Jpn J Pediatr Hematol 23: 244 250, 2009

Original

Outcomes of 59 Children with Lymphoblastic Lymphoma Receiving an Intensive ALL-Type Therapy without Prophylactic Cranial Irradiation

A Report from Japanese Children's Cancer and Leukemia Study Group NHL 960 Trial

Masahito TSURUSAWA, Takashi TAGA, Yasuo HORIKOSHI, Atsushi OGAWA, Atsushi KIKUTA, Hirokazu KANEGANE, Masahiko OKADA, Nobuyuki HYAKUNA, Motoaki CHIN, Asayuki IWAI, Arata WATANABE, Tsutomu WATANABE, Yasuto SHIMOMURA and Koichi OHSHIMA for the Japanese Childhood Cancer and Leukemia Study Group

予防的頭蓋照射を省いた強力なALL型治療を受けた59例の小児リンパ芽球性リンパ腫の予後

——CCLSG-NHL960臨床試験の報告——

鶴澤　正仁，多賀　　崇，堀越　泰雄，小川　　淳，菊田　　敦，
金兼　弘和，岡田　雅彦，百名　伸之，陳　　基明，岩井　朝幸，
渡辺　　新，渡辺　　力，下村　保人，大島　孝一

小児癌白血病研究グループ

要　旨　予防的頭蓋照射を省いた強力なALL型治療を受けた59例の小児リンパ芽球性リンパ腫の長期予後を報告する．患者は限局例（8例：Murphy分類　I期2例，II期6例）と進行例（51例：Murphy分類　III期28例，IV期23例）に層別化され異なる治療レジメンを受けた．初診時に32例に縦隔腫瘤を認め，20例は骨髄浸潤，3例は中枢神経浸潤を有していた．59例中58例（98%）が完全寛解を得て，9例が再発し，維持療法中に1例が死亡した．7年累積生存率と累積無イベント生存期間は全体で86%±4%と81%±5%，進行例のみではIII期症例が93%±5%と86%±7%，IV期症例が83%±8%と74%±9%であった．これらの結果は予防的頭蓋照射を省いた強力なALL型治療によりIII期の小児リンパ芽球性リンパ腫症例に対して90%を超える治癒が可能であることを示唆している．

Abstract　Here, we report the long-term outcome of 59 children with lymphoblastic lymphoma (LBL) treated with an intensified ALL-type protocol without prophylactic cranial irradiation. They were stratified into the two treatment groups of the localized arm with stage I/II (n=2/6) or the advanced arm with stage III/IV (n=28/23). 32 had mediastinal mass, 20 had BM involvement and 3 had CNS diseases. 58/59 (98%) children achieved a complete remission. Nine patients recurred and one patient died during the maintenance therapy. Overall OS and EFS at 7 years were 86%±4% and 81%±5%, respectively. 7-year OS and EFS for advanced stages were 93%±5% and 86%±7% for stage III, and 83%±8% and 74%±9% for stage IV, respectively. These results suggest that the intensive ALL-like therapy without prophylactic cranial irradiation could achieve a cure rate of more than 90% for LBL with stage III disease.

Key words:　lymphoblastic lymphoma, NHL, Children, NHL960

I. Introduction

Lymphoblastic lymphoma (LBL) accounts for about 30% of childhood non-Hodgkin's lymphoma (NHL) and is the most frequent pathological subtype registered in Japanese pediatric lymphoma studies.[1] Since the original studies based on the LSA_2L_2 protocol by Wollner et al,[2,3] the treatment outcome of the disease

Received December 4, 2008, Accepted May 19, 2009
Reprint requests to Masahito Tsurusawa, Department of Pediatrics, Aichi Medical University, Aichi-gun, Aichi, 480-1195 Japan
別刷請求先：〒480-1195 愛知県愛知郡長久手町　愛知医科大学医学部小児科　鶴澤正仁

has improved and 70–90% of patients with advanced-stage LBL achieve a long remission through the use of an acute lymphoblastic leukemia (ALL)-like therapy such as LSA_2L_2-type or BFM-type protocols.[4,15]

Although the Japanese Children's Cancer and Leukemia Study Group (JCCLSG) conducted two trials (NHL855 and NHL890) for children with LBL in the 1980's, their survival outcomes were rather disappointing. The first NHL855 protocol employed a single arm regimen with the induction of five drugs cyclophosphamide (CPA), vincristine (VCR), prednisolone (PSL), doxorubicin (DOX), and high-dose methotrexate (HDMTX)-for patients with all stages and histologies of NHL. This trial showed very poor results for patients with LBL as compared with those of patients with non-LBL.[16] The following NHL890 study adopted histology-oriented therapy, and the regimens for advanced LBL newly incorporated L-asparaginase (LASP) and etoposide (VP-16), which had proved efficient for both leukemia and lymphoma with T-cell phenotype.[17] With this approach, survival rates of advanced LBL increased to 56% event-free survival (EFS) at 4 years,[18] but about a half of patients with stage III disease still recurred within the first 18 months after onset of treatment.

These results prompted us to intensify the chemotherapy regimens during the early treatment period in the succeeding NHL960 protocol, in which high-dose MTX in the induction phase was inverted by repeated doses of LASP, and the re-induction phase including LASP and dexamethazone (DEX) was newly added after the consolidation phase. In addition, we deleted prophylactic cranial radiotherapy (CRT) for CNS-negative patients, because CNS protective chemotherapy without CRT was adopted by other study groups.[6,8]

Thus, this study was performed to improve outcomes of advanced-stage LBL, especially for stage III disease, and to omit CRT safely without jeopardizing patient outcome. We conclude that the intensive ALL-like therapy without prophylactic cranial irradiation could achieve a long-term survival rate of more than 90% for LBL with stage III disease. Herein, we report the results of this multi-institutional, prospective nonrandomized study.

II. Patients and Methods

1. Patients' criteria

Patients were selected according to their ages and histological subtypes of tumors. Children less than 16 years of age, newly diagnosed with LBL who were under the care of a JCCLSG member institution, were entered in the study. Informed consent was given by patients or their parents. The diagnostic material was reviewed centrally by one of the authors (K.O.) whenever possible, or otherwise by the pathologist of each institution. The Revised European American Lymphoma classification system was used for histological classification.[19] Immunophenotypic subtyping was performed by the reactivity to T-cell antigens (CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD45), B-cell antigens (CD19, CD20, CD22, CD79a), and others (CD34, CD38, CD56, HLA-DR, and Tdt). Murphy's classification system was used for clinical staging.[20] Patients with BM blasts $\geqq$25% were registered when they had lymphomatous features, such as mediastinal mass, marked nodal enlargement, marked hepatomegaly, and marked splenomegaly.[21] CNS involvements were diagnosed by the presence of one or more of the following criteria: any lymphoma cells in CSF, isolated intracerebral masses or intra-spinal extensions.

2. Treatments

Patients received either the localized treatment or the advanced treatment according to their clinical stages. Figure 1 shows the treatment design of the present NHL960 study and the previous NHL890 study. The localized treatment for patients with stage I/II diseases consisted of the induction phase (regimen A) and the maintenance phase of 4 cycles of four

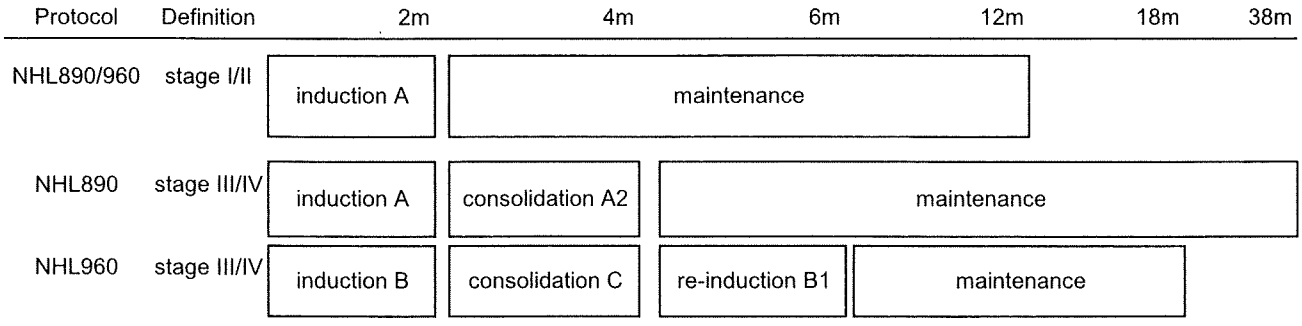

Fig. 1 Schema of the treatment of NHL960 and NHL890

Table 1 NHL960 treatment course for patients with localized disease

	Regimen	Administration	Daily dose	Days
Induction phase				
A	Cyclophosphamide	IV	1.2 g/m^2	1
	Pirarubicin	IV	30 mg/m^2	15, 16
	Vincristine	IV	2 mg/m^2	1, 8, 15, 22
	Prednisolone	Orally	60 mg/m^2	1-28
	Methotrexate	IV (24 hours)*	3 g/m^2	29, 36, 43
	Methotrexate	IT	12 mg/m^2	1, 29
	Hydrocortisone	IT	50 mg/m^2	1, 29
Maintenance phase				
M1	Pirarubicin	IV	30 mg/m^2	1
	Vincristine	IV	2 mg/m^2	1
	Prednisolone	Orally	120 mg/m^2	1-5
	6-Mercaptopurine	Orally	175 mg/m^2	1-5
M2	Cytarabine	IV	100 mg/m^2×2	1-4
	6-Mercaptopurine	Orally	175 mg/m^2	1-5
	Methotrexate	IT	12 mg/m^2	1
	Hydrocortisone	IT	50 mg/m^2	1
M3	Methotrexate	IV (24 hours)*	3 g/m^2	1, 8
M4	Cyclophosphamide	IV	1 g/m^2	1
	6-Mercaptopurine	Orally	175 mg/m^2	1-5

*Folinic acid 15 mg/m^2 was given orally every 6 hours for a total 7 doses. The rescue begins 36 hours from the start of methotrexate infusion. IV: intravenous, IT: intrathecal.

courses (M1-M4) of seven drugs (Table 1). The advanced treatment for stage III/IV patients consisted of the induction phase (regimen B), consolidation phase (regimen C), and re-induction phase (regimen B-1) followed by the maintenance therapy of 4 cycles of five courses (M1-M5) of 10 drugs (Table 2). Induction regimen B was almost identical to regimen A except for the addition of LASP (Kyowa, Japan) instead of HDMTX. Consolidation regimen C consisted of CPA, cytarabine (Ara-C), 6-mercaptopurine (6-MP), and HDMTX (3 g/m^2). Re-induction regimen B-1 consisted of CPA, daunorubicin (DNR), VCR, LASP, and DEX. For CNS prophylaxis, a total of 8 doses of intrathecal (IT) injections of MTX were performed: 2 each in the induction/consolidation phase and 4 doses in the maintenance phase.

We omitted all kind of radiotherapy except for patients with initial CNS disease who received cranial radiation of 18 Gy after the re-induction phase. The standard duration of treatment was 12 months for stage I/II patients and 18 months for stage III/IV patients.

3. Statistics

Final analysis was statistically performed based on the data obtained in June 2007. Survival curves were prepared by the Kaplan-Meier method, and a log-rank test was used to detect significant differences between groups. Overall survival (OS) was defined as the time between diagnosis of the disease and death. EFS was defined at the time to first occurrence of induction failure, relapse at any site, death or second malignant neoplasm. For patients who did not experience an event, EFS was the time to the last follow-up. SPSS statistical analysis software (SPSS 12.0J) was used for all computations.

