surgical resection. After surgical resection, these patients were randomly assigned to either receive post-surgical chemotherapy or not. The tumor was judged to be unresectable when damage to adjacent organs and blood vessels was expected. The final decision for resectability of tumors depended on the judgment of each institution. Patients with unresectable dumb-bell type tumors received the same chemotherapy given to patients in stage 3. Patients with MNA were classified in a high-risk group and underwent intensive treatment, with high-dose chemotherapy and stem cell transplantation. Patients with MNA were treated with 6 cycles of regimen D2, which reduced the need for high-risk induction chemotherapy [10].

Babies less than 6 months old were treated with reduced dosages: 1/3 dose for infants of less than 2 months, 1/2 dose for infants of 2–4 months, and 2/3 dose for infants of 4–6 months.

The therapeutic evaluation before surgery included a computed tomography scan or magnetic resonance imaging every three cycles. Tumors were evaluated according to the International Neuroblastoma Response Criteria (INRC) [7].

Statistical analysis

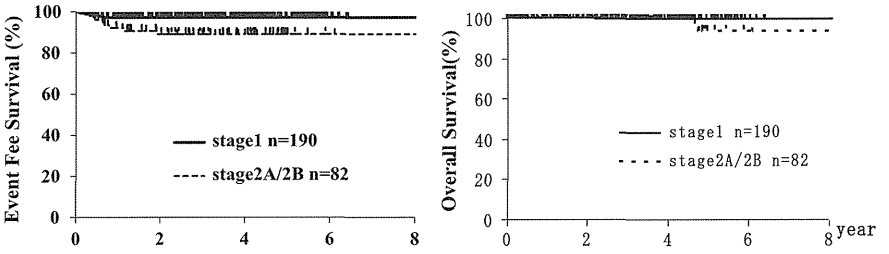
Kaplan–Meier product limit methods were used to estimate the event-free survival (EFS) and overall survival (OS) from the time of diagnosis. The exact permutation test of the log-rank statistics was used to compare the EFS and OS probabilities between subgroups of patients.

Results

There were 190 and 82 patients in stages 1 and 2 without MNA, respectively. Thirty-seven of the 82 stage 2 patients were in stage 2A and 45 were in stage 2B. Eleven unresectable patients in stage 2 were treated with 3 courses of regimen A as preoperative chemotherapy. Macroscopic residual tumors were found in 39 of all 272 patients after surgical resection. Nine patients with residual tumors received postoperative chemotherapy that was requested by the patient's family. Twenty-one patients had residual tumors at the time of prognosis.

The 5-year EFS rates of the stage 1 and stage 2 patients were 97.2 and 89.0%, respectively ($p = 0.02$). The 5-year OS rates (99.2 and 93.3%, respectively) were not significantly different ($p = 0.85$), which is excellent (Fig. 3). As to the cause of death, a stage 1 patient and a stage 2 patient died of their recurrent progressive disease after surgical resection without post-surgical chemotherapy. Surgical complications occurred in 25 infants with stage 1 and 2 neuroblastoma (9.1%). The most frequent surgical complications involved the renal urinary system ($n = 12$, 48%; Table 1).

A total of 68 patients met the criteria for stage 3 neuroblastoma without MNA in the #9805 study. Ten of 19 patients with resectable tumors received post-surgical chemotherapy and 9 patients did not receive any chemotherapy. The tumors disappeared with chemotherapy alone in 7 of the 49 patients with unresectable tumors. The remaining 42 patients underwent delayed primary surgical resection after pre-surgical chemotherapy. Twenty-one of these patients received post-surgical chemotherapy and 21 did not. A total of 31 patients received post-surgical chemotherapy and 30 did not. The 5-year EFS rates of these two groups (96.0 and 96.2%, respectively) were not significantly different ($p = 0.869$). The 5-year OS survival rates (100 and 95.8%, respectively) were not significantly different ($p = 0.306$), which is excellent (Fig. 4). Eighteen of the stage 3 patients had residual tumors at the time of prognosis. One of the stage 3 patients died of recurrent disease after surgical resection. Though the tumor sample



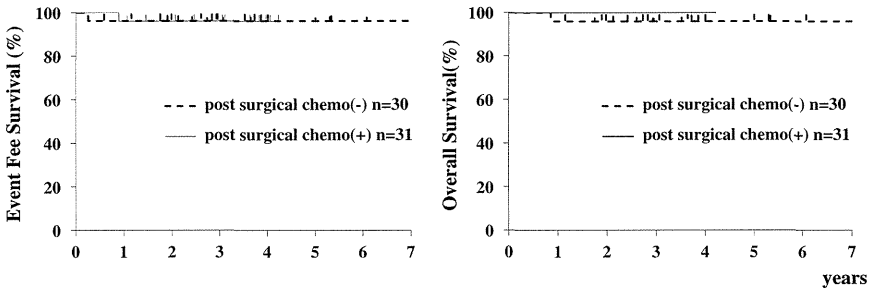
	n	5y-EFS	
Stage 1	190	97.2%	
Stage 2A/2B	82	89.0%	P=0.020
		5y-OS	
Stage 1	190	99.2%	
Stage 2A/2B	82	93.3%	P=0.850

Fig. 3 Survival rates of neuroblastoma infants in stage 1 and 2A/2B without MNA in the #9805 study. The curve was generated using the Kaplan–Meier product limit method. The 5-year event-free survival rate in stage 1 was 97.2% and 89.0% for stage 2A/2B patients ($p = 0.02$). The 5-year overall survival rate in stage 1 was 99.2% for patients and 93.3% for stage 2A/2B patients ($p = 0.85$)

Table 1 Complications of therapy

Treatment	Type of complication	Stage 3		Stage 1, 2A, 2B
		Post chem.+ 4/25 (16.0%)	Post chem.− 5/24 (20.8%)	25/272 (9.1%)
Surgery	Ileus	2	1	5
	Invagination			1
	Horner syndrome			3
	Kidney infarction			3
	Vanishing kidney			5
	Kidney resection		1	
	Hydronephrosis			1
	Renal hypertension			2
	Dysuria		1	
	Urinary tract amputation			1
	Post-operative haemorrhage			1
	Abscess			2
	Lidocaine poisoning			1
	Pulmonary effusion		1	
Chemotherapy	Neutropenia		1	
	Infectious disease	2		

Post chem.+/–: with/without post-surgical chemotherapy



Post surgical chemo	n	5y-EFS	
(-)	30	96.2%	
(+)	31	96.0%	P=0.869
		5y-OS	
(-)	30	95.8	
(+)	31	100%	P=0.306

Fig. 4 Survival rates of infants with stage 3 neuroblastoma without MNA based on post-surgical chemotherapy in the #9805 study. The curve was generated using the Kaplan–Meier product limit method. The 5-year event-free survival rate was 96.2% for patients with post-surgical chemotherapy and 96.0% for patients without chemotherapy. The 5-year overall survival rates for patients with post-surgical chemotherapy and without chemotherapy were 95.8 and 100% respectively. The 5-year event-free and overall survival rates of these two groups were not significantly different ($p = 0.869$ and $p = 0.306$, respectively)

was judged not to have MNA at diagnosis, the other sample was judged to have MNA in the later examination. It was speculated that this tumor showed heterogeneity. Four (16.0%) of the stage 3 patients who received post-surgical chemotherapy had therapy complications (Table 1). Two patients had mechanical ileus, and two had an infectious disease. Five (20.8%) of the patients who did not receive post-surgical chemotherapy had therapy complications. There were single cases of mechanical ileus, a complicated kidney resection, pulmonary effusion, dysuria and neutropenia. The frequencies of complication between patients with and without post-surgical chemotherapy were not significantly different.

There were 6 localized neuroblastoma patients with MNA (Table 2). Four patients underwent primary surgical resection at the onset, and the remaining 2 patients had stage 3 neuroblastoma. All but one of the patients received myeloablative chemotherapy with stem cell transplantation, but one infant could not receive this treatment because of his condition. The 5-year EFS rate for patients with MNA was 50.0%, and for patients without MNA was 95.0% ($p < 0.001$). The 5-year OS survival rates of patients with MNA were significantly better than without MNA (66.7 and 97.7%, respectively; $p < 0.001$) (Fig. 5).

Table 2 Characteristics of neuroblastoma patients registered with #9805 protocol

	n	MNA	%
Registered	429		
Eligible	414		
Male	238		57.5
Female	176		42.5
MS	344		83.1
Clinical	70		17.0
Median age (months)	7.35		
Stage			
1	193	3	46.7
2A/2B	38/45	1	20.0
3	70	2	16.9
4	39	3	9.4
4S	29	0	7.0
Dumb-bell	10		2.4
MNA	6		1.7
MYCN evaluable	346		

MS, mass screening; MNA, *MYCN* amplification

Discussion

The Children's Oncology Group (COG) Study treated all stage 1 and 2 patients with surgery alone regardless of the presence of MNA, although chemotherapy was recommended for patients with threatening symptoms [11, 12]. In the COG study, only 2% of stage 1 and 2 tumors exhibited MNA, and therefore no definitive conclusions were made regarding the need of the chemotherapy. However, we reported that *MYCN* is a powerful prognostic factor even in infants [13]. Recently, the International Neuroblastoma Risk Group (INRG) classification system was recommended [14]. According to the INRG classification, localized tumors with MNA were classified as high risk. Furthermore, in patients with MNA, low-stage neuroblastoma was shown to have a poor prognosis based on the INRG data [15]. This study was conducted to evaluate treatment strategies in the presence or absence of MNA in infants with localized neuroblastoma. Patients with *MYCN* amplified tumors were classified as high-risk patients. According to our results, the patients with *MYCN* amplified localized neuroblastoma actually had a poorer prognosis than patients without MNA, in spite of receiving intensive treatment. The treatment strategy using MNA is therefore appropriate in infants with localized neuroblastoma.

