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Phase I and II pharmacokinetic and pharmacodynamic study of the proteasome inhibitor bortezomib in Japanese patients with relapsed or refractory multiple myeloma

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The purpose of this phase I and II study was to evaluate the safety, pharmacokinetics, pharmacodynamics, and efficacy of bortezomib in Japanese patients with relapsed or refractory multiple myeloma. This was a dose-escalation study designed to determine the recommended dose for Japanese patients (phase I) and to investigate the antitumor activity and safety (phase II) of bortezomib administered on days 1, 4, 8, and 11 every 21 days. Thirty-four patients were enrolled. A dose-limiting toxicity was febrile neutropenia, which occurred in one of six patients in the highest-dose cohort in phase I and led to the selection of 1.3 mg/m^2 as the recommended dose. Adverse events ≥ grade 3 were rare except for hematological toxicities, although there was one fatal case of interstitial lung disease. The overall response rate was 30% (95% confidence interval, 16–49%). Pharmacokinetic evaluation showed a biexponential decline, characterized by a rapid distribution followed by a longer elimination, after dose administration, whereas the area under the concentration–time curve increased proportionately with the dose. Bortezomib was effective in Japanese patients with relapsed or refractory multiple myeloma. A favorable tolerability profile was also seen, although the potential for pulmonary toxicity should be monitored closely. The pharmacokinetic and pharmacodynamic profiles of bortezomib in the present study warrant further investigations, including more relevant administration schedules. (*Cancer Sci* 2008; 99: 140–144)

Multiple myeloma, one of the B-cell lymphatic tumors, is a malignant hematopoietic tumor with poor prognosis for which a cure cannot ever be expected. The peak age of onset is high at 65–70 years, and its onset in patients younger than 40 years is rare. The median survival of patients with multiple myeloma is approximately 6–12 months if untreated, but it is prolonged to approximately 3 years with the administration of chemotherapy; the 5-year survival rate has been reported to be approximately 25% and the 10-year survival rate is <5%.[1,2] As initial therapy for multiple myeloma, melphalan + prednisolone therapy and vincristine + doxorubicin + dexamethasone therapy have been used as global standards.[3,4] High-dose chemotherapy combined with autologous hematopoietic stem-cell transplantation is reported to be significantly superior to multiagent chemotherapy in terms of response rate and progression-free survival,[5] and is considered to be a standard therapy primarily for patients who are 65 years old or younger. However, no consensus has been reached on the standard therapy for relapsed or chemotherapy-refractory multiple myeloma patients.[6–8] Multiple myeloma is an intractable disease with poor prognosis that continues to relapse, and the duration to relapse becomes shorter in patients who repeatedly receive treatment. There are no available treatment options in which durable efficacy can be expected after relapse, and therefore effective therapeutic choices with new mechanisms of action have been long awaited.

Bortezomib is a novel small molecule that is a potent selective and reversible inhibitor of the proteasome, and has been approved for the treatment of recurrent or refractory multiple myeloma in the USA and Europe. The pharmacokinetics (PK) of bortezomib were reported in a phase I study in which it was administered in combination with gemcitabine twice weekly for 2 weeks followed by a 10-day rest period,[9] and in another phase I study in which it was administered once weekly for 4 weeks followed by a 13-day rest period.[10] Both studies were conducted in patients with advanced solid tumors and not patients with multiple myeloma. Therefore, the present phase I and II study was designed to assess the PK and pharmacodynamic (PD) effects of bortezomib in multiple myeloma patients, particularly in a Japanese population. In addition, efficacy and safety were evaluated to determine the recommended dose (RD).

Patients and Methods

Eligibility. The main eligibility criteria were: confirmed multiple myeloma according to the South-west Oncology Group diagnostic criteria;[11] had received at least previous standard front-line therapy (including melphalan and predonisone, vincristine, doxorubicin, and dexamethasone chemotherapy, and high-dose chemotherapy with autologous stem cell transplantation); had documentation of relapse or refractoriness to the last line of therapy and required therapy because of progressive disease at enrolment. Progressive disease was defined as at least one of the following: more than 25% increase in monoclonal immunoglobulin in the serum or urine; development of new osteolytic lesions or soft tissue tumors, or worsening of existing lesions; hypercalcemia (corrected serum calcium value of >11.5 mg/dL); relapse from complete response (CR); the presence of measurable disease lesions; Karnofsky performance status ≥ 60; 20–74 years of age; adequate bone marrow function (absolute neutrophil count ≥ 1000/mm^3, platelets ≥ 75 000/mm^3, and hemoglobin ≥ 8 g/dL),

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hepatic function (aspartate aminotransferase and alanine aminotransferase levels ≤2.5 times the upper limit of institutional normal range, total bilirubin ≤1.5 times the upper limit of institutional normal range), renal function (creatinine clearance ≥ 30 mL/min), and cardiac function (left ventricular ejection fraction ≥ 55% by echocardiography without New York Heart Association class III to IV congestive heart failure) in the previous 2 weeks; and had received no systemic chemotherapy or radiotherapy in the previous 4 weeks. This study was approved by the Institutional Review Board of each participating hospital. All patients gave written informed consent and the study was conducted in accordance with Good Clinical Practice for Trials of Drugs and the Declaration of Helsinki.

Study design. The RD was determined based on the occurrence of dose-limiting toxicity (DLT) in Japanese patients and in the dose-escalating phase I of the study. The safety and efficacy of bortezomib at the RD were assessed in phase II. In phase I, three patients were enrolled in the 0.7 mg/m^2-dose group, and six patients each in the 1.0 and 1.3 mg/m^2-dose groups. DLT was defined as ≥grade 3 non-hematological toxicity or grade 4 hematological toxicity for which the relation to bortezomib could not be ruled out. The RD was defined as a dose level with a DLT incidence closest to but lower than the estimated (expected) value of 30%. Bortezomib was administered for up to six cycles.

Drug administration. Bortezomib, supplied by Janssen Pharmaceutical (Tokyo, Japan) in vials containing 3.5 mg, was administered by intravenous push over 3–5 s on days 1, 4, 8, and 11, followed by a 10-day rest period, with this 3-week period comprising one cycle. There was an interval of at least 72 h between doses.

Response and safety assessments. Patients were monitored for response after every two treatment cycles by quantitation of serum immunoglobulins, serum protein electrophoresis and immunofixation (IF), and collection of a 24-h urine specimen for total protein, electrophoresis, and IF. Response was evaluated using the European Group for Blood and Marrow Transplantation criteria,[12] after cycles 2, 4, and 6.

Adverse events were assessed and graded according to the National Cancer Institute Common Toxicity Criteria version 2.0 from the first dose until 28 days after the last dose of bortezomib.

Pharmacokinetic and pharmacodynamic analysis. Plasma bortezomib concentrations and blood 20S proteasome activity were measured in phase I. Blood samples were collected before each dose, at 5, 15, and 30 min, and 1, 2, 4, 6, 8, 12, 24, and 48 h after treatment on days 1 and 11. The measurement of plasma bortezomib concentration was conducted at Advion BioSciences (Ithaca, NY, USA) using liquid chromatography/tandem mass spectrometry (LC/MS/MS).[13] The measurement of blood 20S proteasome activity was conducted at Millennium Pharmaceuticals (Cambridge, MA, USA) using the synthetic fluorescence substrate method validated for the chymotrypsin-like activity/trypsin-like activity ratio.[14]

Results

Patients and dose escalation. The study was conducted from May 2004 to January 2006, and 34 patients were enrolled. Patient characteristics are shown in Table 1. All patients had secretory-type myeloma, and the breakdown was 20 patients (59%) with IgG type, eight patients (24%) with IgA type, three patients (9%) with light-chain type, and three patients (9%) with IgA and light-chain type. Most patients had received prior therapy with steroids, alkylating agents, and/or vinca alkaloids. Ten patients (29%) had received stem cell transplantation including high-dose therapy. The median number of lines of prior therapy was two (range: one to eight). Osteolytic lesions were observed in 30 patients (88%) and soft-tissue tumors were observed in seven (21%). The median number of treatment cycles was four (range: one to six), and the median duration of treatment was 79 days (range: 1–152 days). Ten patients (29%) completed all six cycles. The reasons for discontinuation of therapy in 25 patients were progressive disease in 11 patients, patient's own request in six patients, serious adverse events in four patients, DLT in two patients, and others in three patients. Three patients were enrolled in the 0.7 mg/m^2 group and six in the 1.0 mg/m^2 group, and no DLT were observed at any dose level. In the 1.3 mg/m^2 group, DLT (grade 3 febrile neutropenia) occurred in one of the six patients. Therefore, 1.3 mg/m^2 was determined to be the RD in subsequent phase II, in which 18 patients were enrolled.

