Fig. 1. A clinical course of the patient performed haploidentical transplantation using in vivo alemtuzumab. The CMV antigenemia was first detected early after transplantation and preemptive antiviral treatment with standard dose of intravenous ganciclovir was initiated. Because it was not effective, we changed antiviral agents from ganciclovir to foscarnet and then to the combination therapy of ganciclovir and foscarnet. After failing the increased dose of ganciclovir, we finally administered cidofovir, which resulted in the clearance of CMV reactivation.

activity [Chou, 1999]. Therefore, the mutations in UL97, especially at codons 460, 592, 594, and 595, are closely related to the resistance to ganciclovir [Chou et al., 1995; Chou, 1999]. On the other hand, UL54 encodes DNA polymerase, the main inhibitory target of antiviral agents including ganciclovir, foscarnet, and cidofovir. Therefore, the mutations in UL54 may be involved in resistance to all of these antiviral agents. However, ganciclovir resistance due solely to UL54 is rare [Smith et al., 1997], whereas foscarnet resistance is closely related to UL54 mutations. The appearance of UL54 mutations following UL97 mutations has been shown to be a higher level of resistance to ganciclovir [Erice et al.,

1997; Smith et al., 1997; Wolf et al., 2003; Hantz et al., 2005]. The clinical isolate of the current patient showed four and two amino acid differences from the reference strains (Towne and AD169) in the UL54 and UL97 regions, respectively. The UL54 mutations included Q578H mutation, that is located in the δ-region C. The Q578H mutation has not been identified in a clinical isolate but has been reported to cause 10 folds resistance to foscarnet but only twice to ganciclovir in the isolate selected after in vitro passage under drug [Mousavi-Jazi et al., 2003]. However, the high-level foscarnet resistance suggested that the other three mutations (V11L, S655L, and G874R), which were not in the conserved

TABLE II. Difference Between Towne and AD169 Versus CMV Isolate

(A) Detected mutations between Towne and AD169 versus CMV isolate

Mutation	Number	%	Common mutations with the AD169 and Towne strains	%
T to C/A to G	56	43	19	54.3
C to T/G to A	56	43	9	25.7
C to A/G to T	11	7.70	1	2.9
G to C/C to G	4	3.10	1	2.9
T to G/A to C	3	2.30	5	14.3
T to A/A to T	0	0	0	0
Total	130	99	35	100
UL54	3,729 bases	95/7458 = 1.27%	24/7458 = 0.3%	
UL97	2,124 bases	35/4248 = 0.82%	11/4248 = 0.26%	

(B) Nucleotide and amino acid substitutions between Towne and AD169 versus CMV isolate
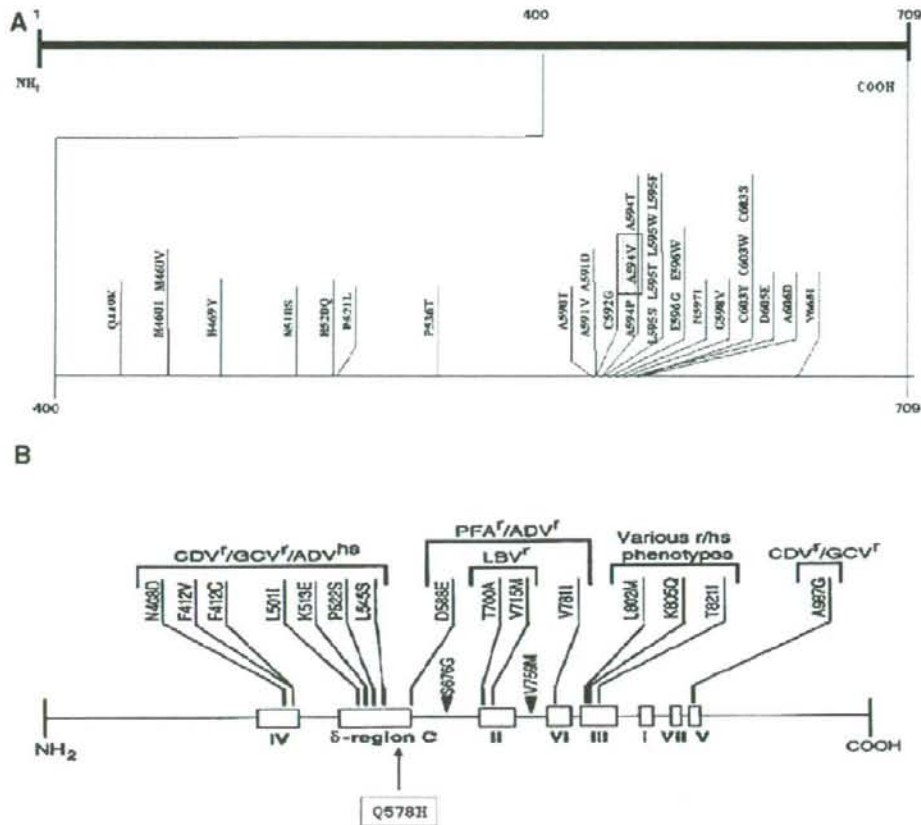| UL54	V11L, Q578H, S655L, G874R
UL97	A140V, A594V

Fig. 2. Mutations responsible for drug resistance in the CMV gene. Many mutations responsible for drug resistance in the UL97 and UL54 genes were reported. A: Mutations responsible for resistance to GCV in the UL97 gene [Erice, 1999; Lurain et al., 2001; Eckle et al., 2004]. B: Mutations responsible for drug resistance in the UL54 gene. GCV, ganciclovir; ADV, adefovir; PFA, foscarnet; CDV, cidofovir; LBV, lobucavir.

regions of DNA polymerase among different herpesviruses, might also have affected the susceptibility to foscarnet. Mutations in the UL54 region may cause cross-resistance to all of these antiviral agents, but interestingly, the mutations in the UL54 region in this strain did not affect the susceptibility to cidofovir. Similar ganciclovir-resistant mutants with resistance to ganciclovir and foscarnet but sensitivity to cidofovir have been reported [Erice et al., 1997; Smith et al., 1997; Erice, 1999; Lurain et al., 2001; Ducancelle et al., 2004]. The UL97 mutations included A594V, that has been reported to be associated with ganciclovir resistance [Abraham et al., 1999; Erice, 1999; Gilbert et al., 2001; Lurain et al., 2001; Ducancelle et al., 2004; Eckle et al., 2004; Scott et al., 2004]. Therefore, ganciclovir resistance was mainly caused by A594V mutation in the UL97 region and probably enhanced by Q578H in the UL54

region. While the other four mutations in the UL97 and UL54 regions might have been involved in the development of resistance, these mutations have not been reported before and further studies are required to clarify the impact of these substitutions.

In conclusion, the emergence of the resistant CMV strain was observed in a patient who had undergone haploidentical hematopoietic stem cell transplantation with in vivo T- and B-cell depletion. Profound immunosuppression as well as the prolonged use of antiviral agents might have affected the emergence of resistant strains. However, the CMV reactivation and CMV disease were successfully treated with cidofovir, selected according to the in vitro susceptibility assay. Therefore, as the Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation recommended, resistance testing should be

performed for patients who failed first-line antiviral treatment allowing selection of the correct second-line antiviral therapy [Ljungman et al., 2004].

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Autologous Stem Cell Transplantation with PCR-Negative Graft Would Be Associated with a Favorable Outcome in Core-Binding Factor Acute Myeloid Leukemia

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Although core-binding factor acute myeloid leukemia (CBF-AML) is generally considered to be a low-risk form of AML, the survival rate is still 50% to 60%. To evaluate the effectiveness of autologous stem cell transplantation (ASCT) with a PCR-negative graft we analyzed a series of consecutive CBF-AML patients. Between 1997 and 2006, 18 patients aged <60 years were referred under a diagnosis of CBF-AML. Peripheral blood stem cells (PBSC) were collected after a second or further course of postremission therapy. When >2.0 × 10^6/kg CD34-positive cells with minimal residual disease (MRD) undetectable by nested polymerase chain reaction (PCR) had been collected, ASCT was performed with busulfan, etoposide, and cytarabine combined with granulocyte colony-stimulating factor. Event-free survival (EFS) and complications of ASCT were then assessed. Fourteen of the 18 patients received ASCT. The median observation period was 4.4 years. The 5-year EFS was 93% for ASCT patients, despite the presence of adverse factors. In 8 of 10 patients who had detectable MRD in the bone marrow before ASCT, MRD became undetectable after ASCT. Neutrophils recovered promptly within 2 weeks, but platelets recovered relatively slowly. Half of the patients suffered from varicella zoster virus infection. Although 1 case of myelodysplastic syndrome occurred, there was no case of relapse. ASCT with a PCR-negative graft was associated with excellent EFS. For patients with CBF-AML, especially with adverse factors or remnant MRD in the bone marrow, this strategy is the treatment of choice.

