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Primary Bone Lymphoma: A New and Detailed Characterization of 28 Patients in a Single-Institution Study

Dai Maruyama[1], Takashi Watanabe[1], Yasuo Beppu[2], Yukio Kobayashi[1], Sung-Won Kim[1], Kazuki Tanimoto[1], Atsushi Makimoto[3], Yoshikazu Kagami[4], Takashi Terauchi[5], Yoshihiro Matsuno[6] and Kensei Tobinai[1]

[1]Hematology and Stem Cell Transplantation, [2]Orthopedic Surgery, [3]Pediatric Oncology, [4]Radiation Oncology and [6]Clinical Laboratory Divisions, National Cancer Center Hospital, Tokyo and [5]Diagnostic Division, Research Center for Cancer Prevention and Screening, National Cancer Center, Tokyo, Japan

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Background: The incidence of primary bone lymphoma (PBL) is so rare that many of its aspects remain unknown. A number of studies have been reported from Western countries, but only a few reports are available from Asia.
Methods: We retrospectively analyzed 28 consecutive patients diagnosed with PBL initially treated at our hospital between 1995 and 2004. All patients underwent chemotherapy with half receiving radiotherapy as their initial treatment. A log-rank test was used in a univariate analysis to identify factors affecting overall survival.
Results: Fifteen (54%) patients were male and 13 (46%) female with a median age of 47 (range: 5–81). Although 19 (68%) patients had diffuse large B-cell lymphoma (DLBCL), other histopathological subtypes (three B-lymphoblastic lymphoma, two anaplastic large cell lymphoma, two indolent B-cell lymphoma, one NK/T-cell lymphoma (NTCL) and one Hodgkin lymphoma) were also included. The pelvis was the most frequently involved site (54%). While 68% of patients had stage IV disease, none of them showed bone marrow involvement at their initial diagnosis. Despite 61% high intermediate-risk and high-risk patients based on the International Prognostic Index, the estimated 3-year overall and progression-free survival rates were 84% and 77%, respectively. Only 'histopathological subtype (immunoblastic variant of DLBCL or NTCL versus others)' and 'response to initial treatment (progression versus remission)' were factors significantly affecting overall survival.
Conclusions: Although the total number of patients was relatively small, the detailed clinical data analyses presented here revealed several new characteristics of PBL and some aspects that may be unique to Japanese patients.

Key words: primary bone lymphoma – DLBCL – radiotherapy – bone tumor

INTRODUCTION

Primary bone lymphoma (PBL) is a rare disease that was first described by Oberling in 1928 (1). Parker and Jackson (2) published their series on 'reticulum cell sarcoma of bone' in 1939 and established PBL as a distinct clinical entity.

The incidence of PBL is 7% of all malignant bone tumors, 4–5% of all extranodal non-Hodgkin lymphoma (NHL) and less than 1% of all malignant lymphomas (3–5). Previous reports showed its particular tendency to affect senior adults although PBL can occur at any age. There is also a male predominance and the femur has been reported to be the most commonly involved location as a single site.

Many PBL patients have had early clinical stage diseases (3, 6–14) and the most important prognostic factor has been the disease stage (6, 15). Histopathologically, the majority of PBL cases have been diffuse large B-cell lymphoma (DLBCL) according to the World Health Organization (WHO) classification (16).

There are several problems, however, that should be noted with respect to the previous PBL reports: (i) the number of patients in each study was small; (ii) the definitions of PBL and the response criteria for PBL were heterogeneous and/or ambiguous in some of the reports; (iii) in most PBL studies,

For reprints and all correspondence: Takashi Watanabe, Hematology and Stem Cell Transplantation Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo, 104-0045, Japan. E-mail: takawata@ncc.go.jp

only patients with early stage disease were regarded as those meeting the criteria of PBL; (iv) treatment modalities were also heterogeneous; and (v) most of the case series were reported from Western countries with only a few studies available from Asia including Japan (17).

In an effort to help clarify the various uncertain aspects as to the character and nature of PBL, we now report the results of detailed clinicopathological analyses conducted on 28 consecutive patients with PBL who received their initial treatments at our hospital during the past 10 years.

PATIENTS AND METHODS

Patients

We retrospectively analyzed 28 consecutive patients who were diagnosed with PBL and received their initial treatment at the National Cancer Center Hospital (NCCH) between April 1995 and September 2004. All pathological materials were obtained from surgical biopsies and the histopathological diagnoses were made according to the WHO classification. A number of clinical data were analyzed based on clinical records including age, gender, B symptoms, performance status according to the Eastern Cooperative Oncology Group (ECOG) scale, serum lactate dehydrogenase (LDH) level, clinical stage (CS), primary bone site, number of bone lesions, other involved sites with lymph-node lesions or extranodal lesions (except for the bone), maximal tumor diameter, treatment, response to initial treatment and histopathological subtype.

Definition of PBL

According to the WHO classification (3), lymphoma involving bone can be classified into four groups: Group 1, lymphoma with a single bone site with or without regional lymph-node involvement; Group 2, lymphoma with multiple bones involved, but no visceral or lymph-node involvement; Group 3, bone tumor with involvement of other visceral sites or lymph nodes at multiple sites; and Group 4, lymphoma involving any other sites and found by bone biopsy which was done to rule out possible involvement. We defined PBL in our study as consisting of WHO classification Groups 1 and 2 as well as Group 3 cases when the bone tumor was the largest lesion, which is supposed to be the initial site involved by lymphoma. This is because lymphomas with the same histology should have the same growth rate, and therefore we cannot expect lymphoma to spread more easily and rapidly inside the hard and compact bone than in other free space.

Clinical Staging

CS was determined according to the revised American Joint Committee on Cancer (AJCC) staging system for lymphoid neoplasms (18). All patients underwent chest X-ray; computed tomography (CT) scans of the neck, chest, abdomen and pelvis; magnetic resonance image (MRI) of bone lesions; histological examination of clot obtained by bone marrow aspiration or bone marrow biopsy; total body scintigraphy (Ga-67 scintigraphy [gallium scan], technetium-99 m bone scintigraphy or fluorine-18-2-fluoro-2-deoxy-D-glucose positron emission tomography [FDG-PET]); upper gastrointestinal tract endoscopy; blood smears; and physical examinations. All stage IIE disease was defined as lymph-node involvement adjacent to the bone lesion according to the AJCC staging system.

Response Criteria

For assessment of response, we used response criteria based on the International Workshop Response Criteria (IWRC) (19) combined with the MRI and total body scintigraphy findings. Complete response (CR) was defined as CR, CR-unconfirmed (CRu), partial response (PR) or stable disease (SD) in accordance with the IWRC without any abnormal accumulation as determined by either an FDG-PET or gallium scan. PR was defined as CR or CRu in accordance with the IWRC with either a positive FDG-PET or gallium scan or PR in accordance with the IWRC with or without evaluation by either an FDG-PET or gallium scan. SD was defined as SD in accordance with the IWRC with or without either a positive FDG-PET or gallium scan. Progressive disease (PD) was defined as PD in accordance with the IWRC with or without either an FDG-PET or gallium scan or the appearance of a new lesion by either an FDG-PET or gallium scan.

Statistical Analyses

For the full-set analysis ($n = 28$), overall survival (OS) was defined as the interval between the dates of diagnosis and death from any cause. Progression-free survival (PFS) was defined as the interval from the date of diagnosis to the date of disease progression, relapse or death from any cause. All survival curves were evaluated by the Kaplan–Meier analysis method. A log-rank test was used in a univariate analysis to identify factors affecting OS. A P value of 0.05 or less was considered to be indicative of a statistical significance. All statistical analyses were performed using SPSS version 11.0J (Dr. SPSS II for Windows).

RESULTS

Patient Characteristics

The demographic and clinical characteristics of the 28 patients at the time of diagnosis are summarized in Table 1. The median age was 47 years (range: 5–81) with 15 male and 13 female patients. Seven (25%) patients had B symptoms, 23 patients (82%) showed elevated serum LDH levels, 19 (68%) had CS IV disease and 17 (61%) were high

intermediate-risk or high-risk patients based on the International Prognostic Index (IPI) (20).

The primary involved sites of the bone are shown in Fig. 1. Notably, the pelvis was the most frequently involved site (15 patients; 54%) rather than the extremities (six patients; 21%). The femur, which had been the most common site involved as a single PBL site in most of the previous reports (6, 7, 9–11, 13–15), was involved in only two (7%) patients. A total of 12 (43%) patients presented with a solitary lesion, nine (32%) had two lesions and seven (25%) had multifocal bone lesions. Nine male (32%) and nine female (32%) patients were classified as WHO Group 1 or Group 2 while six male (21%) and four (14%) female patients were classified as Group 3. In our study, there was no male preponderance although this had been the case in many previous reports (6, 8–12, 15, 17) in every category.

Table 1. Patient demographic and clinical characteristics

	Median (range)
Age	47 years (5–81)
Radiation dose	40 Gy (30–50)
	n (%)
Gender M / F	15 (54) /13 (46)
B symptoms	7 (25)
ECOG performance status 2–4	13 (46)
Serum LDH level >1 × normal	23 (82)
Clinical stage I	4 (14)
II	5 (18)
III	0 (00)
IV	19 (68)
WHO Group 1 or 2	18 (64)
Group 3*	10 (36)
IPI: high-intermediate or high risk	17 (61)
Bone lesions ≥2 sites	16 (57)
Lymph node involvement	13 (46)
Extranodal lesions†	3 (11)
Bulky disease‡	7 (25)
Initial CTx: CHOP regimen	22 (79)
Initial treatment: CTx and RT	14 (50)
Response to initial treatment: CR or PR	25 (89)

M, male; F, female; LDH, lactate dehydrogenase; WHO, World Health Organization; IPI, international prognostic index; CHOP, cyclophosphamide, doxorubicin, vincristine and prednisolone; CTx, chemotherapy; RT, radiotherapy; CR, complete response; PR, partial response.
*If the bone lesion was the largest tumor in a patient with multiple lymphoma sites, the case was defined as primary bone lymphoma in this study.
†The bone was excluded from extranodal lesions.
‡Bulky disease was defined as lymphoma greater than 10 cm in the maximal diameter.