The Cooperative German Neuroblastoma trial NB90 treated patients in stage 1 with surgical resection and patients in stage 2 received surgical resection with 4 cycles of chemotherapy. Localized cases without symptoms and MNA did not receive chemotherapy in the new trials NB95 and NB97 [16]. Many neuroblastoma studies have therefore not tended to administer chemotherapy for neuroblastoma patients in stages 1 or 2 [17, 18]. However, the main strategy for neuroblastoma infants in stage 1 or 2 in Japan until the mid 1990s was to use chemotherapy with surgical resection. The first prospective study (#9405) was

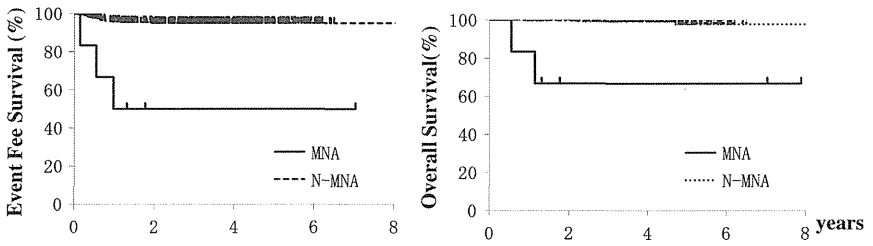


Fig. 5 Survival rates of infants with localized neuroblastoma based on *MYCN* status in the #9805 study. The 5-year event-free survival rates for patients with and without MNA were 50.0% and 95.0%, respectively. The 5-year overall survival rates for patients with and without MNA were 66.7% and 97.7%, respectively. The 5-year event-free and overall survival rates of patients with MNA were significantly better than those without MNA ($p < 0.001$)

Abbreviation: MNA, *MYCN* amplification
N-MNA, without *MYCN* amplification

	n	5y-EFS	
MNA	6	50%	
N-MNA	340	95.0%	P<0.001
		5y-OS	
MNA	6	66.7%	
N-MNA	340	97.7%	P<0.001

therefore initiated to examine the effectiveness of post-surgical chemotherapy for patients in stage 2 without MNA. Study #9405 showed that post-surgical chemotherapy is unnecessary, and many neuroblastoma infants in the early stages were not given excessive chemotherapy. Study #9805 found the 5-year overall survival rates of stage 1 and 2 patients without MNA to be excellent.

The COG Study classified infants with stage 3 neuroblastoma without MNA as an intermediate-risk group. These patients were treated with 8–19 cycles of chemotherapy containing carboplatin, etoposide, doxorubicin and cyclophosphamide. Though the prognosis of patients under 1 year old was good in the COG study (4-year EFS, 98%), its regimen was more intensive than the regimens in the current study #9805 [19]. The French Society of Pediatric Oncology (SFOP) found low-dose chemotherapy to be sufficiently effective in infants presenting with unresectable neuroblastoma and without MNA, thus allowing for a safe surgical resection and preventing long-term late side effects. This low-dose chemotherapy included three regimens, CV (cyclophosphamide and vincristine), CE (carboplatin and etoposide) and CAdO (doxorubicin, vincristine and cyclophosphamide) [16]. Study #9805 treated unresectable patients in stage 3 with 6 cycles of regimen A, which contained a lower dose of cyclophosphamide and vincristine than was used in regimen CV. Patients received 3 cycles of regimen C2, which included pirarubicin, if the tumor was still unresectable after 3 cycles of regimen A. Only 3 patients needed to be changed to regimen C2 with pirarubicin. The low-dose chemotherapy efficiently shrank the tumor volume and prevented side effects in infants with unresectable neuroblastoma. The effectiveness of chemotherapy after surgical resection for infants with stage 3 neuroblastoma was unclear before the #9805 study. The results of study #9805 show no significant difference in survival rates of stage 3 neuroblastoma infants between patients that were and were not administered post-surgical chemotherapy.

It is clear that chemotherapy after surgical resection was unnecessary for infants with localized neuroblastoma without MNA.

Temporary neutropenia and infection were the only complications observed in these studies, and there were no regimen-related long-term late side effects. Though this is a retrospective analysis and there are several biases of institutional judgment with surgical resection, the incidence of surgical side effects in stage 3 patients was 8.8% (6/68), which was similar to that observed in another report [20]. However, some patients had long-term side effects such as kidney dysfunction and pulmonary effusion. The number of cases requiring nephrectomy among children undergoing initial resection was more than twice that in children undergoing resection after chemotherapy [21]. The International Society of Pediatric Oncology Europe Neuroblastoma Group (SIOPEN) has classified localized tumors as resectable or unresectable depending on the absence or presence of surgical risk factors. As a result, considering the surgical risk factors therefore allowed them to predict the surgical outcome [20]. The surgical risk factors are based on radiographic images at the time of diagnosis, and image-defined risk factors (IDRF) have been proposed [22]. Future studies are planned to establish surgical guidelines that consider surgical risk factors.

In Kanagawa Children's Medical Center, an observational study was tried for selected Evans stage I and II cases. From this report, in 32% of patients the tumor became undetectable and in 42% of patients the tumor was detectable without surgical resection. In 26% of patients, the tumor increased and finally underwent tumor resection [23]. Moreover, a German group reported the phenomenon of spontaneous regression of unresected neuroblastoma in infants [24]. As for some localized infantile cases, the observational strategy without surgery may be possible in expectation of spontaneous regression or maturation. It cannot be concluded that surgery is unnecessary in all patients without MNA, because some patients ultimately underwent surgical resection, since that tumor grew during observation. For the next step, we think it appropriate that we can follow up without surgery in suitable selected patients. Further investigation of biological factors during spontaneous regression is necessary.

In conclusion, this study demonstrated an effective strategy for infants with localized neuroblastoma without MNA that can result in a good clinical outcome without any side effects. The treatment strategy using *MYCN* status is therefore considered to be useful even for infants presenting with localized neuroblastoma.

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Conflict of interest All authors have no conflict of interest.

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Ijichi O); Tokyo Metropolitan Kiyose Children's Hospital, Tokyo (Hayahi A, Kamagata S); Gunma Children's Medical Center, Gunma (Tsuchida Y, Kuroiwa M); University of Tsukuba, Tsukuba (Kaneko M); Fukushima Medical University, Fukushima (Kikuta A); University of Yamagata, Yamagata (Kanazawa C); St. Marianna University of Medicine, Kawasaki (Shimada A, Okubo M); National Sapporo Hospital, Hokkaido (Hatae Y, Naito H); Hiroshima University, Hiroshima (Hiyama E, Nishimura S); Kurume University School of Medicine, Kurume (Tsuru T); Osaka University, Osaka (Fukuzawa M, Kusafuka T, Yoneda M); Nihon University School of Medicine, Tokyo (Mugishima H); National Hospital Organization, Kure Medical Center, Hiroshima (Tanaka T); Shiga University of Medicine, Shiga (Oota S).

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ORIGINAL ARTICLE

Detection of *MYCN* amplification using blood plasma: noninvasive therapy evaluation and prediction of prognosis in neuroblastoma

Masato Kojima · Eiso Hiyama · Ikuko Fukuba · Emi Yamaoka · Yuka Ueda · Yoshiyuki Onitake · Shou Kurihara · Taijiro Sueda

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Abstract
Purpose Amplification of neuroblastoma derived (avian) v-myc myelocytomatosis viral related oncogene (*MYCN*) is an important risk-stratified indicator in neuroblastoma. To evaluate the feasibility of noninvasive measurement of *MYCN* amplification, we analyzed *MYCN* amplification in stored blood plasma samples.
Methods We used quantitative real-time PCR to determine *MYCN* copy numbers in plasma-derived DNA of 10 healthy volunteers and 50 neuroblastoma cases. The copy number was calculated as the ratio of copies of *MYCN* to those of a reference gene. Plasma samples obtained after surgery or neoadjuvant therapy were also analyzed in five cases and four cases, respectively.
Results In 34 neuroblastoma cases, *MYCN* was non-amplified in both tumor tissue and blood plasma. In 16 neuroblastoma cases, *MYCN* was amplified in both tumor tissue and blood plasma; 13 of the 16 cases showed poor outcomes. *MYCN* amplification was undetectable in blood plasma shortly after surgery or neoadjuvant therapy. The correlation coefficient between *MYCN* copy numbers in tumor tissue and in blood plasma was approximately 0.9.
Conclusion We can detect *MYCN* amplification of tumor tissue noninvasively and quantitatively by measuring the *MYCN* copy number in blood plasma. Determination of *MYCN* copy number in plasma may be useful when evaluating surgery and neoadjuvant chemotherapy.