Table 1. Patient characteristics

Patient characteristic	*n*	%
Patients	34	
Sex		
Female	12	35
Male	22	65
Age (years)		
Median	60	
Range	34–72	
Durie-Salmon stage		
I	0	
II	15	44
III	19	56
Time since diagnosis (years)		
Median	3.4	
Range	1.0–13.7	
Karnofsky performance status		
100	15	44
90–80	18	53
70–60	1	3
Serum interleukin-6 (pg/mL)		
Mean	4.2	
Range	0.5–30.2	
Cytogenetics		
Karyotype abnormal	4	12
del(13)(q14)	7	21
t(11; 14)	4	12
Prior therapy		
Chemotherapy	34	100
Steroids	34	100
Alkylating agents	33	97
Vinca alkaloids	27	79
Anthracyclines	22	65
Thalidomide	8	24
Interferon	7	21
Radiation therapy	6	18
Autologous hematopoietic stem cell transplantation	10	29

Adverse events. The safety analysis dataset consisted of all patients who received at least one dose of bortezomib (34 patients). Adverse events observed in ≥20% of patients are shown in Table 2. The events observed at a high frequency (≥50%) were lymphopenia, neutropenia, leukopenia, thrombocytopenia, anemia, asthenia, diarrhea, constipation, nausea, anorexia, and pyrexia. At least one ≥grade 3 adverse event was observed in 88% of the patients. Major ≥grade 3 adverse events were hematological toxicities including lymphopenia, neutropenia, leukopenia, thrombocytopenia, and anemia. Grade 4 hematological toxicities included neutropenia in six patients (18%), three of which experienced this adverse event during cycle 1. At least grade 3 non-hematological toxicities occurred in fewer than 10%, and no DLT during cycle 1 were observed. Grade 4 non-hematological toxicities included hematuria, blood amylase

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Table 2. All adverse events occurring in at least 20% of patients ($n = 34$)

Dose (mg/m²)	0.7		1.0		1.3		All		Total	%
No. of Patients	($n = 3$)		($n = 6$)		($n = 25$)		($n = 34$)			
NCI-CTC grade	1/2	3/4	1/2	3/4	1/2	3/4	1/2	3/4		
Adverse event										
Hematologic										
Lymphopenia	3	0	4	2	8	17	15	19	34	100
Neutropenia	1	1	2	4	7	16	10	21	31	91
Leukopenia	2	0	6	0	11	12	19	12	31	91
Thrombocytopenia	1	0	4	0	12	11	17	11	28	82
Anemia	2	0	2	3	10	8	14	11	25	74
Nonhematological										
Asthenia[†]	3	0	3	0	15	0	21	0	21	62
Diarrhea	1	0	2	0	15	1	18	1	19	56
Constipation	2	0	3	0	14	0	19	0	19	56
Nausea	2	0	2	0	14	0	18	0	18	53
Anorexia	3	0	2	0	14	0	18	0	18	53
Pyrexia	0	0	4	0	14	0	18	0	18	53
Peripheral neuropathy[‡]	0	0	3	0	12	1	15	1	16	47
AST increased	1	0	1	0	11	2	13	2	15	44
LDH increased	1	0	1	0	12	1	14	1	15	44
Vomiting	1	0	0	0	9	1	10	1	11	32
Rash	0	0	1	0	10	0	11	0	11	32
ALP increased	0	0	2	0	8	0	10	0	10	29
Headache	0	0	1	0	8	0	9	0	9	27
ALT increased	1	0	1	0	7	0	9	0	9	27
Hyperglycaemia	0	0	2	0	5	0	7	0	7	21
Hyponatremia	1	0	0	1	5	0	6	1	7	21
Renal impairment	1	0	1	0	5	0	7	0	7	21
CRP increased	0	0	1	0	6	0	7	0	7	21
Weight decreased	0	0	0	0	7	0	7	0	7	21

[†]Including fatigue and malaise. [‡]Including peripheral sensory neuropathy, peripheral motor neuropathy, and hypoesthesia. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; LDH, lactate dehydrogenase; NCI-CTC, National Cancer Institute Common Toxicity Criteria.

increase, and blood uric acid increase in one patient (3%) each. Hematuria was attributed to prostate cancer and judged as not related to bortezomib. The underlying disease was considered to be involved in the blood uric acid increase; this event was judged unlikely to be related to bortezomib. At the occurrence of grade 4 blood amylase increase, blood amylase isozymes were pancreatic-type in 86% and salivary-type in 14%. There were no gastrointestinal symptoms, such as abdominal pain, associated with amylase increase. Abdominal echography revealed no finding suggesting pancreatitis or pancreatolithiasis, and the relevant events recovered 5 days after the onset. The causality of the grade 4 blood amylase increase with bortezomib was evaluated as 'probable', and therefore treatment was continued at a reduced dose from 1.3 to 1.0 mg/m².

One case of interstitial lung disease (ILD) that resulted in a fatal outcome was observed in phase II. The patient with grade 5 ILD had developed the event on day 10 in cycle 2 after receiving seven doses of bortezomib in total. Pyrexia, non-productive cough, hypoxia, and dyspnea were observed as early symptoms, and antibiotics, antimicrobials, steroid pulse therapy, and oxygen inhalation were initiated to treat it. However, respiratory failure worsened, so the patient was put on a ventilator, and the study was discontinued. After the onset of ILD, bronchoalveolar lavage was conducted, but the causative pathogen could not be identified. The available examinations for β-D-glucan, cytomegalovirus antigenemia, influenza virus, and urinary antigen of *Legionella* were found to be negative. The diagnosis from the pathological findings was diffuse alveolar damage. A retrospective analysis of the pretreatment computed tomography (CT) images indicated that the patient had subtle interstitial shadows in the basal region of both lungs. In response, the protocol was amended to exclude patients with abnormal pretreatment bilateral interstitial shadows on CT. No cases of fatal pulmonary toxicity were observed thereafter.

Efficacy. Thirty-three patients were evaluable for efficacy, excluding one ineligible patient who had another malignancy (prostate cancer). Objective responses were observed in 10 of 33 patients (30%; 95% confidence interval 16–49%), including five IF-positive complete responses (CR^{IF+}) and five partial responses. Of the 10 responders, five patients had one line of prior therapy, two patients had three lines of prior therapy, and three patients had four or more lines of prior therapy. It is noteworthy that one patient who had received eight lines of prior therapy, including high-dose chemotherapy with autologous stem-cell transplantation, showed CR^{IF+}. Of the 10 patients who had received prior autologous hematopoietic stem cell transplantation, two patients showed CR^{IF+}, and three patients showed PR. With respect to osteolytic lesions, which is one of the efficacy endpoints, partial regression in five patients, partial disappearance in one patient, and regression of soft-tissue tumors in two patients were observed.

Pharmacokinetics and pharmacodynamics. The mean plasma bortezomib concentration–time profiles on days 1 and 11 obtained from 16 patients enrolled in phase I are shown in Fig. 1a. PK parameters obtained using non-compartmental analysis are shown in Table 3. The plasma bortezomib concentration–time

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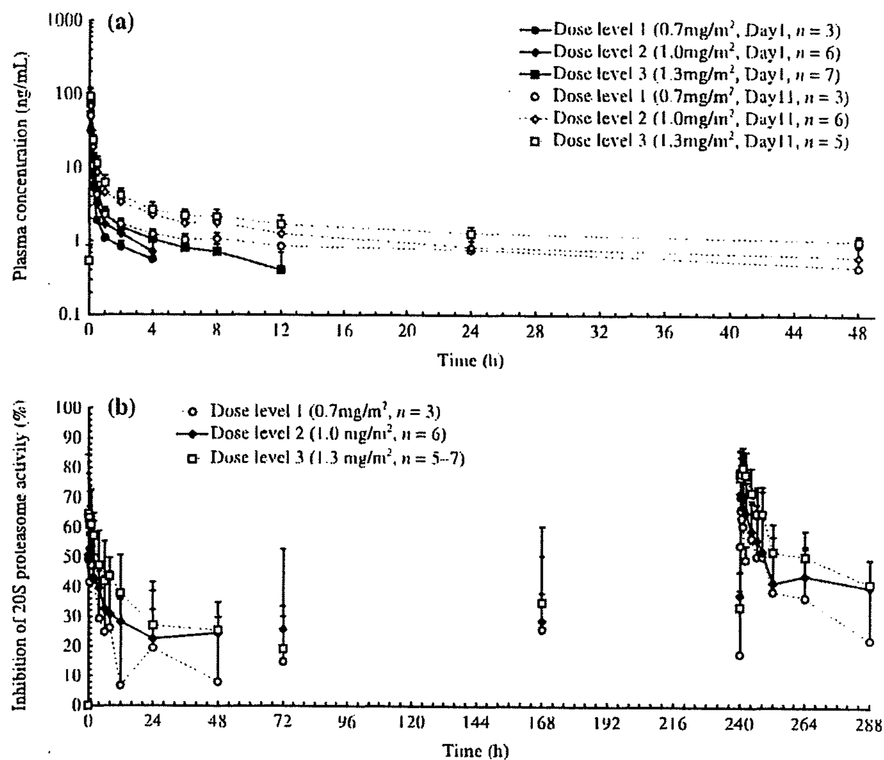

Fig. 1. (a) Plasma bortezomib concentrations (mean + SD). (b) Inhibition of blood 20S proteasome activity (mean + SD).

Table 3. Pharmacokinetic parameters (non-compartmental analysis)

Parameter	Day	Dose (mg/m²) 0.7 (n = 3)	1.0 (n = 6)	1.3 (n = 5–7)†
C_0 (ng/mL)	1	73.75 ± 7.89	144.92 ± 179.31	185.84 ± 57.65
	11	130.68 ± 71.97	147.19 ± 72.33	187.03 ± 54.31
AUC	1	14.04 ± 0.70	28.58 ± 24.86	46.50 ± 19.89
(ng · h/mL)	11	112.01 ± 47.74	108.39 ± 52.32	186.60 ± 49.79
Half life (h)	1	3.31 ± 0.88	6.81 ± 8.81	16.11 ± 20.75
	11	64.59 ± 30.29	32.46 ± 12.91	57.39 ± 24.92
Clearance	1	83.35 ± 10.52	105.41 ± 75.66	51.97 ± 18.99
(L/h)	11	11.77 ± 4.67	19.63 ± 14.50	12.10 ± 3.73
V_z (L)	1	406.92 ± 154.03	520.08 ± 349.87	894.41 ± 682.35
	11	978.51 ± 263.13	731.69 ± 242.35	957.81 ± 350.40
V_{ss} (L)	1	186.46 ± 85.02	288.90 ± 260.74	507.75 ± 558.30
	11	812.60 ± 202.03	540.03 ± 218.72	763.81 ± 271.64
C_0 ratio	11/1	1.789 ± 0.973	1.848 ± 1.133	1.103 ± 0.249
AUC ratio	11/1	7.940 ± 3.247	5.363 ± 2.970	5.142 ± 0.543

†Day 1, n = 7; day 11, n = 5. Values are mean ± SD. AUC, area under the concentration–time curve from time zero to infinity; AUC ratio, AUC on day 11/AUC on day 1; C_0, plasma concentration at the end of administration; C_0 ratio, C_0 on day 11/C_0 on day 1; V_z, the apparent volume of distribution during the terminal phase; V_{ss}, the apparent volume of distribution at steady state.

profiles showed a biphasic elimination profile, characterized by rapid distribution followed by a longer elimination at all dose levels. At any dose level, the elimination half-life ($t_{1/2}$) on day 11 was prolonged, and systemic clearance (CL) was lower compared with day 1. Therefore, delayed elimination of bortezomib from plasma associated with repeated administrations was observed, and the plasma bortezomib concentration after administration (C_0, estimated value) and area under the plasma concentration–time curve (AUC) showed higher values on day 11 compared with day 1. AUC showed dose dependency, whereas C_0 did not.