Clinical Staging

Four (14%) patients presented with stage I disease, five (18%) patients with stage IIE, no patients (0%) with stage III and 19 (68%) patients with stage IV (Table 1). Nine of 19 patients with stage IV disease had multifocal bone lesions without visceral or lymph-node involvement (WHO classification Group 2) and other patients were classified as Group 3 when the bone tumor was the largest lesion. Three of 10 patients with a solitary bone lesion had either distant lymph node (two patients) or an extranodal site other than the bone (one patient, stomach). Seven of 10 patients with multifocal bone lesions had either distant lymph node (five patients) or some extranodal site involvement (one patient, stomach; one patient, skin). None of the 19 CS IV patients had bone marrow involvement at their initial diagnosis.

Histopathological Subtypes

The results of the histopathological diagnoses of all the bone lymphomas are summarized in Table 2. DLBCL was the most common histopathological PBL subtype (19 of 28 patients; 68%) as previously reported, but two of these 19 DLBCL patients revealed an immunoblastic variant.

We also found a variety of histopathological subtypes other than DLBCL. Three patients (11%) had precursor B-lymphoblastic leukemia/lymphoma (B-ALL/LBL). Another patient (4%) had follicular lymphoma (FL), grade 1 without any transformed component in her bone biopsy sample. The bone biopsy sample that had been diagnosed as just 'low grade B-cell lymphoma' was considerably crushed so it was difficult to judge the precise histopathological subtype. An invasive and diffuse proliferation of a mixture of small-sized and medium-sized round cells was found in this sample and its immunohistochemical stainings showed that CD20, CD79a and Bcl-2 were positive and CD3, CD5, CD10, CD23, cyclin D1 and terminal deoxynucleotidyl transferase were negative. Two (7%) pediatric patients were diagnosed as anaplastic large cell lymphoma (ALCL). Both of them were positively stained with anaplastic lymphoma kinase with one positive and the other negative for CD56 stainings. One (4%) patient diagnosed as extranodal NK/T-cell lymphoma, nasal type (NTCL) did not have any nasal lesions, but had a bone lesion in the right tibia and regional lymph-node involvement while one (4%) other patient with lymphocyte-depleted Hodgkin lymphoma (LDHL) had multiple bone lesions accompanied by a skin lesion.

Initial Treatment

All 28 patients underwent chemotherapy with most of them (21; 75%) receiving the CHOP regimen (cyclophosphamide [CPA], doxorubicin [DOX], vincristine [VCR] and prednisolone [PSL]). Two adult patients with B-ALL/LBL received a treatment regimen (Lymphoma Study Group of Japan Clinical Oncology Group 16) for acute lymphoblastic

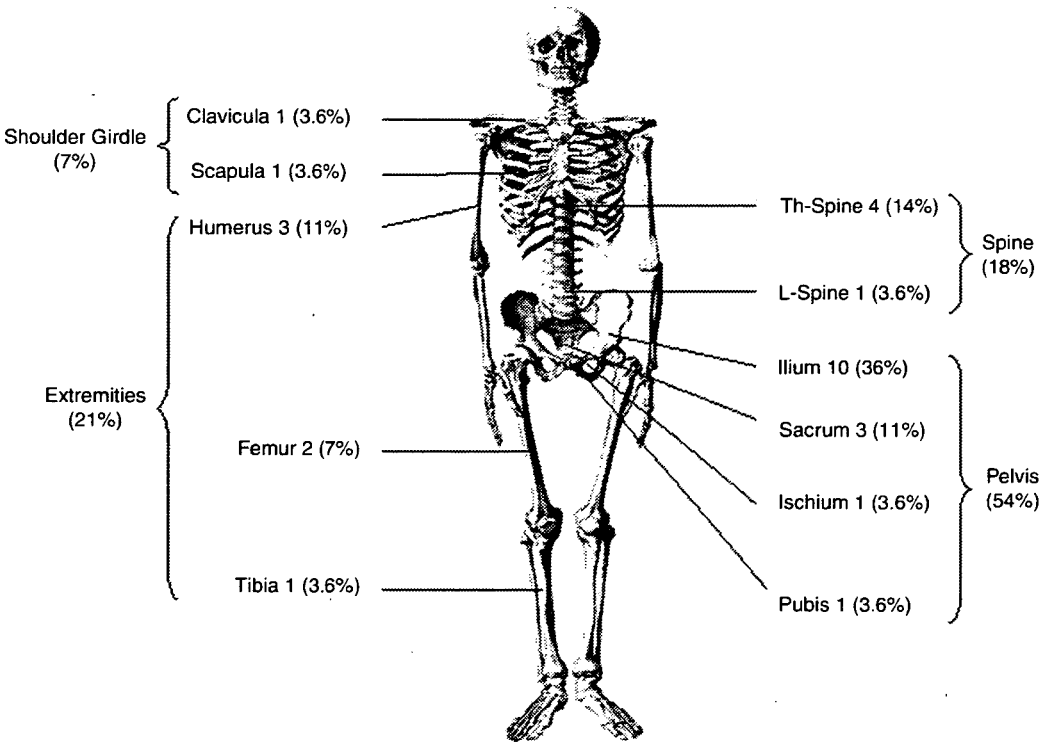

Figure 1. Primary involvement sites of primary bone lymphoma ($n = 28$). The most common primary bone site was the pelvis. Th, thoracic; L, lumbar.

leukemia. The patient with FL had been previously diagnosed as having DLBCL in her former hospital so she received a dose-intensified third-generation regimen (MACOP-B) consisting of methotrexate, DOX, CPA, VCR, PSL and bleomycin (BLM). The patient with LDHL received the ABVD regimen (DOX, BLM, vinblastine and dacarbazine) and three pediatric patients (one B-ALL/LBL and two ALCL cases) received a regimen used in pediatrics for short-term intensive treatment. Fourteen (50%) of the 28 patients received combined modality treatment of sequential chemotherapy and radiotherapy with a median dose of 40 gray (Gy) (range: 30–50). The majority (11 patients) of them received chemotherapy followed by radiotherapy with the other three initially receiving radiotherapy.

Table 2. Histopathologic subtypes

	n (%)
DLBCL	17 (61)
DLBCL, immunoblastic variant	2 (7)
B-ALL/LBL	3* (11)
FL, Grade 1	1 (4)
Low grade B-cell lymphoma ($CD5^-/CD10^-/CD20^+$ /Cyclin $D1^-/TdT^-/bcl\text{-}2^+$)	1 (4)
ALCL	2* (8)
NTCL	1 (4)
LDHL	1 (4)

DLBCL, diffuse large B-cell lymphoma; B-ALL/LBL, precursor B-lymphoblastic leukemia/lymphoma; FL, follicular lymphoma; CD, cluster of differentiation; TdT, terminal deoxy-nucleotidyl transferase; ALCL, anaplastic large cell lymphoma; NTCL, extranodal NK/T-cell lymphoma, nasal type; LDHL, lymphocyte-depleted Hodgkin lymphoma.
*One of B-ALL/LBL and two ALCL were pediatric patients.

Response to Treatment, Survival Rates and Statistical Analyses

All 28 patients could be evaluated for their response to initial treatment, OS and PFS. The median follow-up period was 31 months (range: 15–126). The overall response rate (ORR) for all the PBL patients was 89% as 19 patients (68%) achieved CR and six patients (21%) achieved PR. Three patients (11%) showed PD during initial treatment. For the patients classified as either WHO classification Group 1 or Group 2, the ORR was 88% (15 of 17 patients) and the ORR for the patients classified as Group 3 was 90% (10 of 11).

Twenty-five patients (89%) were alive at the time of the last follow-up and all remain in CR including 18 patients who achieved CR after initial treatment, four with PR following initial treatment and two patients who achieved CR after salvage chemotherapy but one patient who relapsed after achieving CR by salvage chemotherapy followed by radiotherapy. Six patients (21%) experienced recurrences of the lymph nodes (one patient), bone marrow (one patient), central nervous system (one patient) and bone (three patients). The three patients with bone relapses received chemotherapy followed by radiotherapy as the initial treatment and two of the recurrences occurred outside the

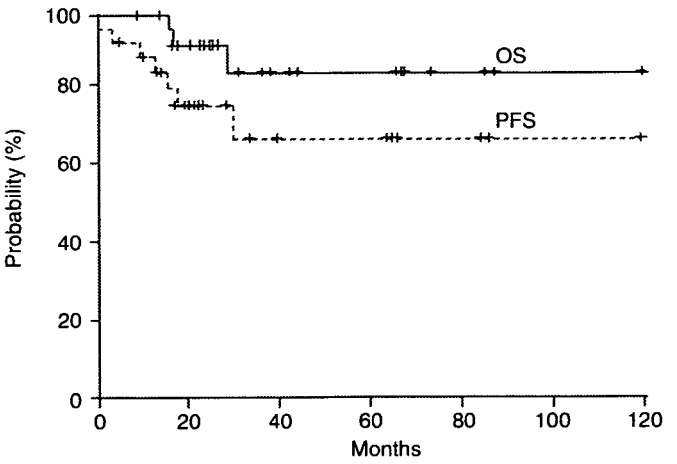

Figure 2. Overall survival (OS) and progression-free survival (PFS) for all patients with primary bone lymphoma.

radiation field. The bone relapse locus in the remaining patient was unclear because the patient was transferred to another hospital and detailed information was unavailable.

