TABLE 2. The Characteristics of the Cell Lines with t(12;22)(p13;q11–12)

		UCSD/AML1	MUTZ-3	AMU-AML1
Abnormalities in karyotypes (G-banding)		t(3;3)(q21;q26) −7 t(12;22)(p13;q12)	inv(3)(q21;q26) inv(7)(p14;q35) t(12;22)(p13;q11–12) others	t(12;22)(p13;q11) only
Disease types, the age and sex of the patients, the source of the sample, and the year of establishment		Mixed acute leukemia, 73-year-old female, bone marrow at relapse and in 1989	Acute myelomonocytic leukemia, 20-year-old-male, peripheral blood at diagnosis and in 1993	Acute myeloid leukemia, 60-year-old-male, bone marrow at diagnosis and in 2003
Breakpoints	Chromosome 12p13	Intron 2 of *TEL*	5′ untranslated region of *TEL*	5′ untranslated region or exon1 or intron 1 of *TEL*
	Chromosome 22q11–12	Intron 1 of *MN1*	3′ untranslated region of *MN1*	3′ untranslated region of *MN1*
Transcripts (RT-PCR)	*MN1-TEL*	*MN1-TEL* type I transcript (382bp)	–	–
	MN1	–	*MN1* transcript (479bp)	*MN1* transcript (479bp)
Proteins (Western blot)	MN1-TEL	MN1-TEL protein (200kDa)	–	–
	MN1	–	–	Wild type (136kDa)
	TEL	Wild type (53 and 57kDa)	Wild type (53 and 57kDa)	Wild type (53 and 57kDa)

(Valle et al., 2004), the *MN1-TEL* fusion transcript could be detected in UCSD/AML1 cells but not in MUTZ-3 cells by using *MN1*-1 and *TEL*-4B primers (Table 1). The *MN1-TEL* fusion transcript was not detected in AMU-AML1, HL-60, and THP-1 cells (Fig. 6a). The *MN1* transcript was detected in AMU-AML1 and MUTZ-3 cell lines but not in the UCSD/AML1, HL-60, and THP-1 cell lines by using *MN1* sense 5 and *MN1* antisense 1 primers (Fig. 6a, Table 1). The expression level of *MN1* mRNA in AMU-AML1 cells was ~3.8-times higher than the expression level in MUTZ-3 cells, as determined by quantitative real-time RT-PCR (Fig. 6b).

Western Blot Analysis

To confirm the detection of MN1-TEL fusion protein, we performed Western blot analysis in three leukemia cell lines that contain t(12;22), UCSD/AML1, MUTZ-3, and AMU-AML1. Concurrently, we examined the expression of TEL and MN1 proteins (Fig. 6c). As previously reported (Valle et al., 2004), the MN1-TEL protein (200 kDa) was detected in UCSD/AML1 cells with n-MN1 antibody, but it was not detected in the AMU-AML1 or MUTZ-3 cell line. The equivalent of a normally sized MN1 protein (136 kDa) was detected only in the AMU-AML1 cell line with c-MN1 antibody. The TEL antibody reacted with protein species corresponding to normally sized TEL proteins (53 and 57 kDa) in all three cell lines (Fig. 6c).

DISCUSSION

We have established a novel human myeloid leukemia cell line, AMU-AML1, from a patient with AML with multilineage dysplasia before the initiation of chemotherapy. The cell line had the same karyotype and immunophenotype as the patient's leukemia cells. AMU-AML1 cells grew relatively slowly, and their proliferation was stimulated by several cytokines (Fig. 2), which may reflect a characteristic of these cells in the early stage of disease.

The patient's leukemia cells had a single chromosomal abnormality, t(12;22)(p13;q11.2), at the time of diagnosis. Therefore, this translocation was not related to chemotherapy. t(12;22)(p13;q11-13) is a recurrent but infrequent abnormality seen in both the early and late stages of various hematological malignancies (Mitelman et al., 2010). As far as we know, this translocation

