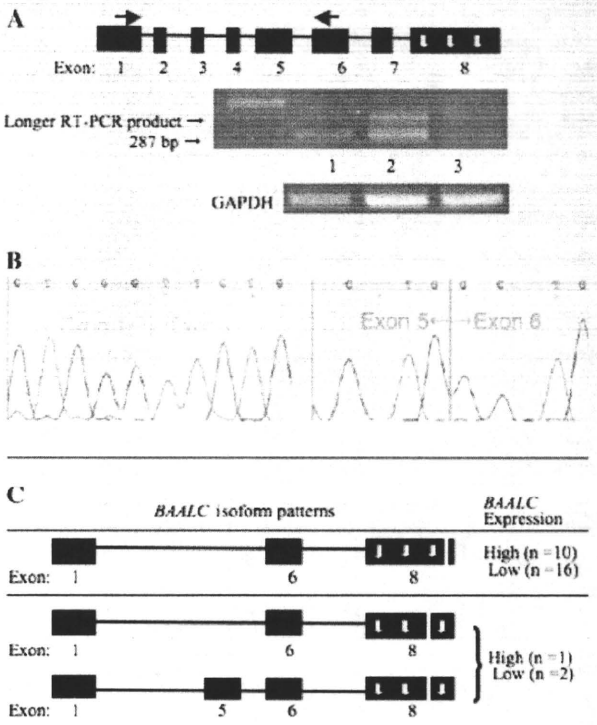

Fig. 3 **a** Schematic depiction of the *BAALC* gene, which consists of eight exons (indicated by *black boxes*). The three polyadenylation signals in the 3′ untranslated region (UTR) of exon 8 that lead to three differently sized transcripts are indicated by *down arrows*. Comparative RT-PCR using primers in exons 1 and 6 revealed that three samples had a longer RT-PCR product in addition to the expected 287 bp product. **b** A partial sequence trace of exons 1, 5, and 6 in the longer RT-PCR product. **c** Schematic depiction of the relationship between *BAALC* isoform patterns and expression levels. Three (10.3%) of the 29 cases had the 1-5-6-8 isoform

3.2 *BAALC* isoform pattern and its relationship to *BAALC* expression levels

The *BAALC* gene consists of eight exons and its transcription followed by alternative splicing yields several different transcripts. Five stable isoforms have been detected in leukemic blasts, namely 1-8, 1-6-8, 1-5-6-8, 1-4-5-6-8, and 1-5-6-7-8 [5]. To investigate the prognostic relevance of *BAALC* isoform patterns for pediatric AML patients, we subjected 29 pediatric AML patients with normal karyotype to RT-PCR and direct sequencing. All samples had the predicted product, which consisted of exon 1, 6, and 8 sequences (1-6-8). However, three samples (10.3%) also had a longer RT-PCR product that contained in addition the exon 5 sequence (1-5-6-8) (Fig. 3a, b). How these isoform patterns relate to the *BAALC* expression levels of the 29 patients is depicted schematically in Fig. 3c. One of three patients with the

Table 2 Characteristics of patients with the 1-5-6-8 *BAALC* isoform

Case no.	*BAALC* expression value	Relapse	Clinical outcome	FAB classification	Age (years)	Sex
1	High (2.43)	+	Dead	M4	13	Female
2	Low (0.39)	–	Dead	M5a	6	Male
3	Low (0.21)	+	Dead	M4	15	Male

Table 3 *CEBPA* mutations

FAB subtype	All ($n = 49$)	*CEBPA* mutations	
M0	4	0	
M1	6	2	c. 1074_1075insAGA
			c. 1092_1093insCAC
M2	12	2	c. 214_224delCCCCGCACGCG
			c. 212_213insC and
			c. 720_726insCGCACC
M4	12	0	
M5	12	0	
M7	3	0	
Total	49	4	

Sequence numbering is according to GenBank accession number U34070

1-5-6-8 isoform had high *BAALC* expression. Of the 26 patients with the 1-6-8 isoform only, 10 and 16 showed high and low *BAALC* expression, respectively. The three patients with the 1-5-6-8 isoform had a poor prognosis (Table 2).

3.3 *CEBPA* mutation

CEBPA mutations were detected in four of the 49 AML with normal karyotype patients (8.2%). Two of these belonged to the M1 subset, and the remaining two belonged to the M2 subset. N-terminal frameshift mutations and in-frame insertions in the basic-leucine zipper (bZIP) domain were detected (Table 3). Novel mutations were identified, namely, c. 212_213insC, c. 214_224delCCCCGCACGCG, c. 720_726insCGCACC, c. 1074_1075insAGA, and c. 1092_1093insCAC. One patient had biallelic mutations in both the N-terminal part and the bZIP domain of *CEBPA*.

4 Discussion

High *BAALC* expression was associated with the M2 subset and the more immature M0/M1 FAB subtypes, while the monocytic-differentiated M4 and M5 FAB subtypes correlated with low *BAALC* expression (Fig. 1). This relationship between *BAALC* expression and FAB subtypes is generally consistent with previously reported observations of adult AML cases [7], although the high *BAALC* expression in the M2 subtype cases was only observed in the pediatric AML patients. *BAALC* expression level was not associated with WBC counts at diagnosis [all AML patients ($n = 104$), $P = 0.91$; AML with normal karyotype ($n = 29$), $P = 0.97$]. The *BAALC* gene is normally expressed by neuroectoderm-derived tissues and CD34-positive hematopoietic progenitor cells and has been implicated in AML and chronic myelogenous leukemia in blast crisis [5]. Recently, quantification of *BAALC* gene expression made it possible to assess MRD in patients with CD34-positive acute leukemia [29]. Little is known about the functions of the *BAALC* gene, but it has been reported to mediate the anabolic action of PTH (parathyroid hormone) on bone cells [30]. It also serves as a marker of the mesodermal lineage (especially muscle) [31] and synaptogenesis [32], and a study on hematopoietic progenitor cells has shown that *BAALC* downregulation occurs upon cell differentiation [33]. Thus, while the functions of the *BAALC* gene remain unclear, our observations are consistent with the notion that it may be associated with monocytic cell differentiation.

We did not observe significant differences between normal karyotype patients with high ($n = 11$) and low ($n = 18$) *BAALC* expression with regard to their OS (54.6 vs. 61.1%, $P = 0.55$, Fig. 2a) or EFS (61.4 vs. 50.0%, $P = 0.82$, Fig. 2b). These results are not consistent with those of a previous study that examined the *BAALC* expression of adult normal karyotype AML patients [7]. In that study, high *BAALC* expression was significantly associated with a poor OS and a higher cumulative incidence of relapse. The discrepancy between this study and ours could reflect the fact that in the other study, the *BAALC* expression values of the patient group were divided at the median value of twelve healthy volunteers, which served as the cutoff [15]. We compared the results according to two different cutoff levels. AML samples were dichotomized at the median value (0.15) of nine healthy volunteers, but we also observed no significant differences of two expression groups (date not shown). To resolve this apparent discrepancy, a larger number of pediatric AML patients will need to be studied.

The *BAALC* gene consists of eight exons and its transcription followed by alternative splicing yields several

different transcripts in leukemic blasts, namely, 1-8, 1-6-8, 1-5-6-8, 1-4-5-6-8, and 1-5-6-7-8 [5]. In our study, all samples of 29 pediatric AML patients with normal karyotype had the *BAALC* isoform that consisted of exon 1, 6, and 8 sequences (1-6-8). However, three samples (10.3%) also had the *BAALC* isoform that contained in addition the exon 5 sequence (1-5-6-8) (Fig. 3a, b). One of the three patients with the 1-5-6-8 isoform also had high *BAALC* expression. With regard to prognosis, all three patients with the 1-5-6-8 *BAALC* isoform have died (Table 2). Two relapsed after complete remission and the third died after intracranial hemorrhage during induction therapy. Of the 29 normal karyotype patients who were subjected to *BAALC* isoform and expression analyses, *FLT3*-ITD were found in eight (27.6%), *FLT3*-D835Mt in two (6.9%), *NRAS* mutations in two (6.9%), and *KRAS* mutations in four (13.8%), but no *NPM1* gene mutations were found [4]. There are no differences of *FLT3*-ITD, *MLL*-PTD and *CEBPA* in high and low *BAALC* expression group (data not shown). Of the three 1-5-6-8 *BAALC* isoform-bearing patients, one had the *FLT3*-ITD mutation and another patient had a *RAS* mutation. A previous study did not detect a difference in outcome between cytogenetically normal adult AML patients with and without *NRAS* mutations [34]. Thus, it seems that the possession of the 1-5-6-8 *BAALC* isoform by pediatric AML patients with normal karyotype may be associated with a candidate for some adverse prognostic factor. Studies with greater patient numbers should be performed to confirm this. Recent reports have suggested that the isoform patterns of other genes (i.e., *AML1-ETO9a* [21], *WT1* [22], *PML/RARa* [23], *Ikaros* [24], and *FHIT* [25]) are of prognostic significance, which supports the significance of investigating the *BAALC* isoform patterns.

CEBPA mutations were detected in four of the 49 AML patients with normal karyotype (8.2%). Two of these belonged to the M1 subset and the remaining two belonged to the M2 subset. One patient had biallelic mutations in both the N-terminal part and bZIP domain of *CEBPA*. To date, two categories of *CEBPA* mutations have been reported: out-of-frame ins/del mutations that often occur in the N-terminal region, and in-frame ins/del mutations that often occur in the C-terminal region [12, 13]. The mutations in both the N-terminal part and bZIP domain have been described in adult AML, but the reported frequencies vary considerably, ranging between 11 and 47% [35]. In a study of pediatric AML patients, of whom six had a normal karyotype, four of the six (67%) had one or more *CEBPA* mutations [36], but the clinical outcomes associated with these mutations are unclear. Notably, in the absence of poor prognostic factors, adult patients with *CEBPA* mutations have been shown to have favorable clinical outcomes [33, 37]. In our study, none of the patients exhibiting a *CEBPA* mutation also had the *FLT3*-ITD mutation and all maintained complete remission for at least 5 years. The statistical significance was not indicated for insufficient sample numbers in AML 99 protocol between normal karyotype patients with CEBPA mutation (+) ($n = 4$) and mutation (−) ($n = 45$) with regard to their OS (100 vs. 55.4%, $P = 0.14$) or EFS (100 vs. 48.9%, $P = 0.09$) (supplementary Fig. 1). But differing from previous report about pediatric AML patients with *CEBPA* mutations, the presentation of clinical information about them may be evaluated. Thus, in the absence of other adverse factors, *CEBPA* mutations may also be suspected to favorable prognostic factors for pediatric AML with normal karyotype.

In summary, we report here for the first time that the presence of the 1-5-6-8 *BAALC* isoform may be associated with a poor prognosis for pediatric AML patients with normal karyotype. In contrast, *CEBPA* mutations are suspected to a good prognosis.

Acknowledgments The authors are grateful to all members of the Japanese Childhood AML Cooperative Study Group. We also thank to Ken Tabuchi for statistical analysis. This work was supported in part by a Grant-in-Aid for Cancer Research (16-3) from the Ministry of Health, Labor and Welfare of Japan, and in part by a Scientific Research (C) grant (17591083) from the Ministry of Education, Science, Technology, Sports, and Culture of Japan.

