TABLE 4. Affected Joints and Clinical Course of Patients With ON

Cases	Affected Lesion	Corticosteroids	Management	Outcome
941-1	Right hip	Reduced	Avoidance of weight-bearing	Improve
2	Bilateral hips	Continue	Prohibit hard exercise	Stable
3	Bilateral hips	Continue	Avoidance of weight-bearing	Improve
4	Bilateral hips	Reduced	Avoidance of weight-bearing	Improve
2000-1	Left hip	Continue	Bracing	Improve
2	Bilateral knees, right talus	Withhold	Observation	Improve
2004-1	Bilateral hips	Withhold	Avoidance of weight-bearing	Stable
2	Bilateral hips	Withhold	Observation	Improve
3	Bilateral knees	Continue	Surgery	Improve
4	Left femur	Continue	Avoidance of weight-bearing	Improve
5	Right femur	Continue	Avoidance of weight-bearing	Improve
6	Bilateral hips and knees	Continue	Observation	ALL relapse
7	Right knee	Continue	Observation	Improve
8	Right knee	Withhold	Surgery	Improve
9	Bilateral hips and knees	Reduced	Bisphosphonate	Stable
10	Bilateral knees	Continue	Surgery	Improve

ALL indicates acute lymphoblastic leukemia; ON, osteonecrosis.

ALL2004, indicating DEX exposure was the risk for ON in ALL chemotherapy.

The overall incidence of ON was 1.5% (16/1095), which was comparable with that in a previous study by the Japan Association of Childhood Leukemia Study (JACLS) (2.4%, Hiroki H, Yasushi I, Teruaki H, Makoto Y, Megumi O, Tooru K, Shinichiro N, Junichi H, Keizo H, Keiko Y, and Tatsutoshi N; unpublished data). In studies from Europe and the United states, the ON incidence was highly variable (1% to 2% up to 9%) and dependent on patient characteristics and treatment intensity.[5–7] Furthermore, the detection methods of ON have significantly affected the incidence. Recent report from St Jude Total XV study showed that 17.6% of patients had the symptomatic ON, whereas the asymptomatic ON was detected in > 50% of patients by the prospective screening with MRI test.[15] With regard to the effects of race, the incidence of ON is reportedly higher in whites than in patients of African descent.[7] Although it remains unclear whether the Asian race is related to an increased risk of ON, our results showed that the incidence of ON in Japanese children seemed to be comparable with that in European and American children. However, it should be taken into account the limitation of the present assessment: the possible missing of asymptomatic cases and the diagnosis partly depending on physician's discretion.

In this study, female sex, age 10 years and above, and the use of DEX as a corticosteroid were significant risk factors for ON. Of the 33 female patients aged over 10 years who received DEX, 7 developed ON (cumulative incidence, 25.6%). This was the extremely higher incidence of ON comparing with the rest of patients. Although females were found to be at a higher risk of developing ON in the Children's Cancer Study Group (CCG) and Italian studies,[5,7] there was no such correlation in studies performed in the UK and Germany and at the Dana Farber Cancer Institute (Boston, MA).[6,16,17] In addition, a Japanese study conducted by the JACLS failed to show a significant female predominance (male to female ratio, 7:9). Therefore, the effects of sex on ON pathogenesis remain unclear.

A significant contribution of age to ON onset has been robustly documented by most retrospective and prospective studies.[5–7,9,16,18–21] Among children aged 10 years and above, those aged 16 to 20 years were at the highest risk of ON. The eligible patient age was 1 to 15 years in ALL941/2000 and 1 to 18 years in ALL2004; therefore, we may have underestimated the incidence of ON. Further monitoring is necessary when ALL treatment protocols designed for children are extended to adolescence and young adulthood.

The potential effect of DEX on ALL is 6.5 times that of PSL, resulting in an increase in the use of DEX for ALL treatment. Because DEX is more toxic to bone tissues,[14,22] a higher incidence of ON has been a major concern in the design of treatment protocols. In ALL2004, DEX was incorporated only in the reinduction phase because an increased incidence of ON and mortality was reported with the use of DEX in the induction phase.[23] Nonetheless, our data revealed a higher cumulative incidence of ON associated with DEX administration; this finding was comparable with the results of the Dana Farber Consortium study DFCI 00-01, wherein DEX was used in postremission intensification therapy and/or in the maintenance phase.[24] Although the total corticosteroid dose (analyzed as PSL equivalents) at therapy completion were slightly lower in ALL2004 than in ALL941/2000 (Table 2), ON was most frequent in patients who had received only DEX in the HR group in ALL2004. These results suggest that DEX administration at any dose (as PSL equivalents) and in any treatment phase affects the incidence of ON. A recent report from the CCG found that DEX administration could influence the risk of ON[21] and that alternate-week DEX administration during delayed intensification therapy decreased ON incidence compared with continuous DEX. In our ALL2004 protocol, DEX was administered continuously for 2 weeks, and it would have been beneficial to modify the DEX schedule from continuous administration to alternate-week administration.

Recently, biological and genetical basis for ON development has been extensively investigated. Children's Oncology Group tested 12 polymorphisms of candidate genes and identified children with *PAI-1* GA/AA genotypes were significantly associated with ON.[25] Another study from St Jude Children's Research Hospital showed polymorphisms of *ACP1* were associated with risk of

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symptomatic ON as well as with lower serum albumin and higher cholesterol levels.[15] These results suggest that some patients are prone to develop ON and individualized therapy should be needed in the future ALL studies.

In the present report, cases with ON were retrospectively collected by the questionnaire, and most of the ON patients were identified by symptoms and confirmed with imaging studies (x-ray/MRI) without central review. Despite such limitations, the clinical features of all 16 ON patients in our study were virtually comparable with those of patients in previous studies.[6,7,16] Weight-bearing joints were commonly affected, whereas asymptomatic lesions might have been overlooked.[15] Once ON is confirmed, the physician must decide whether steroids should be withheld or continued, considering that no consensus guideline is available thus far. Most of our patients were prescribed a planned dose of steroids without compromising functional outcomes after ON development. We believe that it may not be necessary to withhold steroids at the risk of leukemia relapse.

Bisphosphonates, which are structurally similar to pyrophosphates, inhibit osteoclast activity and bone turnover, thus exerting beneficial effects on bone mineralization.[26] Alendronate, a third-generation bisphosphonate, is reportedly effective in the prevention of femoral head collapse in ON patients.[27] Wiernikowski et al[28] showed that alendronate-induced changes in bone mineral metabolism/homeostasis benefited bone mineralization in children with ALL or non-Hodgkin lymphoma with steroid-induced osteopenia. Another bisphosphonate, pamidronate, was shown to be effective in the management of pain and motor function recovery in symptomatic ON occurring in children with ALL.[29] In the present study, alendronate was administered to 1 patient with symptomatic ON of the bilateral hip and knee joints; this resulted in no further deterioration of functional outcome and no treatment-induced side effects. However, further studies are required to clarify the potential benefits of concomitant bisphosphonate and steroid use for ON treatment.

In summary, the overall incidence of ON was 1.5% in the JCCLSG ALL studies, which was comparable with that reported in previous studies conducted in the Unites States and Europe. The known risk factors of age above 10 years, female sex, and DEX use were all significantly associated with an increase in the cumulative incidence of ON. In our future studies, we are intending to routinely screen for ON development with MRI test, especially those incorporating DEX in the treatment protocol. Although an ON management regimen remains to be established, steroids should not be withheld at the risk of ALL relapse.