The median time to progression from the beginning of treatment was 21 months (range: 10–30). Histopathological subtypes for the six relapsed cases were DLBCL (three patients), the immunoblastic variant of DLBCL (two patients) and FL (one patient). Three patients (11%) showed PD during initial treatment and their histopathological subtypes were DLBCL, the immunoblastic variant of DLBCL and NTCL, respectively. The DLBCL patient with PD during first-line treatment achieved CR after salvage chemotherapy (CODOX-M/IVAC regimen (21) with rituximab) followed by radiotherapy. Subsequently, this same patient underwent high-dose chemotherapy and autologous stem cell transplantation (HDT/ASCT) but his lymphoma relapsed in the central nervous system four months later. The second PD patient with the immunoblastic variant of DLBCL relapsed 10 months after achieving PR from an ESHAP salvage regimen (22) followed by radiotherapy and died of lymphoma progression. The third patient with NTCL had PD despite receiving an ESHAP salvage regimen and eventually died of lymphoma. It is important to note that the two PD patients diagnosed with either the immunoblastic variant of DLBCL or the NTCL both died of lymphoma progression.

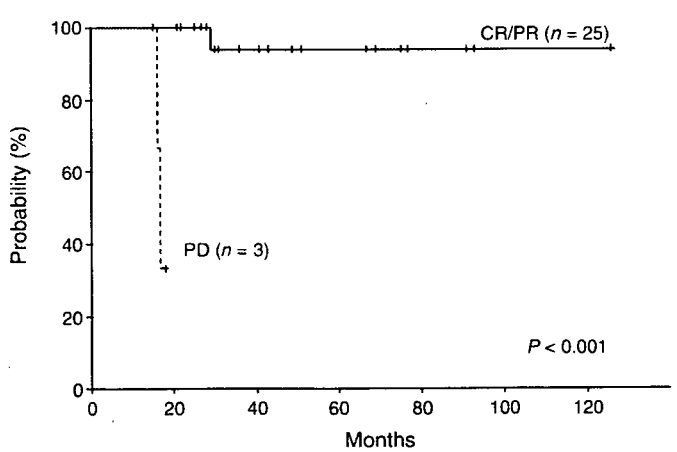

Figure 3. Overall survival (OS) curves of different groups of patients with primary bone lymphoma according to response to initial treatment. The 3-year rate of OS for patients with a complete response (CR) or partial response (PR) was 94%. PD, progressive disease.

The estimated 3-year OS and PFS rates were 84% and 77%, respectively (Fig. 2). The results of our univariate analysis of various factors affecting OS are shown in Table 3. Only two variables were found to be significant adverse factors related to OS: either the immunoblastic variant of DLBCL or the NTCL histopathological subtype and PD at initial treatment. OS classified according to patient response to their initial treatment is shown in Fig. 3. There was a significant difference between the two groups ($P < 0.01$). The estimated 3-year OS rate for patients in WHO classification Groups 1/2 and Group 3 was 84% and 91%, respectively.

DISCUSSION

The incidence of PBL is so rare that many aspects remain controversial, particularly the definition of PBL, appropriate treatment strategies, response criteria and prognostic factors.

There were a number of unique findings in our series compared with most of the previous reports. First, there was a divergence from DLBCL in histopathological subtypes. Second, the pelvis was the most commonly involved site compared to the majority of previous reports from Western countries identifying the femur as the most frequently involved site (Table 4). Horsman *et al.* reported that the most commonly presented site was the pelvis, although they described that the femur was the most frequently involved bone (13). Because another Japanese report also found that the pelvis was the most frequently involved site (17), this pelvic preponderance may be a characteristic peculiar to Japanese patients with PBL. Third, there was a predominance of advanced-stage disease despite defining PBL as including Group 3 of the WHO classification in any patient whose bone lesion was the largest, but neither the ORR to initial treatment nor the OS in this study was inferior to previously published reports. Fourth, there were no PBL patients with bone marrow involvement at the time of their initial diagnosis although stage IV disease was predominant in our study. Fifth, histopathological existence of either the immunoblastic variant of DLBCL or NTCL and response to initial treatment were both significant prognostic factors. Horsman *et al.* found that patients older than 60 years of age and other than complete response to initial treatment had a worse chance of survival (13), which is partially consistent with our results.

According to the WHO classification, lymphoma involving the bone can be classified into four groups. The WHO classification and some previous reports have

Table 3. Univariate analysis of variables adversely related to overall survival

Characteristics (*n*)	Three-year OS (%)	*P* value	Characteristics (*n*)	Three-year OS (%)	*P* value
Age		0.37	No. of bone lesions		0.64
<60 years (18)	86.0		1(12)	79.0	
>60 years (10)	80.0		≥2 (16)	93.0	
Gender		0.1	Lymph node involvement		0.54
male (15)	100.0		no (13)	79.0	
female (13)	73.0		yes (15)	92.0	
B symptoms		0.33	Extranodal lesions†		0.55
no (21)	80.5		no (25)	83.0	
yes (7)	100.0		yes (3)	100.0	
ECOG performance status		0.49	Bulky disease‡		0.95
0–1 (15)	86.0		no (18)	84.0	
2–4 (13)	83.0		yes (10)	90.0	
Serum LDH level		0.39	Initial treatment		0.11
≤1 × normal (5)	100.0		CTx and RT (14)	74.0	
>1 × normal (23)	80.0		CTx alone (14)	100.0	
Cinical stage		0.89	Histopathologic subtypes		<0.01
I or II (9)	80.0		others (25)	100.0	
IV (19)	87.0		immunoblastic variant or NTCL (3)	0.0	
WHO classification		0.74	Response to initial treatment		<0.01
Group 1 and 2 (18)	84.0		CR or PR (25)	94.0	
Group 3* (10)	91.0		PD (3)	0.0	
IPI		0.8			
low or low-intermediate risk (11)	83.0				
high-intermediate or high risk (17)	87.0				

OS, overall survival; ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; WHO, World Health Organization; IPI, International Prognostic Index; CTx, chemotherapy; RT, radiotherapy; NTCL, extranodal NK/T-cell lymphoma, nasal type; CR, complete response; PR, partial response; PD, progressive disease.
*If the bone lesion was the largest tumor in a patient with multiple lymphoma sites, the case was defined as PBL in this study.
†The bone was excluded from extranodal lesions.
‡Bulky disease was defined as a lymphoma greater than 10 cm in terms of the maximal diameter.

indicated that Groups 1 and 2 should be considered as PBL, but Group 3 should be excluded from PBL and considered to be systemic lymphoma regardless of the bone lesion size. An appropriate definition has not been established by verification, however, so the subject continues to be controversial. In clinical practice, we usually consider the largest lesion, which is supposed to be initially involved by lymphoma in cases with lymphomas in multiple lesions. In this study, therefore, we included Group 3 in our definition of PBL when the bone lesion was the largest as well as Groups 1 and 2 and found that there was no significant difference in the estimated 3-year OS rate between Groups 1/2 and Group 3 ($P = 0.74$).

Histopathologically, the previous studies reported that the majority of patients with PBL were DLBCL particularly the multilobated subtype (3, 6, 11, 23). In our series, there was a variety of diagnoses although the majority of histopathological subtypes were DLBCL (68%). It is important to note that PBL with certain specific histopathological subtypes had a poor prognosis despite the majority having a good prognosis. Heyning *et al.* found that patients older than 60 years of age and patients with the immunoblastic variant subtype had a worse chance of survival (6) which is partially consistent with our results. All the patients with either the immunoblastic variant of DLBCL or the NTCL died early of lymphoma progression in our study. Although ALCL as PBL is extremely rare, it has been reported that CD56-positive ALCL has had a tendency to involve the bone and its prognosis has been poor (24).

