研究成果の刊行に関する一覧表

書籍

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Bhattacharya K, Balasubramaniam S, Choy Y, Fietz M, Fu A, Jin D, Kim OH, Kosuga M, Kwun Y, Inwood A, Lin HY, McGill J, Mendelsohn NJ, Okuyama T, Samion H, Tan A, Tanaka A, Thamkunanon V, Toh TH, Yang AD, Lin SP.	Overcoming the barriers to diagnosis of Morquio A syndrome..	Orphanet J Rare Dis	Nov 30;9(1)	192.	2014
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Significance of CD66c expression in childhood acute lymphoblastic leukemia

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ABSTRACT

Upon analyzing 696 childhood B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cases, we identified the characteristics of CD66c expression. In addition to the confirmation of strong correlation with *BCR-ABL* positivity and hyperdiploid, we further observed that CD66c is frequently expressed in CRLF2-positive (11/15, $p < 0.01$ against chimeric gene-negative) as well as hypodiploid cases (3/4), whereas it is never expressed in *ETV6-RUNX1*, *MLL-AF4*, *MLL-AF9*, *MLL-ENL*, and *E2A-PBX1*-positive cases. Although the expression of CD66c itself is not directly linked to the prognosis, the accompanying genetic abnormalities are important prognostic factors for BCP-ALL, indicating the importance of CD66c expression in the initial diagnosis of BCP-ALL.

1. Introduction

Although most leukemic cells retain the characteristics of their normal counterparts and exhibit commitment to any one of the hematopoietic lineages, they frequently show lineage-uncommitted antigen expression, referred to as "aberrant antigen expression" or "lineage infidelity". For example, both T-cell and B-cell precursor (BCP) acute lymphoblastic leukemia (ALL) cells commonly express aberrant myeloid lineage antigens, while acute myeloid leukemia (AML) cells often exhibit the expression of T- or B-cell lineage antigens. Several possibilities to explain this phenomenon have been postulated, whereas the precise mechanism is still unclear [1–3].

CD66c, also called CEACAM6, KOR-SA3544 antigen, and NCA 90/50, is a heavily glycosylated glycosylphosphatidylinositol (GPI)-anchored protein belonging to the carcinoembryonic antigen family, having two constant Ig-like domains and one variable Ig-like domain [4]. The expression of CD66c is observed only in granulocytes and its precursors among normal hematopoiesis [5], while it is known as the most frequently observed aberrant myeloid

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Table 1
The summary of the characteristics of patients.

	n=	Age (mean ± SD)	Range	Initial WBC/μl (mean ± SD)	Range	Gender (male:female)	NCI risk group (SR:HR)
BCR-ABL	35	8.4 ± 4.1	2–15	141,950.0 ± 202,731.2	1220–881,700	0.60:0.40	0.80:0.20
MLL-chimera	20	5.5 ± 4.8	0–15	233,237.2 ± 336,648.4	3300–1,165,400	0.45:0.55	0.80:0.20
E2A-PBX1	65	6.5 ± 4.5	1–15	39,857.4 ± 44,938.8	1730–223,300	0.52:0.48	0.94:0.06
ETV6-RUNX1	154	4.8 ± 2.8	1–15	26,426.4 ± 73,112.6	1600–788,000	0.56:0.44	1.00:0.00
Neardiploid	267	6.1 ± 4.3	1–17	31,153.1 ± 71,020.0	700–597,000	0.53:0.47	0.96:0.04
CRLF2+	15	7.7 ± 4.7	1–16	95,105.5 ± 134,773.6	4200–368,700	0.60:0.40	0.93:0.07
Hypodiploid	4	7.3 ± 5.0	2–12	10,075.0 ± 7605.4	4900–23,200	0.75:0.25	1.00:0.00
Hyperdiploid	136	4.4 ± 2.9	1–15	14,256.6 ± 27,170.5	1100–259,000	0.54:0.46	1.00:0.00
Total	696	5.6 ± 3.9		39,318.0 ± 102,794.7	700–1,165,400	0.54:0.46	0.96:0.04
Neardiploid							
CD66c+	106	5.7 ± 3.8	1–15	24,402.5 ± 51,598.1	700–379,500	0.54:0.46	0.96:0.04
CD66c-	161	6.3 ± 4.6	1–17	35,555.7 ± 80,919.0	800–597,000	0.53:0.47	0.97:0.03
Hyperdiploid							
CD66c+	91	4.3 ± 2.4	1–12	12,339.3 ± 17,488.1	1100–116,900	0.58:0.42	1.00:0.00
CD66c-	45	4.5 ± 3.9	1–15	18,278.3 ± 40,265.9	1700–259,000	0.44:0.56	1.00:0.00

antigen in B-cell precursor acute lymphoblastic leukemia (BCP-ALL) [6]. CD66c was initially reported to be expressed highly selectively in *BCR-ABL*-positive BCP-ALL, while some *BCR-ABL*-negative cases also express this antigen [7]. Later, it was reported that CD66c was correlated strongly with *ETV6-RUNX1* and *MLL-AF4* negativity and was found at high levels in hyperdiploidy [6,8]. A number of studies to clarify the function of this molecule have been performed, and it has been reported that CD66c is involved in homo- and heterotypic adhesion [9], contributes to Ca2+-mediated signaling [10], and is involved in apoptosis induction [11]. However, the biological significance of this molecule in BCP-ALL is still not fully understood.

In an attempt to explore the significance of the expression of CD66c in BCP-ALL, we precisely characterized the properties of CD66c-positive ALL in a large cohort. In this study, we further extend previous findings and indicate that CD66c expression has a close correlation with a definite set of genetic abnormalities, although it is not limited to a specific one. The detection of CD66c at the initial diagnosis of BCP-ALL is important for the prediction of the presence and absence of certain genetic abnormalities. Although the expression of CD66c itself is not directly linked to the prognosis, the genetic abnormalities accompanying CD66c expression are important prognostic factors for BCP-ALL, and, thus, the genetic findings need to be investigated carefully with the presence of CD66c expression.

2. Materials and methods

2.1. Case selection

A total of 696 patients aged between 1 and 18 years (male: female; 0.54: 0.46) who had been newly diagnosed with BCP-ALL and consecutively enrolled on the Tokyo Children's Cancer Study Group (TCCSG) L16 study from December 2004 to August 2012 were included in this study. The characteristics of patients, including age, initial white blood cell (WBC) count, and NCI risk group, were summarized in Table 1. The investigations were approved by the institutional review boards of all participating institutions. Informed consent was obtained from parents or guardians, and informed assent was obtained from the patients when appropriate given their age and understanding.