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IKZF1 and *CRLF2* Gene Alterations Correlate With Poor Prognosis in Japanese *BCR-ABL1*-Negative High-Risk B-Cell Precursor Acute Lymphoblastic Leukemia

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Background: Genome-wide analysis studies have demonstrated that *IKZF1*, *CRLF2*, and *JAK2* gene alterations correlate with poor prognosis in pediatric B-cell precursor acute lymphoblastic leukemia (BCP-ALL). However, the prognostic significance for these gene alterations has not been clarified in Japanese patients. ***Procedure:*** A total of 194 patients with BCP-ALL enrolled in the Japanese Children's Cancer & Leukemia Study Group ALL 2004 clinical trial were assessed for the presence of three different gene alterations: *IKZF1* deletions, *CRLF2* expression and *JAK2* mutation. ***Results:*** *IKZF1* deletions and *CRLF2*-high expression were identified in 22 of 177 (12%) patients and in 15 of 141 (11%) patients, respectively. However, *JAK2* R683 mutation was detected only one of 177 patients. The 4-year event-free survival (4y-EFS) was different when comparing patients with or without *IKZF1* deletions (68.2% vs. 85.2%; $P = 0.04$) and was also different when comparing patients with different *CRLF2* expression levels (high, 66.7% vs. low, 88.1%; $P = 0.03$). The differences in 4y-EFS were statistically significant in patients with ALL in the National Cancer Institute (NCI)-high risk group (HR-ALL) (*IKZF1* deletions: yes, 58.3% vs. no, 87.0%, $P = 0.02$; *CRLF2* expression: high, 55.6% vs. low, 85.3%, $P = 0.04$) but not in patients with ALL in the NCI-standard risk group (SR-ALL; *IKZF1* deletions: yes, 80.0% vs. no, 84.4%, $P = 0.75$; *CRLF2* expression: high, 83.3% vs. low, 89.2%, $P = 0.77$). Coexistence of *IKZF1* deletions and *CRLF2*-high expression associated with poor outcomes. ***Conclusions:*** *IKZF1* deletions and *CRLF2*-high expression predicted poor outcomes in patients with HR-ALL but not in patients with SR-ALL in our Japanese cohort. Pediatr Blood Cancer 2013;60:1587–1592. © 2013 Wiley Periodicals, Inc.

Key words: acute lymphoblastic leukemia; *CRLF2*; *IKZF1*; *JAK2*

INTRODUCTION

Improvements in overall survival in patients with pediatric B-cell precursor acute lymphoblastic leukemia (BCP-ALL) have been achieved via the institution of risk-adapted multi-agent chemotherapy [1]. However, about 20% of patients still show persistent disease or experience relapse. This observation underscores the need for a better understanding of the pathophysiology of the disease as well as the identification of factors that predict outcomes or predict the response to specific therapies.

Findings from genome-wide analysis have demonstrated that alterations in *IKZF1*, which encodes the lymphoid transcription factor, IKAROS, are prevalent in patients with *BCR-ABL1*-positive ALL [2,3]. *IKZF1* alterations have also been demonstrated in patients with high-risk *BCR-ABL1*-negative ALL and are associated with poor prognosis [4–10]. Further, Mullighan et al. [11] identified Janus kinases (*JAKs*) mutations in approximately 10% of the *BCR-ABL1*-negative subgroup and reported that these mutations were associated with *IKZF1* alterations. Recent studies have also revealed that increased expression of *CRLF2*, which is predominantly caused by fusion of *P2RY8-CRLF2* or *IGH-CRLF2*, was found in approximately 5–10% of patients with high-risk ALL and in 50–60% of patients with Down syndrome-associated ALL [12–14]. Alterations in *CRLF2* often coexist with alterations in *IKZF1* and/or *JAK2*, and these gene alterations are associated with poor outcomes [15–17]. However, the prognostic significance for these gene alterations has not been clarified in Japanese patients. Therefore, the incidence and clinical significance of *IKZF1* deletions, *CRLF2* expression and *JAK2* mutations were assessed in Japanese pediatric patients with *BCR-ABL1*-negative BCP-ALL in this study.

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MATERIALS AND METHODS

Patients and Samples

A total of 194 patients were selected from 264 pediatric *BCR-ABL1*-negative BCP-ALL patients who were enrolled in the Japanese Children's Cancer & Leukemia Study Group (JCCLSG) ALL 2004 clinical trial from 2004 to 2008. One hundred seventy-seven DNA and 141 RNA samples were available and extracted from total bone marrow (BM) or peripheral blood (PB) at the time of diagnosis. These samples contained over 50% (median, 95%; range, 53.3–100%) blasts. The analyzed cohort included 131 patients with ALL classified as NCI-SR and 63 patients classified as NCI-HR. Treatment stratification in this clinical trial

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was based on age and white blood cell (WBC) count; patients with HR-ALL were treated with a HR- or very-high-risk regimen, and patients with SR-ALL were treated with a SR-regimen, except for 10 patients who were treated with intensified chemotherapy due to positivity for minimal residual disease. There were no statistical differences in the clinical characteristics (e.g., age, initial WBC, gender, and cytogenetic abnormalities) when comparing the analyzed cohort and original cohort (Supplementary Table I). The median (range) follow-up period from diagnosis was 6.0 (0.1–8.2) years. DNA samples from three healthy donors and from seven patients with solid tumor that were free from BM invasion were used as controls. This study was approved by the institutional review board at Nagoya Medical Center. Informed consent to participate in this study was obtained from patients and their guardians.

Genetic Analysis

The multiplex ligation-dependent probe amplification (MLPA) method (IKZF1 P-335, MRC-Holland, Amsterdam, NL) was used to detect *IKZF1* deletion, according to the manufacturer's instructions [18]. A total of 60 ng of DNA was used per reaction. Fragment analysis was performed using GeneScan v.3.5 (ABI310, Applied Biosystems, Foster City, CA). A probe ratio below 1.3 was considered indicative of deletion, as per the manufacturer's instructions [19]. For detection of *Ik6* and *Ik10* of *IKZF1* and the *P2RY8-CRLF2* fusion gene transcript, cDNA synthesis was performed using SuperScript II (Invitrogen Corporation, Carlsbad, CA) with 1 μg of RNA per 20 μl reaction with random primer (Invitrogen), and reverse transcription polymerase chain reaction (RT-PCR) was performed with following primers; *IKZF1* fwd, 5'-CTCCGAGGTTGCTCTT; *IKZF1* rev.: 5'-AGGTAGTT-GATGGCGTTGTTGATG; *P2RY8-CRLF2* primers were as previously reported [12]. To measure *CRLF2* mRNA levels, real-time quantitative (RQ)-PCR was performed using the TaqMan Gene Expression Assay (*CRLF2*, Hs00845692_m1; *GAPDH*, #4310884E, Applied Biosystems). RQ-PCR was performed in duplicate, using 1 μl of cDNA per reaction. The comparative C_t method was used to quantify relative mRNA levels using the endogenous control gene, *GAPDH*. To detect *JAK2* mutations, exon 12, 16, 20, and 21 were amplified and directly sequenced by Sanger sequencing with the ABI310 sequencing system, as previously reported [20].

Statistical Analysis

Descriptive statistical analyses to assess baseline characteristics of patients diagnosed with *BCR-ABL1*-negative BCP-ALL were performed. Event-free survival (EFS) and relapse-free interval (RFI) were analyzed by the Kaplan–Meier method [21], and log-rank tests [22] were used for group comparisons. EFS was defined as the time from the diagnosis to induction failure, relapse, or death from any cause, whichever occurred first. RFI was estimated for patients who achieved complete remission (CR). Cox proportional hazards regression models [23] were used to investigate factors associated with survival in univariate and multivariate analysis. A two-sided *P*-value of more than 0.05 should be interpreted with care. All data analysis was performed using SAS statistical software (version 9.1.3; SAS Institute, Inc., Cary, NC).

Results

Frequencies of *IKZF1, CRLF2,* and *JAK2* Alterations in BCP-ALL

IKZF1 deletions were detected in 22 (12%) of 177 DNA samples, and various deletion patterns were detected by MLPA (Supplementary Table II). Homozygous deletion was not detected. To confirm the results of MLPA, RT-PCR was performed for six patients whose RNA was available. The isoform type in the cases with the deletion of *IKZF1* exon 4–7 and 2–7 was confirmed to be the *Ik6* and *Ik10* isoform variants, respectively (Supplementary Fig. S1). *IKZF1* deletions were significantly associated with older age ($P < 0.01$) and NCI-HR ($P = 0.02$; Supplementary Table SIII). However, no association was determined between *IKZF1* deletions and any known chromosomal abnormalities.

CRLF2 expression was measured by RQ-PCR in 141 RNA samples. The median expression value was 13.8 copies (range: 0.07–35,100). Fifteen (10%) samples showed *CRLF2* expression that was ≥10-fold of the median value (Fig. 1A). The clinical features of patients with high *CRLF2* expression are shown in Supplementary Table SIII. High *CRLF2* expression was more prevalent in patients with HR-ALL (9/43, 21%) than in patients with SR-ALL (6/98, 6%; $P < 0.01$). *P2RY8-CRLF2* fusion was detected in five of 141 patients. Two of these five patients had high *CRLF2* expression, while the other three patients had low *CRLF2* expression (Fig. 1B). Sequencing of predominant fusion transcripts demonstrated that the non-coding exon 1 of *P2RY8* bound to the start of *CRLF2* exon 1 in all five patients (Fig. 1C) [15]. In addition, transcript variants were demonstrated in three patients. One of the clones of unique patient number (UPN) 035, 099, and 219 showed *P2RY8* exon 1 fused to *CRLF2* exon 2. Sequencing of another clone of UPN219 demonstrated that *P2RY8* exon 1 bound to the 34 bp upstream sequence of *CRLF2* exon 1 (Fig. 1C).