III. Results

1. Patients' characteristics

From November 1996 to April 2003, A total of 68 children were registered in the NHL960 LBL study and 59 children with LBL were evaluable in this study. The other 9 patients were excluded from evaluation for lack of confirmed diagnosis of LBL or previous treatment. Ages ranged from one to 15 years of age with a median of 8 years of age. There were 35 boys and 24 girls, for a sex ratio of 1.46 : 1. The immunophenotypes of blast cells were precursor B-cell in 18, T-cell in 40, and not determined in one. The most common primary sites of tumor were mediastinum in T LBL and cervical in pre-B LBL (Table 3). Clinical stages were stage I in 2, stage II in 6, stage III in 28, and stage IV in 23. Seven of 8 patients with stage I/II had precursor-B cell immunophenotypes. In the patients with advanced diseases, 32 had mediastinal mass, 20 had BM involvements (BM$<$25%, 5; BM$\geqq$25%, 15), and 3

Table 2 NHL960 treatment course for patinets with advanced disease

	Regimen	Administration	Daily dose	Days
Induction phase				
B	Cyclophosphamide	IV	1.2 g/m^2	1
	Pirarubicin	IV	30 mg/m^2	15, 16
	Vincristine	IV	2 mg/m^2	1, 8, 15, 22
	Prednisolone	Orally	60 mg/m^2	1–28
	L-asparaginase	IV	6,000 U/m^2	15, 17, 19, 22, 24, 26, 29, 31, 33
	Methotrexate	IT	12 mg/m^2	1, 29 (14, 42)***
	Hydrocortisone	IT	50 mg/m^2	1, 29 (14, 42)***
Consolidation phase				
C	Cyclophosphamide	IV	400 mg/m^2	1, 15
	Cytarabine	IV	50 mg/m^2×2	1–4, 14–17
	6-mercaptopurine	Orally	60 mg/m^2	1–28
	Methotrexate	IV (24 hours)*	3 g/m^2	29, 36, 43
	Methotrexate	IT	12 mg/m^2	1, 29 (15, 43)***
	Hydrocortisone	IT	50 mg/m^2	1, 29 (15, 43)***
Re-Induction phase				
B1	Cyclophosphamide	IV	1.2 g/m^2	1
	Daunorubicin	IV	40 mg/m^2	1, 8, 15
	Vincristine	IV	2 mg/m^2	1, 8, 15
	Dexamethasone	Orally	10 mg/m^2	1–14
	L-asparaginase	IV	6,000 U/m^2	8, 10, 12, 15, 17, 19, 22, 25, 27
Maintenance phase				
M1	Pirarubicin	IV	30 mg/m^2	1
	Vincristine	IV	2 mg/m^2	1
	Prednisolone	Orally	120 mg/m^2	1–5
	6-mercaptopurine	Orally	175 mg/m^2	1–5
M2	Cytarabine	IV	100 mg/m^2×2	1–4
	6-mercaptopurine	Orally	175 mg/m^2	1–5
	Methotrexate	IT	12 mg/m^2	1
	Hydrocortisone	IT	50 mg/m^2	1
M3	Methotrexate	IV (6 hours)**	500 mg/m^2	1
	L-asparaginase	IV	2,000 U/m^2	1, 3, 5
M4	Cyclophosphamide	IV	1 g/m^2	1
	6-mercaptopurine	Orally	175 mg/m^2	1–5
M5	Cytarabine	IV	100 mg/m^2×2	1–4
	Etoposide	IV	150 mg/m^2	1, 2

*Folinic acid 15 mg/m^2 was given orally every 6 hours for a total 7 doses. The rescue begins 36 hours from the start of methotrexate infusion. **no folinic acid rescue, ***for CNS positive patients. IV: intravenous, IT: intrathecal.

Table 3 Primary tumor sites according to immunophenotype

Primary sites	T-cell	Precursor B	Undeteremined
Skin (head)	0	2	0
Jaw	1	2	0
Parotid	1	0	0
Cervical	8	7	0
Mediastinal	28	0	1
Abdomen	0	1	0
Retroperitoneum	0	1	0
Femoral	0	2	0
Testis	1	0	0
Pelvis	0	1	0
Bone	1	2	0
	40	18	1

had CNS involvements. Eight patients with a mediastinal mass showed superior mediastinal syndrome and/or superior vena cava syndrome. All 15 patients with BM blasts ≧25% fulfilled the criteria of lymphoma syndrome. Their immunophenotypes were precursor-B cell in 4 and T-cell in 11.

2. Events

Eleven patients experienced an event and eight patients died (Table 4). Of all patients, 58/59 (98%) achieved complete remission (CR). Nine relapses occurred at a single site in 7 (2 BM, 3 CNS, 2 locoregional) and multiple sites in 2 (1 BM+CNS, 1 locoregional+BM). Time to relapse ranged from 4.3 months to 28.1 months with a median of 14 months.

Of the 9 patients that relapsed, 3 patients are alive without disease, today. Those patients (two isolated CNS relapses and one mediastinal relapse) responded to the salvage therapy and were rescued by autologous peripheral blood stem cell transplantation.

3. Toxicity

During the protocol treatment period, a twelve-year-old girl with stage IV disease failed to achieve a remission due to pancreatitis by LASP. A nine-year-old girl with stage III disease achieved a remission, but then died of hemophagocytic syndrome during the maintenance therapy. This case showed no evidence of recurrence of lymphoma or severe infection by autopsy, so it was considered to be a malignancy-related hemophagocytic syndrome. Severe septic infection was observed in two patients with advanced diseases. Other adverse events such as hepatic toxicity or mucositis were all at acceptable levels. During the follow-up time, no patients experienced secondary malignancies.

4. Outcome

Overall OS and EFS at 7-years were 86%±4% (mean±SE) and 81%±5%, respectively (Fig. 2). The follow-up time for survivors ranged from 46 to 137 months with a median of 91 months. The 7-year EFS according to the treatment groups were 88%±11% for the localized arm and 80%±5% for the advanced arm. The 7-year OS and EFS according to the clinical stages were 100% (stage I), 83%±15% (stage II), 93%±5% and 86%±7% (stage III), and 83%±8% and 74%±9% (stage IV), respectively (Fig. 3).

In addition, we studied the effects of some factors on the survival rates. First, we studied whether the immunophenotypic subtypes of tumors affect outcomes of all patients, but no significant difference was detected between precursor-B LBL and T-LBL: 7y-EFS, 88%±8% vs. 76%±7%, $p=0.519$. Secondly, we studied the specific effects of a mediastinal mass on the survival rates of stage III/IV patients, but no significant difference was found between the patients with a mediastinal mass and those without a mediastinal mass: 7y-EFS, 84%±9% vs. 78%±7%, $p=0.572$. Moreover, the survival rates of the patients with lymphomatous features with BM ≧25% was compared with patients with BM<25%, and their EFS were almost identical: 7y-EFS, 72%±12% vs.71%±17%.

IV. Discussion

In this study, we confirmed a marked improvement in the survival outcomes of patients with advanced stage LBL as compared with the results obtained from our previous studies.[15,17,18] Equally importantly, we showed that there has been no recurrence at 29 months after the start of treatment during the long observation period of 10 years. These results imply that this therapy is intensive enough to achieve a cure in more than 80% of patients with the advanced diseases. Although the number of patients in our study is small, the long-term outcome with EFS of 80%±5% and OS of 86%±5% for the advanced LBL are favorably comparable to the results obtained by other study groups.[6-14]

Table 4 Events and outcomes according to immunophenotypes and clinical stages

	Immunophenotype*		Stage				
	T-cell	Precursor B	I	II	III	VI	Total
No. of patients	40	18	2	6	29	22	59
Events							
Initial treatment failure	1	0	0	0	1	0	1
Therapy-related death	1	0	0	0	0	1	1
Relapse	7	2	0	1	3	5	9
BM	0	2	0	1	0	1	2
CNS	3	0	0	0	2	1	3
Local	2	0	0	0	0	2	2
BM+CNS	1	0	0	0	1	0	1
BM+Local	1	0	0	0	0	1	1
Late event	0	0	0	0	0	0	0
Outcome							
Death	6	2	0	1	3	4	8
Alive in CR1	31	16	2	5	24	17	48
Alive after event	3	0	0	0	2	1	3

*Immunophenotype is not determined for one patient with stage III who are alive in first complete remission. BM: bone marrow, CNS: central nerveous system, CR1: first complete remission status.

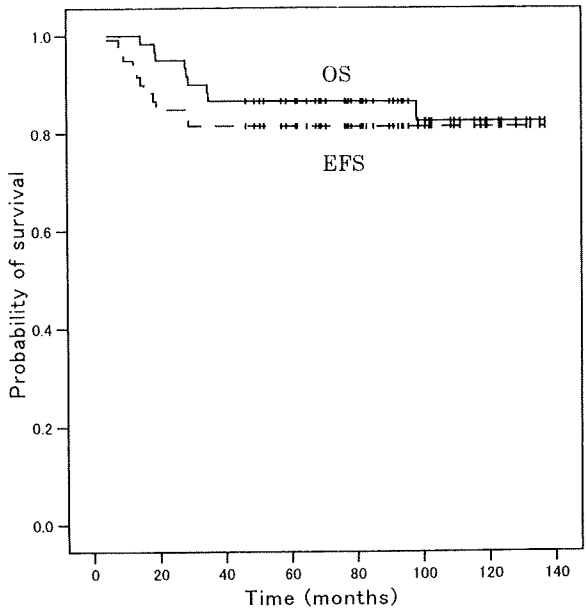

Fig. 2 Kurplen-Meier curves for the survival of 59 children with lymphoblastic lymphoma
OS (——) , EFS (·····).

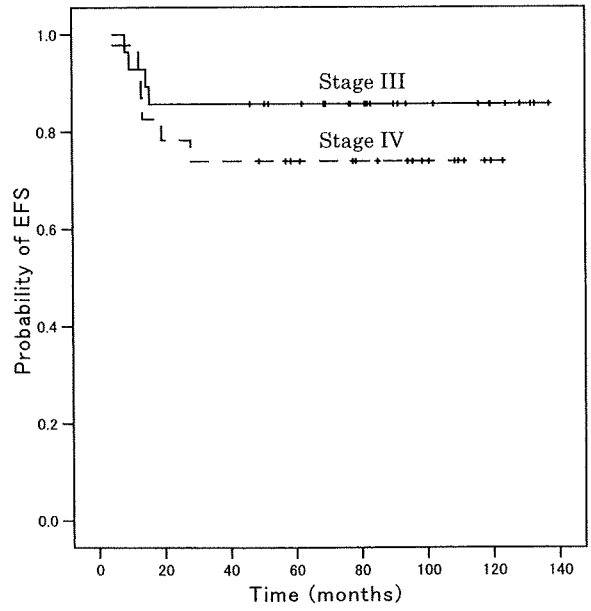

Fig. 3 Kurplen-Meier curves for the event-free survival of the advanced-stage LBL
Stage III (——) , stage IV (·····).

As in the LMT81 study,[8] we did not find significantly different results with stage IV patients when they had less or more than 25% blast cells in bone marrow. Diffuse node enlargement including mediastinum has been called "lymphoma syndrome leukemia" and such patients are considered to be a high risk group for early treatment failure.[21] In the current protocol studies, those patients with more than 25% blasts in marrow are registered as lymphoblastic leukemia sufferers and should be treated by intensive ALL-type therapy. However, the chemotherapy regimens are very similar between LBL and ALL. In the Berlin-Frankfurt-Munster (BFM) trials on childhood NHL, patients with T-cell LBL are treated according to the strategy of acute lymphoblastic leukemia.[13]

The extended use of LASP throughout the induction, re-induction, and maintenance phases is one of the new components of the NHL 960 protocol, which is common to the BFM-type protocol providing the most excellent results for advanced-stage LBL.[13] These are in contrast to the LSA_2L_2-based protocols, in which LASP has been used on a limited basis for the short duration of the induction or the consolidation phase.[6 12] Moreover, the benefit of prolonged use of LASP during the early maintenance phase was shown in the large-scale comparative trials by Amylon.[11] Therefore, intensified LASP therapy throughout the first 6–9 months of treatment may be a key component to improve the treatment outcome of advanced-stage LBL.

In this study, HDMTX in the consolidation phase seemed to favorably affect the survival outcome as compared with the previous NHL890 study in which HDMTX was used in the end of induction phase. Until now, few data have been available on the clinical benefit of the addition of HDMTX in the LBL treatment. For example, Patte et al. suggested the value of HDMTX for not only CNS prophylaxis, but also systemic treatment in the LMT81 trial, in which they added ten doses of HDMTX (3 g/m^2) to the early treatment phase of LSA_2L_2 regimens,[8] while HDMTX has been used as the CNS protective phase which comprises an important component of the early intensive treatment in the BFM-typed protocols.[13-15] Thus, it is likely that HDMTX is an important component during the early therapy for advanced LBL.

The incidence of CNS relapse with 3/48 (6.2%) in the patients with stage III/IV is unsatisfactory as compared with other studies without prophylactic CRT: 1/77 (1.3%) in LMT81,[8] and 1/156 (0.6%) in BFM95.[22] One of the possible reasons for the high CNS relapse rate may be the few doses of ITMTX in the NHL960 regimen. The dose of ITMTX during the first 7 months of treatment in NHL960 was 5 doses, which was apparently smaller than the 10 doses in LMT81, or 11 doses in BFM95. Since the isolated CNS relapse in this study occurred at 4, 8, and 16 months of the treatment, more extended IT treatments throughout the treatment phase may be effective as shown in our ALL studies.[23]

In this study, we treated precursor-B LBL as T LBL with the same protocol. The EFS rate of precursor-B LBL was not inferior to those of the T LBL. These results are in line with the previous report on the BFM86 and 90 NHL studies.[14] This report also mentioned the very low incidence (27/1075=2%) of precursor-B LBL in the BFM86 and 90 studies. However, a recent report on the BFM95 study showed a relatively high-incidence (7%) of precursor-B LBL,[24] which is very similar to our findings.

In conclusion, with the NHL960 protocol we greatly improved the survival of advanced LBL, and we have obtained results comparable to those now obtained by other groups. In a further study, it will be necessary to reduce the CNS recurrence without increasing the regimen-related toxicity.

