from donors and patients, but the Amicus product had fewer contaminating platelets (PLTs).[13] We then demonstrated that yield and collection efficiency (CE) of CD34+ cells were greater in the Amicus than in the automated program of the Spectra (Spectra-Auto, Software Version 6.1).[14] After these studies, the Amicus was withdrawn from the Japanese market, and we have used either Spectra-Auto or Spectra-MNC, according to the circumstance.

The reported advantages of Spectra-Auto, comparing with other apheresis platforms, are decreased citrate toxicity, less PLT contamination, and feasible CEs.[14-19] However, there are few direct comparisons of Spectra-MNC and Spectra-Auto,[9,17] and uncertainty remains. Thus, we aimed to compare Spectra-Auto and Spectra-MNC to evaluate various aspects of efficiency and safety. Here, we investigated apheresis collections of a whole cohort containing both donors and patients in our care. Next, we focused on allodonors whose PBPCs were mobilized by G-CSF. In addition, we evaluated donors and patients who underwent apheresis for 2 consecutive days using both programs with a crossover paired comparison.[13,14]

MATERIALS AND METHODS

Patients and donors

Records were investigated from all PBPC collections performed from 2007 to 2012 at Fukushima Medical University Hospital with allodonors and patients 13 years or older who were scheduled to undergo auto-PBPCT. The investigation was approved by the Ethics Review Board of Fukushima Medical University, which is guided by local policy, national law, and the World Health Association Declaration of Helsinki. All procedures were performed on donors and patients who met the criteria of the Japanese Society of Hematopoietic Cell Transplantation, after informed consent was obtained covering PBPCT, chemotherapy, administration of G-CSF, and apheresis.

G-CSF administration

Allodonors were given G-CSF (400 μg/m^2/day filgrastim, Kirin, Tokyo, Japan; or 10 μg/kg/day lenograstim, Chugai, Tokyo, Japan) subcutaneously daily starting 3 days before PBPC collection. Patients received G-CSF after their mobilizing chemotherapy. Donors and patients continued receiving G-CSF until the last day of apheresis.

Apheresis collections

Apheresis collections with the Spectra-Auto (Software Version 6.1) and the Spectra-MNC (Software Version 6.1) were performed according to the manufacturer's protocols.[13,14] In brief, Spectra-Auto processes a quantity of blood to accumulate mononuclear cells (MNCs) and then transfers cells into a collection bag with nearly total automation, whereas Spectra-MNC collects cells with continuous flow semiautomatically. Spectra-Auto was set for six to 10 harvests, corresponding to two more harvests than the default to prevent overflow of the accumulated MNCs per each harvest, with an initial inlet flow rate of 35 to 75 mL/min. A 12:1 to 18:1 ratio of whole blood to ACD-A solution (Terumo BCT) was used in both Spectra-Auto and Spectra-MNC, with no complications related to aggregation at any ratio. In Spectra-MNC, the blood–plasma interface was determined by adjusting outflow hematocrit to 1% to 2% using a WBC colorgram (COBE, Lakewood, CO) and a collecting rate of 1.0 to 2.0 mL/min. The target volume processed was twice the circulating volume. Apheresis was deemed complete when a target dose of CD34+ cells, usually at least 2×10^6 cells/kg of recipient's body weight, was obtained. We used Spectra-Auto or Spectra-MNC on an arbitrary schedule. From 2007 through early 2009, we used only Spectra-Auto. Spectra-MNC was then used on Monday, Wednesday, and Friday, and Spectra-Auto on Tuesday and Thursday, to maintain operator skill with both programs.

Evaluation of operational settings

For each procedure, we evaluated the volume of ACD used, running time, and processed volume of whole blood without correction for volume of ACD used.

Examination of peripheral cell counts

Peripheral cell counts and hemoglobin (Hb) concentrations were examined by an electronic cell counter (K-2000, Sysmex, Kobe, Japan).

Analysis of collected PBPC products

The product yields and CE of white blood cells (WBCs), MNCs, and CD34+ cells, and residual PLTs were enumerated. CE calculations were based on the total blood volumes (TBVs) of donors and patients (TBV-CE)[14,20] or the cell numbers passed through the instrument (CE1).[15]

$$\text{TBV-CE} = \text{number of collected cells} \times 100 / \text{preapheresis cell count} \times \text{TBV}.$$

$$\text{CE1} = \text{number of collected cells} \times 100 / [(\text{processed volume} - \text{ACD used}) \times (\text{pre-} + \text{postapheresis cell count})/2].$$

Flow cytometry

The number of CD34+ cells was determined by flow cytometry (Cytomics FC 500, Beckman Coulter, Fullerton, CA) with anti-CD34 and anti-CD45 fluorescent markers (both Beckman Coulter).[21]

Statistical analysis

The unpaired t test was used to compare Spectra-Auto and Spectra-MNC. The paired t test was used in the

TABLE 1. Pre- and postapheresis peripheral cell counts and decreases of Hb concentrations and PLT counts after the apheresis*

Variables	All donors and patients[a] Spectra-Auto[c] (n = 118)	Spectra-MNC[d] (n = 70)	p value	Allodonors[b] Spectra-Auto[c] (n = 49)	Spectra-MNC[d] (n = 41)	p value
WBC count ($\times 10^9$/L)						
Before apheresis	28.2 ± 14.5	34.1 ± 17.1	0.0116	39.4 ± 8.9	44.1 ± 10.4	0.0241
Median, range	27.1, 1.9-58.7	37.1, 3.4-64.0		40.6, 21.7-58.2	45.1, 23.0-64.0	
MNC count ($\times 10^9$/L)						
Before apheresis	4.08 ± 2.32	4.53 ± 2.45	NS	5.24 ± 2.16	5.25 ± 2.08	NS
Median, range	3.69, 0.61-12.4	4.51, 0.47-12.9		5.00, 2.02-12.4	4.86, 2.07-12.9	
CD34+ cell ($\times 10^6$/L)						
Before apheresis	68 ± 105	64 ± 76	NS	40 ± 23	48 ± 42	NS
Median, range	33, 4-796	41, 4-363		35, 4-124	41, 8-240	
Hb (g/dL)						
Before apheresis	11.5 ± 2.4	12.0 ± 2.4	NS	13.7 ± 1.3	13.5 ± 1.2	NS
Median, range	11.5, 6.8-16.9	12.5, 8.1-15.5		14.0, 11.1-16.9	13.4, 10.8-15.5	
After apheresis	10.5 ± 2.3	10.9 ± 2.3	NS	12.6 ± 1.4	12.4 ± 1.4	NS
Median, range	10.5, 6.3-15.6	11.2, 6.9-15.0		13.1, 9.5-15.5	12.7, 9.3-14.6	
Decrease (%)	10.3 ± 5.2	10.1 ± 6.1	NS	8.8 ± 3.3	7.9 ± 4.9	NS
95% CI	9.4-11.3	8.6-11.6		7.8-9.8	6.4-9.5	
PLT count ($\times 10^9$/L)						
Before apheresis	129 ± 62	166 ± 81	0.0004	176 ± 54	199 ± 50	0.0440
Median, range	118, 43-359	159, 35-519		174, 93-359	203, 110-301	
After apheresis	81 ± 49	100 ± 49	0.0092	112 ± 49	119 ± 37	NS
Median, range	70, 23-297	93, 28-288		109, 38-297	112, 57-216	
Decrease (%)	31.1 ± 12.0	34.9 ± 11.9	0.0368	34.6 ± 10.9	40.4 ± 8.1	0.0063
95% CI	28.9-33.3	32.1-37.8		31.5-37.8	37.8-43.0	

* Data are shown as means ± SDs. Indicated cell counts or variables were compared among all donors and patients[a] or exclusively among donors[b] between the Spectra-Auto[c] and the Spectra-MNC[d].
NS = not significant.

crossover comparisons.[13,14] The frequencies were compared by the chi-square test. The relationships between preapheresis cell counts and yields or CE were analyzed using a correlation coefficient, of which differences were determined by Fisher's z transformation. All data are shown as mean ± standard deviation (SD) unless otherwise specified. All p values are two-sided, with p values of less than 0.05 considered significant.

RESULTS

Baseline variables

A total of 188 apheresis collections were performed on 65 donors (38 male, 27 female) and 69 patients (39 male, 30 female), including three with acute leukemia, 34 with malignant lymphoma, 27 with multiple myeloma, and five with solid tumors. Donors and patients were 37.5 ± 11.1 or 49.6 ± 14.2 years, respectively. The 188 procedures consisted of 118 with Spectra-Auto (49 for donors, 69 for patients), and 70 with Spectra-MNC (41 for donors, 29 for patients); thus Auto-PBPC was more frequently used for patients (p = 0.0235). Comparing age (44.6 ± 14.1 years in Spectra-Auto vs. 43.7 ± 13.3 years in Spectra-MNC), sex (56% male vs. 50% male), and TBV (3.8 ± 1.0 L vs. 3.9 ± 1.0 L), all were insignificantly different, with p values greater than 0.05.

We were able to study 29 donors and patients (16 donors and 13 patients), who underwent apheresis collection with two different programs for 2 consecutive days, using a paired comparison. Of course, age, sex, and TBV were technically the same between these two programs in this comparison.

In both the whole cohort and the donors, the starting peripheral WBC and PLT counts were higher in Spectra-MNC compared with Spectra-Auto, whereas MNC and CD34+ cell counts were equivalent (Table 1). However, in the paired comparison, there were no differences in initial WBC ($27.8 \times 10^9 \pm 15.3 \times 10^9$/L in Spectra-Auto vs. $30.0 \times 10^9 \pm 16.4 \times 10^9$/L in Spectra-MNC), MNC ($3.78 \times 10^9 \pm 1.68 \times 10^9$/L vs. $3.73 \times 10^9 \pm 1.58 \times 10^9$/L), CD34+ cell ($33.1 \pm 25.0$ vs. $30.2 \pm 31.1 \times 10^6$/L), and PLT ($128 \times 10^9 \pm 67 \times 10^9$/L vs. $145 \times 10^9 \pm 65 \times 10^9$/L) counts, or Hb concentration (12.1 ± 2.0 g/dL vs. 12.0 ± 2.1 g/dL).