In contrast, a *JAK2* R683 mutation was demonstrated in only one of the 177 patients. In this case, *P2RY8-CRLF2* fusion transcript, high *CRLF2* expression and *IKZF1* deletion were also recognized. Furthermore, the patient failed to achieve remission after induction therapy. We further analyzed *JAK2* exons 12, 20, and 21 in 15 patients with high *CRLF2* expression, but no mutation was detected except for a single nucleotide polymorphism (rs10974955) in two patients.

IKZF1 and *CRLF2* Alterations Are Associated With Poor Outcomes in Patients With HR-ALL

In survival analysis, the 4-year EFS was significantly lower for patients with *IKZF1* deletions than for patients without *IKZF1* deletions ($68.2 \pm 9.9\%$ vs. $85.2 \pm 2.9\%$; $P = 0.04$; Fig. 2A). Interestingly, the difference in this parameter was statistically significant in patients with HR-ALL ($58.3 \pm 14.2\%$ vs. $87.0 \pm 5.0\%$; $P = 0.02$) and not in patients with SR-ALL ($80.0 \pm 12.7\%$ vs. $84.4 \pm 3.5\%$; $P = 0.75$; Fig. 2B). Similarly, 4-year EFS for the patients with high *CRLF2* expression was also significantly worse than that for those with low *CRLF2* expression ($62.7 \pm 12.1\%$ vs. $88.1 \pm 2.9\%$; $P = 0.03$, Fig. 2C), and a statistical difference between these groups was recognized only in patients with HR-ALL ($55.6 \pm 16.6\%$ vs. $85.3 \pm 6.1\%$; $P = 0.04$ for HR; $83.3 \pm 15.2\%$ vs. $89.2 \pm 3.3\%$; $P = 0.77$ for SR, Fig. 2D). Similar findings for *IKZF1* and *CRLF2* were noted in the analysis for relapse-free interval (RFI).

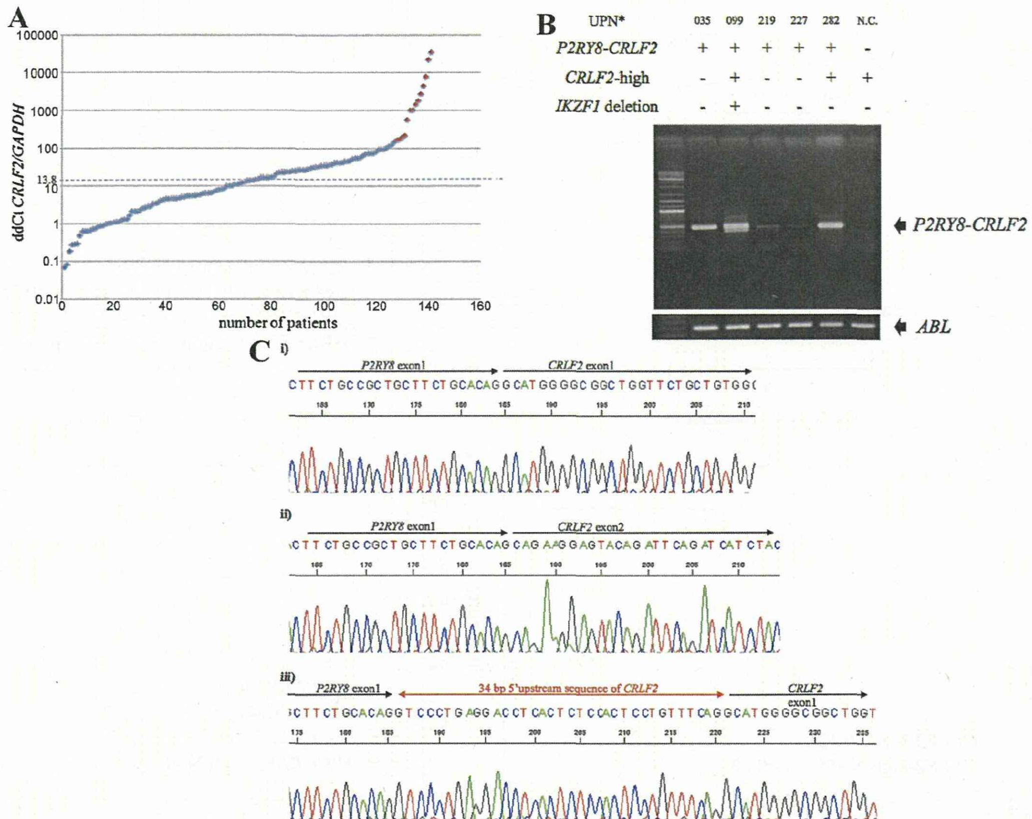

Fig. 1. Assessment of *CRLF2* expression and alterations in *CRLF2*. **A**: Measurement of *CRLF2* expression by RQ-PCR. The median *CRLF2* expression value (normalized to *GAPDH* expression) was 13.8. Red diamonds represent high *CRLF2* expression, defined as *CRLF2* expression that was ≥10-fold higher than the median *CRLF2* expression. Blue diamonds represent low *CRLF2* expression. **B**: *P2RY8-CRLF2* rearrangement detected by RT-PCR. The level of *ABL* transcription served as control. NC indicates negative control. Samples from five patients showed *P2RY8-CRLF2* fusion by RT-PCR. Two of the five samples also showed high *CRLF2* expression, and the remaining three samples showed low *CRLF2* expression. Interestingly, only one patient showed *P2RY8-CRLF2* fusion, *CRLF2* high expression and *IKZF1* deletion. **C**: Sequence results of *P2RY8-CRLF2* fusion. Representative sequences are shown. (i) The major sequence of all samples shows that the 3' end of non-coding *P2RY8* exon 1 bound to the 5' end of *CRLF2* exon 1. Sequence of No. 035 is shown as a representative patient. (ii) One of the No. 035 clones showed that the 3' end of non-coding *P2RY8* exon 1 bound to the 5' end of *CRLF2* exon 2 (skipped *CRLF2* exon 1). This variant was also found in No. 099 and No. 219. (iii) Thirty-four base pair upstream sequence of *CRLF2* exon 1 fused to *P2RY8* exon 1. This fusion was found in No. 219. *UPN indicated unique patient number.

Among the 124 patients whose samples were analyzed for both the *IKZF1* and *CRLF2* genes, five patients had ALL with *IKZF1* deletions and high *CRLF2* expression, simultaneously. All five patients were classified as NCI-HR, and four of the five patients experienced induction failure or relapse. In Kaplan–Meier analysis, EFS was the lowest among patients with ALL and coexisting *IKZF1* deletions and high *CRLF2* expression when compared with other categories of patients (Supplementary Fig. S3).

In comparison with other known prognostic factors in the full cohort and in the NCI-HR cohort, *IKZF1* deletions and high *CRLF2* expression were significant predictors of outcomes in univariate analysis (Table I). However, no variables retained independent prognostic significance in multivariate analysis.

DISCUSSION

Despite recent improvement in outcomes for patients with pediatric BCP-ALL, the genetic pathophysiology of the failure to respond to therapy or the occurrence of relapse remains unclear. *IKZF1*, *CRLF2*, and *JAK2* gene alterations are prognostic factors in patients with pediatric BCP-ALL [4–10,15–17,25]; therefore, we assessed for the presence of these genetic alterations in Japanese patients with *BCR-ABL1*-negative BCP-ALL.