References

1) Nakagawa A, Nakamura S, Nakamine H, et al: Pathology review for paediatric non-Hodgkin's lymphoma patients in Japan; A report from the Japan association of childhood leukaemia study (JACLS). Eur J Cancer **40**: 725–733, 2004
2) Wollner N, Burchenal JH, Lieberman PH, et al: Non-Hodgkin's lymphoma in children. A comparative study of two modalities of therapy. Cancer **37**: 123–134, 1976
3) Wollner N, Exelby PR, Lieberman PH: Non-Hodgkin's lymphoma in children: A progress report on the original patients treated with the LSA_2-L_2 protocol. Cancer **44**: 1990–1999, 1979
4) Anderson JR, Wilson JF, Jenkin DT, et al: Childhood non-Hodgkin's lymphoma. The results of randomized therapeutic trial comparing a 4-drug regimen (COMP) to a 10-drug regimen (SLA_2-L_2). N Engl J Med **308**: 559–565, 1983
5) Dahl GV, Rivera G, Pui CH, et al: A novel treatment of childhood lymphoblastic non-Hodgkin's lymphoma: Early and intermittent use of teniposide plus cytarabine. Blood **66**: 1110–1114, 1985
6) Hvizdala EV, Berard C, Callihan T, et al: Lymphoblastic lymphoma in children — A randomized trial comparing LSA_2-L_2 with the A-COP+ therapeutic regimen: A Pediatric Oncology Group Study. J Clin Oncol **6**: 26–33, 1988
7) Eden OB, Hann I, Imeson J, et al: Treatment of advanced stage T cell lymphoblastic lymphoma: Results of the United Kingdom Children's Cancer Study Group (UKCCSG) protocol 8503. Br J Haematol **82**: 310–316, 1992
8) Patte C, Kalifa C, Flamant F, et al: Results of the LMT81 protocol, a modified LSA_2L_2 protocol with high dose methotrexate, on 84 children with non-B-cell (lymphoblastic) lymphoma. J Med Pediatr Oncol **20**: 105–113, 1992
9) Anderson JR, Jenkin RD, Wilson JF, et al: Long-term follow-up of patients treated with COMP or LSA_2L_2 therapy for childhood non-Hodgkin's lymphoma: A report of CCG-551 from the Childrens Cancer Group. J Clin Oncol **11**: 1024–1032, 1993
10) Tubergen DG, Krailo MD, Meadows AT, et al: Comparison of treatment regimens for pediatric lymphoblastic non-Hodgkin's lymphoma: A Childrens Cancer Group study. J Clin Oncol **13**: 1368–1376, 1995
11) Amylon MD, Shuster J, Pullen J, et al: Intensive high-dose asparaginase consolidation improves survival for pediatric patients with T cell acute lymphoblastic leukemia and advanced stage lymphoblastic lymphoma: A Pediatric Oncology Group study. Leukemia **13**: 335–342, 1999
12) Mora J, Filippa DA, Qin J, et al: Lymphoblastic lymphoma of childhood and the LSA_2-L_2 protocol: The 30-year experience at Memorial-Sloan-Kettering Cancer Center. Cancer **98**: 1283–1291, 2003
13) Reiter A, Schrappe M, Ludwig WD, et al: Intensive ALL-type therapy without local radiotherapy provides a 90% event-free survival for children with T-cell lymphoblastic lymphoma: A BFM group report. Blood **95**: 416–421, 2000
14) Neth O, Seidemann K, Jansen P, et al: Precursor B-cell lymphoblastic lymphoma in childhood and adolescence: Clinical features, treatment, and results in trials NHL-BFM 86 and 90. Med Pediatr Oncol **35**: 20–27, 2000
15) Millot F, Suciu S, Philippe N, et al: Children's Leukemia Cooperative Group of the European Organiztaion for Research and Treatment of Cancer. Value of high-dose cytarabine during interval therapy of a Berlin-Frankfurt-Munster-based protocol in increased-risk children with acute lymphoblastic leukemia and lymphoblastic lymphoma: Results of the European Organization for Research and Treatment of Cancer 58881 randomized phase III trial. J Clin Oncol **19**: 1935–1942, 2001
16) Shimizu H, Kikuchi M, Takaue Y, et al: Improved treatment results of non-Hodgkin's lymphoma in children: A report from the Children's Cancer and Leukemia Study Group of Japan. Int J Hematol **61**: 85–96, 1995
17) Steinherz PG, Redner A, Steinherz L, et al: Development of a new intensive therapy for acute lymphoblastic leukemia in children at increased risk of early relapse. The Memorial Sloan-Kettering-New York-II protocol. Cancer **72**: 3120–3130, 1993
18) Tsurusawa M, Yamamoto Y, Katano N, et al: Treatment of children with non-Hodgkin's lymphoma with CCLSG NHL 855/890 protocols long-term outcome and incidence of secondary malignancies [article in Japanese]. Rinsho Ketsueki **39**: 281–289, 1998
19) Harris NL, Jaffe ES, Stein H, et al: A revised European-American classification of lymphoid neoplasms: A proposal from the International Lymphoma Study Group. Blood **84**: 1361–1392, 1994
20) Murphy SB: Classification, staging and results of treatment of childhood NHL, dissimilarities from lymphoma in adults. Semin Oncol **7**: 332–339, 1980
21) Steinherz PG, Siegel SE, Bleyer WA, et al: Lymphomatous presentation of childhood acute lymphoblastic leukemia. A subgroup at high risk of early treatment failure. Cancer **68**: 751–758, 1991
22) Burkhardt B, Woessmann W, Zimmermann M, et al: Impact of cranial radiotherapy on central nervous system prophylaxis in children and adolescents with central nervous system-negative stage III or IV lymphoblastic lymphoma. J Clin Oncol **24**: 491–499, 2006
23) Tsurusawa M, Katano N, Yamamoto Y, et al: Improvement in CNS protective treatment in non-high-risk childhood acute lymphoblastic leukemia: Report from the Japanese Children's Cancer and Leukemia Study Group. Med Pediatr Oncol **32**: 259–266, 1999
24) Reiter A: Therapeutic strategies and outcomes in lymphoblastic lymphoma. 2nd International symposium on childhood, adolescent and young adult non-Hodgkin's lymphoma, New York, May 18–20, 2006

bjh research paper

Age-associated difference in gene expression of paediatric acute myelomonocytic lineage leukaemia (FAB M4 and M5 subtypes) and its correlation with prognosis

Aoi Jo,[1,2] Ichiro Tsukimoto,[3] Eiichi Ishii,[4] Norio Asou,[5] Sachiyo Mitani,[1] Akira Shimada,[6] Takashi Igarashi,[2] Yasuhide Hayashi[6] and Hitoshi Ichikawa[1]

[1]Genetics Division, National Cancer Centre Research Institute, Tokyo, [2]Department of Paediatrics, University of Tokyo, Tokyo, [3]Department of Paediatrics, Toho University School of Medicine, Tokyo, [4]Department of Paediatrics, Ehime University, Toon, [5]Department of Haematology, Kumamoto University, Kumamoto, and [6]Department of Haematology/Oncology, Gunma Children's Medical Centre, Shibukawa, Japan

Received 11 September 2008; accepted for publication 23 October 2008

Correspondence: Dr Hitoshi Ichikawa, Section Head, Genetics Division, National Cancer Centre Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan.
E-mail: hichikaw@ncc.go.jp

Summary

Acute myeloid leukaemia, French-American-British M4 and M5 subtypes (AML-M4/M5) is frequently associated with *MLL* gene rearrangement and its incidence is relatively high among infants. Clinically, paediatric AML-M4/M5 has been considered as an intermediate or undefined prognostic group. In this study, we analysed gene expression of 40 paediatric AML-M4/M5 patients excluding inv(16) and t(8;21) patients, and found striking differences among the patients in an age-associated manner. In particular, most of the infants displayed very distinct gene expression. On the basis of this difference, we divided paediatric patients into three subgroups (A, B and C) with the average age of 0·3, 3·1 and 6·6 years old respectively. All subgroups included patients with *MLL* gene rearrangement as well as normal and other karyotypes. Surprisingly, gene expression signatures of *MLL* gene rearrangement differed substantially among these subgroups. In addition, subgroup C presented extremely poor outcome (3-year event-free survival 28%) whilst eight patients with *MLL* gene rearrangement in subgroup C had all relapsed within 18 months. These results suggest that age is an important factor contributing to the biology of AML-M4/M5 and the sub-grouping procedures developed in this study could be a powerful tool to identify unfavourable risk patients within paediatric AML-M4/M5.

Keywords: acute myeloid leukaemia, gene expression profiling, microarray, MLL, prognostic factors.

Acute myeloid leukaemia (AML) is a heterogeneous disease with different morphological features usually classified according to the French-American-British (FAB) classification system into subtypes M0–M7. In addition, a variety of genetic alterations have been ascertained including specific chromosomal translocations, such as t(8;21), t(15;17), inv(16) and 11q23 rearrangement. These major chromosomal translocations are usually associated with specific FAB subtypes. With the introduction of high throughput gene expression analysis using DNA microarrays, many studies have reported unique gene expression signatures for major chromosomal translocations, suggesting that the underlying molecular biology of these leukaemias can be defined by the genetic alterations (Golub *et al*, 1999; Armstrong *et al*, 2002; Schoch *et al*, 2002; Yagi *et al*, 2003; Ross *et al*, 2004). AML classified as FAB M4 or M5 subtypes (AML-M4/M5) have myelomonoblastic or monoblastic morphology. In this subtype, 11q23 rearrangements resulting in the fusion of the *MLL* with other partner genes (*MLL* gene rearrangement) are often observed. Unlike other chromosomal translocations, *MLL* gene rearrangement is also observed in acute lymphoblastic leukaemia (ALL), especially in infant ALL. Different studies have identified common gene expression signatures for acute leukaemia that contain *MLL* gene rearrangement irrespective of their lineage (Armstrong *et al*, 2002; Ross *et al*, 2004; Kohlmann *et al*, 2005).

The biological and clinical features of acute leukaemia differ between children and adults (Downing & Shannon, 2002) and considerable variations exist among different age groups in children. It is well known that infant ALL possess molecular abnormalities of *MLL* gene rearrangement that result in

 First published online 16 December 2008
British Journal of Haematology, **144**, 917–929 doi:10.1111/j.1365-2141.2008.07531.x

extremely poor outcome (Taki *et al*, 1996; Pui *et al*, 2002). For these differences, infant ALL is clinically and statistically handled separately from all the other paediatric ALL. In AML, distribution of FAB subtype differs among the age group. Infant AML generally show myelomonoblastic or monoblasic features (FAB M4 or M5) and often involves *MLL* gene rearrangement (Pui *et al*, 1995; Horibe *et al*, 2001). However, the presence of *MLL* gene rearrangement failed to correlate with treatment response in infant AML and the clinical outcomes were very similar to those of childhood AML (Satake *et al*, 1999; Pui *et al*, 2000; Kawasaki *et al*, 2001). For these reasons, not many studies distinguish infant from older paediatric AML clinically and statistically, and the biological differences between infant AML and childhood AML remain obscure.

In this study, we examined gene expression of paediatric AML-M4/M5, excluding inv(16) and t(8;21) patients, using microarray technology and found that age-associated differences exist among paediatric AML-M4/M5 patients. With the use of expression patterns, we have subdivided the patients into three subgroups and identified differences in the clinical outcome among these subgroups. Also, we have reviewed the gene expression profiles specific to patients with *MLL* gene rearrangement and showed that different signatures exist within different subgroups.

Materials and methods

Patients and samples

Forty paediatric AML patients (≤15 years old) diagnosed as FAB M4 or M5 subtype, between 1995 and 2001 in Japan, were enrolled in this study. Patients with inv(16) or t(8;21) were not included in this study. Characteristics of the 40 patients are shown in Table SI. These patients were treated with different but similar protocols, ANLL91 (Nagao, 1995; Satake *et al*, 1999) or AML99 (Tsukimoto *et al*, 2005; Shimada *et al*, 2006), which combine cytarabine, etoposide and anthracyclines. All leukaemic samples used in this study were obtained at the time of diagnosis. Morphological diagnosis according to FAB classification, immunophenotype and cytogenetic analysis were performed locally. *MLL* gene rearrangement was determined by cytogenetic analysis and multiplex reverse transcription polymerase chain reaction (RT-PCR) analysis (Pallisgaard *et al*, 1998; Salto-Tellez *et al*, 2003) using the HemaVision kit (DNA Technology, Aarhus, Denmark). Southern blot or fluorescence *in situ* hybridization analysis was also performed in some patients. In addition to the paediatric patients, 14 adult patients (20–79 years old), diagnosed as AML FAB M4 or M5 subtype [excluding inv(16) and t(8;21) patients], were also enrolled to compare paediatric AML to adult AML (Table SII).

This study was approved by the ethics committee of the National Cancer Centre. Patient identities were masked, and study numbers were assigned to all collected samples. To maintain anonymity, only age, sex, FAB subtype, chromosomal abnormality, treatment protocol and clinical outcome of the patients were linked to RNA samples. Informed consent was provided and obtained from the patients and/or the parents as appropriate.