Keywords Neuroblastoma · *MYCN* · Amplification · Real-time PCR · Plasma

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Introduction

Neuroblastoma (NBL) occurs in the adrenal cortex or sympathetic ganglia, and is thought to arise from the neural crest. NBL is the most common solid tumor in children and almost all patients are infants under 5 years old. The cause of NBL is not yet known, but some genetic or chromosomal abnormalities have been reported. For example, deletion of 1p and 11q, gain of 17q, activation of anaplastic lymphoma receptor tyrosine kinase (*ALK*), and amplification of neuroblastoma derived (avian) v-myc myelocytomatosis viral related oncogene (*MYCN*) are known to occur in NBL [1–4]. *MYCN* amplification is detected in 30–40 % of patients in advanced stages of NBL and is strongly associated with a poor prognosis [5]. In the risk stratifications of the international neuroblastoma risk group (INRG) and the children's oncology group (COG), *MYCN* amplification is one of the most important indicators of risk assessment [6]. Therefore, knowledge of *MYCN* amplification status is important for current therapeutic planning for NBL patients. Usually, *MYCN* amplification status is assessed using tumor cells obtained invasively from a biopsy or surgical specimen. Circulating DNA in serum has been used as a source to detect *MYCN* amplification non-invasively. In this paper, we used quantitative real-time PCR to measure the *MYCN* copy number in blood plasma. We then compared the data derived from PCR of plasma

DNA to those derived from Southern blot or fluorescent in situ hybridization (FISH) analyses of tumor specimens. In addition, we determined the *MYCN* copy numbers in blood plasma after surgical resection or chemotherapy in patients with *MYCN*-amplified NBL.

Materials and methods

Tissues and blood plasma

During the past two decades, more than 500 NBL tumors derived from more than 400 cases, including approximately 180 cases detected by mass screening, were either collected and archived at the Hiroshima University Hospital or subjected to molecular analysis at our hospital after a consultation request from other hospitals in Japan. All patients were diagnosed with NBL between 1991 and 2010, and most were treated in accordance with the Japanese neuroblastoma protocols for infants or advanced stage NBL (A1, new A1, or A3) [7]. The follow-up period of all patients was more than 5 years. Our study selected 50 cases that had both tumor tissue and blood plasma samples available. In these cases, 43 were treated in our university hospital and 7 were in other 4 affiliated hospitals. This research was planned by our hospital and other 4 affiliated hospitals in Japan and approved by the Ethical Committee of Hiroshima University Hiroshima University-Rin-20). We explained that we would use the tissue and plasma sample in future research and obtained written informed consent from the parents of all patients. In addition, 10 plasma samples obtained 0–5 years old children who had inguinal or umbilical hernia were used as healthy childhood volunteer samples. Under the same written informed consent, these samples were obtained from the remaining blood after clinical examination of the blood test and stored as anonymised ones.

We obtained tumor tissues immediately after surgery and stored the specimens at −80 °C. We obtained peripheral blood samples before any therapy to count the peripheral blood cells. The samples were stored in vials containing disodium-EDTA. After the samples were used to obtain blood cell counts, the remaining blood was centrifuged at 1,000 g for 10 min. Then, blood plasma was isolated from each sample and stored at −20 °C. Tables 1 and 2 shows the patients' ages at the time of diagnosis, tumor stages according to the INSS (international neuroblastoma staging system) at the time of surgery, origins of the primary tumor, outcomes, and the DNA ploidy for all cases analyzed in this study. DNA index of the tumor specimens was measured by flow cytometry as described previously [2]. Triploid was defined when DNA index was between 1.2 and 1.8, and tetraploid was defined when DNA index was more than 1.8. In the nine patients with *MYCN*-amplified tumors, we also collected the plasma samples in five patients after primary surgery and in four patients before second-look surgery following neoadjuvant chemotherapy.

DNA isolation

DNA was isolated from approximately 0.5 g of frozen surgical specimen using a proteinase K treatment followed by phenol/chloroform extraction and ethanol precipitation, as described previously [8]. DNA was isolated from 0.2 ml of each stored blood plasma sample using the QIAmp DNA Blood Mini Kit (Quiagen Valencia, CA) in accordance with the protocol. Genomic DNA was also isolated from the blood plasma samples from 10 healthy volunteers.

Quantitative real-time PCR

We used the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) to perform TaqMan PCR assays, as described previously [9]. Each time PCR was performed, a standard curve was generated from a dilution series containing 100, 50, 5, 1, and 0.5 ng of genomic DNA from healthy volunteers' whole blood. We measured the dosages of genes based on these standard curves. We used the average CT value from three measurements to represent the gene dosage. The N-acetylglucosamine kinase gene (*NAGK*; GenBank accession No. NM 017567), located at 2p12, was used as a single-copy reference gene [10].

Calculation of *MYCN* gene copy number

To calculate the *MYCN* copy number, we compared the dosages of *MYCN* and *NAGK*. First, we calculated the ratio of the *MYCN* gene dosage to the *NAGK* gene dosage (the *MYCN/NAGK* ratio) for each sample. Next, to compensate for the difference between the PCR efficiencies of *MYCN* and *NAGK*, we divided the *MYCN/NAGK* ratio of each sample by the *MYCN/NAGK* ratio of the control sample from healthy people's whole blood. We calculated the *MYCN* copy number of each sample using the equation shown below:

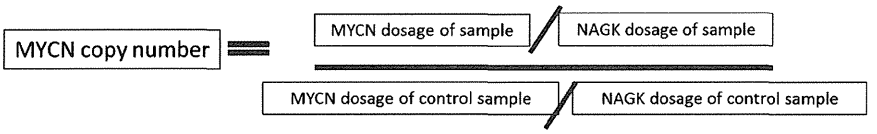

Table 1 *MYCN* non-amplified cases

No.	Pt. ID.	Age at diagnosis (months)	Gender	Origin	INSS	Ploidy	Outcome	*MYCN* copy number	
								Tumor DNA	Plasma DNA
1	013	17	Female	Adrenal	1	Tetraploid	NED	1.17	1.54
2	022	37	Female	Adrenal	3	Diploid	NED	1.05	1.14
3	051	34	Female	Adrenal	4	Tetraploid	NED	1.40	0.67
4	057	6	Female	Retro	2	Diploid	NED	1.15	0.75
5	071	84	Female	Chest	1	Diploid	NED	1.45	0.73
6	101	7	Female	Adrenal	1	Triploid	NED	1.39	1.14
7	146	7	Male	Chest	1	Diploid	NED	1.21	0.69
8	147	8	Male	Adrenal	1	Triploid	NED	0.96	1.11
9	275	20	Female	Multiple	3	Triploid	NED	0.38	1.40
10	288	8	Female	Adrenal	1	Triploid	NED	1.13	1.36
11	296	8	Female	Adrenal	1	Triploid	NED	0.85	1.23
12	311	7	Female	Retro	3	Triploid	NED	0.84	1.21
13	314	1	Male	Adrenal	1	Diploid	NED	0.66	0.57
14	334	46	Female	Adrenal	4	Diploid	NED	1.45	0.97
15	335	8	Female	Adrenal	1	Diploid	NED	1.21	1.04
16	336	7	Female	Retro	4	Diploid	NED	0.56	0.59
17	338	8	Male	Chest	1	Triploid	NED	0.96	0.60
18	342	9	Female	Pelvis	1	Triploid	NED	1.06	0.93
19	365	11	Female	Chest	2	Triploid	NED	0.98	0.65
20	366	8	Female	Retro	2	Triploid	NED	0.99	1.74
21	370	7	Female	Adrenal	1	Diploid	NED	1.18	0.86
22	373	8	Male	Chest	1	Triploid	NED	1.22	0.98
23	396	127	Female	Chest	1	Diploid	NED	1.15	0.70
24	403	66	Female	Retro	4	Diploid	NED	1.15	0.85
25	410	7	Male	Adrenal	4S	Triploid	NED	0.91	0.80
26	441	3	Female	Adrenal	1	Triploid	NED	0.96	1.10
27	448	10	Male	Neck	3	Triploid	DOD	1.12	1.14
28	462	20	Female	Chest	1	Triploid	NED	0.86	1.34
29	463	48	Female	Adrenal	2	Diploid	NED	1.06	0.96
30	470	42	Male	Adrenal	4	Triploid	DOD	1.20	1.22
31	480	61	Female	Retro	3	Diploid	NED	1.26	0.78
32	485	51	Male	Pelvis	4	Diploid	DOD	0.80	0.55
33	487	18	Male	Retro	3	Diploid	NED	1.03	1.01
34	499	106	Male	Retro	3	Diploid	NED	0.92	0.90

No. case number, *Pt. ID* patient identification number, *INSS* international neuroblastoma staging system, *adrenal* adrenal gland, *retro* retroperitonium, *DOD* dead of disease, *NED* no evidence of disease

Measurement error in the *MYCN* copy number.

We speculate that there was measurement error in the *MYCN* copy number, and describe the possible causes of measurement error in the Discussion. To account for this error, we used a boundary to define amplification of the *MYCN* copy number. We used healthy people's whole blood, which was assumed to have an *MYCN* copy number of 1, to make 13 serially diluted samples with a two-fold dilution factor, and then measured the *MYCN* copy number of each sample. The *MYCN* copy numbers ranged from 0.6 to 2.1. Therefore, we defined *MYCN* amplification as a copy number of >2.5 (Fig 1).

PCR primers and TaqMan probes

The PCR primers used were as follows: *MYCN* forward, 5′-GTGCTCTCCAATTCTCGCCT-3′; *MYCN* reverse, 5′-GATGGCCTAGAGGAGGGCT-3′; *NAGK* forward, 5′-TGGGCAGACACATCGTAGCA-3′; *NAGK* reverse, 5′-CACCTTCACTCCCACCTCAAC-3′.