IKZF1 deletions were found in 12% of our cohort (8% of NCI-SR, and 21% of NCI-HR), which is consistent with observations from previous reports (approximately 10–20%) [6–10,15]. Previous studies have reported that *IKZF1* deletions significantly correlated with poor relapse-free survival (RFS). Chen et al. reported that *IKZF1* deletions/mutations retained independent prognostic significance in multivariate analysis in their full cohort and were associated with poor RFS only in NCI-HR patients [9]. In our study, *IKZF1* deletions were significantly associated with outcome in univariate analysis, but not in multivariate analysis within either our full cohort or the NCI-HR cohort. Mi et al. [10] reported that the *Ik6* variant correlated with poor prognosis. In our study, *Ik6* variant was detected in only one-third of *IKZF1*-deletion patients

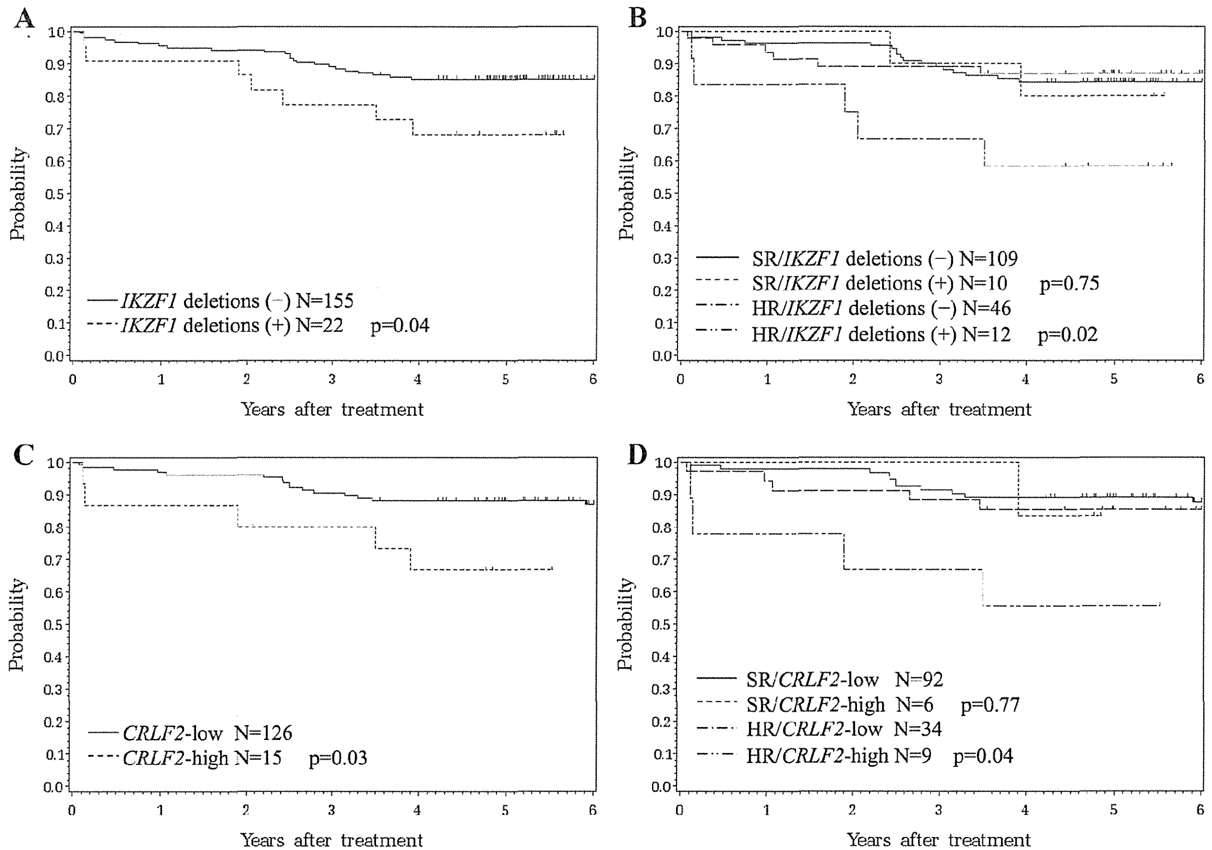

Fig. 2. Probability of EFS according to *IKZF1* deletions, *CRLF2* expression and NCI risk classification. **A**: Probability of EFS for patients with or without *IKZF1* deletions. **B**: Probability of EFS for patients with or without *IKZF1* deletions according to NCI-risk classification. **C**: Probability of EFS for patients with high *CRLF2* expression or low *CRLF2* expression. **D**: Probability of EFS for patients with high *CRLF2* expression or low *CRLF2* expression according to NCI-risk classification.

(Supplementary Table SI), and none of these patients experienced relapsed. The relationship between *Ik6* variant status and patient outcomes remains to be determined.

High *CRLF2* expression was detected in 10% of the patient in this study (6% of SR-ALL, and 21% of HR-ALL), which is consistent with observations from previous reports (5–20% of BCP-ALL) [10–16,24]. Hervey et al. [15] reported that *P2RY8-CRLF2* or *IgH-CRLF2* was highly associated with high *CRLF2* expression. On the other hand, Chen et al. [9] reported that approximately a half of patients with high *CRLF2* expression had these *CRLF2* gene alterations and that these gene alterations were detected only in the patients with high *CRLF2* expression. Palmi et al. [25] reported that *P2RY8-CRLF2* fusion was detected in 45% of patients with high *CRLF2* expression and that it was found in patients with high *CRLF2* expression as well as in patients with low *CRLF2* expression. They also demonstrated that the *P2RY8-CRLF2* fusion was associated with a high incidence of relapse (5-year cumulative incidence of relapse with or without the *P2RY8-CRLF2* fusion: 42.8% vs. 14.5%; $P = 0.001$). In the present study, the *P2RY8-CRLF2* fusion was found in 13% (2 of 15 patients) of ALL samples with high *CRLF2* expression. In addition, the *P2RY8-CRLF2* fusion was also found in 2% (3 of 126 patients) of ALL samples with low *CRLF2* expression, suggesting that a minor population clone had this fusion transcript. Furthermore, five patients with ALL positive for the *P2RY8-CRLF2* fusion are alive in first remission, except for one patient with ALL who had both the *IKZF1* deletion and high *CRLF2* expression. The prognostic impact of the *P2RY8-CRLF2* fusion remains to be clarified in a large-scale study.

Chen et al. [9] also reported that high *CRLF2* expression was associated with poor RFS in a multivariate analysis in HR-ALL patients but not in SR-ALL patients. The present study demonstrated that high *CRLF2* expression was significantly associated with poor outcomes, according to Kaplan–Meier analysis. However, high *CRLF2* expression was associated with only marginal significance for poor EFS, according to univariate and multivariate analysis in the Cox regression model. This discrepancy may be due to the relatively small number of patients analyzed in this study. Some investigators have proposed that ALL patients with high *CRLF2* expression were assigned to the intermediate-risk group because high *CRLF2* expression had no prognostic significance within multivariate analyses [10,17]. The relationship between outcomes and *CRLF2* expression may also be dependent on the specific regimen employed for treatment.

TABLE I. Prognostic Impact of *IKZF1* Deletions and High *CRLF2* Expression in Univariate and Multivariate Analyses

	Full cohort				NCI-HR			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
Factors	HR[a] (95% CI[b])	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age, years								
$\geq$10 versus 10	1.74 (0.81–3.75)	0.16			2.13 (0.58–7.89)	0.26		
Gender								
Male versus female	1.31 (0.66–2.59)	0.44			1.33 (0.43–4.13)	0.62		
WBC[c], $\times 10^9$/L								
$\geq$10 versus 10	0.95 (0.37–2.47)	0.92			0.71 (0.23–2.23)	0.56		
NCI risk classification								
HR[d] versus SR[e]	1.27 (0.62–2.58)	0.51	1.39 (0.50–3.82)	0.53	—	—	—	—
PSL[f] response								
PPR[g] versus PGR[h]	2.63 (0.80–8.60)	0.11	1.56 (0.20–12.08)	0.67	2.70 (0.59–12.34)	0.20	2.03 (0.24–17.49)	0.52
Cytogenetic abnormalities								
Hyperdiploid versus normal	1.48 (0.58–3.76)	0.41			0.49 (0.06–4.23)	0.52		
Other versus normal	1.39 (0.62–3.09)	0.42			0.80 (0.23–2.75)	0.72		
N.D.[i] versus normal	0.83 (0.19–3.69)	0.80			0.74 (0.09–6.29)	0.78		
Fusion genes								
ETV6-RUNX1 versus none	0.74 (0.24–2.34)	0.61			1.71 (0.29–10.21)	0.56		
E2A-PBX1 versus none	1.44 (0.50–4.14)	0.50			1.22 (0.20–7.28)	0.83		
N.D. versus none	1.38 (0.62–3.09)	0.43			2.04 (0.49–8.55)	0.33		
IKZF1 deletions								
Yes versus no	2.38 (1.02–5.55)	0.04	2.78 (0.94–8.27)	0.07	3.61 (1.10–11.84)	0.03	3.93 (0.75–20.75)	0.11
CRLF2 expression								
High versus low	2.97 (1.09–8.11)	0.04	2.24 (0.72–6.95)	0.16	3.56 (0.95–13.27)	0.06	1.97 (0.37–10.37)	0.43

HR[a], hazard ratio; CI[b], confidential interval; WBC[c], white blood cell count; NCI risk classification HR[d], 1–9y. and WBC $<50 \times 10^9$/L; SR[e], $\geq$10y. or WBC $\geq 50 \times 10^9$/L; PSL[f], prednisolone; PGR[g], prednisolone good responder; PPR[h], prednisolone poor responder; N.D.[i], not determined.