Operation of the Spectra with two programs

The two programs processed equivalent volumes, but run time was longer with Spectra-Auto compared with Spectra-MNC, in the whole cohort (p = 0.013, Table 2). In

TABLE 2. Variables of apheresis procedures*

Variables	All donors and patients: Spectra-Auto (n = 118)	All donors and patients: Spectra-MNC (n = 70)	p value	Allodonors: Spectra-Auto (n = 49)	Allodonors: Spectra-MNC (n = 41)	p value
Processed volume (L)	8.19 ± 1.60	8.43 ± 1.95	NS	8.44 ± 1.52	8.03 ± 1.64	NS
95% CI	7.91-8.49	7.97-8.91		8.00-8.87	7.75-9.08	
Run time (min)	180 ± 21	171 ± 21	0.013	178 ± 22	172 ± 24	NS
95% CI	176-183	166-177		172-184	164-179	
ACD used (mL)	547 ± 123	562 ± 136	NS	565 ± 119	566 ± 140	NS
95% CI	524-569	529-594		531-600	521-610	
Volume collected (mL)	100 ± 35	344 ± 92	<0.0001	118 ± 31	358 ± 89	<0.0001
95% CI	93-106	321-365		109-127	331-387	

* Data are shown as means ± SDs.

TABLE 3. Components of collected PBPC products*

Variables	All donors and patients: Spectra-Auto (n = 118)	All donors and patients: Spectra-MNC (n = 70)	p value	Allodonors: Spectra-Auto (n = 49)	Allodonors: Spectra-MNC (n = 41)	p value
WBCs						
$\times 10^9$/bag	23.0 ± 10.3	33.6 ± 19.8	<0.0001	26.0 ± 6.9	37.0 ± 18.2	0.0002
95% CI	21.1-24.9	28.8-38.3		22.4-27.1	29.5-41.9	
Efficiency (%)						
TBV-CE	31.7 ± 30.8	33.4 ± 25.9	NS	17.1 ± 5.3	20.8 ± 7.7	0.0102
CE1	16.3 ± 14.5	18.7 ± 12.8	NS	9.5 ± 2.4	12.6 ± 4.6	0.0001
MNCs						
$\times 10^9$/bag	15.1 ± 7.7	19.1 ± 10.6	0.0036	20.8 ± 5.8	23.1 ± 8.7	NS
95% CI	13.7-16.5	16.6-21.6		19.1-22.4	20.3-25.8	
Efficiency (%)						
TBV-CE	107.3 ± 55.6	114.5 ± 51.6	NS	104.6 ± 28.6	112.8 ± 36.6	NS
CE1	60.9 ± 20.3	66.0 ± 21.6	NS	61.7 ± 10.6	67.3 ± 13.4	0.0320
CD34+ cells						
$\times 10^6$/bag	303 ± 476	294 ± 354	NS	192 ± 118	224 ± 225	NS
95% CI	216-390	210-379		158-226	153-294	
$\times 10^6$/kg†	5.56 ± 9.95	4.76 ± 5.56	NS	3.14 ± 1.85	3.56 ± 3.16	NS
95% CI	3.75-7.37	3.43-6.08		2.60-3.68	2.56-4.56	
Efficiency (%)						
TBV-CE	119.8 ± 43.2	118.5 ± 48.6	NS	123.1 ± 43.3	112.4 ± 41.2	NS
CE1	72.8 ± 24.2	72.1 ± 21.1	NS	74.3 ± 25.3	70.8 ± 20.2	NS
PLTs						
$\times 10^9$/bag	172 ± 125	341 ± 224	<0.0001	271 ± 95	460 ± 181	<0.0001
95% CI	149-194	288-395		244-299	402-517	

* Data are shown as means ± SDs.
† Number of CD34+ cells per donor body weight (kg).

the paired comparison, however, run time was not significantly different (Table 4).

Mild reactions including numbness (n = 13 [10.1%] in Spectra-Auto and n = 5 [7.1%] in Spectra-MNC) and arm pain (n = 2 [1.7%] only in Spectra-Auto) occurred but did not interrupt any procedure. There were no statistical differences in overall frequencies of reactions (11.8% in Spectra-Auto and 7.1% in Spectra-MNC). Hypocalcemic symptoms were relieved promptly by oral or intravenous calcium.[22] The volumes of ACD used were not different, although volumes of collected samples were greater in Spectra-MNC (Tables 2 and 4).

WBCs, MNCs, and CD34+ cells in collected PBPC product

In the whole cohort, total collected WBCs ($p < 0.0001$) and MNCs ($p = 0.0036$), but not CD34+ cells, were more in Spectra-MNC than in Spectra-Auto (Table 3). The CE of WBCs, MNCs, and CD34+ cells were not significantly different (Table 3). However, among donors, CEs of WBCs (TBV-CE, $p = 0.0102$; CE1, $p = 0.0001$) and MNCs (CE1, $p = 0.032$) were higher in Spectra-MNC, although no such differences emerged for CD34+ cells (Table 3). Paired comparison of Spectra-Auto and Spectra-MNC showed no statistical differences in numbers and CE

TABLE 4. Paired comparison of apheresis collections among donors and patients who received PBPC collections with both programs over 2 consecutive days*

Variables	Spectra-Auto (n = 29)	Spectra-MNC (n = 29)	p value
Apheresis procedures			
Used on Day 1/Day 2†	12/17	17/12	NS
Processed volume (L)	8.62 ± 1.78	8.43 ± 1.53	NS
Run time (min)	179 ± 25	174 ± 13	NS
ACD used (mL)	570 ± 141	556 ± 112	NS
PBPC products			
Volume collected (mL)	100 ± 30	329 ± 101	<0.0001
WBCs			
$\times 10^9$/bag	24.7 ± 7.7	27.5 ± 16.4	NS
95% CI	21.7-27.6	21.2-33.7	
Efficiency (%)			
TBV-CE	35.7 ± 36.5	32.8 ± 28.6	NS
CE1	18.3 ± 16.7	17.1 ± 13.2	NS
MNCs			
$\times 10^9$/bag	16.6 ± 6.9	15.5 ± 7.6	NS
95% CI	13.9-19.2	12.6-18.4	
Efficiency (%)			
TBV-CE	118.9 ± 33.4	113.2 ± 39.9	NS
CE1	65.7 ± 13.9	65.8 ± 24.8	NS
CD34+ cells			
$\times 10^6$/bag	164 ± 146	137 ± 169	NS
95% CI	108-220	73-202	
$\times 10^6$/kg‡	2.63 ± 2.16	2.18 ± 2.51	NS
95% CI	1.81-3.46	1.23-3.14	
Efficiency (%)			
TBV-CE	121.8 ± 34.5	114.8 ± 59.2	NS
CE1	72.3 ± 18.0	67.2 ± 22.3	NS
PLTs			
$\times 10^9$/bag	188 ± 95	293 ± 185	0.0008
95% CI	152-224	222-363	

* Data are shown as means ± SDs except number of the programs used on Day 1 or Day 2.
† Was compared by the chi-square test, whereas all other variables were compared by the paired t test.
‡ Number of CD34+ cells per donor body weight (kg).

of WBCs, MNCs, and CD34+ cells in the products (Table 4).

Correlation between preapheresis cell counts and quality of collected products

Regardless of the cohort or apheresis program, preapheresis peripheral WBC, MNC, and CD34+ cell counts were consistently correlated with WBC, MNC, and CD34+ cell yields, respectively (Fig. 1). However, preapheresis WBC counts were negatively correlated with TBV-CE, but not CE1, of CD34+ cells only with Spectra-MNC in the whole cohort (Fig. 2A, $R = -0.3721$, $p = 0.0015$). The donors showed a similar trend in the correlation between preapheresis WBC counts and TBV-CE of CD34+ cells ($R = -0.2895$, $p = 0.07$), although it did not achieve significance. There were no significant correlations between the preapheresis MNC or CD34+ cell counts and TBV-CE or CE1 of CD34+ cells either in the whole cohort or in donors.

Contamination of PLTs and thrombocytopenia after PBPC harvest

The products collected with Spectra-MNC contained more contaminating PLTs compared with Spectra-Auto (Table 3) in the whole cohort ($p < 0.0001$) and among donors ($p < 0.0001$), leading to significant decreases in peripheral PLTs (Table 1). In the paired comparison, numbers of contaminating PLTs were also greater in Spectra-MNC compared with Spectra-Auto ($p = 0.0008$, Table 4). However, decreases in percentages of peripheral PLTs were similar ($p = 0.84$) between Spectra-Auto (34.7 ± 11.3%) and Spectra-MNC (34.2 ± 12.7%).

DISCUSSION

Spectra-Auto operates autonomously with a stable interface and minimal operator supervision, but takes more time.[14,17] As expected, we observed a longer running time with Spectra-Auto compared with Spectra-MNC. We did not find differences in the frequency of ACD-related adverse reactions; indeed the volumes of ACD used were comparable. However, volumes of collected samples were significantly greater in Spectra-MNC. Greater product volume requires more DMSO for frozen storage, which may correlate with infusion reactions.[6,23]

Some studies have speculated that Spectra-MNC may collect greater numbers of CD34+ cells than Spectra-Auto.[9,17] In our present study, although yields of collected WBCs and MNCs were significantly greater, those of CD34+ cells were similar in Spectra-MNC compared to Spectra-Auto, and the cause of this discrepancy is unclear. One explanation is that the quality of products from Spectra-MNC is more dependent on operator skill. Another possibility is that we opted for two more harvests than the default in Spectra-Auto collection, which may contribute to an overall higher yield due to fewer overflown cells in the harvests. In addition, increased preapheresis peripheral WBC counts in Spectra-MNC may have contributed, because it has been reported that the increased peripheral WBC count is associated with decreased CE of CD34+ cells.[9,24-27] In fact, with Spectra-MNC, preapheresis WBC counts were positively correlated with the collection yields of WBCs and inversely correlated with the CE of CD34+ cells. Therefore, among donors and patients with high peripheral WBCs, Spectra-MNC may collect the extra myeloid cells, which potentially lead to infusion reactions.[7] However, our study of 64 allo- and 54 auto-PBPCTs has not shown severe infusion reactions.