Microarray analysis

For microarray gene expression analysis, mononuclear cells were isolated from the bone marrow cells at the time of diagnosis (median percentage of leukaemic blast cells: 82·3%, see Table SI), and then total RNA was prepared using an RNeasy Mini kit (Qiagen, Hilden, Germany). The integrity of the purified RNA was confirmed using a 2100 Bioanalyzer and an RNA 6000 Nano LabChip kit (Agilent Technologies, Santa Clara, CA, USA). The DNA microarray used was a Human Genome U133 plus 2.0 array (Affymetrix, Santa Clara, CA, USA). Target cRNA was prepared from 20 ng of the purified RNA with a two-cycle cDNA synthesis kit and 3′-amplification reagents for IVT labeling (Affymetrix). Hybridization to the microarrays, washing and staining with the antibody amplification procedure and scanning, were performed according to the manufacturer's instructions. Using the GeneChip Operating Software version 1.4 (Affymetrix), the scanned image data were processed and the expression value (Signal) and detection call (Present, Marginal or Absent) of each probe set were calculated. The Signal values were normalized so that the mean in each experiment was set at 100 to adjust for minor differences between the experiments.

Statistical analysis

For statistical analysis, the Signal values were log-transformed after the addition of 10 to reduce adverse effect caused by the noises at low expression levels. Most of the statistical analysis including principal component analysis (PCA), hierarchical clustering analysis, and Student's *t*-test were performed on the GeneSpring GX software version 7.31 (Silicon Genetics, Redwood City, CA, USA) after the log-transformed expression values were normalized to the median of all samples enrolled in each of the analyses. For the hierarchical clustering analysis, Pearson's correlation was used. To select karyotype discriminating genes, Significance Analysis of Microarray (SAM) was also used (Tusher *et al*, 2001). The Kaplan–Meier method and log-rank test were used for comparison of event-free survival (EFS), which was defined as the time from diagnosis to event (relapse or death of any cause).

Quantitative RT-PCR

Quantitative RT-PCR was carried out using the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with FastStart TaqMan Probe Master (Rox) and Universal ProbeLibrary (Roche Applied Science, Mannheim, Germany). cDNA was prepared from 50 ng of total RNA using SuperScript III (Invitrogen), and 1/100 of the cDNA was used as a

template for each PCR reaction. Probes and primers were designed using the Probe Finder software (Roche Applied Science). Sequences of the primers are listed in Table SIII. Transcript levels were normalized to that of the *GAPDH* transcript. Quantitative RT-PCR was performed on 15 of the 40 paediatric patients.

Results

Expression profile of paediatric AML-M4/M5

Gene expression of 40 paediatric AML patients diagnosed as FAB M4 or M5 subtype was analysed with an oligonucleotide microarray composed of 54675 probe sets. Patients with inv(16) or t(8;21) were excluded from this study because they are known to display distinct expression profiles and clinical behavior (Yagi *et al*, 2003; Ross *et al*, 2004; Valk *et al*, 2004). Among the 40 paediatric patients, 14 patients (35%) were infants (<1 year old) and 10 out of 14 infants (71%) possessed *MLL* gene rearrangement (Table I and SI).

To obtain the overview of the variations in gene expression among the 40 patients, PCA was performed. Patients were clearly separated into two clusters by the first principal component, a tight cluster and a relatively loose cluster (Fig 1A). The tight cluster was composed of 10 patients that were all infants. Although the remaining four infants were included in the loose cluster, the gathering of infants into one cluster was statistically significant ($P = 6{\cdot}5^{-07}$ in chi-square test). On the other hand, the distribution of *MLL* gene rearrangement (presence or absence) and FAB subtype (M4 or M5) had no significant correlations between the two clusters ($P = 0{\cdot}097$ and $P = 0{\cdot}58$ respectively). Clinical outcomes were also not significantly different between the two clusters ($P = 0{\cdot}23$ in log-rank test), although the patients in the loose cluster seemed to have poorer outcome.

Table I. AML-M4/M5 patients enrolled in this study.

Variables	Patients (*n*)	Ratio (%)
Total	40	
Gender		
Male	22	55·0
Female	18	45·0
French-American-British subtype		
M4	17	42·5
M5	23	57·5
Age at diagnosis (years)		
<1	14	35·0
1–5	16	40·0
≥6	10	25·0
Karyotype/gene rearrangement		
MLL gene rearrangement	23	57·5
Normal	13	32·5
Others	4	10·0

To assess the differences in gene expression among the 40 patients, we then performed unsupervised two-dimensional hierarchical clustering analysis. In this analysis, the same 10 infants who assembled a strong cluster in PCA again formed a distinct cluster (Fig 1B). This analysis also showed that this infant cluster was characterized by high expression of a very large set of genes (Fig 1B). These results suggest that most of infant AML-M4/M5 possess biological features different from older children with AML-M4/M5. This typical infant cluster comprised both *MLL* gene rearrangement-positive [*MLL*gr(+)] and *MLL* gene rearrangement-negative [*MLL*gr(−)] patients, indicating that a typical infant profile was not restricted by *MLL* gene rearrangement status. In addition, we could not detect any prominent cluster formation of *MLL*gr(+) patients or gene expression signature specific to *MLL* gene rearrangement in this analysis.

Comparison with adult AML-M4/M5

To compare the gene expression of paediatric AML-M4/M5 with that of adult AML-M4/M5, gene expression of 14 adult patients were examined and added to the PCA and hierarchical clustering analysis. PCA with 40 paediatric and 14 adult patients revealed three clusters correlating with patient age. Ten infants and the remaining paediatric patients once again formed separate clusters while 11 out of 14 adult patients also formed a distinct cluster (Fig 1C). These results suggest that older paediatric AML-M4/M5 as well as infant AML-M4/M5 have distinct characteristics that were different from adult AML-M4/M5. In the unsupervised two-dimensional hierarchical clustering analysis, 12 out of 14 adult patients formed a distinct cluster (Fig S1). A large set of genes was commonly overexpressed between adults and infants, and a relatively small set of genes was commonly overexpressed between adults and older children (Fig S1). These results imply that expression patterns are not defined by the linear increment of patients' age. The genes shared by the infant and adult clusters included those encoding transcription factors and regulatory proteins involved in haematopoietic cell differentiation and proliferation (*AFF1*, *MLLT4*, *MLLT10*, *ARHGAP26*, *ELF2*, *ETV6*, *FLI1*, *FOXP1*, *FYN*, *LRRFIP1/2*, *LYN*, *MED13*, *MED13L*, *RUNX1*).

Sub-grouping of paediatric AML-M4/M5

When we looked into the expression patterns of paediatric patients, the solid clustering of 10 infants was inevitable but we also realized that the remaining patients seemed to be heterogeneous. For those genes that were specifically overexpressed in 10 infants, some of the other patients showed quite a different expression while the others showed somewhat closer expression patterns to 10 infants (Fig 1B). Thus, we proceeded with our analysis to separate the 40 paediatric patients into three subgroups based solely on gene expression without any previous knowledge of the patients' clinical

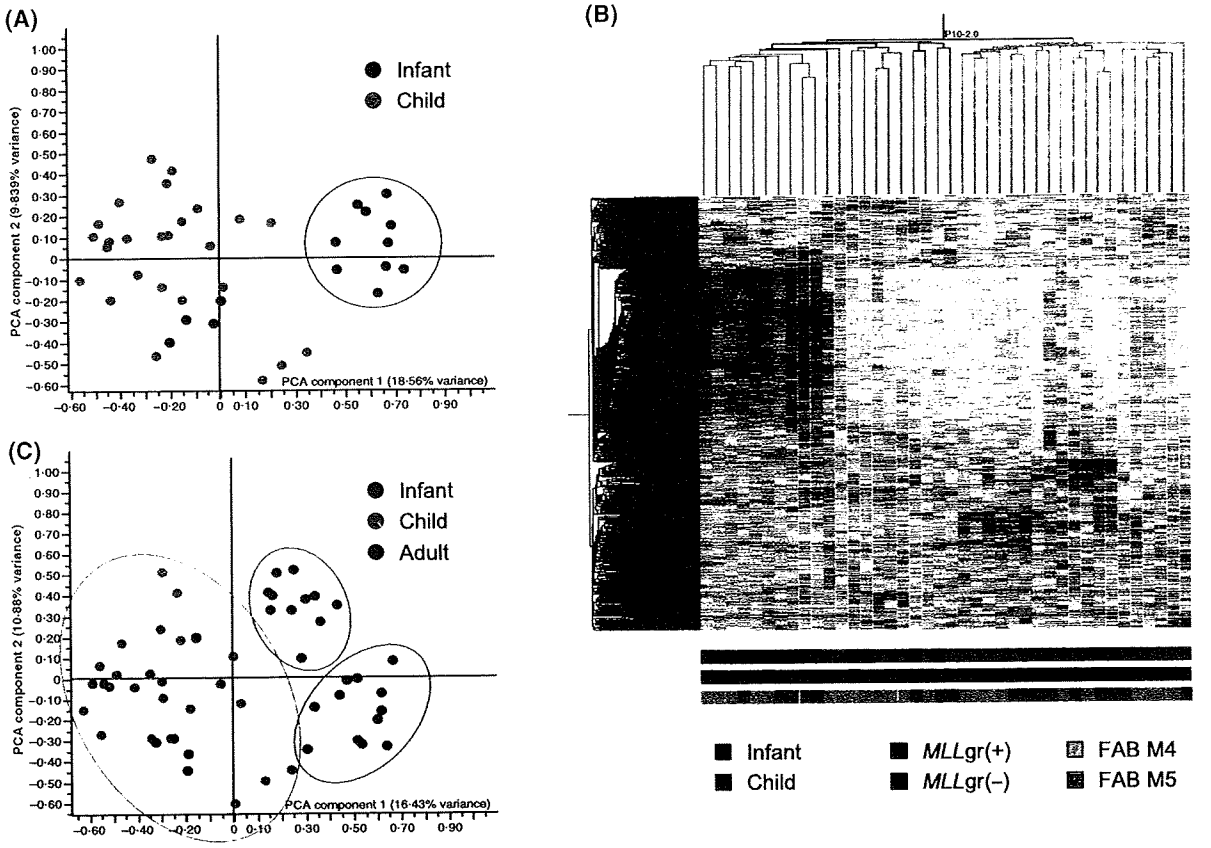

Fig 1. Overview of AML-M4/M5 gene expression. (A) PCA analysis of 40 paediatric AML-M4/M5 patients. 25754 probe sets with Present calls in at least 10 of the 40 patients were used in this analysis. Infant (<1 year old) and child (≥1 year old) patients are indicated by the colours. (B) Two-dimensional hierarchical clustering analysis of 40 paediatric AML-M4/M5 patients. 1865 probe sets with high variation in expression (≥2-fold changes to the median value in at least 10 of the 40 patients) were selected from the 25754 probe sets and used in the analysis. Columns and rows in the matrix represent patients and probe sets, respectively. Red and green indicates high and low expression, respectively. Indicated below the matrix are the age group (infant or child), karyotype [*MLL*gr(+) or *MLL*gr(−)] and FAB subtype (M4 or M5) for each patient by colour codes. (C) PCA analysis of 40 paediatric and 14 adult AML-M4/M5 patients. 25605 probe sets with Present calls in at least 15 of the 54 patients were used in the analysis. Infant, child and adult patients are indicated.

information. First, we isolated 2421 probe sets overexpressed in 10 distinct infants by selecting probe sets that exhibited *P*-values of <0·01 in Students *t*-test and twofold higher expression when the 10 infants were compared with the other remaining patients (Fig 2A and genes listed in Table SIV). With the average normalized expression value of these genes, we then divided the 40 patients into subgroups, designated as A, B and C. Subgroup A had an average normalized value greater than 2·0, whereas subgroup C had an average normalized value less than 1·0 (Fig 2A and B, see also Table SI). All of the 10 distinct infants were selected as subgroup A, and 12 and 18 of the remaining 30 patients were divided into subgroup B and C, respectively.

As shown in Fig 2C and D, age distribution and karyotypes were different among the three subgroups. The average age of subgroup A was 0·3 years old with eight out of 10 patients' possessing *MLL* gene rearrangement. The average age of subgroup B was 3·1 years old, and seven of the 12 patients possessed *MLL* gene rearrangement with t(9;11) as being the dominant karyotype. Subgroup C included many of the older children with the average age of 6·6 years old, and eight of the 18 patients possessed *MLL* gene rearrangement. Interestingly, all the t(6;11) patients (average age 10·0 years old) were placed in subgroup C. FAB subtypes were equally distributed among each subgroup (Fig 2E).

To compare the clinical outcomes among the subgroups, we performed Kaplan–Meier analysis and log-rank test. Subgroup C had the worst outcome (3-year EFS 28%), which was significantly lower than those of subgroup A (3-year EFS 70%) and subgroup B (3-year EFS 75%) ($P = 0·023$) (Fig 2F). The outcome of subgroup C was definitely lower than that of the intermediate-risk patients in AML 99 study (3-year EFS 60%) (Tsukimoto *et al*, 2005).