The inhibition of blood 20S proteasome activity is shown in Fig. 1b. The 20S proteasome inhibition recovered over time at all dose levels, but was prolonged compared with the temporal decrease in plasma bortezomib concentration, and the inhibition was still observed before treatment on days 4, 8, and 11.

Discussion

In the present study, bortezomib was generally well tolerated in the 25 Japanese patients whose treatments were started at the RD of 1.3 mg/m². Hematological toxicities, gastrointestinal toxicities, and peripheral neuropathies observed in our patients were similar to those reported for patients in clinical studies from the USA and Europe.[15,16] Most could be managed without interventions or with the usual symptomatic therapy. Grade 4 neutropenia was observed in 18% of patients, but treatment could be continued with dose reduction. The response rate obtained in the present study was comparable to that reported by Richardson *et al.* in a pivotal phase III study.[16] In addition, patients who had received heavy prior therapy also showed a consistent response. Therefore, 1.3 mg/m² is considered appropriate as an initial dose of bortezomib in Japanese patients. There was a fatal pulmonary disorder event (ILD) in one patient treated with the 1.3 mg/m² dose in which a causal relationship with bortezomib could not be ruled out. Hence, special care should be taken prior to initiating treatment with bortezomib to evaluate patients (e.g. chest X-ray or chest CT scan) and during and after bortezomib treatment if they develop subjective symptoms such as dyspnea, cough, and fever.

The assessment of PK and PD in multiple myeloma patients treated with bortezomib twice weekly for 2 weeks was conducted for the first time in Japanese patients. A decrease in CL associated with increased exposures and subsequently longer $t_{1/2}$ values were observed after repeated administration and dose escalation. The relatively large volume of distribution suggests that bortezomib may be distributed extensively into the extravascular tissues. It can be postulated that CL values on day 1 are apparent values observed due to rapid tissue distribution, whereas

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saturation of proteasome binding sites and tissue distribution occur after multiple dosing, and the CL value on day 11 may be a better representation of the true value.

It was also found that the blood 20S proteasome inhibition at each dose level recovered over time, but was prolonged compared with the temporal decrease in plasma bortezomib concentration. Similarly to CL, this could be due to the large distribution volume of bortezomib and its slow return from tissues to plasma.

Delayed elimination and enhanced proteasome inhibition were observed with repeated administration and dose increase, but no clear tendency in the incidence or degree of adverse reactions was observed. However, the PD results of the present study in Japanese patients demonstrate that the inhibition of 20S proteasome activity does not recover even after 72 h, which is specified as a minimum interval for bortezomib dosing. Accordingly, when bortezomib is used in clinical practice, it is important to determine the optimal dosage and determine whether it is appropriate to administer bortezomib while considering the balance between safety and efficacy.

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Acquired C1-esterase inhibitor deficiency and positive lupus anticoagulant accompanied by splenic marginal zone B-cell lymphoma

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Key words: Systemic lupus erythematosus, hypocomplementemia, ^{18}F-fluorodeoxyglucose positron emission tomography.

Competing interests: none declared.

ABSTRACT

A 70-year-old woman complained of mild shortness of breath. Laboratory findings revealed pancytopenia, positive lupus anticoagulant and severe hypocomplementemia without antinuclear or anti-DNA antibodies. After the failure of prednisolone treatment, an acquired C1-esterase inhibitor (C1-INH) deficiency was diagnosed. There were no episodes of angioedema or deep vein thrombosis. Three months later, extreme splenomegaly was detected. Lymph node biopsy suggested splenic marginal zone B-cell lymphoma. Acquired C1-INH deficiency due to a lymphoproliferative disorder should be considered as a possible diagnosis for patients with severe hypocomplementemia.

Introduction

C1-esterase inhibitor (C1-INH) deficiency is a rare disorder, which usually causes episodes of angioedema, and is classified as either hereditary or acquired (1). The acquired form is thought to be associated with lymphoproliferative disorders (1) or autoimmune disorders such as systemic lupus erythematosus (SLE) (2).

Here, we report the first known case of acquired C1-INH deficiency and positive lupus anticoagulant (LAC) with splenic marginal zone B-cell lymphoma (SMZL).

Case report

A 70-year-old previously healthy woman was referred to our hospital in August 2005 with mild shortness of breath. The patient had never complained of Raynaud's phenomenon, xerostomia, xerophthalmia or arthralgia. Skin rash was not observed. There was no palpable lymphadenopathy. Laboratory data were as follows: hemoglobin, 7.3 g/dL; white blood cell count, 1800 /μL without atypical cells; platelet count, 11.8×10^4/μL; reticulocytes, 2.7%; lactate dehydrogenase (LDH), 224 IU/L. Hepatitis C virus antibody was negative. Bone marrow aspirate was normocellular without atypical cells. International normalized ratio (derived from the prothrombin time) and activated partial thromboplastin time were increased at 1.49 and 85.8 sec, respectively. C4 and CH_{50} levels were below detectable limits. C3, haptoglobin, immunoglobulin (Ig) G, IgA and IgM were within normal limits. Direct and indirect Coombs tests were negative. Assays did not detect rheumatoid factor, anti-nuclear antibody, anti-DNA, anti-RNP, anti-Sm, anti-Ro, anti-La or anti-cardiolipin/beta-2-glycoprotein-1 complex antibody. Although the patient did not suffer from deep vein thrombosis, LAC (based on diluted Russell's viper venom time) was positive at 1.61. These findings suggested a diagnosis of elderly-onset SLE with anti-phospholipid antibody syndrome. Treatment with oral prednisolone (PSL) 40 mg/day and aspirin 100 mg/day was started in mid-October 2005. The symptom was ameliorated at the beginning of November, but treatment was considered ineffective because of persistent pancytopenia and hypocomplementemia. The patient was found to have a low C1q level (2.7 mg/dL) and low C1-INH activity (47%). Consequently, her diagnosis was changed to acquired C1-INH deficiency. Although the cause of the C1-INH deficiency was unclear, PSL administration was terminated in late November 2005. No episodes of angioedema occurred. After termination of PSL administration, LAC was positive with a titer similar to that of the earlier test.

In December 2005, examination revealed enlargement of the spleen to 10 fingers below the left subcostal margin. Bone marrow contained aggregation nests consisting of atypical lymphocytes. In early February 2006, a biopsy specimen was obtained from a pretracheal lymph node, which was found to be positive for ^{18}F-fluorodeoxyglucose (^{18}F-FDG) accumulation on ^{18}F-FDG positron emission tomography (PET)/computed tomography (CT) (Fig. 1). ^{18}F-FDG-PET/CT also revealed strong ^{18}F-FDG accumulation in the spleen (Fig. 2). Lymph node histopathology showed vaguely nodular lesions comprising marginal zone proliferation consisting of neoplastic cells surrounding a germinal center. These cells were strongly positive for CD20 (Fig. 3), but negative for CD5, CD10,

CD23, CD43 and cyclin D1, which is consistent with SMZL. Serum LDH and beta-2-microglobulin levels were 256 IU/L and 6.7 mg/L, respectively. Serum immunoelectrophoresis using the Onchterlony method showed no monoclonal component. The international prognostic index score of lymphoma was 3, indicating intermediate-high risk. Because of the advanced clinical stage (IVA) and the mechanical discomfort caused by splenomegaly, the patient received 6 cycles of R-CHOP therapy consisting of rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone, from March to July 2006. The splenomegaly was markedly regressed following chemotherapy, and the levels of complement components and LAC recovered to their normal ranges.

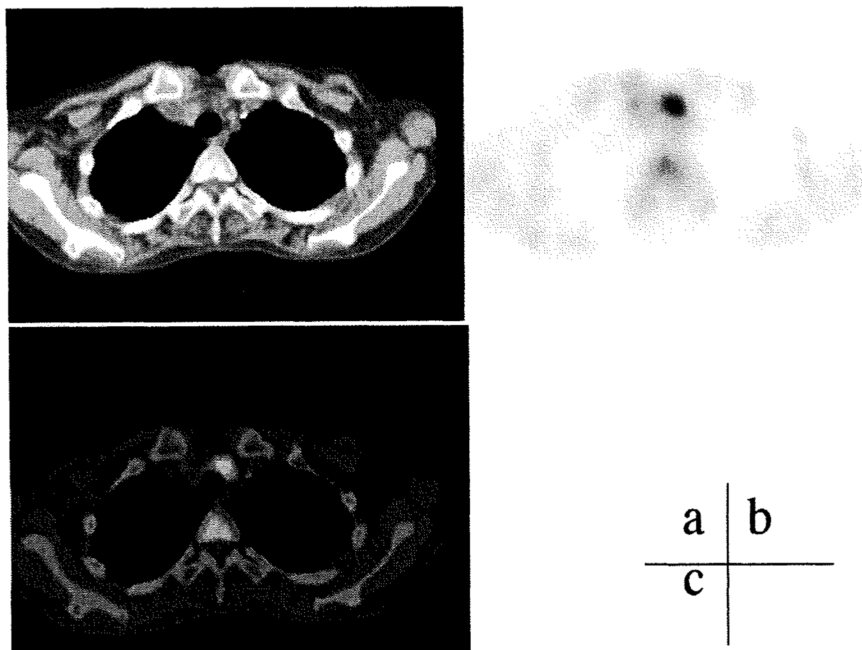


Fig. 1. CT (a), PET (b) and PET/CT (c) images of the chest. Although CT did not show a distinct enlarged lymph node, PET/CT revealed significant FDG accumulations in pretracheal lymphnode.