Although both Spectra-Auto and Spectra-MNC collected greater numbers of contaminating PLTs than the Amicus in our previous studies,[13,14] the numbers were even greater in Spectra-MNC in this study, as well as other studies.[9,17] Associated with this, we observed more substantial decreases in PLT counts after apheresis using Spectra-MNC than Spectra-Auto. Consistent with this

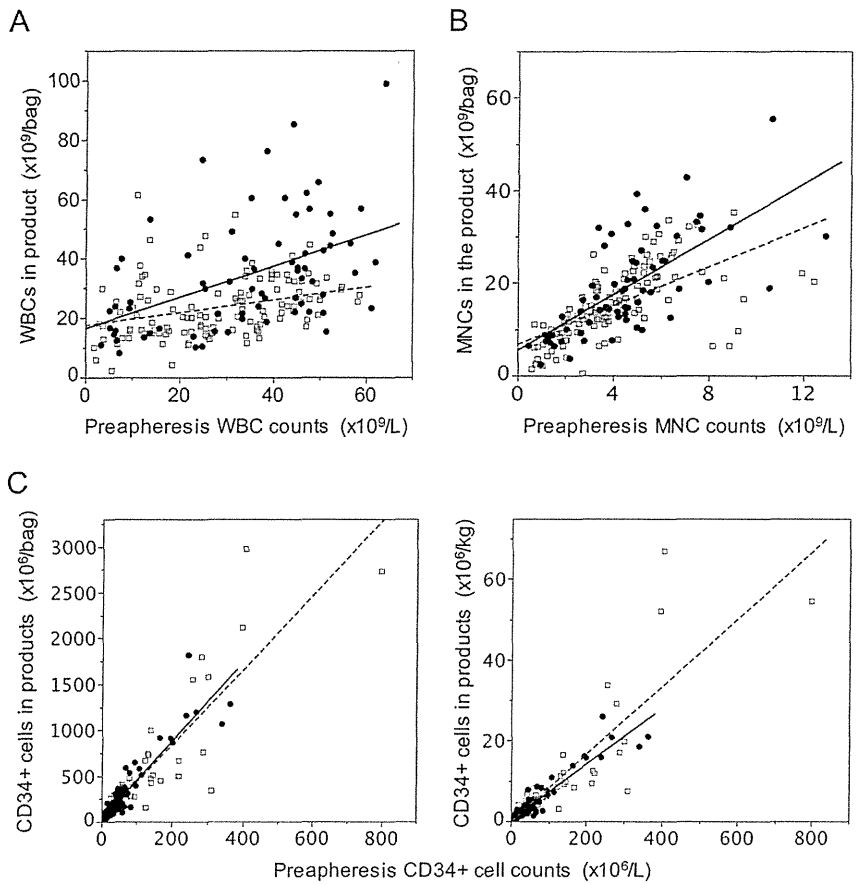

Fig. 1. Correlation of preapheresis cell counts and collection yields. Yields of indicated cells relative to preapheresis cell counts of all donors and patients collected using the Spectra-MNC (•, —) and the Spectra-Auto (□, - -) are shown. (A) Preapheresis WBC counts were significantly correlated with the numbers of collected WBCs in the Spectra-MNC ($r = 0.4072$, $p < 0.0001$) and the Spectra-Auto ($r = 0.3096$, $p = 0.0006$). (B) Preapheresis MNC counts were significantly correlated with the numbers of collected MNCs in the Spectra-MNC ($r = 0.6896$, $p < 0.0001$) and the Spectra-Auto ($r = 0.6287$, $p < 0.0001$). (C) Preapheresis CD34+ cell counts were significantly correlated with the numbers of collected CD34+ cells per bag and donor body weight in the Spectra-MNC ($r = 0.9225$, $p < 0.0001$; and $r = 0.8815$, $p < 0.0001$, respectively) and the Spectra-Auto ($r = 0.8952$, $p < 0.0001$; and $r = 0.9399$, $p < 0.0001$, respectively). There were no significant differences between these two programs in the slopes of correlation coefficients in each of WBCs, MNCs, and CD34+ cells.

observation, it has been reported that donors sometimes require PLT transfusion after apheresis using Spectra-MNC.[27] However, our donors and patients did not require PLT transfusion. Possibly because the number of contaminating PLTs correlated with preapheresis peripheral PLT counts, the number of contaminating PLTs in patients with low peripheral PLT counts are fewer than those with high peripheral PLT counts. This might also be associated with similar decrease of peripheral PLTs in the paired comparison between these two programs.

Reduced contamination of unnecessary cells including myeloid cells and PLTs is an advantage not only with Spectra-Auto, but also with newer collection platforms,[28,29] including the Spectra Optia (Terumo BCT), which features a real-time automated interface as a machine and software upgrade of the original Spectra. Brauninger and colleagues[28] recently reported that apheresis collection with the Optia reduced contamination of lymphocytes and showed a 6% decline in attrition of peripheral PLTs (from 50% to 44%) in the donors, compared with Spectra-MNC. In another study,[29] there were no significant differences in attrition of PLTs after the apheresis collections between the Optia and the Spectra-MNC. This study showed a 3.8 or 5.8% decline difference in attrition of peripheral PLTs in the whole cohort and among donors, respectively, in Spectra-Auto, compared with Spectra-MNC. Moreover, our data, which showed similar CD34 but lower MNC in Spectra-Auto versus Spectra-MNC, may indicate less contamination with undesired cells. Despite not being prospectively randomized, our study suggests that the Optia should be compared with the Spectra-Auto, as well as the Spectra-MNC.

In summary, we report more PLTs in products collected by Spectra-MNC compared with Spectra-Auto. However, we did not observe higher collection yields of CD34+ cells in Spectra-MNC, even though this has been posited as a benefit of Spectra-MNC. This might be attributable to the negative correlation between preapheresis cell counts and CE of CD34+ cells. In the future, Spectra-Auto should be carefully compared with newer automated programs to guide decisions about which system to prefer for safe and efficient apheresis collections.

ACKNOWLEDGMENTS

The authors are grateful to Miyoko Goto, certified apheresis nurse for her great help and Kinuyo Kawabata, MT and Satoshi Ono, MT for their skillful assays.

CONFLICT OF INTEREST

The authors report no conflicts of interest or funding sources.

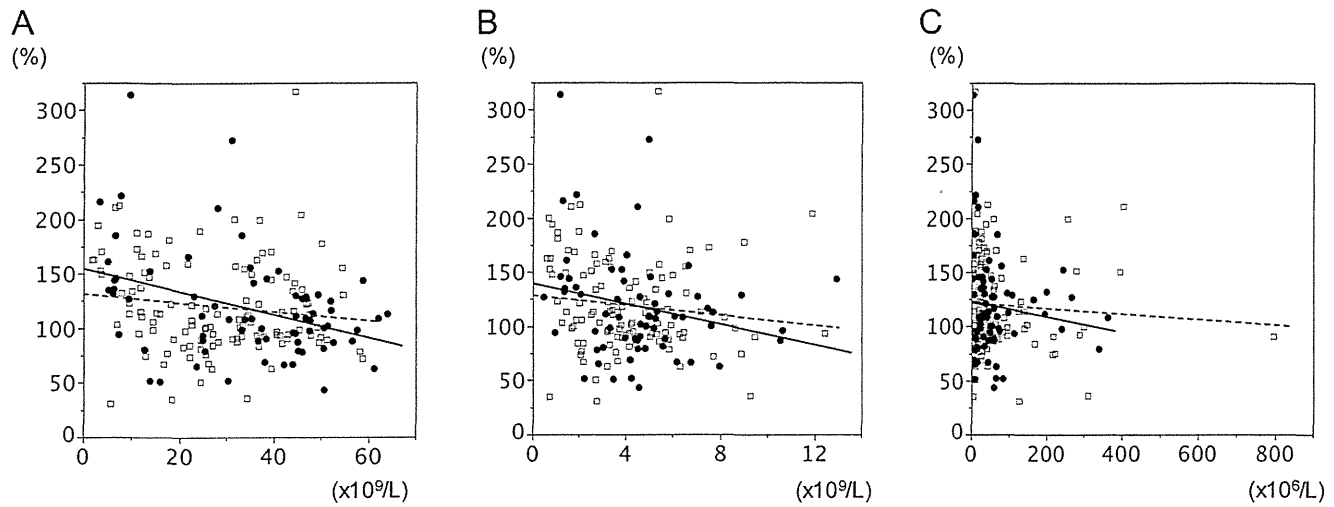

Fig. 2. Correlation of preapheresis peripheral cell counts and CEs of CD34+ cells. Collection efficiencies calculated based on TBV of donors and patients (TBV-CE) of CD34+ cells relative to preapheresis cell counts of all donors and patients collected using the Spectra-MNC (•, —) and the Spectra-Auto (□, - - -) are shown. (A) Preapheresis WBC counts were negatively correlated with the TBV-CE of CD34+ cells in the Spectra-MNC, but not the Spectra-Auto. There were no significant correlations between preapheresis MNC (B) or CD34+ cell counts (C) and TBV-CE of CD34+ cells either in the Spectra-MNC or the Spectra-Auto.

REFERENCES

1. Copelan EA. Hematopoietic stem-cell transplantation. N Engl J Med 2006;354:1813-26.
2. Körbling M, Freireich EJ. Twenty-five years of peripheral blood stem cell transplantation. Blood 2011;117:6411-6.
3. Goldberg SL, Mangan KF, Klumpp TR, et al. Complications of peripheral blood stem cell harvesting: review of 554 PBSC leukaphereses. J Hematother 1995;4:85-90.
4. Ohto H. Efficient and safe stem cell apheresis. Transfus Apher Sci 2004;31:203-5.
5. Moog R. Mobilization and harvesting of peripheral blood stem cells. Curr Stem Cell Res Ther 2006;1:189-201.
6. Zambelli A, Poggi G, da Prada GA, et al. Clinical toxicity of cryopreserved circulating progenitor cells infusion. Anticancer Res 1998;18:4705-8.
7. Calmels B, Lemarie C, Esterni B, et al. Occurrence and severity of adverse events after autologous hematopoietic progenitor cell infusion are related to the amount of granulocytes in the apheresis product. Transfusion 2007; 47:1268-75.
8. Martín-Henao GA, Resano PM, Villegas JM, et al. Adverse reactions during transfusion of thawed haematopoietic progenitor cells from apheresis are closely related to the number of granulocyte cells in the leukapheresis product. Vox Sang 2010;99:267-73.
9. Cooling L, Hoffmann S, Herrst M, et al. A prospective randomized trial of two popular mononuclear cell collection sets for autologous peripheral blood stem cell collection in multiple myeloma. Transfusion 2010;50:100-19.
10. Vogelsang GB, Lee L, Bensen-Kennedy DM. Pathogenesis and treatment of graft-versus-host disease after bone marrow transplant. Annu Rev Med 2003;54: 29-52.
11. Welniak LA, Blazar BR, Murphy WJ. Immunobiology of allogeneic hematopoietic stem cell transplantation. Annu Rev Immunol 2007;25:139-70.
12. Ngoma AM, Ikeda K, Hashimoto Y, et al. Impaired regulatory T cell reconstitution in patients with acute graft-versus-host disease and cytomegalovirus infection after allogeneic bone marrow transplantation. Int J Hematol 2012;95:86-94.
13. Ikeda K, Ohto H, Nemoto K, et al. Collection of MNCs and progenitor cells by two separators for PBPC transplantation: a randomized crossover trial. Transfusion 2003;43:814-9.
14. Ikeda K, Ohto H, Kanno T, et al. Automated programs for collection of mononuclear cells and progenitor cells by two separators for peripheral blood progenitor cell transplantation: comparison by a randomized crossover study. Transfusion 2007;47:1234-40.
15. Schreiner T, Wiesneth M, Krug E. Collection of allogeneic peripheral blood progenitor cells by two protocols on an apheresis system. Transfusion 1998;38:1051-5.
16. Ravagnani F, Siena S, De Reys S, et al. Improved collection of mobilized CD34+ hematopoietic progenitor cells by a novel automated leukapheresis system. Transfusion 1999;39:48-55.
17. Rowley SD, Prather K, Bui KT, et al. Collection of peripheral blood progenitor cells with an automated leukapheresis system. Transfusion 1999;39:1200-6.
18. Green RH, Watson D, Buchanan S, et al. A prospective randomized concurrent comparison of the COBE Spectra Version 4.7, COBE Spectra Version 6 (auto PBSC™) and