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KEY WORDS: Core binding factor acute myeloid leukemia, Autologous stem cell transplantation, Minimal residual disease, Polymerase chain reaction

INTRODUCTION

Translocation (8;21) (q22;q22) or inversion (16) occurs in approximately 7% to 8% of patients with de novo acute myeloid leukemia (AML) [1]. These leukemia entities are associated with aberration of core-binding factors (CBF), which are heterodimeric transcriptional regulators containing a common β (CBFβ) and 1 of 3 α (CBFα) subunits. Translocation fuses the AML1 (CBFα2) gene located on chromosome 21 to the ETO (MTG8) gene located on chromosome 8. The CBFβ gene located at 16q22 fuses with the MYH11 gene located at 16p13. The AML1-ETO or CBFβ-MYH11 fusion protein represses and alters the function of CBF during normal differentiation [2].

Both cytogenetic groups (referred to as CBF-AML) have a relatively favorable prognosis compared with most other forms of adult AML [1,3-5]. In younger patients, repeated cycles of high-dose cytarabine (HDAC) therapy can prolong survival [6,7].

Prognostic factors of CBF-AML have been evaluated in several studies. In t(8;21) AML patients, inferior outcome has been associated with a high white blood cell (WBC) count [8], a low platelet count [8,9], a high WBC index [10], loss of sex chromosomes [8], expression of CD56 antigen [11], extramedullary disease [12], non-White race [9], and older age [9]. In inv(16) AML patients, a high WBC count [13,14],

older age [9,15], a low platelet count [9,15], and absence of the additional aberration of trisomy 22 [8] have been considered to be adverse factors. In addition, c-*KIT* mutations have recently been identified as adverse factors for CBF-AML [16].

Although opinion about these adverse factors varies, disease-free survival (DFS) is estimated to be <50% [8-15] when patients with CBF-AML have at least 1 adverse factor. In addition, response rate and survival after first relapse are low and short in t(8;21) AML [8,9].

The European Group for Bone and Marrow Transplantation and the Japanese Society of Hematopoietic Stem Cell Transplantation have recommended autologous stem cell transplantation (ASCT) for selected AML patients achieving first complete remission (CR1) [17,18]. Based on these guidelines, ASCT has been performed at our center for younger AML patients with favorable risk other than acute promyelocytic leukemia or with intermediate risk without a human leukocyte antigen (HLA)-matched sibling. In addition, we have infused peripheral blood stem cells (PBSC) in which minimal residual disease (MRD) is undetectable using a nested polymerase chain reaction (PCR) (ASCT with a PCR-negative graft).

We have retrospectively analyzed a series of younger consecutive CBF-AML patients and evaluated ASCT using a PCR-negative graft.

MATERIALS AND METHODS

Patients

The analysis included all consecutive patients aged <60 years with CBF-AML diagnosed and treated at our institution between October 1997 and November 2006. In this survey, CBF-AML was defined by the presence of either t(8;21)(q22;q22) or inv(16)(p13q22)/t(16;16)(p13;q22) chromosomal rearrangement, or by the presence of the AML1-ETO (MTG8) fusion gene or the presence of the CBFβ-MYH11 fusion gene confirmed by PCR. Although this study was not a formal clinical trial, all events and information were systematically recorded and available. This analysis was approved by our institutional review board.

Therapy

Induction therapy for younger patients aged <60 years was started with idarubicin (12 mg/m^2 days 1-3) and cytarabine (100 mg/m^2 days 1-7). After achievement of complete remission, the patients received postremission chemotherapy as follows: first postremission chemotherapy, idarubicin (12 mg/m^2, days 1-2) and cytarabine (2 g/m^2, every 12 hours, days 1-4); second therapy, enocitabine (200 mg/m^2,

days 1-7), etoposide (100 mg/m^2, days 1-5), daunorubicin (50 mg/m^2, days 1-3) and mercaptopurine (70 mg/m^2, days 1-7); third therapy, mitoxantrone (10 mg/m^2, days 1-2), etoposide (100 mg/m^2, days 1-4), and cytarabine (1 g/m^2, every 12 hours, days 1-4); fourth to seventh therapy, cytarabine (3 g/m^2, every 12 hours, days 1,3,5).

If a patient had neither active infection nor sepsis, PBSC were mobilized by granulocyte colony-stimulating factor (G-CSF), and a collection of PBSC was attempted in the phase of recovery from myelosuppression after second or further postremission chemotherapy. One cycle of PBSC collection was defined as a sequential collection course after 1 chemotherapy course. PBSC collection was repeatedly attempted within a maximum of 3 cycles. When more than 2.0 × 10^6/kg CD34-positive cells in which MRD was undetectable by nested PCR had been collected, ASCT was attempted after a third or further session of postremission chemotherapy. Although the ideal doses were >2.0 × 10^6/kg CD34-positive cells, in fact, ASCT was performed when at least 1.5 × 10^6/kg CD34-positive cells were collected after 3 cycles of PBSC collections had been attempted.

The conditioning regimen for ASCT was G-CSF combined with BEA [19] as follows: busulfan (4 mg/kg/day for 4 days as 1 mg/kg four times a day for 16 doses on days −9∼−6), etoposide (20 mg/kg on days −5∼−4), cytarabine (100 mg/m^2 on days −10∼−4, 3 g/m^2 every 12 hours on days −3∼−2), and filgrastim 200 μg/m^2 on days −12∼−4). PBSC were administered on day 0. Filgrastim (300 μg) was started on day 1 until recovery of granulocytes. Prophylactic levofloxacin or tosufloxacin 300 mg/day, fluconazole 200 mg/day, and acyclovir 1000 mg/day were administered from day −7 until neutrophils had recovered to >0.5 × 10^9/L.

Minimal Residual Disease Monitoring

We evaluated the bone marrow (BM) and harvested PBSC for assessment of MRD. From 2001, we used quantitative real-time reverse transcriptase PCR (RQ-PCR) for detection of MRD in BM. Before 2000, MRD in BM was evaluated by fluorescence in situ hybridization or nested reverse transcriptase PCR (nested RT-PCR). MRD in PBSC were evaluated by nested RT-PCR.

For PCR, total RNA was extracted from mononuclear cells in BM and transcribed to cDNA in accordance with the manufacturer's instructions. RT-PCR assay [20-23] was performed by Taqman technology using the following primers: for AML1/MTG8 chimeric mRNA, forward 5'...GAG CCA TCA AAA TCA CAG TGG A ...3', reverse 5'...ATG AAC TGG TTC TTG GAG CTC CTT ...3', and probe 5' FAM (6-carboxylfluorescein)...CAC CTG TGG

ATG TGA AGA CGC AAT CTA GGC TG...TAMRA (6-carboxy-tetramethyl-rhodamine) 3'; for CBFβ/MYH11 chimeric mRNA, forward 5'...CTC CAA AGA CTG GAT GGT ATG GGC ...3', reverse 5'...CTT GGA CTT CTC CAG CTC ATG G ...3', and probe 5' FAM...TCT GGA GTT TGA TGA GGA GCG AGC CC...TAMRA 3'. Nested RT-PCR was performed in accordance with previous reports [21-23]. For RQ-PCR, the number of transcript copies was normalized relative to glycer-aldehyde 3-phosphate dehydrogenase, and converted into molecules/μg RNA. The detection threshold of RQ-PCR was 50 copies/μg RNA and the sensitivity was 10^{-4}. The threshold of nested RT-PCR was 10^{-5}.

Mutational Analysis of c-KIT

Mutational analysis of the extracellular (EC) do-main (exons 8 and 9), transmembrane (TM) domain (exon 10), juxtamembrane (JM) domain (exon 11), and the second intracellular kinase (TK) 2 domain (exons 17 and 18) of the c-KIT gene was performed with PCR followed by direct sequencing. The geno-mic DNA from Wright-Giemsa-stained or unstained blood smears was extracted with a Gentra Puregene Tissue Kit (Qiagen, Hilden, Germany). TaKaRa LA Taq DNA polymerase (Takara, Shiga, Japan) was used to amplify the genes from genomic DNA. PCR products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced bidirectionally using the Big Dye Termination 3.1 kit and the ABI Prism 310 system (Perkin-Elmer Cetus, Norwalk, CT). Specific sequences of primers used for PCR and sequencing are available upon request. To validate the sequencing results, PCR products were inserted into the pCR2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Recombinant plasmids isolated from 8 to 12 white colonies were sequenced.

Statistical Analysis

The major indicator of outcome was event-free survival (EFS), defined as the period from initial diag-nosis to relapse (failure), secondary malignancy (fail-ure), death because of any cause (failure), and alive at last follow-up (censored). Overall survival (OS) was also assessed, and was defined as the period from initial diagnosis to death because of any cause (failure), and alive at last follow-up (censored). Estimations of EFS and OS distributions were performed by the Kaplan-Meier method. Comparisons of patient characteristics were performed by χ^2 test for categorical variables and by the Mann-Whitney U test for continuous variables. A Cox hazard model was used for univariate analysis of prognostic factors. Estimates of hazard ratios (HR) and corresponding 95% confidence intervals (CIs) were obtained for each of the following variables:

gender, age, WBC, hemoglobin, platelets, lactate dehydrogenase (LDH), percentage of blasts in periph-eral blood or BM, WBC index, karyotype aberration, CD56 positivity, and total dose of cytarabine. WBC index was derived as the product of WBC and the ratio of marrow blasts at diagnosis [10]. To assess the impact on EFS of a cytarabine dosage exceeding 1 g/m², the actual cumulative dosage was calculated and entered as a continuous variable for univariate analysis. For all analyses, statistical significance was defined as a 2-sided value of $P < .05$. Statistical analyses were performed with StatView version 5.0.