Table 2 *MYCN*-amplified cases

No.	Pt. ID.	Age at diagnosis (months)	Gender	Origin	INSS	Ploidy	Outcome	MYCN copy number	
								Tumor DNA	Plasma DNA
35	004	13	Female	Adrenal	3	Diploid	DOD	17.29	11.05
36	029	9	Female	Adrenal	4	Triploid	DOD	12.57	7.54
37	055	15	Male	Adrenal	4	Diploid	DOD	22.21	6.45
38	066	14	Male	Adrenal	4	Diploid	DOD	61.57	24.29
39	322	14	Male	Adrenal	4	Diploid	DOD	11.99	9.89
40	380	29	Male	Adrenal	4	Tetraploid	DOD	28.00	58.83
41	445	19	Female	Adrenal	4	Triploid	DOD	29.19	35.80
42	452	30	Male	Adrenal	4	Diploid	DOD	18.69	29.20
43	459	24	Male	Adrenal	4	Diploid	DOD	55.58	82.41
44	474	27	Male	Adrenal	4	Diploid	NED	65.92	56.53
45	479	26	Male	Adrenal	4	Diploid	DOD	25.08	18.03
46	484	36	Male	Adrenal	4	Diploid	NED	12.47	14.01
47	488	28	Male	Adrenal	4	Diploid	DOD	56.95	89.20
48	504	27	Male	Adrenal	3	Tetraploid	DOD	44.31	50.85
49	510	19	Male	Adrenal	4	Triploid	DOD	27.25	6.13
50	521	18	Male	Adrenal	4	Diploid	NED	110.92	67.24

No. case number, *Pt. ID* patient identification number, *INSS* international neuroblastoma staging system, *adrenal* adrenal gland, *DOD* dead of disease, *NED* no evidence of disease

The TaqMan probe for *MYCN* was 5′-FAM-CACTAAAGTTCCTTCCACCCTCTCCT-3′; the TaqMan probe for *NAGK* was 5′-VIC-TGTTGCCCGAGATTGACCCGGT-3′.

Real-time PCR conditions

Real-time PCRs were carried out in a final volume of 20 μl with the FastStart Universal Probe Master (ROX).(Applied Biosystems) All PCRs were performed with 1 cycle of 50 °C for 2 min and 1 cycle of 95 °C for 10 min, followed by an amplification reaction composed of 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Statistical analysis

Statistical analyses were performed using Wilcoxon signed rank test. A P of less than 0.05 was considered significant.

Results

MYCN copy numbers in plasma DNA and tumor tissue

We measured the *MYCN* copy numbers in tumor tissues and in the corresponding blood plasma samples of 50 NBL cases, whose *MYCN* amplification status was previously determined by Southern blot analysis or FISH analysis. Our results are shown in Tables 1 and 2. The *MYCN/NAGK* ratios could be determined in approximately 2 h using real-time quantitative TaqMan PCR. Among the 50 plasma DNA samples, 34 samples had a non-amplified *MYCN* copy number and 16 samples had an amplified *MYCN* copy number. These data are in complete agreement with those obtained by Southern blot or FISH analyses.

In the present study, we reanalyzed the *MYCN* copy numbers of DNA derived from tumor specimens using quantitative real-time PCR. The *MYCN* copy numbers in the tumor tissues of the 34 non-*MYCN*-amplified cases ranged from approximately 0.4–1.4; the average was approximately 1.0 and the standard deviation was approximately 0.2. In the blood plasma samples of the 34 non-*MYCN*-amplified cases, the copy numbers ranged from approximately 0.5–1.7; the average was approximately 1.0 and the standard deviation was approximately 0.3 (Figs. 1, 2). Among the 34 cases, 7 of 18 aneuploid (triploid or tetraploid) cases (DNA index > 1.3) had slightly higher *MYCN* copy numbers (copy number > 1.2) in the plasma DNA samples than in the 16 diploid cases in which only one showed slightly higher *MYCN* copy number ($P = 0.042$) (Table 2, Fig. 1). Thirty-one of the 34 cases with a non-amplified *MYCN* copy number showed a good prognosis.

The copy numbers in the tumor tissues of the 16 *MYCN*-amplified cases ranged from approximately 11.99–110.92, while those of the blood plasma samples ranged from approximately 6.45–89.20 (Table 2, Fig. 1). Thirteen of the 16 cases showed a poor prognosis. The correlation

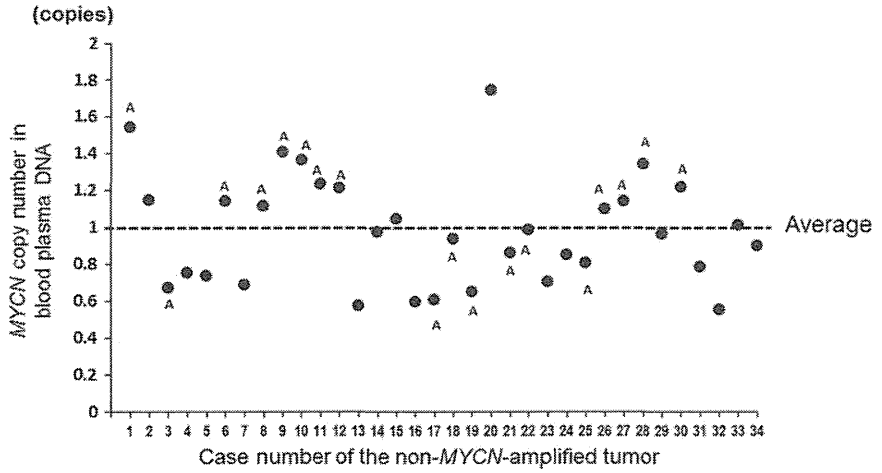

Fig. 1 *MYCN* copy numbers in blood plasma DNA of 34 non-*MYCN*-amplified cases. The *MYCN* copy numbers of these cases ranged from 0.5 to 1.8; the average was 1.0. Among these 34 cases, 7 of 18 aneuploid cases (DNA index > 1.2, indicated with an "A") had higher copy numbers (range 1.2–1.8) than those obtained by 16 diploid cases (DNA index ≤ 1.2). A: aneuploid (triploid or tetraploid), *MYCN*: neuroblastoma derived (avian) v-myc myelocytomatosis viral related oncogene

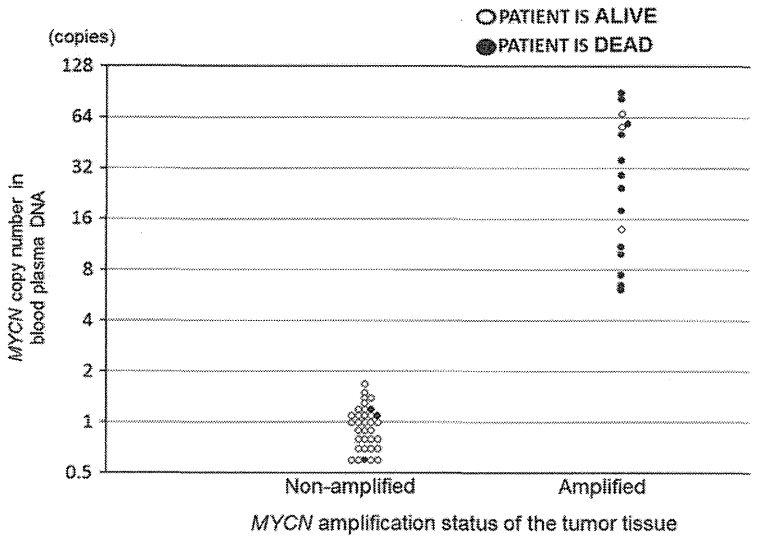

Fig. 2 *MYCN* copy numbers in plasma DNA isolated from patients with neuroblastoma a scatter plot of the plasma *MYCN* copy numbers in patients with neuroblastomas. The scatter plot is segregated according to the *MYCN* amplification status by Southern or FISH analysis. The copy numbers were calculated using the plasma *MYCN*/*NAGK* ratio. The copy numbers were significantly higher in *MYCN*-amplified cases than in non-*MYCN*-amplified cases ($P < 0.001$). *NAGK*: N-acetylglucosamine kinase, *MYCN*: neuroblastoma derived (avian) v-myc myelocytomatosis viral related oncogene open circle (*open circle*): alive cases with disease-free, closed circle (*filled circle*): dead cases of disease

coefficient between the *MYCN* copy numbers in the tumor tissues and those in the blood plasma samples was approximately 0.9 (Fig. 3). In 2 cases whose *MYCN* copy numbers in the plasma DNA was higher than those in the tumor DNA, it is possible that PCR inhibitors such as phenol may have been present in the tumor DNA samples. Conversely, in one case whose *MYCN* copy number in the plasma DNA was lower than that in the tumor DNA, the plasma DNA concentration was too low to be estimated precisely.