Haemonetics MCS+ cell separators for leucapheresis in patients with haematological and non-haematological malignancies. Transfus Sci 2001;24:85-90.
19. Adorno G, Del Proposto G, Palombi F, et al. Collection of peripheral progenitor cells: a comparison between Amicus and Cobe-Spectra blood cell separators. Transfus Apher Sci 2004;30:131-6.
20. Hitzler WE, Wolf S, Runkel S, et al. Comparison of intermittent- and continuous-flow cell separators for the collection of autologous peripheral blood progenitor cells in patients with hematologic malignancies. Transfusion 2001;41:1562-6.
21. Ngoma A, Saito S, Ohto H, et al. CD34+ cell enumeration by flow cytometry: a comparison of systems and methodologies. Arch Pathol Lab Med 2011;135:909-14.
22. Kishimoto M, Ohto H, Shikama Y, et al. Treatment for the decline of ionized calcium levels during peripheral blood progenitor cell harvesting. Transfusion 2002;42: 1340-7.
23. Konuma T, Ooi J, Takahashi S, et al. Cardiovascular toxicity of cryopreserved cord blood cell infusion. Bone Marrow Transplant 2008;41:861-5.
24. Benjamin RJ, Linsley L, Fountain D, et al. Preapheresis peripheral blood CD34+ mononuclear cell counts as predicators of progenitor cell yield. Transfusion 1997;37: 79-85.
25. Ford CD, Pace N, Lehman C. Factors affecting the efficiency of collection of CD34-positive peripheral blood cells by a blood cell separator. Transfusion 1998;38:1046-50.
26. Heuft HG, Dubiel M, Rick O, et al. Inverse relationship between patient peripheral blood CD34+ cell counts and collection efficiency for CD34 cells in two automated systems. Transfusion 2001;41:1008-13.
27. Gidron A, Verma A, Doyle M, et al. Can the stem cell mobilization technique influence CD34+ cell collection efficiency of procedures in patients with hematologic malignancies? Bone Marrow Transplant 2005;35: 243-6.
28. Brauninger S, Bialleck H, Thorausch K, et al. Allogeneic donor peripheral blood "stem cell" apheresis: prospective comparison of two apheresis systems. Transfusion 2012; 52:1137-45.
29. Reinhardt P, Brauninger S, Bialleck H, et al. Automatic interface-controlled apheresis collection of stem/progenitor cells: results from an autologous donor validation trial of a novel stem cell apheresis device. Transfusion 2011;51:1321-30.

Pediatr Blood Cancer 2013;60:1587–1592

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

Yuka Yamashita, MS,[1] Akira Shimada, MD, PhD,[1,2] Tomomi Yamada, PhD,[3] Kazutaka Yamaji, MD, PhD,[3] Toshinori Hori, MD, PhD,[3] Masahito Tsurusawa, MD,[3] Arata Watanabe, MD,[4] Atsushi Kikuta, MD,[5] Keiko Asami, MD,[6] Akiko M. Saito, MD, PhD,[1] and Keizo Horibe, MD, PhD[1]*

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 (*JAK*s) 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.

DOI 10.1002/pbc.24571
Published online 27 June 2013 in Wiley Online Library (wileyonlinelibrary.com).

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

[1]National Hospital Organization Nagoya Medical Center Clinical Research Center, Nagoya, Japan; [2]Department of Pediatrics, Okayama University Hospital, Okayama, Japan; [3]Department of Pediatrics, Aichi Medical University, Nagakute, Japan; [4]Department of Pediatrics, Nakadoori General Hospital, Akita, Japan; [5]Division of Pediatric Oncology, Cancer Center, Fukushima Medical University, Fukushima, Japan; [6]Department of Pediatrics, Niigata Cancer Center Hospital, Niigata, Japan

Grant sponsor: Clinical Cancer Research; Grant sponsor: Grant-in-Aid for Cancer Research; Grant sponsor: The Ministry of Health, Labour and Welfare of Japan

Conflict of interest: Nothing to declare.

*Correspondence to: Keizo Horibe, National Hospital Organization Nagoya Medical Center Clinical Research Center, 4-1-1, Sanno-maru, Naka-ku, Nagoya, Aichi 460-0001, Japan. E-mail: horibek@nnh.hosp.go.jp

Received 10 September 2012; Accepted 25 March 2013

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).

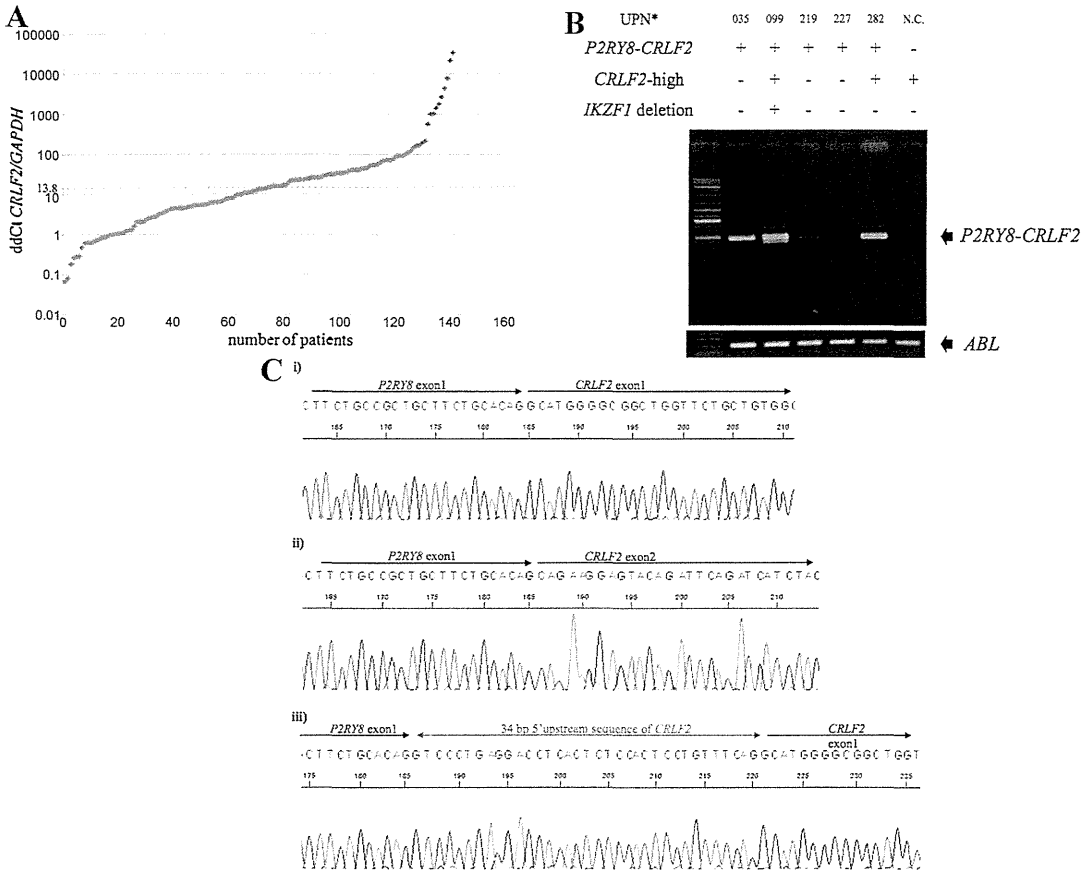

Fig. 1. Assessment of *CRLF2* expression and alterations in *CRLF2*. **A**: Measurement of *CRLF2* expression by RQ-PCR. The median *CRLF2* expression value (normalized to *GAPDH* expression) was 13.8. Red diamonds represent high *CRLF2* expression, defined as *CRLF2* expression that was ≥10-fold higher 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