To investigate the underlying biology in paediatric AML-M4/M5, we selected the genes that differentially expressed in subgroups A and C (Table II and SV). Three thousand and thirty-three probe sets were identified by selecting genes with *P*-values of <0·0001 and fold change values of >2·0 or <0·5. Among these genes, 2487 probe sets (82%) were higher in subgroup A and 546 probe sets (18%) were higher in subgroup C. Almost all of

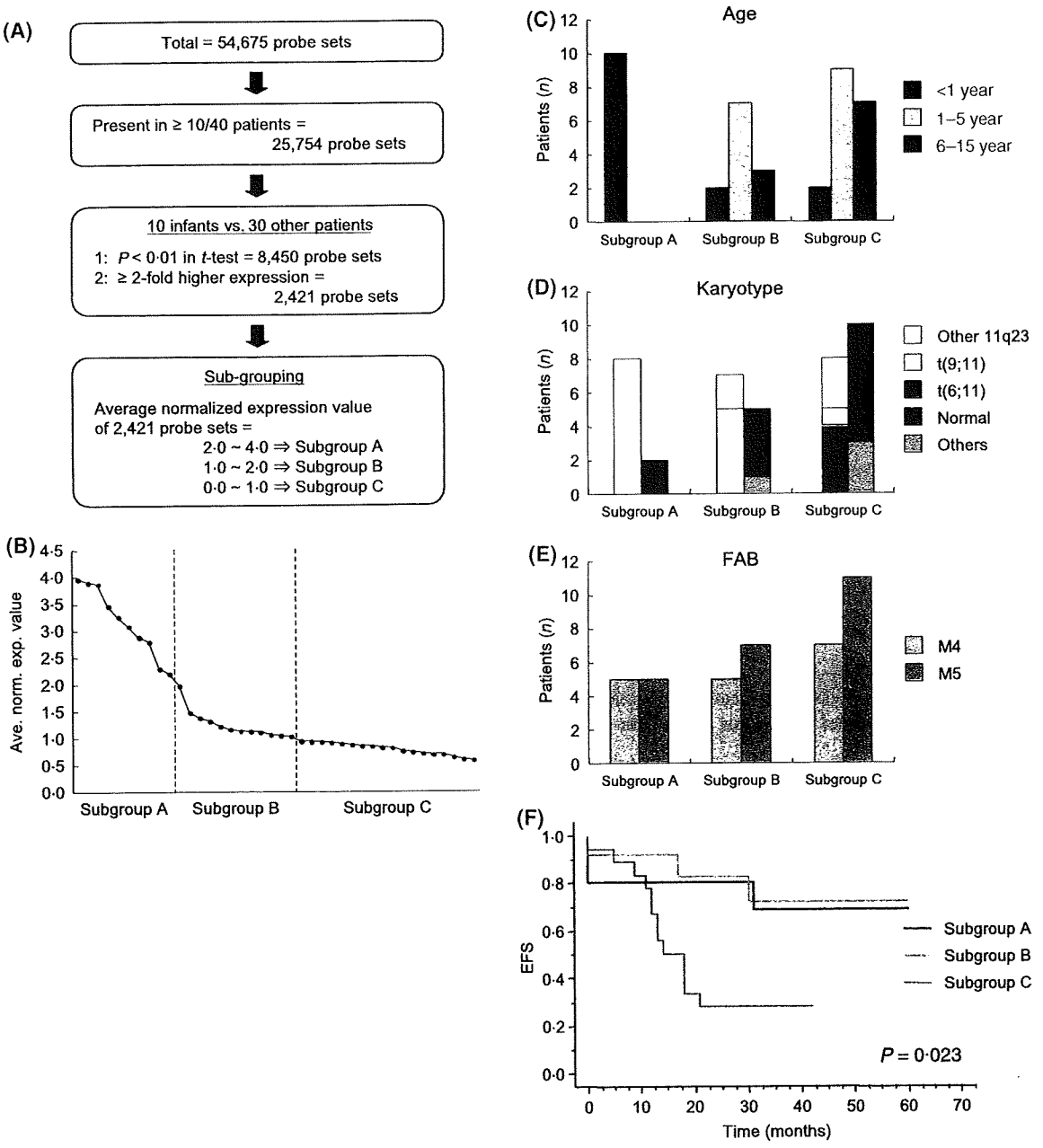

Fig 2. Sub-grouping of paediatric AML-M4/M5. (A) Sub-grouping scheme. First, subgroup-discriminating probe sets were selected as follows: (i) probe sets without Present calls in at least 10 of 40 paediatric patients were removed (25754 probe sets); (ii) probe sets with *P*-value of <0·01 in Student's *t*-test comparing 10 distinct infants and the remaining 30 patients were selected (8450 probe sets) and (iii) probe sets with ≥2-fold higher expression in the 10 distinct infants were chosen (2421 probe sets). Then, the average of normalized expression values of the 2421 subgroup-discriminating probe sets was calculated for each patient and used for sub-grouping. 10 patients with the average values between 2·0 and 4·0 were assigned as subgroup A, 12 patients with the average values between 1·0 and 2·0 as subgroup B, 18 patients with the average values lower than 1·0 were assigned as subgroup C. (B) Distribution of the average normalized expression values of 40 patients. (C) Age distribution of each subgroup. The average age was 0·3, 3·1 and 6·6 years old for subgroup A, B and C, respectively. (D, E) Karyotype and FAB subtype distribution of each subgroup. (F) Kaplan–Meier analysis of event-free survival (EFS). *P*-value was obtained by log-rank test.

the selected genes (2995 probe sets, 99%) showed medium expression level in subgroup B whether their expression were higher in subgroup A or in subgroup C, confirming that subgroups A, B and C have a linear correlation in gene expression. Among the genes overexpressed in subgroup A, the top-ranked gene (25·3-fold) was a DNA-binding zinc finger homeobox protein gene *ZEB2* (Table II). The expression of this gene was higher in adult patients than in subgroup C patients (Fig 3A). Interestingly, *MLL* translocation partner genes such as *ARHGAP26* and *FOXO3* were also overexpressed

Table II. Genes expressed differentially between subgroups A and C.

Fold rank	Probe set ID	Gene symbol	Fold change
Highly expressed in subgroup A			
1	239296_at	*ZEB2*	25·3
2	242008_at	*AGTPBP1*	21·3
3	240652_at	Unknown	21·0
4	1556590_s_at	Unknown	18·1
5	1562194_at	*ZEB2*	17·3
6	244548_at	*ARHGAP26*	17·0
7	1557797_a_at	*ZEB2*	16·6
8	239555_at	*LYN*	16·5
9	1569477_at	*FOXO3*	16·4
10	232307_at	*MED13L*	16·4
Highly expressed in subgroup C			
1	206310_at	*SPINK2*	23·8
2	203373_at	*SOCS2*	13·5
3	201427_s_at	*SEPP1*	12·8
4	205051_s_at	*KIT*	9·6
5	209160_at	*AKR1C3*	8·9
6	206067_s_at	*WT1*	7·1
7	206772_at	*PTH2R*	6·8
8	210140_at	*CST7*	6·7
9	200923_at	*LGALS3BP*	6·5
10	236738_at	*LOC401097*	6·2

Probe sets were selected with Student's *t*-test and fold changes. Top 10 probe sets were listed in the order of their fold change values.

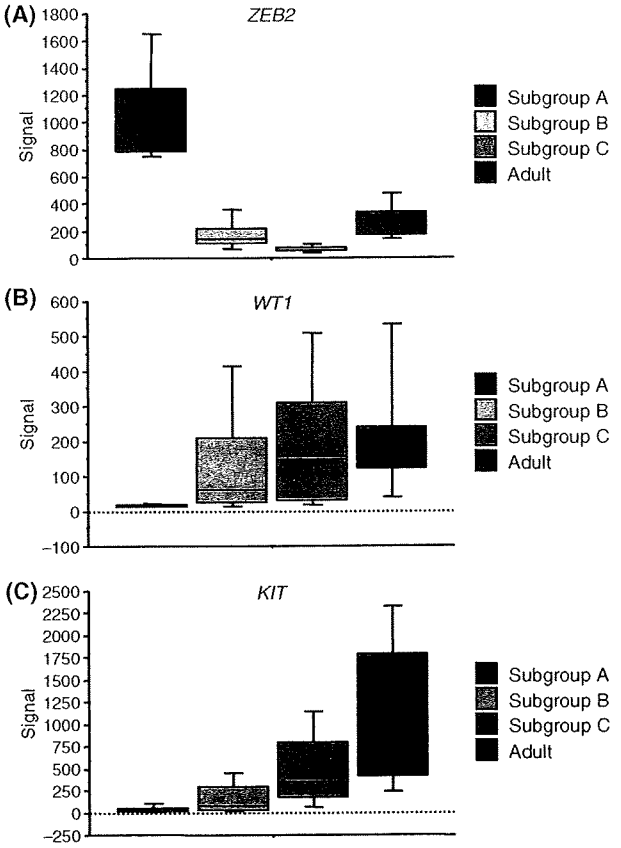

Fig 3. Differentially expressed genes between subgroup A and subgroup C. Box and whiskers plots are shown for *ZEB2* (A), *WT1* (B) and *KIT* (C). Signal values were compared among subgroup A, B and C paediatric patients and adult patients.

in subgroup A regardless of the karyotype. In subgroup C, poor prognostic factors such as *WT1* and *KIT* were overexpressed, coinciding with its poor clinical outcome (Table II and Fig 3B and C). *WT1* and *KIT* exhibited linear increment with the adult patients expressing highest of all.

Gene expression signatures of MLL gene rearrangements

More than a half of the patients enrolled in this study possessed *MLL* gene rearrangement (Table I), but with unsupervised hierarchical clustering analysis, no prominent cluster was observed for *MLL*gr(+) patients (Fig 1B). To evaluate the differences between *MLL*gr(+) AML-M4/M5 and *MLL*gr(−) AML-M4/M5, we used SAM and selected differentially expressed probe sets, at 5% false discovery rate (FDR), between *MLL*gr(+) and *MLL*gr(−) patients among all patients and also within each subgroup (Table III and SVI). When all the patients were compared, 112 probe sets were selected. Comparison within subgroup A did not exhibit any significant difference between *MLL*gr(+) and *MLL*gr(−) patients and this was probably due to the scarcity of *MLL*gr(−) patients. Only 11 and 55 probe sets were selected at 5% FDR when compared within subgroup B and subgroup C, respectively (Table III). Surprisingly, commonly selected probe sets were very small (Fig 4A), indicating that the gene expression signatures of *MLL* gene rearrangement differed considerably among the different subgroups identified. Expression patterns of selected genes are shown in Fig 4B. Among the 112 probe sets selected in comparison of all patients, only 17 probe sets (15%) were upregulated by *MLL* gene rearrangement. On the other hand, among the 55 probe sets selected within subgroup C, 53 probe sets (96%) were upregulated by *MLL* gene rearrangement, and some of these probe sets were also upregulated in some *MLL*gr(+) patients of subgroup B but not in subgroup A patients (Fig 4B), indicating a clear difference of *MLL* gene rearrangement signatures among the subgroups.

Table III. The numbers of probe sets showing differential expression between *MLL*gr(+) and *MLL*gr(−).

	Patient no. *MLL*gr(+) vs. *MLL*gr(−)	5% FDR
All patients	(23 vs. 17)	112
Subgroup A	(8 vs. 2)	0
Subgroup B	(7 vs. 5)	11
Subgroup C	(8 vs. 10)	55

Probe sets were selected using SAM with 5% false discovery rate (FDR).