Several studies have suggested that a combination of chemotherapy and radiotherapy was the best treatment for patients with PBL (12, 25–27). Zinzani *et al.* conducted a retrospective analysis of 52 patients with stage I to stage IV PBL. The CR rates for patients treated by radiotherapy

Table 4. Previous reports of PBL

First author, Year of publication (reference number)	Country	No. of patients	Years of enrollment into study	M/F (No.)	Median age	Most common primary site (%)	DLBCL, %	CS I–II, %	Ratio of RT in treatment, %	ORR to initial treatment, %	OS, % (years)
Ostrowski, 1986 (15)	USA	422	1907–1982	260/162	46	Femur (21)	NA	42	86	NA	CS I 53 (10)
Ueda, 1989 (17)	Japan	34	1961–1988	24/10	56	Pelvis (29)	NA	44	NA	NA	CS I 75 (5) CS IV 50 (5)
Heyning, 1999 (6)	Netherlands	60	1943–1996	39/21	48	Femur (24)	92	62	66	56	61 (5)
Bayrakci, 2001 (7)	Turkey	20	1986–1997	8/12	48	Femur (24)	NA	70	65	65	CS I 78 CS IV 16
Stein, 2003 (8)	Israel	19	1979–2000	12/7	54	NA	95	58	58	89	NA
Zinzani, 2003 (9)	Italy	52	1982–1998	30/22	58	Femur (27)	85	79	85	90	68 (9)
Lewis, 2003 (10)	USA	28	1984–1994	19/9	45	Femur (39)	89	71	64	NA	58 (5)
Leval, 2003 (11)	USA	29	1990–2000	23/6	44	Femur (31)	100	62	61	NA	74 (5)
Barbieri, 2004 (12)	Itary	77	1983–2001	56/21	42	Extremities (51)	97	100	100	95	88
Horsman, 2006 (13)	UK	37	1970–2003	17/20	55	Pelvis (24)	73	100	78	57	64 (5)
Beal, 2006 (14)	USA	82	1963–2003	41/41	48	Femur (27)	80	81	70	NA	88 (5)
Present study	Japan	28	1995–2004	15/13	47	Pelvis (41)	68	32	50	88	84 (3)

No., number; M, male; F, female; DLBCL, diffuse large B-cell lymphoma; CS, clinical stage; RT, radiotherapy; ORR, overall response rate; OS, overall survival; NA, not available.

alone and chemotherapy with or without radiotherapy were 64% and 85%, respectively. The relapse rates between the two groups were 57% and 6%, respectively (9). These previous reports confirmed the superiority of chemotherapy to radiotherapy alone as the initial treatment for PBL patients. Beal *et al.* concluded that PBL patients treated with a combination of chemotherapy and radiotherapy were found to have a significantly better survival than the patients treated with single modality therapy (chemotherapy or radiotherapy alone), but the 5-year OS rate for patients treated with combined modality therapy versus chemotherapy alone was not significantly different (14). The addition of radiotherapy did not affect the survival rate in either the total of all PBL patients (Table 3) or those with early stage disease (data not shown) in our univariate analysis. Bacci *et al.* reported that four of six patients who underwent radiotherapy of less than 30 Gy had a relapse in their radiation fields and that a combination of chemotherapy and radiotherapy of more than 40 Gy was needed (27). Among the 28 patients in our study, three patients had relapses on the bone with each of them having received chemotherapy followed by radiotherapy. The patient transferred to another hospital received only 30 Gy while the others received more than 35 Gy as their initial treatment including two patients with bone relapses that occurred outside their radiation fields who then received additional radiotherapy. No bone marrow involvement at initial diagnosis might be beneficial to HDT/ASCT, however, PFS for most of PBL with DLBCL histology is quite good and only a few cases underwent HDT/ASCT. Additional studies will be necessary to help clarify the significance of this finding. Because the number of patients in this study was relatively small, further studies are needed to clarify the characteristics of PBL and its optimal treatment strategy.

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Conflict of interest statement

None declared.

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Phase I Trial of FLAGM with High Doses of Cytosine Arabinoside for Relapsed, Refractory Acute Myeloid Leukemia: Study of the Japan Adult Leukemia Study Group (JALSG)

Shuichi Miyawaki,[a] Yasukazu Kawai,[b] Akihiro Takeshita,[c] Norio Komatsu,[d] Noriko Usui,[e] Yukihiro Arai,[f] Fumihiro Ishida,[g] Takeshi Morii,[h] Yasuhiko Kano,[i] Michinori Ogura,[j] Noriko Doki,[a] Ryuzo Ohno[k]

[a]Leukemia Research Center, Saiseikai Maebashi Hospital, Gunma, Japan; [b]Divison of Hematology & Oncology University of Fukui Hospital, Fukui, Japan; [c]Department of Medicine III, Hamamatsu University School of Medicine, Shizuoka, Japan; [d]Division of Hematology, Department of Internal Medicine, Jichi Medical University, Tochigi, Japan; [e]Division of Oncology & Hematology, Department of Internal Medicine, The Jikei University School of Medicine, Tokyo, Japan; [f]Department of Hematology, Dokkyo Medical University School of Medicine, Tochigi, Japan; [g]2nd Department of Internal Medicine, Shinshu University School of Medicine, Nagano, Japan; [h]2nd Department of Internal Medicine, Nara Medical University, Nara, Japan; [i]Division of Hematology, Tochigi Cancer Center, Tochigi, Japan; [j]Department of Hematology and Cell Therapy, Aichi Cancer Center, Aichi, Japan; [k]Aichi Cancer Center, Aichi, Japan

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Abstract

This study was designed to determine the optimal high dose for cytosine arabinoside (ara-C) in combination with fludarabine, granulocyte colony-stimulating factor, and mitoxantrone (FLAGM) in adult patients with relapsed or refractory acute myeloid leukemia. Nine patients were enrolled at increasing dosage levels of ara-C (8, 12, and 16 g/m^2 per dose level). Ara-C and fludarabine were administered once a day at level 1, once or twice a day at level 2, and twice a day at level 3. All patients had grade 4 hematologic toxicity. The most common adverse events were of grade 2 or less, with nausea and vomiting being the most common (6 events), followed by diarrhea (5 events), and rash (5 events). Of the 13 grade 3 nonhematologic toxicities reported, the 2 most common were febrile neutropenia (6 events) and disseminated intravascular coagulation (3 events). No early deaths were observed. FLAGM with high-dose ara-C was considered safe for patients, and the recommended dosage of ara-C in this study was 2 g/m^2 every 12 hours for a total dose of 16 g/m^2.
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Key words: AML; Ara-C; FLAGM therapy; Cytarabine; High-dose ara-C; Phase I study

1. Introduction

Treatment for acute myeloid leukemia (AML) has improved over the years since the addition of cytosine arabinoside (ara-C) to anthracycline therapy, which has enabled 70% to 80% of patients to achieve complete remission (CR). Even patients treated with this combination show long-term survival rates of only approximately 30%, however, and relapses occur in many patients [1,2]. In patients who relapse or have refractory disease, salvage therapy is imperative for long-term survival [3]. One type of salvage therapy is high-dose ara-C. Rudnick and colleagues [4] reported that 1 to 7.5 g/m^2 of ara-C is effective in refractory AML patients. Miyawaki et al [5] conducted a phase II study in which 2 g/m^2 of ara-C administered every 12 hours for a total of 24 g/m^2 was shown to be effective in patients with relapsed and refractory AML.

Arabinosylcytosine 5′-triphosphate (ara-CTP) is a metabolite of ara-C; studies have shown that fludarabine, a purine nucleoside analogue, can augment ara-CTP

N.K. is currently at the Department of Hematology, University of Yamanashi, Yamanashi, Japan.

Correspondence and reprint requests: S. Miyawaki, Leukemia Research Center, Saiseikai Maebashi Hospital, 564-1, Kamisinden-cho, Maebashi, Gunma 371-0821, Japan; 81-27-252-6011; fax: 81-27-253-0390 (e-mail: miyawaki@mail.wind.ne.jp).

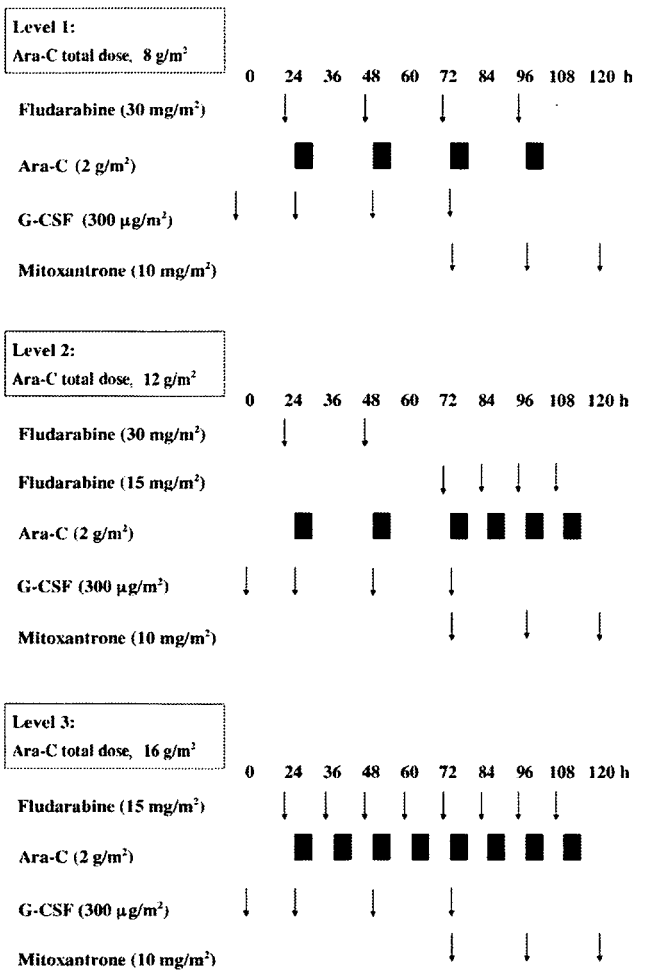

Figure 1. Dosing schedule for the 3 dosage levels. At all dosage levels, granulocyte colony-stimulating factor (G-CSF) was administered in 4 doses every 24 hours, beginning at the start of therapy, and mitoxantrone was administered in 3 doses every 24 hours, beginning 72 hours after the start of therapy. Ara-C (total dose per dosage level: 8, 12, and 16 g/m^2) was administered every 12 or 24 hours, beginning 24 hours after the start of therapy, and fludarabine (30 mg/m^2 once daily or 15 mg/m^2 twice daily) was administered 4 hours before each ara-C dose.

accumulation in leukemic cells [6-9]. This combination of fludarabine and ara-C was studied by Estey et al [10] in patients with newly diagnosed AML or myelodysplastic syndromes, and a CR rate of 53% was achieved. Other investigators also examined this combination therapy in patients with relapsed and refractory AML and achieved similar CR rates (28%-59%) [11-13]. The total-dose range of ara-C administered in these studies was 3 to 10 g/m^2. Because higher doses of ara-C have successfully been used to treat AML patients, the current phase I study was designed to determine the optimal high dose for ara-C in FLAGM, a combination with fludarabine, granulocyte colony-stimulating factor (G-CSF), and mitoxantrone, in patients with recurrent or refractory AML. In a high-dose ara-C regimen, ara-C is generally administered every 12 hours; therefore, fludarabine was administered twice a day. The optimal doses derived from the results of this study will be used in phase II studies.