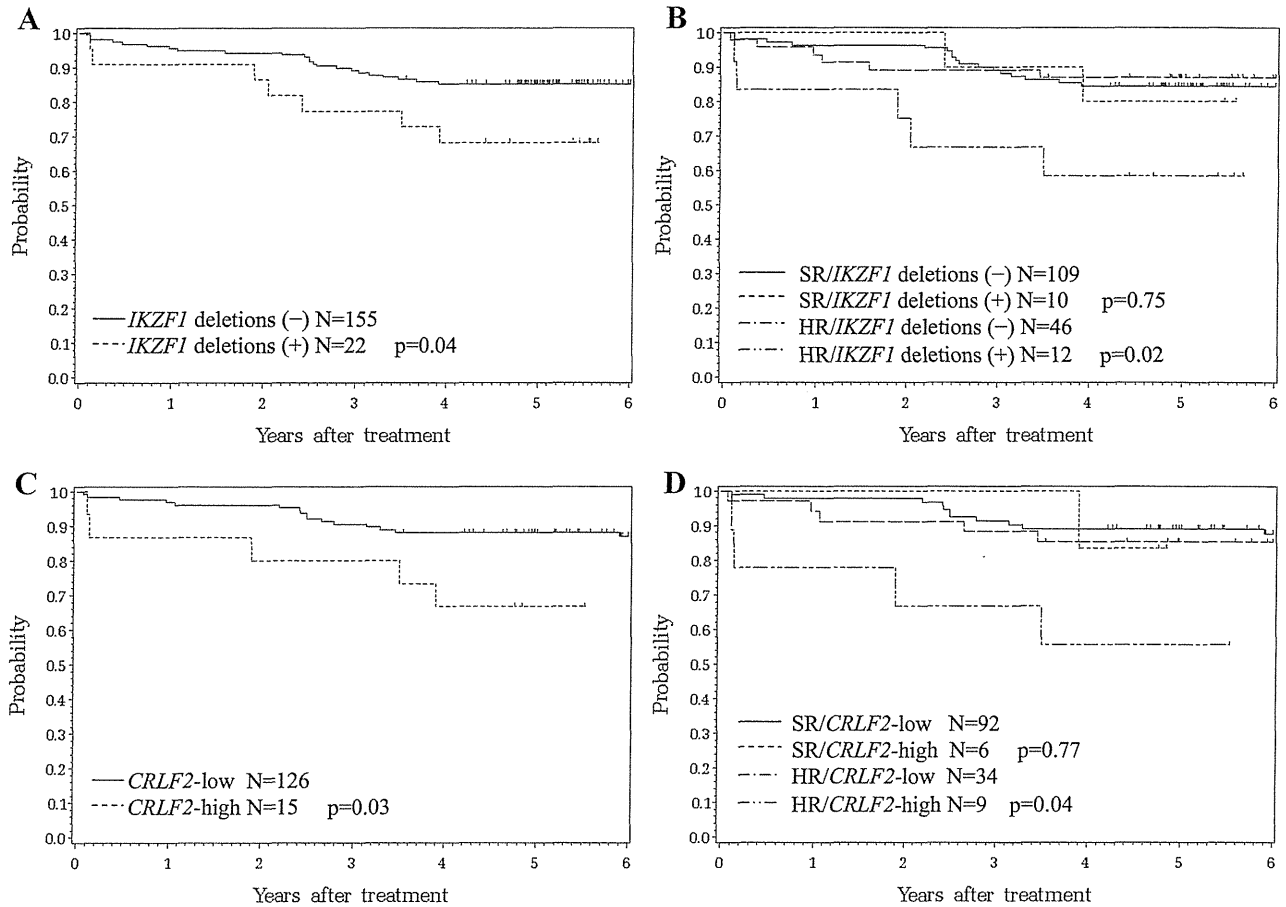

Fig. 2. Probability of EFS according to *IKZF1* deletions, *CRLF2* expression and NCI risk classification. **A**: Probability of EFS for patients with or without *IKZF1* deletions. **B**: Probability of EFS for patients with or without *IKZF1* deletions according to NCI-risk classification. **C**: Probability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

ACKNOWLEDGMENTS

We thank all participants of JCCLSG ALL 2004, and we thank Ms. Miho Yamada for her excellent support in sequencing analysis.

REFERENCES

1. Jeha S, Pui CH. Risk-adapted treatment of pediatric acute lymphoblastic leukemia. Hematol Oncol Clin North Am 2009;23:973–990.
2. Mullighan CG, Miller CB, Radtke I, et al. BCR-ABL1 lymphoblastic leukemia is characterized by the deletion of Ikaros. Nature 2008;453:110–114.
3. Iacobucci I, Lonetti A, Messa F, et al. Expression of spliced oncogenic Ikaros isoforms in Philadelphia-positive acute lymphoblastic leukemia patients treated with tyrosine kinase inhibitors: Implications for a new mechanism of resistance. Blood 2008;112:3847–3855.
4. Den Boer ML, van Sleqtenhorst M, De Menezes RX, et al. A subtype of childhood lymphoblastic leukemia with poor treatment outcome: A genome-wide classification study. Lancet Oncol 2009;10:125–134.
5. Mullighan CG, Goorha S, Radtke I, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukemia. Nature 2007;446:758–764.
6. Mullighan CG, Su X, Zhang J, et al. Deletion of *IKZF1* and Prognosis in Acute Lymphoblastic Leukemia. N Engl J Med 2009;306:470–480.
7. Kuiper RP, Waanders E, van der Velden VH, et al. IKZF1 deletions predict relapse in uniformly treated pediatric precursor B-ALL. Leukemia 2010;24:1258–1264.
8. Yung YL, Hung CC, Chen JS, et al. *IKZF1* deletions predict a poor prognosis in children with B-cell progenitor acute lymphoblastic leukemia: A multicenter analysis in Taiwan. Cancer Sci 2011;102:1874–1881.
9. Chen IM, Harvey RC, Mullighan CG, et al. Outcome modeling with CRLF2, IKZF1, JAK, and minimal residual disease in pediatric acute lymphoblastic leukemia: A Children's Oncology Group Study. Blood 2012;119:3512–3522.
10. Mi JQ, Wang X, Yao Y, et al. Newly diagnosed acute lymphoblastic leukemia in China (II): Prognosis related to genetic abnormalities in a series of 1091 patients. Leukemia 2012;26:1507–1516.

11. Mullighan CG, Zhang J, Harvey RC, et al. JAK mutations in high-risk childhood acute lymphoblastic leukemia. Proc Natl Acad Sci U S A 2009;106:9414–9418.
12. Mullighan CG, Collins-Underwood JR, Phillips LA, et al. Rearrangement of *CRLF2* in B-progenitor and down syndrome associated acute lymphoblastic leukemia. Nat Genet 2009;41:1243–1246.
13. Russell LJ, Capasso M, Vater I, et al. Deregulated expression of cytokine receptor gene, CRLF2, is involved in lymphoid transformation in B-cell precursor acute lymphoblastic leukemia. Blood 2009;114:2688–2698.
14. Hertzberg L, Vendramini E, Ganmore I, et al. Down syndrome acute lymphoblastic leukemia, a highly heterogeneous disease in which aberrant expression of CRLF2 is associated with mutated JAK2: A report from the international BFM Study Group. Blood 2010;115:1006–1017.
15. Harvey RC, Mullighan CG, Chen IM, et al. Rearrangement of CRLF2 is associated with mutation of JAK kinases, alteration of IKZF1, Hispanic/Latino ethnicity, and a poor outcome in pediatric B-progenitor acute lymphoblastic leukemia. Blood 2010;115:5312–5321.
16. Cario G, Zimmermann M, Romey R, et al. Presence of the *P2RY8-CRLF2* rearrangement is associated with a poor prognosis in non-high-risk precursor B-cell acute lymphoblastic leukemia in children treated according to the ALL-BFM 2000 protocol. Blood 2010;115:5393–5397.
17. Ensor HM, Schwab C, Russell LJ, et al. Demographic, clinical and outcome features of children with acute lymphoblastic leukemia and CRLF2 deregulation: Results from the MRC ALL97 clinical trial. Blood 2010;117:2129–2136.
18. Schwab CJ, Jones LR, Morrison SL, et al. Evaluation of multiplex ligation-dependent probe amplification as a method for the detection of copy number abnormalities in B-cell precursor acute lymphoblastic leukemia. Genes Chromosomes Cancer 2010;49:1104–1113.
19. MLPA procedure, Result interpretation. MRC Holland Home Page. [Interpretation of MLPA results] http://www.mlpa.com/WebForms/WebFormMain.aspx?Tag=wl2zCjiVrCGANQgZPuTixrMkcAVlTx-SUI1uxaJsuta9GGaLIE8p67QllDescription version 02; 11-02-2010.
20. Bercovich D, Ganmore I, Scott LM, et al. Mutations of *JAK2* in acute lymphoblastic Leukemia associated with Down's syndrome. Lancet 2008;372:1484–1492.
21. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Am Stat Assoc 1958;53:457–481.
22. Mantel N. Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother Rep 1966;50:163–170.
23. Cox DR. Regression models, life tables. J R Stat Soc 1972;B34:187–220.
24. Collins-Underwood JR, Mullighan CG. Genomic profiling of high-risk acute lymphoblastic leukemia. Leukemia 2010;24:1676–1685.
25. Palmi C, Vendramini E, Silvestri D, et al. Poor prognosis for P2RY8-CRLF2 fusion but not for CRLF2over-expression in children with intermediate risk B-cell precursor acute lymphoblastic leukemia. Leukemia 2012;26:2245–2253.
26. Roberts KG, Morin RD, Zhang J, et al. Genetic alterations activating kinase and cytokine receptor signaling in high-risk acute lymphoblastic leukemia. Cancer Cell 2012;22:153–166.

SUPPORTING INFORMATION

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

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

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

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

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

Table SII. *IKZF1* Deletion Patterns Detected by MLPA

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

PEDIATRICS INTERNATIONAL Official Journal of the Japan Pediatric Society

Pediatrics International (2013) **55**, 65–71 doi: 10.1111/ped.12025

Original Article

Clinical analysis of combination therapy for febrile neutropenic patients in childhood cancer

Shogo Kobayashi,[1] Masaki Ito,[1] Hideki Sano,[1] Kazuhiro Mochizuki,[1] Mitsuko Akaihata,[1] Tomoko Waragai,[1] Mitsuaki Hosoya[1] and Atsushi Kikuta[2]

[1]*Department of Pediatrics, Fukushima Medical University School of Medicine, and* [2]*Division of Pediatric Oncology, Fukushima Medical University Cancer Center, Fukushima Medical University Hospital, Fukushima, Japan*

Abstract ***Background***: The objective of this study was to evaluate the efficacy and safety of our combination therapy in febrile neutropenic children with cancer.

Methods: A total of 109 patients with 251 episodes of febrile neutropenia received antibiotic therapy between January 2003 and December 2008 at a single institution.

Results: Blood cultures were positive in 35 episodes (14%). Gram-positive organisms predominated (23/38 organisms isolated). There were 15 gram-negative isolates and no fungal isolates. The recommended empirical first-line antibiotics (cefepime or cefozopran + piperacillin + amikacin) were used in 206 (82%), second-line antibiotics (piperacillin-tazobactam + carbapenem + amikacin + micafungin) in 73 (29%), and third-line antibiotics (meropenem + glycopeptides + micafungin) in 24 (10%) episodes. The overall response rates were 71.4%, 50.7%, and 62.5% for the first-, second-, and third-line antibiotic therapies, respectively. Granulocyte transfusion was performed in seven patients, and the response rate was 57%. Four deaths were recorded.

Conclusions: Although a significant improvement of mortality was not observed, our regimen of empirical antibiotic therapies led to a significant and clinically relevant decrease in glycopeptide use, and it is safe and well tolerated by pediatric neutropenic patients.

Key words bone marrow transplantation, chemotherapy, children, empirical antibiotic therapy, febrile neutropenia.

Pediatric cancer patients who become severely neutropenic as a result of intensive myelosuppressive chemotherapy are at high risk of developing life-threatening infections, and unless they are treated at the first sign of infection, the rate of mortality is high.[1,2] Empirical antibiotic therapy should be administered promptly to all neutropenic patients at the onset of fever.

Routine use of empirical antibiotic therapy for febrile neutropenic oncology patients has decreased infection-related morbidity and mortality. Originally, most regimens targeted gram-negative organisms, the major cause of morbidity and mortality;[3–5] however, with the increased use of central venous catheters, gram-positive organisms have become an increasing concern.[6,7] Mucositis-inducing chemotherapy regimens have also been shown to increase the risk of infections by gram-positive organisms, particularly those by viridans streptococci and coagulase-negative staphylococci.[8,9] In response, many oncologists have added vancomycin to the empiric therapy of febrile neutropenia (FN), and as a result, vancomycin use has increased 20-fold over a 10-year period in some institutions and overall vancomycin use was found to increase over the last decade.[10] Because of the susceptibility patterns of these organisms, many of which are methicillin-resistant, this agent has become the empirical therapy of choice in combination with cephalosporins.