RESULTS

Patient Characteristics

Between October 1997 and November 2006, 25 patients were diagnosed as having CBF-AML. Of these patients, 18 were <60 years old and eligible for ASCT. Sixteen had t(8;21) AML and 2 had inv(16) AML (Table 1). Fourteen patients actually received ASCT. The remaining 4 who did not received ASCT included 2 with relapse and secondary myelodysplasia during postremission chemotherapy, 1 with poor mobilization of PBSC, and 1 who withdrew consent to treatment. The relapsed and MDS patients received allogeneic transplantation at other hospitals and were lost to follow-up.

Between patients with and without ASCT, there were no significant differences in additional chromo-some aberrations, CD56 positivity or WBC index. However, patients with ASCT had moderately lower platelet counts as well as more blasts in their peripheral blood and BM at diagnosis, and received a lower cu-mulative dose of cytarabine, although the differences were not statistically significant.

Survival Analysis

The median period of observation of survivors was 4.4 years. All of 18 patients achieved complete remis-sion after induction therapy. The estimated 5-year EFS for these patients and the patients with ASCT was 83.0% ± 9.0% (±standard error) and 92.9% ± 6.9%, respectively (Figure 1). The estimated 5-year OS for the 18 patients was 100%, although follow-up details were lost for relapsed and MDS patients who later underwent allogeneic transplantation at other hospitals. Univariate analysis of prognostic fac-tors for EFS showed that ASCT was the only signifi-cant factor (HR 12.9 [CIs: 1.05-157, $P = .045$]), and that age, WBC, cumulative dose of cytarabine, CD56 positivity, loss of sex chromosomes, and lower platelet count had no prognostic value for EFS. Anal-ysis of OS was not done because none of the patients died.

Table I. Patient Characteristics and Clinical Features

	Total	With ASCT	Without ASCT	P Value
Number of patients	18	14	4	
Gender				
male	15	11	4	*.80
female	3	3	0	
Age (years)				
median	44	44	51	†.2
range	20–59	20–59	43–53	
WBC (x10⁹/L)				
median	5.5	5.5	6.8	†.91
range	1.7–82	1.7–82	2.5–37	
Hb (g/L)				
median	85.5	78	99	†.46
range	38–131	38–131	77–12.6	
Plt (x10⁹/L)				
median	35	24	58	†.089
range	7.0–101	7.0–101	44–81	
LDH (IU/L)				
median	661	872	644	†.75
range	245–6090	245–6090	420–1865	
PB blast (%)				
median	47	49.5	32.8	†.22
range	13–90	24.5–90	18–82.5	
BM blast (%)				
median	67.3	71.2	61.25	†.24
range	26.4–84.4	26.4–84.4	50.4–64.3	
WBC index				
Low	5	5	2	*.80
Intermediate	6	6	1	
High	3	3	1	
Karyotype				
t(8;21)	16			*.50
only t(8;21)	7	5	2	
-X or -Y	8	7	1	
del(9q)	3	2	1	
inv(16)	2			
only inv(16)	2	2	0	
trisomy 22	0	0	0	
CD56‡				
+	8	5	3	*.67
–	4	3	1	
Extramedullary involvement	0	0	0	
Cumulative AC (g/m²)	48 (12–114)	48 (12–78)	60 (30–114)	†.3

ASCT indicates autologous stem cell transplantation; WBC, white blood cell; Hb, hemoglobin; plt, platelet; LDH, lactate dehydrogenase; PB, peripheral blood; BM, bone marrow; cumulative AC, the actual cumulative dosage of cytarabine exceeding 1 g/m²; WBC index, WBC x [% of marrow blast] low index <2.5, intermeditate index between 2.5 and 20; high index 20 or more.

P-value was calculated by x^2 exact test(*) or U exact test of Mann Whitney(†). P < .05 was considered as significant value. ‡ CD56 expression was investigated in 12 of total 18 patients.

ASCT and Clinical Features

Fourteen patients received ASCT using an identical conditioning regimen and PBSC with undetectable MRD. The median period from diagnosis to ASCT was about 9 months (Table 2). A median of 5 chemotherapy courses were given before ASCT. Neutrophil counts recovered to more than 0.5×10^9/L within a median period of 13 days (range: 11~36 days). Platelet counts recovered to $>20 \times 10^9$/L without transfusion within a median period of 27.5 days (range:
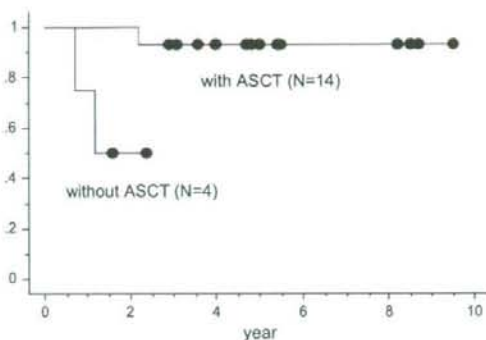

Figure I. Kaplan-Meier curves of EFS of the subgroup of patients with (n = 14) and without (n = 4) autologous stem cell transplantation. ASCT: autologous stem cell transplantation.

12~217 days) and to $>50 \times 10^9$/L within a median period of 4 months (range: 15–378 days). Infusion of more CD34 cells was associated with prompter platelet recovery ($P = .027$ for platelets $>20 \times 10^9$/L and $P = .038$ for platelets $>50 \times 10^9$/L, calculated by Pearson's correlation coefficient). As late adverse events, 5 patients suffered varicella zoster virus (VZV) reactivation, which was promptly resolved with acyclovir. These infections occurred at a median of 130 days after ASCT (range: 85-567 days). Although 1 case of secondary myelodysplasia was observed after ASCT, no relapse occurred.

Details of PBSC Collection Among ASCT Patients

In total, 23 cycles of PBSC collection were performed in 14 patients. A median of 1.85×10^6/kg CD34-positive cells were collected in each cycle. Eight cycles of PBSC collection were performed after the fourth session of postremission chemotherapy, 4 cycles after the fifth and sixth sessions, and 3 cycles after the second and third sessions. In 1 cycle, collections

Table 2. Clinical Features of Patients with ASCT

Total number	14 cases
Conditioning regimen Bu+VP16+AraC	14 cases
Median time to stem cell transplantation*	273 days (167–375)*
Median number of prior chemotherapy sessions*	5 times (3–8)*
Median number of cycle of PBSCH*	2 times (1–3)*
Median number of infused CD34 positive cells*	2.6×10^6/kg (1.5–4.4)*
Recovery of neutrophil count (>0.5 × 10⁹/L)	13 days (11–36)*
Recovery of platelet count (>20×10⁹/L)	27.5 days (12–217)*
Recovery of platelet count (>50×10⁹/L)*	123 days (15–378)*
Median observation duration after transplantation*	4.3 years (1.3–8.9)*
Late adverse events	
MDS/sAML	1 case
VZV	5 cases
pneumonia	1 case
meningitis	1 case

PBSCH indicates harvest of peripheral blood stem cell; MDS/sAML, myelodysplastic syndrome/secondary acute myeloid leukemia.
*Ranges are shown in parentheses.

Table 3. Details of Timings and Doses of CD34 Positive Cells in Peripheral Blood Stem Cell Collections

| | | | | CD34 Doses/days of Collection in Each Cycle | | | | |
| | | | | Post-remission chemotherapy | | | | |
Pt	1st	2nd	3rd	4th	5th	6th	7th	Actually infused CD34 Cell Dose
1				1.0×10^6/kg/2	1.9×10^6/kg/1			2.9×10^6/kg
2					1.8×10^6/kg/2	0.28×10^6/kg/1		2.0×10^6/kg
3				0.68×10^6/kg/3	2.9×10^6/kg/2			3.4×10^6/kg
4				2.4×10^6/kg/1				2.4×10^6/kg
5				(×)1.2×10^6/kg/2	(×) 0.62×10^6/kg/3	1.5×10^6/kg/4		1.5×10^6/kg
6						(×) 4.6×10^6/kg/1	2.2×10^6/kg/2	2.2×10^6/kg
7						3.7×10^6/kg/4		3.7×10^6/kg
8		8.3×10^6/kg/3						2.7×10^6/kg
9			3.5×10^6/kg/2					3.5×10^6/kg
10				4.4×10^6/kg/2				4.4×10^6/kg
11				2.9×10^6/kg/2				2.9×10^6/kg
12		0.37×10^6/kg/3	0.88×10^6/kg/4	0.15×10^6/kg/1				1.6×10^6/kg
13			2.4×10^6/kg/1	1.3×10^6/kg/2				2.6×10^6/kg
14		2.0×10^5/kg/1						2.0×10^6/kg

Pt indicates patient number; (X) means that MRD was detected in collected peripheral blood stem cells.

were performed for median 2 days (range: 1~4 days) (Table 3).