References

1. Brown P, McIntyre E, Rau R, Meshinchi S, Lacayo N, Dahl G, et al. The incidence and clinical significance of nucleophosmin mutations in childhood AML. Blood. 2007;110:979–85.
2. Shimada A, Taki T, Tabuchi K, Taketani T, Hanada R, Tawa A, et al. Tandem duplications of MLL and FLT3 are correlated with poor prognosis in pediatric acute myeloid leukemia: a study of the Japanese childhood AML Cooperative Study Group. Pediatr Blood Cancer. 2008;50:264–9.
3. Meshinchi S, Alonzo TA, Stirewalt DL, Zwaan M, Zimmerman M, Reinhardt D, et al. Clinical implications of FLT3 mutations in pediatric AML. Blood. 2006;108:3654–61.
4. Shimada A, Taki T, Kubota C, Tawa A, Horibe K, Tsuchida M, et al. No nucleophosmin mutations in pediatric acute myeloid leukemia with normal karyotype: a study of the Japanese Childhood AML Cooperative Study Group. Leukemia. 2007;21:1307.
5. Tanner SM, Austin JL, Leone G, Rush LJ, Plass C, Heinonen K, et al. BAALC, the human member of a novel mammalian neuroectoderm gene lineage, is implicated in hematopoiesis and acute leukemia. Proc Natl Acad Sci USA. 2001;98:13901–6.
6. Baldus CD, Tanner SM, Ruppert AS, Whitman SP, Archer KJ, Marcucci G, et al. BAALC expression predicts clinical outcome of de novo acute myeloid leukemia patients with normal cytogenetics: a Cancer and Leukemia Group B Study. Blood. 2003;102:1613–8.
7. Baldus CD, Thiede C, Soucek S, Bloomfield CD, Thiel E, Ehninger G, et al. BAALC expression and FLT3 internal tandem duplication mutations in acute myeloid leukemia patients with

normal cytogenetics: prognostic implications. J Clin Oncol. 2006;24:790–7.
8. Marcucci G, Mrózek K, Bloomfield CD. Molecular heterogeneity and prognostic biomarkers in adults with acute myeloid leukemia and normal cytogenetics. Curr Opin Hematol. 2005;12:68–75.
9. Mrózek K, Marcucci G, Paschka P, Whitman SP, Bloomfield CD. Clinical relevance of mutations and gene-expression changes in adult acute myeloid leukemia with normal cytogenetics: are we ready for a prognostically prioritized molecular classification? Blood. 2007;109:431–48.
10. Baldus CD, Mrózek K, Marcucci G, Bloomfield CD. Clinical outcome of de novo acute myeloid leukaemia patients with normal cytogenetics is affected by molecular genetic alterations: a concise review. Br J Haematol. 2007;137:387–400.
11. Gombart AF, Hofmann WK, Kawano S, Takeuchi S, Krug U, Kwok SH, et al. Mutations in the gene encoding the transcription factor CCAAT/enhancer binding protein in myelodysplastic syndromes and acute myeloid leukemias. Blood. 2002;99:1332–40.
12. Preudhomme C, Sagot C, Boissel N, Cayuela JM, Tigaud I, de Botton S, et al. Favorable prognostic significance of CEBPA mutations in patients with de novo acute myeloid leukemia: a study from the Acute Leukemia French Association. Blood. 2002;100:2717–23.
13. Fröhling S, Schlenk RF, Stolze I, Bihlmayr J, Benner A, Kreitmeier S, et al. CEBPA mutations in younger adults with acute myeloid leukemia and normal cytogenetics: prognostic relevance and analysis of cooperating mutations. J Clin Oncol. 2004;22:624–33.
14. Basecke J, Whelan JT, Griesinger F, Bertrand FE. The MLL partial tandem duplication in acute myeloid leukaemia. Br J Haematol. 2006;135:438–49.
15. Bienz M, Ludwig M, Leibundgut EO, Mueller BU, Ratschiller D, Solenthaler M, et al. Risk assessment in patients with acute myeloid leukemia and a normal karyotype. Clin Cancer Res. 2005;11:1416–24.
16. Marcucci G, Baldus CD, Ruppert AS, Radmacher MD, Mrózek K, Whitman SP, et al. Overexpression of the ETS-related gene, ERG, predicts a worse outcome in acute myeloid leukemia with normal karyotype: a Cancer and Leukemia Group B study. J Clin Oncol. 2005;23:9234–42.
17. Barjesteh van Waalwijk van Doorn-Khosrovani S, Erpelinck C, van Putten WL, Valk PJ, van der Poel-van de Luytgaarde S, Hack R, et al. High EVI1 expression predicts poor survival in acute myeloid leukemia: a study of 319 de novo AML patients. Blood. 2003;101:837–45.
18. Damiani D, Tiribelli M, Calistri E, Geromin A, Chiarvesio A, Michelutti A, et al. The prognostic value of P-glycoprotein (ABCB) and breast cancer resistance protein (ABCG2) in adults with de novo acute myeloid leukemia with normal karyotype. Haematologica. 2006;91:825–8.
19. Heuser M, Beutel G, Krauter J, Döhner K, von Neuhoff N, Schlegelberger B, et al. High meningioma 1 (MN1) expression as a predictor for poor outcome in acute myeloid leukemia with normal cytogenetics. Blood. 2006;108:3898–905.
20. Radmacher MD, Marcucci G, Ruppert AS, Mrózek K, Whitman SP, Vardiman JW, et al. Independent confirmation of a prognostic gene-expression signature in adult acute myeloid leukemia with a normal karyotype: a Cancer and Leukemia Group B study. Blood. 2006;108:1677–83.
21. Yan M, Kanbe E, Peterson LF, Boyapati A, Miao Y, Wang Y, et al. A previous unidentified alternatively spliced isoform of t(8;21) transcript promotes leukemogenesis. Nat Med. 2006;12:945–9.
22. Hossain A, Nixon M, Kuo MT, Saunders GF. N-terminally truncated WT1 protein with oncogenic properties overexpressed in Leukemia. J Biol Chem. 2006;281:28122–30.
23. Jurcic JG, Nimer SD, Scheinberg DA, DeBlasio T, Warrell RP Jr, Miller WH Jr, et al. Prognostic significance of minimal residual disease and PML/RARa isoform type: long-term follow-up in acute promyelocytic leukemia. Blood. 2001;98:2651–6.
24. Yagi T, Hibi S, Takanashi M, Kano G, Tabata Y, Imamura T, et al. High frequency of Ikaros isoform 6 expression in acute myelomonocytic and monocytic leukemias: implications for up-regulation of the antiapoptotic protein Bcl-XL in leukemogenesis. Blood. 2002;99:1350–4.
25. Yang HW, Piao HY, Taki T, Chen T, Hashizume K, Ohnishi H, et al. Pattern of FHIT gene expression in normal and leukaemic cells. Int J Cancer. 1999;81:897–901.
26. Shimada A, Taki T, Tabuchi K, Tawa A, Horibe K, Tsuchida M, et al. KIT mutations, and not FLT3 internal tandem duplication, are strongly associated with a poor prognosis in pediatric acute myeloid leukemia with t(8;21): a study of the Japanese Childhood AML Cooperative Study Group. Blood. 2006;107:1806–9.
27. Tsukimoto I, Tawa A, Horibe K, Tabuchi K, Kigasawa H, Tsuchida M, et al. Risk-stratified therapy and the intensive use of cytarabine improves the outcome in childhood acute myeloid leukemia: the AML99 trial from the Japanese Childhood AML Cooperative Study Group. J Clin Oncol. 2009;27:4007–13.
28. Pabst T, Mueller BU, Zhang P, Radomska HS, Narravula S, Schnittger S, et al. Dominant-negative mutations of CEBPA, encoding CCAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. Nat Genet. 2001;27:263–70.
29. Najima Y, Ohashi K, Kawamura M, Onozuka Y, Yamaguchi T, Akiyama H, et al. Molecular monitoring of *BAALC* expression in patients with CD34-positive acute leukemia. Int J Hematol. 2010. doi:10.1007/s12185-010-0550-8.
30. Robinson JA, Susulic V, Liu YB, Taylor C, Hardenburg J, Gironda V, et al. Identification of a PTH regulated gene selectively induced in vivo during PTH-mediated bone formation. J Cell Biochem. 2006;98:1203–20.
31. Satoskar AA, Tanner SM, Weinstein M, Qualman SJ, de la Chapelle A. BAALC, a marker of mesoderm and muscle. Gene Expr Patterns. 2005;5:463–73.
32. Wang X, Tian QB, Okano A, Sakagami H, Moon IS, Kondo H, et al. BAALC 1-6-8 protein is targeted to postsynaptic lipid rafts by its N-terminal myristoylation and palmitoylation, and interacts with alpha, but not beta, subunit of Ca/calmodulin-dependent protein kinase II. J Neurochem. 2005;92:647–59.
33. Baldus CD, Tanner SM, Kusewitt DF, Liyanarachchi S, Choi C, Caligiuri MA, et al. BAALC, a novel marker of human hematopoietic progenitor cells. Exp Hematol. 2003;31:1051–6.
34. Bacher U, Haferlach T, Schoch C, Kern W, Schnittger S. Implications of NRAS mutations in AML: a study of 2502 patients. Blood. 2006;107:3847–53.
35. Leroy H, Roumier C, Huyghe P, Biggio V, Fenaux P, Preudhomme C. CEBPA point mutations in hematological malignancies. Leukemia. 2005;19:329–34.
36. Liang DC, Shih LY, Huang CF, Hung IJ, Yang CP, Liu HC, et al. CEBPA mutations in childhood acute myeloid leukemia. Leukemia. 2005;19:410–4.
37. Wouters BJ, Löwenberg B, Erpelinck-Verschueren CA, van Putten WL, Valk PJ, Delwel R. Double CEBPA mutations, but not single CEBPA mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. Blood. 2009; 113:3088–91.