We designed empirical antibiotic therapies for FN with aims that included the restricted use of glycopeptides. The aim of this study was to evaluate the efficacy and safety of our combination therapy in febrile neutropenic children with cancer.

Methods

Procedures

In the Department of Pediatrics at the Fukushima Medical University Hospital, the 251 eligible episodes analyzed were those that occurred in 109 children (210 episodes in the conventional chemotherapy [CCT] group, and 41 in the hematopoietic stem cell transplantation [HSCT] group). These 251 episodes that occurred between January 2003 and December 2008 were evaluated to determine the differences in the durations of neutropenia. The characteristics of the patients are shown in Table 1. The patients in the CCT group were cared for in single-bed or four-bed rooms, with observance of routine precautions against infections, such as hand washing. The patients in the HSCT group were cared for in single-bed rooms with HEPA-air filtration devices.

Correspondence: Atsushi Kikuta, MD, PhD, Division of Pediatric Oncology, Fukushima Medical University Cancer Center, Fukushima Medical University Hospital, 1 Hikarigaoka, Fukushima 960-1247, Japan. Email: akikuta@fmu.ac.jp

Received 7 March 2012; revised 19 September 2012; accepted 3 October 2012.

Table 1 Clinical characteristics of patients

Characteristics	
Total number of patients	109
Total number of episodes	251
Median age (median [range])	8.9 (0–22)
Sex (male/female)	57/52
Diagnosis (patients [episodes])	
Acute lymphoblastic leukemia	41 (101)
Acute myeloid leukemia	11 (29)
Non-Hodgkin's lymphoma	15 (37)
Solid tumor	35 (75)
Others	7 (9)
Disease status (CR/non-CR) (episodes)	151/100
Chemotherapy/transplantation (episodes)	210/41
Neutrophil count at entry (neutrophils /μL)	
Mean ± SD	70 ± 281
Median (range)	5 (0–1590)
Days of neutropenia (<500 neutrophils/μL)	
Mean ± SD	16.2 ± 14.4
Median (range)	11 (1–106)
Days of neutropenia at entry	
Mean ± SD	9.3 ± 10.9
Median (range)	5 (1–51)
Type of infection episodes (% of all episodes)	
Microbiologically defined	78 (31.0)
With bacteremia	38 (15.1)
Without bacteremia	40 (15.9)
Clinically defined	17 (6.8)
Unexplained fever	156 (62.2)

CR, complete remission.

Broviac catheters were inserted in all the patients for blood sampling, as well as administration of medications and blood products.

Prophylaxis

The CCT group received oral fluconazole as prophylaxis for *Candida* infection and TMP/SMX as prophylaxis for *Pneumocystis* pneumonia. The HSCT group received vancomycin at 15 mg/kg bodyweight every 12 h from day −7 until resolution of neutropenia, and acyclovir at 10 mg/kg bodyweight/day from day +1 to +35.

Growth factors

All the patients who underwent HSCT received granulocyte-colony stimulating factor at a dose of 300 $\mu g/m^2$/day, starting from 24 h after the stem cell transplantation procedure to the time the neutrophil count was above 0.5×10^9/L for 3 consecutive days; the first day was considered as the day of neutrophil engraftment.

Microbiological investigations

Appropriate clinical examinations were performed once daily and body temperature was measured at least three times a day. Routine cultures of blood, stool, and mouth samples from the HSCT patients were carried out once weekly to examine for infecting pathogens. If the body temperature of the patient was over 38°C, catheter culture samples were obtained from both groups; in patients with established bacteremia, blood culture was repeated until results became negative. Blood was drawn from the lumens of an indwelling central venous catheter or from a peripheral vein. Blood culture was carried out as soon as neutropenic fever was detected, and antibiotic therapy was started as soon as possible without waiting for the results of the culture.

Treatment

Fever was defined as a single temperature above 38.0°C. Body temperature was measured at an axillary fossa after wiping off perspiration, in accordance with routine practice in Japan. Neutropenia was defined as an absolute neutrophil count of $<0.5 \times 10^9$/L, or when $>0.5 \times 10^9$/L, it was expected to fall below 0.5×10^9/L within 24–48 h because of the preceding chemotherapy. Cefepime or cefozopran (160 mg/kg/day in two portions; i.v.; maximum, 4 g/day), piperacillin (200 mg/kg/day in two portions; i.v.; maximum, 8 g/day), and amikacin (8 mg/kg/day in two portions; i.v.; maximum, 200 mg/day) were administered initially (Fig. 1). The drug therapy was started on day 0 and was given for 3 days, unless the patient's clinical condition worsened or microorganisms resistant to these antibiotics were isolated.

When the patient became afebrile within 72 h and a causative microorganism was not identified by day 4, the initial treatment was continued until the neutrophil count was higher than 0.5×10^9/L. When the cause was established, the antibiotic therapy was adjusted in accordance with the susceptibility profile of the isolate, and treatment with broad-spectrum antibiotics was maintained until the neutrophil count became higher than 0.5×10^9/L.

When fever persisted for >72 h or recurred after an initial response, the causes of fever were reassessed by thorough history and physical examinations, serological testing for fungal antigens (e.g., β-D-glucan), and blood culture, in addition to the tests carried out on day 0.

When the causative agent was not identified, cephalosporin was changed to panipenem betamipron (100 mg/kg/day in two portions; i.v.; maximum 2 g/day), piperacillin was changed to piperacillin-tazobactam (150 mg/kg/day in two portions; i.v.; maximum 5 g/day) and micafungin was added to the antibiotics (second-line treatment). The drug therapy was also started on day 4 and was administered for 3 days, unless the patient's clinical condition worsened or microorganisms resistant to these antibiotics were isolated.

When the patient became afebrile within 72 h and a causative microorganism was not identified by day 7, the second-line treatment was continued until the neutrophil count became higher than 0.5×10^9/L. When the cause was established, the antibiotic therapy was adjusted in accordance with the susceptibility profile of the isolate, and treatment with broad-spectrum antibiotics was maintained until the neutrophil count became higher than 0.5×10^9/L.

When fever persisted for >72 h or recurred after an initial response, the causes of fever were reassessed by thorough history and physical examinations, serological testing for fungal antigens (e.g., β-D-glucan), and blood culture, in addition to the tests performed on days 0 and 4.

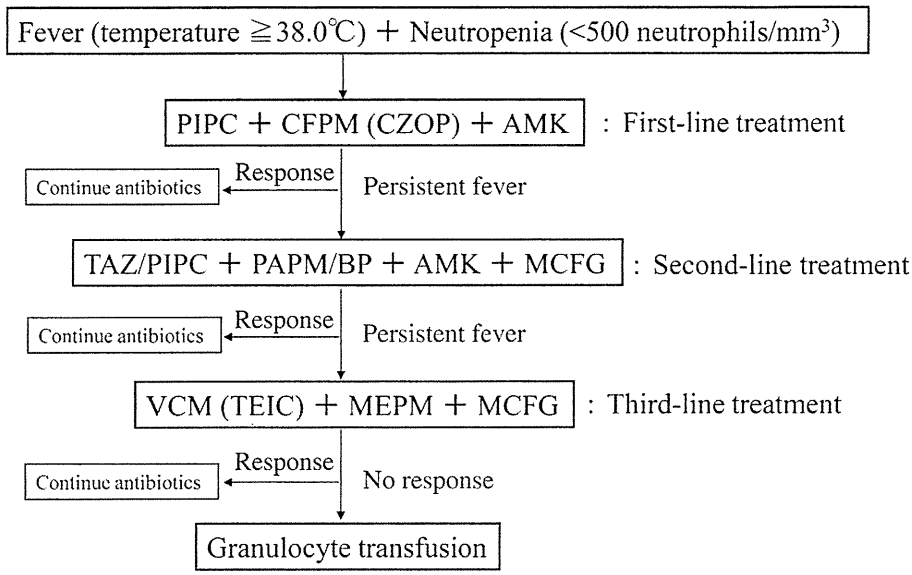

Fig. 1 Algorithm for management of febrile neutropenic patients. When the cause was established, the antibiotic therapy was adjusted in accordance with the susceptibility profile of the isolate. AMK, amikacin; CFPM, cefepime; CZOP, cefozopran; MCFG, micafungin; MEPM, meropenem; PAPM/BP, panipenem–betamipron; PIPC, piperacillin; TAZ, tazobactam; TEIC, teicoplanin; VCM, vancomycin.

When the causative agent was not identified, panipenem betamipron was changed to meropenem (100 mg/kg/day in two porions; i.v.; maximum 2 g/day), piperacillin-tazobactam was stopped, and a glycopeptide (vancomycin or teicoplanin) was started (third-line treatment). The drug therapy was also started on day 7 and was administered for 3 days, unless the patient's clinical condition worsened or microorganisms resistant to these antibiotics were isolated. When fever persisted despite the dosages of these broad-spectrum antibiotics, we considered their administration in combination with granulocyte transfusion.

All febrile episodes were subdivided into three categories as follows: microbiologically defined infections (MDI), clinically defined infections (CDI), and unexplained fever (UF). An MDI was the diagnosis when the infecting organism(s) were isolated. Febrile episodes that were attributed to a clinical site of infection were classified as CDI. UF was the diagnosis when clinical, microbiological, or radiographic evaluation failed to attribute the patient's fever to any infected site or microbial organism.

The study was conducted so that individual patients could not be identified. Informed consent was obtained from all patients.

Results

The 251 eligible episodes analyzed occurred in 109 children. Demographic characteristics of all the enrolled patients are shown in Table 1.

The underlying diagnoses of the 109 children were as follows: acute lymphoblastic leukemia (n = 41), acute myeloid leukemia (n = 11), non-Hodgkin's lymphoma (n = 15), solid tumors (n = 35), and others (n = 7). The age distribution was 0–22 years (median, 8.9 years).

The recommended first-line antibiotics were used in 82% (206) of episodes.

A total of 128 bacterial or fungal pathogens were isolated from 78 patients, of which 34 had a mixed infection (Table 2).

Table 2 Strains isolated from patients (n = 78) with microbiologically documented infections

Isolated strains	Number of isolates (%)		
	Chemotherapy (n = 111)	SCT (n = 17)	Total (n = 128)
Gram-positive	67 (60.4)	8 (47.1)	75 (58.6)
MSSA	2	3	5
MRSA	2	0	2
Coagulase-negative staphylococci	12	1	13
Streptococcus sp.	33	2	35
Enterococcus sp.	10	0	10
Corynebacterium sp.	4	2	6
Bacillus sp.	2	0	2
Lactococcus sp.	1	0	1
Clostridium sp.	1	0	1
Gram-negative	40 (36.0)	7 (41.2)	47 (36.7)
Pseudomonas aeruginosa	13	1	14
Escherichia coli	4	0	4
Klebsiella pneumoniae	3	1	4
Klebsiella oxytoca	1	0	1
Burkholderia cepacia	1	0	1
Bacteroides fragilis	0	1	1
Gram-negative rod (not identified)	1	0	1
Haemophilus influenzae	3	0	3
Acinetobacter sp.	7	0	7
Enterobacter sp.	3	1	4
Stenotrophomonas maltophilia	3	0	3
Alcaligenes sp.	1	0	1
Chryseobacterium menigosepticum	0	2	2
Serratia marcescens	0	1	1
Fungi	4 (3.6)	2 (11.8)	6 (4.7)
Candida sp.	4	2	6

MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *Staphylococcus aureus*; SCT, stem cell transplantation.

