11. Mullighan CG, Zhang J, Harvey RC, et al. JAK mutations in high-risk childhood acute lymphoblastic leukemia. Proc Natl Acad Sci U S A 2009;106:9414–9418.
12. Mullighan CG, Collins-Underwood JR, Phillips LA, et al. Rearrangement of *CRLF2* in B-progenitor and down syndrome associated acute lymphoblastic leukemia. Nat Genet 2009;41:1243–1246.
13. Russell LJ, Capasso M, Vater I, et al. Deregulated expression of cytokine receptor gene, CRLF2, is involved in lymphoid transformation in B-cell precursor acute lymphoblastic leukemia. Blood 2009;114:2688–2698.
14. Hertzberg L, Vendramini E, Ganmore I, et al. Down syndrome acute lymphoblastic leukemia, a highly heterogeneous disease in which aberrant expression of CRLF2 is associated with mutated JAK2: A report from the international BFM Study Group. Blood 2010;115:1006–1017.
15. Harvey RC, Mullighan CG, Chen IM, et al. Rearrangement of CRLF2 is associated with mutation of JAK kinases, alteration of IKZF1, Hispanic/Latino ethnicity, and a poor outcome in pediatric B-progenitor acute lymphoblastic leukemia. Blood 2010;115:5312–5321.
16. Cario G, Zimmermann M, Romey R, et al. Presence of the *P2RY8-CRLF2* rearrangement is associated with a poor prognosis in non-high-risk precursor B-cell acute lymphoblastic leukemia in children treated according to the ALL-BFM 2000 protocol. Blood 2010;115:5393–5397.
17. Ensor HM, Schwab C, Russell LJ, et al. Demographic, clinical and outcome features of children with acute lymphoblastic leukemia and CRLF2 deregulation: Results from the MRC ALL97 clinical trial. Blood 2010;117:2129–2136.
18. Schwab CJ, Jones LR, Morrison SL, et al. Evaluation of multiplex ligation-dependent probe amplification as a method for the detection of copy number abnormalities in B-cell precursor acute lymphoblastic leukemia. Genes Chromosomes Cancer 2010;49:1104–1113.
19. MLPA procedure, Result interpretation. MRC Holland Home Page. [Interpretation of MLPA results] http://www.mlpa.com/WebForms/WebFormMain.aspx?Tag=wl2zCjiVrCGANQgZPuTixrMkcAVITx-SUI1uxaJsuta9GGaLIE8p67QllDescription version 02; 11-02-2010.
20. Bercovich D, Ganmore I, Scott LM, et al. Mutations of *JAK2* in acute lymphoblastic Leukemia associated with Down's syndrome. Lancet 2008;372:1484–1492.
21. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Am Stat Assoc 1958;53:457–481.
22. Mantel N. Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother Rep 1966;50:163–170.
23. Cox DR. Regression models, life tables. J R Stat Soc 1972;B34:187–220.
24. Collins-Underwood JR, Mullighan CG. Genomic profiling of high-risk acute lymphoblastic leukemia. Leukemia 2010;24:1676–1685.
25. Palmi C, Vendramini E, Silvestri D, et al. Poor prognosis for P2RY8-CRLF2 fusion but not for CRLF2over-expression in children with intermediate risk B-cell precursor acute lymphoblastic leukemia. Leukemia 2012;26:2245–2253.
26. Roberts KG, Morin RD, Zhang J, et al. Genetic alterations activating kinase and cytokine receptor signaling in high-risk acute lymphoblastic leukemia. Cancer Cell 2012;22:153–166.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Fig. S1. Detection of Ik6/lk10 Isoform by RT-PCR. Five patients had the deletion of *IKZF1* exon 4–7 (Ik6), while patient No. 182 showed deletion of exon 2–7 (Ik10) by MLPA. The 358-bp band (open arrow head) indicates Ik6 isoform, and the 184-bp band (closed arrow head) indicates the Ik10 isoform. The sample (WT) without *IKZF1* deletion was used as a negative control.

Fig. S2. Probability of RFI according to *IKZF1* deletion, *CRLF2* expression and NCI risk classification. **A**: Probability of RFI for patients with or without *IKZF1* deletion (4-year RFI: $76.2 \pm 10.9\%$ vs. $91.7 \pm 2.5\%$; $P = 0.047$). **B**: Probability of RFI for patients with or without *IKZF1* deletion according to NCI-risk classification (4-year RFI: $53.3 \pm 23.4\%$ vs. $93.2 \pm 3.8\%$; $P = 0.03$ for NCI HR; $90.0 \pm 9.5\%$ vs. $91.0 \pm 3.1\%$; $P = 0.82$ for NCISR). **C**: Probability of RFI for patients with high *CRLF2* expression or low *CRLF2* expression (4-year RFI: $71.8 \pm 14.0\%$ vs. $92.4 \pm 2.4\%$; $P = 0.06$). **D**: Probability of RFI for patients with high *CRLF2* expression or low *CRLF2* expression according to NCI-risk classification (4-year RFI: $68.6 \pm 18.6\%$ vs. $90.6 \pm 5.2\%$; $P = 0.18$ for NCI HR; $75.0 \pm 21.7\%$ vs. $93.1 \pm 2.7\%$; $P = 0.35$ for NCI SR).

Fig. S3. Probability of EFS according to *IKZF1* deletions and *CRLF2* expression. The probability of EFS was much lower for the patients with *IKZF1* deletions and high *CRLF2* expression (4y-EFS: $20.0 \pm 17.9\%$) when compared with patients with other *IKZF1*/*CRLF2* statuses (4y-EFS: $88.0 \pm 3.2\%$ for del.(−)/low, $90.0 \pm 9.5\%$ for del.(−)/high, $88.9 \pm 10.5\%$ for del.(+)/low, $77.8 \pm 6.2\%$ for del.(−)/missing, $75.0 \pm 15.3\%$ for del.(+)/missing, $88.2 \pm 7.8\%$ for missing/low).

Table SI. Patient Characteristics of the BCP-ALL Patients Enrolled in the CCLSG ALL 2004 Clinical Study Versus the Analyzed Cohort

Table SII. *IKZF1* Deletion Patterns Detected by MLPA

Table SIII. Clinical Features of the Patients With *IKZF1* Deletions and High *CRLF2* Expression

Improved Treatment Results of Children With B-Cell Non-Hodgkin Lymphoma: A Report From the Japanese Pediatric Leukemia/Lymphoma Study Group B-NHL03 Study

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Background. Previous Japanese studies of childhood B-cell non-Hodgkin lymphoma (B-NHL) have shown a favorable outcome, though the study size was too small to effectively assess the efficacy and safety of treatment for childhood B-NHL. ***Procedure.*** We performed a nation-wide prospective B-NHL03 study to assess the efficacy and safety of short-pulse intensive chemotherapy for children with B-NHL. They were stratified into four treatment groups according to disease stage, tumor resectability and bone marrow/CNS involvement: Group 1 with all resected stage I/II, Group 2 with non-resected stage I/II, Group 3 with stage III & CNS-negative stage IV, and Group 4 with CNS-positive stage IV & Burkitt leukemia. Treatment duration was 2 courses for Group 1, 4 courses for Group 2, and 6 courses for Groups 3 and 4, respectively. CNS irradiation was omitted in all patients. ***Results.*** The follow-up time ranged from 0.8 to 88 months, with a median of being 45 months. For 321 patients analyzed in this study, overall survival and event-free survival (EFS) at 4 years was 92.7% and 87.4%, respectively. The 4-year EFS according to treatment group were 94% for Group 1 (n = 17), 98% for Group 2 (n = 103), 84% for Group 3 (n = 111), and 78% for Group 4 (n = 90). There was no significant difference in outcome by histology. Therapy-related death occurred in three patients in remission. ***Conclusions.*** Our nationwide large-scale study resulted in a cure rate above 90% with <1% toxic death in childhood B-NHL.

Key words: B-NHL03; childhood; JPLSG; non-Hodgkin lymphoma

INTRODUCTION

Childhood B-cell non-Hodgkin Lymphoma (B-NHL) consists mainly of two histological subtypes, namely Burkitt lymphoma (BL), which includes Burkitt leukemia (B-ALL), and diffuse large B-cell lymphoma (DLBCL). The cure rate of childhood BL has been markedly improved over the past 30 years, and long-term event-free survival (EFS) of patients has reached to approximately 90%. This is largely due to prospective studies of European and North American groups that developed a short intensive chemotherapy regimen, including a high-dose methotrexate (HDMTX), an intermediate dose of cyclophosphamide (CPA), and anthracyclines [1–6]. Although DLBCL is a distinct disease entity from BL, the treatment is the same as that for patients with Burkitt histology, and excellent outcome has been reported [1–6]. Previously most clinical experiences of childhood B-NHL were reported by European and North American study groups, and there were few data on Japanese or Asian patients with B-NHL. In the1990s, we conducted group-wide trials for childhood B-NHL [7–10]: Horibe et al. showed a 4-year EFS with 70% for 57 patients (BL 31, B-ALL 17, DLBCL 9) [8], Kikuchi et al. showed a 6-year EFS with 82% for 91 patients (BL 45, B-ALL 9, DLBCL 26, others 11) [10], and Tsurusawa et al. showed a 7-year EFS with 93% for 30 patients with DLBCL [9]. In addition, Lee et al. has recently shown a 5-year EFS with 95% for 61 patients (BL 46, DLBCL 15) [11]. However, the treatment duration of these studies was relatively long and the number of patients was small compared to the European and North American studies [1–6].

Here, we report on the results of the nation-wide large prospective study for children with B-NHL. The primary object was to evaluate the efficacy and safety of short-pulse intensive chemotherapy regimen designed by the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG).

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PATIENTS AND METHODS

Study Design and Diagnostic Criteria

The B-NHL03 study was a prospective nonrandomized trial that investigated the efficacy and safety of short-pulse intensive chemotherapy in childhood B-NHL. The chief aim was to improve the outcomes of patients enrolled in the B-NHL03 study to the level of those of European and North American studies.

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

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Conflict of interest: Nothing to declare.

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The diagnosis of B-NHL was based on histopathology, immunocytochemistry, and cytogenetics. All histopathological specimens were first classified by the institutional pathologist and finally each of them were reviewed by a group of seven pathologists of a central pathological review committee according to WHO classification, that is, BL or Burkitt-like lymphoma (BLL), DLBCL, mediastinal large B-cell lymphoma (MLBCL), and mature B-cell neoplasm, NOS (not otherwise specified) [12]. A mature B-cell phenotype was primarily defined as positive for C20 and/(or) CD79a and negative for CD3 and terminal deoxynucleotidyl transferase. When an immunophenotype study was not available, specific translocations t(8;14)(q24;q32), t(2;8)(p11;q24), t(8;22)(q24;q11) at cytogenetic analysis were included. CNS involvement was diagnosed by the presence of one or more of the following: any blasts with FAB L3 morphology in CSF, isolated intracerebral mass, or intra-spinal extension. The clinical stage was defined by Murphy's classification [13].