Leukemia (2010) 24, 1018–1024
© 2010 Macmillan Publishers Limited All rights reserved 0887-6924/10 $32.00
www.nature.com/leu

ORIGINAL ARTICLE

AID-induced T-lymphoma or B-leukemia/lymphoma in a mouse BMT model

Y Komeno[1,2], J Kitaura[1,2], N Watanabe-Okochi[3,4], N Kato[1,2], T Oki[1,2], F Nakahara[1,2], Y Harada[5], H Harada[6], R Shinkura[7], H Nagaoka[7], Y Hayashi[8], T Honjo[7] and T Kitamura[1,2]

[1]Division of Cellular Therapy, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo, Japan; [2]Division of Stem Cell Signaling, Center for Stem Cell Therapy, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo, Japan; [3]Department of Hematology and Oncology, Graduate School of Medicine, University of Tokyo, Bunkyo-ku, Tokyo, Japan; [4]Department of Transfusion Medicine, Graduate School of Medicine, University of Tokyo, Bunkyo-ku, Tokyo, Japan; [5]International Radiation Information Center, Research Institute for Radiation Biology and Medicine, Hiroshima University, Minami-ku, Hiroshima, Japan; [6]Department of Hematology and Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Minami-ku, Hiroshima, Japan; [7]Department of Immunology and Genomic Medicine, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto, Japan and [8]Department of Hematology/Oncology, Gunma Children's Medical Center, Shibukawa, Gunma, Japan

Activation-induced cytidine deaminase (AID) diversifies immunoglobulin through somatic hypermutation (SHM) and class-switch recombination (CSR). AID-transgenic mice develop T-lymphoma, indicating that constitutive expression of AID leads to tumorigenesis. Here, we transplanted mouse bone marrow cells transduced with AID. Twenty-four of the 32 recipient mice developed T-lymphoma 2–4 months after the transplantation. Surprisingly, unlike AID-transgenic mice, seven recipients developed B-leukemia/lymphoma with longer latencies. None of the mice suffered from myeloid leukemia. When we used nude mice as recipients, they developed only B-leukemia/lymphoma, presumably due to lack of thymus. Analysis of AID mutants suggested that an intact form with SHM activity is required for maximum ability of AID to induce lymphoma. Except for a K-ras active mutant in one case, specific mutations could not be identified in T-lymphoma; however, Notch1 was constitutively activated in most cases. Importantly, truncations of Ebf1 or Pax5 were observed in B-leukemia/lymphoma. In conclusion, this is the first report on the potential of AID overexpression to promote B-cell lymphomagenesis in a mouse model. Aberrant expression of AID in bone marrow cells induced leukemia/lymphoma in a cell-lineage–dependent manner, mainly through its function as a mutator.
Leukemia (2010) **24**, 1018–1024; doi:10.1038/leu.2010.40; published online 1 April 2010
Keywords: AID; BMT; T-lymphoma; B-leukemia/lymphoma

Introduction

Under physiological conditions, activation-induced cytidine deaminase (AID) is expressed in germinal center (GC) B-cells and initiates somatic hypermutation (SHM) and class-switch recombination (CSR) by deaminating a cytosine to create a uracil.[1,2] Structurally, the N-terminal or C-terminal domain of AID is indispensable for SHM or CSR, respectively.[3–5] Interestingly, expression of AID is increased in B-lymphoid leukemia or GC-derived B-lymphoma, with frequent hypermutation of proto-oncogenes and reciprocal chromosomal translocation.[6–9] In fact, recent studies have shown that AID is required for GC-derived lymphomagenesis and c-Myc/IgH chromosomal translocations.[10,11] In addition, elevated expression of endogenous AID and aberrant somatic mutations in tumor-related genes have also been observed in cancerous tissues related to inflammation.[12] Analysis of AID-transgenic (Tg) mice has revealed that constitutive expression of AID leads to tumorigenesis; ubiquitous and constitutive expression of AID induced lethal T-lymphoma with no apparent chromosomal translocation, occasionally accompanied by lung, liver, and gastric cancers,[13,14] and specific expression of AID in double-positive thymocytes also induced T-lymphoma.[15] However, neither AID-Tg mice specifically expressing AID in single-positive thymocytes and mature T-cells nor AID-Tg mice with $CD19^+$ B-cell-specific expression of AID developed lymphoma/leukemia.[15,16] These results suggest that susceptibility to AID-induced tumorigenesis depends on tissue or cell lineage, but the underlying mechanism remains obscure. Importantly, sequencing analysis in AID-Tg mice indicated that AID is an organ-specific mutator of non-Ig genes.[14] To prevent accumulation of unfavorable mutations induced by AID, its activity is tightly regulated by several mechanisms.[17]

In this study, we focused on AID-mediated leukemogenesis and created a mouse bone marrow transplantation (BMT) model, using BM cells retrovirally transduced with AID. Notably, recipient mice developed B-leukemia/lymphoma, albeit less frequently as compared with thymic T-lymphoma.

Correspondence: Dr T Kitamura, Division of Cellular Therapy, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan.
E-mail: kitamura@ims.u-tokyo.ac.jp
Received 23 November 2009; revised 8 January 2010; accepted 9 February 2010; published online 1 April 2010

Materials and methods

Retroviral constructs, transfection, and retrovirus production

Murine AID (mAID), mAID mutants (G23S[4] and Δ189–198[5]), human AID (hAID), and hAID mutants (P20[3] and JP8B[3]) were subcloned into the pMYsIG vector as described in 'Supplementary Materials and methods'. All constructs were verified by DNA sequencing. Expression of wild-type or mutant AID was recognized in 293T cells transiently transfected with each construct. Retroviruses were generated by transient transfection of Plat-E packaging cells with FuGene 6 (Roche Diagnostics, Mannheim, Germany), as described earlier.[18–20]

Mouse BMT

Mouse BMT was performed as described earlier.[20] C57BL/6 CD45.1 or CD45.2 mice were used as donors or recipients,

respectively. Infected cells (2×10^5) were intravenously injected into recipient mice, which had been administered a sublethal dose of γ-irradiation. Overall survival was estimated using the Kaplan–Meier method and log-rank test. Data are presented as the means ± s.d. PB smears and cytospin slides were stained with Hemacolor (Merck, Darmstadt, Germany). Tissues were fixed in 4% w/v buffered formalin, embedded in paraffin, and then sliced and stained with standard hematoxylin and eosin. All animal studies were approved by the Animal Care Committee of the Institute of Medical Science, The University of Tokyo.

Flow cytometric analysis

Cells were stained with phycoerythrin-conjugated monoclonal antibodies (eBiosciences, San Diego, CA, USA), as described.[20] Flow cytometric analysis was performed with a FACSCalibur equipped with CellQuest software (BD Biosciences, San Jose, CA, USA) and Flowjo software (Tree Star, San Carlos, CA, USA).

Western blotting

Equal numbers of cells were denatured in pre-heated sample buffer. Western blotting was performed as described.[20] Anti-AID mAb raised against the N-terminus of mAID, and anti-α-tubulin Ab (T6074, Sigma-Aldrich, St Louis, MO, USA) were used.

Southern blotting

Southern blotting was performed as described.[20] Briefly, 10 μg of genomic DNA digested with *EcoRI* was electrophoresed on a 0.7% agarose gel. Proviruses were probed with a ^{32}P-labeled GFP probe.

Reverse transcription and real-time PCR

Real-time PCR was performed using LightCycler (Roche Diagnostics), as described.[20] cDNA was amplified using a SYBR Premix EX Taq (Takara, Shiga, Japan). Primer pairs and conditions used for real-time PCR are listed in 'Supplementary Materials and methods'. Informed consent for the use of the human leukemia/lymphoma cells was obtained from patients in accordance with the Declaration of Helsinki, and study approval was obtained from the ethics committee of the Institute of Medical Science, the University of Tokyo (Approval Number 20-10-0620).

Sequencing of target genes

Genomic PCR was performed by using AmpliTaq Gold (Roche Molecular Systems Inc., Branchburg, NJ, USA) and the primer pairs described in 'Supplementary Materials and methods'. The PCR products were gel-purified and directly sequenced. If necessary, PCR products were subcloned and sequenced.

Treatment of AID-induced T-lymphoma cell lines with γ-secretase inhibitor

Cell lines of AID-induced T-lymphoma were established by culturing tumor cells in RPMI1640 with 20% FBS. Human T-acute lymphoblastic leukemia (T-ALL) cell lines Jurkat and HPB-ALL were cultured in RPMI1640 with 10% FBS. Various concentrations of γ-secretase inhibitor (DAPT, 565770, Calbiochem, Darmstadt, Germany) or vehicle (DMSO, Wako, Osaka, Japan) were added to 5×10^3 cells for 72 h. Cell growth was estimated by using CellTiter-Glo (Promega, Madison, WI, USA). Cleavage of intracellular domains of Notch1 (ICN) was detected by anti-ICN antibody (2421, Cell Signaling, Beverly, MA, USA) in western blotting.

Results

Transduction with AID into BM cells causes B-leukemia/lymphoma as well as thymic T-lymphoma in a mouse BMT model

First, we asked whether transduction of wild-type mAID (WT) into BM cells caused leukemia/lymphoma other than T-lymphoma in a BMT model ($n = 32$) (Figure 1). We confirmed efficient retrovirus infection: 50–70% and 76–84% of the BM cells transduced with WT or mock, respectively, were GFP-positive before transplantation. The recipient mice of WT-transduced cells developed thymic T-lymphoma more frequently than did AID-Tg mice[13] (75 vs 35%). The disease was associated with hepatosplenomegaly, killing the mice in 2–4 months after transplantation (Figure 1a; Supplementary Figure 1; Supplementary Table 1). Histological and flow cytometric analyses showed that the thymus was filled with the T-lymphoblastic cells $CD3^{dull}$, $CD4^+$, $CD8^+$, and $Thy1.2^+$, indicating the differentiation block at early stages of T-cell development in thymus (Figures 1b–d). The complete blood counts of these mice were usually normal, except for the increase of T-lymphoblastic cells or mature granulocytes in some cases (Supplementary Figure 1; Supplementary Table 1, and data not shown). Notably, 7 of 32 transplanted mice (22%) developed B-leukemia/lymphoma with pancytopenia and splenomegaly, and died with significantly longer latencies as compared with those of T-lymphoma (Figures 1a–d; Supplementary Figure 1; Supplementary Table 1). Spleen and BM were filled with B-lymphoblastic cells in most cases, while affected lymph nodes differed in size among cases. B-lymphoma cells were $B220^+$, $CD19^+$, $CD43^{dull/+}$, $c\text{-}kit^{dull/+}$, and IgM^- (Figure 1d). Neither Bcl-6 induction[21,22] nor abnormal Ikaros deletions[23] were detected in these cells (data not shown). There was a wide range of GFP-positive ratios among AID-induced T- or B-lymphoma cells, irrespective of disease severity (Supplementary Figure 2 and data not shown). Sequencing analysis of GFP gene integrated into the genome revealed multiple mutations, resulting in reduced green fluorescence. Interestingly, one recipient developed both T- and B-lymphoma. None of the mice suffered from myeloid leukemia. The lymphoma cells, irrespective of T- or B-lineage, were serially transplantable and developed T- or B-leukemia with shorter latencies, respectively (data not shown). The recipient mice of mock-transduced cells did not develop any leukemia/lymphoma (Figure 1a). Collectively, transduction with AID into BM cells led to thymic T-lymphoma or B-leukemia/lymphoma, but not myeloid leukemia in a mouse BMT model. Similar results were obtained when Balb/c mice were used (data not shown).

We next asked whether the integration of retroviruses influenced the different phenotypes (T- or B-lymphoma) in AID-induced leukemogenesis. Southern blot analysis demonstrated a single or several proviral integrations in T-lymphoma samples (Figure 1e, left panel). On the other hand, we found that a single integration was predominant in B-lymphoma samples (Figure 1e, right panel). In one recipient harboring both T- and B-lymphoma, a distinct integration was confirmed in each sample (Figure 1e, right panel, lanes *B and *T), indicating the double cancer in this case. We identified a single or several retroviral integration sites (RIS) from lymphoma samples by inverse PCR method (Supplementary Table 2). However, we could not find any specific relationship between RIS and different phenotypes of lymphomas. In addition, a common integration site was identified only in one recipient (ID69) (Supplementary Table 2). These results suggested that AID-induced lymphomagenesis mainly depended on its intrinsic function, but not RIS.