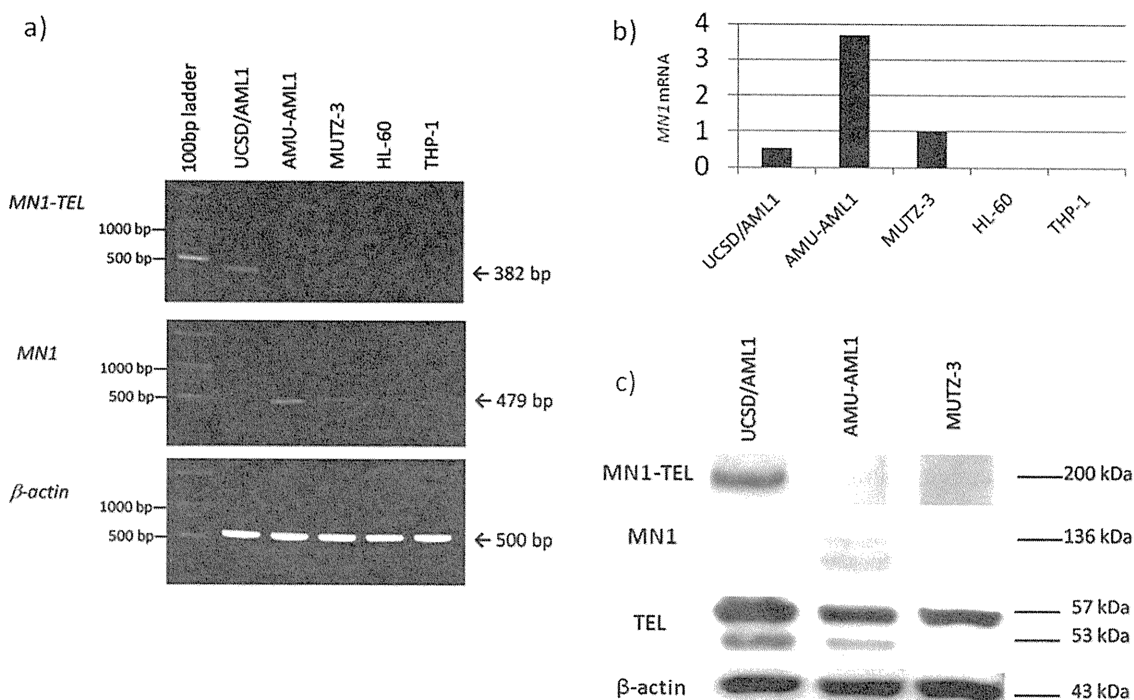

Figure 6. Expression of *MN1-TEL*, *MN1*, and *TEL* by RT-PCR and Western blotting in AMU-AML1 and other leukemic cell lines. Detection of the transcripts of *MN1-TEL* and *MN1* by RT-PCR in various human myeloid leukemic cell lines. *MN-1-TEL* fusion transcript (382 bp) was detected only in UCSD/AML1 cells, and *MN1* (479 bp) was detected in AMU-AML1 and MUTZ-3 cell lines. The expression level of *MN1* was higher in AMU-AML1 cells when compared with MUTZ-3 cells. UCSD/AML1, AMU-AML1, and MUTZ-3 cell lines had t(12;22)(p13;q11-13), and HL-60, and THP-1 cell lines had no t(12;22). b) Relative expression levels of *MN1* mRNA detected by quantitative real-time RT-PCR in UCSD/AML1, AMU-AML1, MUTZ-3, HL-60, and THP-1. (c) The proteins of MN1-TEL, MN1, and TEL detected by Western blotting in the cells with t(12;22)(p13;q11-12). MN1-TEL protein was observed only in UCSD/AML1 cells. MN1 was found only in the AMU-AML1 cell line, but TEL was found in all three cell lines.

has been reported in at least 17 patients with hematological malignancies, but a chimeric fusion of *MN1* and *TEL* has been found in only seven patients to date. These translocations can result in the chimeric fusion of *MN1* and *TEL*, the partial disruption of *TEL*, or no disruption in these genes. The oncogenic activities of *MN1-TEL* include the upregulation of *HOXA9* and the inhibition of RAR-RXR-mediated transcription (Kawagoe et al., 2005; van Wely et al., 2007). The biological significance of the partial disruption of *TEL* caused by t(12;22) is unclear.

Only two of the previously established leukemia cell lines, UCSD/AML1 and MUTZ-3, carry t(12;22)(p13;q11-12) (Buijs et al., 1995; Hu et al., 1996). UCSD/AML1 cells contain both the fusion transcript and protein of *MN1-TEL*, while MUTZ-3 cells do not contain either of the fusion products (Valle et al., 2004). The *MN1-TEL* transcript detected in UCSD/AML1 cells was the type 1 pattern (Buijs et al., 1995) and led to MN1-TEL fusion protein (Valle et al., 2004). On the other hand, MUTZ-3 cells had no *MN1-TEL* but a partial deletion of *TEL*, which might be involved in the pathogenetic events in this leukemia (Hu et al., 1996).