Treatments

The treatment outline is shown in Figure 1 and chemotherapy regimens are shown in Table I. They were stratified into four treatment groups according to disease stage, tumor resectability and bone marrow/CNS involvement: Group 1 with all resected stage I/II, Group 2 with non-resected stage I/II, Group 3 with stage III & CNS-negative stage IV, and Group 4 with CNS-positive stage IV & B-ALL. All groups except Group 1 received a pre-phase therapy of prednisolone (PSL), vincristine (VCR), CPA and it (intrathecal) MTX to reduce tumor volume. As shown in Figure 1, Group 1 received two courses (1A × 2), Group 2 received 4 courses (2A × 2 + 2B × 2), Group 3 received 6 courses (3A × 4 + 3B × 2), and Group 4 received 6 courses (4A1 × 2 + 4A2 × 2 + 4B × 2), respectively. No patients received prophylactic cranial irradiation. Patients with CNS involvements received HDMTX (5 g/m^2) plus an extended it regimen (14 times), but no therapeutic cranial irradiation. The schedule of HDMTX administration was identical

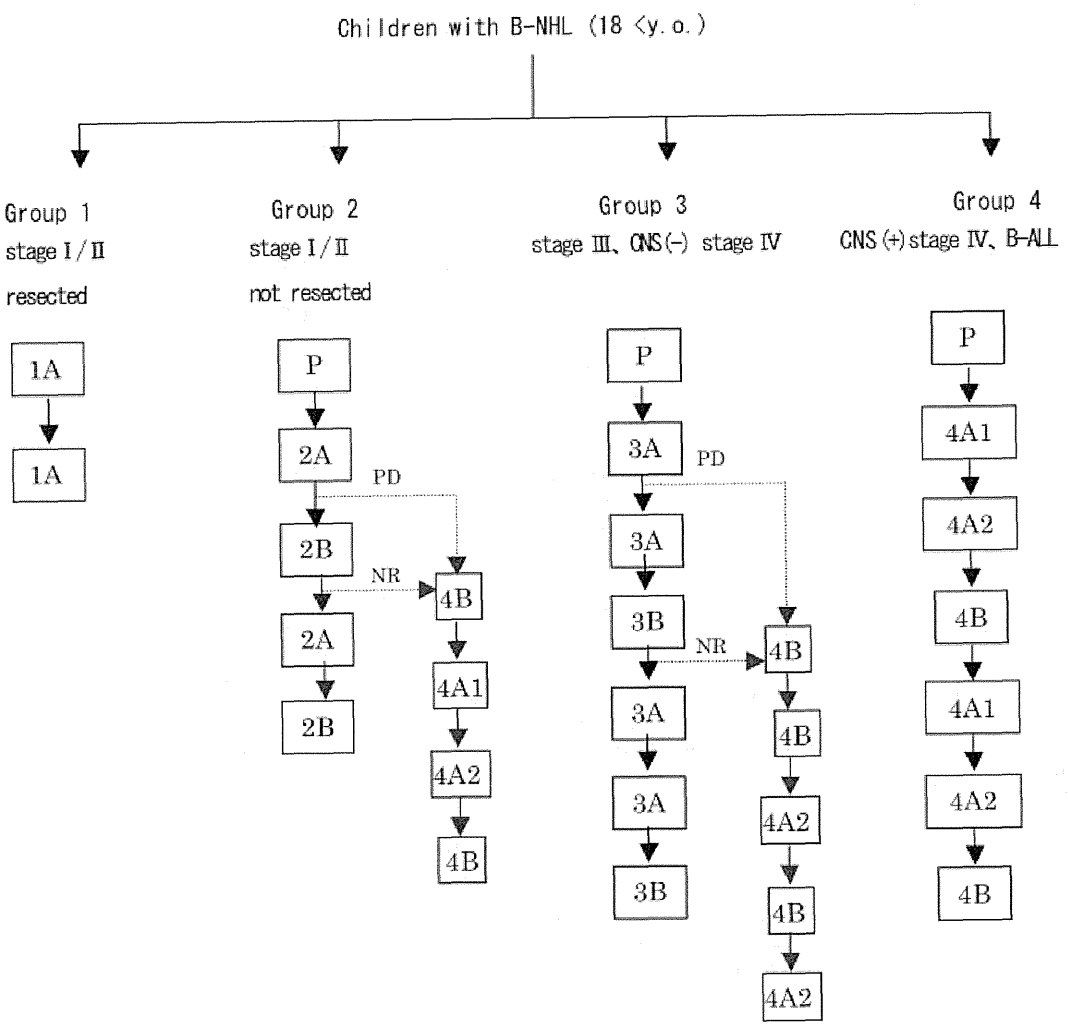

Fig. 1. Treatment framework of the B-NHL03 study. Patients were stratified into four treatment groups according to disease stage, tumor resectability, and BM/CNS involvement. All groups except Group 1 received pre-phase therapy. Group 1 received two courses of chemotherapy, Group 2 received 4 courses, Groups 3 and 4 received 6 courses, respectively. When patients in Group 2 or 3 did not achieve CR or CRu during the first 2 or 3 courses, they received salvage therapy consisting of 4B and 4A1/2 courses.

TABLE I. B-NHL03 Treatment Schedules

Regimen	Administration	Daily dose	Days
Pre-phase			
Prednisolone	Orally	30 mg and 60 mg/m^2	Days 1–3 and 4–7
Vincristine	IV	1 mg/m^2	Day 3
Cyclophosphamide	IV	150 mg/m^2	Days 4–6
Methotrexate	TIT	12 mg/m^2	Day 1, (4)[a]
Hydrocortisone	TIT	25 mg/m^2	Day 1, (4)[a]
Cytarabine	TIT	30 mg/m^2	Day (4)[a]
Regimen 1A			
Prednisolone	Orally	60 mg/m^2	Days 1–5
Methotrexate	IV	1 g/m^2	Day 1
Vincristine	IV	1.5 mg/m^2	Day 2
Cyclophosphamide	IV	250 g/m^2 × 2	Days 2–4
THP-adriamycin	IV	30 mg/m^2	Days 3, 4
Methotrexate	DIT	12 mg/m^2	Day 1
Hydrocortisone	DIT	25 mg/m^2	Day 1
Regimen 2A			
Same as 1A except for dexamethasone	Orally	10 mg/m^2	Days 1–7
Methotrexate	IV 24 hours with LV rescue	3 g/m^2	Day 1
Regimen 3A			
Same as 2A except for *t.i.t* at day 1			
Regimen 4A1			
Same as 3A except for methotrexate	IV 24 hours with LV rescue	5 g/m^2	Day 1
Methotrexate	TIT	12 mg/m^2	Day 1, (5),[a] 8
Hydrocortisone	TIT	25 mg/m^2	Day 1, (5),[a] 8
Cytarabine	TIT	30 mg/m^2	Day 1, (5),[a] 8
Regimen 4A2			
Same as 4A1 except for cyclophosphamide	IV	1 g/m^2	Days 4, 5
Regimen 2B			
Methotrexate	IV 6 hours	500 mg/m^2	Day 1
Cytarabine	cIV	150 mg/m^2	Days 1–5
Methotrexate	DIT	12 mg/m^2	Day 1
Hydrocortisone	DIT	25 mg/m^2	Day 1
Regimen 3B			
Same as 2B except for TIT at day 1, and cytarabine	cIV	150 mg/m^2	Days 1–6
Etoposide	IV	100 mg/m^2 × 2	Days 3–5
Regimen 4B			
Same as 3B except for without methotrexate, DIT at day 1 and TIT at day 8, and dexamethasone	Orally	10 mg/m^2	Days 1–7
Cytarabine	IV	2 g/m^2 × 2	Days 2–4
Etoposide	IV	150 mg/m^2	Days 2–5
Vincristine	IV	1.5 mg/m^2	Day 1

LV, leucovorin; IV, intravenous; cIV, continuous intravenous; DIT, double intrathecal; TIT, triple intrathecal. [a]For CNS positive patients.

to that of the B-NHL960 study [9]: HDMTX was administered for the first 24 hours, and 12 hours later, leucovorin (LV) 15 mg/m^2 was given orally every 6 hours, for a total of seven doses [9]. Blood MTX concentration was measured 24, 48, and 72 hours after the MTX administration. When patients showed delayed MTX clearance ($\geqq$0.2 μM after 72 hours), LV rescue was continued until MTX concentration level decreased to less than 0.2 μM.

Induction failure (IF) was defined as patients who did not achieve complete remission (CR) or unconfirmed remission (CRu) until the last evaluation time (before the second course of 2A in Group 2, before the third course of 3A in Group 3, before the second course of 4A1 in Group 4). When patients in Group 2 or 3 were evaluated to have progressive disease or no response during the first 2 or 3 courses, they received salvage therapy consisting of regimens 4B and 4A1/2. The cumulative dose of cytotoxic drugs for treatment groups was as follows: CPA 3 g/m^2, THP 120 mg/m^2 for Group 1; CPA 3.45 g/m^2, THP 120 mg/m^2 for Group2; CPA 6.45 g/m^2, THP 240 mg/m^2, VP16 0.6 g/m^2 for Group 3; CPA 7.45 g/m^2, THP 240 mg/m^2, VP16 1.2 g/m^2 for Group 4.

Statistical Analysis

Final statistical analyses were performed based on data obtained in June 2012. Overall survival (OS) was defined as the time between diagnosis and death from any causes, and EFS was defined as the time to first events defined as an occurrence of induction failure, relapse at any site, death from any causes, or second malignant neoplasm. For patients who did not experience an event, EFS was defined as the time to the last follow-up. Survival curves were prepared using the Kaplan–Meier method and standard errors (SEs) with the Greenwood formula. The significance of differences in survival outcomes was determined by means of the log-rank test.

STATA® statistical analysis software (version 11.0; StataCorp LP, College Station, TX) was used for all computations.

RESULTS

Patients

The protocol was conducted in 112 hospitals of the JPLSG after approval by each institution's review board, and written informed consent was provided by patients or legal guardians before treatment. Between November 2004 and January 2011, 346 cases of newly diagnosed B-NHL were enrolled in this study. Of these, 25 cases were excluded: 14 due to ineligible pathology, 8 for late enrollment, 2 for ineligible clinical stage, and 1 for prior chemotherapy. A total of 321 cases of four treatment groups were analyzed (Fig. 2).

Patient characteristic are shown in Table II. There were few protocol deviations: 10 patients in the Group 3/4 skipped or postponed HDMTX therapy in the A course, 5 because of retention of ascites or pleural effusion, 2 because of renal dysfunction, 2 due to septic infection, and one for stomatitis.

EFS and OS

The follow-up time ranged from 0.8 to 88 months, with a median 47 months. For the 321 patients analyzed in this study, 4-year OS was 92.7% ± 1.4% and 4-year EFS was 87.3% ± 1.8% (Fig. 3A). There was no significant difference in outcome by gender (4-year EFS, male 87.5% ± 2.2% vs. female 87.0% ± 3.8%, P =0.864). The 4-year OS and EFS according to treatment subgroup were 100% and 94.1% ± 5.7% for Group 1, 100% and 98.6% ± 1.4% for Group 2, 93.6% ± 2.3% and 83.6% ± 3.5% for Group 3, and 82.1% ± 4.1% and 77.8% ± 4.4% for Group 4 (Fig. 3B). The 4-year OS and EFS according to clinical stage were 100% and 97.7% ± 2.3% for stage I, 100% and 97.8% ± 2.0% for stage II, 92.0% ± 2.9% and 82.9% ± 4.0% for stage III, 84.6% ± 5.8% and 71.8% ± 7.2% for stage IV. The 4-year OS and EFS of B-ALL were 86.2% ± 4.0% and 83.6% ± 4.3%. The 4-year EFS by histology was 86.1% ± 2.6% for BL/BLL, 87.3% ± 3.5% for DLBCL, 92.1% ± 4.3% for others, and 100% for MLBCL (P = 0.717) (Fig. 3C). When we analyzed the outcome of patients who had BM or CNS disease, the 4-year EFS was 83.8% ± 4.3% for patients (n = 74) with BM involvement only (BM+/CNS−), 60.0% ± 1.5%

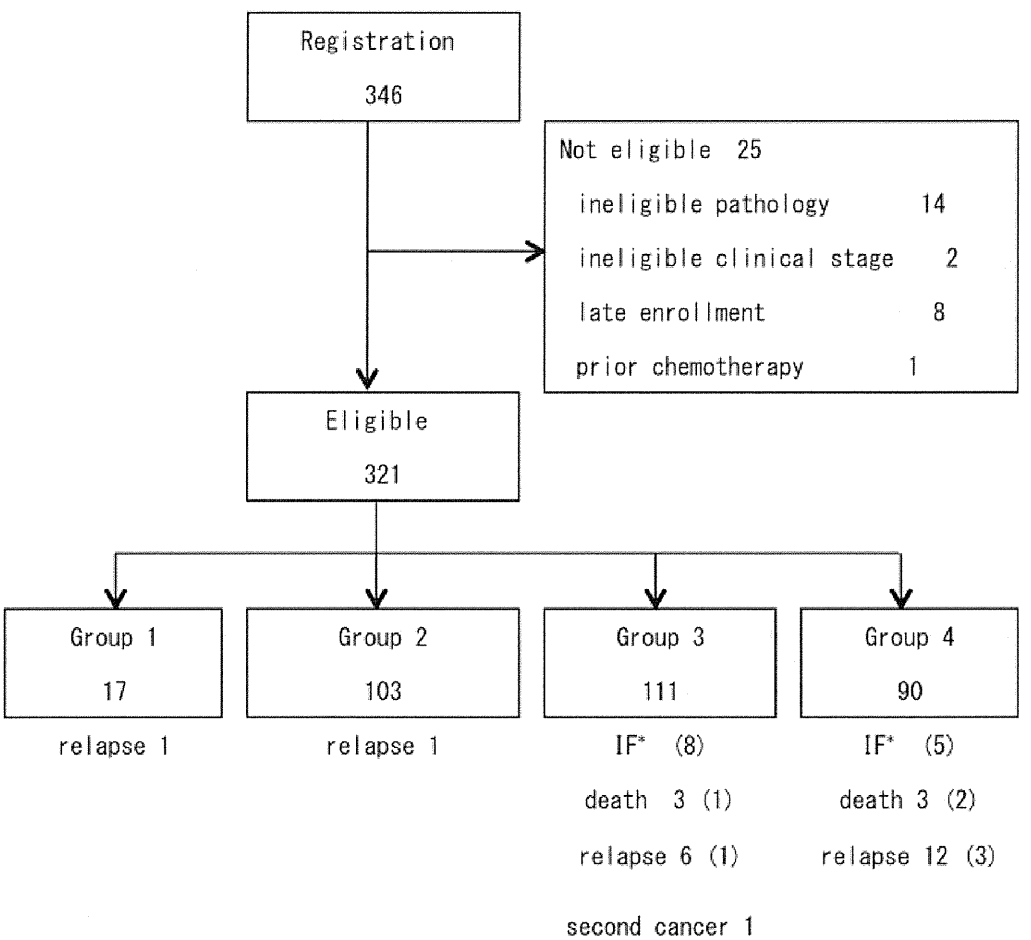

Fig. 2. Patient flow chart and events according to the treatment group. There were 40 events which consisted of each one in Group 1 and 2, 18 in Group 3, and 20 in Group 4. Number in parentheses indicates events occurred during protocol chemotherapy. Number in parenthesis indicates events occurred during protocol chemotherapy. *IF, induction failure defined as patients did not achieve complete remission or unconfirmed remission at the last evaluation time in group 3/4.