Some studies have reported that high *CRLF2* expression was highly associated with *JAK2* mutation and *IKZF1* deletions [15,16]. In the report by Chen et al. [9], *JAK* mutations were found in 21.8% of patients with high *CRLF2* expression and in 4.4% of their full cohort. Mullighan et al. reported that *JAK* mutations were detected in 20 of 187 patients with high-risk childhood BCP-ALL. A total of 16 cases had *JAK2* mutations, with 13 located in exon 16, and three located within exon 20 or 21. Another four cases had *JAK1* or *JAK3* mutations [11]. In our study, *JAK2* mutation in exon 16 was detected in only 1 of 177 cases. In addition, no mutations in *JAK2* exons 12, 20, and 21 were found in any cases of ALL with high *CRLF2* expression. These results suggest that *JAK2* mutations might be rare in Japanese patients. Further analysis of screening mutations of *JAK1* and *JAK3* as well as other sites of *JAK2* should be performed to confirm this finding.

In the Kaplan–Meier analysis from the present study, the coexistence of *IKZF1* deletions and high *CRLF2* expression, which was found only in patients with HR-ALL and not in patients with SR-ALL, was related to poor outcomes. One of the five patients with ALL and coexisting of *IKZF1* deletions and high *CRLF2* expression was positive for *JAK2* mutation, but the others were not. Therefore, they might have additional genetic alterations similar to those seen in patients with Ph-like ALL [26].

In conclusion, the present study suggests that *IKZF1* deletions and high *CRLF2* expression (and particularly, the combination of these two variables) predicted poor outcome in patients with HR-ALL but not in patients with SR-ALL in our Japanese cohort. However, the small sample size might have limited the statistical power of this study. A large-scale nationwide cohort study is planned to clarify the prognostic significance of these genetic abnormalities in Japan.

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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/Ik10 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 ± 10.9% vs. 91.7 ± 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 ± 23.4% vs. 93.2 ± 3.8%; $P = 0.03$ for NCI HR; 90.0 ± 9.5% vs. 91.0 ± 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 ± 14.0% vs. 92.4 ± 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 ± 18.6% vs. 90.6 ± 5.2%; $P = 0.18$ for NCI HR; 75.0 ± 21.7% vs. 93.1 ± 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 ± 17.9%) when compared with patients with other *IKZF1*/*CRLF2* statuses (4y-EFS: 88.0 ± 3.2% for del.(−)/low, 90.0 ± 9.5% for del.(−)/high, 88.9 ± 10.5% for del.(+)/low, 77.8 ± 6.2% for del.(−)/missing, 75.0 ± 15.3% for del.(+)/missing, 88.2 ± 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

Pediatr Blood Cancer

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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for the lymphoma committee of the Japanese Pediatric Leukemia/Lymphoma Study Group

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. Pediatr Blood Cancer © 2014 Wiley Periodicals, Inc.

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 $\times$ 2), Group 2 received 4 courses (2A $\times$ 2 + 2B $\times$ 2), Group 3 received 6 courses (3A $\times$ 4 + 3B $\times$ 2), and Group 4 received 6 courses (4A1 $\times$ 2 + 4A2 $\times$ 2 + 4B $\times$ 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

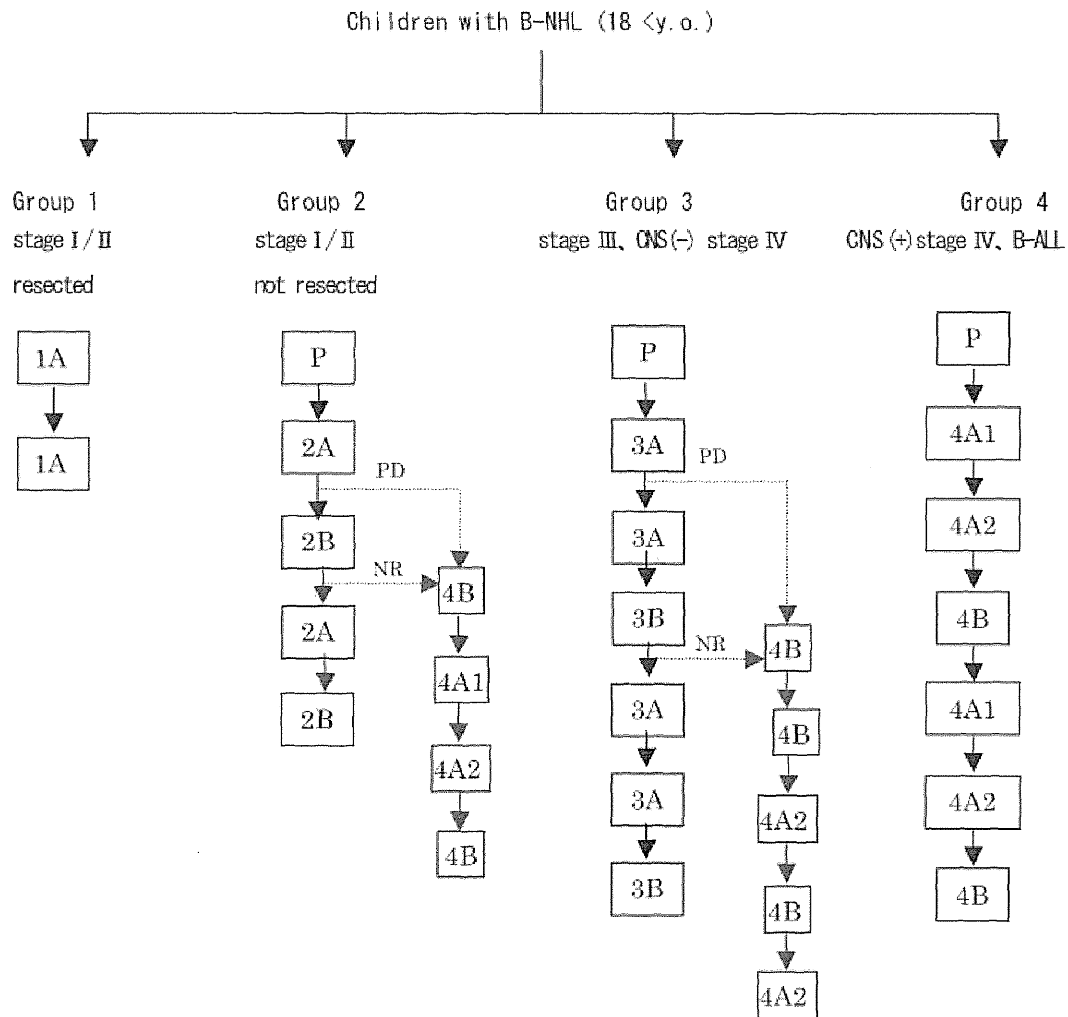

Fig. 1. Treatment framework of the B-NHL03 study. Patients were stratified into four treatment groups according to disease stage, tumor resectability, and BM/CNS involvement. All groups except Group 1 received pre-phase therapy. Group 1 received two courses of chemotherapy, Group 2 received 4 courses, 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 ($\geqq 0.2\ \mu$M 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%