2. Materials and Methods

The present study was conducted from October 2001 to June 2002 at 8 institutions belonging to the Refractory Leukemia Committee of the Japan Adult Leukemia Study Group (JALSG). Registration of the participants began after consent was obtained by the Ethics Committee or the Institutional Review Board of each institution.

2.1. Study Population

Patients who had recurrent AML (excluding M3 and hybrid leukemia) after a CR or who had failed 2 courses of standard induction therapy were enrolled in the study. M3 was excluded because this disease entity was treated with a specific regimen, and hybrid leukemia was excluded because it was not included in the AML category.

To be eligible, patients were required to meet the following criteria: having had an interval of ≥4 weeks before treatment; having a performance status of 0 to 2; being older than 18 years but younger than 65 years; having a life expectancy of ≥2 months and no major organ dysfunction (hemoglobin ≥9.0 g/dL; platelets ≥20,000 × 10^9/L; leukocytes ≥2000 × 10^9/L; total bilirubin ≤1.5 mg/dL; liver function tests ≤3 times the normal maximum value used by each institution; and serum creatinine ≤1.5 mg/dL); and having an arterial blood oxygen saturation ≥90%. All patients were required to provide written consent at the start of receiving the study medication.

2.2. Study Design and Treatment

As shown in Figure 1, 3 cohorts at 3 ara-C dosage levels (8 g/m^2, 12 g/m^2, and 16 g/m^2) were set in this study, and the administration of ara-C was started at 8 g/m^2 by the dose-escalation method. Each ara-C administration was given as a 3-hour infusion. G-CSF was subcutaneously administered at every dosage level at the start of treatment and was given every 24 hours for a total of 4 doses. Fludarabine (30 mg/m^2 once daily or 15 mg/m^2 twice daily) was administered as a 30-minute infusion 4 hours before the ara-C dose. The total fludarabine dose administered at each level was 120 mg/m^2. Lastly, 10 mg/m^2 mitoxantrone was administered as a 30-minute infusion for a total of 3 doses every 24 hours, beginning 72 hours after the start of therapy.

The sample size for each cohort was set at 3 patients. When the critical toxicity was observed in 1 of the 3 patients, 3 more patients were added to that cohort. When the critical toxicity was not seen in any of the 3 patients or was seen in 1 of the 6 patients, the dose was increased for the next cohort. Finally, when the critical toxicity was encountered in 2 of the 6 patients, the maximum tolerated dose was considered to have been reached in that cohort.

The treatment schedule for this study was derived from that of a phase II study in which 2 g/m^2 of ara-C was administered every 12 hours for a total of 12 doses

Table 1.
Demographic and Baseline Clinical Characteristics*

Dosage Level	Age, y	FAB Classification	Status	Karyotype (MRC Classification)	Duration of CR, mo	WBC, $\times 10^9$/L (blasts, %)	Nucleated Cell Count in BM, $\times 10^9$/L (blasts, %)
1	21	M4	Relapse 1	46,XY,del(12)(p?) (I)	11	9890 (47.0)	3.8 (40.4)
1	37	M2	Relapse 1	46,XY,t(6;9)(p23;q34),47,idem,+13 (I)	4	14,470 (50.0)	12.6 (78.8)
1	33	M2	Relapse 1	46,XY,del(1)(p?),add(3)(q21),add(5)(q22) 46,idem,add(7)(q32),add(9)(p13) 47,idem,+Y (P)	19	3800 (13.5)	19.7 (15.0)
2	57	M5b	Relapse 1	46,XY,t(2;3)(p23;q29) (I)	14	5770 (38.0)	7.5 (94.0)
2	60	M4	Relapse 2	46,XY (I)	18	20,630 (75.0)	7.6 (72.8)
2	29	M2	Relapse 1	46,XX,t(8;21)(q22;q22) (F)	24	2600 (34.0)	NA (30.0)
3	41	M5a	Relapse 1	46,X,add(Y)(p11),del(5)(p?),add(8)(q22) (I)	34	2100 (14.5)	8.9 (94.0)
3	51	M2	Relapse 1	46,XY (I)	16	5400 (0)	91.7 (10.4)
3	55	M4	Relapse 1	46,XY (I)	13	40,100 (80.0)	NA

*FAB indicates French-American-British; MRC, Medical Research Council; CR, complete remission; WBC, white blood cells; BM, bone marrow; I, intermediate; P, poor; F, favorable; NA, not available.

(total dose, 24 g/m^2) [5]. Treatment-related deaths occurred in 5 of 46 patients in that study. Therefore, fludarabine and mitoxantrone were given concurrently in the present study, and 16 g/m^2 was taken to be the maximum administrable dose of ara-C. If none of the 3 patients or 1 of the 6 patients showed critical toxicity at dose level 3, the trial was terminated without further increase in the dose.

2.3. Supportive Care

Inhaled amphotericin B, amphotericin B syrup, and nystatin were administered to neutropenic patients to prevent airway, oral, and esophageal fungal infections. Oral polymyxin B sulfate was administered to limit colonization in the gastrointestinal tract, and the prophylactic use of isoniazid was prescribed to patients who had a history of tuberculosis. Platelets were supplemented as needed to maintain a platelet count ≥20,000 × 10^9/L, and G-CSF was administered within the scope of the protocol guidelines.

2.4. Safety Evaluations and Study End Points

Safety was the primary study end point, and adverse events were graded according to the National Cancer Institute Common Toxicity Criteria. The critical toxicity was decided as follows: (1) grade 3 or higher nonhematologic toxicity (except for nausea and vomiting, loss of appetite, diarrhea, infection, or fever of grade 4); and (2) early death (defined as death occurring within 2 months after the start of treatment). The secondary end points included the type, degree, and frequency of adverse events of grade 1 or 2, and the efficacy of treatment. For the assessment of efficacy, the JALSG criteria were followed [2]. A CR was established when observations of fewer than 5% blasts in normocellular marrow were accompanied by a normal level of peripheral blood neutrophils (>1200 × 10^9/L) and a normal platelet count (>100,000 × 10^9/L). The definition of partial remission was established when a decrease of at least 50% in the percentage of blasts, to between 5% and 25%, was observed in the bone marrow aspirate.

Table 2.
Summary of Adverse Events*

	Dosage Level 1 (n = 3)					Dosage Level 2 (n = 3)					Dosage Level 3 (n = 3)				
	Grade, n					Grade, n					Grade, n				
	1	2	3	4	Total, n (%)	1	2	3	4	Total, n (%)	1	2	3	4	Total, n (%)
Diarrhea	2	0	0	0	2 (67)	0	1	0	0	1 (33)	2	0	0	0	2 (67)
DIC	0	0	1	0	1 (33)	0	0	0	0	0 (0)	0	0	2	0	2 (67)
Fever (allergy)	0	0	0	0	0 (0)	2	0	0	0	2 (67)	0	1	0	0	1 (33)
Hyperglycemia	0	0	0	0	0 (0)	0	0	0	0	0 (0)	0	0	1	0	1 (33)
Nausea/vomiting	3	0	0	0	3 (100)	0	2	0	0	2 (67)	0	1	0	0	1 (33)
Febrile neutropenia	0	0	2	0	2 (67)	0	0	1	0	1 (33)	0	0	3	0	3 (100)
Rash	1	0	0	0	1 (33)	0	2	0	0	2 (67)	1	1	0	0	2 (67)
Sepsis	0	0	1	0	1 (33)	0	0	0	0	0 (0)	0	0	0	0	0 (0)
SGOT elevation	0	0	0	0	0 (0)	0	0	1	0	1 (33)	1	0	0	0	1 (33)
SGPT elevation	1	0	0	0	1 (33)	0	0	1	0	1 (33)	2	0	0	0	2 (67)
Stomatitis	0	0	0	0	0 (0)	1	0	0	0	1 (33)	0	1	0	0	1 (33)

*DIC indicates disseminated intravascular coagulation; SGOT, serum glutamic-oxaloacetic transaminase; SGPT, serum glutamic-pyruvic transaminase.

Table 3.
Overall Outcome*

Dosage Level	Early Death, n	Response			WBC Nadir, $\times 10^9$/L	Duration of WBC $<1000 \times 10^9$/L, d	Duration of Plt $>10 \times 10^{13}$/L, d	Death, n (%)
		CR, n	PR, n	Overall, n (%)				
1 (n = 3)	0	1	0	1 (33)	70, 100, 270	15, 36, 43	—, 75, —	3 (100)
2 (n = 3)	0	3	0	3 (100)	50, 150, 160	14, 18, 22	18, 22, 35	1 (33)
3 (n = 3)	0	1	2	3 (100)	100, 100, 110	15, 18, 30	22, 33, 38	0 (0)
Total	0	5	2	7 (78)	Median = 100	Median = 18	Median = 33	4 (44)

*CR indicates complete remission; PR, partial remission; WBC, white blood cells; Plt, platelets.