TABLE II. Patients Characteristics

Therapy groups	G1	G2	G3	G4	Total (%)
No. of patients	17	103	111	90	321
Sex					
Male	12	72	90	71	245 (76)
Female	5	31	21	19	76 (24)
Age					
0–4	2	12	18	16	48 (15)
5–9	3	45	42	39	129 (40)
10–14	8	42	42	27	119 (37)
15–	4	4	9	8	25 (8)
Histology					
BL/BLL/B-ALL	5	33	62	80	180 (56)
DLBCL	12	58	26	5	101 (31.4)
MLBCL	0	0	2	0	2 (0.6)
Others	0	12	21	5	38 (12)
Primary sites					
Thorax	5	30	7	1	43
Head & neck	5	39	12	2	58
Peripheral lymph nodes	0	3	3	0	6
Abdomen	7	29	75	11	122
Mediastinum	0	0	8	0	8
B-ALL	0	0	0	73	73
CNS	0	0	0	2	2
Other tumor site	0	2	5	0	7
Not specified	0	0	1	1	2
BM involvement	0	0	22	80	102 (32)
CNS involvement	0	0	0	38	38 (12)

BL, Burkitt lymphoma; BLL, Burkitt-like lymphoma; B-ALL, Burkitt leukemia; DLBCL, diffuse large B-cell lymphoma, MLBCL, mediastinal large.

for patients (n = 10) with CNS involvement only (BM−, CNS+), and 75.0% ± 8.2% for patients (n = 28) with BM and CNS involvements (BM+/CNS+), ($P = 0.102$) (Fig. 3D). Outcome by treatment response to initial A courses were as follows: The 4-year OS and EFS for patients who achieved CR (n = 236) or CRu (n = 54) at the last evaluation time were 95.7% ± 1.6% and 93.5% ± 1.6%, and 96.1% ± 2.7% and 86.9% ± 4.6%, respectively, while the 4-year OS and EFS for patients (n = 13) who did not achieve CR/CRu was 69.2% ± 12.8% and 15.4% ± 10.1% ($P < 0.001$), respectively.

Treatment Failure Events

Forty patients experienced an event and 25 have died (Fig. 2). The cause of death was tumor progression in 14, infection in 7, stem cell transplantation-related death in 3, and pulmonary bleeding in 1. The 40 events consisted of 13 induction failures, 6 deaths, 20 relapses, and one second cancer. Of the13 patients (6 in Group 3 and 7 in Group 4) who failed the initial treatment, 4 patients in Group 3 received salvage therapy and achieved CRu. At the time of the last analysis, 8 patients (4 in Group 3 and 4 in Group 4) were alive without tumor. Death in remission occurred in 3/321 (1%) patients: two died of infection and one died of pulmonary bleeding. The longest duration before relapse from the start of therapy was 38.9 months in DLBCL and 13.6 months in Burkitt histology. Relapse sites were 10 in local, 6 in BM, 2 in BM+CNS, one in local + CNS, and one in CNS. All CNS relapse occurred in patients with BL, but not with DLBCL. Thus, isolated CNS failure was only one among 38 patients with CNS involvement. Of the 20 relapsed patients, 11 died and 9 survived without tumor. A second cancer occurred among the patients who failed the initial treatment: a 12-year-old male with BL developed a secondary malignancy with acute myeloid leukemia (FAB M5) 17 months after the initial diagnosis.

Toxicity

Acute toxicity of treatment courses (A and B) was evaluated by the scale of NCI-CTC version 2.0., and rates of acute toxicity Grade 3 among patients in Groups 2, 3, and 4 are shown in Supplemental Table I. Anemia and neutropenia were the most frequent hematological toxicities with grade III or IV in all groups. In particular, grade IV neutropenia occurred in almost all patients (>98%) during A courses. In nonhematologic toxicity, infection was the single most frequent occurring with grade III or IV at least once in 70% of patients although the rate of grade IV infection was very small (<1%). Stomatitis and hepatotoxicity were also frequent, occurring with grade III or IV at least once in 20–35% and 24–38% of patients, respectively. The rate of renal toxicity grade III was very low. Leukoencephalopathy was reported in two patients of Group 3, and their MRI findings disappeared within 2 months without neurological symptoms. The overall incidence of renal insufficiency associated with tumor lysis syndrome was 2 out of 96 (2%) in Group 4, and these required assisted renal support with continuous hemodiafiltration.

DISCUSSION

During the last two decades, the survival outcome of children with B-NHL has been markedly improved through consecutive

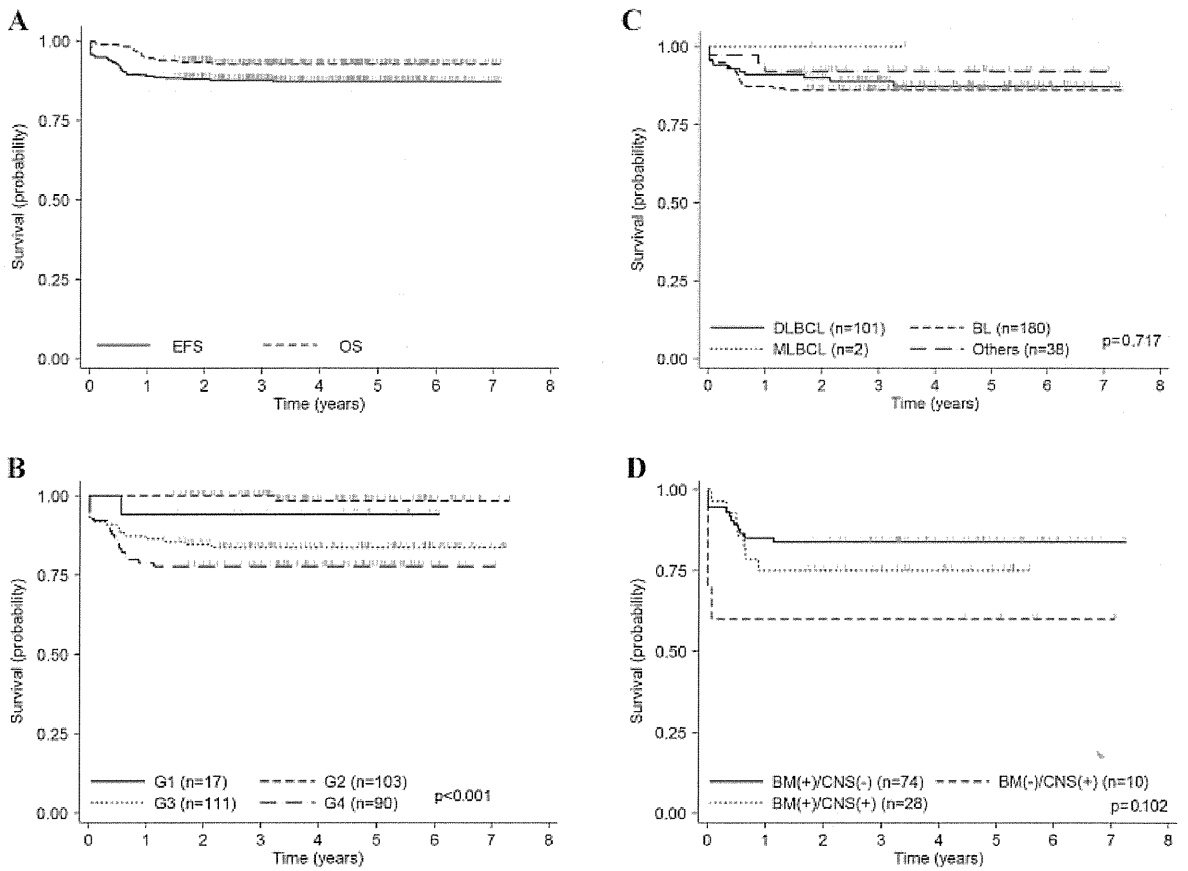

Fig. 3. Kaplan–Meier curves for OS and EFS of all patients (**A**). Kaplan–Meier curves for EFS according to treatment group (**B**), histology (**C**), and BM/CNS involvement (**D**).

clinical trials in large study groups, and the cure rate of childhood B-NHL has reached 90% [1–6]. In the present study, we showed an excellent survival outcome with 4-year OS 93% in children with B-NHL. In our study, the 4-year EFS 84% of Group 3 patients was considerably lower than the 4-year EFS 90% of intermediate risk group in the FAB/LMB96 study [5] or the 6-year EFS 88% of stage III patients in the BFM90 study [2], whereas, the 4-year EFS 78% of Group 4 patients compared favorably with the 4-year EFS 79% of high-risk group in the FAB/LMB96 study [5] and the 6-year EFS 74% of stage IV/B-ALL patients in the BFM90 study [2]. This outcome was obtained via the short-intensive chemotherapy regimen based on COPAD (CPM, VCR, PSL, and ADR) regimen plus the HDMTX of the lymphomas malin B (LMB) studies [3]. We omitted cranial irradiation for all patients, because recent studies have suggested the possibility of deleting radiotherapy in treating CNS diseases as well as CNS prophylaxis [2,3,5,9]. However, having no experience in administrating 8 g/m^2 HDMTX, we employed 5 g/m^2 HDMTX over 24 hour-infusion and not the 8 g/m^2 HDMTX over 4 hour-infusion in the LMB protocols for treating patients with CNS disease [3,5]. The treatment result for CNS disease was satisfactory, because CNS failure was only one of 38 patients with primary CNS disease in the present study.

This suggests that the 5 g/m^2 HDMTX over 24 hour-infusion is equally as effective to the CNS-positive disease as the aforementioned 8 g/m^2 HDMTX over 4 hours infusion, and reinforces the possibility that CNS irradiation could be omitted without jeopardizing the outcome of patients with CNS disease by using systemic and it MTX therapy [3,5,9].

The treatment of DLBCL as well as BL was another important focus of our study, because the incidence of DLBCL in childhood B-NHL is relatively more frequent than that of Western countries: the number of DLBCL was almost similar to that of BL (excluding B-ALL) in the present study and our recent national survey for childhood hematological malignancies has shown that the ratio of DLBCL to BL was 0.79 [14]. In our study, according to the strategy that DLBCL was treated by short-pulse chemotherapy as well as BL [15], we followed the same protocol, and achieved a favorable outcome of 4-year EFS with 87% for DLBCL which was not inferior to that of BL. This outcome can be partly explained by shared biological features, that is, that more than half of childhood DLBCL has the molecular subtypes of BL [16].

Several factors associated with poor outcome in the high-risk group in childhood B-NHL have been reported. Cairo et al. has shown a significantly inferior outcome (4-year EFS 61% ± 6%) of the subgroup of children with combined BM and CNS involvement at diagnosis as compared with children with BM or CNS only [5]. However, our results in Group 4 showed that the outcome (4-year EFS 75% ± 8%) of this subgroup with BM+/CNS+ was not significantly inferior than that of the subgroup with BM+ (83% ± 4%) or CNS+ (60% ± 1%). Failure to initial therapy is

also known to be a strong, unfavorable prognostic factor. Past studies in LMB 89/96 have shown that non-responders to pre-phase therapy (COP regimen) suffer a significantly inferior outcome as compared with responders or incomplete responders [3,5]. In our study, an appropriate evaluation of tumor regression just after pre-phase therapy was difficult for many patients, such that we compared the outcome according to response at the final evaluation time after two or three courses of therapy. These results showed that 4-year EFS of patients who did not achieve CR/CRu was only $15\% \pm 10\%$, which was as dismal as the outcome of poor-responders to COP regimen in the FAB/LMB 96 study [5]. To rescue the poor-responders in our study, we employed salvage therapy with high-dose Ara-C and VP16 to patients who did not achieve remission after 2 or 3 courses of therapy in Group 2 or 3, as in the BFM90 or FAB96 study [2,4]. As a result, 4 of 6 patients in Group 3 received salvage therapy and survived without tumor. This response rate was similar to that of FAB96 study, in which 10 out of 16 patients who received the second phase treatment intensification after the consolidation phase were alive. Thus, our results reconfirmed the efficacy of the salvage therapy.