Discussion

Acquired C1-INH deficiency, also known as acquired angioedema (AAE), is a rare syndrome with only about 150 reported cases (1). Although clinical manifestations of AAE are similar to those of hereditary C1-INH deficiency, also known as hereditary angioedema (HAE), AAE is characterized by elderly onset and a lack of family history. The serum C1q level is considered to be useful for distinguishing AAE from HAE (3). In the present case, the serum C1q level was significantly decreased, indicating AAE. Sub-classification of AAE is controversial; generally, AAE is divided into 2 pathogenic types (1, 3). In type-I AAE, an increase in consumption of available C1q molecules is induced by high levels of idiotype-anti-idiotype immune complexes produced by proliferating lymphocytes in underlying lymphoproliferative disorders (3-5). In type-II AAE, autoantibodies appear to inactivate C1-INH function (1), suggesting that immunosuppressive therapy such as PSL, which reduces autoantibody levels, would be effective against type-II AAE. In the present case, although assays for autoantibodies against C1q could not be performed, the patient was classified as type-I AAE because SMZL was present and PSL treatment was ineffective.

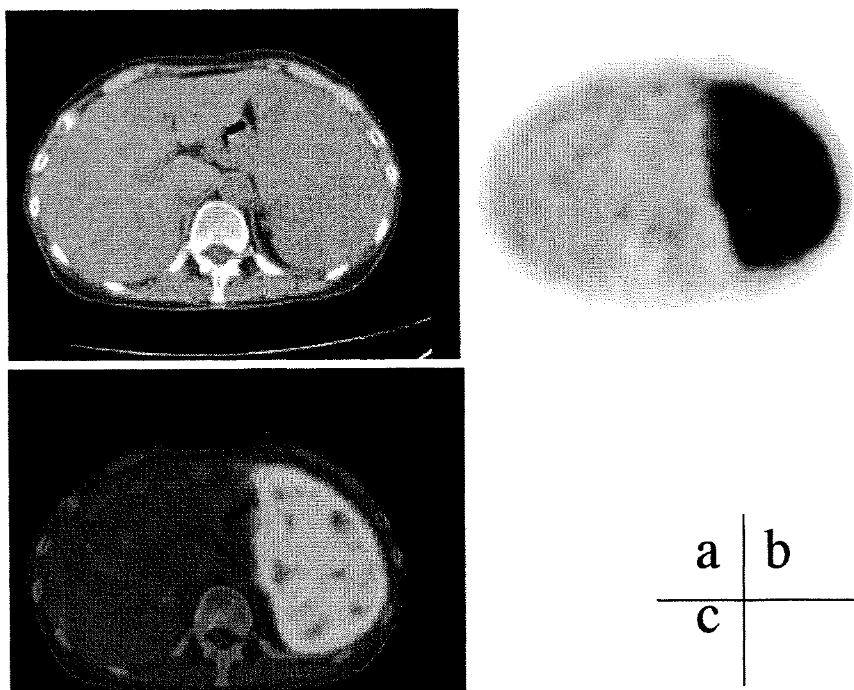
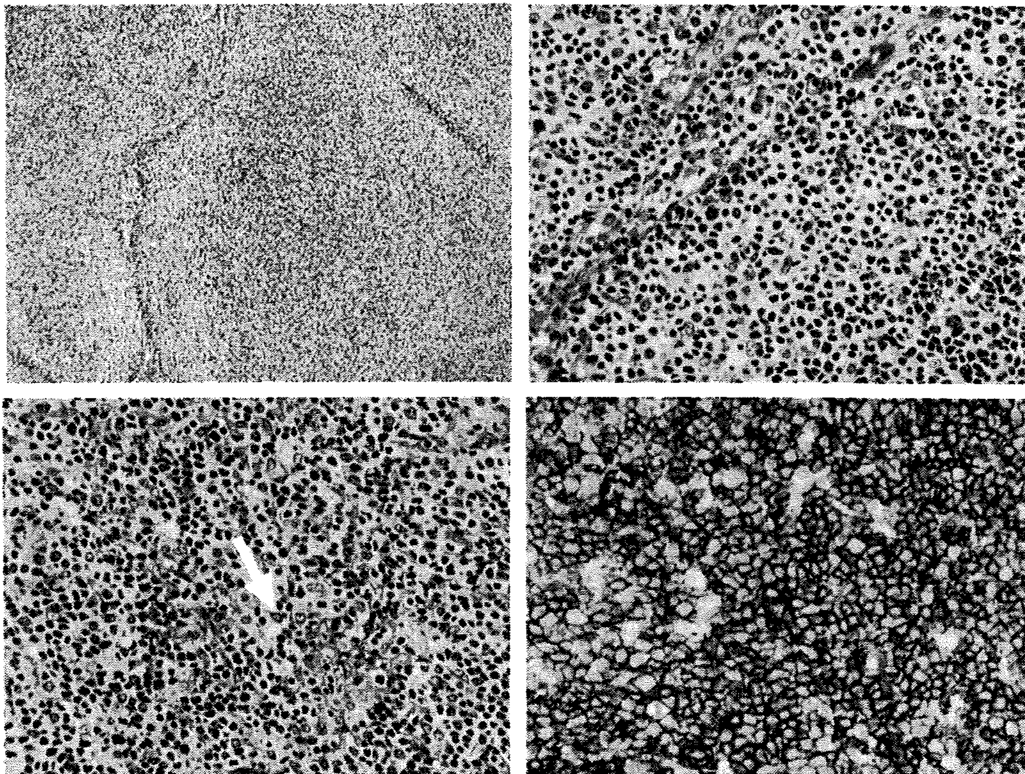


Fig. 2. CT (a), PET (b) and PET/CT (c) images of the abdomen. Strong FDG accumulations were detected in the greatly enlarged spleen. No accumulations in lymph nodes were observed.

SMZL comprises less than 1% of non-Hodgkin's lymphoma, and its most frequent and characteristic manifestations are extreme splenomegaly and lymphocytosis (6). SMZL is also often accompanied by autoimmune disorders, such as autoimmune hemolytic anemia (AIHA) and Sjögren's syndrome (7, 8). Anti-phospholipid antibodies, such as anti-cardiolipin antibody and LAC, have only rarely been detected in SMZL patients (9, 10). However, anti-phospholipid antibodies appear to be reliable markers of SMZL activity (11). In the present case, AIHA and Sjögren's syndrome were not observed, but LAC was initially positive in repeated exam-



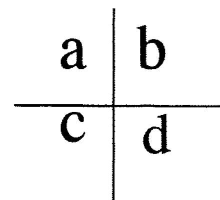


Fig. 3. Photomicrograph of the lymph node. Low- (**a**) and high-power (**b**, **c**) images and immunostaining for CD20 (**d**) suggested marginal zone B-cell lymphoma. Dutcher's body was occasionally observed (c, arrow).

inations and became negative after chemotherapy. Although it is unclear why the C1-INH deficiency coincided with positive LAC, both C1-INH deficiency and positive LAC may occur independently as a result of SMZL, as previously suggested (11). Moreover, it is unclear why angioedema symptoms did not occur in the present patient. Only 1 case of acquired C1-INH deficiency and positive LAC accompanied by germinal center lymphoma has been reported (12). To our knowledge, the present patient is the first reported case of SMZL coexisting with acquired C1-INH deficiency and positive LAC.

Similar to reports indicating that levels of complement components gradually recover following spontaneous remission of SMZL (4) and that C1-INH activity recovers after treatment of lymphoproliferative disorders in AAE cases (1), the levels of complement components in the present patient recovered after chemotherapy.

Although no episodes of angioedema were observed in the present case, the present findings suggest that acquired C1-INH deficiency based on a lymphoproliferative disorder should be considered as a possible diagnosis for patients with severe hypocomplementemia.

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悪性腫瘍について知っておきたいこと

悪性リンパ腫の最新の治療法は？

伊藤国明*
Kuniaki ITOH

● Key Words ●悪性リンパ腫，ABVD 療法，R-CHOP 療法●

I．治療の前に

悪性リンパ腫は，ホジキンリンパ腫とB細胞性・T細胞性などの非ホジキンリンパ腫（WHO分類）に分類されている。悪性リンパ腫は化学療法感受性が高く，抗癌剤治療によって治癒も可能であるが，病理組織診断に基づいて治療方針が決定されるので，リンパ節あるいは腫瘍部位などの病変組織の生検が必須である。細胞診も実施されているが診断に至らないことも多く，リンパ腫を疑う場合は生検を行うべきである。また，外科的処置は診断のための生検にとどめるべきであるが，診断に迷う場合や診断が確定できない場合には再生検をためらうべきではない。

悪性リンパ腫は診断時に病変が多発していることも多く，全身症状（B症状），bulky disease の有無とともに，頸部・胸部・腹部CT，PET，骨髄生検などの全身的検索を行い，臨床病期を確認することが極めて重要である。病期分類は，Ann-Arbor 分類あるいは Ann-Arbor 分類を改訂した Cotswolds meeting[1)]に基づいて行う（**表1**）。

表1　悪性リンパ腫の臨床病期分類

I期	病変が1つのリンパ節領域，または1つのリンパ節外臓器の限局性病変の場合
II期	病変が横隔膜の一方に限局し，2個以上のリンパ節領域に存在する場合，または病変リンパ節と関連した1つのリンパ節外臓器（または部位）への限局性の浸潤がある場合
III期	病変が横隔膜の両側のリンパ節領域に進展している場合，または病変リンパ節領域に関連するリンパ節外臓器（または部位）への限局性浸潤を伴っている場合。脾臓浸潤も含む
IV期	リンパ節病変と，それに関連しない遠隔のリンパ節外臓器へびまん性に浸潤している場合