The PBSC in 3 cycles (patients 5 and 6) were inappropriate as grafts because MRD was detected by nested RT-PCR, and the PBSC were therefore discarded. For collection of PBSC with undetectable MRD, 1 cycle of PBSC collection was sufficient for 7 patients, and 2 cycles were needed in 5 patients (Table 3). In the remaining 2 patients, 3 cycles were performed but no more than 2.0×10^6/kg CD34-positive cells could be collected.

MRD in BM was assessed by RT-PCR before chemotherapy in each of 20 cycles (Table 4). MRD remained in BM in 18 of the investigated 20 cycles. However, PBSC with undetectable MRD were col-

lected in 15 of the 18 cycles. Finally, all 14 patients received PBSC with undetectable MRD.

MRD Monitoring of ASCT Patients

Table 4 shows the time courses of MRD and the timing of PBSC collections among ASCT patients. All of the patients sustained complete remission after ASCT, and MRD in BM remained at <100 copies/ μgRNA in all 14 patients and became undetectable by RT-PCR in 12 patients (patients 1-3 and 6-14). MRD in BM became undetectable by RT-PCR after ASCT in 8 (patients 1-3, 6, 8, 11, 13, and 14) of 10 patients (patients 1-6, 8, 11, 13, and 14) whose MRD in BM before ASCT was detectable by RT-PCR.

Table 4. Time Course of Minimal Residual Disease (MRD) among Those with Autologous Stem Cell Transplantation (ASCT)

| | | Postremission Chemotherapy‡ | | | | | | | | After ASCT | | | |
Pt	AML type	1st	2nd	3rd	4th	5th	6th	7th	ASCT‡	1 year	2 year	3 year	4 year
1	t(8;21)	●	⊗	⊗	⊗	⊗	—	—	⊗	○			
2	t(8;21)	NA	●	●	●	⊗	●	—	⊗	⊗	○		
3	t(8;21)	●●	●	●	⊗	—	—	—	⊗	○	○		
4	t(8;21)	NA	●●	●	●●	—	—	—	●●	⊗	⊗	⊗	
5	t(8;21)	NA	●	●	▦	▦	●	—	●	⊗	⊗	⊗	
6	t(8;21)	NA	●	●	⊗	⊗	▦	⊗	●	⊗	⊗	○	
7	t(8;21)	NA	⊗†	⊗	○	○	○	—	○	○	○	○	○
8	t(8;21)	●		●●	—	—	—	—	●	○	○		
9	t(8;21)	NA*	●	NA*	NA*	—	—	—	NA*	○	○	○	○
10	t(8;21)	NA	NA*	NA*	⊗†	—	—	—	NA*	○	○	○	○
11	t(8;21)	NA*	NA*	⊗†	⊗†	—	—	—	⊗†	○	○	○	○
12	t(8;21)	⊗†	⊗†	NA	○	—	—	—	○	NA	○	○	○
13	inv(16)	●	⊗	⊗	⊗	—	—	—	⊗	○	○	○	○
14	inv(16)	NA	NA	NA	—	—	—	—	⊗†	○	○	○	○

Pt indicates patient number; AML, acute myeloid leukemia; ASCT, autologous stem cell transplantation; —, postremission therapy was not performed; ●●, MRD was 10^3-10^4 copies by RQ-PCR; ●, MRD was 10^2-10^3 copies by RQ-PCR; ⊗, MRD was less than 10^2 copies by RQ-PCR or detectable by nested PCR; †, detectable by nested PCR; ○, MRD was undetectable by nested PCR or RQ-PCR; N.A, PCR was not performed; *, negative for t(8;21) by FISH; ‡, PCR sample was obtained just before the start of each postremission chemotherapy and conditioning of ASCT; □, Harvest of peripheral blood stem cells with MRD undetectable by nested PCR; ▦, Peripheral blood stem cells were not used because MRD was detectable by nested PCR.

Table 5. Analysis of c-kt Mutations of Leukemia Cells at Diagnosis

	Exon 8	Exon 10	Exon 11	Exon 12	Exon 13	Exon 17	Exon 18	Exon 19	Exon 20
Pt1	normal	normal	normal	normal	normal	normal	normal	normal	normal
Pt2	normal	normal	normal	normal	L813_A814 ins	normal	normal	normal	normal
Pt3	normal	normal	normal	normal	normal	normal	normal	normal	normal

Interestingly, in patients 2 and 6, molecular disappearance of AML1/ETO(MTG8) was confirmed by RT-PCR 2 and 3 years after each ASCT, respectively (Table 4). In the other 6 patients [patients 1, 3, 8, 11, 13, and 14) MRD was undetectable by RT-PCR 1 year after ASCT.

Analysis of c-KIT Mutations and MRD

c-KIT mutations were analyzed in 3 patients (patients 1-3). Pt.2 had c-KIT mutations on exon 17, and the others had no mutations (Table 5). All 3 patients remained in CR1. AML1/MTG8 chimera was undetectable by RQ-PCR in all 3 patients.

DISCUSSION

We analyzed the survival of 18 consecutive young patients with CBF-AML treated between 1997 and 2006 at our center, and revealed that EFS of ASCT with a PCR-negative graft was 93% with no incidence of relapse.

Neutrophils recovered promptly within 2 weeks, but platelets tended to recover more slowly, although severe hemorrhage was not a complication. After ASCT, half of the patients suffered late infections, especially VZV reactivation, at a median of 4 months after ASCT. Prolonged prophylaxis with acyclovir is reportedly effective for prevention of VZV reactivation [24]. The high rate of VZV reactivation in the present series may have been because of the short duration of prophylaxis.

Until more than 2.0×10^6/kg CD34-positive cells were collected, 1 cycle of PBSC collection was sufficient for half of the patients and 3 cycles were necessary for only 2. This number of collections seems average in comparison with other studies (median 2 times) [25,26]. Although MRD remained in the BM in most patients, a PCR-negative graft was obtainable, except in 3 cycles. This was consistent with other studies [27,28] in which MRD was observed less frequently in PBSC than in BM. Also in an animal model, leukemic contamination was reportedly not enhanced by G-CSF mobilization, and a different mechanism for mobilization of leukemic cells into peripheral blood was suggested [29].

Previous studies of ASCT in CBF-AML have indicated a survival of about 45% to 66% [8-15], which was not superior to that achieved with chemotherapy alone

[8,9]. Our present result was excellent in comparison with previous studies. This may have been because of the characteristics of the patients; our series might include only patients without adverse factors [8-15] or c-KIT mutations [16]. In fact, however, those with ASCT had at least 1 adverse prognostic factor other than non-White race (Table 1), including a case showing c-KIT mutation of exon 17 (Table 5), which is associated with a high rate of relapse, although the number of cases analyzed was too small to allow any conclusion to be drawn. The main reason for the good outcome in our series was probably because our ASCT strategy was based on MRD in PBSC (ASCT with a PCR-negative graft). A gene-marking study has suggested that relapse after autologous bone marrow transplantation originates from the graft [30]. In addition, graft contamination of leukemic cells is associated with rapid relapse and poor prognosis [31]. We employed grafts in which absence of MRD was confirmed by nested RT-PCR, and this would have contributed to the good outcome.

As our analysis was retrospective and involved a very small population, it might have included variable bias. Although the present study included truly consecutive patients, there might have been an institutional bias because of the discrepancy in the number of patients between t(8;21)-AML and inv(16)-AML and because only 2 patients as yearly average were referred as having CBF-AML in our institution. In addition, in general, ASCT was used for patients with good performance status and good control of leukemia, which would have contributed to the good outcome. However, the actual OS and EFS for ASCT with a PCR-negative graft were surprisingly good in our series (100% and 93%, respectively), and EFS for patients overall exceeded 80%. Therefore, a prospective trial will be needed to investigate further confirmation of ASCT with a PCR-negative graft.

MRD in BM before ASCT was detectable in 10 of the 12 investigated patients. In 8 of these, MRD in BM became undetectable by RT-PCR after ASCT. Interestingly, in patients 2 and 6 (Table 4), the AML1/ETO(MTG8) fusion transcript disappeared 2 and 3 years after each ASCT, respectively, without further therapy. Although the reason for the late disappearance of MRD was unclear, 1 possibility was an enhanced and reconstructed immune response after ASCT. The myeloablative conditioning regimen would also have eradicated leukemic stem cells with

self-renewal potential, so that the latent AML1/ETO fusion transcript may have been correlated with daughter leukemic cells without self-renewal potential [27].

The significance of MRD in CBF-AML has not yet been precisely evaluated because of the persistence of AML1/ETO and CBFβ/MYH11 in long survivors [13,32,33]. However, a lower frequency of gene fusion, especially undetectable MRD, is reportedly associated with long relapse-free survival (RFS) [13,34-36]. Therefore, our results suggest that ASCT with a graft that is PCR-negative for CBF-AML could be indicated not only for patients with adverse factors but also those with persistent MRD detectable by RQ-PCR after postremission therapy.