Management of acute toxicity by short-pulse intensive chemotherapy is essential to successfully carry out the treatment protocol for childhood B-NHL. In our study, grade IV neutropenia occurred in almost all patients, but the rate of grade IV infection was quite low. Consequently, therapy-related death was less than 1% in all patients, and 2.1% in Group 4 patients. These results show the safety and feasibility of our treatment protocol. Anthracycline cardiotoxicity and secondary malignancy by alkylating agents are serious late events in pediatric cancer treatment [17,18]. To reduce the risk of cardiotoxicity, we employed THP-adriamycin (pirarubicin) instead of ADR. Pirarubicin is a derivative of ADR with reportedly less cardiotoxicity in adults [19–24]. Recently, we have reported that no significant cardiac dysfunction was detected in long-term survivors of children with acute lymphoblastic leukemia who received THP treatment [25–27]. In the present study, there were no patients with cardiac insufficiency or cardiac myopathy during the 7-year observation period. These results suggest that late-onset cardiotoxicity induced by pirarubicin is uncommon in childhood lymphoid malignancies, at least up to the cumulative dose of 240 mg/m^2. In our study, there was one male with a second cancer with acute myeloid leukemia, although the correlation between his second cancer and the protocol treatment is uncertain because he was resistant to the pre-phase followed by arbitrary treatment.

As shown above, chemotherapy-related toxicity of our protocol treatment was within acceptable range. However, a 6-course treatment for Group 3 seemed to be more intensive as compared with a 4-course treatment for intermediate risk group in the FAB96 study [4]. In order to reduce the total dose of cytotoxic drugs without impairing the survival outcome, new approaches including targeted monoclonal antibody therapy in combination with chemotherapy [28,29], are needed for children with an advanced or resistant disease in coming studies.

In conclusion, our nationwide study resulted in a cure rate above 90% with <1% toxic death in childhood B-NHL.

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REFERENCES

1. Reiter A, Schrappe M, Parwaresch R, et al. Non-Hodgkin's lymphomas of childhood and adolescence: Results of a treatment stratified for biologic subtypes and stage. A report of the Berlin Frankfurt-Münster Group. J Clin Oncol 1995;13:359–372.
2. Reiter A, Schrappe M, Tiemann M, et al. Improved treatment results in childhood B-cell neoplasms with tailored intensification of therapy: Reports of the Berlin–Frankfurt–Münster Group trial NHL–BFM 90. Blood 1999;94:3294–3306.
3. Patte C, Auperin A, Michon J, et al. The Société Française d'Oncologie Pédiatrique LMB89 protocol: Highly effective multiagent chemotherapy tailored to the tumor burden and response in 561 unselected children with B-cell lymphomas and L3 leukemia. Blood 2001;97:3370–3379.
4. Patte C, Auperin A, Gerrard M, et al. Results of the randomized international FAB/LMB96 trial for intermediate risk B-cell non-Hodgkin lymphoma in children and adolescents. It is possible to reduce treatment for the early responding patients. Blood 2007;109:2773–2780.
5. Cairo MS, Gerrard M, Sposto R, et al. Results of a randomized international study of high-risk central nervous system B non-Hodgkin lymphoma and B acute lymphoblastic leukemia in children and adolescents. Blood 2007;109:2736–2743.
6. Gerrard M, Cairo MS, Weston C, et al. Excellent survival following two courses of COPAD chemotherapy in children and adolescents with resected localized B-cell non-Hodgkin's lymphoma: Results of the FAB/LMB 96 international study. Br J Haematol 2008;141:840–847.
7. 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 1995;61:85–96.
8. Horibe K, Akiyama Y, Kobayashi M, et al. Treatment outcome of AT-B88 regimen for B-cell non-Hodgkin's lymphoma and surface immunoglobulin-positive acute lymphoblastic leukemia in children. Int J Hematol 1997;66:89–98.
9. Tsurusawa M, Taga T, Horikoshi Y, et al. Favourable outcomes in children with diffuse large B-cell lymphoma treated by a short-term ALL-like regimen: A report on the NHL960 study from the Japanese Childhood Cancer and Leukemia Study Group. Leuk Lymphoma 2008;49:734–739.
10. Kikuchi A, Mori T, Fujimoto J, et al. Outcome of childhood B-cell non-Hodgkin lymphoma and B-cell acute lymphoblastic leukemia treated with the Tokyo Children's Cancer Study Group NHL B9604 protocol. Leuk Lymphoma 2008;49:757–762.
11. Lee SH, Yoo KH, Sung KW, et al. Should children with non-Hodgkin lymphoma be treated with different protocols according to histopathologic subtype? Pediatr Blood Cancer 2013;60:1842–1847.
12. Jaffe ES, Harris NL, Stein H, et al., editors. WHO classification of tumors, pathology and genetics of tumors of haematopoietic and lymphoid tissues. Lyon: IARC Press; 2001.
13. Murphy SB. Classification, staging and results of treatment of childhood NHL, dissimilarities from lymphoma in adults. Seminars in Oncology 1980;7:332–339.
14. Horibe K, Saito AM, Takimoto T, et al. Incidence and survival rates of hematological malignancies in Japanese children and adolescents (2006-2010): Based on registry data from the Japanese Society of Pediatric Hematology. Int J hematol 2013;98:74–88.
15. Reiter A, Klapper W. Recent advances in the understanding and management of diffuse large B-cell lymphoma in children. Br J Haematol 2008;142:329–347.
16. Klapper W, Szczepanowski M, Burkhardt B, et al. Molecular profiling of pediatric mature B-cell lymphoma treated in population-based prospective clinical trials. Blood 2008;112:1374–1381.
17. Sorensen K, Levitt GA, Bull C, et al. Late anthracycline cardiotoxicity after childhood cancer: A prospective longitudinal study. Cancer 2003;97:1991–1998.
18. Davies SM. Subsequent malignant neoplasms in survivors of childhood cancer: Childhood Cancer Survivor Study (CCSS) studies. Pediatric Blood Cancer 2007;48:727–730.
19. Umezawa H, Takahashi Y, Kinoshita M, et al. Tetrahydropyranyl derivatives of daunomycin and adriamycin. J Antibiot 1979;32:1082–1084.
20. Takagi T, Sakai C, Oguro M. Combination chemotherapy with pirarubicin (THP), cyclophosphamide, vincristine, and prednisolone (VEP-THP therapy) in the treatment of non-Hodgkins lymphoma. Oncology 1990;47:25–28.
21. Niitsu N, Umeda M. Biweekly THP-COPBLM (pirarubicin, cyclophosphamide, vincristine, prednisone, bleomycin and procarbazine) regimen combined with granulocyte colony-stimulating factor (G-CSF) for intermediate- and high-grade non-Hodgkins's lymphoma. Leukemia 1998;12:1457–1460.
22. Niitsu N, Umeda M. Response and adverse drug reactions to combination chemotherapy in elderly patients with aggressive non-Hodgkin's lymphoma: Comparison of CHOP, COP-BLAM, COP-BLAM III, and THP-COPBLM. Eur J Haematol 1999;63:337–344.
23. Tsurumi H, Yamada T, Sawada M, et al. Biweekly CHOP or THP-COP regimens in the treatment of newly diagnosed aggressive non-Hodgkin's lymphoma. A comparison of doxorubicin and pirarubicin: A randomized phase II study. J Cancer Res Clin Oncol 2004;130:107–113.
24. Zhai L, Guo C, Cao Y, et al. Long-term results of pirarubicin versus doxorubicin in combination chemotherapy for aggressive non-Hodgkin's lymphoma: Single center, 15-year experience. Int J Hematol 2010;91:78–86.
25. Tsurusawa M, Shimomura Y, Asami K, et al. Long-term results of the Japanese Childhood Cancer and Leukemia Study Group studies 811, 874 and 911 on childhood acute lymphoblastic leukemia. Leukemia 2010;24:335–344.
26. Yamaji K, Okamoto T, Yokota S, et al. Minimal residual disease-based augmented therapy in childhood acute lymphoblastic leukemia: A report from the Japanese Childhood Cancer and Leukemia Study Group Study. Pediatr Blood Cancer 2010;55:1287–1295.
27. Shimomura Y, Baba R, Watanabe A, et al. Japanese Childhood Cancer and Leukemia Study Group (JCCLSG). Assessment of late cardiotoxicity of pirarubicin (THP) in children with acute lymphoblastic leukemia. Pediatr Blood Cancer 2011;57:461–466.
28. Meinhardt A, Burkhardt B, Zimmermann M, et al. Phase II window study on rituximab in newly diagnosed pediatric mature B-cell non-Hodgkin's lymphoma and Burkitt leukemia. J Clin Oncol 2010;28:3115–3121.
29. Barth MJ, Goldman S, Smith L, et al. Rituximab pharmacokinetics in children and adolescents with de novo intermediate and advanced mature B-cell lymphoma/leukaemia: A Children's Oncology Group report. Br J Haematol 2013;162:678–683.

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BRIEF REPORT
Two Cases of Neuroblastoma Comprising Two Distinct Clones

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We report two cases of high-risk metastatic neuroblastoma, comprising two biologically distinct components in the adrenal primary tumor, which showed clear differences not only histologically but also in *MYCN* amplification and *HA-RAS/TRKA* immunoreactivity (Case 1), anaplastic lymphoma kinase (ALK) immunoreactivity (Case 2). These two cases with multiple separated components were similar to cases classified as ganglioneuroblastoma, nodular subtype (GNBn), in terms of composite tumor. Comparable to the GNBn category, the prognosis of the patients described here may depend on the components with unfavorable histology according to International Neuroblastoma Pathology Classification. Further analyses of such composite neuroblastoma cases are important for assessing disease prognosis. Pediatr Blood Cancer 2014;61:760–762. © 2013 Wiley Periodicals, Inc.

Key words: ganglioneuroblastoma; international neuroblastoma pathology classification; neuroblastoma

INTRODUCTION

The International Neuroblastoma Pathology Classification (INPC) classifies peripheral neuroblastic tumors into four categories: neuroblastoma (Schwannian stroma-poor); ganglioneuroblastoma, intermixed (Schwannian stroma-rich); ganglioneuroma (Schwannian stroma-dominant); and ganglioneuroblastoma, nodular (GNBn) (composite: Schwannian stroma-rich/stroma-dominant, and stroma-poor) [1,2]. Among these four categories, only GNBn tumors are defined as composite tumors comprising histologically and biologically different clones. By definition, one of the tumor components in GNBn has features consistent with ganglioneuroblastoma, intermixed or ganglioneuroma, and the other has an appearance of neuroblastoma. Here, we report two cases of neuroblastoma comprising two histologically and biologically distinct clones, similar to the GNBn category in terms of having two or more different clones. However, this type of composite neuroblastoma could not be classified according to the current INPC system.

CASE PRESENTATION

Case 1. A 14-month-old male presented with intermittent fever for 2 months. Abdominal computed tomography (CT) revealed an approximately 4-cm left adrenal mass. After complete resection of the mass, he was diagnosed with neuroblastoma. Other sites of involvement included the lymph nodes and bone marrow: International Neuroblastoma Risk Group Classification (INRG) stage M. After surgery, the patient underwent induction/high-dose chemotherapy with autologous peripheral blood stem cell transplantation, radiation, and 13-cis-retinoic acid treatment. Complete remission has been maintained for 3 years since diagnosis.

Microscopically, the resected left adrenal gland tumor comprised two neuroblastoma nodular components separated by a fibrillary matrix (components A and B in Fig. 1a). Component A was classified as neuroblastoma, a poorly differentiated subtype with a high mitosis-karyorrhexis index (MKI); small round cells with almost bare nuclei surrounded by a few neuropils were observed (Fig. 1b). Component B was classified as neuroblastoma, poorly differentiated subtype with a low MKI; relatively large nuclear cells surrounded by abundant neuropils were observed. Component B was more differentiated compared with component A. Some cells in component B had abundant eosinophilic cytoplasm and were observed to be in the process of differentiating into ganglion cells (Fig. 1c). Schwannian cells were not identified by S-100 immunostaining in both component A and B. Lymph nodes around the left adrenal tumor and bone marrow contained metastatic tumor cells similar in appearance to component A cells. According to INPC, component A was classified into Unfavorable Histology Group and component B into Favorable Histology Group.