Bone marrow (BM) and/or peripheral blood (PB) smears of the patients were stained by standard techniques, and the diagnosis of ALL was made according to the morphologic and cytochemical (myeloperoxidase and nonspecific esterase) criteria of the French-American-British (FAB) classification. All cases had fewer than 3% myeloperoxidase-positive, 3% Sudan black B-positive (myeloid pattern), or 20% butyrate esterase-positive (myeloid pattern) blast cells and no Auer rods. Basically, children with ALL of the mature B-cell type were not enrolled in this trial. BM aspirate or PB was immediately mixed with anti-coagulant and sent by overnight transport to the flow cytometry and fusion transcript laboratories, National Research Institute for Child Health and Development (NCH) and Univ. of Tsukuba, respectively, as part of routine pretreatment studies.

2.2. Flow cytometry

Four-color flow cytometric immunophenotyping with CD45-gating was performed on a flow cytometer (FC500, Beckman-Coulter, Brea, CA). The panel monoclonal antibodies (MoAbs) used for immunophenotyping are presented in Supplementary information. Whole blood samples were stained with various combinations of fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, PE-cyanin 5.1 (PC5)-, and PE-cyanine 7 (PC7)-conjugated MoAbs in the presence of electron-coupled dye (ECD)-conjugated CD45, following RBC-lysis treatment. For the detection of cytoplasmic (cyCD3, cyCD22, cyCD79a, cy-μ, and MPO) and nuclear TdT antigens, the cells were permeabilized with the Intraprep Permeabilization reagent kit (Beckman-Coulter). Analysis was done by collecting 10,000 gated list mode events, and selecting an appropriate blast gate for the combination of CD45 and side scatter. An antigen was considered positively expressed when at least 20% of the gated cells expressed that antigen.

DNA contents were examined by Propidium Iodide (PI)-staining. Following RBC-lysis treatment, 2.5×10^5 cells were suspended in phosphate-buffered saline (PBS) containing 0.2% of Triton X-100, 20 μg/ml of PI, and 100 ng/ml of RNase (Sigma–Aldrich, St. Louis, MO). PI fluorescence was collected through a 645-nm dichroic long-pass filter and a 620-nm band-pass filter. Upon appropriate gating, at least 10,000 events were collected and analyzed.

The detection of BCR-ABL protein by flow cytometry was performed by Cytometric Bead Array (CBA) for BCR-ABL protein (Becton Dickinson, BD, Franklin Lakes, NJ) according to manufacturer's instruction.

2.3. Detection of fusion transcripts and conventional cytogenetic analysis

The expression of 8 fusion transcripts: *MLL-AF4*, *MLL-AF9*, *MLL-ENL*, major *BCR-ABL*, minor *bcr-abl*, *ETV6-RUNX1*, *E2A-PBX*, and *SIL-TAL1*, was detected by real-time PCR using appropriate primer sets. Cytogenetic analysis was performed on bone marrow or peripheral blood specimens using standard techniques. At least 20 metaphases were examined for each case. Actual examinations were performed by Special Reference Laboratory (SRL, Tachikawa, Tokyo, Japan). In the present study, we have defined BCP-ALL cases with more than 51 chromosomes or DNA-index > 1.16 (corresponding to 51 chromosomes) as hyperdiploid (high-hyperdiploid) based on the previous reports [12,13]. Similarly, we have defined the cases with fewer than 44 chromosomes [13–15] or DNA-index < 0.95 (corresponding to 43 chromosomes) [16] as hypodiploid (near-haploid, low-hypodiploid and high-hypodiploid). The cases with 44–50 chromosomes have designated as neardiploid.

2.4. Statistical analysis

Statistical analysis was performed by means of Student's *t*-test. A *p*-value less than 0.05 was considered significant. Principal components analysis (PCA) was performed by using TriSP version2.1 developed by Yamasaki H (http://www014.upp.so-net.ne.jp/acremaker/).

3. Results

3.1. Close correlation between CD66c expression and nonrandom genetic abnormalities

We analyzed CD66c expression in 696 unselected patients' specimens with a diagnosis of BCP-ALL and available information on the presence of well-established chimeric genes, including major and minor *BCR-ABL*, *ETV6-RUNX1*, *E2A-PBX1*, *MLL-AF4*, *MLL-AF9*, and *MLL-ENL* and/or cytogenetic findings, including DNA ploidy. As shown in Table 2, CD66c was expressed in 34.9% of all BCP-ALL cases

Table 2
Expression of myeloid antigens in B-cell precursor acute lymphoblastic leukemia.

	CD66c	CD33	CD13	CD15	CD65	CD117
>20% (%)	34.91	21.73	9.20	3.44	2.46	1.62
Number	(243/696)	(151/695)	(64/696)	(23/668)	(17/692)	(11/679)
Mean (%)	23.18	13.53	6.40	3.35	2.80	1.88
SD (%)	31.26	21.85	14.11	8.57	9.59	5.08
Median (%)	4.87	2.58	1.26	0.79	0.70	0.35

and appeared to be most frequently aberrantly expressed in BCP-ALL compared to other myeloid antigens, including CD33 (21.7%), CD13 (9.2%), CD15 (3.4%), CD65 (2.5%), and CD117 (1.6%).

Consistent with previous reports, CD66c expression showed a close correlation with nonrandom genetic abnormalities and was expressed only in *BCR-ABL*-positive (91.4%, 32/35) or specific chimeric gene-negative cases (50.0%, 211/422), while none of the *ETV6-RUNX1*-positive cases expressed CD66c (Fig. 1A). In addition, not only the *MLL-AF4*-positive cases, but also *MLL-AF9* and *MLL-ENL*-positive cases were negative for CD66c. Furthermore, it is noteworthy that none of the *E2A-PBX1*-positive cases expressed CD66c.

3.2. High rate expression of CD66c in CRLF2-positive and hyperdiploid cases

Next, we further analyzed CD66c expression in BCP-ALL cases without specific chimeric genes (Fig. 1B). The chimeric gene-negative BCP-ALL cases can be subdivided into near-, hyper-, and hypodiploid based on the number of chromosomes. The abnormalities in chromosome number have been shown to have prognostic significance in BCP-ALL and hyperdiploid ALL (more than 51 chromosomes) exhibit a superior outcome [12,13], whereas hypodiploid ALL (fewer than 44 chromosomes) is characterized by extremely poor outcomes when compared with their nonhyperdiploid counterparts (44–50 chromosomes) [13–15]. As shown in Fig. 1B, hyperdiploid cases exhibited high frequency of CD66c expression (66.9%, 91/136). Interestingly, although the number of cases was small, three out of four hypodiploid cases were positive for CD66c.