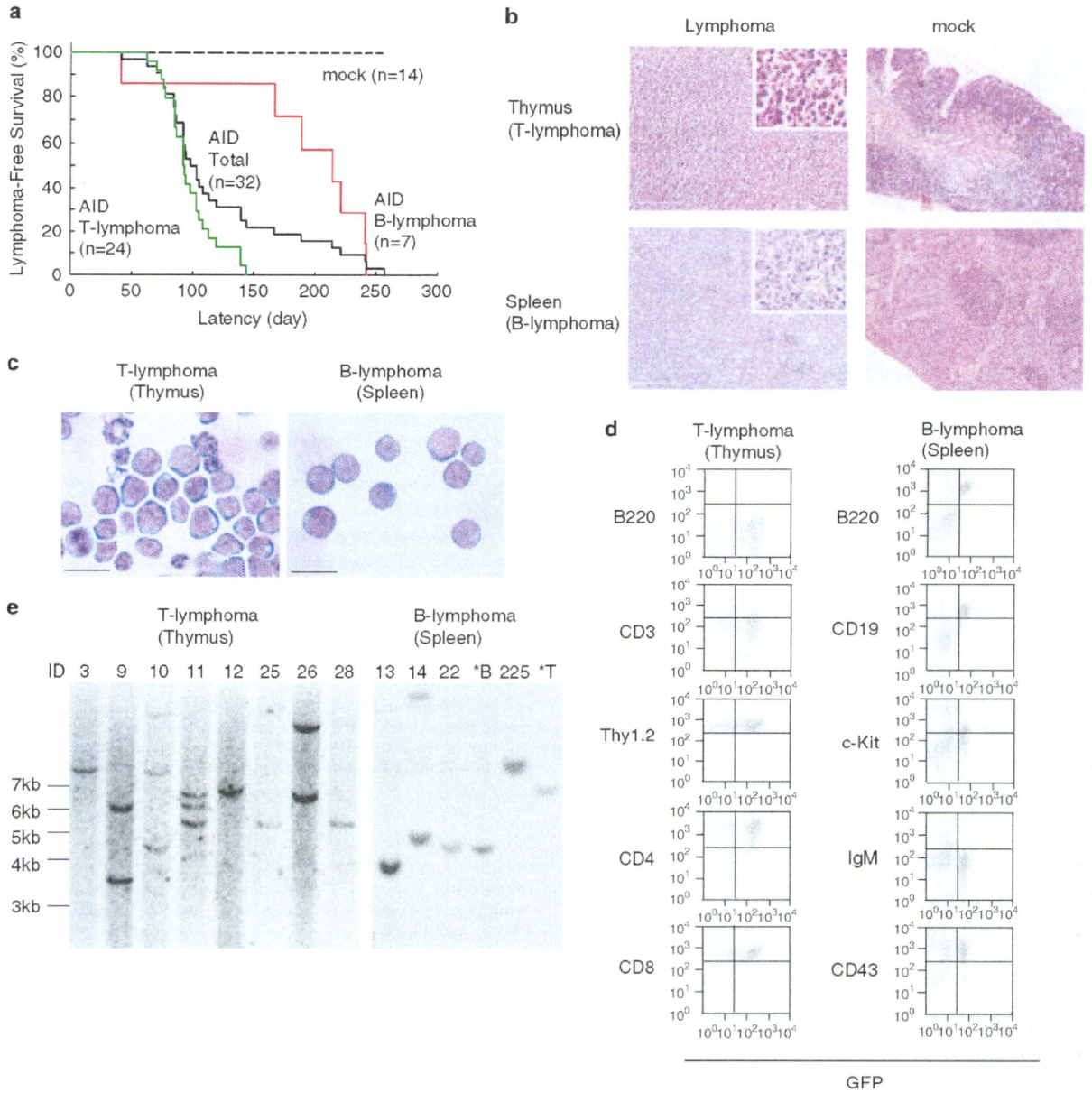

Figure 1 AID-induced T-lymphoma and B-leukemia/lymphoma in a mouse BMT model. (**a**) Kaplan–Meier plot of survival (black lines). Survival curve for AID recipient mice that developed T-lymphoma or B-lymphoma is indicated by green or red line, respectively. (**b**) Hematoxylin and eosin staining of AID-induced T-lymphoma (thymus, upper/left panel), control thymus (upper/right panel), AID-induced B-lymphoma (spleen, lower/left panel), or control spleen (lower/right panel). Magnifications × 200 (overview) and × 400 (insert). Scale bars: 200 μm. (**c**) Cytospin preparations of T-lymphoma (left panel) and B-lymphoma (right panel). Magnification × 1000. Scale bars: 20 μm. (**d**) Flow cytometric analysis of lymphoma cells. (**e**) Southern blotting of T-lymphoma (left panel) and B-lymphoma (right panel). *B or *T in the right panel indicates B- or T-lymphoma, respectively, which was found in the same mouse (ID 29).

We then asked whether other hematopietic malignancies, including myeloid leukemia, are induced in the absence of thymus. We used athymic nude mice as recipients of a BMT model, finding that 5 of 8 nude mice transplanted with AID-transduced BM cells developed B-leukemia/lymphoma, but no other hematopoietic diseases were observed (Supplementary Figure 3). Thus, B-leukemia/lymphoma was predominantly induced in the absence of thymus, but transduction of AID into BM cells did not induce myeloid leukemia. Altogether, these results suggested that the oncogenic transformation of AID-transduced BM cells requires *in vivo* environment suitable for differentiation and proliferation of immature lymphoid cells.

Impaired lymphomagenesis by SHM-defective AID mutants

To examine how SHM and/or CSR activities of AID contribute to lymphomagenesis, we constructed a BMT model using mutant forms of AID: missense mutant G23S with decreased SHM activity[4]; and truncation mutant Δ189–198 defective for CSR

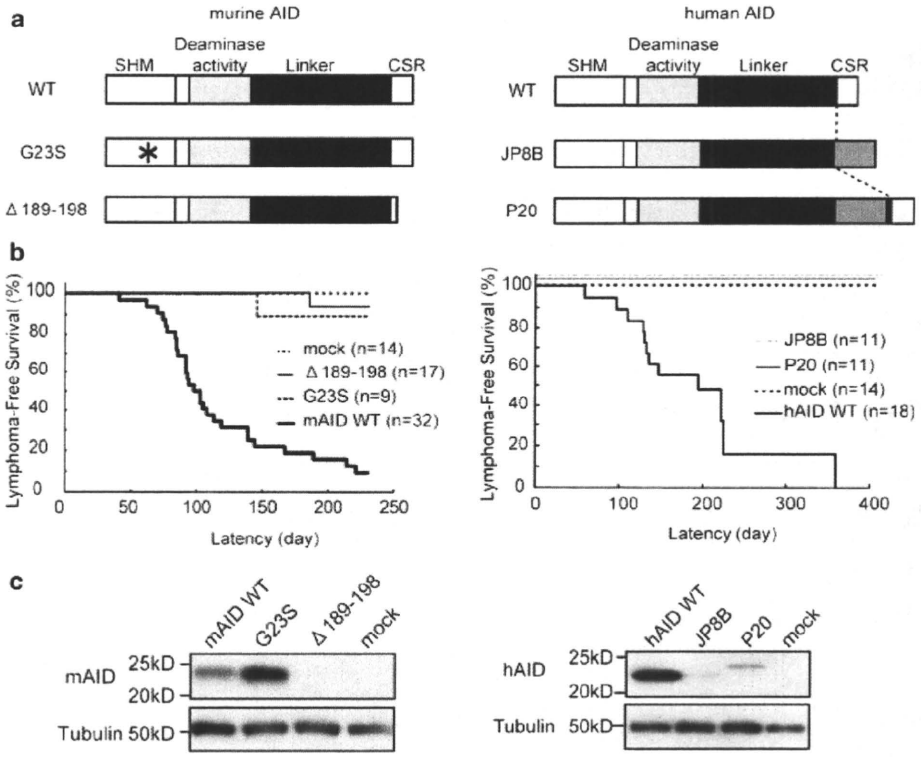

Figure 2 A BMT model using mutant forms of AID. (**a**) Diagrams of WT or mutant forms of AID. Left panel, mAID. G23S harbors a missense mutation (asterisk). Δ189–198 mutant lacks C-terminal 10 residues. Right panel, human AID (hAID). JP8B has a frameshift replacement of the C-terminus with 26 residues. P20 has an insertion of 34 residues. (**b**) Kaplan–Meier curves for the survival. Left panel, mAID. Right panel, hAID. (**c**) Expression of WT or mutant forms of AID in GFP-sorted BM cells by western blotting. Left panel, mAID. Right panel, hAID.

activity (Figure 2a, left panel).[5] Interestingly, recipients of these mutants showed a significantly decreased incidence of lymphoma as compared with those of WT (G23S $n=1/9$, 11%; Δ189–198 $n=1/17$, 6%) (Figure 2b, left panel). Each mutant developed only T-lymphoma. The expression level of G23S was comparable with that of WT in GFP-sorted BM cells (Figure 2c, left panel). On the other hand, the expression of Δ189–198 mutant in GFP-sorted BM cells was hardly detectable (Figure 2c, left panel). Similar results were obtained when hAID and its C-terminal mutants JP8B and P20 were transduced into BM cells (Figures 2a–c, right panels). Therefore, it was difficult to evaluate the effect of the CSR activity of AID on lymphomagenesis. Altogether, these results suggest that the intact form of AID with SHM activity is required to maximally exert its oncogenic activity.

An active mutation of K-ras in one case as well as multiple point mutations of Notch1, PTEN, and c-Myc was observed in AID-induced T-lymphoma

As point mutations are introduced into non-Ig genes such as *TCR* or *c-Myc* gene in T-lymphoma cells of AID-Tg mice,[13] we next asked which mutations caused by AID were responsible for T-lymphomagenesis in a mouse BMT model. On the basis of the fact that AID-mediated mutations occur after about 100 nucleotides downstream of the promoter and extend to 1–2 kb,[24] we performed genomic sequencing of several possible target genes in the region encompassing 0.5–1 kb from the transcription start site, in addition to the mutational hotspots implicated in tumorigenesis (Supplementary Table 3 and data not shown). Similar to the results on AID-Tg mice,[13] multiple mutations were observed in the *c-Myc* gene. An activating mutation of *K-ras* (G13D) was detected in 1 out of 14 analyzed samples, although no mutation was found in *N-ras*, *H-ras*, or *p53* tumor suppressor gene. As for genes involved in human T-ALL, *Notch1*[25] and *Pten*, but not *Fbxw7*, had multiple mutations. Intriguingly, we found several mutations in exon 27 (HD domain) and exon 34 (PEST domain), mutational hotspots of *Notch1* far downstream from the transcription start site. Consistent with the earlier report,[13] detected mutations were predominantly transition mutations and strongly biased to GC bases. Collectively, multiple mutations in T-lymphoma were introduced by AID, probably in association with lymphomagenesis.