Changes of the *MYCN* copy numbers in plasma DNA after therapy

We followed the postsurgical changes of the *MYCN* copy numbers in the blood plasma of five patients with *MYCN*-amplified tumors. Three patients underwent total resection of their primary tumors, but the remaining two patients underwent subtotal resection due to either extensive invasion by the primary tumor or residual distant metastasis. The changes of the *MYCN* copy numbers over the course of

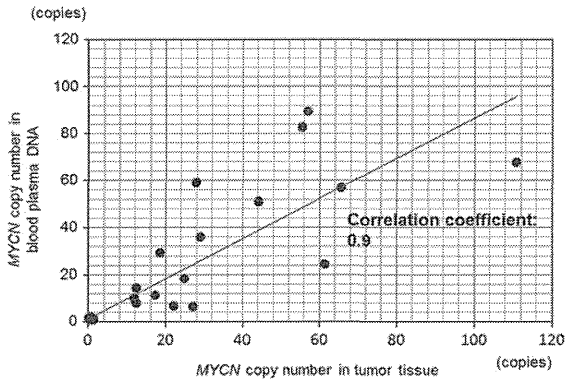

Fig. 3 Correlation between *MYCN* copy numbers in tumor tissue and blood plasma. The copy numbers were calculated using the serum *MYCN/NAGK* ratio. The correlation coefficient between the *MYCN* copy numbers in tumor tissue and those in blood plasma was approximately 0.9. *NAGK*: N-acetylglucosamine kinase, *MYCN*: neuroblastoma derived (avian) v-myc myelocytomatosis viral related oncogene

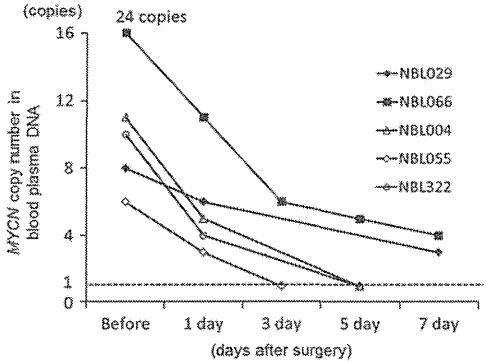

Fig. 4 Postsurgical changes of the *MYCN* copy numbers in the blood plasma of five patients with *MYCN*-amplified tumors In 3 patients (cases NBL004, 055, and 322) whose tumors were totally resected by surgery, the *MYCN* copy numbers decreased to 1.0 by 3–5 days after surgery. In the remaining 2 patients (cases NBL029 and 066), who still had metastases after surgery, the *MYCN* copy numbers did not decrease to 1.0 after resection of their primary tumors. *MYCN*: neuroblastoma derived (avian) v-myc myelocytomatosis viral related oncogene; NBL: neuroblastoma

1 week are shown in Fig. 4. The *MYCN* copy numbers of the three patients who received total resections decreased to <1.5 by 6 days after surgery. The *MYCN* copy numbers of the two patients who received subtotal resections did not decrease to <3 copies by 1 week after surgery. We also examined the plasma DNA samples of four patients before second-look surgery following neoadjuvant chemotherapy: the *MYCN* copy numbers of the samples were 0.97, 1.23, 1.56, and 2.01. These copy numbers are similar to those of patients with non-*MYCN*-amplified tumors.

Discussion

The clinical usefulness of assessing *MYCN* amplification in tumor tissue as a prognostic factor depends on the reliability, rapidity, and simplicity of the procedure. Usually, gene amplification is detected by Southern blot or in situ hybridization procedures, which require microgram quantities of high quality DNA or fresh tumor specimens derived from the resected tissues [11]. The ability to rapidly detect *MYCN* gene amplification, especially in small samples obtained by aspiration or biopsy, has stimulated considerable interest in improved clinical management of NBL patients.

PCR is a powerful procedure to amplify small amounts of DNA or mRNA for molecular analyses. This procedure is very beneficial for several reasons: (1) large amounts of specimen are not required; (2) the amplification reaction is rapid; and (3) partially degraded DNA may be amplified. Recently, gene amplification of several oncogenes has been detected with PCR [9, 11, 12]. Since almost all *MYCN*-amplified NBLs have been reported to have more than 10 copies [9, 11], we previously established differential PCR with co-amplification of two reference gene fragments. Then, quantitative PCR was developed and has become so popular that this method will no doubt become one of the routine methods to detect *MYCN* amplification in NBLs. In patients with adult cancers, genetic aberrations such as amplification have been detected for several genes using the DNA isolated from serum [13, 14]. Following this discovery, serum DNA was used to detect *MYCN* amplification in patients with NBL, because detection of *MYCN* amplification in circulating DNA is less invasive as well as simpler [10, 15]. In these previous reports, circulating DNA was usually isolated from serum. In this paper, we examined *MYCN* amplification in circulating DNA isolated from plasma, and discovered that plasma DNA samples are suitable to evaluate *MYCN* amplification in NBL. Since blood samples containing anticoagulants such as disodium-EDTA are frequently examined in cases of NBL to determine blood cell counts, plasma samples are easily obtained from the remaining blood samples. Therefore, this method is more convenient than invasive surgical approaches for screening or surveillance of *MYCN*-amplified tumors.

The *MYCN* copy numbers in plasma-derived circulating DNA exhibited significant concordance with those of DNA isolated from *MYCN*-amplified tumor tissues. In these cases, we wondered why there were differences between the *MYCN* copy number in the tumor tissue and that in the blood plasma of the same patient. We can think of two causes of the measurement error. First, DNA from normal cells mingles with that from tumor cells when we collect surgical specimens or blood plasma. If cells from the neighboring normal tissue mingle with those of tumor

tissue in a surgical specimen, or if DNA from normal cells such as leukocytes mingles with tumor DNA in blood plasma, the *MYCN* copy number will be underestimated. Second, measurement error can occur during real-time PCR. There are technical errors, including pipetting errors and underestimation of DNA density or the reactions may contain inhibitors such as heparin or protein.

Among the cases of non-*MYCN*-amplified tumors in our study, the *MYCN* copy numbers in blood plasma DNA samples were less than 1.8. Interestingly, 7 of 18 aneuploid cases exhibited more than 1.2 copies. These copy numbers might reflect the number of *MYCN* present in these cases. In these aneuploid cases, chromosomal aberrations or imbalance might occur in the tumor cell. Therefore, 3 or 4 *MYCN* signals might exist in the tumor cells, whereas the reference gene was 2 signals. Data from FISH analyses of these tumors with an *MYCN* probe supported this hypothesis (data not shown). These data suggested that slightly high number of *MYCN* copy number might indicate aneuploidy of tumor cells in NBLs.

In this study, we followed the postsurgical changes of the *MYCN* copy numbers in the plasma DNA of five patients with *MYCN*-amplified tumors. Our results indicate that the copy numbers decreased after extirpation of tumors from almost all patients, but did not decrease to one copy in the blood of patients with residual tumors. Furthermore, after chemotherapy, the *MYCN* copy numbers in the plasma DNA of patients with *MYCN*-amplified tumors were similar to those in patients with non-*MYCN*-amplified tumors, indicating that effective chemotherapy might reduce the amount of tumor-derived circulating DNA. Taken together, these results suggest that the *MYCN* copy numbers in plasma DNA may be useful for evaluating therapeutic efficacy and the effects of surgery. To confirm this speculation, more samples from patients with *MYCN*-amplified tumors should be analyzed in the future.

In conclusion, we have determined that measuring the *MYCN* copy number in blood plasma by quantitative real-time PCR can detect *MYCN* amplification in tumor tissue noninvasively and quantitatively. Hence, our results indicate that this method may be useful not only to predict a prognosis and plan a therapeutic approach, but also to evaluate the therapeutic efficacy of chemotherapy and surgery.

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Conflict of interest The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Appropriate dose reduction in induction therapy is essential for the treatment of infants with acute myeloid leukemia: a report from the Japanese Pediatric Leukemia/Lymphoma Study Group

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Abstract Infants (<1 year old) with acute myeloid leukemia (AML) are particularly vulnerable to intensive cytotoxic therapy. Indeed, the mortality rate was high among infants enrolled in the Japanese Pediatric Leukemia/Lymphoma Study Group AML-05 study, which prompted us to temporarily suspend patient enrollment and amend the protocol. Forty-five infants with AML were enrolled. For patients aged <2 years, drug doses were adjusted for body weight. Following the protocol amendments, doses for infants were reduced by a further 33 % in the initial induction course. Six infants died during the induction phase (including five early deaths), mainly due to pulmonary complications. The 3-year probability of overall survival (pOS) in all 45 infants [55.9 %, 95 % confidence interval (CI) 37.9–70.6 %] was significantly lower than that of patients aged 1 to <2 years (77.0 %, 95 % CI 62.7–86.3 %) and those aged ≥2 years (74.7 %, 95 % CI 69.2–79.4 %) ($P = 0.037$), mainly due to the higher non-relapse mortality rate in infants. No early deaths occurred after the protocol amendments, and the 3-year pOS of the 17 infants enrolled thereafter was 76.4 % (95 % CI 48.8–90.4 %). In conclusion, appropriate dose reduction is

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essential to avoid early deaths when treating infants with AML.

Keywords Acute myeloid leukemia · Infants · Early death · Acute respiratory distress syndrome

Introduction

Infants aged <1 year with acute myeloid leukemia (AML) show distinct clinical features compared with older children with AML, including a higher white blood cell (WBC) count, extramedullary involvement at diagnosis, and a higher frequency of M4/M5 or M7 leukemic cells classified by the French–American–British (FAB) system, as well as unique cytogenetic characteristics [1, 2]. Relatively few infants have favorable cytogenetic characteristics, such as t(8;21)(q22;q22)/*RUNX1-RUNX1T1*, inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/*CBFB-MYH11*, or t(15;17)(q22;q12)/*PML-RARA*. Most infants have cytogenetic characteristics that are associated with poor prognosis, including rearrangement of the mixed lineage leukemia (*MLL*) gene on chromosome 11q23, t(1;22)(p13;q13)/*RBM15-MKL1*, which is highly associated with acute megakaryocytic leukemia in non-Down syndrome infants, and t(7;12)(q36;p13)/*HLXB9-ETV6* [3–5]. Hence, infants with AML are usually classified in intermediate (IR) or high-risk (HR) groups, and are treated with an intensive combination chemotherapy regimen based on cytarabine and anthracyclines that is also used for older children. However, as infants are particularly vulnerable to intensive cytotoxic treatment, many study groups have modified the doses of chemotherapeutic drugs administered to infants [6, 7].