In our study, we examined the expression of CRLF2 using specific monoclonal antibody retrospectively and prospectively, and found 15 CRLF2-positive cases in the neardiploid cases (2.2% in our total cohort). As shown in Fig. 1B, CRLF2-positive cases exhibited a significantly high frequency of CD66c-expression and 73.3% (11/15) were CD66c-positive. No significant difference was observed between hyperdiploid and CRLF2-positive cases in CD66c-expression. In contrast, the remaining neardiploid cases exhibited less frequent CD66c-expression (39.7%, 106/267).

3.3. Correlation between CD66c expression and that of other myeloid antigens and CD21/CD27 expression

It was reported that the expression of myeloid antigens tended to be mutually exclusive with CD66c [6]. Therefore, we next examined the correlation between the expression of CD66c and other myeloid antigens. As presented above, BCP-ALL cases possessing specific chimeric genes except *BCR-ABL* never express CD66c. Since it was also reported that *ETV6-RUNX1*-positive ALL frequently expressed CD33 and CD13 [17], ALLs expressing these two antigens should be enriched in CD66c-negative/neardiploid cases. Therefore, we compared *BCR-ABL*-positive and chimeric gene-negative cases by excluding BCP-ALL cases possessing other specific chimeric genes from this analysis.

As shown in Fig. 2A and B, the expression of CD33 and CD13 was concentrated in *BCR-ABL*-positive and neardiploid cases. As described above, the vast majority of BCR-ABL-positive cases expressed CD66c and they exhibited a higher frequency of both CD33 (37.5%, 12/32) and CD13 (18.8%, 6/32) expression compared to CD66c-positive cases with neardiploid and hyperdiploid states. In contrast, although we excluded *ETV6-RUNX1*-positive cases from the analysis, neardiploid/CD66c-negative cases still exhibited a significantly higher expression of CD33 (23.6%, 38/161) compared to neardiploid/CD66c-positive (17.9%, 19/106) and hyperdiploid/CD66c-negative (4.6%, 2/44) cases. In CRLF2-positive/CD66c-positive cases, frequent expression of CD33 (36.4%, 4/11) but not CD13 was observed. Since positivity for CD15 and CD65 was low in BCP-ALL, with the exception of *MLL*-related chimeric gene-positive cases [18], no significant differences in the expression of these antigens depending on CD66c expression were observed (data not shown).

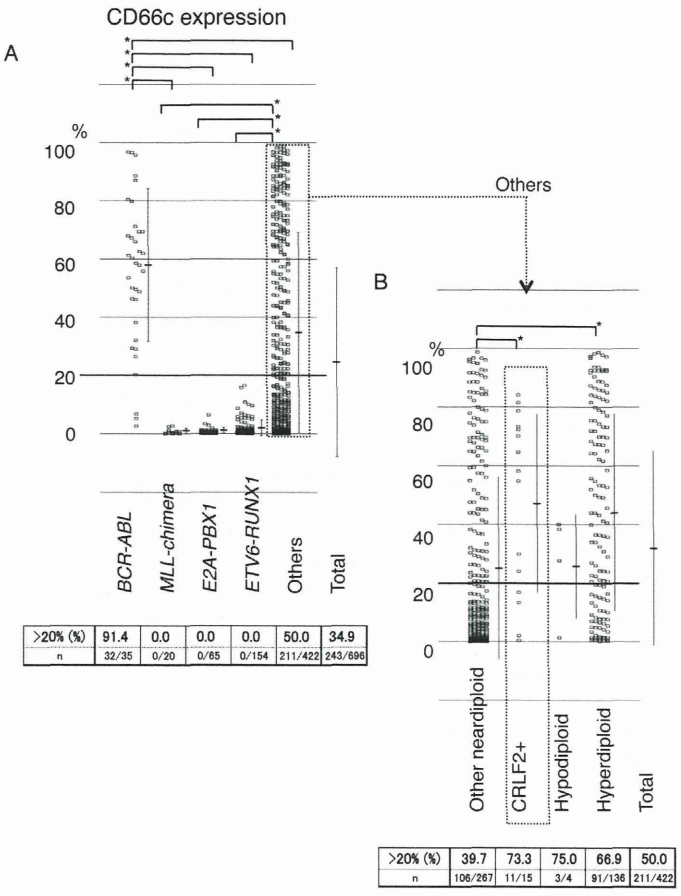

Fig. 1. Correlation between percentage CD66c positivity and acute lymphoblastic leukemia (ALL) genotype categories. (A) CD66c positivity (percentage) of B-cell precursor ALL ($n = 696$) was plotted on a scattergram categorized by the presence of well-known chimeric genes. Percentage of CD66c-positive cases (more than 20% expression in blasts) in each genotype group is listed below. (B) CD66c positivity (percentage) of B-cell precursor ALL without chimeric genes listed above ($n = 422$) was further subclassified based on the DNA-ploidy and CRLF2 expression and presented as in (A). *$p < 0.01$, using Student's t-test.