3. Results

3.1. Demographic and Baseline Characteristics

Nine AML patients were enrolled, and all were eligible for this study. Their demographics and baseline characteristics are shown in Table 1. The median age was 41 years (range, 21-60 years). Eight of the 9 patients were in their first relapse, and 1 patient had a karyotype aberration involving core-binding factor [14].

3.2. Safety

No early deaths occurred within 2 months after the start of treatment. Grade 4 leukopenia ($<1000 \times 10^9$/L) was seen in all patients. The median leukocyte count was 100×10^9/L (range, 50-270 $\times 10^9$/L), and the median period for which the count was $<1000 \times 10^9$/L was 18 days (range, 14-43 days). The leukocyte count and the period over which that count was $<1000 \times 10^9$/L were not related to the ara-C dose.

The most commonly reported adverse events were of grade 2 or less, with nausea and vomiting being the most common (6 events), followed by diarrhea (5 events) and rash (5 events) (Table 2). Of the 13 grade 3 nonhematologic toxicities reported, the most common were febrile neutropenia (6 events) and disseminated intravascular coagulation (DIC) (3 events). Of the 3 DIC events, 2 were considered to be related to AML, and 1 was related to cytomegalovirus infection. In addition, 1 case of grade 3 sepsis was seen. Because DIC is a type of hematologic toxicity and because sepsis is caused by an infection, these adverse events were judged not to fall under the heading of critical toxicities as defined in the present study. Grade 3 hyperglycemia was detected after the administration of steroid drugs, and grade 3 increases in serum glutamic-oxaloacetic transaminase and serum glutamic-pyruvic transaminase concentrations were seen after the administration of antibiotics. One patient developed hepatotoxicity 1 month following chemotherapy. This patient had experienced hepatotoxicity and skin eruptions caused by the same antibiotics during previous chemotherapy. Accordingly, these events were considered to have had no causal relationship to the FLAGM therapy. Therefore, no critical toxicity attributable to this study was seen in any cohort.

3.3. Response to Dosing Regimens

The overall response rate in the study was 78% (7 cases), and the overall responses are summarized in Table 3. One patient (33%) achieved CR at dose level 1, 3 patients (100%) achieved CR at dose level 2, and 1 patient (33%) achieved a CR at dose level 3. In addition, 2 patients at dose level 3 had a partial response, and 2 patients at dose level 1 showed resistant disease. During the follow-up period, 2 patients who received doses at level 1 died from progressive disease, and 2 patients (1 each from dose levels 1 and 2) died from complications arising from a transplant received after having achieved CR.

4. Discussion

With the goal of improving response rates and long-term survival in patients with AML, treatments with new drugs such as mitoxantrone [11-13] and idarubicin [15-17] have recently been added to FLAG therapy (fludarabine, ara-C, and G-CSF). Because idarubicin is used in Japan as induction therapy for AML, this study developed the FLAGM regimen to determine the optimal dose for high-dose ara-C.

The fludarabine dosage in this study was 30 mg/m^2 administered once a day or 15 mg/m^2 twice daily. This dosing regimen was based on the results of Gandhi et al [18], who determined that both regimens would maximize ara-CTP accumulation in AML blasts. Studies have shown that numerous central nervous system adverse events can occur at ara-C dosages of 3 g/m^2 administered twice daily for 6 days (total dose, 36 g/m^2) [19]. In our previous phase II study of ara-C in which 2 g/m^2 was administered twice daily for 6 days (total dose, 24 g/m^2), we observed 5 deaths that were attributable to the treatment among a total of 46 cases [5]. In the present study, when we considered that fludarabine and mitoxantrone were to be administered concurrently with ara-C, we strictly fixed the maximum dose at 16 g/m^2.

As expected, the main adverse events were hematologic toxicities and febrile neutropenia, but both were manageable with supportive care. Neither reduction of the leukocyte count nor prolongation of the period of leukopenia due to higher ara-C doses was observed. In a study with a dosing regimen similar to that in our study, Hanel et al [13] demonstrated that the median period during which the leukocyte count was $<500 \times 10^9$/L was 21 days (range, 4-51 days), which was similar to the present results (ie, $\leq 1000 \times 10^9$/L

leukocytes for 18 days; range, 14-43 days). In another study of high-dose ara-C in patients with relapsed or refractory AML, the median period during which the leukocyte count was $<1000 \times 10^9$/L was 19 days [5]; this period was also similar to our results. The nonhematologic toxicities were manageable, and no central nervous system toxicity was observed. Koller et al [12] conducted a study in which fludarabine, ara-C, and comparable doses of mitoxantrone were administered concurrently; hyperbilirubinemia was reported in approximately 60% of the patients. In the present study, although 1 case of grade 3 liver failure was reported, no adverse events of a high bilirubin concentration were observed. Clavio et al [11] treated poor-risk AML patients with the same drug combination and the same mitoxantrone doses that we used in our protocol, and they found no patient with hyperbilirubinemia, as was the case in our study. The difference between the protocol of Koller et al and those used in the study of Clavio et al and our study is the dosage of mitoxantrone administered. Given this difference, a low mitoxantrone dose may be closely correlated with an absence of patients with hyperbilirubinemia.

Although the CR rate achieved in this study was 56%, the number of patients was small. In another study of high-dose ara-C therapy, however, the reported CR rate was 45.7%, and the remission rate was 51.4%. Therefore, it is possible that the FLAGM therapy regimen used in this study is more efficacious than the regimen of high-dose ara-C therapy. We conducted a phase I study for the purpose of selecting doses of high-dose ara-C for FLAGM therapy in patients with relapsed or refractory AML. The results of the study showed a high degree of effectiveness at dose levels 2 and 3, and we observed no treatment-related mortality at any dosage level. Therefore, the treatment was considered well tolerated. At the ara-C dose that we presumed to be the maximum administrable dose, 16 g/m^2, we observed no critical toxicity attributable to the study, and we therefore concluded that the recommended dosage for ara-C for phase II clinical trials should be 2 g/m^2 administered twice daily for 4 days, for a total dose of 16 g/m^2.

This regimen is currently being evaluated in phase II studies. However, the safety of this regimen should be continually evaluated in the phase II study because of the relatively small number of patients included in the phase I study.

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Molecular Characterization of 8-Methoxyfluoroquinolone Resistance in a Clinical Isolate of Methicillin-Resistant *Staphylococcus aureus*

Toshinobu Horii[a, b] Yasuhiro Suzuki[c] Akihiro Takeshita[a] Masato Maekawa[a]

[a]Department of Laboratory Medicine and [b]Division of Infectious Diseases and Control, Hamamatsu University School of Medicine, Hamamatsu, and [c]National Hospital Organization Nagoya Medical Center, Nagoya, Japan

Key Words
Methicillin-resistant *Staphylococcus aureus* · Fluoroquinolone · 8-Methoxyfluoroquinolone · Antibiotic resistance · Septicemia

Abstract
Background: Activity of gatifloxacin against clinical isolates of fluoroquinolone-resistant *Staphylococcus aureus* is more potent than that of other fluoroquinolones such as norfloxacin and levofloxacin. To date, few reports have described high-level resistance to gatifloxacin in clinical isolates of *S. aureus,* although in vitro studies have shown that mutations in both DNA gyrase and topoisomerase IV were required for gatifloxacin resistance in *S. aureus.* ***Methods:*** Minimum inhibitory concentrations were determined for fluoroquinolones and other antimicrobials in a methicillin-resistant *S. aureus* isolate that was cultured from blood of a patient with septicemia. Fluoroquinolone resistance was characterized by DNA sequencing and microbiologic assay. ***Results:*** The isolate showed high-level resistance to fluoroquinolones including an 8-methoxyfluoroquinolone, gatifloxacin (minimum inhibitory concentration 64 μg/ml). Amino acid mutations of Ser80Tyr and Glu84Lys in GrlA and Ser84Leu and Ser85Pro in GyrA were possibly related to this resistance in methicillin-resistant *S. aureus* HU2000-062, although efflux may play a minor role in resistance as well. ***Conclusion:*** GyrA and GrlA mutations mainly conferred to 8-methoxyfluoroquinolone resistance in this isolate.

Introduction

Prevalence of fluoroquinolone resistance in *Staphylococcus aureus,* including methicillin-resistant *S. aureus* (MRSA), has increased worldwide [1–7]. Fluoroquinolone resistance in *S. aureus* is associated with mutations within the *gyrA* and *gyrB* genes, which encode subunits of DNA gyrase, mutations in the *grlA* and *grlB* genes, which encode subunits of DNA topoisomerase IV, increased expression of the *norA* gene, which encodes a drug efflux protein, NorA and mutations in the *norA* coding region [1–3, 8–19].

Mutations associated with increased resistance to fluoroquinolones have been documented in specific regions of the *gyrA, gyrB, grlA* and *grlB* genes, which are referred to as the quinolone resistance-determining regions

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Dr. Toshinobu Horii, MD, PhD
Department of Laboratory Medicine, Hamamatsu University School of Medicine
1-20-1 Handa-yama
Hamamatsu 431-3192 (Japan)
Tel. +81 53 435 2788, Fax +81 53 435 2096, E-Mail horiihm@hama-med.ac.jp

(QRDRs) [1–3, 10–19]. Recently, Takei et al. [20] demonstrated preferential targeting of DNA gyrase or DNA topoisomerase IV among individual fluoroquinolones, with norfloxacin, ofloxacin, ciprofloxacin and levofloxacin preferring topoisomerase IV, sparfloxacin preferring DNA gyrase, and gatifloxacin, moxifloxacin and pazufloxacin targeting both enzymes.