In the nationwide multicenter AML-05 study conducted by the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG), the early mortality rate was unacceptably high among the first 32 infants (28 eligible infants) enrolled in the study, mainly because of acute respiratory distress syndrome (ARDS). This issue prompted us to temporarily suspend patient enrollment and amend the protocol. Here, we report the outcomes of 45 infants with AML who were enrolled and treated in the AML-05 study.

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Materials and methods

Patients

Between November 2006 and December 2010, 485 consecutive patients aged <18 years old with suspected AML treated at 118 institutions in Japan were registered in the AML-05 study. Patients with acute promyelocytic leukemia, Down syndrome, secondary AML, myeloid/natural killer cell leukemia, and myeloid sarcoma were not eligible. AML was diagnosed using the World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissues (3rd edition) [8] with a comprehensive central diagnostic review system that used morphology, immunophenotyping [9], and cytogenetic analysis of diagnostic bone marrow specimens. Overall, 38 patients (including 5 infants) were excluded, mainly because of misdiagnosis, while 4 additional patients were excluded because the guardian refused to participate ($n = 1$), there was a significant protocol violation during the initial induction course ($n = 1$), the hospital withdrew its membership from the JPLSG ($n = 1$), and the patient was transferred to a non-JPLSG member hospital ($n = 1$). Among the 443 eligible patients, 45 (10.1 %) were infants aged <1 year at initial diagnosis. Written informed consent, provided according to the Declaration of Helsinki, was obtained from the guardians of each patient. All aspects of the study were approved by the institutional review boards at all participating institutions.

Treatments

The therapeutic regimens used in the AML-05 study are presented in Fig. 1. After the second induction course, the patients were stratified to one of three risk groups according to their cytogenetic characteristics and treatment response following the initial induction course, and received three additional intensified chemotherapeutic courses. Patients who failed to achieve complete remission (CR) after the second course were removed from the study. Allogeneic hematopoietic stem cell transplantation (HSCT) was indicated for all of the high-risk (HR) patients after three or more treatment courses. For patients aged <2 years, the drug doses were reduced by taking into account body weight rather than body surface area throughout the treatment course. Because of the high early mortality rate in infants, we temporarily suspended the enrollment of infants between April 2 and August 11, 2009. At this time, the following amendments were made: (1) an

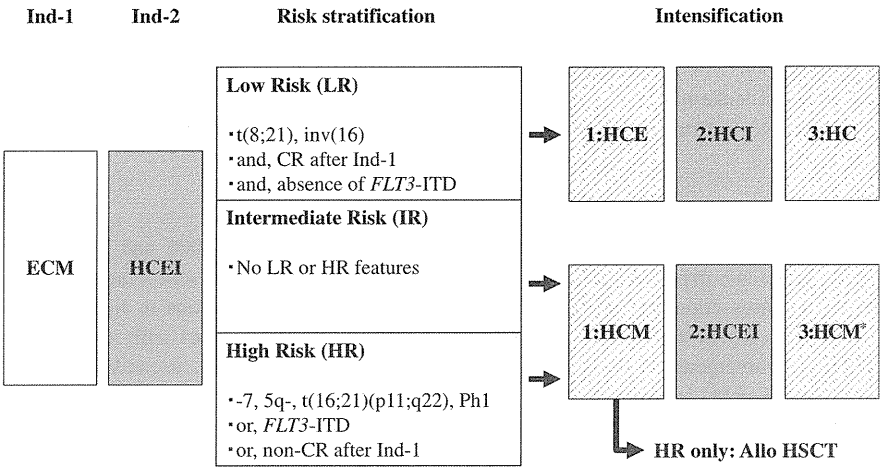

Fig. 1 Treatment schedule in the AML-05 study. ECM consisted of etoposide (150 mg/m^2 per day on days 1–5), cytarabine [200 mg/m^2/day via 12 h continuous intravenous (CIV) infusion on days 6–12], mitoxantrone (5 mg/m^2/day on days 6–10), and an age-adjusted dose of triple intrathecal chemotherapy (TIT) on day 6. HCEI consisted of high-dose cytarabine (HDCA; 3 g/m^2 every 12 h on days 1–3), etoposide (100 mg/m^2/day on days 1–5), idarubicin (10 mg/m^2 on day 1), and TIT on day 1. HCE consisted of HDCA (2 g/m^2 every 12 h on days 1–5), etoposide (100 mg/m^2/day on days 1–5), and TIT on day 1. HCI consisted of HCEI without etoposide. HC consisted of HCE without etoposide. HCM consisted of HDCA (2 g/m^2 every 12 h on days 1–5), mitoxantrone (5 mg/m^2/day on days 1 and 2), and TIT on day 1. *Ind-1* induction course 1, *Ind-2* induction course 2, *Allo HSCT* allogeneic hematopoietic stem cell transplantation. *Asterisk* indicates patients in the intermediate-risk or high-risk groups who experienced Grade 4 infection during intensification course 1 with HCM received HC for intensification course 3

additional dose reduction by 33 % during induction phase 1 for infants; (2) avoidance of the prophylactic administration of granulocyte-colony stimulating factor (G-CSF), considering its possibility as a risk factor for developing ARDS; (3) introduction of enhanced guidelines for supportive care in relation to infection prevention; and (4) close prospective safety monitoring during the induction phase.

Statistical analyses

The baseline characteristics and the clinical course of patients were analyzed using the χ^2 test or Fisher's exact test for categorical variables, and the Wilcoxon rank-sum test for continuous variables. Early death was defined as any-cause death occurring within 42 days of enrollment. Event-free survival (EFS) was defined as the time from the diagnosis of AML to the last follow-up or the first event (failure to achieve remission, relapse, secondary malignancy, or any-cause death). Overall survival (OS) was defined as the time from the diagnosis of AML to any-cause death. The probabilities of EFS (pEFS) and OS (pOS) were estimated using the Kaplan–Meier method. Standard errors (SEs) were calculated using the Greenwood formula and curves were compared using the log-rank test. Confidence intervals (CIs) were calculated at the 95 % confidence level. Gray's methods were used to estimate and compare the cumulative incidence of important events (relapse, non-relapse death). All analyses were performed using STATA® statistical software (version 11.0; StataCorp LP, College Station, TX). Follow-up data were actualized as of May 1, 2012. This trial is registered with the UMIN Clinical Trials Registry (UMIN-CTR, URL: http://www.umin.ac.jp/ctr/index.htm), number UMIN000000511.

Results

Patient characteristics and clinical outcomes according to age group

The characteristics of the patients are reported in Table 1 for three age groups: infants (<1 year old), 1 to <2 years old, and ≥2 years old. Distributions of FAB categories and cytogenetic characteristics differed among the three groups. In particular, there were more patients with monocytic (M5a/M5b) and megakaryocytic (M7) leukemia, but less with M1/M2 leukemia in the younger age groups. Regarding cytogenetic characteristics, there were more patients with *MLL* gene rearrangements [t(9;11) and other 11q23 abnormalities], but fewer with core-binding factor AML [t(8;21) and inv(16)/t(16;16)] among infants compared with the other age groups. None of the infants were positive for *FLT3* internal tandem duplications.

Table 1 Patient characteristics according to age group

	<1 year (n = 45) n (%)	1 to <2 years (n = 58) n (%)	≥2 years (n = 340) n (%)	P value
Sex				
Male	20 (44.4)	32 (55.1)	186 (54.7)	0.419
Female	25 (55.5)	26 (44.8)	154 (45.2)	
WBC at diagnosis (/μL)				
<10,000	8 (17.7)	20 (34.4)	128 (37.6)	0.051
10,000–50,000	25 (55.5)	23 (39.6)	117 (34.4)	
>50,000	12 (26.6)	15 (25.8)	95 (27.9)	
FAB classification				
M0	0 (0.0)	0 (0.0)	8 (2.3)	<0.001
M1	2 (4.4)	3 (5.1)	52 (15.2)	
M2	1 (2.2)	2 (3.4)	114 (33.5)	
M3	1 (2.2)	0 (0.0)	0 (0.0)	
M4	3 (6.6)	4 (6.8)	40 (11.7)	
M4Eo	1 (2.2)	5 (8.6)	9 (2.6)	
M5a	15 (33.3)	11 (18.9)	49 (14.4)	
M5b	4 (8.8)	4 (6.8)	11 (3.2)	
M6	0 (0.0)	5 (8.6)	5 (1.4)	
M7	14 (31.1)	21 (36.2)	13 (3.8)	
RAEB[a]	1 (2.2)	1 (1.7)	1 (0.2)	
RAEB-T[a]	1 (2.2)	2 (3.4)	36 (10.5)	
ND	2 (4.4)	0 (0.0)	2 (0.5)	
Cytogenetic characteristics				
t(8;21)	0 (0.0)	1 (1.7)	121 (35.5)	<0.001
inv(16)	1 (2.2)	6 (10.3)	25 (7.3)	
t(9;11)	8 (17.7)	9 (15.5)	22 (6.4)	
Other 11q23 abnormalities	11 (24.4)	4 (6.8)	15 (4.4)	
t(6;9)	0 (0.0)	0 (0.0)	3 (0.8)	
inv(3)	0 (0.0)	0 (0.0)	2 (0.5)	
t(1;22)	3 (6.6)	0 (0.0)	0 (0.0)	
t(7;12)	2 (4.4)	1 (1.7)	0 (0.0)	
Normal karyotype	6 (13.3)	6 (10.3)	68 (20.0)	
Others	13 (28.8)	31 (53.4)	82 (24.1)	
ND	1 (2.2)	0 (0.0)	2 (0.5)	
FLT3-ITD status				
Positive	0 (0.0)	3 (5.1)	44 (12.9)	0.002
Negative	44 (97.7)	55 (94.8)	296 (87.0)	
ND	1 (2.2)	0 (0.0)	0 (0.0)	

WBC white blood cell count, *FAB* French–American–British, *RAEB* refractory anemia with excess blasts, *RAEB-T* refractory anemia with excess blasts in transformation, *ND* not detected, *ITD* internal tandem duplications

[a] As the World Health Organization classification (3rd edition) was used, patients with <30 % bone marrow blasts were included in this study

The results of induction therapies are described in Table 2. The proportion of patients with <5 % bone marrow blasts following initial induction therapy and the complete remission (CR) rate were significantly worse in infants than in patients aged 1 to <2 years or in those aged ≥2 years, respectively. This was due to the higher early mortality rate in infants than in other age groups. CR could not be evaluated in two infants who discontinued the study because of an adverse event (patient #2 in Table 3) and at the physician's decision.