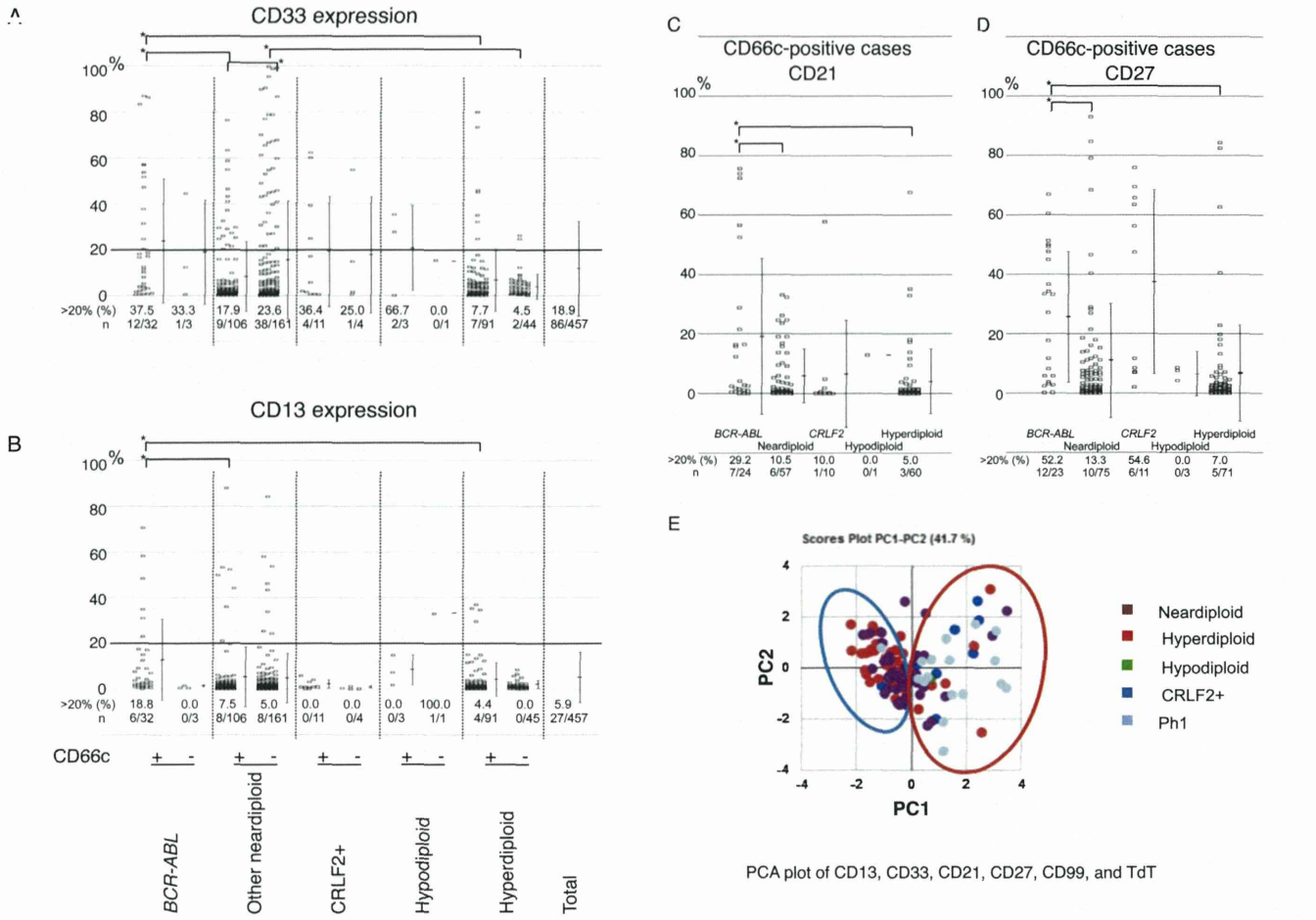

Fig. 2. Correlation between CD66c positivity and the expression of myeloid antigens, CD21 and CD27 in different genotype categories of acute lymphoblastic leukemia (ALL). The positivity (percentage) of CD33 (A) and CD13 (B) of B-cell precursor ALL was plotted on a scattergram categorized by the CD66c expression and genotype, as in Fig. 1. The positivity (percentage) of CD21 (C) and CD27 (D) of CD66c-positive B-cell precursor ALL was plotted on a scattergram categorized by the genotype as indicated in the figure. The percentage of positive cases (more than 20% expression in blasts) in each group is listed below. *$p < 0.01$, using Student's *t*-test. (E) Principal components analysis (PCA) was performed on CD66c-positive B-cell precursor ALL cases. PCA plot of 6 antigen expression, including CD13, CD33, CD21, CD27, CD99, and TdT, is presented with two PCA axes (PC1 vs PC2).

In an attempt to explore immunophenotypic characteristics distinguishing *BCR-ABL*-positive ALLs and other CD66c-positive ALLs, we observed relatively high expression of CD21 and CD27 in *BCR-ABL*-positive ALLs. The molecule CD21 is a mature B-cell antigen and its expression in BCP-ALL is very limited [19]. The CD27 molecule is a member of the TNF receptor family and known as a marker of mature memory B cells, while some malignant and nonmalignant B precursors also express this antigen [20]. Among CD66c-positive cases, CD21 expression was revealed to be relatively high in *BCR-ABL*-positive cases (Fig. 2C), and CD27 expression was high in both *BCR-ABL*-positive and CRLF2-overexpressing cases (Fig. 2D).

To further assess the biological relevance of the expression of above antigens in CD66c-positive ALL, we performed multivariate analyses by employing PCA. As shown in Fig. 2E, PCA plot using the expression data of 6 antigens, including CD13, CD33, CD21, CD27, CD99, and TdT, could roughly separate BCR-ABL-positive and CRLF2-positive cases from remaining chimeric gene-negative cases expressing CD66c.

3.4. Correlation between risk factors and CD66c expression

We next examined the correlation between CD66c expression and risk classification in chimeric gene-negative cases. In our protocol, the patients were stratified into three risk groups, namely, standard risk (SR), intermediate risk (IR), and high risk (HR), based on presenting features (age and the leukocyte count before starting the treatment) and, then, reclassified into them three categories 7 days later according to the sensitivity to oral prednisolone monotherapy, using the cut-off counts of 1000 blasts/μL [21]. As shown in Table 3, hyperdiploidy/CD66c-positive cases were more frequently classified into SR at diagnosis, while no difference was observed at reclassification on Day 8, indicating that hyperdiploid/CD66c-positive cases tend to have favorable initial presenting features but exhibit poor response for steroid. On the other hand, neardiploid/CD66c-negative cases tended to be more frequently classified into IR and HR at the initial classification, and the tendency appeared to be more significant at reclassification on Day 8 (Table 3), indicating that neardiploid/CD66c-negative have unfavorable presenting features as well as poor steroid sensitivity. After 5-year observation, however, no significant difference in the subsequent prognosis between CD66c-positive and -negative groups was observed (data not shown).

4. Discussion

Upon precisely analyzing CD66c expression in a large cohort of childhood BCP-ALL, we further extended the previous findings, and clearly identified the characteristics of CD66c expression as follows: First, among BCP-ALL possessing well-known chimeric genes, CD66c expression is highly selective in *BCR-ABL*-positive

Table 3
Risk classification and CD66c expression.

Hyper/ CD66c+	HR (Case no.) 1		IR 12		SR 27			Total 40	HR+IR/SR (Ratio) 0.48	Initial
	HR-SCT 0	HR 1	HR 4	IR 8	HR 7	IR 0	SR 20		1.00	Day-8
Hyper/ CD66c-	HR 0		IR 8		SR 8			16	1.00	Initial
	HR-SCT 0	HR 0	HR 1	IR 7	HR 0	IR 0	SR 8		1.00	Day-8
Diploid/ CD66c+	HR 7		IR 24		SR 30			61	1.03	Initial
	HR-SCT 4	HR 3	HR 2	IR 22	HR 0	IR 0	SR 30		1.03	Day-8
Diploid/ CD66c-	HR 14		IR 48		SR 31			93	2.00	Initial
	HR-SCT 8	HR 6	HR 4	IR 44	HR 3	IR 0	SR 28		2.32	Day-8

Hyper, hyperdiploid; Diploid, neardiploid; HR, high risk; IR, intermediate risk; SR, standard risk; SCT, stem-cell transplantation; Initial, risk classification based on presenting features; Day-8, re-risk classification after 7-day oral prednisolone monotherapy.

cases, while CD66c is never expressed in cases possessing not only *ETV6-RUNX1* and *MLL-AF4*, but also *MLL-AF9*, *MLL-ENL*, and *E2A-PBX1*. Second, among BCP-ALL cases without well-known chimeric genes, CD66c expression also exhibits some selectivity that correlates with genetic abnormalities and CRLF2-positive and probably hypodiploid states, and, as in hyperdiploidy cases tend to express CD66c at a high frequency. The results were schematically summarized in Fig. 3A. Above data indicate that CD66c expression has a close correlation with definite set of genetic abnormalities, although it is not limited to a specific one.