下記の全身症状の有無によってA（症状なし）とB（症状あり）に分ける。
- 6カ月以内に原因不明の10%以上の体重減少が認められる場合
- 原因不明の38℃以上の発熱
- 多量の盗汗（下着を着替えるような発汗）

II．ホジキンリンパ腫[1)]

ホジキンリンパ腫はほとんどの場合リンパ節原発で，7〜8割は治癒可能であり，現時点では病理組織の亜型によって治療方針が変更されることはない。I・II期ホジキンリンパ腫の予後因子としては，50歳未満，リンパ節病変数が3個以下，bulky disease がない，B症状がなく赤沈（1時間値）が50 mm 未満またはB症状はあるが赤沈（1時間値）が30 mm 未満，の4つの項目を満たすものが予後良好グループであり，進行期では，国際予後予測スコア（international prognostic score：IPS）(**表2**)[1)]が予後因子として使われている。

治療は，放射線療法・抗癌剤による化学療法・放射線と抗癌剤の併用療法（combined modality therapy）があり，化学療法としては ABVD 療法（doxorubicin-アドリアシン®，bleomycin-ブレオ®，vinblastine-エクザール®，dacarbazine-ダカルバジン®）が標準的治療である。一般的には，I・II期でも試験開腹の必要性や広範囲照射による毒性を考慮して ABVD 療法4コースを施行し，その後病変領域照射（involved-field irradiation）を追加する。III・IV期では，4コースまで

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表 2　進行期ホジキンリンパ腫の予後不良因子数による生存率（Hasenclever index）

予後不良因子数*	5 年無増悪生存率 (%)	5 年生存率 (%)
0	84	89
1	77	90
2	67	81
3	60	78
4	51	61
5 以上	42	56

*各予後不良因子
- 血清アルブミン値：4.0 g/dl 未満
- ヘモグロビン：10.5 g/dl 未満
- 男性
- 年齢：45 歳以上
- 病期：IV 期
- 白血球数：15,000/μL 以上
- リンパ球数：600/μL 未満，または 8%未満

に完全寛解になった早期奏効例では 6 コースを，他の場合は ABVD 療法 8 コースを施行し，bulky disease や残存病変があれば病変領域照射を追加する。交叉耐性のない薬剤の追加と治療強度を高めた治療法（increased dose BEACOPP）の優位性も報告されているが，まだ広く行われているわけではない。

化学療法，または combined modality therapy 後の再発では，救援化学療法後に自家造血幹細胞移植併用大量化学療法を施行することが無増悪生存率で有意に優れている。

III. 非ホジキンリンパ腫

本邦で多くを占めるのは非ホジキンリンパ腫であるが，病理組織型によって初期の治療目標がまったく異なっていることに注意しなければならない。濾胞性リンパ腫では，濾胞性リンパ腫国際予後予測因子（follicular lymphoma international prognostic index：FLIPI）[2]が，aggressive lymphoma では国際予後予測因子（international prognostic index：IPI）[3]が予後予測に使用されている（**表 3**）。

濾胞性リンパ腫のような低悪性度リンパ腫（indolent lymphoma）は，限局期（I/II期）であれば放射線療法により治癒を目指すが，進行期

表 3　非ホジキンリンパ腫の予後因子分類

濾胞性リンパ腫国際予後予測因子（FLIPI）

	予後不良因子数*	5 年生存率 (%)	10 年生存率 (%)
Low	0〜1	90.6	70.7
intermediate	2	77.6	50.9
High	3〜5	52.5	35.5

*各予後不良因子
- 年齢：61 歳以上
- 臨床病期（III・IV 期）
- hemoglobin（<12 g/dl）
- nodal sites（>4）
- LDH（>正常範囲）

国際予後予測因子（IPI）

	予後不良因子数*	完全寛解率 (%)	5 年無再発生存率 (%)	5 年生存率 (%)
Low	0〜1	87	70	73
Low-intermediate	2	67	50	51
High-intermediate	3	55	49	43
High	4〜5	44	40	26

*各予後不良因子
- 年齢（>60 歳）
- 全身状態（Performance Status 2〜4）
- 臨床病期（III・IV 期）
- LDH（>正常範囲）
- 節外病変数（>1）

（III/IV期）では，年単位の経過であっても現状では薬物療法による治癒は証明されていない。したがって，無治療で注意深く経過をみることもひとつの選択肢であり，rituximab（リツキサン®）による抗体単独療法・抗体＋抗癌剤併用療法などを選択する場合もある。近日中には，経口 fludarabin（フルダラ®）や免疫放射線療法（zevalin）も保険診療で使用が可能になる予定であり，さらに選択肢は広がると考えられる。

びまん性リンパ腫のような中悪性度リンパ腫（aggressive lymphoma）は，現在でも CHOP 療法（cyclophosphamide-エンドキサン®，doxorubicin-アドリアシン®，vincristine-オンコビン®，prednisolone-プレドニン®）が初回治療の標準的治療である。限局期では，CHOP 療法 3 コースに引き続き放射線療法を行う。ただし，長期生存については CHOP 療法 8 コースと差がな

いと報告されており，予後不良因子を持つ場合や放射線治療後毒性を考慮し，頭頸部領域ではCHOP療法6〜8回を選択する場合も多い。進行期では，CHOP療法6〜8回が標準的治療法であり，多くを占めるCD20抗原陽性のB細胞性リンパ腫（diffuse large B-cell lymphoma）ではrituximabを併用したR-CHOP療法の優位性が証明されている[4~6]。

初回寛解後に再発した場合は，65歳未満で救援化学療法が有効であれば，自家造血幹細胞移植併用大量化学療法が標準的治療である。悪性リンパ腫に対する同種移植は長期生存が得られる可能性もあるが合併症などのリスクも高く，初回再発では対象にならない[7]。

リンパ芽球性リンパ腫などの高悪性度リンパ腫は急性リンパ性白血病などと同様に強力な寛解導入療法を行う。

まとめ

悪性リンパ腫は化学療法で治癒しうる疾患であることを認識し，早期に血液・腫瘍内科医に相談することが重要である。

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ONCOGENOMICS

Identification of the novel *AML1* fusion partner gene, *LAF4*, a fusion partner of *MLL*, in childhood T-cell acute lymphoblastic leukemia with t(2;21)(q11;q22) by bubble PCR method for cDNA

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The *AML1* gene is frequently rearranged by chromosomal translocations in acute leukemia. We identified that the *LAF4* gene on 2q11.2–12 was fused to the *AML1* gene on 21q22 in a pediatric patient having T-cell acute lymphoblastic leukemia (T-ALL) with t(2;21)(q11;q22) using the bubble PCR method for cDNA. The genomic break points were within intron 7 of *AML1* and of *LAF4*, resulting in the in-frame fusion of exon 7 of *AML1* and exon 8 of *LAF4*. The *LAF4* gene is a member of the *AF4/FMR2* family and was previously identified as a fusion partner of *MLL* in B-precursor ALL with t(2;11)(q11;q23), although *AML1-LAF4* was in T-ALL. *LAF4* is the first gene fused with both *AML1* and *MLL* in acute leukemia. Almost all *AML1* translocations except for *TEL-AML1* are associated with myeloid leukemia; however, *AML1-LAF4* was associated with T-ALL as well as *AML1-FGA7* in t(4;21)(q28;q22). These findings provide new insight into the common mechanism of *AML1* and *MLL* fusion proteins in the pathogenesis of ALL. Furthermore, we successfully applied bubble PCR to clone the novel *AML1-LAF4* fusion transcript. Bubble PCR is a powerful tool for detecting unknown fusion transcripts as well as genomic fusion points.
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Keywords: *AML1/RUNX1*; *LAF4*; T-cell acute lymphoblastic leukemia; *MLL*

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Introduction

A large number of leukemias have been found to be associated with specific chromosomal aberrations. Recent studies have demonstrated that several chromosomal rearrangements and molecular abnormalities are strongly associated with distinct clinical subgroups and can predict clinical features and therapeutic responses (Rowley, 1999; Taki and Taniwaki, 2006). Some genes have been associated with recurrent rearrangements and have many fusion partner genes, such as *MLL* at 11q23, *TEL (ETV6)* at 12p13 and *NUP98* at 11p15; *AML1* (*RUNX1*, *CBFA2*) at 21q22 is one of the most frequent targets of these chromosomal rearrangements in both acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) (Miyoshi *et al.*, 1991; Hayashi, 2000; Kurokawa and Hirai, 2003). To date, a number of in-frame fusion partners of *AML1* have been cloned: *YTHDF2* at 1p35 (Nguyen *et al.*, 2006), *ZNF687* at 1q21.2 (Nguyen *et al.*, 2006), *MDS1/EVI1* at 3q26 (Mitani *et al.*, 1994), *FGA7* at 4q28 (Mikhail *et al.*, 2004), *SH3D19* at 4q31.3 (Nguyen *et al.*, 2006), *USP42* at 7p22 (Paulsson *et al.*, 2006), *MTG8* (*ETO*, *CBFA2T1*) at 8q22 (Erickson *et al.*, 1992; Miyoshi *et al.*, 1993), *FOG2* at 8q23 (Chan *et al.*, 2005), *TRPS1* at 8q24 (Asou *et al.*, 2007), *TEL* (*ETV6*) at 12p13 (Golub *et al.*, 1995), *MTG16* at 16q24 (Gamou *et al.*, 1998) and *PRDX4* at Xp22 (Zhang *et al.*, 2004). Most *AML1* translocations, except for *TEL-AML1*, are associated with AML, involving the N-terminus Runt domain and lacking the C-terminus transactivation domain (Kurokawa and Hirai, 2003). AML1 fusion proteins are associated with leukemogenesis by dominantly interfering with normal *AML1*-mediated transcription and acting as a transcriptional repressor (Okuda *et al.*, 1998; Wang *et al.*, 1998). Clinically, patients with AML harboring t(8;21) in both children and adults show a high rate of complete remission, and its prognosis is considered better than that of patients with a normal karyotype or other chromosomal aberrations (Grimwade *et al.*, 1998).