In conclusion, we have analyzed a series of consecutive CBF-AML patients, and found that those with ASCT had excellent EFS. Even if MRD was detectable in BM, it was possible to harvest a PCR-negative graft. Our ASCT strategy was based on graft MRD, and this was thought to have contributed to the excellent EFS and overcome other adverse factors. A large trial of ASCT with a PCR-negative graft is warranted for CBF-AML, especially in patients with adverse factors or with remnant MRD in BM after postremission therapy.

CONFLICT OF INTEREST

The authors report no potential conflicts of interest.

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Serum autotaxin measurement in haematological malignancies: a promising marker for follicular lymphoma

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Summary

Autotaxin (ATX) is a tumour cell motility-stimulating factor originally isolated from melanoma cell supernatants. ATX is identical to lysophospholipase D, which produces a bioactive lipid mediator, lysophosphatidic acid (LPA), from lysophosphatidylcholine. ATX is overexpressed in various malignancies, including Hodgkin lymphoma, and ATX may stimulate tumour progression via LPA production. The present study measured the serum ATX antigen levels in patients with haematological malignancies using a recently developed automated enzyme immunoassay. The serum ATX antigen levels in patients with B-cell neoplasms, especially follicular lymphoma (FL), were higher than those in healthy subjects. Serum ATX antigen levels in FL patients were associated with tumour burden and changed in parallel with the patients' clinical courses. The serum ATX antigen levels were little affected by inflammation, unlike the soluble interleukin-2 receptor and β2-microglobulin levels. As expected, the plasma LPA levels in FL patients were correlated with the serum ATX antigen levels. Given that leukaemic tumour cells from FL patients expressed ATX, the shedding of ATX from lymphoma cells probably leads to the elevation of serum ATX antigen levels. Our results suggest that the serum ATX antigen level may be a promising and novel marker for FL.

Keywords: autotaxin, lysophospholipase D, lysophosphatidic acid, lysophospholipid, follicular lymphoma.

Lysophosphatidic acid (LPA: monoacyl-sn-glycero-3-phosphate) is a lipid mediator with a variety of biological actions, such as cell proliferation and survival, cell migration and invasion, platelet activation and aggregation and smooth muscle cell contraction (Moolenaar et al, 2004; Birgbauer & Chun, 2006). In addition, mounting evidence points to a role for LPA in cancer progression (Mills & Moolenaar, 2003). Recent studies have shown that LPA is related to the

initiation or progression of ovarian (Xu *et al*, 1995), prostate (Xie *et al*, 2002), and other cancers (Xu *et al*, 1995; Shida *et al*, 2003). LPA acts via specific G protein-coupled receptors (GPCRs) on the cell surface and activates a variety of signalling pathways (Valentine *et al*, 2008). Five LPA-specific GPCRs are as follows: LPA$_{1-3}$, which belong to the endothe-lial differentiation gene (EDG) family (Hecht *et al*, 1996; An *et al*, 1998; Bandoh *et al*, 1999), LPA$_4$/p2y9/GPR23 (Noguchi *et al*, 2003), and LPA$_5$/GPR92 (Lee *et al*, 2006). The aberrant expression of LPA receptors has been detected in various cancers (Mills & Moolenaar, 2003), while an association between LPA signalling and tumour progression in mouse models has been reported (Boucharaba *et al*, 2004; Yang *et al*, 2005).

Lysophosphatidic acid is present in human serum, plasma, saliva, follicular fluid and malignant effusions at a physio-logically significant level (Moolenaar *et al*, 2004; Nakamura *et al*, 2007a). Several pathways contribute to LPA production, and it is now clear that extracellular LPA is mainly produced through the action of lysophospholipase D (lysoPLD), which converts lysophosphatidylcholine (LPC) to LPA (Meyer zu Heringdorf & Jakobs, 2007). Recently, lysoPLD was purified from human plasma (Tokumura *et al*, 2002) and fetal bovine (Umezu-Goto *et al*, 2002) and was found to be identical to autotaxin (ATX).

Autotaxin is a 125-kDa glycoprotein and a potent cell motility-stimulating factor originally isolated from the condi-tioned medium of A2058 human melanoma cells (Stracke *et al*, 1992). ATX belongs to the ecto-nucleotide pyrophosphatase/phosphodiesterase (ENPP) family and is known as ENPP2. ATX is widely expressed, with the highest mRNA levels detected in the brain, placenta, ovary, and small intestine (Lee *et al*, 1996). ATX is also overexpressed in several human malignancies, such as glioblastoma multiforme (Kishi *et al*, 2006), prostate cancer (Zhao *et al*, 2007), and Hodgkin lymphoma (HL) (Baumforth *et al*, 2005). Based on the cloning of lysoPLD and the resultant finding that ATX is identical to plasma/serum lysoPLD, ATX has been confirmed to regulate cell motility through the production of LPA and LPA's effects on LPA receptors. Indeed, recent studies have shown that ATX stimulates the cell motility of various cancer cells *in vitro* through LPA formation and its interaction with LPA$_1$ (Hama *et al*, 2004; Kishi *et al*, 2006) although a key role for ATX in vascular development has also been reported (van Meeteren *et al*, 2006; Tanaka *et al*, 2006).

Several studies have shown that the addition of exogenous LPA stimulates cell proliferation and protects tumour cells from apoptosis in haematological malignancies. However, little is known about the association between the mechanism of LPA production via ATX and haematological malignancies. LPA acts as a survival factor in B-cell neoplasms. LPA stimulates the proliferation and immunoglobulin formation of B lympho-blasts (Rosskopf *et al*, 1998). LPA also protects B-cell lines and primary chronic lymphocytic leukaemia (CLL) cells from apoptosis (Hu *et al*, 2005; Satoh *et al*, 2007). Furthermore, the

induction of ATX by the Epstein Barr virus (EBV) has been suggested to promote the growth and survival of HL cells; the up-regulation of ATX increased the generation of LPA and led to the enhanced growth and survival of HL cells (Baumforth *et al*, 2005).

Recently, we, for the first time, developed an automated enzyme immunoassay for measuring serum ATX antigen levels (Nakamura *et al*, 2008a). Using this new assay system, we previously reported the clinical significance of serum ATX measurements. In the present study, we measured the serum ATX antigen levels in patients with haematological malignan-cies and evaluated the usefulness of this parameter for clinical laboratory testing.

Materials and methods

Patients

We enrolled 161 patients with haematological malignancies who were treated at the Department of Haematology and Oncology, the University of Tokyo Hospital (Tokyo, Japan) and the Division of Transfusion, Tokyo Metropolitan Fuchu Hospital (Tokyo, Japan) between 2005 and 2007. The serum samples used in this study were residual samples from after the completion of the requested clinical laboratory tests. Plasma samples for the measurement of LPA and LPC were available from six patients with follicular lymphoma (FL). Informed consent was obtained from the patients for the usage of the samples. This study was approved by the Institutional Research Ethics Committee of the Faculty of Medicine, the University of Tokyo, and that of Tokyo Metropolitan Fuchu Hospital.

Table I shows the clinical diagnoses (according to the World Health Organization classification) (Jaffe *et al*, 2001) of the patients enrolled in the study. The patients with lymphoma were staged according to Ann Arbor classifi-cation (Carbone *et al*, 1971) by means of a physical examination, a computed tomography examination of the neck, chest, abdomen and pelvis, a bone marrow aspiration and biopsy, a haemogram and differential cell count, and routine biochemistry tests. Performance status was assessed using the Eastern Cooperative Oncology Group (ECOG) scale. In the patients with FL, the prognostic factors and the tumour burden were assessed according to the Follicular Lymphoma International Prognostic Index (FLIPI) (Solal-Céligny *et al*, 2004), and the Groupe d'Etude des Lymphomas Folliculaires (GELF) criteria (Brice *et al*, 1997) respectively.

As a control group, blood was collected from the antecubital vein of 120 healthy adult volunteers who had given their informed consent. To obtain the serum samples, whole blood specimens were directly collected into glass tubes and left to stand for 15 min at room temperature to allow blood clots to form. Then, the serum was separated by centrifugation at $1500 \times g$ for 5 min.