For each of the two components observed in the case, fluorescent in situ hybridization (FISH) using the LSI *MYCN* (2p24) SpectrumGreen/CEP 2 Spectrum Orange Probe (Vysis) was performed on formalin-fixed, paraffin-embedded material, and *HA-RAS/TRKA* expression patterns were immunohistochemically examined. Component A exhibited *MYCN* amplification and low *HA-RAS/TRKA* expression, whereas component B demonstrated *MYCN* non-amplification and high *HA-RAS/TRKA* expression. In contrast, array-based comparative genomic hybridization profiles of two components demonstrated similar patterns for chromosomal events as a prognostic marker, including deletions of chromosomes 1p and 11q and gain of chromosome 17q. *MYCN* amplification was presented only in component A, which was the same as shown in FISH analysis. Both components were classified within the genetic group of partial chromosomal gains and/or losses that indicate poor disease prognosis [3].

Case 2. A 21-month-old male presented with right hip pain and gradual bulging of his eyes for several days. Abdominal CT showed an approximately 8-cm right adrenal mass. He was diagnosed with

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Conflict of interest: Nothing to declare.

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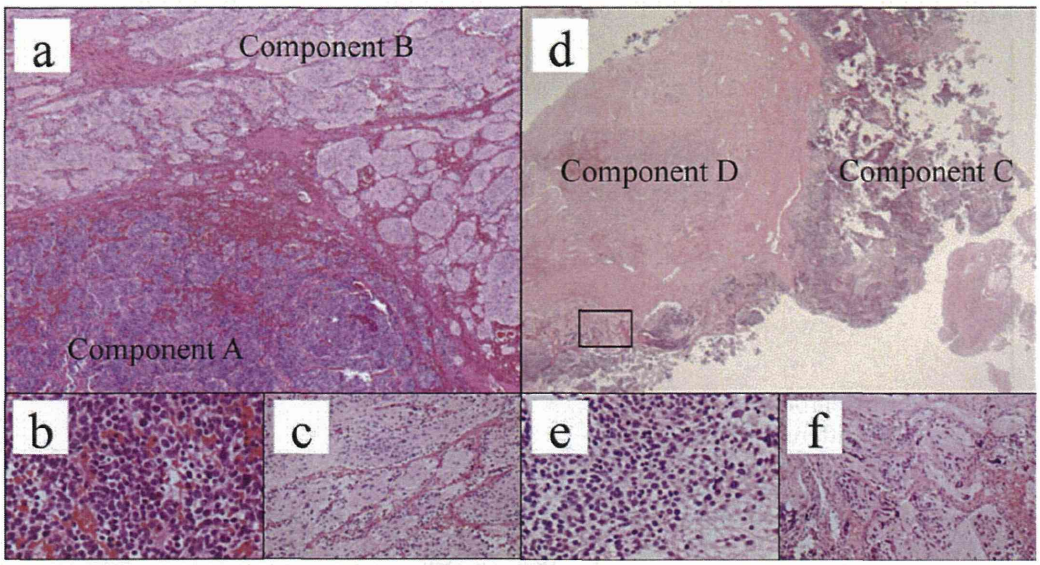

Fig. 1. **a**: The resected tumor in the left adrenal gland comprised two distinct components: component A (lower) and component B (upper). Case 1, H&E, 40×. **b**: Component A: neuroblastoma, a poorly differentiated subtype with a high mitosis-karyorrhexis index (MKI). H&E, 400×. **c**: Component B: neuroblastoma, a poorly differentiated subtype with a low MKI, more differentiated compared with component A.H&E, 400×. **d**: The tissue obtained at biopsy in the right adrenal gland comprised two distinct components: component C (right) and component D (left). Most Component D consisted of fibrous tissue with small foci of neuroblasts. A portion of Component D (square, Figure f) was classified as neuroblastoma, a differentiating subtype. Case 2, H&E, 40×. **e**: Component C: neuroblastoma, a poorly differentiated subtype with a high MKI. H&E, 400×. **f**: Component D: neuroblastoma, a differentiating subtype. H&E, 400×.

neuroblastoma following biopsy of the mass. Other sites of involvement included the lymph nodes, multiple bones, and bone marrow (INRG stage M). Because of the poor response to multidrug chemotherapy, a tandem transplantation was planned after abdominal tumor resection. Unfortunately, the patient died from sudden respiratory failure before the second transplantation.

Microscopically, the tissue obtained by biopsying the right adrenal gland before treatment comprised two neuroblastoma nodular components separated by a fibrillary matrix (components C and D in Fig. 1d). Component C was classified as neuroblastoma, a poorly differentiated subtype with a high MKI (Fig. 1e). Most of component D consisted of fibrous tissue with small foci of neuroblasts. A portion of component D was classified as neuroblastoma, a differentiating subtype. MKI could not be assessed because the number of neuroblastic cells was insufficient (<5,000 cells; Fig. 1f). Schwannian cells were not identified by S-100 immunostaining in both component C and D. Lymph nodes around the right adrenal tumor and bone marrow contained metastatic tumor cells similar in appearance to the cells of component C. According to INPC, component C was classified into Unfavorable Histology Group and component D into Favorable Histology Group. Both components exhibited *MYCN* non-amplification and high *HA-RAS/TRKA* expression. However, different patterns were observed in ALK expression analysis; components C and D showed high and low ALK immunoreactivity, respectively.

DISCUSSION

We present two cases of neuroblastoma comprising two histologically and biologically distinct neuroblastoma components. A similar case was previously reported by Sano et al. [4]; one component showed neuroblastoma, poorly differentiated subtype with a high MKI and *MYCN* amplification, whereas the other showed neuroblastoma, poorly differentiated subtype with a low MKI and *MYCN* non-amplification. INPC distinguished the two components into Unfavorable and Favorable Histology Groups. In addition to clear histological differences between the two components in Sano's case and our two cases, the components differed in *MYCN* amplification in Sano's case, *MYCN* amplification and *HA-RAS/TRKA* immunoreactivity in our Case 1, and ALK immunoreactivity in our Case 2. Similar to *MYCN* amplification and *Ha-ras/trkA* gene expression [5], ALK immunoreactivity is a significant predictor of disease prognosis [6,7]. These three cases indicate that the combination of two separate neuroblastoma components that histologically, immunohistochemically, and biologically vary depending on a case-by-case basis.

Our two cases were similar to tumors classified as GNBn in that they also showed multiple separated components (clones). Based on the similarity with GNBn, we propose describing the composite neuroblastomas reported here as neuroblastoma, nodular (NBn), as previously proposed by Sano et al. [4]. Nonaggressive components (ganglioneuroblastoma, intermixed or ganglioneuroma) of GNBn are classified as Favorable Histology in INPC and are considered to have differentiated from neuroblastoma based on the age-linked maturational sequence. If we can observe a GNBn tumor at an earlier age, the nonaggressive components may have the appearance of neuroblastoma with Favorable Histology in the first or second step of the age-linked maturational sequence, that is, poorly differentiated or differentiating neuroblastoma. This subtype is considered to correspond to NBn (Fig. 2).

Patients with GNBn can be divided into two prognostic subsets by applying the same age-linked morphological criteria used in

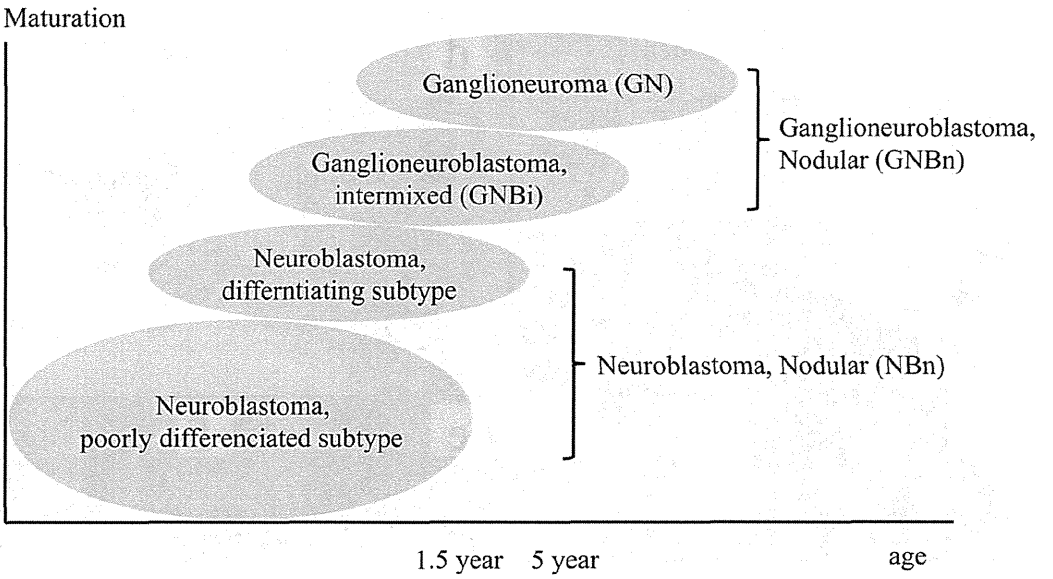

Fig. 2. The nonaggressive components classified as Favorable Histology of composite neuroblastic tumors (ganglioneuroblastoma, nodular (GNBn) and Neuroblastoma, nodular (NBn)) are considered to differentiate from poorly differentiated (<1.5 years of age at diagnosis) to differentiating (<5 years of age) neuroblastomas to ganglioneuroblastoma, intermixed and to ganglioneuroma based on the age-linked maturational sequence. Ganglioneuroblastoma, intermixed or ganglioneuroma are the feature of nonaggressive components in GNBn, while poorly differentiated or differentiating neuroblastoma are the feature of those in NBn.

neuroblastoma category to their neuroblastoma components: favorable and unfavorable subsets. The difference in estimated survival between these two subsets of GNBn patients is statistically significant [8]. Therefore, the INPC system was modified to account for this difference [9]. If the same principle is applied, NBn patient prognosis should depend on the components with unfavorable histology. Although histological and molecular intratumoral heterogeneity was previously reported in neuroblastoma with transition from one component to the other [10,11], two different tumor components (Favorable and Unfavorable Histology Groups) were clearly distinguishable and separately identifiable in NBn, including our cases. *MYCN* analysis by FISH with paraffin sections and immunohistochemical analysis for ALK were critical for precisely identifying the two histologically and biologically different neuroblastoma components. Further analyses of NBn patients are needed to assess disease prognosis by obtaining a sufficient amount of tumor tissues for precise phenotypic and genotypic evaluations.

REFERENCES

1. Shimada H, Ambros IM, Dehner LP, et al. The international neuroblastoma pathology classification (the Shimada system). Cancer 1999;86:364–372.
2. Shimada H, Ambros IM, Dehner LP, et al. Terminology and morphologic criteria of neuroblastic tumors: Recommendations by the International Neuroblastoma Pathology Committee. Cancer 1999;86:349–363.
3. Ohira M, Nakagawara A. Global genomic and RNA profiles for novel risk stratification of neuroblastoma. Cancer Sci 2010;101:2295–2301.
4. Sano H, Gonzalez-Gomez I, Wu SQ, et al. A case of composite neuroblastoma composed of histologically and biologically distinct clones. Pediatr Dev Pathol 2007;10:229–232.
5. Kyo Y, Tanaka T, Hayashi K, et al. Identification of therapy-sensitive and therapy-resistant neuroblastoma subtypes in stages III, IVs and IV. Cancer Lett 2011;1:27–33.
6. Duijkers FA, Gaal J, Meijerink JP, et al. High anaplastic lymphoma kinase immunohistochemical staining in neuroblastoma and ganglioneuroblastoma is an independent predictor of poor outcome. Am J Pathol 2012;180:1223–1231.
7. Passoni L, Longo L, Collini P, et al. Mutation-independent anaplastic lymphoma kinase overexpression in poor prognosis neuroblastoma patients. Cancer Res 2009;69:7338–7346.
8. Umehara S, Nakagawa A, Matthay KK, et al. Histopathology defines prognostic subsets of ganglioneuroblastoma, nodular. Cancer 2000;89:1150–1161.
9. Peuchmaur M, d'Amore ES, Joshi VV, et al. Revision of the international neuroblastoma pathology classification: Confirmation of favorable and unfavorable prognostic subsets in ganglioneuroblastoma, nodular. Cancer 2003;98:2274–2281.
10. Ambros PF, Ambros IM, Kerbl R, et al. Intratumoral heterogeneity of 1p deletions and MYCN amplification in neuroblastomas. Med Pediatr Oncol 2001;36:1–4.
11. Thomer PS, Ho M, Chilton-MacNeill S, et al. Use of chromogenic in situ hybridization to identify MYCN gene copy number in neuroblastoma using routine tissue sections. Am J Surg Pathol 2006;30:635–642.