In the present study, we analysed pediatric T-ALL with t(2;21)(q11;q22) and identified the *LAF4* gene,

which is one of the fusion partners of *MLL*, as a novel fusion partner of the *AML1* gene.

Results

Case report

A 6-year-old boy with a high leukocyte count (64 700 μl^{-1}), containing 84% blasts in peripheral blood and with a mediastinal mass, was diagnosed as having T-ALL. A bone marrow smear was hypercellular with 69% blasts and negative for myeloperoxidase. The leukemic cells, after gating of CD45-positive cells, were positive for CD5 (90.7%), CD7 (90.7%), CD58 (69.9%) and cytoplasmic CD3 (92.8%), and negative for HLA-DR, IgG, IgM, Igκ, Igλ, CD8, CD13, CD14, CD19, CD20 and CD33. He was treated on the Tokyo Children's Cancer Study Group (TCCSG) L04-16 extremely high-risk (HEX) protocol, including stem cell transplantation, because the response to initial 7-day prednisolone (60 mg m^{-2}) monotherapy was poor. He achieved complete remission after the induction phase. After the early consolidation phase and two courses of the consolidation phase, he received allogeneic bone marrow transplantation from an unrelated HLA-matched donor 4 months after diagnosis. He has been in complete remission for 17 months.

The patient's leukemic cells at diagnosis were analysed after written informed consent was obtained from his parents, and the ethics committee of Kyoto Prefectural University of Medicine approved this study.

Identification of the AML1-LAF4 *fusion transcript*

Cytogenetic analysis of the leukemic cells of the patient using routine G-banding revealed 47, XY, add(1)(p36), +der(2)t(2;21)(q13;q22), t(2;21)(q13;q22), −9, −9, +mar1, +mar2, and spectral karyotyping (SKY) analysis revealed 47, XY, der(1)t(1;17)(p36.1;q23), der(2)t(2;21)(q11.2;q22), +der(2)t(2;21)(q11.2;q22), del(5)(p15.1), del(9)(q22), del(9)(p13), der(21)t(2;21)(q11.2;q22) (Supplementary Figure S1). Since *AML1* is located at 21q22, we inferred that *AML1* was rearranged in this case. Fluorescence *in situ* hybridization analysis using *AML1*-specific BAC (bacterial artificial chromosome) clones showed split signals of *AML1* on two der(2)t(2;21)(q11.2;q22) and der(21)t(2;21)(q11.2;q22) chromosomes (Figure 1a).

To isolate fusion transcripts of *AML1*, we performed the bubble PCR method for cDNA (Figure 2) and obtained various-sized products (Figure 3a). Four different-sized products were sequenced and two products contained *AML1* sequences fused to unknown sequences. Basic local alignment search tool (BLAST) search revealed that the unknown sequences were part of the *LAF4* gene and both products had the same in-frame junctions (Figure 3b). *LAF4* was located on chromosome 2q11.2–12, which was compatible with the result of spectral karyotyping analysis. We next performed reverse transcription-PCR to confirm *AML1-LAF4* fusion transcripts, and obtained three different-sized *AML1-LAF4* fusion products, including only one in-frame product (Figures 3c and d); however, reciprocal *LAF4-AML1* fusion transcripts were not generated (Figure 3c). Type 2 transcript is an out-of-frame fusion and generated premature termination in exon 9 of *LAF4* (Figure 3d). On the other hand, type 3 transcript is an in-frame fusion of exon 7 of *AML1* and exon 8 of *LAF4*, the same as the type 1 transcript; however, the type 3 transcript contained an 85-bp intronic sequence between exons 9 and 10 of *LAF4*, which might be due to splicing error, and appeared as a premature termination codon within the intronic sequences (Figure 3d). *AML1-LAF4* fusions were also confirmed by fluorescence *in situ* hybridization analysis (Figure 1b).

Detection of AML1-LAF4 *genomic junctions*

Southern blot analysis using a cDNA probe within exon 7 of *AML1* detected a rearranged band derived from an

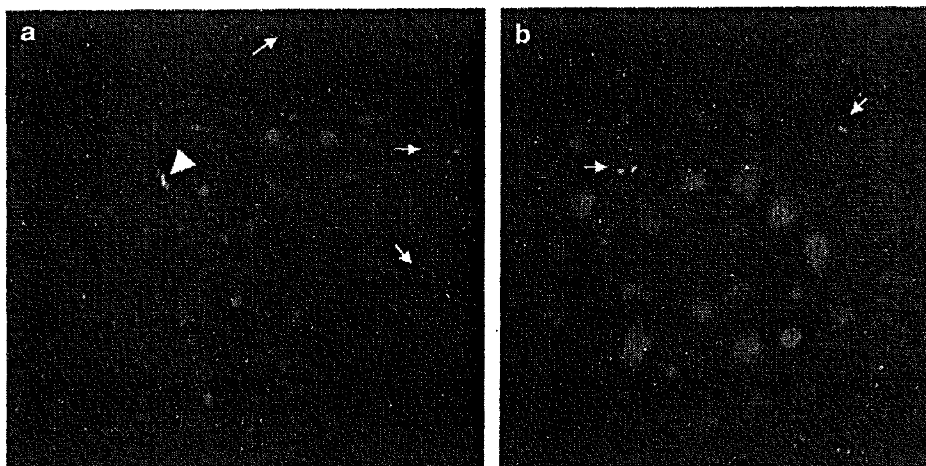

Figure 1 Fluorescence *in situ* hybridization analysis of the leukemic metaphase. (**a**) Both RP11-272A3 (green, 3′ side of *AML1*) and RP11-994N6 (red, 5′ side of *AML1*) were hybridized to normal chromosome 21 (arrowhead), RP11-272A3 to der(21)t(2;21)(q11.2;q22) (arrow, green signal) and RP11-994N6 to two der(2)t(2;21)(q11.2;q22) chromosomes (arrows, red signal). (**b**) Two fusion signals of RP11-994N6 (5′ of *AML1*, red signals) and RP11-527J8 (3′ of *LAF4*, green signals) were detected on two der(2)t(2;21)(q11.2;q22) chromosomes (arrows).

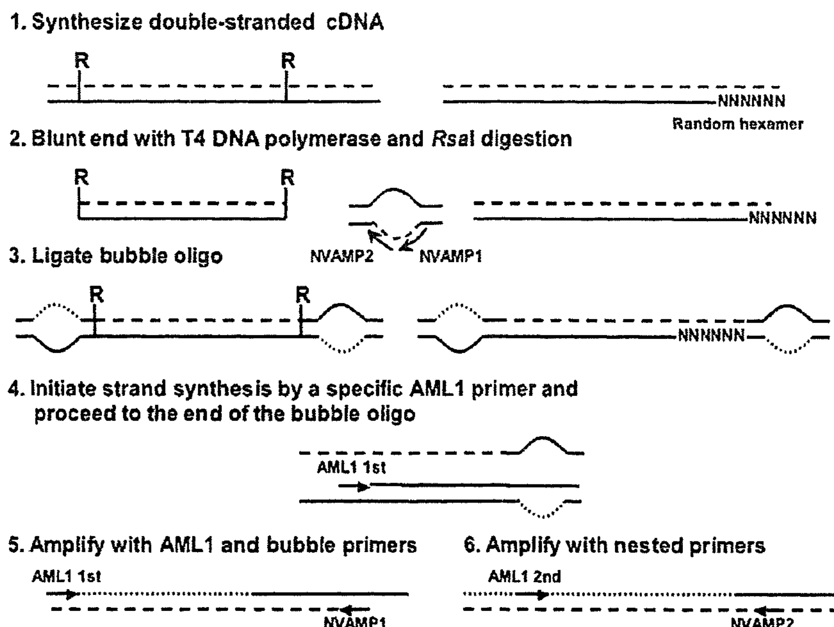

Figure 2 Outline of bubble PCR for cDNA. Bubble PCR primers (NVAMP-1 and NVAMP-2) can only anneal with one complementary sequence for bubble oligo synthesized with *AML1* primer, but not bubble oligo itself; therefore, this single-stranded bubble provides the specificity of the reaction.