Diagnosis	No. of patients (M/F)	Age, years (mean ± SD)	Serum ATX antigen, mg/l (mean ± SD)
AML	26 (14/12)	53·2 ± 16·2	0·864 ± 0·293
MDS	5 (2/3)	48·2 ± 17·9	0·929 ± 0·301
CML-BC	3 (2/1)	60·0 ± 14·7	1·019 ± 0·266
B-cell neoplasms			
Precursor B-ALL	7 (3/4)	52·3 ± 16·9	1·088 ± 0·345
CLL	14 (10/4)	64·0 ± 12·8	1·037 ± 0·355
FL	25 (15/10)	63·0 ± 11·2	1·471 ± 0·693
DLBCL	28 (18/10)	64·5 ± 10·6	0·936 ± 0·387
MLBCL	1 (1/0)	31·0	0·704
MCL	8 (7/1)	64·8 ± 11·7	1·107 ± 0·320
LPL	3 (3/0)	75·3 ± 6·8	1·750 ± 0·989
Plasma cell myeloma	8 (5/3)	67·4 ± 11·9	0·816 ± 0·175
HCL	1 (0/1)	69·0	1·291
Burkitt lymphoma	1 (1/0)	59·0	1·382
T-cell and NK-cell neoplasms			
T-ALL	4 (3/1)	38·0 ± 12·1	0·795 ± 0·179
T-LGL	1 (1/0)	67·0	0·768
ATL	3 (2/1)	49·0 ± 8·2	1·047 ± 0·047
Extra-nodal NK/T-cell lymphoma, nasal type	2 (0/2)	60·0 ± 5·7	0·769 ± 0·127
AITL	3 (3/0)	66·0 ± 8·2	2·021 ± 0·667
ALCL	2 (2/0)	38·0 ± 24·0	1·133 ± 0·533
Primary cutaneous-ALCL	2 (1/1)	61·0 ± 15·6	0·992 ± 0·205
MF	1 (1/0)	57·0	0·814
Sezary syndrome	1 (0/1)	58·0	1·713
PTCL, unspecified	1 (0/1)	60·0	1·240
Hodgkin lymphoma	11 (5/6)	37·8 ± 18·0	0·952 ± 0·273
Healthy subjects	120 (74/46)	40·4 ± 10·3	0·731 ± 0·176

Table I. Serum ATX antigen levels in patients with haematological malignancies.

AML, acute myeloid leukaemia; MDS, myelodysplastic syndrome; CML-BC, chronic myeloid leukaemia blastic crisis; Precursor B-ALL, precursor B lymphoblastic leukaemia; CLL, chronic lymphocytic leukaemia; FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma; MLBCL, mediastinal large B-cell lymphoma; MCL, mantle cell lymphoma; LPL, lymphoplasmacytic lymphoma; HCL, hairy cell leukaemia; T-ALL, precursor T lymphoblastic leukaemia; T-LGL, T-cell large granular lymphocytic leukaemia; ATL, adult T-cell leukaemia; NK, natural killer cell; AITL, angioimmunoblastic T-cell lymphoma; ALCL, anaplastic large cell lymphoma; Primary cutaneous-ALCL, primary cutaneous anaplastic large cell lymphoma; MF, mycosis fungoides; PTCL, unspecified, peripheral T-cell lymphoma, unspecified.

Measurement of serum ATX antigen

Anti-human ATX monoclonal antibodies were produced by immunization with recombinant human ATX expressed in a baculovirus system. An automated immunoassay for the quantitative determination of ATX was then established, and human serum samples were assayed using an automated immunoassay analyzer AIA-system (TOSOH Corp., Tokyo, Japan), as previously described (Nakamura *et al*, 2008a).

Serum ATX antigen levels were previously found to be significantly higher among females than among males in healthy subjects (Nakamura *et al*, 2008a). Therefore, to compare the serum ATX antigen levels in both sexes, we defined the ATX ratio as follows: the individual's serum ATX antigen level divided by the mean of the serum ATX antigen levels in healthy subjects of the same sex (males, 0·656 mg/l; females, 0·852 mg/l).

Measurement of plasma LPA

Blood samples were treated with ethylene-diamine-tetra-acetic acid and citrate-theophylline-adenosine-dipyridamole (BD Biosciences, Tokyo, Japan). The samples were centrifuged at 2500 × g for 30 min at 4°C and the plasmas obtained were stored at −80°C until LPA measurement (Nakamura *et al*, 2007a). The plasma LPA level was determined using a colorimetric assay with an enzymatic cycling method, as previously described (Kishimoto *et al*, 2003).

Measurement of plasma LPC

The plasma LPC level was determined using a specific enzymatic assay, as previously described (Kishimoto *et al*, 2002).

Flow cytometry

Flow cytometry was performed by following the Guidelines for Performing Surface Antigen Analysis on Haematopoietic Malignant Cells (Japanese Committee for Clinical Laboratory Standards (JCCLS) H2-P V1.0), proposed by the Subcommittee on Flow Cytometry, Area Committee on Haematology (Japanese Committee for Clinical Laboratory Standards; JCCLS Area Committee on Haematology Subcommittee on Flow Cytometry, 2003). Blood samples were adjusted to obtain white blood cell counts of 5 to 10×10^9/l. For indirect immunofluorescence staining, the cells were incubated with anti-ATX monoclonal antibody (3D1), which was generated as previously described (Tanaka *et al*, 2004). Then, the samples were washed with phosphate-buffered saline and incubated with fluorescein isothiocyanate (FITC)-labelled anti-rat IgG (BD Biosciences, San Jose, CA, USA), followed by washing with phosphate-buffered saline.

The cells were further stained with specific antibody solution to identify the blood cell types and leukaemic tumour cells. The following antibodies were employed to distinguish the cell types: phycoerythrin (PE)-conjugated mouse anti-human CD20 (BD Biosciences) for B-lymphocytes; and PE-conjugated mouse anti-human CD10 (BD Biosciences) for FL. Subsequent two-colour flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences), and the data were analysed using the CELLQUEST software (BD Biosciences).

Statistical analysis

The Steel–Dwass test, a non-parametric multiple comparison procedure, was performed to compare medians among groups (Dwass, 1960; Steel, 1960). Comparisons between two groups were performed using the non-parametric Wilcoxon rank-sum test. Correlations between serum ATX antigen levels and clinical laboratory data were obtained using linear regression analysis. All data are expressed as the mean ± standard deviation (SD) unless indicated otherwise. *P* values less than 0·05 were considered statistically significant. All analyses were performed using JMP6 (SAS Institute, Cary, NC, USA).

Results

Serum ATX antigen levels in patients with haematological malignancies

Serum ATX antigen levels were measured in 161 patients with various haematological malignancies and in 120 healthy

subjects. The patient characteristics and the values of the serum ATX antigen levels are summarized in Table I. Patients who had been treated just before the serum ATX measurement was obtained were excluded. As shown in Table I, the mean value of the serum ATX antigen levels in 120 healthy subjects (74 males and 46 females) was 0·731 ± 0·176 mg/l. Elevated ATX antigen levels were found in most patients with B-cell neoplasms, especially in those with FL, when compared with the levels in healthy subjects.

We then compared the values among the groups that contained over 10 patients using the Steel–Dwass test (Fig 1). The serum ATX antigen levels in the patients with FL ($P < 0·001$), CLL ($P < 0·001$), diffuse large B-cell lymphoma (DLBCL) ($P = 0·03$), and HL ($P = 0·01$) were significantly higher than those in the healthy subjects. On the other hand, no significant difference was found between the serum ATX antigen levels in the patients with acute myeloid leukaemia (AML) and the healthy subjects ($P = 0·08$). The serum ATX antigen levels in the patients with FL were significantly higher than those in the patients with AML ($P = 0·01$) or DLBCL ($P = 0·01$).

In the healthy subjects, the serum ATX antigen levels were significantly higher ($P < 0·001$) among females (0·852 ± 0·184 mg/l) than among males (0·656 ± 0·121 mg/l), as evaluated using the Wilcoxon rank-sum test. Therefore, we compared the serum ATX antigen levels among the groups according to sex. In males, the serum ATX antigen levels in the patients with FL ($P < 0·001$), CLL ($P < 0·001$), and DLBCL ($P = 0·02$) were significantly higher than those in the healthy subjects. In females, the serum ATX antigen levels in the patients with FL ($P < 0·001$) and AML ($P = 0·03$) were significantly higher than those in the healthy subjects. In addition, the serum ATX antigen levels in the patients with FL were significantly ($P = 0·03$) higher than those in the patients with AML.

Because of the difference in serum ATX antigen levels between females and males, we also compared the ATX ratios among the groups. Similar results were obtained when the ATX ratios were compared among the groups (data not shown), as was the case with the ATX antigen levels.

These results indicate that serum ATX antigen levels in patients with B-cell neoplasms, especially those with FL, are specifically higher than those in healthy subjects.