NorA is a membrane protein that actively transports norfloxacin and other hydrophilic fluoroquinolones out of the bacterial cell, thus effectively decreasing the intracellular concentration of the drugs [8, 9]. Previous studies have shown that a point mutation in the *norA* gene and also *norA* overexpression caused by mutations in the promoter region can lead to increases in minimum inhibitory concentrations (MICs) [8, 9, 12].

The newly developed 8-methoxyfluoroquinolones include gatifloxacin and moxifloxacin. Gatifloxacin has increased potency and bactericidal activity that can be attributed in part to the 8-methoxy substituent [16]. Activity of gatifloxacin against clinical isolates of fluoroquinolone-resistant *S. aureus* is more potent than that of other fluoroquinolones such as norfloxacin and levofloxacin [2, 21]. To date, few reports have described high-level resistance to gatifloxacin in clinical isolates of *S. aureus*, although in vitro studies have shown that mutations in both DNA gyrase and topoisomerase IV were required for gatifloxacin resistance in *S. aureus* [16].

In the present study, we characterized high-level resistance to fluoroquinolones including the 8-methoxyfluoroquinolone gatifloxacin in a clinical MRSA isolate.

Patients and Methods

Patient

A 69-year-old man was admitted to the oral surgery ward of our hospital to undergo chemotherapy for cancer of the tongue. After completion of chemotherapy, an abdominal aortic aneurysm had suddenly ruptured. Replacement of a stent and bypass grafting were performed. A central venous catheter was placed, and treatment with intravenous piperacillin was initiated. On the second postoperative day, acute respiratory distress syndrome developed. Antibiotic therapy was changed to intravenous imipenem/cilastatin sodium. On postoperative day 13, aerobic blood culture (BactecPlus, Nippon Becton Dickinson, Tokyo, Japan) yielded MRSA (isolate HU2000-062). Then, antimicrobial therapy was changed to intravenous vancomycin 0.5 g. The patient died of septic shock and multiple organ failure on postoperative day 15.

Antimicrobials

The antimicrobials used were cloxacillin, biapenem, arbekacin and prulifloxacin (Meiji Seika Kaisya, Tokyo, Japan), cefdinir, teicoplanin, quinupristin/dalfopristin and telithromycin (Astellas Pharma, Tokyo, Japan), norfloxacin, fleroxacin and gatifloxacin (Kyorin Pharmaceutical, Tokyo, Japan), ofloxacin, levofloxacin and sitafloxacin (Daiichi Pharmaceutical, Tokyo, Japan), ciprofloxacin (Bayer Yakuhin, Osaka, Japan), sparfloxacin and meropenem (Dainippon Sumitomo Pharma, Osaka, Japan), pazufloxacin (Taishotoyama Pharmaceutical, Tokyo, Japan), cephaloridine, cefpirome and vancomycin (Shionogi Pharmaceutical, Osaka, Japan), cefepime (Bristol-Myers Squibb, Tokyo, Japan), sulbactam/cefoperazone and linezolid (Pfizer Japan, Tokyo, Japan), ceftazidime and mupirocin (GlaxoSmithKline, Tokyo, Japan), ampicillin and imipenem (Banyu Pharmaceutical, Tokyo, Japan) and panipenem (Sankyo, Tokyo, Japan).

Susceptibility Testing

The MICs were determined by an agar dilution method as described by the Clinical and Laboratory Standards Institute (formerly, National Committee for Clinical Laboratory Standards) (CLSI/NCCLS) [22]. Susceptibility testing was performed on Mueller-Hinton agar (Nippon Becton Dickinson) in accordance with the manufacturer's instructions. MIC interpretative criteria for ofloxacin, norfloxacin, ciprofloxacin, levofloxacin, fleroxacin, sparfloxacin, gatifloxacin, ampicillin, ceftazidime, cefepime, cefdinir, imipenem, meropenem, vancomycin, teicoplanin, linezolid, quinupristin/dalfopristin and telithromycin were referred to the CLSI/NCCLS [22]. The MIC breakpoints of other antimicrobials were not defined.

Amplification and DNA Sequencing of QRDRs and the norA *Gene*

Chromosomal DNA was extracted from the clinical MRSA isolate and *S. aureus* RN4220 as previously described [2, 23]. *S. aureus* RN4220 was used as a reference strain. Primer sets used for amplification of the QRDRs of the *grlA*, *grlB*, *gyrA* and *gyrB* genes, and the *norA* gene, have been reported previously [2]. Amplifications were carried out with KOD dash enzyme (Toyobo, Osaka, Japan) according to the manufacturer's recommendations. The QRDRs and the *norA* promoter and coding regions were sequenced using methods described previously [2, 24].

Determination of Fluoroquinolone Concentration Accumulated in S. aureus

The effect of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was determined by the method of Hirai et al. [25], with modifications as previously described [2]. In brief, bacteria were grown to mid-exponential phase in antibiotic medium 3 broth (Nippon Becton Dickinson) and resuspended in fresh 50 mM sodium phosphate buffer (pH 7.0) at a concentration equivalent to an A_{660} of 20. CCCP was added to a final concentration of 100 mM before the addition of levofloxacin, gatifloxacin or sitafloxacin to a final concentration of 16 μg/ml. After 0, 5, 15 and 30 min of incubation, the cells harvested by centrifugation were resuspended in phosphate-buffered saline and boiled. The concentration of fluoroquinolone in the supernatant was determined by a microbiologic assay with the indicator strains *Escherichia coli* XL1-Blue for levofloxacin and gatifloxacin and *E. coli* NIHJ JC2 for sitafloxacin [2]. *S. aureus* RN4220 was used as a fluoroquinolone-susceptible reference strain.

	-177	-167	-157	-147	-137	-127
HU2000-062	GTAGAAATGG	TAAAAACATT	GTATAGCATT	TTACACAGGA	GTCTGGACTT	GACTAATGTA
RN4220	TA*TT*TACA	ATT***TGGA	AA****TGA*	AATT***AAG	AAAAAATA**	*T*A******
SA-1199B	TA*TT*TACA	ATT***TGGA	AA****TGA*	AATT***AAG	AAAAAATA**	*T*A******
TK2566	TA*TT*TACA	ATT***TGGA	AA****TGA*	AATT***AAG	AAAAAATA**	*T*A******

Fig. 1. Nucleotide sequence comparison in the region from -124 to -177 bp upstream of the *norA* initiation codon between MRSA HU2000-062, *S. aureus* RN4220, SA-1199B [8] and TK2566 [9].

Results

Antimicrobial Susceptibility

The MICs for *S. aureus* HU2000-062 were as follows: >128 μg/ml for ofloxacin, >128 μg/ml for norfloxacin, >128 μg/ml for ciprofloxacin, >128 μg/ml for levofloxacin, >128 μg/ml for fleroxacin, 64 μg/ml for sparfloxacin, >128 μg/ml for pazufloxacin, >128 μg/ml for prulifloxacin, 64 μg/ml for gatifloxacin, 8 μg/ml for sitafloxacin, >128 μg/ml for cloxacillin, 64 μg/ml for ampicillin, 16 μg/ml for cephaloridine, >128 μg/ml for ceftazidime, 128 μg/ml for sulbactam/cefoperazone, 64 μg/ml for cefpirome, >128 μg/ml for cefepime, >128 μg/ml for cefdinir, 64 μg/ml for imipenem, 32 μg/ml for panipenem, 32 μg/ml for meropenem, 64 μg/ml for biapenem, 0.5 μg/ml for vancomycin, 1 μg/ml for teicoplanin, 1 μg/ml for arbekacin, 2 μg/ml for linezolid, 0.25 μg/ml for quinupristin/dalfopristin, 0.25 μg/ml for mupirocin and 0.25 μg/ml for telithromycin. MRSA HU2000-062 showed resistance to fluoroquinolones and β-lactams and was susceptible to vancomycin, teicoplanin, linezolid, quinupristin/dalfopristin and telithromycin. MICs of fluoroquinolones examined in this study were high (≥64 μg/ml) except for that of sitafloxacin (8 μg/ml).

Genetic Analysis of the grlA, grlB, gyrA *and* gyrB *QRDRs*

We determined the QRDR nucleotide sequences of the *grlA, grlB, gyrA* and *gyrB* genes. The mutations identified in the QRDRs included Ser80Tyr and Glu84Lys in GrlA and Ser84Leu and Ser85Pro in GyrA. No mutation affecting the amino acid sequence was found in the QRDRs of GrlB and GyrB.

Genetic Analysis of the norA *Promoter and Encoding Region*

We compared the nucleotide sequences of the *norA* promoter and coding regions of MRSA HU2000-062 and those from *S. aureus* RN4220, SA-1199B and TK2566 as reference sequences [2, 8, 9]. Mutations were found in the region from -124 to -177 bp upstream of the *norA* initiation codon (fig. 1). No difference in nucleotide sequences in the -35 and -10 *norA* promoter regions or the coding region was found between MRSA NU2000-062 and *S. aureus* SA-1199B or TK2566. In the coding region, an amino acid mutation, Gly291Asp, was found in *S. aureus* RN4220 [2].

Effects of CCCP on Fluoroquinolone Concentration Accumulated in S. aureus

Amounts of active levofloxacin, gatifloxacin or sitafloxacin in the presence and absence of CCCP are compared in table 1 as effect of CCCP. After a 30-min incubation with fluoroquinolones, MRSA HU2000-062 accumulated 4.6, 1.8 and 1.1 times more levofloxacin, gatifloxacin and sitafloxacin, respectively, than did RN4220. The accumulation of gatifloxacin was the least among these fluoroquinolones after a 30-min incubation in MRSA HU2000-062.