Most AID-induced T-lymphoma cells exhibited constitutive activation of Notch1 and were susceptible to a γ-secretase inhibitor

The finding that Notch1 mutations were observed in AID-induced T-lymphoma led us to address the question of whether these mutations caused the activation of Notch1, leading to T-lymphomagenesis. Interestingly, western blot analysis demonstrated that cleavage of intracellular Notch1 (ICN) was evident in most T-lymphoma samples tested (Figure 3a), indicating that constitutive activation of Notch1 occurred in most T-lymphoma cells. In support of this finding, real-time PCR analysis showed increased expression of Hes1 and c-Myc and decreased expression of PTEN in most samples (Figure 3b). Expression levels of Notch1 did not significantly vary among these samples, except for a few cases. Interestingly, when we treated two cell

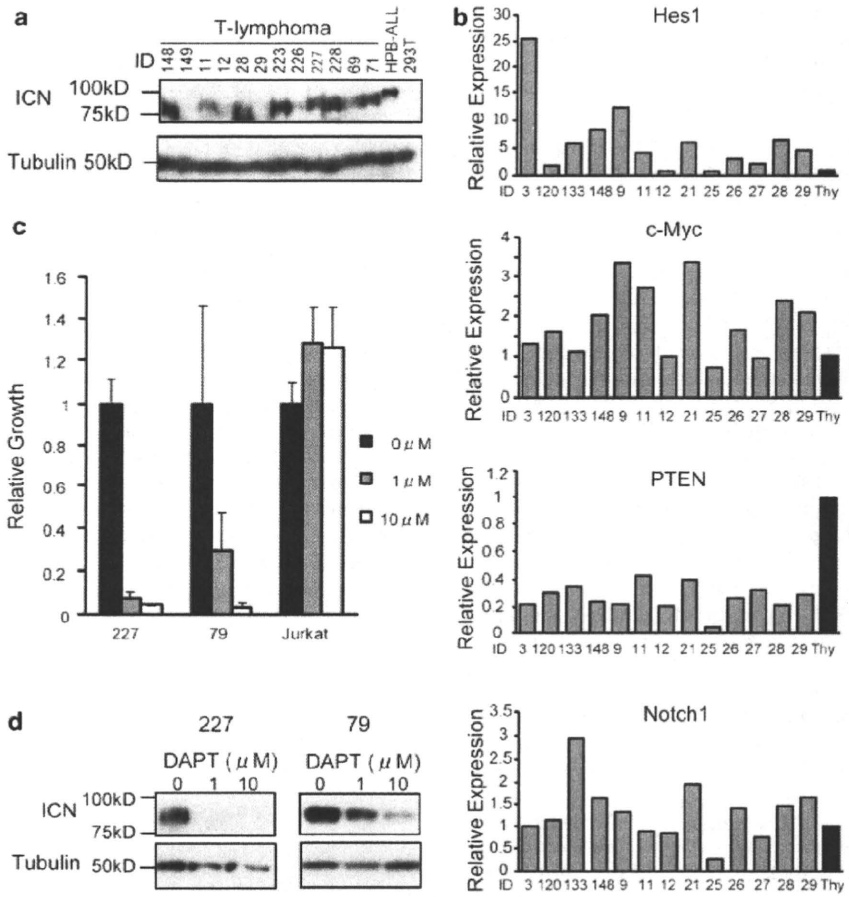

Figure 3 Notch1 is constitutively activated in AID-induced T-lymphoma. (**a**) Cleavage of intracellular Notch1 (ICN) in AID-induced T-lymphoma confirmed by western blotting. (**b**) Relative expression levels of Hes1, c-Myc, PTEN, or Notch1 in T-lymphoma samples and normal thymocytes (Thy) measured by real-time PCR. (**c**) The relative growth estimated by colorimetric assay. Two cell lines 227 and 79 established from AID-induced T-lymphoma, or Jurkat cells were treated with indicated concentrations of DAPT for 72 h. The means ± s.d. of triplicate measurements are shown. Data are representative of three independent experiments. (**d**) Cleavage of ICN examined by western blotting. Cells were treated as described in (**c**). Data are representative of three independent experiments.

lines established from AID-induced T-lymphoma with a γ-secretase inhibitor, DAPT, the growth as well as the cleavage of ICN of these cell lines was dose dependently inhibited by DAPT (Figures 3c and d). Although Notch1 mutations did not account for constitutive activation of Notch1 in all cases, these results indicated that T-lymphomagenesis in most cases was induced by Notch1 activation, probably in conjunction with AID-introduced mutations of the related genes.

Truncation mutations of Ebf1 and Pax5 were found in AID-induced B-lymphoma

To investigate the relevant mechanism of AID-induced B-lymphomagenesis, we performed genomic sequencing of the *Ebf1* and *Pax5* genes of AID-induced B-leukemia/lymphoma of seven recipient mice (Supplementary Table 4 and data not shown). Intriguingly, sequencing of *Ebf1* revealed a 23-base deletion and a 4-base insertion in exon 2, which together resulted in truncation in one sample. In addition, multiple point mutations were found in three samples. As for *Pax5*, we found a truncation caused by a couple of 2-base deletions and a point mutation in exon 1a in one case, and point mutations in two cases. On the basis of the recent study,[26] the aberrations of *Pax5* and *Ebf1* genes described above might have some function in B-lymphomagenesis in the recipient mice. On the other hand, we did not find c-Myc/IgH chromosomal translocations in B-lymphoma samples analyzed by PCR combined with Southern blotting (data not shown).[27] Collectively, these results suggested that B-lymphomagenesis in a BMT model was, at least in part, due to AID-introduced mutations/deletions of the genes regulating B-cell differentiation.

Discussion

In this study, we constructed a mouse BMT model to test whether AID is implicated in the pathogenesis of leukemia/lymphoma including myeloid leukemia. Our results revealed that aberrant expression of AID in BM cells led to T-lymphoma and less frequently B-leukemia/lymphoma, but not myeloid leukemia (Figure 1). The recipient mice developed 'thymic' T-lymphoma, but not 'peripheral type' T-lymphoma observed in 65% of AID-Tg mice.[13] It was noteworthy that B-leukemia/lymphoma was observed in our BMT model, but not in AID-Tg

mice,[13,15,28] because AID is implicated in the pathogenesis of human B-cell malignancy.[6–9] Interestingly, nude mice developed only B-leukemia when used as recipients, probably due to lack of thymus (Supplementary Figure 3). The differences between AID-Tg mice and the BMT model were probably caused by the expression levels in the different types of cells, although we could not completely exclude the possibility that RIS affected the phenotypes. We have two hypotheses for AID-induced lymphomagenesis in the BMT model: (1) AID-transduced stem/progenitor cells may move to thymus, where they would be susceptible to AID-mediated mutations and rapidly acquire oncogenic properties at an early stage of T-lineage development; (2) AID could introduce some mutations and transform cells at stem/progenitor levels, which would commit to T-lineage in thymus. Otherwise, AID-transduced stem/progenitor cells would be transformed during the early B-lineage development in BM and spleen, which result in B-leukemia/lymphoma. Both hypotheses would explain why no lymphoma was observed in AID-Tg mice with its expression restricted to mature lymphocytes.[15,28] However, it is not clear why AID overexpression did not induce myeloid leukemia. We found scarcely detectable levels of AID in human myeloid malignancy (Supplementary Figure 4). Recent study showed that chronic myeloid leukemia (CML) does not express AID unless CML cells are forced into B-lineage conversion by Pax5.[9] It is possible that the protective machinery efficiently works against AID functioning as a mutator in myeloid cells, but not in lymphoid cells. Indeed, we confirmed that AID overexpression did not affect myeloid cell development in BM one month after transplantation (data not shown). In addition, sorted myeloid progenitors (common myeloid progenitors and granulocyte-macrophage progenitors) transduced with AID did not cause myeloid leukemia in our BMT model (data not shown). According to the recent studies, the balance between error-prone repair (EPR) and high-fidelity repair (HFR) determines the outcome of AID-generated uracils, that is, accumulation or elimination of mutations.[29] It is tempting to speculate that the frequencies of uracils generated by AID are not different between the myeloid- and lymphoid-lineage, but that HFR overcomes EPR in the myeloid-lineage. In any case, solving the riddle of how AID induces leukemia/lymphoma in a cell-lineage–dependent manner will help understand AID functions.

It is generally accepted that the N-terminal or C-terminal domain of AID is important for SHM or CSR activity, respectively,[3–5] but neither activity is regulated in an exclusively distinct way. Our results showed that AID mutants with decreased SHM or CSR activity have impaired oncogenic activity (Figure 2). It must be noted that expression levels of mouse mutant Δ189–198 as well as human mutants JP8B and P20 in GFP-sorted BM cells were lower than those of WTs, possibly due to protein instability of these C-terminal mutants.[30] Therefore, we cannot answer the question whether CSR activity is indispensable for oncogenicity of AID, but we assume that the maximum ability of AID to cause lymphoma requires an intact form of AID with SHM activity.

We clarified to some extent the mechanism by which AID-introduced mutations of tumor-related genes led to lymphomagenesis (Supplementary Table 3). As reported on AID-Tg mice,[13] multiple mutations of the *c-Myc* gene were found in T-lymphoma samples. The *Notch1* gene was mutated in exon 1 and mutational hotspots (HD and PEST domains) in four cases. Intriguingly, Notch1 was constitutively activated in T-lymphoma more frequently than expected from the mutation frequency of *Notch1* (Figure 3). We speculate that AID-mediated mutations of other genes caused secondary Notch1 activation, resulting in T-lymphoma. However, one such candidate, *Fbxw7*,[31] did not have significant mutations. Further examination will identify unknown mutations responsible for human T-lymphoma/leukemia.

Sequencing analysis of AID-induced B-leukemia/lymphoma samples revealed frequent mutations in the *Ebf1* and *Pax5* genes (Supplementary Table 4 and data not shown). Importantly, we found truncation mutations in *Ebf1* and *Pax5* that probably have oncogenic properties; mono-allelic deletions of these genes were observed in human B-ALL.[26] As for chromosomal instability, c-Myc/IgH translocation was not detected (data not shown). The presence of TCR translocations was unlikely, as no chromosomal translocation was detected in AID-induced T-lymphoma observed in AID-Tg mice.[13,15] The present results suggest that, like thymic T-lymphoma, B-leukemia/lymphoma was induced by AID-introduced mutations/deletions of the key molecules regulating B-cell differentiation and/or proliferation.

In conclusion, this is the first report on the potential of AID overexpression to promote B-cell lymphomagenesis. Aberrant expression of AID in bone marrow cells induced leukemia/lymphoma in a cell-lineage–dependent manner, probably because an intact form of AID efficiently introduced mutations into the responsible genes, thereby disrupting normal development of lymphoid progenitors.

Conflict of interest

TK serves as a consultant for R&D Systems.