Correlations between serum ATX antigen levels and clinical parameters in patients with FL

Next, we analysed the correlations between the serum ATX antigen levels and clinical parameters, including prognostic factors, in 25 patients with FL. Because the patients with FL showed various disease statuses, the patients were divided into two groups according to each individual's clinical parameters; the groups were then compared using the
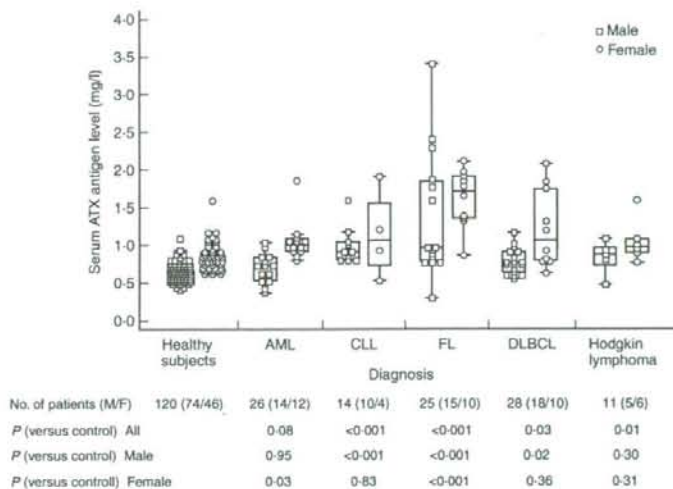
Fig 1. Serum ATX antigen levels in patients with haematological malignancies compared with healthy subjects. Serum ATX antigen levels were measured using an automated enzyme immunoassay. We compared the serum ATX antigen levels among healthy subjects and disease groups that contained over 10 patients. Samples from males and females are shown as open squares and open circles, respectively. The central boxes represent the values from the lower to upper quartiles (25 to 75 percentiles), and the middle lines represent the medians. The vertical lines extend from the minimum to the maximum value, excluding the outside values, which are displayed as separate points. An outside value is defined as a value that is smaller than the lower quartile minus 1·5 times the interquartile range, or larger than the upper quartile plus 1·5 times the interquartile range. To compare the levels among the groups, the Steel–Dwass test, a non-parametric multiple comparison procedure, was performed. *P* values less than 0·05 indicate significant differences.

Wilcoxon rank-sum test. Thirteen patients were analysed at the time of diagnosis, before any treatment had been administered. Twelve patients had been previously diagnosed and treated for FL, but had either progressive disease or disease relapse at the time of the serum ATX antigen measurement. The performance status was grade 0 or 1 in all the patients. Table II gives the clinical characteristics and the individual serum ATX antigen levels.

The serum ATX antigen levels were significantly higher among patients with a lactate dehydrogenase (LDH) level greater than the upper normal limit ($P = 0.02$), a β2-microglobulin level greater than 3 mg/l ($P = 0.01$), a tumour diameter greater than 7 cm ($P = 0.03$), or the presence of a high tumour burden ($P = 0.008$), compared with their counterparts. The levels were also significantly higher among patients with disease relapse or disease progression, compared with those in patients at the time of diagnosis ($P = 0.02$). No significant relationships were found when sex, age groups, clinical stage, the presence of B symptoms, the presence of bone marrow involvement, histological subgroups, or the FLIPI scores were evaluated. The serum ATX antigen levels in patients with stage IV disease were higher than those in patients with stages I to III, although the difference was not significant ($P = 0.12$; 1·643 ± 0·763 and 1·213 ± 0·500 mg/l respectively). Considering these results, we regarded the serum ATX antigen levels to be associated with tumour burden in patients with FL.

Correlations of serum ATX antigen levels with soluble interleukin-2 receptor, β2-microglobulin, and LDH in patients with FL

Next, we analysed the relationship between the serum ATX antigen levels and the biomarkers for lymphoma, such as soluble interleukin-2 receptor (sIL-2R), β2-microglobulin, and LDH, in the patients with FL. As shown in Fig 2, the serum ATX antigen levels were significantly and positively correlated with sIL-2R ($r = 0.594$, $P < 0.001$, $n = 115$), β2-microglobulin ($r = 0.465$, $P < 0.001$, $n = 58$), and LDH ($r = 0.495$, $P < 0.001$, $n = 154$), as determined using linear regression analysis. Because the serum levels of sIL-2R and β2-microglobulin are known to be elevated in patients with inflammatory or infectious conditions (Bethea & Forman, 1990; Rubin & Nelson, 1990), we analysed the relationship of serum ATX antigen, sIL-2R, and β2-microglobulin levels to inflammation markers, such as C-reactive protein (CRP), to evaluate the specificity of this marker as a laboratory test. The serum sIL-2R levels and the β2-microglobulin levels were significantly correlated with CRP ($r = 0.566$, $P < 0.001$, $n = 91$; $r = 0.516$, $P < 0.001$, $n = 55$, respectively). In contrast, no significant correlation was found between the serum ATX antigen levels and CRP (Fig 2D). These results suggest that the serum ATX antigen levels are little affected by inflammation and are a more specific biomarker for lymphoma than sIL-2R and β2-microglobulin.

Table II. Correlations between serum ATX antigen levels and clinical parameters in the patients with FL.

Characteristic	No. of patients	Serum ATX antigen, mg/l (mean ± SD)	P
Sex			
Male	15	1·369 ± 0·836	0·17
Female	10	1·624 ± 0·385	
Age			
<60 years	10	1·495 ± 0·521	0·68
≥60 years	15	1·455 ± 0·804	
Disease status			
At diagnosis	13	1·149 ± 0·542	0·02*
At relapse or in progression	12	1·819 ± 0·687	
Ann Arbor stage			
I–III	10	1·213 ± 0·500	0·12
IV	15	1·643 ± 0·763	
B symptoms			
Absence	22	1·443 ± 0·717	0·45
Presence	3	1·679 ± 0·531	
Serum LDH			
Less than or equal to ULN	10	1·105 ± 0·615	0·02*
Greater than ULN	15	1·715 ± 0·648	
Serum β2-microglobulin†			
Less than or equal to 3 mg/l	9	1·054 ± 0·516	0·01*
Greater than 3 mg/l	4	2·005 ± 0·278	
Bone marrow involvement			
Absence	14	1·311 ± 0·514	0·19
Presence	11	1·674 ± 0·852	
Histological findings†			
Grade 1, 2	19	1·492 ± 0·722	0·64
Grade 3	5	1·534 ± 0·634	
Tumour diameter			
Less than or equal to 7 cm	13	1·253 ± 0·801	0·03*
Greater than 7 cm	12	1·706 ± 0·479	
High tumour burden (GELF criteria)			
Absence	7	0·928 ± 0·201	0·008*
Presence	18	1·682 ± 0·703	
FLIPI score			
Low/intermediate risk	12	1·245 ± 0·513	0·14
High risk	13	1·680 ± 0·787	

*Statistically significant as determined using the Wilcoxon rank-sum test.
†Data were missing for serum β2-microglobulin (12 patients) and histological findings (one patient).
LDH, lactate dehydrogenase; ULN, upper limit of normal; GELF, Groupe d'Etude des Lymphomas Folliculaires; FLIPI, Follicular Lymphoma International Prognostic Index.

Relationship between serum ATX antigen levels, clinical laboratory data and clinical course in patients with FL

We further analysed the relations of serum ATX antigen levels to laboratory data and clinical course in patients with FL (Fig 3). The serum ATX antigen levels were measured at various times during the clinical courses of four patients with FL.

Patient 1 was a 51-year-old man who had been newly diagnosed as having FL. He was treated with R-CHOP therapy (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone) and obtained a partial response. Because of progressive disease, rituximab was administered once a week eight times as salvage therapy. In this patient, the serum ATX antigen levels and sIL-2R levels changed in parallel with the clinical course. Patient 2 was a 65-year-old man with refractory FL who had been treated repeatedly until the time of the serum ATX antigen measurement. Although this patient's serum ATX antigen levels and sIL-2R levels decreased after R-2CDA-MIT therapy (rituximab, cladribine, and mitoxantrone), the levels increased in parallel with tumour progression. The levels once again decreased after modified R-ESHAP therapy (rituximab, etoposide, carboplatin, cytarabine and methylprednisolone). Patients 3 and 4 were females with refractory FL. The serum ATX antigen levels and sIL-2R levels in these patients decreased after R-2CDA-MIT therapy. These results suggest that the serum ATX antigen levels change in parallel with the clinical course in patients with FL.

Correlations between serum ATX antigen levels and plasma LPA levels

As described above, ATX exerts a lysoPLD activity, which converts LPC to LPA (Tokumura et al, 2002; Umezu-Goto et al, 2002). To examine whether the serum ATX antigen levels play a role in determining the plasma LPA levels, we measured the plasma LPA levels in patients with FL; note that it is LPA, not ATX that actually plays a pathophysiological role. The plasma LPA levels were significantly and positively correlated with the serum ATX antigen levels ($r = 0·905$, $P = 0·01$, $n = 6$) (Fig 4A). On the other hand, no significant correlation was found between the plasma LPA levels and the plasma LPC levels ($r = -0·402$, $P = 0·43$, $n = 6$) (Fig 4B). These results suggest that the plasma LPA levels depend on the levels of ATX (the enzyme that produces LPA) but not LPC (the substrate).