Table 3 Bacterial isolates from blood cultures in 35 episodes

Gram-positive	*n*	Gram-negative	*n*
MSSA	2	*Pseudomonas aeruginosa*	4
MRSA	1	*Escherichia coli*	4
Staphylococcus epidermidis	7	*Klebsiella pneumoniae*	3
Streptococcus spp.	7	*Klebsiella oxytoca*	1
Corynebacterium	2	*Burkholderia cepacia*	1
Enterococcus faecalis	1	*Bacteroides fragilis*	1
Bacillus cereus group	1	Gram-negative rod	1
Lactococcus spp.	1	(not identified)	
Clostridium spp.	1		

A total of 38 organisms were isolated from 35 positive blood cultures. Three positive blood cultures were polymicrobial. MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *Staphylococcus aureus*.

Positive results of blood culture were documented in 35 episodes (14%), with a total of 38 organisms isolated. The overwhelming majority (23/38; 61%) of the isolates were gram-positive bacteria, with 15 (39%) gram-negative bacteria and no fungi (0%) (Table 3). Most of these blood culture samples were taken prior to commencement of empirical antibiotics; however, the date of the culture was not always recorded. Therefore, some positive results of blood culture may have been emergent infection. Of 128 bacterial or fungal isolates, 75 (58.6%) were gram-positive bacteria, 47 (36.7%) were gram-negative bacteria, and six (4.7%) were fungi. Streptococci (35 strains) were the most common isolate, followed by coagulase-negative staphylococci (13). There were two methicillin-resistant isolates (MRSA) amongst seven *Staphylococcus aureus* isolates and no vancomycin-resistant enterococci were detected.

UF (62.2%) was the most common type of febrile episode, followed by MDI (31.0%) and CDI (6.8%). MDI without bacteremia were found in 43 episodes (17%), including urinary tract infection (n = 1); skin (n = 13) and oropharynx (n = 27) infections (as indicated by bacteria-positive swabs); and respiratory tract infection (n = 2) (as indicated by bacteria-positive sputum). The most frequent MDI were severe oropharynx mucositis (higher than or equal to grade 2 according to the World Health Organization definition). There were seven episodes of herpes infections, three episodes of shingles, one episode of chickenpox, one episode of hemorrhagic cystitis, two episodes of *Aspergillus* pneumonia, and three episodes of sepsis diagnosed clinically. Overall, 37.8% of all the episodes were associated with a clinical and/or microbiological focus of infection. Oropharynx infection (29 episodes) was the most frequent infection, followed by perianal abscess/infection (n = 11), pneumonia (n = 5), local-site abscess/infection without perianal infection (n = 4), and urinary tract infection (n = 1).

Neutropenia

The median duration of neutropenia was 16 (1–106) days.

Clinical outcomes

Clinical outcomes are summarized in Figure 2.

The recommended first-line antibiotics (cefepime or cefozopran, plus piperacillin plus amikacin) were used in 82% (n = 206) of the 251 episodes. The rate of response achieved without a need for treatment modification was 63%. The overall response rate with or without modification of therapy was 71.4%. Persistent or relapsing fever after 72 h occurred in 49 (23.8%) episodes.

Second-line treatment (piperacillin-tazobactam plus carbapenem plus amikacin and micafungin) was chosen for the treatment of 73 (29.1%) out of 251 episodes. The overall response rate with or without modification of therapy was 50.7%. Persistent or relapsing fever after 72 h occurred in 34 (46.6%) episodes.

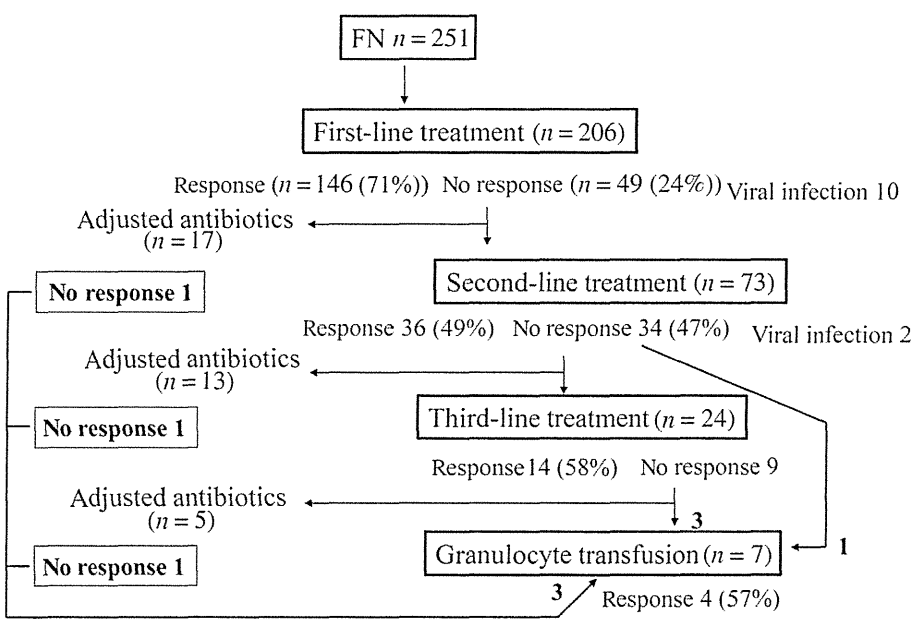

Fig. 2 Clinical outcomes, adjusted antibiotics, antibiotics adjusted according to the culture results. FN, febrile neutropenia.

Third-line treatment (meropenem plus glycopeptides and micafungin) was chosen for the treatment of 24 (9.6%) out of 251 episodes; the overall response rate with or without modification of therapy was 62.5%. Persistent or relapsing fever after 72 h occurred in nine (37.5%) episodes.

Antibiotic therapy in combination with granulocyte transfusion was administered in seven patients, and the response rate was 57%. Two patients died owing to progressive malignancy. Four patients died owing to an infection. As for the cause of deaths, one patient died of septic shock caused by viridans streptococci, the other three patients developed organ deficiency caused by the worsening of an infectious disease despite the administrations of antibiotics adjusted according to the culture results in combination with granulocyte transfusion.

Discussion

The results show that our regimen of empirical antibiotic therapies is a safe and tolerable option for febrile neutropenic episodes in pediatric hematological disorder patients. We have used the combination therapy of cefepime or cefozopran, plus piperacillin plus amikacin in febrile neutropenic patients. Cefepime or cefozopran was used on the basis of its efficacy against sepsis due to *Pseudomonas aeruginosa* and showed good activity against methicillin-susceptible staphylococci, enterococci, and viridans-group streptococci, all of which are refractory to other cephalosporins. The advantages of combination therapy with amikacin are potential synergistic effects against some gram-negative bacilli and minimal emergence of drug-resistant strains during treatment.[11,12]

The major disadvantages are the lack of activity of these combinations, such as cephalosporin plus an aminoglycoside, against some gram-positive bacteria. Some strains of viridans streptococci are resistant to or tolerant of penicillin, but piperacillin has excellent activity against most strains detected in our institution. Therefore we added piperacillin to the initial therapy of febrile neutropenic episodes.

Similarly to other recent reports,[13,14] the rate of bacteremia due to *P. aeruginosa* was not very high. The low number of patients with central venous catheters may explain the low percentage of episodes with positive culture results. The detection rate of gram-positive cocci, such as *Staphylococcus epidermidis*, is higher than that of gram-negative rods in our unit. This finding agrees with the reports that there has been an apparent reduction in the incidence of sepsis due to gram-negative bacteria, such as *Escherichia coli*, *P. aeruginosa*, and *Klebsiella pneumoniae* over the last 10–15 years among neutropenic hosts. The sepsis due to multidrug-resistant and mild toxic gram-positive bacteria, such as *S. epidermidis*, *S. aureus*, and *Enterococcus spp.*, has replaced sepsis due to gram-negative bacteria.

Bacteremia rates as low as 12–14% have been reported in some studies.[15,16] A review of 757 episodes of neutropenic sepsis in four European studies of empirical antibiotic therapy revealed a bacteremia rate of 22% in children.[17] In that review, 49% of the children had neither a clinical nor microbiological focus of infection documented. The prevalence of bacteremia was 14% in the current study; 62.2% of patients had neither a clinical nor microbiological focus of infection. As in a previous study,[17] we found that among isolates from bacteremic patients, gram-positive organisms predominated; the high rate of gram-positive bacteria may reflect the almost universal use of central venous lines in our population.

Mortality from neutropenic sepsis in children has progressively fallen in recent years.[18] European data from the 1990s suggested 1% mortality from infections during febrile neutropenic episodes in children[17] and a recent large North American study showed an all-cause mortality of 3% in children with FN.[18] A low mortality rate (1.6%) was also observed by our regimen of empirical antibiotic therapies.

In our study, the response rate was comparable to those of other beta-lactam-aminoglycoside regimens tested in such a pediatric population. In a prospective non-comparative open study of piperacillin plus gentamicin for the treatment of 239 febrile episodes in neutropenic children, Fleischhack *et al.* reported an overall response rate of 55.2%.[19] In a prospective, open-labeled, single-center study of single-daily dose of ceftriaxone plus amikacin for the treatment of 191 febrile episodes in neutropenic children, Ariffin *et al.* reported an overall response rate of 55.5%.[20] In a randomized study in which ceftriaxone plus amikacin was compared with ceftazidime plus amikacin for the treatment of 364 febrile episodes in neutropenic children, Charnas *et al.* found an overall response rate of 66% in both therapeutic strategies.[21] Chastagner *et al.* reported a success rate of 67% of fever of unknown origin in a series of 64 febrile neutropenia episodes in cancer children treated with cefepime plus amikacin.[22]

In our study, antimicrobial modifications were required in 66 (32%) of 206 episodes, in which the recommended first-line antibiotics (cefepime or cefozopran, plus piperacillin plus amikacin) were used; 49 (24%) of the 206 episodes that required treatment modification performed were due to persistent fever lasting longer than 72 h from the beginning of the antimicrobial therapy. An overall response rate of 70.9% was achieved by the first-line treatment, including the cases in which the antibiotics used were adjusted in accordance with the susceptibility profile of the isolate.