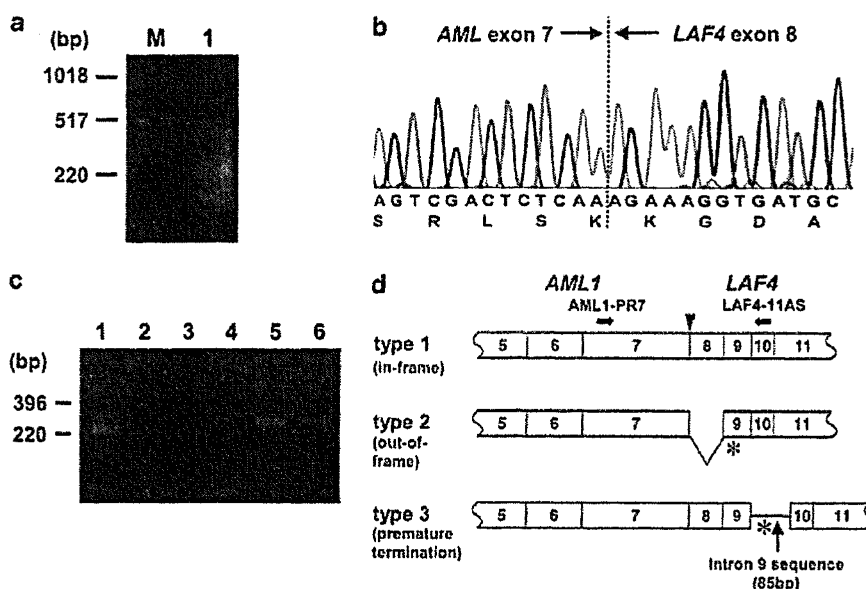

Figure 3 Identification of *AML1-LAF4* fusion transcript. (**a**) Bubble PCR products by nested PCR using AML1-5S and NVAMP1 for first PCR, and AML1-E6S and NVAMP2 for second PCR (lane 1). M, size marker. (**b**) Sequence analysis of *AML1-LAF4* fusion transcript. The single letter amino-acid sequences surrounding the fusion point are shown at the bottom of the figure. (**c**) Detection of *AML1-LAF4* fusion transcripts by reverse transcription-PCR. Primers were AML1-PR7 and LAF4-11AS (lanes 1 and 3), AML1-PR8 and LAF4-PR5 (lanes 2 and 4), and β-actin, respectively. Lanes 1, 3 and 5, patient's leukemic cells; lanes 2, 4 and 6, normal peripheral lymphocytes. (**d**) Three fusion transcripts of *AML1-LAF4* are schematically depicted. Gray/dotted boxes denote predicted *AML1* exons and white boxes represent predicted *LAF4* exons. Type 3 contains the *LAF4* intron 9 splicing donor site. AML1-PR7 and LAF4-11AS indicate the primers used for reverse transcription-PCR. Asterisk shows the termination codon.

approximately 11 kb *Bgl*II germline fragment on chromosome 21 (data not shown). To isolate the fusion point of chromosomes 2 and 21, we next performed bubble PCR on genomic DNA and detected nested PCR products using primers AML1-GNM8-2S and NVAMP2 (Figure 4a). Sequence analysis of the subcloned PCR product revealed the genomic junction of 5′-*AML1-LAF4*-3′ (Figures 4c and d), and the result

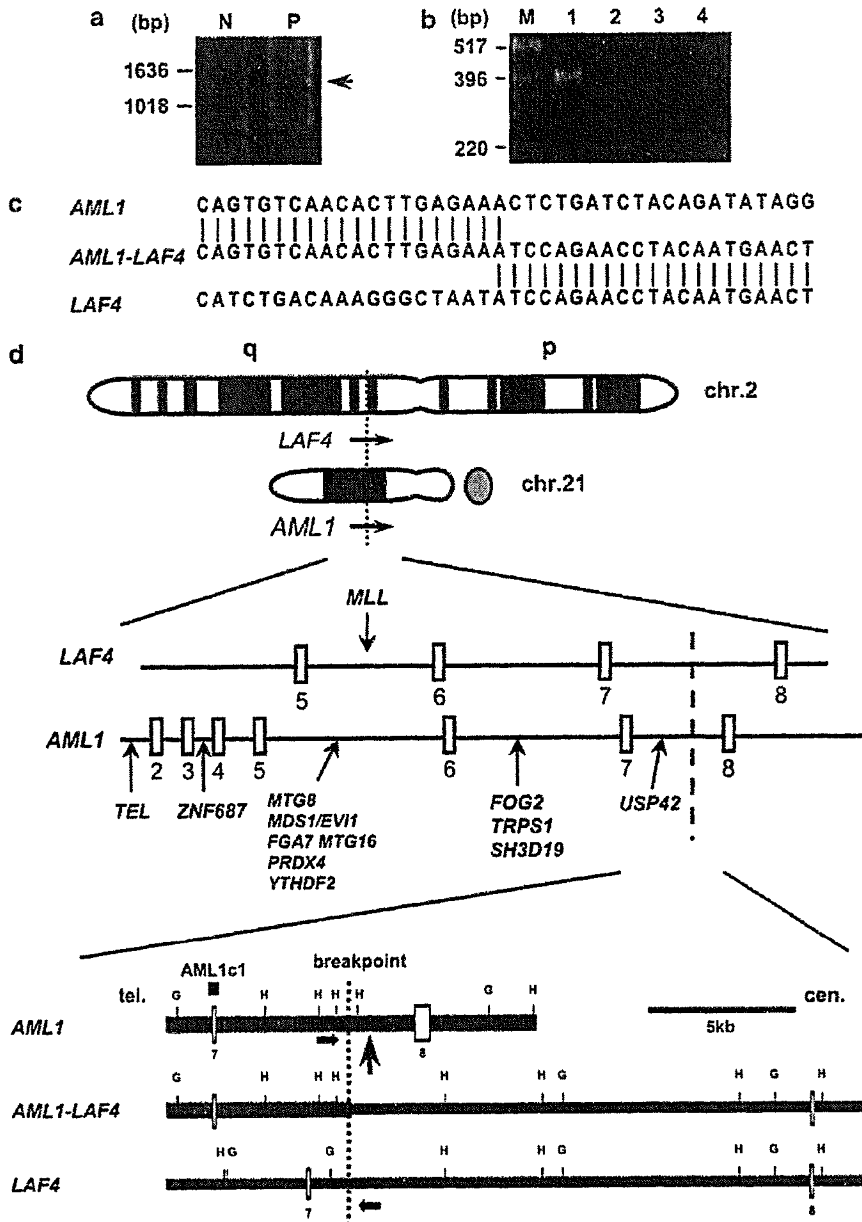

Figure 4 Cloning of the genomic junction of *AML1* and *LAF4*. (**a**) Bubble PCR for genomic DNA. N, normal human lymphocytes; P, patient's leukemic cells. (**b**) Detection of the genomic fusion point of *AML1-LAF4* by PCR. Primers were AML1-GNM8-4S and LAF4-GNM11-2AS (lanes 1 and 3), and LAF4-GNM11-2S and AML1-GNM8-2AS (lanes 2 and 4). Lanes 1 and 2, patient's leukemic cells; lanes 3 and 4, normal peripheral lymphocytes. M, size marker. (**c**) Sequences of breakpoints in the patient's leukemic cells. (**d**) Physical map of the breakpoint regions. Open vertical boxes represent defined exons in each gene. Horizontal arrows show the primers used. Restriction sites are indicated by capital letters: G, *Bgl*II; H, *Hin*dIII. AML1c1 indicates the position of the cDNA probes for Southern blot analysis. A vertical arrow shows *AML1-USP42* breakpoint.

was confirmed by PCR analysis using primers AML1-GNM8-4S and LAF4-GNM11-2AS (Figure 4b); however, no 5′-*LAF4-AML1*-3′ product was generated, suggesting interstitial deletion near genomic break points (Figure 4b). These sequences near the break points did not contain any lymphoid heptamer/nonamer sequences, *Alu* sequences or consensus topoisomerase II cleavage sites.

Discussion

In this study, we identified that *LAF4* was fused to *AML1* in pediatric T-ALL with t(2;21)(q11;q22). Other regions with chromosomal aberrations in this patient were not considered to be associated with recurrent cytogenetic changes involving T-ALL, except for the deletion of the short arm of chromosome 9. Spectral

Table 1 Comparison between bubble PCR and panhandle PCR

Characteristics	*Bubble PCR*	*Panhandle PCR*
Available orientation of fusion transcript	5′–3′, 3′–5′	Only 5′–3′
AML1-specific random hexamer[a]	Not necessary	Necessary
Self-annealing	Not necessary	Necessary
Number of required polymerase reaction	2	4
Number of final products	Many (smear)	A few
Nonspecific product	Few	Few
Number of extra sequences other than targeted sequences in cloned product	50–60 bp	> 100 bp
Search for other targeted exons	Easy	Hard[b]

[a]30-mers *AML1*-specific oligonucleotide with random hexamer (AML1-N). [b]Necessary to use another *AML1*-specific random hexamer if the target exons are 5′ region of the initial target.

handle any exons fused to unknown partners for amplification. Once-ligated cDNAs are also available for cloning any genes, other than *AML1*, as the target. We demonstrated the efficiency and specificity of bubble PCR for cDNA (Table 1 and Supplementary Figure S2).

To date, a great number of fusion genes associated with chromosomal translocations have been cloned, although these fusion genes are found as a minor part of various malignancies. Recently, high frequencies of mutations in *NOTCH1* in T-ALL (James *et al.*, 2005), *NPM* in AML with normal karyotype (Weng *et al.*, 2004) and *JAK2* in myeloproliferative disorders (polycythemia vera, essential thrombocythemia and idiopathic myelofibrosis) (James *et al.*, 2005) have been reported, and these mutations are considered to be a good target for therapy. These genes were first identified as associated with chromosomal translocations in a small subset of specific phenotypes of hematologic malignancies (Ellisen *et al.*, 1991; Morris *et al.*, 1994; Lacronique *et al.*, 1997). These findings suggest that continuing attempts to identify genes associated with chromosomal translocations can be expected to provide further insights into the significance of various gene alterations in cancer and the development of novel-targeted therapies (Taki and Taniwaki, 2006). The bubble PCR method for cDNA will contribute to identifying numerous novel translocation partners more easily and further functional analysis of chimeric transcripts.

Materials and methods

Spectral karyotyping analysis
Spectral karyotyping analysis was performed with a Sky-Painting kit (Applied Spectral Imaging, Migdal Ha'Emek, Israel). Signal detection was performed according to the manufacturer's instructions.

Fluorescence in situ *hybridization analysis*
Fluorescence *in situ* hybridization analysis of the patient's leukemic cells using *AML1*-specific BAC clones (RP11-272A3, 3′ of *AML1* and RP11-994N6, 5′ of *AML1*) was carried out as described previously (Taniwaki *et al.*, 1994). Fusion of *AML1* and *LAF4* was analysed with the patient's leukemic cells using RP11-994N6 (5′ of *AML1*) and RP11-527J8 (3′ of *LAF4*).

Bubble PCR for cDNA
We modified the original bubble PCR method to apply for cDNA cloning (Figure 2; Supplementary Figure S2) (Smith, 1992; Zhang *et al.*, 1995).