Expression of ATX in leukaemic tumour cells from patients with FL

Autotaxin is synthesized as a secreted protein and is released into the extracellular space (Jansen et al, 2005; Koike et al, 2006). To examine the source of the elevated serum ATX antigen level, we determined the surface expression of ATX in peripheral blood cells from healthy subjects and patients with FL. The leukaemic tumour cells from the patients with FL were found to express ATX (Fig 5B). On the other hand, normal peripheral blood cells, including B-cells, from healthy subjects failed to express ATX (Fig 5A). Although the results were highly reproducible, i.e., similar results were obtained in three patients with FL and in five healthy subjects, the ATX fluorescence observed by flow cytometry may reflect the cell
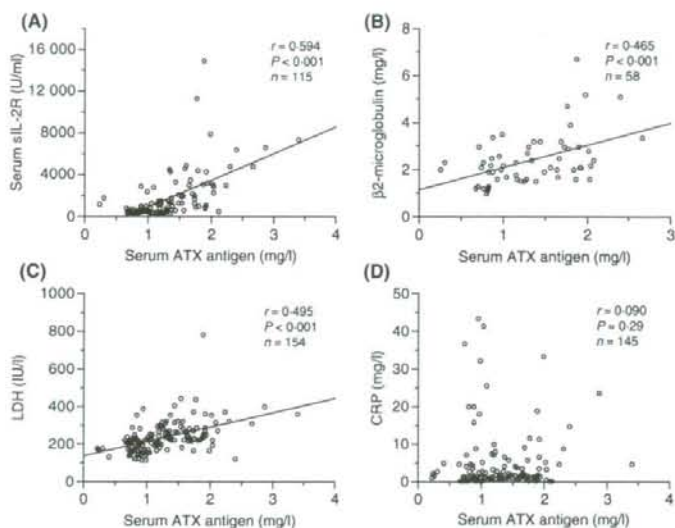
Fig 2. Correlations of serum ATX antigen levels with sIL-2R (A), β2-microglobulin (B), LDH (C), and CRP (D) in patients with FL. The correlations of serum ATX antigen levels with clinical laboratory data were examined using linear regression analysis in 25 patients with FL.

targeting of secreted ATX by cell surface molecules such as integrins, as reported recently (Kanda *et al*, 2008).

Discussion

This study demonstrated that the serum ATX antigen levels in patients with B-cell neoplasms, especially those with FL, were higher than those in healthy subjects. The serum ATX antigen levels in the patients with FL were found to be associated with the tumour burden and to change in parallel with the clinical course. In addition, the serum ATX antigen levels were little affected by inflammation, in contrast to other biomarkers for lymphoma, such as sIL-2R and β2-microglobulin. Our study is the first to report the usefulness of serum ATX measurements not only in B-cell neoplasms (especially FL), but also in haematological malignancies. As expected, as ATX is a key enzyme for converting LPC to LPA and as plasma LPA levels are correlated with the serum ATX activity in patients with chronic liver disease (Watanabe *et al*, 2007a), the serum ATX antigen levels and the plasma LPA levels were correlated in patients with FL. In the plasma (van Meeteren *et al*, 2006) and serum (Tanaka *et al*, 2006) from ATX-deficient heterozygous mice, both the ATX activity and LPA levels have been shown to be about half of those from wild-type mice, while transgenic overexpression of lipid phosphate phosphatase-1 reportedly failed to affect plasma LPA levels in mice (Yue *et al*, 2004). Although the plasma LPA level can be controlled by balance between LPA production (through the action of ATX activity) and degradation (through the action of lipid phosphate phosphatases), the former may be more important *in vivo*.

We have attempted to apply the measurement of serum ATX to clinical laboratory testing (Nakamura *et al*, 2007a,b,c; Nakamura *et al*, 2008a,b; Watanabe *et al*, 2007a). We previously reported the levels of serum ATX activity and serum ATX antigen in patients with various diseases and conditions: levels were elevated in patients with chronic liver disease (Watanabe *et al*, 2007a; Nakamura *et al*, 2008a), decreased in postoperative prostate cancer patients (Nakamura *et al*, 2007c), and the application to hypoalbuminemia differentiation (Nakamura *et al*, 2008a). Reportedly, the serum ATX activity level was significantly higher in normal pregnant females than in non-pregnant healthy females (Tokumura *et al*, 2000, 2002). From the results of this study, B-cell neoplasms, especially FL, should be added to the list of pathophysiological conditions in which serum ATX levels are altered.

Autotaxin was originally identified as a tumour cell motility factor (Stracke *et al*, 1992), and mounting evidence points to a link between ATX and cancer, such as tumour progression, metastasis, and angiogenesis (Mills & Moolenaar, 2003; Moolenaar *et al*, 2004; Birgbauer & Chun, 2006). However, elevated ATX activity in cancer patients has rarely been reported. Many studies have suggested a causal link between LPA and ovarian cancer (Mills & Moolenaar, 2003), and plasma LPA levels were found to be elevated in patients with ovarian cancer (Xu *et al*, 1998). Because ATX is a key enzyme producing LPA, the association between ATX and ovarian cancer once received attention. However, whether plasma LPA levels are elevated in patients with ovarian cancers is controversial (Baker *et al*, 2002), and a recent study suggests that no difference in serum ATX activity exists between ovarian cancer
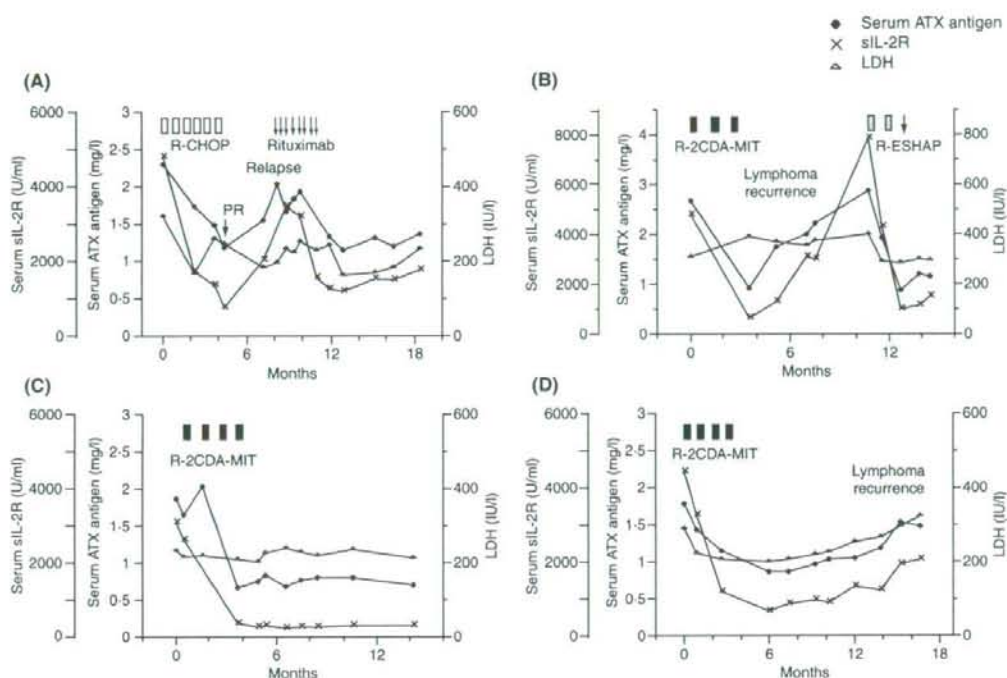
Fig 3. Relationship between serum ATX antigen levels, clinical laboratory data and clinical course in patients with FL. Closed circles, crosses, and open triangles represent serum ATX antigen, sIL-2R, and LDH levels, respectively. (A) Clinical course of Patient 1, a 51-year-old man newly diagnosed with FL. (B) Clinical course of Patient 2, a 65-year-old man with refractory FL. (C) Clinical course of Patient 3, a 59-year-old woman with refractory FL. (D) Clinical course of Patient 4, a 73-year-old woman with refractory FL. R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone; R-2CDA-MIT, rituximab, cladribine and mitoxantrone; R-ESHAP, rituximab, etoposide, carboplatin, cytarabine and methylprednisolone.
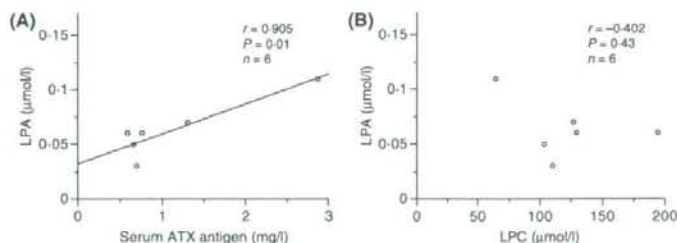

Fig 4. Correlations of plasma LPA levels with serum ATX antigen levels (A) and plasma LPC levels (B) in patients with FL. The plasma LPA levels were measured using a colorimetric assay, and the plasma LPC levels were measured using a specific enzymatic assay. The correlations of the plasma LPA levels with the serum ATX antigen levels and the plasma LPC levels were examined using linear regression analysis in six patients with FL.

patients and healthy females (Tokumura *et al*, 2007). LPA is also an autocrine mediator in prostate cancer cells (Xie *et al*, 2002), and ATX is up-regulated in stromal cells from prostate cancer patients (Zhao *et al*, 2007). Although LPA and ATX are thought to be associated with prostate cancer, no significant difference in serum ATX activity has been found between prostate cancer patients and healthy subjects (Nakamura *et al*, 2007c). Accordingly, the present study is the first report to show an elevation in serum ATX antigen levels in patients with malignancies.

In this study, the serum ATX antigen levels in patients with FL were found to be significantly higher than those in healthy