In the second-line therapy (panipenem, plus piperacillin-tazobactam plus amikacin), antimicrobial modifications were required in 47 (64%) of 73 episodes, which included first-line therapy (cefepime or cefozopran, plus piperacillin plus amikacin) and resulted in no response. Thirty-four (47%) of the 73 episodes that required treatment modification were due to persistent fever lasting longer than 72 h from the beginning of the antimicrobial therapy.

Although vancomycin has not been shown to affect overall mortality due to gram-positive cocci as a group, mortality due to viridans streptococci may be higher among patients not initially treated with vancomycin.[23,24] Inclusion of vancomycin in the initial empirical therapy may be prudent for selected patients with the following clinical findings: (i) clinically suspected serious catheter-related infections (e.g., bacteremia, cellulitis); (ii) known colonization by penicillin- and

cephalosporin-resistant pneumococci or methicillin-resistant *S. aureus*; (iii) positive results of blood culture for gram-positive bacteria before final identification and susceptibility testing; or (iv) hypotension or other evidence of cardiovascular impairment.[25] Intensive chemotherapy that produces substantial mucosal damage (e.g., high-dose cytarabine) or increases the risk of penicillin-resistant streptococcal infections (e.g., infection with viridans streptococci), is also considered an indication for vancomycin to be included in the initial regimen. A sudden increase in temperature to >40°C has, to some extent, been predictive of sepsis due to viridans streptococci.[24] In our study, one patient died of septic shock caused by viridans streptococci. The use of glycopeptides is not routine but should be considered in certain cases with predicted worsening of course, particularly when the bacillemia with viridans streptococci is suspected, because only glycopeptides are clinically effective in such a case. We should treat with glycopeptides in combination with FN, meeting the use-adaptive condition of vancomycin s Starting from the early days.

There is usually no indication for the empirical use of antiviral drugs in the treatment of febrile neutropenic patients without evidence of a viral disease; however, if skin or mucous membrane lesions due to herpes simplex or varicella-zoster viruses are present, even if they are not the cause of fever, treatment with acyclovir (ACV) is indicated. In certain patients with hematologic malignancies, fever, and neutropenia, the administration of acyclovir for the treatment or suppression of herpes simplex virus infection has been associated with a more favorable febrile response than that in untreated patients.[26] In our study, there were seven episodes of herpes and three episodes of varicella-zoster virus infections. When stomatitis by the treatment is severe, stomatitis caused by the herpes simplex virus can be overlooked, and there are cases of FN that are unresponsive to broad-spectrum antimicrobial agents. Therefore, it is worth considering the empirical treatment dosage of ACV for the febrile neutropenic patients who have extensive stomatitis caused by the chemotherapy and antimicrobial agent unresponsiveness.

In management of high-risk febrile patients with neutropenia, the Infectious Disease Society of America (IDSA) 2010 guideline update recommends monotherapy with an antipseudomonal β-lactam agent, such as cefepime, a carbapenem (meropenem or imipenem-cilastatin), or piperacillin-tazobactam. Other antimicrobials (aminoglycosides, fluoroquinolones, and/or vancomycin) may be added to the initial regimen for management of complications (e.g., hypotension and pneumonia) or if antimicrobial resistance is suspected or proven. Vancomycin (or other agents active against aerobic gram-positive cocci) is not recommended as a standard part of the initial antibiotic regimen for fever and neutropenia. These agents should be considered for specific clinical indications, including suspected catheter-related infection, skin or soft-tissue infection, pneumonia, or hemodynamic instability.[27] Although our combination therapy provided satisfactory efficacy and a good tolerance, three antibiotics may be too much compared with the guideline. When febrile patients with neutropenia are given antibiotics according to the IDSA guideline in our institution, we must review it in comparison with our combination therapy results.

In conclusion, patients with febrile neutropenia might respond better to the combination therapy of cefepime or cefozopran, plus piperacillin plus amikacin as the initial treatment than to other combination therapies that have been reported. A significant improvement of mortality was not shown; however, our combination therapy led to a significant and clinically relevant decrease in glycopeptide use. Findings of this study suggest that our combination therapy provides an alternative option in treating febrile neutropenic episodes in pediatric neutropenic patients with satisfactory efficacy and a good tolerance. Using this strategy, we can expect the improvement of results by identifying patients for whom glycopeptides are effective at the early-onset stage on the basis of the patients' background and symptom course, and reduce mortality due to infections caused by some gram-positive bacteria, such as viridans streptococci and MRSA.

References

1 Bodey GP, Buckley M, Sathe YS *et al.* Quantitative relationships between circulating leukocytes and infection in patients with acute leukemia. *Ann. Intern. Med.* 1996; **64**: 328–40.
2 Klastersky J. Febrile neutropenia. *Curr. Opin. Oncol.* 1993; **5**: 625–32.
3 Rodriguez V, Burgess M, Bodey GP. Management of fever of unknown origin in patients with neoplasms and neutropenia. *Cancer* 1973; **32**: 1007–12.
4 Tattersall MH, Hutchinson RM, Gaya H *et al.* Empirical antibiotic therapy in febrile patients with neutropenia and malignant disease. *Eur. J. Cancer* 1973; **9**: 417–23.
5 Tattersall MH, Spiers AS, Darrell JH. Initial therapy with combination of five antibiotics in febrile patients with leukaemia and neutropenia. *Lancet* 1972; **1**: 162–5.
6 Wischnewski N, Kampf G, Gastmeier P *et al.* Prevalence of primary bloodstream infections in representative German hospitals and their association with central and peripheral vascular catheters. *Zentralbl. Bakteriol.* 1998; **287**: 93–103.
7 Karthaus M, Doellmann T, Klimasch T *et al.* Central venous catheter infections in patients with acute leukemia. *Chemotherapy* 2002; **48**: 154–7.
8 Gamis AS, Howells WB, DeSwarte-Wallace J *et al.* Alpha hemolytic streptococcal infection during intensive treatment for acute myeloid leukemia. A report from the Children's Cancer Group Study CCG-2891. *J. Clin. Oncol.* 2000; **18**: 1845–55.
9 Lex C, Korholz D, Kohlmuller B *et al.* Infectious complications in children with acute lymphoblastic leukemia and T-cell lymphoma—a rationale for tailored supportive care. *Support. Care Cancer* 2001; **9**: 514–21.
10 Ena J, Dick RW, Jones RN *et al.* The epidemiology of intravenous vancomycin usage in a university hospital. *JAMA* 1993; **269**: 598–602.
11 Sepkowitz KA, Brown AE, Armstrong D. Empirical therapy for febrile, neutropenic patients: persistence of susceptibility of gram-negative bacilli to aminoglycoside antibiotics. *Clin. Infect. Dis.* 1994; **19**: 810–1.
12 Brown AE, Kiehn TE, Armstrong D. Bacterial resistance in the patient with neoplastic disease. *Infect. Dis. Clin. Pract.* 1995; **4**: 136–44.
13 Hughes WT, Armstrong D, Bodey GP *et al.* 2002 guidelines for the use of antimicrobial agents in neutropenic patients with cancer. *Clin. Infect. Dis.* 2002; **34**: 730–51.

14 Kutluk T, Kurne O, Akyüz C *et al.* Cefepime vs. meropenem as empirical therapy for neutropenic fever in children with lymphoma and solid tumours. *Pediatr. Blood Cancer* 2004; **42**: 284–6.
15 Klaassen RJ, Goodman TR, Pham B *et al.* "Low-risk" prediction rule for pediatric oncology patients presenting with fever and neutropenia. *J. Clin. Oncol.* 2000; **18**: 1012–9.
16 Baorto EP, Aquino VM, Mullen CA *et al.* Clinical parameters associated with low bacteraemia risk in 1100 paediatric oncology patients with fever and neutropenia. *Cancer* 2001; **92**: 909–13.
17 Hann I, Viscoli C, Paesmans M *et al.* A comparison of outcome from febrile neutropenic episodes in children compared with adults: results from four EORTC studies. *Br. J. Haematol.* 1997; **99**: 580–8.
18 Basu SK, Fernandez ID, Fisher SG *et al.* Length of stay and mortality associated with febrile neutropenia among children with cancer. *J. Clin. Oncol.* 2005; **23**: 7958–66.
19 Fleischhack G, Schmidt-Niemann M, Wulff B *et al.* Piperacillin, beta-lactam inhibitor plus gentamicin as empirical therapy of a sequential regimen in febrile neutropenia of pediatric cancer patients. *Support. Care Cancer* 2001; **9**: 372–9.
20 Ariffin H, Arasu A, Mahfuzah M *et al.* Single-daily ceftriaxone plus amikacin versus thrice-daily ceftazidime plus amikacin as empirical treatment of febrile neutropenia in children with cancer. *J. Paediatr. Child Health* 2001; **37**: 38–43.
21 Charnas R, Lüthi AR, Ruch W. Once daily ceftriaxone plus amikacin vs. three times daily ceftazidime plus amikacin for treatment of febrile neutropenic children with cancer. Writing Committee for the International Collaboration on Antimicrobial Treatment of Febrile Neutropenia in Children. *Pediatr. Infect. Dis. J.* 1997; **16**: 346–53.
22 Chastagner P, Plouvier E, Eyer D *et al.* Efficacy of cefepime and amikacin in the empiric treatment of febrile neutropenic children with cancer. *Med. Pediatr. Oncol.* 2000; **34**: 306–8.
23 Shenep JL, Hughes WT, Roberson PK *et al.* Vancomycin, ticarcillin, and amikacin compared with ticarcillin-clavulanate and amikacin in the empirical treatment of febrile, neutropenic children with cancer. *N. Engl. J. Med.* 1988; **319**: 1053–8.
24 Elting LS, Bodey GP, Keefe BH. Septicemia and shock syndrome due to viridans streptococci: a case-control study of predisposing factors. *Clin. Infect. Dis.* 1992; **14**: 1201–7.
25 Centers for Disease Control and Prevention. Recommendations for preventing the spread of vancomycin resistance: recommendations of the Hospital Infection Control Practices Advisory Committee (HICPAC). *MMWR Morb. Mortal. Wkly Rep.* 1995; **44** (RR-12): 1–13.
26 Baglin TP, Gray JJ, Marcus RE *et al.* Antibiotic resistant fever associated with herpes simplex virus infection in neutropenic patients with haematological malignancy. *J. Clin. Pathol.* 1989; **42**: 1255–8.
27 Freifeld AG, Bow EJ, Sepkowitz KA *et al.* Clinical practice guideline for the use of antimicrobial agents in neutropenic patients with cancer: 2010 Update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* 2011; **52**: e56–e93.