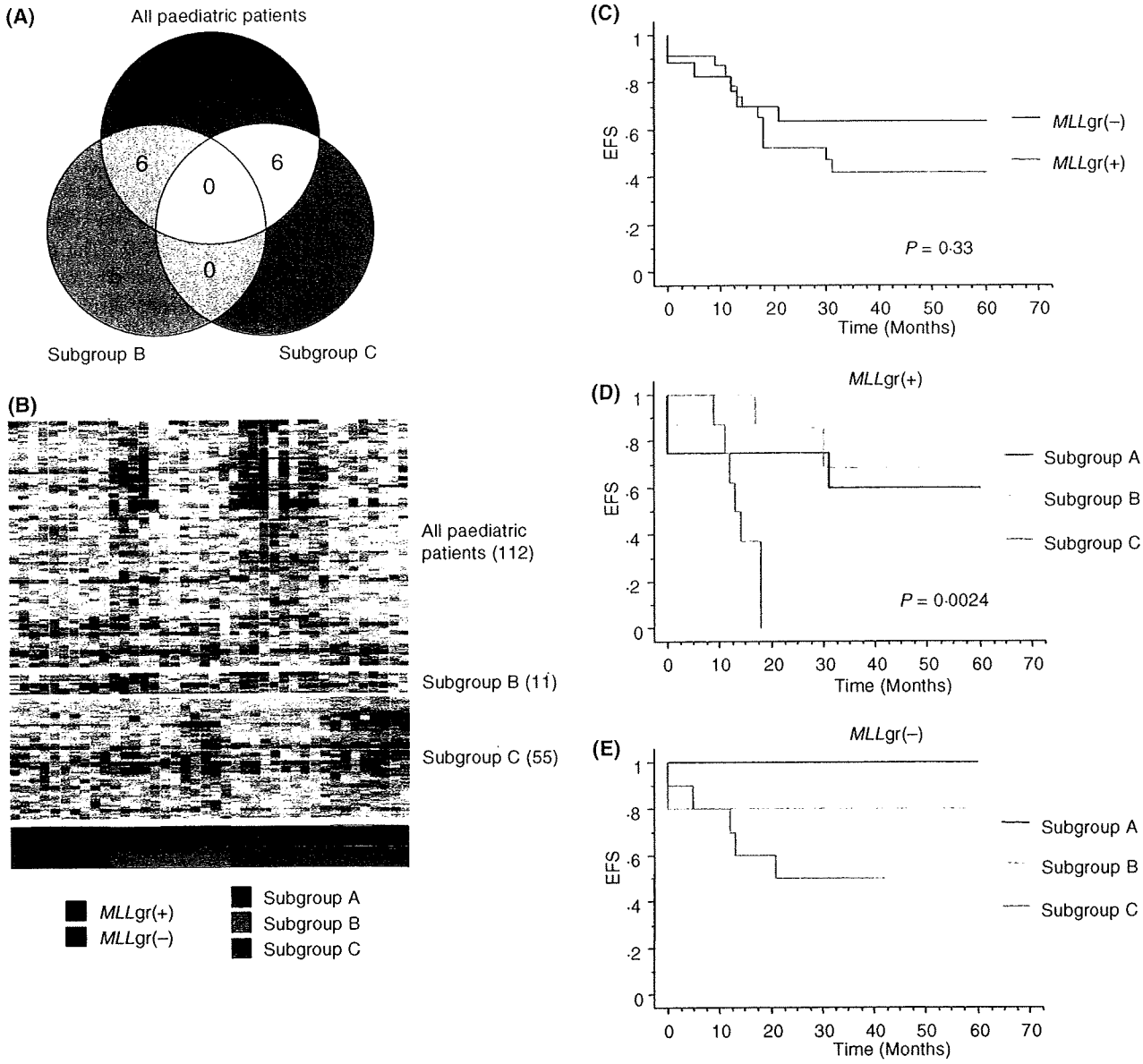

Fig 4. The significance of *MLL* gene rearrangements in paediatric AML-M4/M5. (A) Venn diagram comparing the differentially expressed genes between *MLL*gr(+) and *MLL*gr(−) patients. The probe sets selected by SAM with 5% FDR were compared among subgroup B, subgroup C and all paediatric patients. (B) Expression pattern of the SAM-selected probe sets. Columns and rows in the matrix represent patients and probe sets, respectively. The normalized expression level for each probe set is represented by colour, with red representing deviation above the median and green representing deviation below the median. The *MLL* gene rearrangement [*MLL*gr(+) or *MLL*gr(−)] and paediatric AML-M4/M5 subgroup (A, B or C) of each patient are indicated by colour codes below the matrix. (C–E) Kaplan–Meier analysis of event-free survival (EFS). *MLL*gr(+) and *MLL*gr(−) patients within paediatric patients (C), subgroups A, B and C within *MLL*gr(+) patients (D) and subgroups A, B and C within *MLL*gr(−) patients (E) were compared. *P*-value was obtained by log-rank test.

Genes specifically overexpressed throughout all of the *MLL*gr(+) patients included a zinc finger protein gene *ZNF521* (Table IV and Fig 5A), which has been reported previously (Kohlmann *et al*, 2005). However, frequently reported genes such as *HOXA* family members and *MEIS1* (Armstrong *et al*, 2002; Yeoh *et al*, 2002; Tsutsumi *et al*, 2003; Bullinger *et al*, 2004; Ross *et al*, 2004; Valk *et al*, 2004; Andersson *et al*, 2005) were not selected with SAM and *HOXA9* had no correlation with *MLL* gene rearrangement (Fig 5D). Rather, the *HOXB2* and *HOXB3* genes were selected as underexpressed genes among *MLL*gr(+) patients (Table IV and Fig 5B). A transcription factor gene *EVI1*, whose overexpression is known to associate with poor prognosis, was upregulated in subgroup C *MLL*gr(+) patients (Table IV and Fig 5C) (Doorn-Khosrovani *et al*, 2003; Valk *et al*, 2004). These data were also confirmed by quantitative RT-PCR (Fig 6).

Table IV. Genes expressed differentially between *MLL*gr(+) and *MLL*gr(−) patients.

Fold rank	Probe set ID	Gene symbol	Fold change
Highly expressed in *MLL*gr(+) patients among all patients			
1	226676_at	*ZNF521*	19·0
2	226677_at	*ZNF521*	15·8
3	1552665_at	*MGC14425*	7·9
4	227929_at	Unknown	4·9
5	217520_x_at	*LOC646278*	4·9
6	206440_at	*LIN7A*	3·1
7	205472_s_at	*DACH1*	3·0
8	1557450_s_at	*WHDC1L1*	2·8
9	225785_at	*REEP3*	2·7
10	205471_s_at	*DACH1*	2·5
Highly expressed in *MLL*gr(−) patients among all patients			
1	228904_at	*HOXB3*	13·1
2	205453_at	*HOXB2*	10·8
3	205033_s_at	*DEFA1*	10·2
4	236892_s_at	Unknown	8·5
5	223828_s_at	*LGALS12*	5·1
6	202833_s_at	*SERPINA1*	5·0
7	200923_at	*LGALS3BP*	4·8
8	211429_s_at	*SERPINA1*	4·5
9	206761_at	*CD96*	4·4
10	239791_at	*LOC404266*	4·3
Highly expressed in *MLL*gr(+) patients among subgroup C patients			
1	235700_at	*CT45-4*	28·2
2	1567912_s_at	*CT45-4*	25·4
3	226676_at	*ZNF521*	22·3
4	236858_s_at	Unknown	17·9
5	226677_at	*ZNF521*	16·9
6	226420_at	*EVI1*	12·1
7	1552665_at	*MGC14425*	11·2
8	1559266_s_at	*LOC730417*	10·5
9	236859_at	*RUNX2*	10·0
10	1557261_at	*WHDC1L1*	10·0
Highly expressed in *MLL*gr(−) patients among subgroup C patients			
1	209298_s_at	*ITSN1*	2·7
2	209357_at	*CITED2*	2·6

Probe sets were selected with SAM at 90th percentile with 5% false discovery rate (FDR). Top 10 or all selected probe sets were listed in the order of their fold change values.

When compared between all patients, EFS did not differ statistically ($P = 0·33$) between *MLL*gr(+) and *MLL*gr(−) patients whilst *MLL*gr(+) patients seemed to have a poorer prognosis (Fig 4C). The EFS of each subgroup was compared within each *MLL* gene arrangement status and subgroup C presented the poorest outcome for both *MLL*gr(+) and *MLL*gr(−) karyotypes (Fig 4D and E). It was astounding to see that all subgroup C *MLL*gr(+) patients (eight patients) had relapsed within 18 months (3-year EFS 0%). Subgroup C represented an extremely poor prognostic subgroup with early relapse especially with *MLL* gene rearrangement.

Discussion

Microarray-based gene expression profiling has been applied to the diagnosis of many different types of leukaemia and used to explore their underlying molecular pathology (Golub *et al*, 1999; Armstrong *et al*, 2002; Schoch *et al*, 2002; Yeoh *et al*, 2002; Yagi *et al*, 2003; Ross *et al*, 2004). However, no previous study has been reported that focussed solely on the gene expression profile of AML-M4/M5 or on the difference between infants and older children with AML. The present study analysed the gene expression of 40 paediatric AML-M4/M5 patients and found that typical infants displayed very unique and distinct expression patterns that are clearly different from the older patients. These distinctly expressed genes were used to separate 40 patients into three subgroups without any previous knowledge of the patients' clinical information, and showed that these subgroups differed in age distribution and clinical outcome. In addition, the gene expression signature of *MLL* gene rearrangement and outcomes of *MLL*gr(+) patients were different among the different subgroups. These results indicate that paediatric AML-M4/M5 is heterogeneous in an age-associated manner and this heterogeneity is correlated with their prognosis.

The three subgroups that were separated by gene expression were different in age distribution and in clinical outcome, but not in FAB subtype (Fig 2). The difference between subgroup A and subgroup C was extremely prominent. A large set of differentially expressed genes was isolated and these genes were medianly expressed in subgroup B. Among the genes overexpressed in subgroup A, many transcription factor genes involved in haematopoietic cell differentiation and proliferation were included such as *FOXO3*, *MLL3* and *ELF2* (Table II and S5). These observations suggest that underlying biology is transitionally different among the three subgroups. The top-ranked gene overexpressed in subgroup A is a DNA-binding zing finger homeobox protein gene *ZEB2*. *ZEB2* mutations are responsible for Mowat–Wilson syndrome and Hirschsprung disease (Wakamatsu *et al*, 2001; Mowat *et al*, 2003). It functions in the transforming growth factor β signaling pathway and may promote tumor invasion in hepatic, renal and breast cancer (Miyoshi *et al*, 2004; Elloul *et al*, 2005; Krishnamachary *et al*, 2006), but the association between *ZEB2* and leukaemia is still unknown. Genes that were overexpressed in subgroup C included *WT1* and *KIT*; overexpression of these genes is known to be associated with poor prognosis (Ashman *et al*, 1991; Inoue *et al*, 1994; Weisser *et al*, 2005; Paschka *et al*, 2006; Shimada *et al*, 2006) and this overexpression may be responsible for the dismal outcome of subgroup C patients.

When adult AML-M4/M5 patients were included in the analysis, it was very interesting to find that adult patients not only shared common expression pattern with older children (subgroup C) but many of the genes upregulated in infants (subgroup A) were similarly highly expressed among the adult patients (Fig S1). It is well known that certain types of leukaemia are common only in the paediatric population and

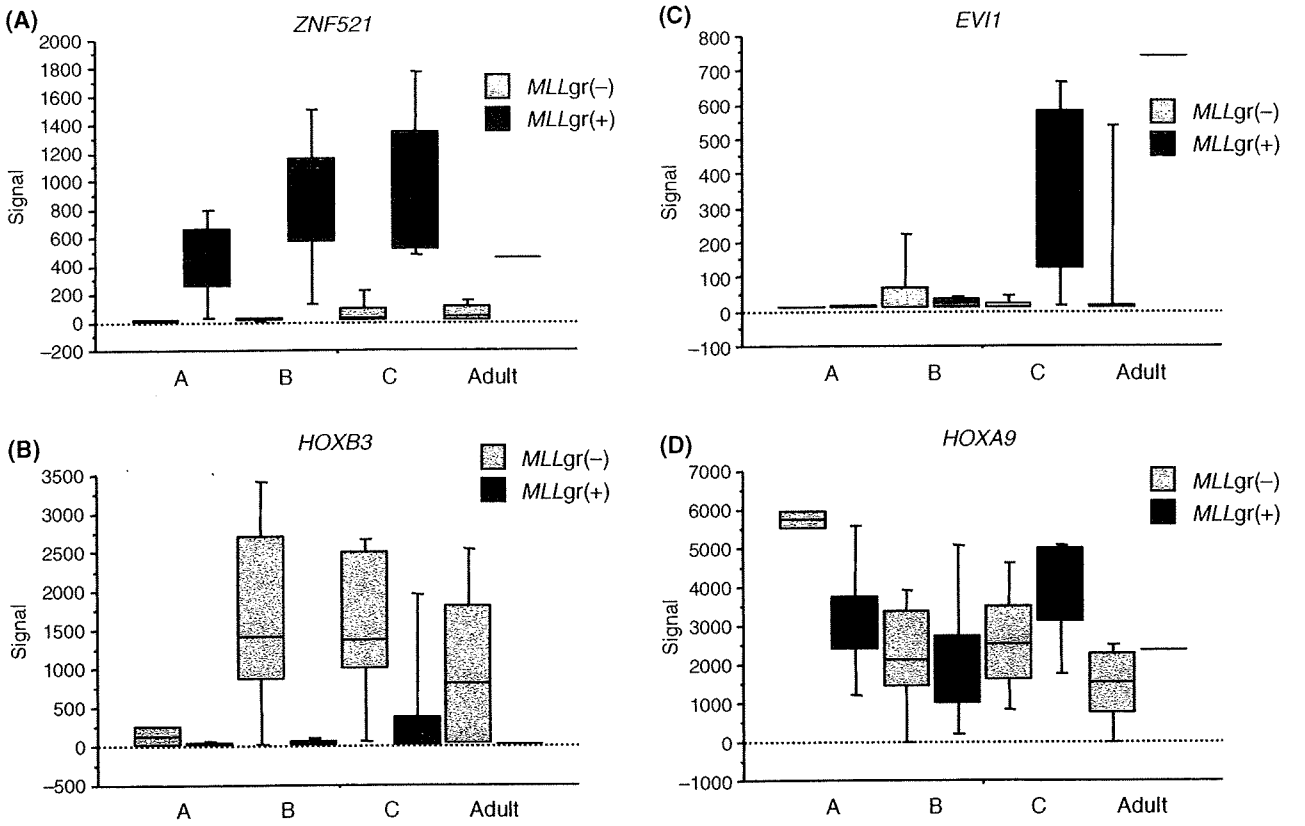

Fig 5. Differentially expressed genes between *MLL*gr(+) and *MLL*gr(−) patients. Box and whiskers plot are shown for *ZNF521* (A), *HOXB3* (B), *EVI1* (C) and *HOXA9* (D). Signal values were compared between *MLL*gr(+) and *MLL*gr(−) patients within subgroup A, B and C paediatric patients and adult patients.

vice versa. For example, *MLL* gene rearrangements are much more frequent among younger populations while *NPM1* mutations and *FLT3*-internal tandem duplication (ITD) are typically prevalent in the adult population (Iwai *et al*, 1999; Meshinchi *et al*, 2001; Cazzaniga *et al*, 2005; Falini *et al*, 2005; Brown *et al*, 2007). With these differences in genetic alterations, we had expected that gene expression would be transitional in an age-increasing manner. In previous studies, the gene expression signatures of genetic alterations were compared between adult and paediatric leukaemia. Kohlmann *et al* (2004) have reported that gene expression signatures identified in paediatric ALL were applicable to adult ALL patients. Ross *et al* (2004) have also shown that the expression signatures generated from paediatric AML were able to accurately classify adult AML with the same genetic lesions. The similarities or differences between infant AML and adult AML has never been thoroughly discussed before, and thus we have identified for the first time that infant AML-M4/M5 and adult AML-M4/M5 share expression patterns. Among the commonly shared genes between infant AML-M4/M5 (subgroup A) and adult AML-M4/M5, transcription factor genes involved in haematopoietic cell differentiation and proliferation such as *ETV6* and *RUNX1* were included, and this may suggest that these two groups might share common underlying biology.