The overexpression of *CRLF2* arises from a translocation juxtaposing *CRLF2* to the *IGH* enhancer or an interstitial deletion (*CRLF2-P2RY8*) and has been reported to be found in 4.7% to 17.5% of BCP-ALL cases as assessed by real-time PCR [22–29]. In this study, however, we found only 15 CRLF2-positive cases (2.2%) in our cohort by flow cytometry. Although the precise reason for the inconsistency in the frequency of CRLF2 overexpression between previous reports by real-time PCR and our data of flow cytometry is remaining unclear, it is possibly due to the difference of detection methods including diagnostic criteria for positive case.

Most recently, a subtype of BCP-ALL including CRLF2-overexpressing cases has been called "Ph-like ALL" and identified to be sharing a transcriptional signature that significantly overlaps with a *BCR-ABL*-positive ALL and accompanied by high rates of relapse and poor overall survival [30]. Besides CRLF2-overexpressing cases, our preliminary results indicate that other Ph-like ALL cases also tend to frequently express CD66c (data not shown).

As well as CRLF2 overexpression [22–29], both *BCR-ABL*-positive [13,31] and hypodiploid patients are well known to show a poor prognosis [13–15]. In contrast, hyperdiploid BCP-ALL patients are generally accompanied by a relatively favorable therapeutic outcome [12,13]. Therefore, the expression of CD66c itself is not directly linked to the prognosis, whereas the genetic abnormalities accompanying CD66c expression are important to make a prognosis for BCP-ALL patients. Concerning the chimeric gene-negative cases, our data further indicated that the combination of CD66c expression and chromosome number abnormalities is closely related to risk classification and steroid sensitivity. Thus the genetic findings must be paid attention when CD66c expression is detected.

Since *CRLF2*-overexpressing BCP-ALLs and *BCR-ABL*-positive cases share overlaped transcriptional signature as we described above [24], the transcription of CD66c might be regulated by a common downstream factor in both pathways. Similarly, CD66c expression in hyper- and hypodiploid cases might also share the same pathway, whereas the precise mechanism that induces the aberrant expression of CD66c in BCP-ALL is unclear. In the

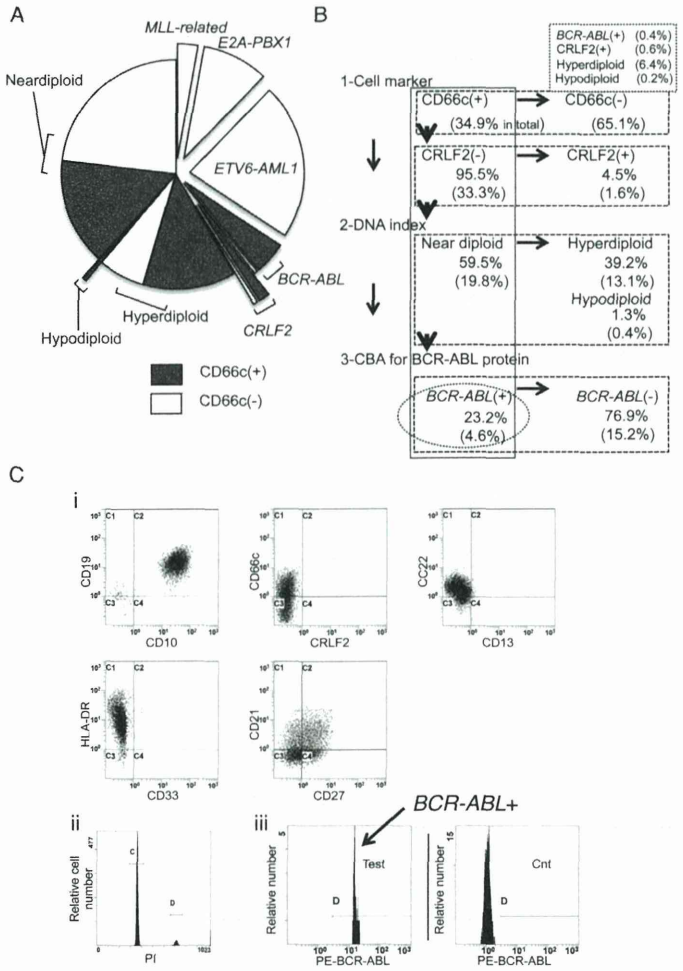

Fig. 3. Summary of CD66c expression and diagnostic flow to detect BCR-ABL-positive acute lymphoblastic leukemia (ALL) by flow cytometry. (A) Summary of CD66c expression and correlation with genetic abnormalities. (B) An initial diagnostic flow of the subclassification of BCP-ALL using flow cytometry is indicated. In the case of neardiploid B-cell precursor ALL that is CD66c-positive and CRLF2-negative, it is recommended to perform the Cytometric Bead Array (CBA) for BCR-ABL protein. Cases expressing any of the myeloid antigens, CD21, or CD27 and exhibiting extreme elevation of peripheral blood white blood cell counts are highly probable of being BCR-ABL-positive. The frequency (%) of each subclass in our study is indicated as a percentage. The number in parentheses indicates the frequency in the total cases. (C) Case diagnosed as BCR-ABL-positive ALL by flow cytometry. The leukemic cells exhibit CD19+, CD10+, HLA-DR+, CD22+, CD66c+, CRLF2-, myeloid-antigen-, CD21+, and CD27+. The DNA-index analysis revealed neardiploidy, and CBA for BCR-ABL protein was positive.

case of hyperdiploid ALLs, a significant correlation between the chromosomal location of upregulated genes and the presence of trisomies/tetrasomies was observed, and, thus, the reflection of a gene-dosage effect has been suggested [32]. On the other hand, hyperdiploid ALL is characterized by a nonrandom gain of chromosomes commonly including chromosomes X, 4, 6, 10, 14, 17, 18, and 21, but CD66c is located on chromosome 19 [33]. Considering the findings, the expression of CD66c in hyperdiploid cases should not be mediated by the gene-dosage effect.