Mutations of the *GATA2* and *CEBPA* genes in paediatric acute myeloid leukaemia

Hereditary *GATA2* mutations show predisposition to acute myeloid leukaemia (AML) and myelodysplastic syndrome (MDS) (Hahn *et al*, 2011). These mutations have also been reported in chronic myeloid leukaemia (Zhang *et al*, 2008) and monocytopenia and mycobacterial infection (MonoMAC) syndrome (Hsu *et al*, 2011). More recently, *GATA2* mutations have been identified in *de novo* AML, especially in adult patients with biallelic *CEBPA* mutations (Greif *et al*, 2012; Green *et al*, 2013). GATA2 and CEBPA are transcription factors that are crucial for haematopoietic development. These findings prompted us to identify possible *GATA2* and *CEBPA* mutations in patients with various paediatric leukaemias.

Direct Sequencing of *GATA2* was performed in 157 *de novo* AML patients, including 13 patients with acute promyelocytic leukaemia (APL; French–American–British type-M3) and 10 with Down syndrome (DS; Table S1), 22 secondary AML patients, 40 juvenile myelomonocytic leukaemia (JMML) patients, 50 acute lymphoblastic leukaemia (ALL) patients, 70 cell lines (25 B-cell precursor-ALL, 15 T-cell-ALL, 22 AML, and 8 neuroblastomas), and 60 healthy subjects. *GATA2* mutation analysis was performed by direct sequencing for all coding exons (exons 2–6) using an ABI PRISM 3130 Genetic Analyser (Applied Biosystems, Branchburg, NJ, USA) (Table S2). For AML patients, *CEBPA* and *NPM1* mutations were also examined. Mutational analyses of *FLT3*, *KIT*, *WT1* and *RAS* genes in our AML patients was performed as described previously (Shimada *et al*, 2006). Informed consent was obtained from the patients or the patients' parents according to guidelines based on the tenets of the revised Helsinki protocol. The institutional review boards of Gunma Children's Medical Centre approved this project.

GATA2 mutations were found in eight out of 157 AML patients (5·1%), including three APL patients (Fig 1A,B), but were absent in 18 patients with acute megakaryocytic leukaemia (FAB-M7; Table S3). Furthermore, there were no *GATA2* mutations in patients with other leukaemias, in the cell lines, or in the 60 healthy subjects, suggesting that *GATA2* mutations were indeed associated with leukaemogenesis in a subset of patients with *de novo* AML.

Germline *GATA2* mutations were also examined in five AML patients whose complete remission (CR) samples were available, and a germline mutation was identified in one patient. Furthermore, we performed *GATA2* mutation analyses of the patient's parents and two siblings, and identified the same *GATA2* mutations in her father (II-4) and brother (III-1) but not in her mother (II-5) or sister (III-2) (Fig 1C). Her father and brother lacked abnormalities in their full blood cell counts, lymphocyte subsets, or episodes of opportunistic infections. The proband experienced severe mycotic pneumonia during induction chemotherapy. Remarkably, she has been in CR for more than 11 years, despite discontinuation of chemotherapy. Three patients, for whom CR samples were not available, had no history of MonoMAC syndrome.

In addition, 16 *CEBPA* mutations (10·2%) and three *NPM1* mutations (1·9%) were found in 157 paediatric AML patients. Thirteen (81·3%) of 16 patients with *CEBPA* mutations had been in CR for more than 4 years, suggesting that *CEBPA* mutations may be associated with favourable outcomes. Although most *GATA2* mutations were found in patients with biallelic *CEBPA* mutations in adult AML (Greif *et al*, 2012; Green *et al*, 2013), only two of eight *GATA2* mutation-positive patients had monoallelic *CEBPA* mutations in this study (Table I).

We compared the clinical and molecular features between patients with and without *GATA2* mutations. However, there were no significant differences in terms of age, initial white blood cell count, gender, and cytogenetics (Table S3). Of the eight patients with *GATA2* mutations, one had a *WT1* mutation, one had a *KIT* mutation, and two patients had *RAS* mutations (Table I). *FLT3*-internal tandem duplication, *MLL*-partial tandem duplication, and *NPM1* mutations were not found in any patients with *GATA2* mutations (Table S3). All of the *GATA2* mutations were found in the intermediate risk subgroup or APL patients with t(15;17), whereas none were found in those with core-binding factor AML [i.e. t(8;21) and inv(16)]. *GATA2* mutations were found in two patients with 11q23 translocations, including t(11;19) and t(7;11), and three patients with complex chromosomal abnormalities, whereas most *GATA2* mutations were found in cytogenetically normal AML patients in previous reports (Table I) (Greif *et al*, 2012; Luesink *et al*, 2012).

GATA2 mutations were previously reported in patients with M1, M2, and M4 subtypes of AML (Greif *et al*, 2012; Luesink *et al*, 2012), which is in accordance with our results. *GATA2* mutations have not been previously reported in APL, but our study found these mutations in three APL patients. Of note, promyelocytic leukaemia protein has been shown to interact with GATA2 and potentiate its transactivation capacity (Tsuzuki *et al*, 2000).

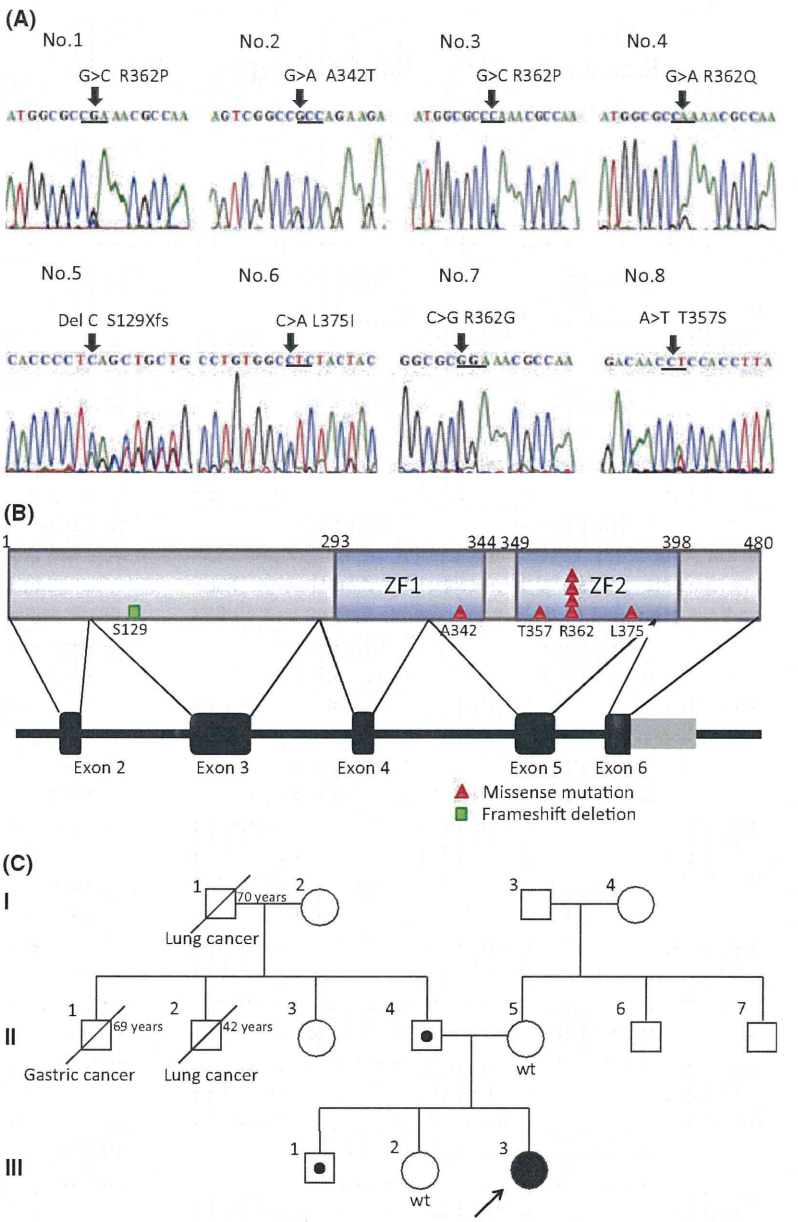

Fig 1. Identification of *GATA2* mutations by direct sequencing. (A) Eight *GATA2* mutations were identified in 157 Japanese paediatric *de novo* acute myeloid leukaemia (AML) patients (5·1%). Major missense mutations were R362 (R362P, R362Q, and R362G). Small vertical arrows indicate the mutated nucleotides. (B) Of the eight mutations, six mutations were identified in the ZF2 domain, one mutation was identified in the ZF1, and a mutation was identified on the outside of the ZF domain. (C) The family pedigree is shown. Squares indicate males and circles indicate females. The proband (III-3) is indicated by an arrow. The proband, her father (II-4), and her brother (III-1) harboured *GATA2* mutations (shown by squares containing dots). Her uncles and grandfather died of lung cancer (I-1 and II-2) and gastric cancer (II-1). wt, wild-type.

The outcomes of our patients with *GATA2* mutations was not poor (3-year overall survival and event free survival: 87·5%), which is in agreement with previous reports on *de novo* AML (Greif *et al*, 2012; Luesink *et al*, 2012): two of eight patients received autologous-stem cell transplantation (Auto-SCT), and one died of gastrointestinal haemorrhage after Auto-SCT. The remaining six patients who did not receive Auto-SCT were still alive (Table I).

In this study, one patient with a germline *GATA2* mutation developed AML. Her paternal grandfather (I-1) and second uncle (II-2) died of lung cancer at the age of 70 and 42 years, respectively, while her first uncle (II-1) died of gastric cancer at 69 years of age (Fig 1C). Increased GATA2 protein expression has been associated with biochemical recurrence and distant metastatic progression in prostate cancer (Böhm *et al*, 2009), as loss of GATA2 reduced the viability of Non-small cell lung cancer cells with RAS-pathway mutations, whereas wild-type cells were unaffected (Kumar *et al*, 2012). These facts indicate that GATA2 upregulation is strongly associated with maintenance of cancer cells. The association between *GATA2* mutations and solid tumours remains to be elucidated.

Our results indicate that *GATA2* mutations are associated with a favourable outcome in paediatric AML. Therefore, less aggressive treatment strategies without SCT may be

Table I. Clinical and molecular characteristics of patients with *GATA2* mutations.

Pt	Sex	Age (years)	FAB	WBC ($\times 10^9$/l)	Chromosome	Risk	Tx	Relapse	Prognosis (months)	*GATA2* mutation	Germline	Additional mutations
1	M	3	M4	23·8	46, XY, t(11;19) (q23; p13·1)	IR	Auto	Yes	16	R362P	N/A	–
2	F	7	M0	3·7	45,XX,add(3)(p13),del(6)(q?), der(8) t(3;8)(p21;q24), −13	IR	Chemo	No	+141	A342T	Yes	*NRAS*
3	F	8	M1	1·8	46, XX	IR	Chemo	No	+56	R362P	No	*KRAS*
4	M	14	M1	440·0	46XY [2/8], 46, XY, del(6) (q15 q21), −7, −9, −10, +3mar[1/8], 46, XY, ?del(3) (p25)[1/8], 47, XY, −5, −8, −10, add(12)(q24·1), −16, −18, +6mar [1/8], 46, XY, −2, −6, −8, +3mer [1/8], 46, XY, −8, +mar [1/8], 46, Y, ?add(X)(p11·2) [1/8]	IR	Auto	No	+51	R362Q	No	*WT1*, *CEBPA*-SM
5	M	11	M3	16·1	46,XX,inv(9)(p11q13),t(15;17)(q22;q11-21)	M3	Chemo	No	+50	S129X	N/A	–
6	M	3	M3	11·6	46,XY,t(15;17)(q22;q11?21)	M3	Chemo	No	+45	L375I	No	*CEBPA*-SM
7	M	10	M3	13·6	47,XY, +8, t(15;17)(q22;q11-21)	M3	Chemo	No	+41	R362G	N/A	*KIT*
8	F	2	M4	12·7	48, XX, +6, +10, t(11?; 7) (q23;q25)	IR	Chemo	No	+38	T357S	No	–

Pt, Patient; FAB, French–American–British classification; WBC, white blood cell count; Tx, Treatment; M, Male; F, Female; IR, Intermediate risk; Auto, Autologous stem cell transplantation; Chemo, Chemotherapy; N/A, not available; +, alive; SM, single mutation.

appropriate for paediatric AML patients with *GATA2* mutations, although most patients with *GATA2* mutations were classified into an intermediate risk group. Furthermore, the association between germline *GATA2* mutations and solid tumours remains to be elucidated.

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Author contributions

Y.H. designed the study; M.F., S.A., M.K., A.K., M.S., A.T., K.H. and I.T. collected patient samples and clinical data; N.S., K.O., M.-J.P., Y.M. and S.M. performed the laboratory research; N.S., M.-J.P. and Y.H. analysed and interpreted the data; N.S. performed the statistical analysis; N.S. and Y.H. wrote the manuscript; H.A. and Y.H. supervised the work; and all authors critically reviewed the manuscript and gave their final approval.

Conflicts of interest

The authors declare no competing financial interests.