Of the 33 infants who achieved CR, 1 patient with inv(16) was included in the low-risk group, 26 in the IR group, and 5 in the HR group. Four patients in the HR group received allogeneic HSCT at the first CR (Fig. 2). One infant discontinued the study because of the physician's decision. When we compared the 3-year pEFS among the three age groups, pEFS was lower in infants (46.1 %, 95 % CI 31.1–59.9 %) than in those aged 1 to <2 years (55.4 %, 95 % CI 41.2–67.5 %) or those aged ≥2 years (55.2 %, 95 % CI 49.4–60.5 %). However, the

Table 2 Initial treatment response according to age group and before/after the protocol amendments

Age group	<1 year				1 to <2 years	≥2 years	*P* value[b]
	Before amendment $n = 28$	After amendment $n = 17$	*P* value[a]	Total $n = 45$	$n = 58$	$n = 340$	
<5 % bone marrow blast after Ind-1	16 (57.1 %)	12 (70.5 %)	0.367	28 (62.2 %)	51 (87.9 %)	290 (85.2 %)	0.001
CR rate (after Ind-2)	19 (67.9 %)	14 (82.4 %)	0.488	33 (73.3 %)	49 (84.4 %)	299 (87.9 %)	0.036
Early death (≤42 days)	5 (17.9 %)	0 (0.0 %)	0.140	5 (11.1 %)	1 (1.7 %)	1 (0.2 %)	<0.001
Non-response	2 (7.1 %)	3 (17.6 %)	0.350	5 (11.1 %)	6 (10.3 %)	32 (9.4 %)	0.922
Other	2 (7.0 %)	0 (0.0 %)	0.519	2 (4.4 %)	2 (3.4 %)	8 (2.3 %)	0.670

Ind-1 induction course 1, *CR* complete remission, *Ind-2* induction course 2

[a] Before versus after the protocol amendments

[b] Comparison among the three age groups

Table 3 Characteristics of the six infants who died during the initial induction phase

Patient	Characteristics at diagnosis					Cause of early death	Death (day+)	Infectious complications	Other complications
	Age (months)	Sex	WBC (/μL)	EMD	AML subtype (WHO-3/ FAB)				
#1	7	F	58,000	Yes	Acute monoblastic leukemia/M5a	Leukemia	5	No	No
#2	7	F	152,130	Yes	AML with 11q23 abnormalities/M5a	ARDS	62	Sepsis	HPS
#3	4	M	7,200	Yes	Acute monocytic leukemia/ M5b	ARDS	39	FN	HPS
#4	7	M	7,900	Yes	Acute monoblastic leukemia/M5a	ARDS	22	RSV infection	No
#5	7	M	7,840	Yes	AML with 11q23 abnormalities/M4	ARDS	39	RSV infection	HPS
#6	2	F	4,400	No	AML with multilineage dysplasia/M1	IP	17	Sepsis (*S. aureus*)	No

WBC white blood cell count, *EMD* extramedullary disease, *AML* acute myeloid leukemia, *WHO-3* World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissues (3rd edition), *FAB* French–American–British, *F* female, *M* male, *ARDS* acute respiratory distress syndrome, *IP* interstitial pneumonia, *FN* febrile neutropenia, *RSV* respiratory syncytial virus, *S. aureus Staphylococcus aureus*, *HPS* hemophagocytic syndrome

difference was not statistically significant ($P = 0.108$) because of the relatively small numbers of patients (Fig. 3a). The median follow-up time of living patients was 3.06 years (range, 0.84–5.36 years). However, the 3-year pOS was significantly worse in infants (55.9 %, 95 % CI 37.9–70.6 %) than in those aged 1 to <2 years (77.0 %, 95 % CI 62.7–86.3 %) and those aged ≥2 years (74.7 %, 95 % CI 69.2–79.4 %; $P = 0.037$; Fig. 3b). The inferior survival rate in infants appeared to be due to the higher non-relapse mortality rate in this age group, rather than recurrent disease (Fig. 3c, d). The non-relapse mortality rates in infants, those aged 1 to <2 years, and those aged ≥2 years were 29.2 % (95 % CI 15.6–50.4 %), 7.4 % (95 % CI 2.8–18.9 %), and 11.8 % (95 % CI 8.3–16.7 %), respectively ($P = 0.007$). The cumulative relapse rates were 44.0 % (95 % CI 29.8–61.2 %), 41.0 % (95 % CI 29.0–55.8 %), and 42.6 % (95 % CI 37.3–48.3 %), respectively ($P = 0.564$).

Remission induction results of infants enrolled before and after the protocol amendments

We next compared the outcomes between 28 infants who were enrolled before the protocol amendments and 17 patients enrolled after these amendments. Before the protocol amendments, there were 5 early deaths plus 1 patient who died on day 62 during the initial induction phase (Table 3). These six deaths that occurred during initial