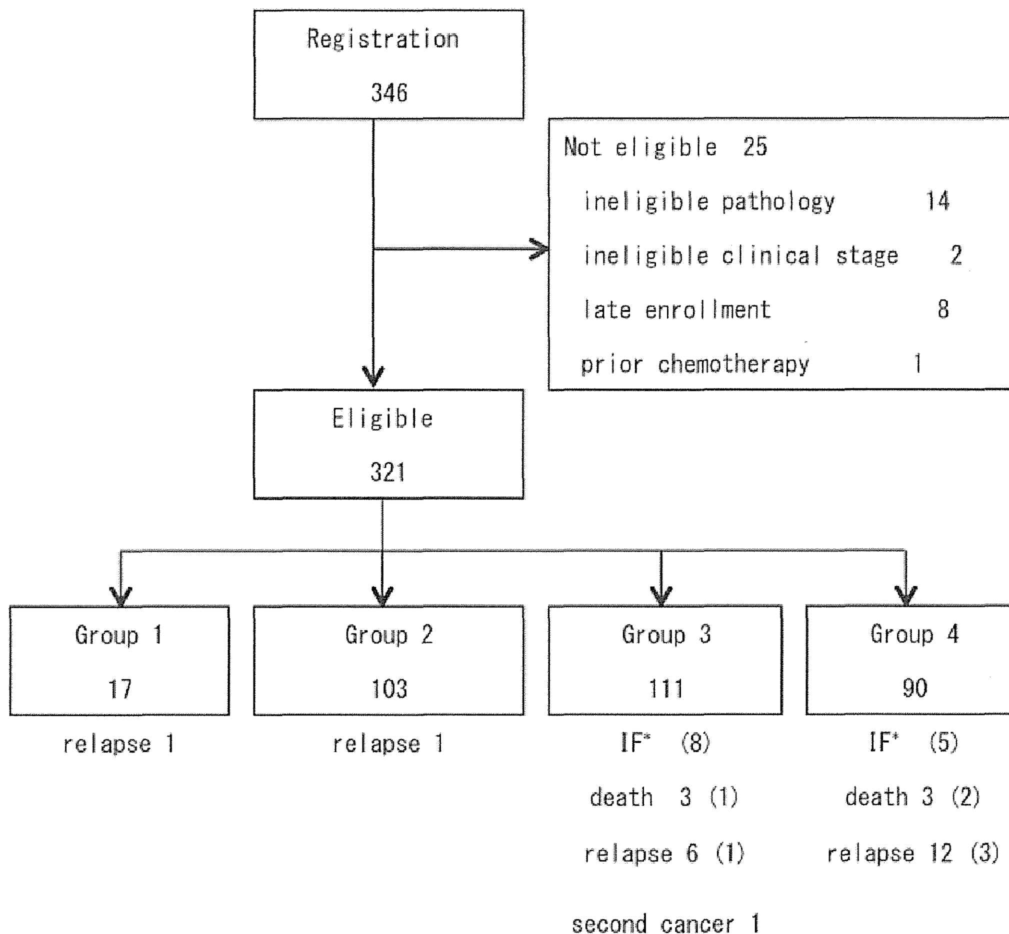

Fig. 2. Patient flow chart and events according to the treatment group. There were 40 events which consisted of each one in Group 1 and 2, 18 in Group 3, and 20 in Group 4. Number in parentheses indicates events occurred during protocol chemotherapy. Number in parenthesis indicates events occurred during 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

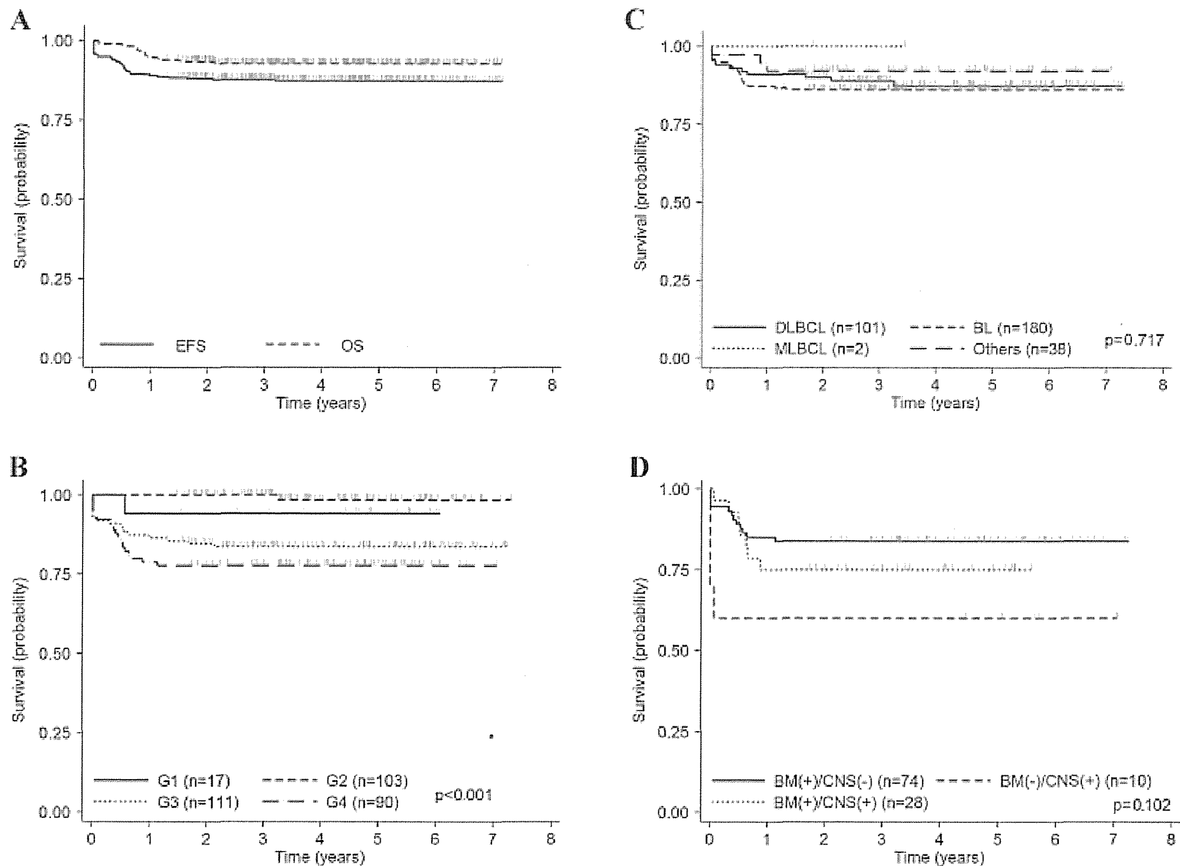

Fig. 3. Kaplan–Meier curves for OS and EFS of all patients (**A**). Kaplan–Meier curves for EFS according to treatment group (**B**), histology (**C**), and BM/CNS involvement (**D**).

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

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Stronger Prognostic Power of the CpG Island Methylator Phenotype than Methylation of Individual Genes in Neuroblastomas

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Objective: The CpG island methylator phenotype is strongly associated with poor survival in neuroblastomas. Neuroblastomas with the CpG island methylator phenotype include almost all neuroblastomas with *MYCN* amplification, and, even among neuroblastomas without *MYCN* amplification, have worse prognosis. At the same time, methylation of individual tumor-suppressor genes is also reported to be associated with poor survival. The purpose of this study was to compare the prognostic power of the CpG island methylator phenotype with that of methylation of individual genes.

Methods: Methylation-specific polymerase chain reaction was performed for five individual genes (*CASP8, EMP3, HOXA9, NR1I2* and *CD44*) in 140 Japanese and 152 German neuroblastomas. Kaplan–Meier analysis and log-rank tests were conducted to compare the survival between groups defined by methylation status.

Results: Among the five individual genes, only *CASP8* methylation had a significant association with poor overall survival both in Japanese (hazard ratio = 3.1; 95% confidence interval = 1.5–6.4; $P = 0.002$) and German (hazard ratio = 4.8; 95% confidence interval = 2.1–11; $P = 0.0002$) neuroblastomas. *HOXA9* and *NR1I2* methylation were associated with poor survival only in German neuroblastomas. On the other hand, the CpG island methylator phenotype had a strong and consistent association in Japanese (hazard ratio = 22; 95% confidence interval = 5.3–93; $P = 1.5 \times 10^{-5}$) and German (hazard ratio = 9.5; 95% confidence interval = 3.2–28; $P = 4.7 \times 10^{-5}$) neuroblastomas.

Conclusion: The CpG island methylator phenotype is likely to have stronger prognostic power than methylation of individual genes in neuroblastomas.