It was reported that the expression of CD13, CD33, CD15, and CD65 tended to be mutually exclusive with CD66c [6]. Since both *ETV6-RUNX1*-positive ALL frequently expressing CD33 and CD13 [17] and ALL with *MLL*-related chimeric genes commonly expressing CD15 and CD65 [18] are highly concentrated in neardiploid/CD66c-negative cases, it is quite reasonable that the expressions of CD66c and other myeloid antigens tend to be mutually exclusive. Therefore, we excluded BCP-ALL cases possessing well-known chimeric genes lacking CD66c expression and then analyzed the correlation between CD66c expression and that of other myeloid antigens. However, upon excluding *ETV6-RUNX1*-positive cases from the analysis, neardiploid/CD66c-negative cases still exhibited a significantly higher expression of CD33. On the other hand, both *BCR-ABL*-positive and CRLF2-expressing cases exhibited frequent expression of CD33 besides CD66c. Further investigation of the underlying mechanisms that induce the aberrant expression of CD66c and other myeloid antigens should be conducted in the future.

Based on our analysis, we propose an initial diagnostic flow of the prognosis-based subclassification of BCP-ALL using flow cytometry. As presented in Fig. 3B and C, in addition to a regular diagnostic panel, the immunocytological detection of CD66c and CRLF2 in combination with PI staining should be a useful tool for the initial diagnosis of BCP-ALL. By assessing cases with PI staining, more than one-third of the patients should be diagnosed as hyperdiploid, and hypodiploid cases might be rarely detected. After the exclusion of hyper- and hypodiploid BCP-ALL cases, less than 10% of the cases will be CRLF2-positive. In the remaining cases, approximately one quarter of the cases should be *BCR-ABL*-positive ALL. As we presented in Fig. 2E by PCA, the coexpression of myeloid antigens, CD21 or CD27 with CD66c, as well as extreme elevation of peripheral blood white blood cell counts, suggests the presence of a *BCR-ABL* chimeric gene at a high probability, while the findings are not definitive. However, by utilizing the recently developed CBA for BCR-ABL protein, we can make a final diagnosis of *BCR-Abl*-positive ALL at the initial presentation of the patient without waiting for the results of RT-PCR or chromosomal analysis. Since the effectiveness of tyrosine kinase inhibitors as first-line treatment has been reported [31,34], the prompt diagnosis of *BCR-ABL*-positive ALL is important. In our pilot study on 20 patients suspected of *BCR-ABL*-positive ALL, including 5 cases subsequently confirmed as true *BCR-ABL*-positive, the results showed a complete concordance between prior CBA for BCR-ABL fusion proteins and following real-time PCR for *BCR-ABL* chimeric genes (a typical result was presented in Fig. 3C).

In conclusion, CD66c expression is not always specific for *BCR-ABL*-positive ALL, whereas it is frequently associated with some genetic abnormalities, which are important for the prognosis. Although further analysis is needed to elucidate the underlying genetic characteristics as well as clinico-pathological features of CD66c-positive neardiploid BCP-ALL cases, our observations should shed light on the significance of CD66c expression in BCP-ALL.

Conflict of interest statement

The authors have no conflict of interest to declare with regards to this work.

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Contributions. NK designed the research study, performed research, analyzed data and wrote the paper. OT, KI, MM, DH, KK, HO, MK, HS, AM, TK, NT and KK performed the research. AK, JF, YH AO analyzed data. TK, AM and KS analyzed data and wrote the paper.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2013.10.008.

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LETTER FROM THE FRONTLINE

Hepatocyte Transplantation Using a Living Donor Reduced Graft in a Baby With Ornithine Transcarbamylase Deficiency: A Novel Source of Hepatocytes

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TO THE EDITORS:

We performed hepatocyte transplantation (HT) in an 11-day-old infant with ornithine transcarbamylase deficiency (OTCD). We used cryopreserved hepatocytes prepared from remnant liver tissue, a byproduct of a hyper-reduced left lateral segment from living donor liver transplantation (LDLT). The patient exhibited hypothermia, drowsiness, and apnea at 3 days of age; these symptoms were accompanied by hyperammonemia (1940 μg/dL at maximum), although there were no abnormalities at birth or an obvious family history (Fig. 1). Further examinations confirmed that the hyperammonemia was the result of OTCD. Multimodal treatments, including alimentotherapy, medications, and continuous hemodiafiltration (CHDF), did not improve the patient's clinical state, and severe hyperammonemia attacks recurred. Because of the patient's small body size (2550 g) and the lack of an available liver donor, HT was indicated. Hepatocytes of the same blood type were chosen from an institutional repository of cryopreserved hepatocytes prepared from the remnant tissue of segment III from unrelated living donors. Thawed hepatocytes were transplanted twice at 11 and 14 days of age with a double-lumen catheter inserted into the left portal vein via the umbilical vein (Fig. 2). The amounts of transplanted hepatocytes were 7.4×10^7 and 6.6×10^7 cells/body, and the viability rates were 89.1% and 82.6%, respectively. The portal flow was kept stable at greater than 10 mL/kg/minute, and the pressure was maintained at less than 20 mm Hg during and after HT. The immunosuppressive treatment followed the same protocol used for LDLT with tacrolimus and low-dose steroids.[1] The patient was weaned from CHDF and the ventilator at 26 and 30 days of age, respectively, with a stable serum ammonia level of 40 μg/dL. The patient was ultimately discharged 56 days after HT. During the 3 months of follow-up, the baby did well with protein restriction (2 g/kg/day), medication for OTCD, and immunosuppression. No neurological sequelae related to hyperammonemia have been observed so far (Fig. 1).

DISCUSSION

For children with metabolic liver disease, HT is indicated as an alternative or bridge to liver transplantation.[2] HT is less invasive than liver transplantation and can be performed repeatedly. Limitations to the widespread application of HT include the poor availability of hepatocytes. Therefore, it is important to find new sources of high-quality hepatocytes. We previously prepared a repository of hepatocytes obtained from remnant liver tissue, a byproduct of hyper-reduced left lateral segmentectomy in LDLT.[1]

The cell donor was an unrelated volunteer with the same blood type who had previously undergone hyper-reduced left lateral segmentectomy. The main unit of segment II was used as a monosegmental liver graft for the primary recipient with end-stage liver disease, and the remnant was used to isolate hepatocytes with fully informed consent. The hepatocytes were isolated according to the collagenase perfusion method, as described elsewhere,[3] with Liberase MTF C/T GMP grade (Roche). All procedures were performed at our cell processing center according to a strictly controlled protocol based on good manufacturing practices. The total number of transplanted live hepatocytes was 1.4×10^8 cells/body; the ammonia removal rate was more than 200 fmol/cell/hour (203.4 and 265.4 fmol/cell/hour with the first and second injections, respectively). The dose was judged to be sufficiently high to obtain therapeutic effectiveness according to our theoretical background.[4]