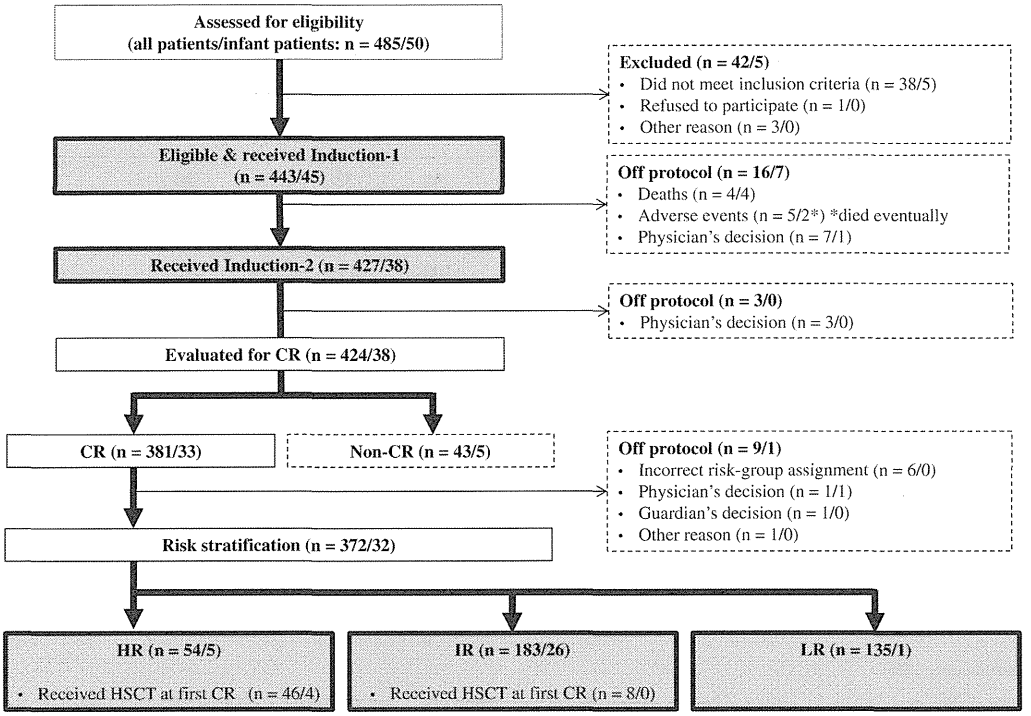

Fig. 2 Patient disposition in the AML-05 study

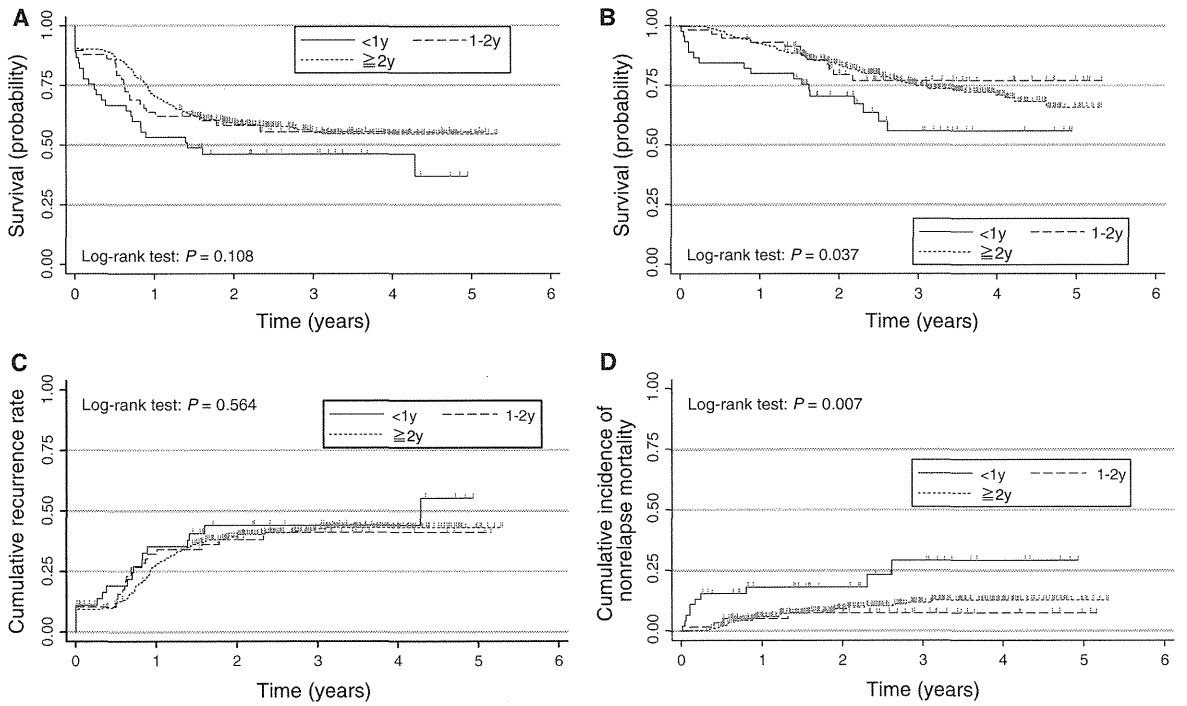

Fig. 3 Comparison of outcomes of patients according to age group. **a** Event-free survival (EFS). **b** Overall survival (OS). **c** Cumulative relapse rate. **d** Non-relapse mortality

Table 4 Incidence of grade ≥3 adverse events among infants

	Induction			Intensification		
	1: ECM		2: HCEI	1: HCE or HCM	2: HCI or HCEI	3: HC or HCM
	Before amendment	After amendment				
Number of patients assessed	28	17	38	32	30	24
Blood/bone marrow (%)						
Hemoglobin	100	100	94	100	86	91
Leukocytes	96	88	100	100	100	100
Neutrophils	96	100	100	100	100	100
Platelets	100	100	100	100	96	100
Cardiac (%)						
LV systolic dysfunction	7	0	0	0	0	0
Coagulation (%)						
DIC	17	5	2	0	0	0
Dermatology/skin (%)						
Rash/desquamation	3	5	0	0	0	0
Gastrointestinal (%)						
Vomiting	7	5	0	3	0	0
Diarrhea	21	11	2	12	6	4
Mucositis	3	0	0	0	3	0
Hemorrhage/bleeding (%)						
Hemorrhage, CNS	3	0	0	0	0	0
Hemorrhage, pulmonary	14	0	0	0	0	0
Infection (%)						
Febrile neutropenia	57	64	34	46	30	33
Infection (documented clinically)	42	17	18	21	23	25
Metabolic/laboratory (%)						
Creatinine	5	0	0	0	0	0
ALT	14	0	10	6	13	12
AST	21	0	10	0	13	12
Bilirubin	3	0	0	0	0	0
Neurology (%)						
Somnolence	5	0	0	0	0	0
Seizure	3	0	0	0	0	0
Pulmonary/upper respiratory (%)						
ARDS	5	0	0	0	0	0
Hypoxia	28	5	0	0	0	4
Syndromes (%)						
Tumor lysis syndrome	14	5	0	0	0	0

Treatment courses are described in Fig. 1

LV left ventricular, *DIC* disseminated intravascular coagulation, *CNS* central nervous system, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *ARDS* acute respiratory distress syndrome

induction were due to rapid progression of leukemia in one patient, ARDS in four patients, and interstitial pneumonia in one patient. All five of the non-leukemic deaths were associated with infectious disease or febrile neutropenia. Notably, two of the patients had respiratory syncytial virus (RSV) infection. Concurrent hemophagocytic syndrome (HPS) was also found in three of the patients who died because of non-leukemic causes.

The overall early treatment responses are summarized in Table 2. Although none of the parameters showed statistically significant differences because of the small number of patients analyzed, there were no early deaths after the protocol amendments. Additionally, the incidence of grade ≥3 non-hematological toxicities, as evaluated by the common terminology criteria for adverse events (3rd version), was lower in patients enrolled after versus before the

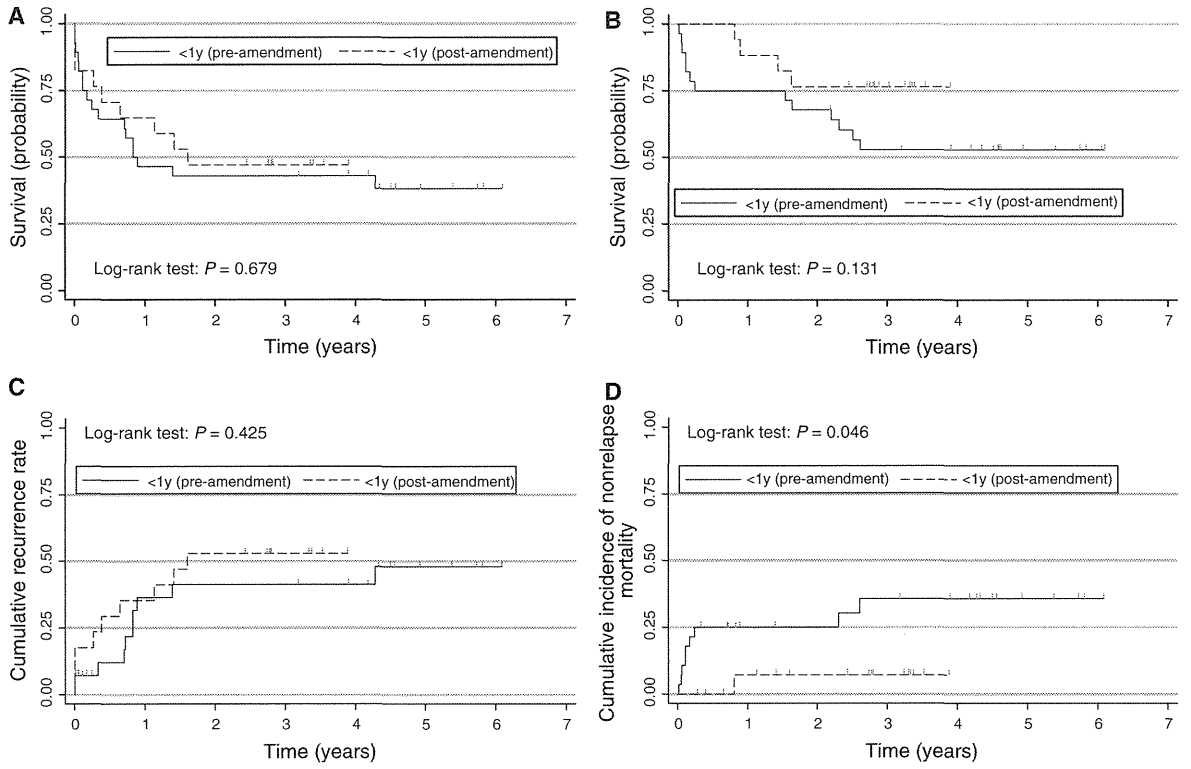

Fig. 4 Comparison of outcomes of infants enrolled before or after the protocol amendments. **a** Event-free survival (EFS). **b** Overall survival (OS). **c** Cumulative relapse rate. **d** Non-relapse mortality

protocol amendments (Table 4). Importantly, it seems that the dose reductions in the initial induction course did not compromise treatment efficacy, as 82.4 % of the infants enrolled after the protocol amendments achieved CR. Additionally, the percentage of patients receiving prophylactic G-CSF use seemed to be lower in infants enrolled after the protocol amendment compared to the pre-amendment cohort; 23.5 % (4/17) vs. 39.2 % (11/28). Median days for G-CSF use in these patients were also shorter in the post-amendment cohort; 9 days (range 5–19 days) vs. 14 days (range 2–25 days).

Overall outcomes of infants enrolled before and after the protocol amendments

There were no significant differences in 3-year pEFS [42.8 % (95 % CI 24.5–59.9 %) vs. 47.0 % (95 % CI 22.9–67.9 %); $P = 0.679$] and the cumulative relapse rate [41.3 % (95 % CI 23.8–64.8 %) vs. 52.9 % (95 % CI 32.0–77.0 %); $P = 0.425$] between infants enrolled before and after the protocol amendments (Fig. 4a, c). However, there was a significant improvement in the reduction of non-relapse mortality rate [35.7 % (95 % CI 19.9–58.3 %) vs. 7.1 % (95 % CI 1.0–40.9 %); $P = 0.046$; Fig. 4d] and achieved 3-year pOS of 76.4 % (95 % CI 48.8–90.4 %) for the post-amendment cohort [vs. 52.7 % (95 % CI 32.8–69.3 %) for the pre-amendment cohort; $P = 0.131$; Fig 4b]. The incidence of grade ≥3 adverse events by treatment courses in infants is described in Table 4. Besides hematologic toxicities and febrile neutropenia/infection that were frequent throughout the whole treatment courses, the other grade ≥3 adverse events that occurred in >10 % of infants beyond the initial induction course were gastrointestinal toxicity (diarrhea) and elevated liver function parameters (aspartate aminotransferase and alanine aminotransferase).

Discussion

Intensive combination chemotherapy with cytarabine and anthracyclines, together with optimal risk stratification based on the cytogenetic characteristics of leukemia cells and the response to the initial induction course, has led to a 70 % probability of survival in childhood AML [10, 11]. Infants with AML aged <1 year have features that are