Acknowledgements

We thank Dr Chiba (Tsukuba University, Ibaraki, Japan) for providing HPB-ALL cell line. We are grateful to Dr Dovie Wylie for her excellent language assistance. This work was supported by grants from the Ministry of Education, Science, Technology, Sports and Culture, Japan to TK.

References

1 Muramatsu M, Sankaranand VS, Anant S, Sugai M, Kinoshita K, Davidson NO *et al.* Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. *J Biol Chem* 1999; **274**: 18470–18476.
2 Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 2000; **102**: 553–563.
3 Ta VT, Nagaoka H, Catalan N, Durandy A, Fischer A, Imai K *et al.* AID mutant analyses indicate requirement for class-switch-specific cofactors. *Nat Immunol* 2003; **4**: 843–848.
4 Shinkura R, Ito S, Begum NA, Nagaoka H, Muramatsu M, Kinoshita K *et al.* Separate domains of AID are required for somatic hypermutation and class-switch recombination. *Nat Immunol* 2004; **5**: 707–712.
5 Barreto V, Reina-San-Martin B, Ramiro AR, McBride KM, Nussenzweig MC. C-terminal deletion of AID uncouples class switch recombination from somatic hypermutation and gene conversion. *Mol Cell* 2003; **12**: 501–508.
6 Pasqualucci L, Neumeister P, Goossens T, Nanjangud G, Chaganti RS, Kuppers R *et al.* Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas. *Nature* 2001; **412**: 341–346.
7 Greeve J, Philipsen A, Krause K, Klapper W, Heidorn K, Castle BE *et al.* Expression of activation-induced cytidine deaminase in human B-cell non-Hodgkin lymphomas. *Blood* 2003; **101**: 3574–3580.

8 Smit LA, Bende RJ, Aten J, Guikema JE, Aarts WM, van Noesel CJ. Expression of activation-induced cytidine deaminase is confined to B-cell non-Hodgkin's lymphomas of germinal-center phenotype. *Cancer Res* 2003; **63**: 3894–3898.
9 Klemm L, Duy C, Iacobucci I, Kuchen S, von Levetzow G, Feldhahn N *et al.* The B cell mutator AID promotes B lymphoid blast crisis and drug resistance in chronic myeloid leukemia. *Cancer Cell* 2009; **16**: 232–245.
10 Pasqualucci L, Bhagat G, Jankovic M, Compagno M, Smith P, Muramatsu M *et al.* AID is required for germinal center-derived lymphomagenesis. *Nat Genet* 2008; **40**: 108–112.
11 Takizawa M, Tolarova H, Li Z, Dubois W, Lim S, Callen E *et al.* AID expression levels determine the extent of cMyc oncogenic translocations and the incidence of B cell tumor development. *J Exp Med* 2008; **205**: 1949–1957.
12 Matsumoto Y, Marusawa H, Kinoshita K, Endo Y, Kou T, Morisawa T *et al.* Helicobacter pylori infection triggers aberrant expression of activation-induced cytidine deaminase in gastric epithelium. *Nat Med* 2007; **13**: 470–476.
13 Okazaki IM, Hiai H, Kakazu N, Yamada S, Muramatsu M, Kinoshita K *et al.* Constitutive expression of AID leads to tumorigenesis. *J Exp Med* 2003; **197**: 1173–1181.
14 Morisawa T, Marusawa H, Ueda Y, Iwai A, Okazaki IM, Honjo T *et al.* Organ-specific profiles of genetic changes in cancers caused by activation-induced cytidine deaminase expression. *Int J Cancer* 2008; **123**: 2735–2740.
15 Rucci F, Cattaneo L, Marrella V, Sacco MG, Sobacchi C, Lucchini F *et al.* Tissue-specific sensitivity to AID expression in transgenic mouse models. *Gene* 2006; **377**: 150–158.
16 Muto T, Okazaki IM, Yamada S, Tanaka Y, Kinoshita K, Muramatsu M *et al.* Negative regulation of activation-induced cytidine deaminase in B cells. *Proc Natl Acad Sci USA* 2006; **103**: 2752–2757.
17 Casellas R, Yamane A, Kovalchuk AL, Potter M. Restricting activation-induced cytidine deaminase tumorigenic activity in B lymphocytes. *Immunology* 2009; **126**: 316–328.
18 Morita S, Kojima T, Kitamura T. Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther* 2000; **7**: 1063–1066.
19 Kitamura T, Koshino Y, Shibata F, Oki T, Nakajima H, Nosaka T *et al.* Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. *Exp Hematol* 2003; **31**: 1007–1014.
20 Watanabe-Okochi N, Kitaura J, Ono R, Harada H, Harada Y, Komeno Y *et al.* AML1 mutations induced MDS and MDS/AML in a mouse BMT model. *Blood* 2008; **111**: 4297–4308.
21 Ye BH, Chaganti S, Chang CC, Niu H, Corradini P, Chaganti RS *et al.* Chromosomal translocations cause deregulated BCL6 expression by promoter substitution in B cell lymphoma. *EMBO J* 1995; **14**: 6209–6217.
22 Pasqualucci L, Migliazza A, Basso K, Houldsworth J, Chaganti RS, Dalla-Favera R. Mutations of the BCL6 proto-oncogene disrupt its negative autoregulation in diffuse large B-cell lymphoma. *Blood* 2003; **101**: 2914–2923.
23 Mullighan CG, Miller CB, Radtke I, Phillips LA, Dalton J, Ma J *et al.* BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. *Nature* 2008; **453**: 110–114.
24 Longerich S, Tanaka A, Bozek G, Nicolae D, Storb U. The very 5′ end and the constant region of Ig genes are spared from somatic mutation because AID does not access these regions. *J Exp Med* 2005; **202**: 1443–1454.
25 Aster JC, Pear WS, Blacklow SC. Notch signaling in leukemia. *Annu Rev Pathol* 2008; **3**: 587–613.
26 Mullighan CG, Goorha S, Radtke I, Miller CB, Coustan-Smith E, Dalton JD *et al.* Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature* 2007; **446**: 758–764.
27 Ramiro AR, Jankovic M, Eisenreich T, Difilippantonio S, Chen-Kiang S, Muramatsu M *et al.* AID is required for c-myc/IgH chromosome translocations *in vivo*. *Cell* 2004; **118**: 431–438.
28 Muto T, Okazaki IM, Yamada S, Tanaka Y, Kinoshita K, Muramatsu M *et al.* Negative regulation of activation-induced cytidine deaminase in B cells. *Proc Natl Acad Sci USA* 2006; **103**: 2752–2757.
29 Liu M, Schatz DG. Balancing AID and DNA repair during somatic hypermutation. *Trends Immunol* 2009; **30**: 173–181.
30 Geisberger R, Rada C, Neuberger MS. The stability of AID and its function in class-switching are critically sensitive to the identity of its nuclear-export sequence. *Proc Natl Acad Sci USA* 2009; **106**: 6736–6741.
31 O'Neil J, Grim J, Strack P, Rao S, Tibbitts D, Winter C *et al.* FBW7 mutations in leukemic cells mediate NOTCH pathway activation and resistance to gamma-secretase inhibitors. *J Exp Med* 2007; **204**: 1813–1824.

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

Leukemia (2010) 24, 383–396
© 2010 Macmillan Publishers Limited All rights reserved 0887-6924/10 $32.00
www.nature.com/leu

EDUCATIONAL REPORT

Long-term results of Tokyo Children's Cancer Study Group trials for childhood acute lymphoblastic leukemia, 1984–1999

M Tsuchida[1], A Ohara[2], A Manabe[3], M Kumagai[4], H Shimada[5], A Kikuchi[6], T Mori[4], M Saito[7], M Akiyama[8], T Fukushima[9], K Koike[1], M Shiobara[10], C Ogawa[3], T Kanazawa[11], Y Noguchi[12], S Oota[13], Y Okimoto[14], H Yabe[15], M Kajiwara[16], D Tomizawa[16], K Ko[17], K Sugita[18], T Kaneko[19], M Maeda[20], T Inukai[21], H Goto[22], H Takahashi[23], K Isoyama[24], Y Hayashi[25], R Hosoya[3] and R Hanada[17] on behalf of Tokyo Children's Cancer Study Group, Tokyo, Japan

[1]Department of Pediatric Hematology and Oncology, Ibaraki Children's Hospital, Mito, Japan; [2]Department of First Pediatrics, Toho University Medical Center, Oomori Hospital, Tokyo, Japan; [3]Department of Pediatrics, St Luke's International Hospital, Tokyo, Japan; [4]Department of Pediatric Hematology/Oncology, National Center for Child Health and Development, Tokyo, Japan; [5]Department of Pediatrics, Keio University, School of Medicine, Tokyo, Japan; [6]Department of Pediatrics, Faculty of Medicine, University of Tokyo, Tokyo, Japan; [7]Department of Pediatrics, Juntendo University, School of Medicine, Tokyo, Japan; [8]Department of Pediatrics, Tokyo Jikei University, School of Medicine, Tokyo, Japan; [9]Department of Pediatrics, School of Medicine, University of Tsukuba, Tsukuba, Japan; [10]Departments of Pediatrics, University of Shinshu, School of Medicine, Matsumoto, Japan; [11]Department of Pediatrics, Gumma University, School of Medicine, Maebashi, Japan; [12]Department of Pediatrics, Japanese Red Cross Narita Hospital, Narita, Japan; [13]Department of Pediatrics, Chiba Medical Center, Teikyo University, Ichihara, Japan; [14]Department of Hematology/Oncology, Chiba Children's Hospital, Chiba, Japan; [15]Department of Pediatrics and Blood Transfusion, Tokai University, School of Medicine, Isehara, Japan; [16]Department of Pediatrics, Tokyo Medical and Dental University, School of Medicine, Tokyo, Japan; [17]Department of Hematology/Oncology, Saitama Children's Medical Center, Iwatsuki, Saitama, Japan; [18]Department of Pediatrics, Dokkyo Medical University, Mibu, Tochigi, Japan; [19]Department of Hematology/Oncology, Tokyo Metropolitan Kiyose Children's Hospital, Tokyo, Japan; [20]Department of Pediatrics, Nippon Medical University, Tokyo, Japan; [21]Department of Pediatrics, University of Yamanashi, School of Medicine, Kohu, Japan; [22]Department of Pediatrics, Yokohama City University, School of Medicine, Yokohama, Japan; [23]Department of Pediatrics, Yokohama Saiseikai Nanbu Hospital, Yokohama, Japan; [24]Department of Pediatrics, Showa University, School of Medicine, Fujigaoka Hospital, Yokohama, Japan and [25]Department of Hematology/Oncology, Gunma Children's Hospital, Maebashi, Japan