Discussion

In the present study, we characterized fluoroquinolone resistance in MRSA HU2000-062, which was isolated from the blood of a patient with ruptured abdominal aortic aneurysm and subsequent septicemia. The isolate was highly resistant to fluoroquinolones including an 8-methoxyfluoroquinolone (gatifloxacin, MIC 64 μg/ml). Amino acid mutations of Ser80Tyr and Glu84Lys in GrlA and Ser84Leu and Ser85Pro in GyrA were possibly related to this resistance in MRSA HU2000-062, although efflux may play a minor role in resistance. The isolate showed susceptibility to vancomycin, teicoplanin, linezolid, quinupristin/dalfopristin and telithromycin.

Table 1. Effects of 100 mM CCCP on fluoroquinolone concentration accumulated in MRSA HU2000-062 and *S. aureus* RN4220

Strain	Fluoroquinolone	Difference of active fluoroquinolone concentration in the presence and absence of CCCP[a] ng/10^9 CFU		
		5 min[b]	15 min[b]	30 min[b]
MRSA HU2000-062	levofloxacin	12.9 ± 0.7	36.3 ± 8.1	230.0 ± 17.3
	gatifloxacin	13.1 ± 2.7	36.0 ± 14.1	142.7 ± 5.8
	sitafloxacin	165.3 ± 36.3	267.7 ± 60.7	404.7 ± 35.2
S. aureus RN4220	levofloxacin	21.7 ± 11.5	58.3 ± 5.8	50.0 ± 30.0
	gatifloxacin	52.7 ± 1.5	50.7 ± 8.1	77.5 ± 26.2
	sitafloxacin	110.0 ± 22.9	125.0 ± 17.3	383.3 ± 164.4

[a] The concentration of active fluoroquinolone was determined in the supernatant of lysed cells after thorough washing. The experiments were repeated three times to present as mean ± standard deviation.
[b] Incubation time with fluoroquinolone.

Amino acid mutations in the QRDRs of topoisomerase IV and DNA gyrase are the main mechanisms involved in fluoroquinolone resistance of *S. aureus* [1–3, 10–19]. Amino acid mutations in the QRDRs responsible for fluoroquinolone resistance are usually restricted to limited positions in clinical isolates [2, 3, 10, 11, 13–15, 17, 18]. Mutations in GrlA conferring fluoroquinolone resistance include Ser80, Glu84 and Asp116, with mutations at positions of 80 and 84 reported most frequently [2, 3]. In GyrA, mutations of Ser84 and Glu88 are frequently encountered [2, 3]. The *grlA* mutations were associated with both high- and low-level resistance to ciprofloxacin in clinical isolates of *S. aureus,* while the *gyrA* mutations were responsible for an increase in resistance in the *grlA* mutant [11]. Previous in vitro studies have shown that multiple mutations in the QRDRs of both *grlA* and *gyrA* genes appeared to confer high-level resistance to fluoroquinolones, including an 8-methoxyfluoroquinolone [11, 12, 26–33]. However, few previous reports have characterized clinical *S. aureus* isolates highly resistant to 8-methoxyfluoroquinolones, including gatifloxacin [18]. We found that a clinical isolate of MRSA showing high-level resistance to gatifloxacin (MIC 64 μg/ml) had double mutations in GyrA, in addition to double mutations in GrlA. The role of the additional mutation of Ser85Pro in GyrA in fluoroquinolone resistance remains unclear. Takei et al. [20] previously showed that antibacterial activity of an 8-methoxyfluoroquinolone was influenced equally by inhibition of topoisomerase IV and DNA gyrase. Accordingly, double mutations involving both GyrA and GrlA most likely contributed to high-level resistance to gatifloxacin in HU2000-062. To determine the contribution of these mechanisms to high-level resistance, it should be confirmed that transformation of plasmids carrying the wild-type *gyrA* or *grlA* gene into these isolates results in low MICs of fluoroquinolones [34]. In the present study, it was not determined whether full complementation is obtained with the wild-type *gyrA* or *grlA* gene in the clinical isolate.

A point mutation in the *norA* gene, and also *norA* overexpression caused by mutations in the promoter region, can increase the MICs of fluoroquinolones [8, 9, 14]. Recently, it was also shown that overexpression of the NorA efflux pump has minimal effect on the MIC of gatifloxacin [16]. In MRSA HU2000-062, we found nucleotide sequences in the upstream region of the initiation codon that differed from those of *S. aureus* RN4220 and those previously described by Kaatz et al. [9] and Yoshida et al. [8]. To know the effect of the mutations in the upstream region of the *norA* gene, further investigations such as quantitative reverse transcriptase-polymerase chain reaction for the *norA* gene are required.

CCCP-related changes in fluoroquinolone accumulation may be caused by energy-dependent efflux, including NorA. In the present study, MRSA HU2000-062 accumulated 1.8 times more active gatifloxacin than *S. aureus* RN4220 after a 30-min incubation. These results suggested that the increased MICs of gatifloxacin were mainly caused by mutations in the QRDRs, although efflux may play some role.

Sitafloxacin, which is one of the newly developed fluoroquinolones, showed good susceptibility for the high-level resistant isolate to gatifloxacin, MRSA HU2000-062 (MIC 8 μg/ml). Although the MIC breakpoint of si-

tafloxacin has not been defined by the CLSI/NCCLS, some resistant bacteria to older fluoroquinolones with alterations in the QRDRs may remain susceptible to a newer fluoroquinolone with increasing potency [19].

Investigations in vitro have shown that some mutants selected by fluoroquinolone treatment acquired resistance via mutations in GrlA, GrlB, GyrA, GyrB and/or the *norA* promoter and coding regions [12, 17, 26, 27, 35]. In *S. aureus,* fluoroquinolone resistance that emerged only shortly after these drugs were introduced has become recognized as a clinical problem [1, 36]. Ince and Hooper [16] suggested that MICs of gatifloxacin for *grlA gyrA* double mutants are the approximate peak serum drug concentrations and that selection of mutants would be more probable in infections with such strains. In the present case, risk factors such as fluoroquinolone use for selection of fluoroquinolone-resistant isolates with multiple mutations remain unclear, since fluoroquinolones had not been given to the patients before isolation of MRSA HU2000-062.

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ORIGINAL ARTICLE

Pharmacokinetics of arsenic species in Japanese patients with relapsed or refractory acute promyelocytic leukemia treated with arsenic trioxide

Shinya Fujisawa · Ryuzo Ohno · Kazuyuki Shigeno · Naohi Sahara · Satoki Nakamura · Kensuke Naito · Miki Kobayashi · Kaori Shinjo · Akihiro Takeshita · Yoshinari Suzuki · Hisakuni Hashimoto · Kenji Kinoshita · Masahito Shimoya · Toshikazu Kaise · Kazunori Ohnishi

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Abstract
Purpose To investigate the pharmacokinetics of arsenic species in Japanese patients with relapsed or refractory acute promyelocytic leukemia (APL) treated with arsenic trioxide (ATO) at a daily dose of 0.15 mg/kg.
Methods Inorganic arsenic (As^{III} and As^{V}) and the major metabolites monomethylarsonic acid (MAA^{V}) and dimethylarsinic acid ($DMAA^{V}$) in plasma and urine collected from 12 Japanese patients were quantified by HPLC/ICP-MS.
Results The plasma concentrations of As^{III} and As^{V} on day 1 reached the similar *C*max (12.4 ± 8.4 and 10.2 ± 3.9 ng/ml) immediately after completion of administration followed by a biphasic elimination. The $AUC_{0-\infty}$ of As^{V} was about twice that of As^{III}. The appearance of methylated metabolites in the blood was delayed. During the repeated administration, the plasma concentrations of inorganic arsenic reached the steady state. In contrast, the MAA^{V} and $DMAA^{V}$ concentrations increased in relation to increased administration frequency. The mean total arsenic excretion rate including inorganic arsenic and methylated arsenic was about 20% of daily dose on day1 and remained at about 60% of daily dose during week 1–4.
Conclusions This study demonstrates that ATO is metabolized when administered intravenously to APL patients and methylated metabolites are promptly eliminated from the blood and excreted into urine after completion of administration, indicating no measurable accumulation of ATO in the blood.

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S. Fujisawa · K. Shigeno · N. Sahara · S. Nakamura · K. Naito · M. Kobayashi · K. Shinjo · K. Ohnishi
Department of Medicine III, Hamamatsu University School of Medicine, Hamamatsu, Japan

R. Ohno
Aichi Cancer Center Hospital, Nagoya, Aichi, Japan

A. Takeshita
Department of Laboratory Medicine, Hamamatsu University School of Medicine, Hamamatsu, Japan

Y. Suzuki · H. Hashimoto
Pharmacy Department, Hamamatsu University Hospital, Hamamatsu, Japan

K. Kinoshita · M. Shimoya · T. Kaise
Laboratory of Environmental Chemodynamics, Tokyo University of Pharmacy and Life Science, Hachioji, Tokyo, Japan

K. Ohnishi (✉)
Division of Clinical Oncology, Hamamatsu University School of Medicine, 1-20-1, Handayama, Hamamatsu 431-3192, Japan
e-mail: kohnishi@hama-med.ac.jp

Introduction

Acute promyelocytic leukemia (APL) is a distinctive type of acute myelocytic leukemia (AML) characterized by chromosome translocations *t* (15; 17) and accounts for approximately 10–15% of all cases of AML. In the 1990s, investigators from China reported that arsenic trioxide (ATO) induces complete remission (CR) in patients with relapsed or refractory APL