Key words: neuroblastoma – methylation – CIMP – poor survival

INTRODUCTION

Neuroblastoma (NBL) is the most frequent extracranial pediatric tumor (1). The CpG island methylator phenotype (CIMP), methylation of multiple CpG islands (CGIs), was associated with poor survival with a hazard ratio (HR) of 22 [95% confidence interval (95% CI) = 5.3–93] in Japanese and 9.5 (95% CI = 3.2–28) in German NBLs, respectively (2,3). The prognostic significance of CIMP was further confirmed in Italian NBLs by a pyrosequencing assay (4). Notably, NBLs with CIMP included almost all NBLs with *MYCN* amplification (37/38 in Japanese and 23/23 in German NBLs), the strongest current prognostic marker (5–7). Even among NBLs without

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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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MYCN amplification, CIMP was a significant and strong prognostic marker with an HR of 12 (95% CI = 2.6–59) in Japanese and 4.5 (95% CI = 1.3–16) in German NBLs.

CIMP is sensitively detected by methylation of marker CGIs, such as CGIs in gene bodies of the *PCDHB* gene family in NBLs. It is known that methylation of CGIs outside promoter regions (non-promoter CGIs) is not associated with loss of expression, and such non-promoter CGIs are more susceptible to methylation induction than promoter CGIs (8). As a model of the close association between methylation of non-promoter CGIs and poor survival, it was considered that CIMP consistently leads to methylation of non-promoter CGIs, such as CGIs of the *PCDHB* gene family in NBLs, and also to methylation of various promoter CGIs with low incidences, which causes poor survival.

At the same time, methylation of an individual gene has been also shown to be associated with poor survival. For example, methylation of *CASP8* was associated with poor survival with an HR of 5.3 (95% CI = 1.5–18; $P = 0.008$) (9). Methylation of *NR1I2*, *EMP3*, *HOXA9* and *CD44* was associated with poor survival with P values of 0.014, 0.03, 0.04 and 0.049, respectively (9–12). Functionally, *CASP8*, an apoptosis-related gene, has been reported to act as a tumor suppressor, and its loss is required for survival of NBL cells overexpressing *MYC* or *MYCN* (13). *NR1I2* (a nuclear receptor gene) and *EMP3* (a myelin-related gene) have been reported to have growth suppressive activity in NBL cells (10,11). However, the prognostic powers of methylation of these individual genes and of CIMP have never been analyzed in identical sets of NBLs.

In the present study, we aimed to compare the prognostic power of CIMP with that of methylation of individual genes.

PATIENTS AND METHODS

DNA Samples and Analysis of CIMP

The 140 Japanese and 152 German NBLs were identical with those analyzed in our previous studies (2,3). These samples were analyzed at the Division of Epigenomics, National Cancer Center Research Institute under the approval of institutional review boards. The presence of CIMP and *MYCN* amplification were determined as in our previous studies (2,3), and this information was used in the present study.

Sodium Bisulfite Modification and Methylation-specific Polymerase Chain Reaction (PCR)

Fully methylated DNA and fully unmethylated DNA were prepared by methylating genomic DNA with *Sss*I methylase (New England Biolabs, Beverly, MA, USA) and by amplifying genomic DNA with the GenomiPhi amplification system (GE Health Care, Buckinghamshire, UK), respectively. Bisulfite modification was performed using 1 μg of *Bam*HI-digested genomic DNA as previously described (14), and the modified DNA was suspended in 40 μl of Tris–ethylenediaminetetraacetic acid buffer (pH 8.0). An aliquot of 1 μl was used for methylation-specific PCR (MSP).

MSP was performed using primers as previously published (11,13,15,16) (Supplementary data, Table S1). For the *NR1I2* gene, although the combined bisulfite restriction analysis was performed in the previous study (10), MSP targeting the same region was used in this study. Using fully methylated and unmethylated DNA, the annealing temperature that specifically amplified only methylated or unmethylated DNA was determined. Also, a minimum number of PCR cycles to obtain visible bands was determined using the (un)methylated DNA, and four cycles were added for the analysis of primary NBLs (Supplementary data, Table S1).

Statistical Analysis

Survival time was defined as the time between initial diagnosis and death, or time between diagnosis and last contact if no event had occurred. Kaplan–Meier analysis and log-rank tests were conducted to compare survival between the groups defined by methylation status. HRs were estimated by the Cox

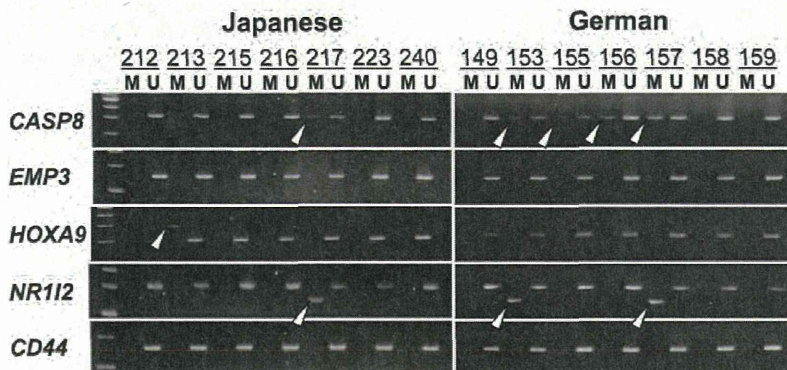

Figure 1. Methylation of promoter CpG islands (CGIs) of five individual genes (*CASP8*, *EMP3*, *HOXA9*, *NR1I2* and *CD44*) in Japanese and German neuroblastomas (NBLs). Representative results of methylation-specific PCR are shown. M and U, primers specific to methylated and unmethylated DNA, respectively. Arrowheads show the presence of methylated DNA molecules.

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proportional hazard model. These statistical analyses were performed using the SPSS software, version 13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Methylation of Individual Genes and their Prognostic Power Compared with CIMP

CASP8, *EMP3*, *HOXA9*, *NR1I2* and *CD44* were methylated in 26, 4, 27, 15 and 3, respectively, of the 140 Japanese NBLs, and in 30, 2, 2, 13 and 2, respectively, of the 152 German NBLs (representative results shown in Fig. 1). The prognostic power of methylation of the five genes was analyzed in Japanese and German NBLs, respectively (Fig. 2A and Table 1). In Japanese NBLs, only *CASP8* methylation had a significant association with poor survival (HR = 3.1; 95% CI = 1.5–6.4; $P = 0.002$). Regarding CIMP, defined by methylation of multiple genes and detected by methylation of the *PCDHB* gene family (2), it had a strong association with poor survival (HR = 22; 95% CI = 5.3–93; $P = 1.5 \times 10^{-5}$), and its prognostic power was stronger than that of *MYCN* amplification (HR = 9.5; 95% CI = 4.4–21; $P = 4.0 \times 10^{-9}$) (Fig. 2B). In the identical set of Japanese NBLs, a stronger prognostic power of CIMP than methylation of an individual gene was clearly shown.

In German NBLs, *CASP8* methylation was also associated with poor survival (HR = 4.8; 95% CI = 2.1–11; $P = 0.0002$) (Fig. 2A and Table 1). In addition, *HOXA9* and *NR1I2* methylation were associated with poor survival with an HR of 14 for *HOXA9* (95% CI = 3.1–62; $P = 0.0006$) and 4.2 for *NR1I2* (95% CI = 1.6–11; $P = 0.003$), respectively. Regarding CIMP and *MYCN*, as shown in our previous study (3), CIMP had a strong association with poor survival (HR = 9.5; 95% CI = 3.2–28; $P = 4.7 \times 10^{-5}$) and it was comparable to that of *MYCN* (HR = 12; 95% CI = 4.9–29; $P = 4.8 \times 10^{-8}$) (Fig. 2B). The stronger prognostic power of CIMP was consistently shown in the identical set of German NBLs.

Association Between CIMP and Methylation of Individual Genes

Among the five individual genes analyzed in this study, two genes (*CASP8* and *NR1I2*) were methylated at a significantly higher incidence in NBLs with CIMP (Fig. 3). In Japanese NBLs with and without CIMP, *CASP8* methylation was found in 24/67 and 2/73, respectively ($P = 5.0 \times 10^{-7}$). *NR1I2* methylation was found in 15/67 and 0/73, respectively ($P = 3.2 \times 10^{-5}$). Also in German NBLs with and without CIMP, *CASP8* methylation was found in 28/50 and 2/95, respectively ($P = 2.6 \times 10^{-14}$). *NR1I2* methylation was found in 11/50 and 1/95, respectively ($P = 1.4 \times 10^{-5}$). These results showed that CIMP was associated with methylation of multiple promoter CGIs, mainly *CASP8* and *NR1I2*.