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Supporting Information

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

Table S1. Clinical and cytogenetically characteristics of 157 AML patients.

Table S2. PCR primers used for mutation screening.

Table S3. Clinical and molecular characteristics of *GATA2* mutation positive patients.

References

Böhm, M., Locke, W.J., Sutherland, R.L., Kench, J.G. & Henshall, S.M. (2009) A role for GATA-2 in transition to an aggressive phenotype in prostate cancer through modulation of key androgen-regulated genes. *Oncogene*, **28**, 3847–3856.

Green, C.L., Tawana, K., Hills, R.K., Bödör, C., Fitzgibbon, J., Inglott, S., Ancliff, P., Burnett, A.K., Linch, D.C. & Gale, R.E. (2013) GATA2 mutations in sporadic and familial acute myeloid leukaemia patients with CEBPA mutations. *British Journal of Haematology*, **161**, 701–705.

Greif, P.A., Dufour, A., Konstandin, N.P., Ksienzyk, B., Zellmeier, E., Tizazu, B., Sturm, J., Benthaus, T., Herold, T., Yaghmaie, M., Dörge, P., Hopfner, K.P., Hauser, A., Graf, A., Krebs, S., Blum, H., Kakadia, P.M., Schneider, S., Hoster, E., Schneider, F., Stanulla, M., Braess, J., Sauerland, M.C., Berdel, W.E., Büchner, T., Woermann, B.J., Hiddemann, W., Spiekermann, K. & Bohlander, S.K. (2012) GATA2 zinc finger 1 mutations associated with biallelic CEBPA mutations define a unique genetic entity of acute myeloid leukemia. *Blood*, **120**, 395–403.

Hahn, C.N., Chong, C.E., Carmichael, C.L., Wilkins, E.J., Brautigan, P.J., Li, X.C., Babic, M., Lin, M., Carmagnac, A., Lee, Y.K., Kok, C.H., Gagliardi, L., Friend, K.L., Ekert, P.G., Butcher, C.M., Brown, A.L., Lewis, I.D., To, L.B., Timms, A.E., Storek, J., Moore, S., Altree, M., Escher, R., Bardy, P.G., Suthers, G.K., D'Andrea, R.J., Horwitz, M.S. & Scott, H.S. (2011) Heritable GATA2 mutations associated with familial myelodysplastic syndrome and acute myeloid leukemia. *Nature Genetics*, **43**, 1012–1017.

Hsu, A.P., Sampaio, E.P., Khan, J., Calvo, K.R., Lemieux, J.E., Patel, S.Y., Frucht, D.M., Vinh, D.C., Auth, R.D., Freeman, A.F., Olivier, K.N., Uzel, G., Zerbe, C.S., Spalding, C., Pittaluga, S., Raffeld, M., Kuhns, D.B., Ding, L., Paulson, M.L., Marciano, B.E., Gea-Banacloche, J.C., Orange, J.S., Cuellar-Rodriguez, J., Hickstein, D.D. & Holland, S.M. (2011) Mutations in GATA2 are associated with the autosomal dominant and sporadic monocytopenia and mycobacterial infection (MonoMAC) syndrome. *Blood*, **118**, 2653–2655.

Kumar, M.S., Hancock, D.C., Molina-Arcas, M., Steckel, M., East, P., Diefenbacher, M., Armenteros-Monterroso, E., Lassailly, F., Matthews, N., Nye, E., Stamp, G., Behrens, A. & Downward, J. (2012) The GATA2 transcriptional network is requisite for RAS oncogene-driven non-small cell lung cancer. *Cell*, **149**, 642–655.

Luesink, M., Hollink, I.H., van der Velden, V.H., Knops, R.H., Boezeman, J.B., de Haas, V., Trka, J., Baruchel, A., Reinhardt, D., van der Reijden, B.A., van den Heuvel-Eibrink, M.M., Zwaan, C.M. & Jansen, J.H. (2012) High GATA2 expression is a poor prognostic marker in pediatric acute myeloid leukemia. *Blood*, **120**, 2064–2075.

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.

Tsuzuki, S., Towatari, M., Saito, H. & Enver, T. (2000) Potentiation of GATA-2 activity through interactions with the promyelocytic leukemia protein (PML) and the t(15;17)-generated PML-retinoic acid receptor alpha oncoprotein. *Molecular and Cellular Biology*, **20**, 6276–6286.

Zhang, S.J., Ma, L.Y., Huang, Q.H., Li, G., Gu, B.W., Gao, X.D., Shi, J.Y., Wang, Y.Y., Gao, L., Cai, X., Ren, R.B., Zhu, J., Chen, Z. & Chen, S.J. (2008) Gain-of-function mutation of GATA-2 in acute myeloid transformation of chronic myeloid leukemia. *Proceedings of the National Academy of Sciences*, **105**, 2076–2081.

NUP98-NSD1 Gene Fusion and Its Related Gene Expression Signature Are Strongly Associated with a Poor Prognosis in Pediatric Acute Myeloid Leukemia

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The cryptic t(5;11)(q35;p15.5) creates a fusion gene between the *NUP98* and *NSD1* genes. To ascertain the significance of this gene fusion, we explored its frequency, clinical impact, and gene expression pattern using DNA microarray in pediatric acute myeloid leukemia (AML) patients. *NUP98-NSD1* fusion transcripts were detected in 6 (4.8%) of 124 pediatric AML patients. Supervised hierarchical clustering analyses using probe sets that were differentially expressed in these patients detected a characteristic gene expression pattern, including 18 *NUP98-NSD1*-negative patients (*NUP98-NSD1*-like patients). In total, a *NUP98-NSD1*-related gene expression signature (*NUP98-NSD1* signature) was found in 19% (24/124) and in 58% (15/26) of cytogenetically normal cases. Their 4-year overall survival (OS) and event-free survival (EFS) were poor (33.3% in *NUP98-NSD1*-positive and 38.9% in *NUP98-NSD1*-like patients) compared with 100 *NUP98-NSD1* signature-negative patients (4-year OS: 86.0%, 4-year EFS: 72.0%). Interestingly, t(7;11)(p15;p15)/*NUP98-HOXA13*, t(6;11)(q27;q23)/MLL-*MLLT4* and t(6;9)(p22;q34)/*DEK-NUP214*, which are known as poor prognostic markers, were found in *NUP98-NSD1*-like patients. Furthermore, another type of *NUP98-NSD1* fusion transcript was identified by additional RT-PCR analyses using other primers in a *NUP98-NSD1*-like patient, revealing the significance of this signature to detect *NUP98-NSD1* gene fusions and to identify a new poor prognostic subgroup in AML. © 2013 Wiley Periodicals, Inc.

INTRODUCTION

Acute myeloid leukemia (AML) is a complex disease caused by mutations, epigenetic modifications, and deregulated expression of genes, leading to increased proliferation and decreased differentiation of hematopoietic progenitor cells (Frohling et al., 2005; Marcucci et al., 2011; Pui et al., 2011). Several important molecular markers have been discovered in AML that have not only helped to characterize better patients, but also to improve risk stratification (Marcucci et al., 2011; Pui et al., 2011). However, in a subset of AML patients, no prognosis-associated cytogenetic aberrations or mutations are known (Frohling et al., 2005; Marcucci et al., 2011; Pui et al., 2011). In

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hematological malignancies, 11p15 translocations involving the nucleoporin 98-kDa (NUP98) protein gene are relatively rare. This notwithstanding, more than 20 different chromosomal rearrangements have been identified (Romana et al., 2006). These translocations fuse *NUP98* with respective partner genes, including many homeobox genes and nuclear non-homeobox genes (Arai et al., 1997; Taketani et al., 2002a,b,c). Although the *NUP98* fusion genes are rare, they have provided valuable information regarding the role of homeobox proteins in leukemogenesis (Nakamura et al., 1996; Nakamura, 2005).

The cryptic t(5;11)(q35;p15.5), which is frequently accompanied by deletion of the long arm of chromosome 5, del(5q), creates a fusion gene between *NUP98* and the nuclear receptor-binding SET-domain protein 1 (*NSD1*) gene (Jaju et al., 2001). This fusion gene has mainly been identified in pediatric AML, by the use of fluorescence in situ hybridization (FISH) with subtelomeric probes (Jaju et al., 2001; Brown et al., 2002; Panarello et al., 2002; Cerveira et al., 2003), it is rare in adult AML (Brown et al., 2002; Casas et al., 2003; Nebral et al., 2005; Walter et al., 2009). Recently, the *NUP98-NSD1* gene fusion, identified by high-resolution genome-wide copy number analysis and reverse transcription (RT)-PCR in pediatric and adult AML patients, was associated with poor prognosis (Hollink et al., 2011). To increase our understanding of this gene fusion, we explored the frequency, clinical significance, and gene expression pattern of *NUP98-NSD1* using DNA microarray in pediatric AML patients.

MATERIALS AND METHODS

Patients and Samples

From January 2000 to December 2002, 318 patients were diagnosed with de novo AML. The diagnosis of AML was based on the French-American-British (FAB) classification, and cytogenetic analysis was performed using conventional G-banding. Of these patients, samples from 124 patients with known mutation status and gene expression profiling data were available, including 10 patients with FAB-M3 and six patients with Down syndrome who were treated on different treatment protocols (Kudo et al., 2007; Tsukimoto et al., 2009; Imaizumi et al., 2011). Age and initial white blood cell (WBC) count were higher, patients with t(8;21) were more frequent, and M7 patients with Down syndrome were fewer in the present study cohort than in the non-analyzed patients (Supporting Information Table S1). There were no significant differences in survival between these two groups [4-year overall survival (OS): 76.6% versus 79.9% and 4-year event-free survival (EFS): 65.3% versus 71.1%]. Informed consent was obtained from the patients or the patients' parents, according to guidelines based on the tenets of the revised Helsinki protocol. The institutional review boards of Gunma Children's Medical Center and National Cancer Center approved this project.

Reverse Transcription-PCR and Sequence Analysis

Total RNA extracted from leukemic cells at diagnosis was reverse transcribed to cDNA with a cDNA Synthesis Kit (GE Healthcare, Tokyo, Japan). PCR was performed with AmpliTaq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ), using a DNA thermal cycler (Applied Biosystems). For the detection of *NUP98-NSD1* and the reciprocal fusion transcript, *NUP98*−5F and *NSD1*−1R, and *NSD1*−2F and *NUP98*−6R were used, respectively (Supporting Information Table S2) (Jaju et al., 2001; Brown et al., 2002). PCR conditions were as follows: initial denaturation at 94°C (9 min), 40 cycles of 96°C (45 sec), 58°C (45 sec), and 72°C (1 min), followed by final elongation at 72°C (7 min). For sequencing, PCR products were amplified using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) under the following conditions: 95°C (2 min) followed by 25 cycles of 95°C (10 sec), 50°C (5 sec), and 60°C (4 min). Direct sequencing was performed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). To confirm and identify the gene fusions precisely, we used various primer sets for RT-PCR followed by direct sequencing (Supporting Information Table S2). Mutations of *NPM1* were also examined as previously reported (Döhner et al., 2005). Mutation analyses of the *DNMT3A*, *FLT3*, *MLL*, *KIT*, *NRAS*, *KRAS*, and *WT1* genes have been reported previously (Shimada et al., 2006, 2008; Sano et al., 2012; Shiba et al., 2012).

Microarray Analysis

Gene expression profiling data for the 124 patients (Gene Expression Omnibus accession number, GSE35784) were obtained and analyzed as follows. Total RNA was re-purified using the RNeasy MinElute cleanup kit (Qiagen, Hilden,

Germany), and the integrity of the purified RNA was confirmed using a 2100 Bioanalyzer and an RNA 6000 Nano LabChip kit (Agilent Technologies, Santa Clara, CA). The DNA microarray used was a Human Genome U133 plus 2.0 array (Affymetrix, Santa Clara, CA). Target cRNA was prepared from 20 ng 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) of each probe set was 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 analyses and fold change calculations were performed using expression values that were log-transformed after addition of 10 to reduce adverse effects caused by noise at low expression levels (Ichikawa et al., 2006; Jo et al., 2009). To identify differentially expressed probe sets between *NUP98-NSD1*-positive and -negative patients, *P*-values in Student's *t*-test and fold change values were used. Hierarchical clustering analysis was performed using the Cluster and Tree View software (Eisen et al., 1998). For this analysis, log-transformed expression values were normalized for each probe set by subtracting the mean, and uncentered correlation metric and complete linkage clustering methods were used. Unsupervised clustering analyses was performed and visualized as previously described (Ichikawa et al., 2006).