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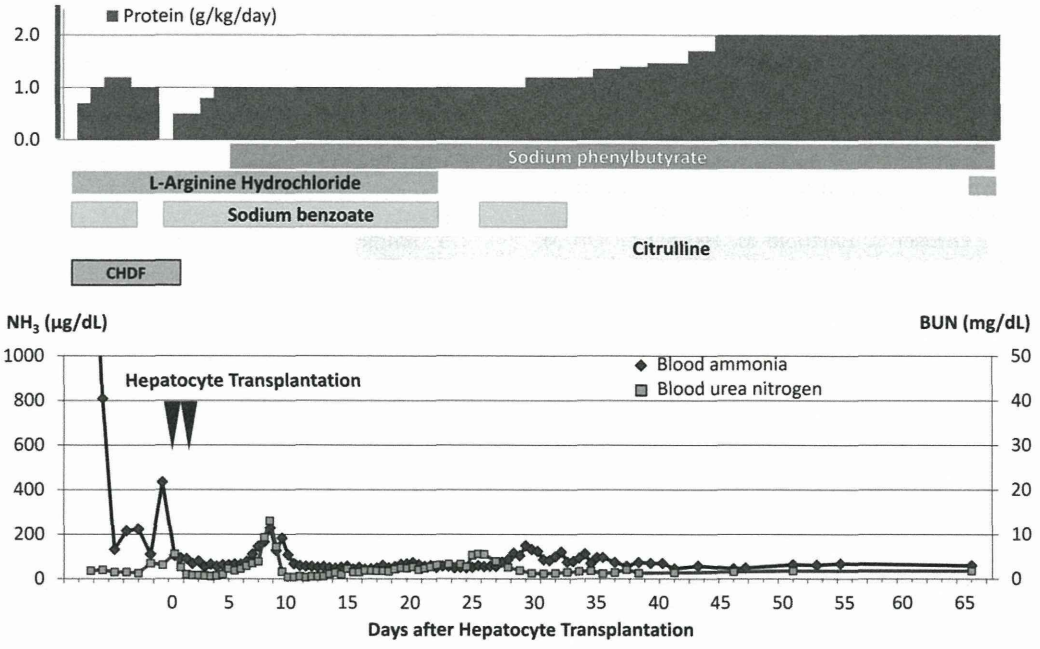

Figure. 1. Treatment schedule (top) and patient condition (bottom). The changes with time for blood ammonia and blood urea nitrogen are shown. The baby was delivered vaginally as a first child. At 3 days of age, hypothermia, low oxygen saturation, and, finally, respiratory arrest occurred. The patient was incubated and given artificial respiration. Concurrently, hyperammonemia (1940 μg/dL) was found, and continuous hemodiafiltration (CHDF) was started in addition to alimentotherapy (protein withdrawal) and medications. Whenever the administration of essential amino acids was restarted, the blood ammonia level became elevated, and at 9 days of age, despite the suspension of essential amino acid administration, the level increased up to 434 μg/dL. At 11 days of age, HT was performed for the first time, and it was performed for the second time at 14 days of age. After HT, amino acid intake was restarted along with the continuation of multimodal treatments, and blood ammonia was controlled well except for episodic increases. The patient was weaned from CHDF and the ventilator at 26 and 30 days of age, respectively, and the patient was ultimately discharged 56 days after HT.

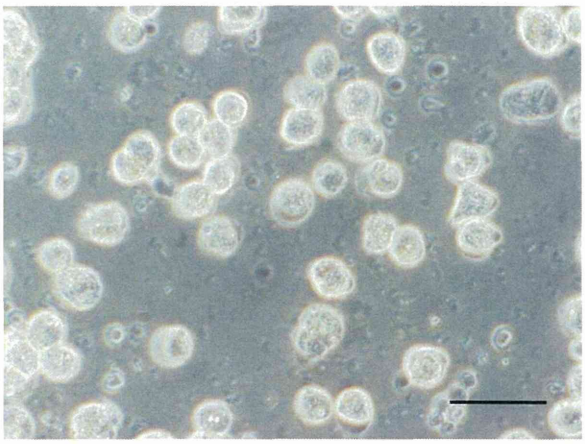

Figure. 2. Hepatocytes transplanted during the first injection. The cells showed a glazed and firm surface. The bar indicates 50 μm.

Because liver transplantation is approved as a treatment for end-stage hepatic failure, donor livers are preferentially allocated for organ transplantation and not for hepatocyte isolation. On rare occasions, the lack of appropriate donor-recipient matching (eg, infant donor livers) provides good-quality hepatocytes.[2] Fetal livers are also considered to be an alternative cell source, although ethical issues remain to be resolved. At present, we have little choice but to use marginal donor tissues, such as livers obtained from donors after cardiac death and organs with steatosis, fibrosis, or a long ischemia time. However, there are unfavorable issues related to the use of marginal donors, including low viability and vulnerability to cryopreservation. In this respect, the remnant liver tissue of hyper-reduction procedures used in LDLT has the same quality as that of left lateral segment grafts. As for availability, there are 5 cases of hyper-reduction per year at our institution on average.[5] The use of remnant liver tissues obtained from hyper-reduced LDLT procedures will, therefore, help to address the shortage of hepatocyte donors.

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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. Pediatr Blood Cancer 2014;61:1215–1221.

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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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