We report the long-term results of Tokyo Children's Cancer Study Group's studies L84-11, L89-12, L92-13, and L95-14 for 1846 children with acute lymphoblastic leukemia, which were conducted between 1984 and 1999. The value of event-free survival (EFS) ± s.e. was 67.2 ± 2.2% at 10 years in L84-11, which was not improved in the following two studies, and eventually improved to 75.0 ± 1.8% at 10 years in L95-14 study. The lower EFS of the L89-12 reflected a high rate of induction failure because of infection and delayed remission in very high-risk patients. The L92-13 study was characterized by short maintenance therapy; it resulted in poor EFS, particularly in the standard-risk (SR) group and boys. Females did significantly better than males in EFS in the early three studies. The gender difference was not significant in overall survival, partly because >60% of the males survived after the testicular relapse. Randomized studies in the former three protocols revealed that intermediate- or high-dose methotrexate therapy significantly reduced the testicular relapse rate. In the L95-14 study, gender difference disappeared in EFS. Contrary to the results of larger-scale studies, the randomized control study in the L95-14 reconfirmed with updated data that dexamethasone 8 mg/m^2 had no advantage over prednisolone 60 mg/m^2 in the SR and intermediate-risk groups. Prophylactic cranial irradiation was assigned to 100, 80, 44, and 44% of the patients in the studies, respectively. Isolated central nervous system relapse rates decreased to <2% in the last two trials. Secondary brain tumors developed in 12 patients at 8–22 years after cranial irradiation. Improvement of the remission induction rates and the complete omission of irradiation are currently main objectives in our studies.
Leukemia (2010) **24,** 383–396; doi:10.1038/leu.2009.260; published online 24 December 2009

Correspondence: Dr M Tsuchida, Department of Pediatric Hematology and Oncology, Ibaraki Children's Hospital, 3-3-1, Futabadai, Mito, #311-4145, Japan.
E-mail: mtsuchida@ibaraki-kodomo.com
Received 19 October 2009; accepted 29 October 2009; published online 24 December 2009

Keywords: acute lymphoblastic leukemia; children; long-term results; cranial irradiation; secondary malignancy

Introduction

We present here the long-term results of four studies for childhood acute lymphoblastic leukemia (ALL) of Tokyo Children's Cancer Study Group (TCCSG) conducted between 1984 and 1999.

Treatment protocol for SR and IR of the L84-11 study[1,2] was based on the early St Jude's total therapy.[3] ALL–BFM 81[4] protocol was modified and introduced to extremely high-risk group regimen for the first time. The protocols of the following three studies L89-12,[1,5] L92-13,[1,6] and L95-14,[7] were designed on the basis of the ALL-BFM framework. All the four protocols contained trials to reduce the number of patients who received irradiation, as had been reported in other studies.[8,9] The second point of analysis was on a gender difference[10–12] with respect to long-term event-free survival (EFS) and overall survival (OS). Randomized studies were mostly designed to test whether or not intermediate-dose methotrexate (ID-MTX) and high-dose methotrexate (HD-MTX) could replace the cranial irradiation. It is needed to describe the further long-term outcome of the patients who were treated in L92-13 study, which was characterized by very short maintenance therapy. We published the discordant results on the randomized comparison between dexamethasone and prednisolone in 2005, which was updated in this analysis.[7]

Materials and methods

Total of 1846 newly diagnosed patients with ALL aged 1–15 years entered into the four studies—that is L84-11 (n=484),

Table 1 Event-free survival, overall survival, and CNS relapse of TCCSG studies L84-11, L89-12, L92-13, and L95-14

Study	*Year*	*Number of patients*	*Complete remission rate (corrected)*[a]	*Event-free survival ± s.e.%*			*Overall survival ± s.e.%*			*Isolated and any CNS relapse rate ± s.e.%*
				5 years	*10 years*	*15 years*	*5 years*	*10 years*	*15 year*	*10 year*
L84-11	1984–1989	484	97.3 (98.6)%	71.2 ± 2.1	67.2 ± 2.2	66.3 ± 2.2	80.7 ± 1.8	74.3 ± 2.0	73.5 ± 2.1	4.1 ± 1.0 5.5 ± 1.1
L89-12	1989–1992	418	92.8 (95.7)%	67.2 ± 2.4	64.4 ± 2.4	62.3 ± 2.6	77.7 (2.1)	73.5 ± 2.2	71.9 ± 2.2	3.7 ± 1.1 5.4 ± 1.3
L92-13	1992–1995	347	96.5 (97.7)%	63.7 ± 2.7	60.1 ± 2.7	57.7 ± 2.9	80.4 (2.1)	77.9 ± 2.2	77.4 ± 2.4	1.0 ± 0.6 2.6 ± 1.0
L95-14	1995–1999	597	95.0 (97.4)%	76.8 ± 1.8	75.0 ± 1.8	—	84.9 (1.5)	82.0 ± 1.6	—	1.7 ± 0.6 2.8 ± 0.7

Abbreviations: CNS, central nervous system; s.e., standard error; TCCSG, Tokyo Children's Cancer Study Group.
[a]Corrected remission (rate %): patients who achieved delayed remission were included in remission, and censored patients during the induction phase were excluded from the total.

L89-12 ($n=418$), L92-13 ($n=347$), and L95-14 ($N=597$)—as shown in Table 1. Diagnoses were made based on morphology, immunophenotype, and cytogenetics in each institution; the ALL committee evaluated these results for eligibility. Patients aged 1–6 years presented with a leukocyte count $<20\times10^9$/l and B-precursor phenotype were classified into the standard-risk (SR) group in all the studies. Definitions of the intermediate-risk (IR) and high-risk (HR) or extremely high-risk groups varied across the four studies. Nonetheless, HR patients were mostly defined as having one of the following: initial leukocyte count $\geqslant100\times10^9$/l, age of $\geqslant10$ years, leukocyte count $\geqslant50\times10^9$/l; Philadelphia chromosome (Ph) or BCR–ABL fusion gene product positive, 11q23 chromosome translocation or MLL gene rearrangements, and T-ALL with otherwise IR-risk factors. The reminder of the SR and HR patients was assigned to the IR group. Analysis of the outcome was based on the risk classification of the NCI/Rome criteria.[13]

Leukemic-cell karyotype was obtained from 20 to 30% of the patients in the first three studies. The DNA index was measured by flow cytometry.

Infants were excluded from these studies, and their treatment results were already published elsewhere.[14–16]

Treatment

The precise regimens of L84-11,[2] L89-12,[5] L92-13,[6] and L95-14[7] studies were available in earlier publications. Table 2 provides a summary of regimens in each study.

L84-11 study (1984–1989). Both the SR and HR groups were randomized at early intensification into two arms—that is S1 and S2, and H1 and H2, respectively. In the S2 and H2 arms, the patients received three courses of ID-MTX (500 mg/m^2) with a single dose of leucovorin rescue (12 mg/m^2) at 48 h, in conjunction with double-drug intrathecal injections (DIT) before cranial irradiation. In the S1and H1 arms, 18 Gy of cranial irradiation with five doses of triple-drug intrathecal injections (TIT) were administered without ID-MTX.

The DIT consisted of methotrexate (MTX) 15 mg/m^2 $\leqslant$15 mg and hydrocortisone 30 mg/m^2 $\leqslant$30 mg, respectively. The TIT consisted of DIT and cytosine arabinoside (CA) 30 mg/m^2 $\leqslant$30 mg.

L89-12 study (1989–1992). The regimen was based on the BFM backbone in all three risk groups. There was a week of prophase treatment with prednisolone alone to evaluate initial steroid response, as BFM group described.[17] The main objective was to determine whether cranial irradiation was essential to the treatment of SR patients or not. To do so, the SR patients were randomly assigned to the SR0 and SR18 arms, and patients in the SR0 arm were given three courses of HD-MTX (3 g/m^2) with three DIT without cranial irradiation. The doses of intrathecal injection were reduced from those of the earlier study, changing to age-adjusted calculation. The patients assigned to the SR18 arm received 18 Gy of cranial irradiation and three doses of TIT. The randomization ratio in SR arms changed from 1:1 to 2:1 in the last half period, so that there were 83 patients enrolled in SR0 arm and 64 in SR18 arm. The HR group was treated with a single arm of BFM-style therapy for 2 years, modified with an insertion of HD-MTX (3 g/m^2, two courses) between the induction (Ia) and early intensification and cranial irradiation (Ib). Four courses of multiple-drug intensifications were given during the first year followed by 1-year maintenance therapy.

L92-13 study (1992–1994). A major objective was to evaluate 1-year therapy in all risk groups. The length of the maintenance therapy was kept to a minimum of 6 months in the SR group and 3 months in each of the IR and HR groups. All three risk regimens had BFM-type structures. This protocol was characterized by the use of intermediate-dose cytosine arabinoside (ID-CA, 500 mg/m^2/day for 4 days) and high-dose cytosine arabinoside (HD-CA, 1 or 2 g/m^2/day for 4 days) in the early intensification and in the re-intensification phases.

The SR regimen had two courses of HD-MTX (3 g/m^2) and two DITs. The early intensification phases were complete before week 28; 24 weeks were left for the continuous therapy. IR group was randomized either to IR18 arm with 18-Gy cranial irradiation, or to IR0 arm with two courses of HD-MTX (3 g/m^2/day) without cranial irradiation. All patients of the HR group were given 2 weekly courses of HD-CA (2 g/m^2, six doses for 3 days) and mitoxantrone (2 days) after remission induction.

L95-14 study (1994–1999). SR and IR groups were randomized into prednisolone arm (PSL) and dexamethasone arm (DEX) not only in the induction, but also in re-induction phase and three courses of late intensification for SR and two courses for IR. During remission induction, prednisolone (60 mg/m^2) or dexamethasone (8 mg/m^2) was given for 4 weeks and tapered. In the re-induction and intensification courses, prednisolone (40 mg/m^2) or dexamethasone (6 mg/m^2) were given for 2 weeks in each arm. For patients presenting with leukocyte count $\geqslant150\times10^9$/l and aged 7 years or older (assigned to allo-stem-cell transplantation (SCT) group), allogeneic bone marrow transplantation from HLA-matched family donor, if any, and autologous blood or marrow SCT or chemotherapy could be elected. For patients presented with