Many studies have already reported that AML with *MLL* gene rearrangement present distinct gene expression profiles (Armstrong *et al*, 2002; Yeoh *et al*, 2002; Tsutsumi *et al*, 2003; Bullinger *et al*, 2004; Ross *et al*, 2004; Valk *et al*, 2004; Andersson *et al*, 2005; Kohlmann *et al*, 2005), but with our unsupervised analysis, no prominent cluster of *MLL*gr(+) patients was observed. This inconsistency may be attributable to the fact that we focused solely on AML-M4/M5, while previous studies investigated the whole AML population to identify the signature of *MLL* gene rearrangements. We speculated that *MLL*gr(+) AML-M4/M5 and *MLL*gr(−) AML-M4/M5 might share similar gene expression especially in paediatric patients. When we examined the differences between *MLL*gr(+) and *MLL*gr(−) patients in AML-M4/M5, relatively small differences (112 probe sets at 5% FDR) were found and almost all of these genes were under-expressed in *MLL*gr(+) patients. In addition, *HOXA* family genes and *MEIS1*, which have been repeatedly identified as overexpressed genes in *MLL*gr(+) leukaemia (Armstrong *et al*, 2002; Yeoh *et al*, 2002; Tsutsumi *et al*, 2003; Bullinger *et al*, 2004; Ross *et al*, 2004; Valk *et al*, 2004; Andersson *et al*, 2005; Kohlmann *et al*, 2005), were not included in the selected gene lists. Rather, under-expression of *HOXB2* and *HOXB3* in *MLL*gr(−) patients was observed (Table IV). Among the

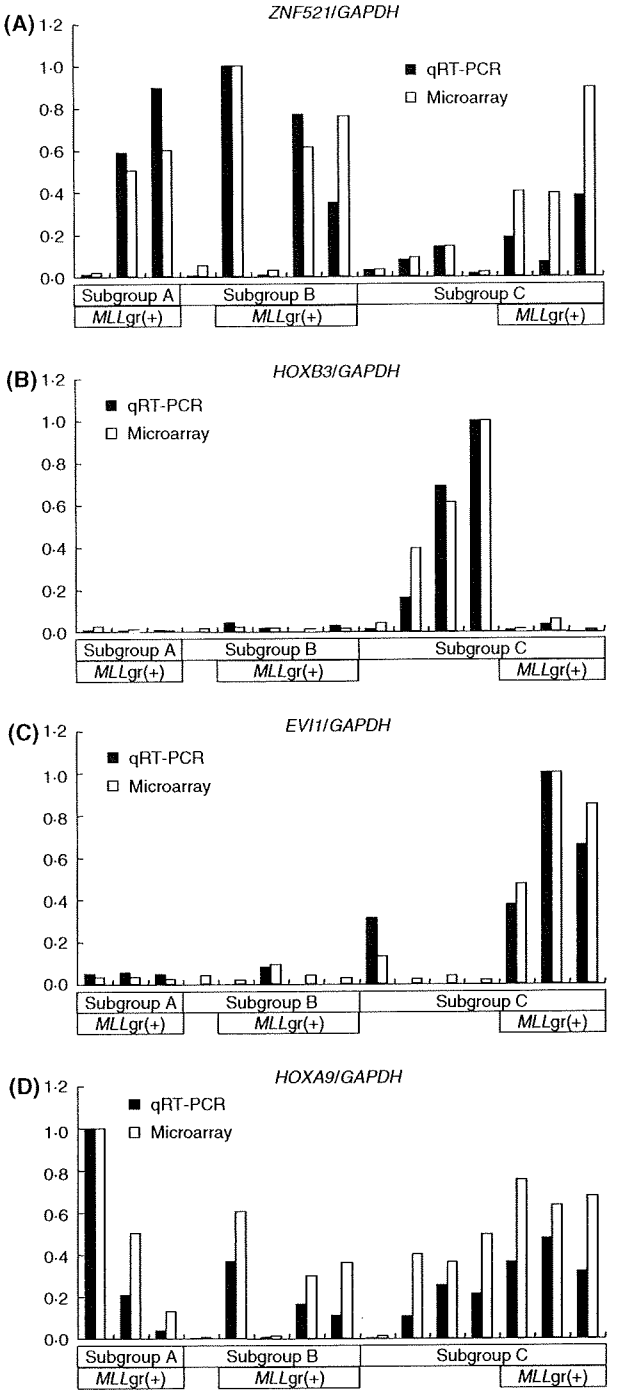

Fig 6. Quantitative RT-PCR confirmation of differential expression between *MLL*gr(+) and *MLL*gr(−) patients. Quantitative RT-PCR was performed for *ZNF521* (A), *HOXB3* (B), *EVI1* (C) and *HOXA9* (D) on RNA samples from 15 patients. Included were 3 subgroup A [3 *MLL*gr(+)], 5 subgroup B [4 *MLL*gr(+) and 1 *MLL*gr(−)] and 7 subgroup C [3 *MLL*gr(+) and 4 *MLL*gr(−)] patients. Transcript levels of those 4 genes were normalized to that of *GAPDH*, and presented as ratios to the highest patient. Their expression levels determined by microarray analysis were also shown as the same ratios after normalization to the *GAPDH* expression level.

previously reported *MLL* gene rearrangement-associated genes, *ZNF521* was specifically overexpressed among all of the *MLL*gr(+) patients in our study. *ZNF521* is an early haematopoietic zinc finger protein gene that is highly expressed in primitive human haematopoietic cells (Bond *et al*, 2004), and *PAX5-ZNF521* gene fusion has been recently reported among B-progenitor ALL (Mullighan *et al*, 2007). This gene, which is likely to be involved in normal haematopoiesis and ALL development, may also be active in the leukaemogenesis of *MLL*gr(+) AML-M4/M5. On the other hand, we also found that some *MLL* gene rearrangement-associated genes were subgroup-specific. When compared within subgroup C, 55 probe sets were selected as differentiating genes for *MLL*gr(+) and *MLL*gr(−) patients at 5% FDR, but the expression patterns of these probe sets were not shared by the other subgroups, especially subgroup A. This is noteworthy because EFS of *MLL*gr(+) patients were very different among different subgroups and subgroup C *MLL*gr(+) patients had the poorest outcome (Fig 4D). These findings suggest that the target genes of *MLL* fusion proteins might be different among each subgroup.

Our gene expression profiling analysis revealed that paediatric AML-M4/M5 consists of different subgroups with distinctively different outcomes. Subgroup C, which comprised older children, presented 3-year EFS of 28%, which was extremely lower than the previously reported EFS of intermediate risk patients (60%) (Tsukimoto *et al*, 2005). Our sub-grouping procedure enabled us to extract these poor prognostic patients from the other intermediate risk patients. It is known that some mutations, such as *FLT3*-ITD, *KIT* mutation and *NPM1* mutation, influence the prognosis of AML and their incidences increase in an age-dependent manner. In our analysis, however, these mutations were rare not only in subgroup B but also in subgroup C (Table SI), indicating that these factors are not the main reason for the dismal outcome of subgroup C. Subgroup C contained both *MLL*gr(+) and *MLL*gr(−) patients but subgroup C *MLL*gr(+) patients especially had a disappointing outcome, as all eight patients had relapsed within 18 months. Subgroup C included different types of *MLL* gene rearrangement, but t(6;11) was present only in subgroup C. Previous reports have also suggested that the prognosis of t(6;11) is dismal (Welbron *et al*, 1993; Martineau *et al*, 1998). In our study, the clinical outcome of t(6;11) was also very disappointing as all four patients had relapsed within 18 months (Fig S2A). In addition, we were able to isolate the genes discriminating t(6;11) and the other *MLL* gene rearrangements with SAM (Table SVII and SVIII). These results provide another possibility that patients with t(6;11) exhibit different expression profiles and dismal outcome. However, some of the upregulated genes in t(6;11) patients were also upregulated in *MLL*gr(−) patients of subgroup C (Fig S2B). So some of the selected genes for t(6;11) represent not only t(6;11) patients but rather represent subgroup C patients. Moreover, the outcome of other subgroup C *MLL*gr(+) patients is also dismal, although they

did not show high expression of those genes. These data suggest that t(6;11) karyotype is not an independent prognostic factor but rather a surrogate marker for subgroup C *MLL*gr(+) patients.

In summary, paediatric AML-M4/M5 was identified as a group of heterogeneous diseases comprised different outcomes. Adverse prognostic subgroup could be distinguished with the use of gene expression profiling. Our results have shown that age is an important factor contributing to the biology of AML-M4/M5. These findings also suggest that gene expression profiling may be very useful for risk estimation and our sub-grouping procedures could be a powerful risk stratification tool to identify unfavourable risk patients within the heterogeneous paediatric AML-M4/M5. Further studies are needed to explain the biology underlying different gene expression between infants and older children and to verify the usefulness of our sub-grouping procedures for risk-stratified therapeutics.

Acknowledgements

The authors thank the members of Japan Infant Leukemia Study Group (MLL96/98) and Japanese Childhood AML Cooperative Study Group (AML99). This work was supported by a Grant-in-Aid for Third Term Comprehensive Control Research for Cancer from the Ministry of Health, Labour and Welfare and by the programme for promotion of Fundamental Studies in Health Sciences of National Institute of Biomedical Innovation (NiBio).

References

Andersson, A., Olofsson, T., Lindgren, D., Nilsson, B., Ritz, C., Eden, P., Lassen, C., Rade, J., Fontes, M., Morse, H., Heldrup, J., Behrendtz, M., Mitelman, F., Hoglund, M., Johansson, B. & Fioretos, T. (2005) Molecular signatures in childhood acute leukemia and their correlations to expression patterns in normal hematopoietic subpopulations. *Proceedings of the National Academy of Sciences of the United States of America*, **102,** 19069–19074.

Armstrong, S.A., Staunton, J.E., Silverman, L.B., Pieters, R., den Boer, M.L., Minden, M.D., Sallan, S.E., Lander, E.S., Golub, T.R. & Korsmeyer, S.J. (2002) MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. *Nature Genetics*, **30,** 41–47.

Ashman, L.K., Cambareri, A.C., To, L.B., Levinsky, R.J. & Juttner, C.A. (1991) Expression of the YB5.B8 antigen (c-kit proto-oncogene product) in normal human bone marrow. *Blood*, **78,** 30–37.

Bond, H.M., Mesuraca, M., Carbone, E., Bonelli, P., Agosti, V., Amodio, N., De Rosa, G., Di Nicola, M., Gianni, A.M., Moore, M.S., Hata, A., Grieco, M., Morrone, G. & Venuta, S. (2004) Early hematopoietic zinc finger protein (EHZF), the human homolog to mouse Evi3, is highly expressed in primitive human hematopoietic cells. *Blood*, **103,** 2062–2070.

Brown, P., McIntyre, E., Rau, R., Meshinchi, S., Lacayo, N., Dahl, G., Alonzo, T.A., Chang, M., Arececi, R.J. & Small, D. (2007) The incidence and clinical significance of nucleophosmin mutations in childhood AML. *Blood*, **110,** 979–985.

Bullinger, L., Dohner, K., Bair, E., Frohling, S., Schlenk, R.F., Tibshirani, R., Dohner, H. & Pollack, J.R. (2004) Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *The New England Journal of Medicine*, **350,** 1605–1616.

Cazzaniga, G., Dell'Oro, M.G., Mecucci, C., Giarin, E., Masetti, R., Rossi, V., Locatelli, F., Martelli, M.F., Basso, G., Pession, A., Biondi, A. & Falini, B. (2005) Nucleophosmin mutations in childhood acute myelogenous leukemia with normal karyotype. *Blood*, **106,** 1419–1422.

Doorn-Khosrovani, S.B., Erpelinck, C., van Putten, W.L., Valk, P.J., Luytgaarde, S., Hack, R., Slater, R., Smit, E.M., Beverloo, B., Verhoef, G., Verdonck, L.F., Ossenkoppele, G.J., Sonneveld, P., de Greef, G.E., Lowenberg, B. & Delwel, R. (2003) High EVI1 expression predicts poor survival in acute myeloid leukemia: a study of 319 de novo AML patients. *Blood*, **101,** 837–845.