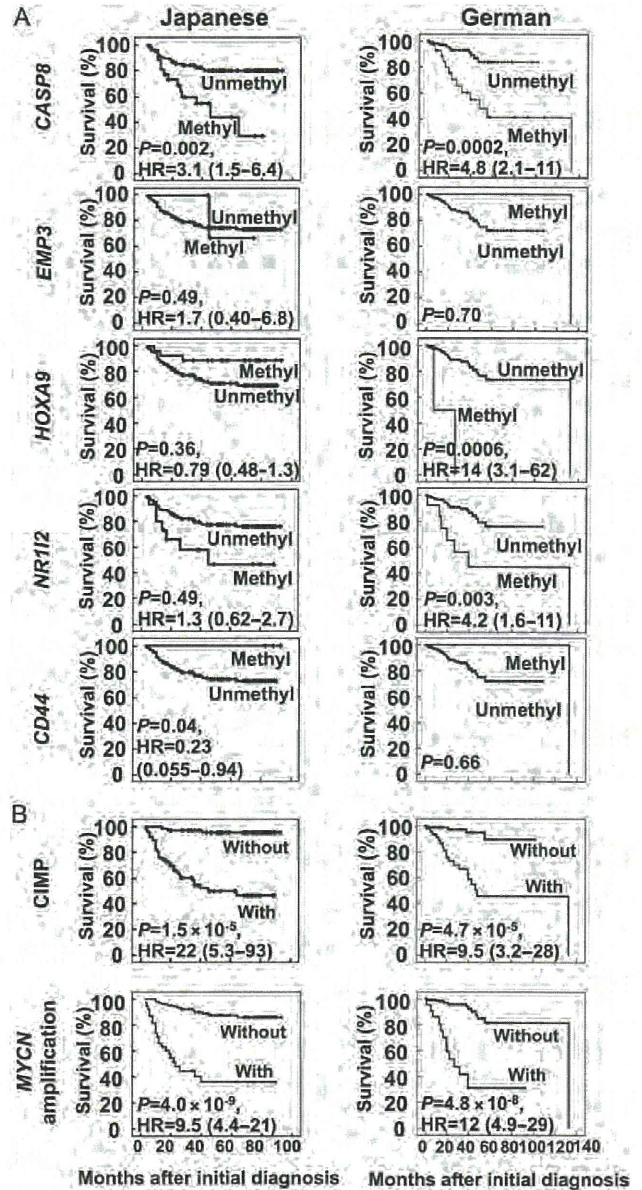

Figure 2. Prognostic power of (A) methylation of five individual genes (*CASP8*, *EMP3*, *HOXA9*, *NR1I2* and *CD44*), and (B) CpG island methylator phenotype (CIMP) and MYCN amplification in Japanese and German NBLs. Kaplan–Meier survival curves were drawn using the SPSS software. Among the five genes, only CASP8 methylation had a significant association with poor survival both in Japanese and German NBLs.

DISCUSSION

The stronger prognostic power of CIMP than methylation of individual genes was shown in this study. Also, the association between CIMP and methylation of multiple promoter CGIs was indicated. These results supported the idea that CIMP leads to a poor prognosis by induction of methylation of promoter CGIs of various tumor-suppressor genes with low incidences.

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Table 1. Prognostic power of methylation of individual genes and CpG island methylator phenotype (CIMP)

Marker	Japanese ($n = 140$)				German ($n = 152$)			
	No. of NBLs with methylation or amplification	HR	95% CI for HR	*P* value	No. of NBLs with methylation or amplification	HR	95% CI for HR	*P* value
CASP8	26	3.1	1.5–6.4	0.002	30	4.8	2.1–11	0.0002
EMP3	4	1.7	0.4–6.8	0.49	2	NA	–	0.70
HOXA9	27	0.79	0.48–1.3	0.36	2	14	3.1–62	0.0006
NR1I2	15	1.3	0.62–2.7	0.49	13	4.2	1.6–11	0.003
CD44	3	0.23	0.055–0.94	0.04	2	NA	–	0.66
CIMP	67	22	5.3–93	1.5×10^{-5}	50	9.5	3.2–28	4.7×10^{-5}
MYCN amplification	38	9.5	4.4–21	4.0×10^{-9}	23	12	4.9–29	4.8×10^{-8}

NBL, neuroblastoma; HR, hazard ratio; CI, confidence interval; NA, not applicable.

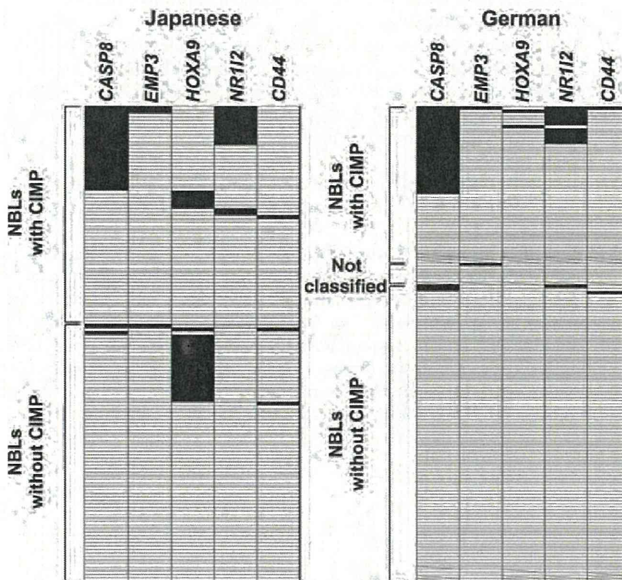

Figure 3. Methylation profiles of the five individual genes in NBLs with and without CIMP. Left panel, 140 Japanese NBLs; and right panel, 152 German NBLs. NBLs were classified by CIMP status determined as in our previous studies (2,3), and then aligned by methylation statuses of the five genes. In German NBLs, seven cases were not classified as NBLs with CIMP or without CIMP (3). The NBLs with CIMP tended to show methylation of multiple promoter CGIs. Closed box, methylated DNA detected; open box, only unmethylated DNA detected; and box with a slash; neither methylated nor unmethylated DNA detected, possibly due to low DNA quality.

Regarding the assessment of CIMP, besides the use of the *PCDHB* gene family, a combination of silenced genes has been proposed. Yang et al. (17) analyzed methylation of eight genes (*HIC-1*, *RASSF1A*, *BLU*, *DCR2*, *CASP8*, *TIG-1*, *HIN-1*, *TMS-1*), and identified that methylation of two and three genes had no effects on survival ($P = 0.719$ and 0.214, respectively), but methylation of ≥4 genes had a trend toward decreased survival ($P = 0.055$). Also, Lau et al. (18) identified that methylation of at least one of three genes (*FOLH1*, *MYOD1* and *THBS1*) was associated with event-free survival (HR = 2.2; 95% CI = 1.1–4.2; $P = 0.022$), and the association was stronger in methylation of all the three genes (HR = 4.5; 95% CI = 1.6–13; $P = 0.006$). These data support the model that CIMP leads to methylation of promoter CGIs of tumor-related genes with low incidences, which leads to poor survival.

Among the individual genes, *CASP8* and *RASSF1A* methylation have been repeatedly shown to be associated with poor survival (9,17,19–23). *CASP8* methylation was consistently associated with poor survival in the present study. By the analysis of methylation and survival data in our previous study (2), *RASSF1A* methylation was also revealed to be associated with poor survival in Japanese NBLs (HR = 4.2; 95% CI = 1.9–9.3; $P = 0.0005$). However, HRs of these genes were smaller than that of CIMP. These data indicated that these two genes play critical roles in a fraction of NBLs but not in the other NBLs. Indeed, a recent genome-wide methylation study revealed that methylation of numerous genes was associated with poor survival in NBLs (24).

In conclusion, the stronger prognostic power of CIMP than of methylation of individual genes was shown, and methylation silencing of various tumor-suppressor genes with low incidences was suggested to be involved in poor survival.

Supplementary data

Supplementary data are available at http://www.jjco.oxfordjournals.org.

Funding

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