Table 2 Treatment protocols of the four studies

Studies	*TCCSG risk*	*Number*	*Therapy period (years)*	*Cranial irradiation***		*Remission induction*	*Early intensification*	*CNS prophylaxis*	*Reindution*	*Intensification*	*Continuation*
L84-11	SR	194	3.5	100%	100%	P V5 Asp	**Randomized S1:CRX18/ itMHC(5) vs S2:IDMTX(3)/ itMH(3)**	**S1:none vs S2:CRX18/itMHC(5)**	Dex V2/itMH..q16m(7) Dex V2 IDMTX/itMH..q12w(4)— 2.5–3.5 years		MTX+6mp (throughout)
	HR	244	3.5		100%	P V5 Asp Cy	**Randomized H1:CRX24/ itMHC(5) vs H2:IDMTX(3)/ itMH(3)**	**H1:none vs H2:CRX24/itMHC(5)**	Dex V2 D2, Dex V2 Cy, Dex B Acr, Dex V2 Asp, Dex V2 MTX(iv)— first, second year Cy(4), HDCA(4), IDMTX/itMHC(4)—third year		MTX+6mp (throughout)
	HEX	48	2.5		100%	P V4 Asp D3 Cy1 itMHC(3)	Cy1 CA(4x4) 6mp	CRX24/itMHC(5)	Dex V4 Ad4 Asp, Cy B(8) itMH(2)	itMHC(8), PV2(4)	MTX+6mp
L89-12	SR	142	2	80%	44%	P V4 Asp T2 itMHC(1)	Vp CA(4x3) 6mp itMHC(3)	**Randomized HDMTX/ itMH(3) vs CRX18/itMHC(3)**	Dex V3 Asp T(3)	Vp4 B4 6mp/itMH(2)	MTX+6mp (1.5 years)
	IR	100	2		100%	P V4 Asp T3 itMHC(1)	CRX18 itMH(3)	Cy1 CA(4x4) 6mp itMHC(3)	Dex V3 Asp T4	P Vp4 B4 Acr(2), P Vp4 Cy4 Aap(2)	MTX+6mp (1 year)
	HR	146	2		100%	P V4 Asp T3 itMHC(1-2)	HDMTX(2)/itMH(2)	CRX18 /itMHC(3) Cy2 CA(4x4) 6mp	Dex V3 Asp T4	mP HDCA Asp Mit(2), P Vp4 B4 Acr(2), P Vp4 Cy4 Asp(2)	MTX+6mp (1 year)
L92-13	SR	124	1	44%	0%	P V4 Asp T2 itMH(1)	Mit CA(4x4) 6mp	HDMTX/itMH(2)	P V3 Asp T2	IDCA0.5gx4/Mit(2)	MTX+6mp (6 months)
	IR	122	1		47%	P V4 Asp T3 itMH(1)	Cy1 CA(4x4) 6mp itMHC(3)	**Randomized HDMTX(2)/ itMH(2) vs CRX18/itMH (3)**	P V3 Asp T2	HDCA1gx4/Mit(2) it(MH), Vp B Asp(2)	MTX+6mp (3–4 months)
	HR	101	1		100%	P V4 Asp T3 itMHC(2-3)	HDCA2gx6/Mit (2) itMH(2)	CRX18/itMHC(3) Cy1 CA(4x4) 6mp	P V3 Asp T(2)	HDCA2gx4/Mit/ it(MH)(2), Vp B Asp(2)	MTX(iv)q4W+ 6mp (3–4 months)
L95-14	SR	231	2	44%	0%	**Randomized* P vs Dex** and V5 Asp T2 itMH(2)	Cy1 CA(5x3) 6mp itMHC(3)	HDMTX/itMH(3)	**P vs Dex*** V3 Asp T3	Cy1 CA(2x5) 6mp(1), **P vs Dex*** V3 Asp(3) IDMTX(no CF)/itMH(3)	MTX(iv)+6mp, MTX+6mp (1 year+)
	IR	129	2		18%	**Randomized* P vs Dex** and V5 Asp T2 Cy1 itMH(2)	Cy1 CA(5x3) 6mp itMHC(3)	**Randomized HDMTX/itMH(3) vs CRX18/itMH(3)** Asp MTX+6mp	**P vs Dex*** V3 Asp T3	Cy1CA(2x5) 6mp(1), **P vs Dex*** V3 Asp T3(2) HDCA/Asp(1), IDMTX(no CF)(2), Cy1 CA(2x5) 6mp(1)	MTX(iv)q2W+ 6mp, MTX+6mp (1 year+)
	HR	237	2		100%	P V5 Asp D4 Cy2/ itMH(2-3)	HDCA2gx4/Asp(2)/itMH(2)	CRX18/itMHC(3) Cy1 CA(5x3) 6mp(1)	Dex V4 Ad4 Asp(1), P V3 Asp Ad2(2)	HDCA2gx8/itMH(2) IDMTX(no CF)(2), Cy1 CA(2x5) 6mp(1)	MTX+6mp (1 year)

Abbreviations: CNS, central nervous system; HEX, extremely high risk; HR, high risk; IR, intermediate risk; SR, standard risk; TCCSG, Tokyo Children's Cancer Study Group.
Acr, aclarubicin; Ad, doxorubicin; Asp, L-asparaginase; B, behenoyl cytosine arabinoside; CA, cytosine arabinoside; CRX18, cranial irradiation 18 Gy; Cy, cytoxan; D, daunorubicin; Dex, dexamethasone (8 mg/m^2 in induction 6 mg/m^2 consolidation of dex arm); HDCA, high-dose cytosine arabinoside (1–2 g/m^2); HDMTX, high-dose methotrexate (3 g/m^2); IDCA, intermediate-dose cytosine arabinoside (500 mg/m^2); itMH, double intrathecal injection of methotrexate and hydrocortisone; itMHC, triple intrathecal injection of methotrexate, cytosine arabinoside, and hydrocortisone; IDMTX, intermediate-dose methotrexate (500 mg/m^2); Mit, mitoxantrone; mP, methy-prednisolone; MTX, oral methotrexate; MTX(iv), intravenous MTX (75 mg/m^2); (noCF), no leucovorine rescue; P, prednisolone (*60 mg/m^2 in induction 40 mg/m^2 consolidation of P arm); T, THP-adriamycin (pirarubicin); V, vincristine; Vp, etoposide; 6mp, oral 6 mercapropurine.
Number after drug-dose, (Numer), repeat. Randomizations were written with bold letters. Randomized*, initially randomized for whole course. **Proportion of the patients who were initially assigned to cranial irradiation arm; actual proportion was lower than the assigned.

leukocyte count $\geqslant 100 \times 10^9$/l, or 10 years old or older with leukocyte count $\geqslant 50 \times 10^9$/l (assigned to auto-SCT group), autologous blood or marrow SCT or chemotherapy could be elected. Each institute declared the choice in advance of the study initiation.

Statistical analysis

The duration of EFS was defined as the time from the initiation of therapy to the date of failure (that is any relapse, death, or diagnosis of secondary malignancy) or to the date when patients were confirmed to be in remission and alive. Patients who did not achieve complete remission at the end of the initial induction phase or who died before the confirmation of remission were considered to have failed at day 0, even if they entered remission later with a second course or through additional treatment. The probability of EFS and s.e. was estimated by the Kaplan–Meier method (Greenwood), and differences were tested by the log-rank test. Analysis was performed with the intent to treat. 'Any central nervous system (CNS) relapse' include both 'isolated CNS relapse' and CNS relapse combined with other sites. Probability of cumulative CNS relapse was estimated by inversed Kaplan–Meier method, which involves subtraction of Kaplan–Meier products from 100%. Only patients who had CNS relapse were failure, and all the others were censored. Cumulative probability of any secondary malignancy was calculated using the same method. Patients who received modified treatment were censored at that point in time. The patients who did not enter complete remission or had died during induction were treated as at the date of the beginning of treatment. Patients who were confirmed as remaining in first remission and alive, or who were lost of follow-up, were censored for EFS analysis; all those who were alive with or without disease were censored in OS analysis at the date of last contact.

Follow-up was updated in 2008. The proportions of patients whose data of the last 5 years were available were 144 of 357 (40.3%) in L84-11 study, 197 of 306 (64.3%) in L89-12, 220 of 266 (82.7%) in L92-13, and 449 of 489 (91.8%) in L95-14.

Results

Probability of EFS, OS, and cumulative CNS relapse rate of each study are shown in Tables 1 and 3. There was no improvement in EFS during the first three studies. The OS of L92-13 improved,

Table 3 Summary of the study results

Studies	*L84-11*	*L89-12*	*L92-13*	*L95-14*
Number of eligible patients (B+T)	484	418	347	597
Number of B/T	420/32	375/43	315/32	539/58
Average age (B/T) year	5.7/8.8	5.9/8.2	5.8/7.7	5.9/7.7
Average WBC (B/T)	20.1/108.0	31.6/137.5	38.4/146.1	30.6/167.0
Number of censored early	0	1 (0.2%)	2 (0.6%)	9 (1.5%)[a]
Death during induction	3 (0.6%)	12 (2.9%)[b]	5 (1.4%)	10 (1.7%)[c]
Failure of initial remission	11 (2.3%)[d]	17 (4.1%)[e]	5 (1.4%)	11 (1.8%)[f]
Complete remission (rate)	470 (97.1%)	388 (92.8%)	335 (96.0%)	567 (95.0%)
Corrected remission (rate)[g]	477 (98.6%)	399 (95.7%)	337 (97.7%)	573 (97.4%)
Death in first remission	19 (3.9%)	7 (1.7%)	6 (1.7%)	22 (3.7%)[h]
Number of censored in first remission	13 (2.7%)	13 (3.1%)[i]	31 (8.9%)[j]	21 (3.5%)[k]
Number of patients at event free	308 (63.6%)	256 (61.2%)	180 (55.3%)	428 (71.7%)
Number of relapse after remission	123 (26.1%)	104 (26.9%)	112 (33.4%)	92 (16.7%)
Site of relapse: total	123 (100%)	104 (100%)	112 (100%)	92 (100%)
Isolated bone marrow (BM)	72 (58.5%)	70 (67.3%)	87 (78.4%)	68 (73.9%)
Isolated CNS	17 (13.8%)	13 (12.5%)	3 (2.7%)	10 (10.9%)
Isolated testis	19 (15.4%)	6 (5.8%)	9 (78.4%)	7 (7.6%)
BM+CNS	6 (4.9%)	4 (3.8%)	3 (7.2%)	5 (5.4%)
BM+testis	7 (5.7%)	7 (6.7%)	6 (3.6%	1 (1.1%)
CNS+testis	1 (0.8%)	1 (0.9%)	0	0 (0%)
Other sites	1 (0.8%)	3 (2.9%)	3 (2.7%)	1 (1.1%)
Secondary AML/MDS	0/1	3/1	0/0	2/1
Brain tumor/Other	5/1[l]	4	2	1
Any BM	85 (69.1%)	81 (77.9%)	97 (87.4%)	74 (80.4%)
Any CNS	24 (19.5%)	18 (17.3%)	6 (5.4%)	15 (16.3%)
Any testis	27 (22.0%)	14 (13.5%)	15 (13.3%)	8 (8.7%)
Any testis/males	27 (10.3%)	14 (5.8%)	15 (8.5%)	8 (2.4%)

Abbreviations: AML, acute myeloid leukemia; CNS, central nervous system; MDS, myelodysplastic syndrome; SCT, stem-cell transplantation; WBC, white blood cells.
[a]Four patients assigned in dexamethasone arm dropped off, one in prednisolone arm, and four in HR risk group dropped off.
[b]Marrow suppression and infection.
[c]Five deaths in dexamethasone arm, two deaths in prednisolone arm, three deaths in HR risk.
[d]7/11 entered into remission in the following phase.
[e]11/17 patients entered remission in the following phase.
[f]All 11 failures in HR risk group; 3 Ph+ALL, 4 chromosomal translocations, 6/11 entered into remission in the following phase.
[g]Corrected remission (rate %): patients who achieved delayed remission were included in remission, and censored patients during the induction phase were excluded from the total.
[h]18/22 deaths in HR risk group, 5 related with transplants.
[i]7/13 patients underwent SCT in CR1.
[j]26/31 patients underwent SCT in CR1.
[k]9/21 patients underwent SCT in CR1.
[l]Olfactory neuroblastoma.