Downing, J.R. & Shannon, K.M. (2002) Acute leukemia: a pediatric perspective. *Cancer Cell*, **2,** 437–445.

Elloul, S., Elstrand, M.K., Nesland, J.M., Trope, C.G., Kvalheim, G., Goldberg, I., Reich, R. & Davidson, B. (2005) Snail, Slug, and Smad-interacting protein 1 as novel parameters of disease aggressiveness in metastatic ovarian and breast carcinoma. *Cancer*, **103,** 1631–1643.

Falini, B., Mecucci, C., Tiacci, E., Alcalay, M., Rosati, R., Pasqualucci, L., La Starza, R., Diverio, D., Colombo, E., Santucci, A., Bigerna, B., Pacini, R., Pucciarini, A., Liso, A., Vignetti, M., Fazi, P., Meani, N., Pettirossi, V., Saglio, G., Mandelli, F., Lo-Coco, F., Pelicci, P.G. & Martelli, M.F. (2005) Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *The New England Journal of Medicine*, **352,** 254–266.

Golub, T.R., Slonim, D.K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J.P., Coller, H., Loh, M.L., Downing, J.R., Caligiuri, M.A., Bloomfield, C.D. & Lander, E.S. (1999) Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science*, **286,** 531–537.

Horibe, K., Tsukimoto, I. & Ohno, R. (2001) Clinicopathologic characteristics of leukemia in Japanese children and young adults. *Leukemia*, **15,** 1256–1261.

Inoue, K., Sugiyama, H., Ogawa, H., Nakagawa, M., Yamagami, T., Miwa, H., Kita, K., Hiraoka, A., Masaoka, T., Nasu, K., Kyo, T., Dohy, H., Nakauchi, H., Ishidate, T., Akiyama, T. & Kishimoto, T. (1994) WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. *Blood*, **84,** 3071–3079.

Iwai, T., Yokota, S., Nakao, M., Okamoto, T., Taniwaki, M., Onodera, N., Watanabe, A., Kikuta, A., Tanaka, A., Asami, K., Sekine, I., Mugishima, H., Nishimura, Y., Koizumi, S., Horikoshi, Y., Mimaya, J., Ohta, S., Nishikawa, K., Iwai, A., Shimokawa, T., Nakayama, M., Kawakami, K., Gushiken, T., Hyakuna, N., Katano, N., Tsurusawa, M. & Fujimoto, T. (1999) Internal tandem duplication of the FLT3 gene and clinical evaluation in childhood acute myeloid leukemia. *Leukemia*, **13,** 38–43.

Kawasaki, H., Isoyama, K., Eguchi, M., Hibi, S., Kinukawa, N., Kosaka, Y., Oda, T., Oda, M., Nishimura, S., Imaizumi, M., Okamura, T., Hongo, T., Okawa, H., Mizutani, S., Hayashi, Y., Tsukimoto, I., Kamada, N. & Ishii, E. (2001) Superior outcome of infant acute myeloid leukemia with intensive chemotherapy: results of the Japan Infant Leukemia Study Group. *Blood*, **98,** 3589–3594.

Kohlmann, A., Schoch, C., Schnittger, S., Dugas, M., Hiddemann, W., Kern, W. & Haferlach, T. (2004) Pediatric acute lymphoblastic

leukemia (ALL) gene expression signatures classify an independent cohort of adult ALL patients. *Leukemia*, **18**, 63–71.

Kohlmann, A., Schoch, C., Dugas, M., Schnittger, S., Hiddemann, W., Kern, W. & Haferlach, T. (2005) New insights into MLL gene rearranged acute leukemias using gene expression profiling: shared pathways, lineage commitment, and partner genes. *Leukemia*, **19**, 953–964.

Krishnamachary, B., Zagzag, D., Nagasawa, H., Rainey, K., Okuyama, H., Baek, J.H. & Semenza, G.L. (2006) Hypoxia-inducible factor-1-dependent repression of E-cadherin in von Hippel-Lindau tumor suppressor-null renal cell carcinoma mediated by TCF3, ZFHX1A, and ZFHX1B. *Cancer Research*, **66**, 2725–2731.

Martineau, M., Berger, R., Lillington, D.M., Moorman, A.V. & Secker-Walker, L.M. (1998) The t(6;11)(q27;q23) translocation in acute leukemia: a laboratory and clinical study of 30 cases. *Leukemia*, **12**, 788–791.

Meshinchi, S., Woods, W.G., Stirewalt, W.D., Sweetser, D.A., Buckley, J.D., Tjoa, T.K., Bernstein, I.D. & Radich, J.P. (2001) Prevalence and prognostic significance of FLT3 internal tandem duplication in pediatric acute myeloid leukemia. *Blood*, **97**, 89–94.

Miyoshi, A., Kitajima, Y., Sumi, K., Sato, K., Hagiwara, A., Koga, Y. & Miyazaki, K. (2004) Snail and SIP1 increase cancer invasion by upregulating MMP family in hepatocellular carcinoma cells. *British Journal of Cancer*, **90**, 1265–1273.

Mowat, D.R., Wilson, M.J. & Goossens, M. (2003) Mowat-Wilson syndrome. *Journal of Medical Genetics*, **40**, 305–310.

Mullighan, C.G., Goorha, S., Radtke, I., Miller, C.B., Coustan-Smith, E., Dalton, J.D., Girtman, K., Mathew, S., Ma, J., Pounds, S.B., Su, X., Pui, C.H., Relling, M.V., Evans, W.E., Shurtleff, S.A. & Downing, J.R. (2007) Genome-wide analysis of genetic alterations in acute lymphoblastic leukemia. *Nature*, **446**, 758–764.

Nagao, T. (1995) Footprints of the Children's Leukemia Study Group of Japan. *The Japanese Journal of Pediatric Hematology*, **9**, 332–340.

Pallisgaard, N., Hokland, P., Riishoj, D.C., Pedersen, B. & Jorgensen, P. (1998) Multiplex reverse transcription-polymerase chain reaction for simultaneous screening of 29 translocations and chromosomal aberrations in acute leukemia. *Blood*, **92**, 574–588.

Paschka, P., Marucci, G., Ruppert, A.S., Mrozek, K., Chen, H., Kittles, R.A., Vukosavljevic, T., Perrotti, D., Vardiman, J.W., Carroll, A.J., Kolitz, J.E., Larson, R.A. & Bloomfield, C.D. (2006) Adverse prognostic significance of KIT mutations in adult acute myeloid leukemia with inv(16) and t(8;21): a Cancer and Leukemia Group B Study. *Journal of Clinical Oncology*, **24**, 3904–3911.

Pui, C.H., Kane, J.R. & Crist, W.M. (1995) Biology and treatment of infant leukemias. *Leukemia*, **9**, 762–769.

Pui, C.H., Raimondi, S.C., Srivastava, D.K., Tong, X., Behm, F.G., Razzouk, B., Rubnitz, J.E., Sandlund, J.T., Evans, W.E. & Ribeiro, R. (2000) Prognostic factors in infants with acute myeloid leukemia. *Leukemia*, **14**, 684–687.

Pui, C.H., Gaynon, P.S., Boyett, J.M., Chessells, J.M., Baruchel, A., Kamps, W., Silverman, L.B., Biondi, A., Harms, D.O., Vilmer, E., Schrappe, M. & Camitta, B. (2002) Outcome of treatment in childhood acute lymphoblastic leukaemia with rearrangements of the 11q23 chromosomal region. *Lancet*, **359**, 1909–1915.

Ross, M.E., Mahfouz, R., Onciu, M., Liu, H., Zhou, X., Song, G., Shurtleff, S.A., Pounds, S., Cheng, C., Ma, J., Ribeiro, R.C., Rubnitz, J.E., Girtman, K., Williams, W.K., Raimondi, S.C., Liang, D.C., Shih, L.Y., Pui, C.H. & Downing, J.R. (2004) Gene expression profiling of pediatric acute myelogeneous leukemia. *Blood*, **104**, 3679–3687.

Salto-Tellez, M., Shelat, S.G., Benoit, B., Rennert, H., Carroll, M., Leonard, D.G., Nowell, P. & Bagg, A. (2003) Multiplex RT-PCR for the detection of leukemia-associated translocations. *Journal of Molecular Diagnostics*, **5**, 231–236.

Satake, N., Maseki, N., Nishiyama, M., Kobayashi, H., Sakurai, M., Inaba, H., Katano, N., Horikoshi, Y., Eguchi, H., Miyake, M., Seto, M. & Kaneko, Y. (1999) Chromosome abnormalities and MLL rearrangements in acute myeloid leukemia of infants. *Leukemia*, **13**, 1013–1017.

Schoch, C., Kohlmann, A., Schnittger, S., Brors, B., Dugas, M., Mergenthaler, S., Kern, W., Hiddemann, W., Eils, R. & Haferlach, T. (2002) Acute myeloid leukemias with reciprocal rearrangements can be distinguished by specific gene expression profiles. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 10008–10013.

Shimada, A., Taki, T., Tabuchi, K., Tawa, A., Horibe, K., Tsuchida, M., Hanada, R., Tsukimoto, I. & Hayashi, Y. (2006) 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*, **107**, 1806–1809.

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

Tsukimoto, I., Tawa, A., Hanada, R., Tabuchi, K., Kigasawa, H., Tsuchiya, S., Tsuchida, M., Yabe, H., Nakayama, H., Kudo, K., Kobayashi, R., Hamamoto, K., Imaizumi, M., Morimoto, A. & Horibe, K. (2005) Excellent outcome of risk stratified treatment for childhood acute myeloid leukemia-AML99 trial. For the Japanese Childhood AML Cooperative Study Group. *Blood*, **106**. (Abstract 889).

Tsutsumi, S., Taketani, T., Nishimura, K., Ge, X., Taki, T., Sugita, K., Ishii, E., Hanada, R., Ohki, M., Aburatani, H. & Hayashi, Y. (2003) Two distinct gene expression signatures in pediatric acute lymphoblastic leukemia with MLL rearrangements. *Cancer Research*, **63**, 4882–4887.

Tusher, V.G., Tibshirani, R. & Chu, G. (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 5116–5121.

Valk, P.J., Verhaak, R.G., Beijen, M.A., Erpelinck, C.A., Doorn-Khosrovani, S.B., Boer, J.M., Beverloo, H.B., Moorhouse, M.J., van der Spek, P.J., Lowenberg, B. & Delwel, R. (2004) Prognostically useful gene-expression profiles in acute myeloid leukemia. *The New England Journal of Medicine*, **350**, 1617–1628.

Wakamatsu, N., Yamada, Y., Yamada, K., Ono, T., Nomura, N., Taniguchi, H., Kitoh, H., Mutoh, N., Yamanaka, T., Mushiake, K., Kato, K., Sonta, S. & Nagaya, M. (2001) Mutations in SIP1, encoding Smad interacting protein-1, cause a form of Hirschsprung disease. *Nature Genetics*, **27**, 369–370.

Weisser, M., Kern, W., Rauhut, S., Schoch, C., Hiddemann, W., Haferlach, T. & Schnittger, S. (2005) Prognostic impact of RT-PCR-based quantification of WT1 gene expression during MRD monitoring of acute myeloid leukemia. *Leukemia*, **19**, 1416–1423.

Welbron, J.L., Jenks, H.M. & Hagemeijer, A. (1993) Unique clinical features and prognostic significance of the translocation (6;11) in acute leukemia. *Cancer Genetics and Cytogenetics*, **65**, 125–129.

Yagi, T., Morimoto, A., Eguchi, M., Hibi, S., Sako, M., Ishii, E., Mizutani, S., Imashuku, S., Ohki, M. & Ichikawa, H. (2003)

Identification of a gene expression signature associated with pediatric AML prognosis. *Blood*, **102**, 1849–1856.

Yeoh, E.J., Ross, M.E., Shurtleff, S.A., Williams, W.K., Patel, D., Mahfouz, R., Behm, F.G., Raimondi, S.C., Relling, M.V., Patel, A., Cheng, C., Campana, D., Wilkins, D., Zhou, X., Li, J., Liu, H., Pui, C.H., Evans, W.E., Naeve, C., Wong, L. & Downing, J.R. (2002) Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. *Cancer Cell*, **1**, 133–143.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Expression profiles of AML-M4/M5.

Figure S2. The significance of *MLL* gene rearrangements partners.

Table SI. Clinical information of paediatric patients enrolled in this study.

Table SII. Clinical information of adult patients enrolled in this study.

Table SIII. Primer sequences used for quantitative RT-PCR.

Table SIV. Top 50 probe sets in overexpressed genes in 10 typical infants.

Table SV. Top 50 probe sets in differentially expressed genes between subgroup A and subgroup C patients.

Table SVI. Differentially expressed genes between *MLL*gr(+) and *MLL*gr(−) patients.

Table SVII. Differentially expressed genes among *MLL*gr(+) patients.

Table SVIII. The numbers of probe sets showing differential expression among *MLL*gr(+) patients.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