Statistical Analysis

All analyses were carried out using the SPSS statistical package program (version 18.0J; SPSS Tokyo, Japan). Survival distributions were assessed using the Kaplan-Meier method and the differences were compared using the log-rank test. EFS and OS were defined as the times from diagnosis to event (relapse or death of any cause) and from diagnosis to death from any cause, respectively. Statistical analyses were performed using Fisher's exact test for categorical variables and Mann-Whitney's U test for continuous variables. For all analyses, the *P*-values were two-tailed, and $P < 0.05$ was considered significant. To identify independent predictors of poor prognosis, multivariate Cox regression analysis was performed using factors that were significant in frequencies between *NUP98-NSD1* signature positive and negative cases.

RESULTS

Detection of *NUP98-NSD1*-Positive Patients

We identified the *NUP98-NSD1* fusion transcript in 6 (4.8%) out of 124 Japanese pediatric AML patients. The reciprocal fusion transcript *NSD1-NUP98* was detected in five of the patients (Supporting Information Fig. S1A). Sequence analysis of the PCR products confirmed that *NUP98* and *NSD1* were fused in-frame, joining *NUP98* exon 12 with *NSD1* exon 6 (Supporting Information Fig. S1B) (Jaju et al., 2001; Brown et al., 2002; Panarello et al., 2002; Casas et al., 2003; Cerveira et al., 2003; Hollink et al., 2011). The reciprocal fusion products were also fused in-frame, joining *NSD1* exon 5 with *NUP98* exon 13 (Supporting Information Fig. S1C).

Detection of *NUP98-NSD1*-Like Patients by Gene Expression Analysis

To investigate the gene expression pattern characteristic of *NUP98-NSD1*-positive patients, we used gene expression profiling data on the 124 patients. In unsupervised hierarchical clustering analyses (Supporting Information Fig. S2), *NUP98-NSD1*-positive patients did not form a cluster, whereas patients with t(8;21)(q22;q22), t(15;17)(q22;q21), and inv(16)(p13;q22) formed unique clusters. Comparing the 6 *NUP98-NSD1*-positive patients with the other 118 patients resulted in the identification of 87 differentially expressed probe sets ($P < 0.001$, fold change ≥ 2) (Supporting Information Table S3). Fifty-one probe sets, including several *HOX* genes, were overexpressed, and 36 probe sets were underexpressed. Interestingly, in supervised hierarchical clustering analysis using those 87 probe sets, a relatively large cluster including 18 *NUP98-NSD1*-negative patients was found, not only a cluster of the 6 *NUP98-NSD1*-positive patients (Fig. 1). These 24 patients also formed a single cluster in similar supervised clustering analyses in which probe sets were selected by slightly different criteria (Supporting Information Fig. S3). The robustness in clustering suggests that these 24 patients have certain common features and that they constitute a distinct subtype. Thus, we termed this characteristic gene expression pattern the *NUP98-NSD1* signature. We also designated the 18 patients lacking the *NUP98-NSD1* gene fusion but displaying the *NUP98-NSD1* signature as *NUP98-NSD1*-like AML patients. Of the 24 patients displaying the *NUP98-NSD1* signature, a common overexpression of 3 *HOXB* genes (*HOXB3*,

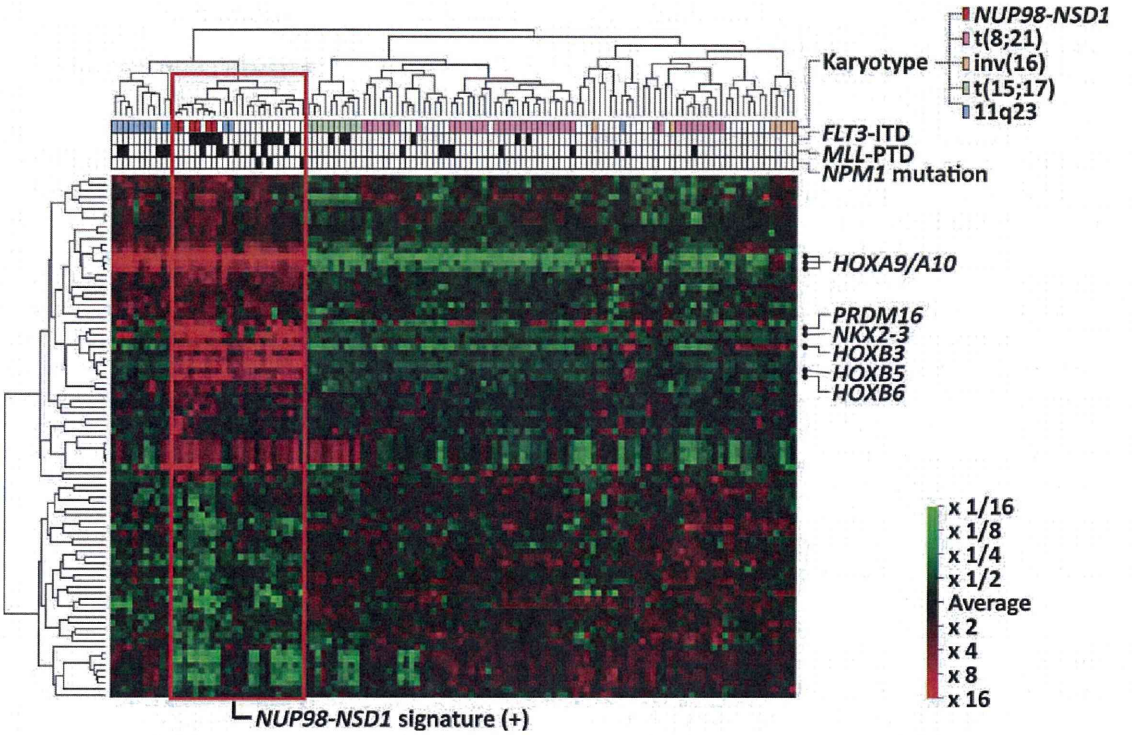

Figure 1. Supervised hierarchical clustering analysis of the 124 patients using differentially expressed genes between *NUP98-NSD1*-positive and -negative patients. Two-dimensional hierarchical clustering analysis of the 124 patients was performed using 87 probe sets that were differentially expressed between the *NUP98-NSD1*-positive and -negative patients (Table S3). Each column represents a patient and each row represents a probe set. The karyotype and the *FLT3*-ITD, *MLL*-PTD, and *NPM1* mutation status of each patient are indicated. Relative expression levels normalized to the average for each probe set are indicated by color, where red and green represent high and low expressions, respectively.

HOXB5, and *HOXB6*), another homeobox gene *NKX2–3*, a zinc finger transcription factor gene *PRDM16*, and a noncoding RNA gene *LOC404266* was particularly marked (Fig. 1). Although *HOXA9* and *HOXA10* were also overexpressed in these patients, their overexpression was shared by many patients with 11q23 abnormalities (Fig. 1).

Clinical and Molecular Characteristics of *NUP98-NSD1*-Positive and *NUP98-NSD1*-Like Patients

We compared the clinical and molecular features of the *NUP98-NSD1*-positive and *NUP98-NSD1*-like patients; there were no significant differences in terms of age, initial WBC count, gender, FAB subtypes, and cytogenetics (Table 1). Frequent gene mutations were also detected in both the *NUP98-NSD1*-positive and -like patients. All six *NUP98-NSD1*-positive patients and 16 *NUP98-NSD1*-like patients had one or several of the mutations analyzed (Table 2). *NUP98-NSD1*-positive patients frequently had class I aberrations, such as *FLT3*-ITD (4/6), and *NRAS* (1/6), *KRAS* (1/6), and *KIT* (1/6) mutations; however, no class II aberrations (*NPM1* or *MLL*-PTD) were detected in *NUP98-NSD1*-positive patients. On the other hand, *NUP98-NSD1*-like patients frequently had class II aberrations [*MLL*-PTD (7/18) and *NPM1* (3/18)] in addition to class I aberrations [*FLT3*-ITD (8/18), *KRAS* (3/18), and *KIT* (2/18)] (Table 2). Furthermore, we identified *DEK-NUP214*, *NUP98-HOXA13* and *MLL-MLLT4* fusion genes, which were generated by t(6;9)(p22;q34), t(1;7;11)(q32;p15;p15), and t(6;11)(q27;q23), respectively, in *NUP98-NSD1*-like patients (Table 2). In addition, we identified another type of *NUP98-NSD1* fusion transcript using another forward primer of *NUP98*-F', located on the 5′ side further from the known *NUP98* junction point (Supporting Information Table S2), in a *NUP98-NSD1*-like patient (Supporting Information Fig. S4).

Clinical and Molecular Characteristics of *NUP98-NSD1* Signature-Positive Patients

When compared with 100 *NUP98-NSD1* signature-negative patients, age (median 8.8 years vs.

TABLE 1. Clinical and Molecular Characteristics of the *NUP98-NSD1*-Positive and *NUP98-NSD1*-Like Patients

	NUP98-NSD1-positive	*NUP98-NSD1*-like	P-value
Total (n = 24)	6	18	
Age (range), y	7.2 (2–15)	9.3 (0–15)	0.28
Mean WBC ($\times 10^9$/l) (range)	115.9 (9.0–329.0)	60.5 (2.3–255.0)	0.31
Gender			0.34
Male	5 (83.3%)	9 (50.0%)	
Female	1 (16.7%)	9 (50.0%)	
FAB subtype			
M0	0 (0.0%)	1 (5.6%)	
M1	1 (16.7%)	4 (22.2%)	
M2	0 (0.0%)	2 (11.1%)	
M3	0 (0.0%)	0 (0.0%)	
M4	2 (33.3%)	6 (33.3%)	
M5	2 (33.3%)	4 (22.2%)	
M6	0 (0.0%)	0 (0.0%)	
M7	0 (0.0%)	1 (5.6%)	
Unclassified	1 (16.7%)	0 (0.0%)	
Cytogenetics			
Normal	4 (66.7%)	11 (61.1%)	1.00
t(8;21)(q22;q22)	0 (0.0%)	0 (0.0%)	1.00
t(15;17)(q22;q12)	0 (0.0%)	0 (0.0%)	1.00
inv(16)(p13q22)	0 (0.0%)	0 (0.0%)	1.00
abnormal 11q23	0 (0.0%)	2 (11.1%)	1.00
del(9q)	2 (33.3%)	0 (0.0%)	0.054
Others	0 (0.0%)	5 (27.8%)	0.28
Gene mutations			
FLT3-ITD	4 (66.7%)	8 (44.4%)	0.64
DNMT3A	0 (0.0%)	0 (0.0%)	1.00
NPM1	0 (0.0%)	3 (16.7%)	0.55
KIT	1 (16.7%)	2 (11.1%)	1.00
NRAS	1 (16.7%)	0 (0.0%)	0.25
KRAS	1 (16.7%)	3 (16.7%)	1.00
WT1	2 (33.3%)	3 (16.7%)	0.57
MLL-PTD	0 (0.0%)	7 (38.9%)	0.13

Abbreviations: y, years; WBC, white blood cell count; FAB,French-American-British subtype.

6.7 years; $P = 0.069$) and initial WBC count (median 74.4 $\times$ 10^9/ L vs. 49.7 $\times$ 10^9/ L; $P = 0.025$) were higher in the 24 *NUP98-NSD1* signature-positive patients. They frequently had a normal karyotype (62.5%) or del(9q) (8.3%), but did not have the favorable chromosomal translocations t(8;21), t(15;17), and inv(16) (Tables 2 and 3). The frequencies of the M4 and M5 subtypes (14/24; $P < 0.001$) were higher than in *NUP98-NSD1* signature-negative patients.

Prognosis of *NUP98-NSD1*-Positive and *NUP98-NSD1*-Like Patients

Both the 4-year OS and EFS were 33.3% in *NUP98-NSD1*-positive patients and 38.9% in *NUP98-NSD1*-like patients, which is significantly worse than for those with *NUP98-NSD1* signature-negative patients (86.0% in OS and 72.0% in EFS; Figs. 2A and 2B). Five of the six *NUP98-NSD1*-positive and 8 of the 18 *NUP98-NSD1*-like patients received allogeneic-stem cell transplantation (allo-SCT) (Table 2). Of the *NUP98-NSD1*-positive patients, only two of the three who underwent allo-SCT in first CR are still alive and, in the *NUP98-NSD1*-like patients, all 4 who received SCT in first CR and 3 of 10 who were treated with only chemotherapy are still alive without relapse. All 12 relapsed patients and two who did not achieve CR died (Table 2). Multivariate Cox regression analysis of OS was used to construct a model including the *NUP98-NSD1* signature, *FLT3*-ITD, *WT1*, *NPM1*, t(8;21), del(9q), and initial WBC, which were statistically significant in univariate analysis. In this model, the *NUP98-NSD1* signature and *FLT3*-ITD were independent poor prognostic factors (Table 4). Among the 24 patients displaying the *NUP98-NSD1* signature, the outcome of the 12 *FLT3*-ITD-positive patients was worse than that of the 12 *FLT3*-ITD-negative