Poly(A)$^+$ RNA was extracted from the patient's leukemic cells using a QuickPrep Micro mRNA Purification Kit (GE Healthcare, Buckinghamshire, UK). Two hundred nanograms of poly(A)$^+$ RNA was reverse transcribed to cDNA in a total volume of 33 µl with random hexanucleotide using the Ready-To-Go You-Prime First-Strand Beads (GE Healthcare). Double-stranded cDNAs were synthesized from 10 µl of single-stranded cDNA with a phosphorylated random hexanucleotide, blunt ended with T4 DNA polymerase, digested with *Rsa*I endonuclease and ligated with bubble oligo. *Rsa*I, a 4-bp blunt-ended cutter, was chosen to shorten the bubble oligo-ligated fragments, so that almost all bubble oligo-ligated fragments would be easy to clone by standard PCR reaction. This suggests that poor-quality samples are also suited to this method, although it is unsuitable for cloning long products.

The sequences of the primers used are listed in Supplementary Table S1 and their positions in the *AML1* gene are shown in Supplementary Figure S2. Nested PCR was performed using primers NVAMP-1 (bubble oligo) and AML1-5S (exon 5) for first round PCR, and NVAMP-2 (bubble oligo) and AML1-E6S (exon 6) for nested PCR. NVAMP1 and NVAMP2 can only anneal to the newly synthesized unique sequence of the bubble oligo by AML1-5S.

We used poly(A)$^+$ RNA in bubble PCR for cDNA with the expectation that this approach could amplify fewer transcripts; however, total RNA is also suitable for this method.

Bubble PCR for genomic DNA
Bubble PCR for genomic DNA was performed as described previously (Smith, 1992; Zhang *et al.*, 1995). Primers were as follows: NVAMP-1 and AML1-GNM8S for first round PCR, and NVAMP-2 and AML1-GNM8-2S for second round PCR (Supplementary Table S1).

Reverse transcription–PCR and genomic PCR analyses
Reverse transcription–PCR and genomic PCR analyses were performed as described previously. After 35 rounds of PCR (30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C), 5µl of PCR product were electrophoresed in a 3% agarose gel. Primers were as follows: AML1-PR7 and LAF4-11AS, and AML1-PR8 and LAF4-PR5 for reverse transcription-PCR; and AML1-GNM8-4S and LAF4-GNM11-2AS, and LAF4-GNM11-2S and AML1-GNM8-2AS for genomic PCR (Supplementary Table S1).

Nucleotide sequencing
Nucleotide sequences of PCR products and, if necessary, subcloned PCR products were analysed as described previously (Hiwatari *et al.*, 2003).

Southern blot analysis
High-molecular-weight DNA was extracted from the patient's leukemic cells by proteinase K digestion and phenol/chloroform extraction. DNA (10 µg) was digested with *Bgl*II, subjected to electrophoresis on 0.7% agarose gel and transferred to a nylon membrane. Blots were hybridized to probes that were labeled by the Dig-labeled PCR method according to the manufacturer's instructions (Roche Applied Science, Tokyo, Japan). Probes

karyotyping analysis detected del(9)(p13), and additional analysis of genome array (Human Mapping 50 K Hind Array, Affymetrix, Tokyo, Japan) revealed homozygous deletion of 4.5 Mb within the 9p21 region, including the *CDKN2A/p16/p14* locus (data not shown), which is frequently deleted in T-ALL (Ohnishi *et al.*, 1995).

Although the patient showed a complex chromosomal abnormality, t(2;21)(q11;q22) can form regular head-to-tail fusion transcripts of both *AML1* and *LAF4*, because the transcription direction of *AML1* and *LAF4* is telomere to centromere. Furthermore, fluorescence *in situ* hybridization analysis revealed two der(2)t(2;21)(q11.2;q22) creating 5′-*AML1-LAF4*-3′, suggesting that 5′-*AML1-LAF4*-3′ is critical for leukemogenesis.

LAF4 was previously reported to be a fusion partner of *MLL* in pediatric B-precursor ALL with t(2;21)(q11;q23) (von Bergh *et al.*, 2002; Bruch *et al.*, 2003; Hiwatari *et al.*, 2003). *LAF4* is the first gene fused to both *AML1* and *MLL*, and both *AML1-LAF4* and *MLL-LAF4* contained the same domains of *LAF4* (Figure 5). During the preparation of this manuscript, we found another pediatric T-ALL patient with *AML1-LAF4* reported in the Meeting Abstract (Abe *et al.*, *Blood* (ASH Annual Meeting Abstracts) 2006; **108**: 4276), suggesting that t(2;21)(q11;q23) is a recurrent cytogenetic abnormality and that the *AML1-LAF4* fusion gene is associated with the T-ALL phenotype. Both putative fusion proteins of AML1-LAF4 observed in two patients contained the Runt domain of AML1, and the transactivation domain, nuclear localization sequence and C-terminal homology domain of LAF4, although the fused exon of *LAF4* differed in the two cases. Several studies have reported that the fusion partners of *MLL* fused with different genes such as *MLL-AF10* and *CALM-AF10*, *MLL-CBP* and *MOZ-CBP* or *MLL-p300* and *MOZ-p300* (Ida *et al.*, 1997; Taki *et al.*, 1997; Chaffanet *et al.*, 2000). Comparison of the structure and function between *AML1-LAF4* and *MLL-LAF4* will facilitate our understanding of the molecular mechanisms underlying *AML1*- and *MLL*-related leukemia.

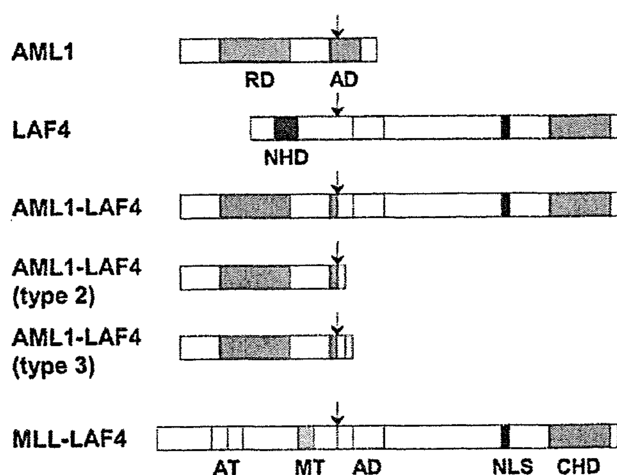

Figure 5 Schematic representation of putative AML1, LAF4 and AML1-LAF4 fusion proteins. Putative MLL-LAF4 fusion protein is also indicated for comparison. Arrows, break points or fusion points; AD, transactivation domain; AT, AT hooks; CHD, C-terminal homology domain; DNA, methyltransferase homology region; RD, RUNT domain; MT, DNA methyltransferase homology region; NLS, nuclear localization sequence.

The only *AML1* fusion partners in T-ALL are *LAF4* and *FGA7*. It is not known how *FGA7* is associated with T-ALL leukemogenesis, because *FGA7* does not show any significant sequence homology to any known protein motifs and/or domains (Mikhail *et al.*, 2004). Both patients with *AML1-LAF4* and *MLL-LAF4* fusions were diagnosed as having ALL, but they have different lymphoid lineages. *MLL-LAF4* is associated with B-lineage ALL; however, *AML1-LAF4* generates T-ALL. Our previous study showed that *LAF4* was expressed not only in B-lineage ALL but also in T-lineage ALL cell lines (Hiwatari *et al.*, 2003). *LAF4* showed strong sequence similarity to *AF4* (Ma and Staudt, 1996), which has a role in the differentiation of both B and T cells in mice (Isnard *et al.*, 2000). Furthermore, it was reported that *AML1* also plays an important role in T- and B-cell differentiation, because *AML1*-deficient bone marrow increased defective T- and B-lymphocyte development (Ichikawa *et al.*, 2004). These findings support that both *AML1* and *LAF4* are associated with T-ALL, respectively. Further functional analysis of the *AML1-LAF4* fusion gene will provide new insights into the leukemogenesis of *AML1*-related T-ALL. Recently, it has been reported that C-terminal truncated AML1-related fusion proteins play critical roles in leukemogenesis (Yan *et al.*, 2004, Agerstam *et al.*, 2007), suggesting that the two additional types of fusion transcripts observed in our patient (types 2 and 3 in Figures 3d and 5) have an additional function in leukemogenesis other than that of the entire AML1-LAF4 fusion protein.

In this study, we first applied the panhandle PCR method, which is usually used for cloning the fusion partners of *MLL* or *NUP98* (Megonigal *et al.*, 2000; Taketani *et al.*, 2002); however, no fusion transcripts could be obtained. Therefore, we searched for another method to clone the fusion transcripts and adapted the bubble PCR method for cDNA cloning. To date, bubble PCR has been performed for cloning unknown genomic fusion points but not fusion cDNAs (Zhang *et al.*, 1995). Using double-stranded cDNA, we could apply the bubble PCR method for cloning fusion cDNA with fewer nonspecific products. The bubble PCR primer can only prime DNA synthesis after a first-strand cDNA has been generated by an *AML1*-specific primer because of the bubble-tag with an internal non-complementary region (Zhang *et al.*, 1995). Although bubble PCR for genomic DNA generated one or two amplification products (Smith, 1992), bubble PCR for cDNA generated a complex set of amplification products that appeared as a smear by SYBR green staining, suggesting that a random hexamer generated various double-stranded cDNA containing the *AML1* sequence. This means that various fusion points can be estimated, even if after bubble oligo ligation was generated. Furthermore, bubble PCR for cDNA could amplify in both 5′–3′ and 3′–5′ directions of the gene or transcript, and easily

were 112 bp *AML1* cDNA fragments (AML1c1, nucleotides 1233–1344; GenBank accession no. NM_001754).

Abbreviations

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia.

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