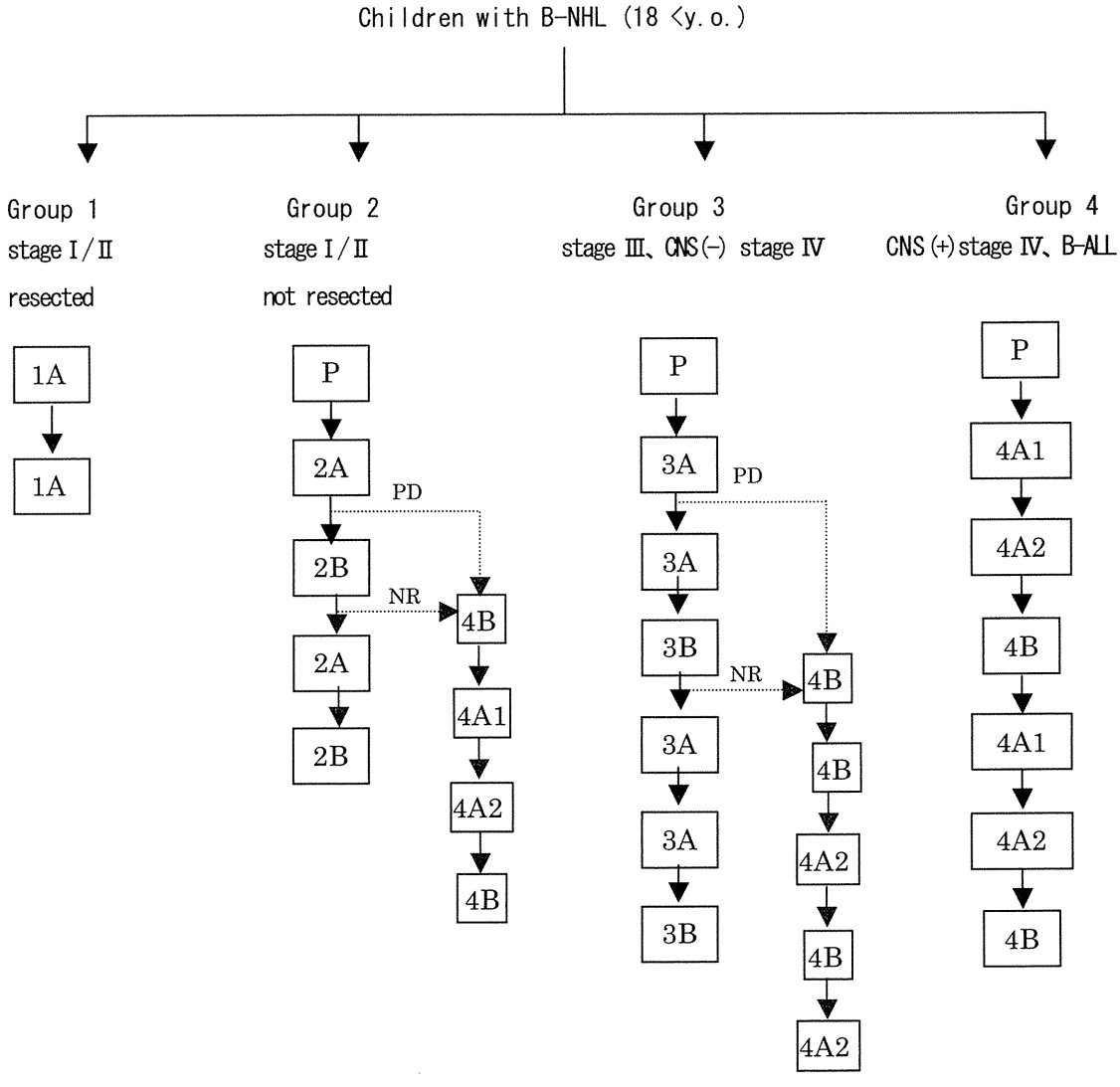

Fig. 1. Treatment framework of the B-NHL03 study. Patients were stratified into four treatment groups according to disease stage, tumor resectability, and BM/CNS involvement. All groups except Group 1 received pre-phase therapy. Group 1 received two courses of chemotherapy, Group 2 received 4 courses,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

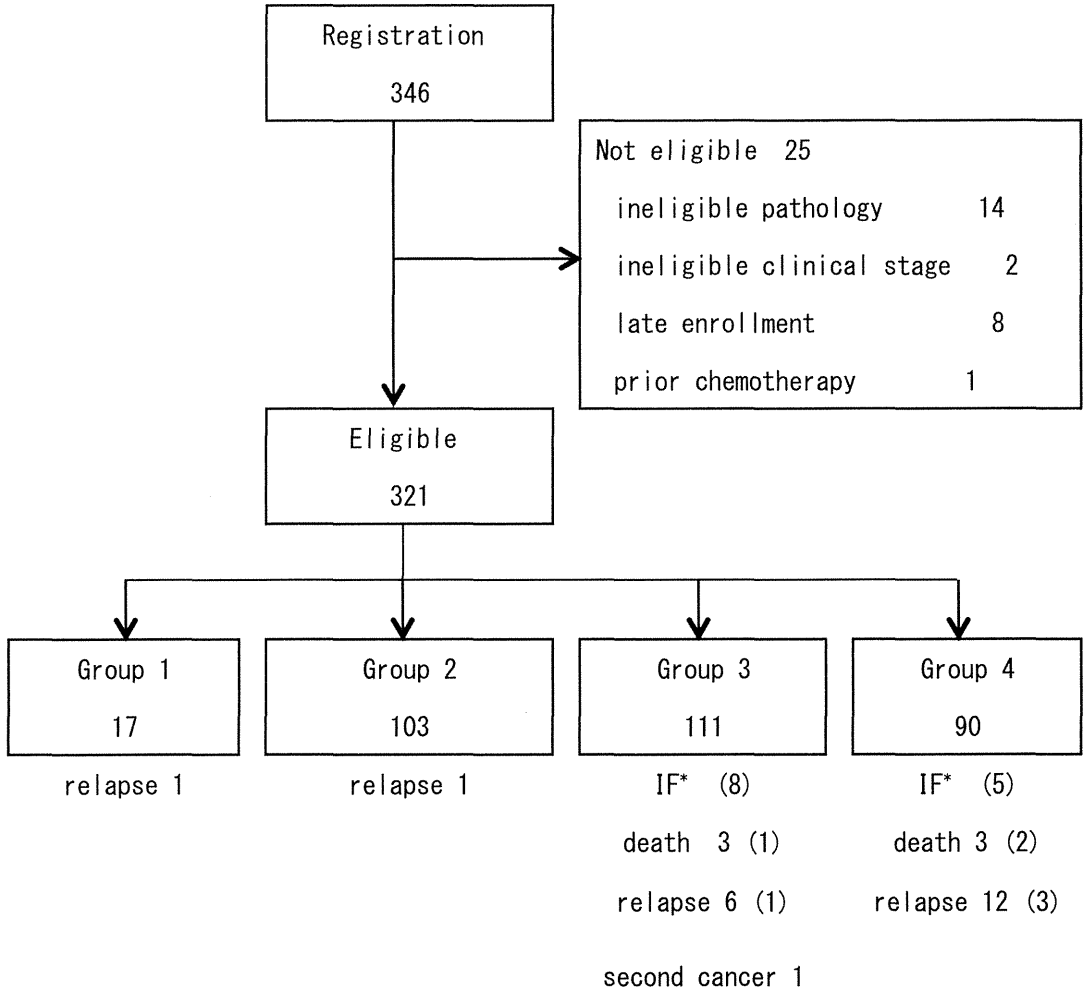

Fig. 2. Patient flow chart and events according to the treatment group. There were 40 events which consisted of each one in Group 1 and 2, 18 in Group 3, and 20 in Group 4. Number in parentheses indicates events occurred during protocol chemotherapy. Number in parenthesis indicates events occurred during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