In the AMU-AML1 cell line that we established, the breakpoints of t(12;22) were within *TEL* or telomeric to 5′ *TEL* in 12p13 and centromeric to 3′ *MN1* in 22q11, resulting in no chimeric *MN1-TEL*. The aCGH data revealed that the region surrounding t(12;22) that contained whole *MN1* and whole or partial *TEL* was amplified to three copies (Fig. 5). We speculate that tandem duplication of this region occurred after translocation of chromosome 12p13 and 22q11. The t(12;22)(p13;q11) in AMU-AML1 cells was unexpectedly unbalanced. The transcript and protein of *MN1-TEL* were not detected in AMU-AML1 cells. However, the transcript and protein of *MN1* were detected (Fig. 6), perhaps due to the increased gene dosage of *MN1* or position effects of the 5′ end of *TEL*, which is similar to the cases of *GSH2* in t(4;12)(q11-12, p13) and *IL-3* in t(5;12)(q31;p13) reported by Cools et al. (2002). High expression levels of *MN1* mRNA

were correlated to a poor prognosis in patients with AML, and *MN1* overexpression induced myeloid malignancy in mice (Heuser et al., 2007; Meester-Smoor et al., 2007). Therefore, *MN1* expression in AMU-AML1 cells might play a role in the disease progression and possibly in leukemogenesis.

Our SNP array data also detected two loci deleted to one copy that corresponded to copy number variation (CNV) regions in chromosome 6p21 (Variation_3599) and 16q22 (Variation_4012) (Redon et al., 2006) and no copy neutral loss of heterogeneity in AMU-AML1 cells (Fig. 5a). A CNV is a DNA segment ~1 kb or larger that is present at variable copy numbers in comparison to a reference genome (Feuk et al., 2006). Some CNVs detected in cancer cells might play an important role in carcinogenesis and cancer development; however, the significance of the CNVs found in AMU-AML1 cells is not clear. In future studies, a more comprehensive cataloging and characterization of CNVs may reveal the significance of the CNVs detected in AMU-AML1 cells.

Considering the karyotypes among the cell lines with t(12;22)(p13;q11-12), a chromosomal abnormality in band 3q26, which contains the *EVI1* oncogene, might be related to t(12;22)(p13;q11-12). The 3q26 abnormality is common to the UCSD/AML1 and MUTZ-3 cell lines (Table 2), although AMU-AML1 cells do not have this abnormality. The mechanisms of leukemogenesis vary among patients with leukemia even if the leukemia cells have the same chromosomal abnormality, because the molecular breakpoints and genes that are dysregulated by translocation might be different, and there can be many epigenetic and genetic alterations influencing the disease. As we have shown in the present study, the UCSD/AML1, MUTZ-3, and AMU-AMl1 cell lines have the same chromosomal abnormality, t(12;22)(p13;q11-12), but *MN1-TEL* was only detected in UCSD/AML1 cells, and only AMU-AML1 cells expressed MN1 protein.

In summary, we have established a novel human myeloid leukemia cell line, AMU-AML1, which can contribute to further study of the biological consequences in hematological malignancies with t(12;22)(p13;q11) lacking a chimeric fusion gene, *MN1-TEL*.

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Incidence, Clinical Features, and Risk Factors of Idiopathic Pneumonia Syndrome Following Hematopoietic Stem Cell Transplantation in Children

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Background. Idiopathic pneumonia syndrome (IPS) is a severe complication that can occur after hematopoietic stem cell transplantation (HSCT) and is often associated with a fatal outcome despite intensive supportive care. ***Procedure.*** To assess the incidence and risk factors of IPS, we reviewed 251 consecutive patients (median age, 7.0 years) who received HSCT at the Department of Pediatrics, Nagoya University Hospital, between January 1990 and July 2009. ***Results.*** Twenty of 251 (cumulative incidence of IPS at 2 years after HSCT, 8.0%; 95% confidence interval (CI), 5.1–12.4%) patients developed IPS. The median duration from HSCT to diagnosis of IPS was 67 days (range, 12–486 days). Patients with IPS had significantly higher 5-year transplant-related mortality compared to patients without IPS (52% (95% CI, 19–77%) vs. 13% (95% CI, 5–25%), $P < 0.001$), and the probability of 5-year overall survival was significantly worse for patients with IPS (42% (95% CI, 25–64%) vs. 68% (95% CI, 59–76%), $P = 0.01$). By multivariate analysis, high risk in underlying disease (HR, 2.5; 95% CI, 1.0–6.7; $P = 0.05$) and a busulfan-containing regimen (HR, 3.5; 95% CI, 1.3–9.9; $P < 0.01$) were identified as the independent risk factors for developing IPS. ***Conclusion.*** The prophylactic strategies for IPS in patients with these risk factors were warranted. Pediatr Blood Cancer 2012;58:780–784. © 2011 Wiley Periodicals, Inc.

Key words: busulfan; complication; idiopathic pneumonia syndrome; pediatrics; stem cell transplantation

INTRODUCTION

Idiopathic pneumonia syndrome (IPS) is a severe complication following hematopoietic stem cell transplantation (HSCT) characterized by the rapid onset of respiratory failure with acute, non-infectious, diffuse lung injury [1,2]. Presence of diffuse lung injury is demonstrated as multi-lobar infiltrates on X-ray or computed tomography (CT) scan, clinical signs of pneumonia, and abnormal pulmonary physiology, such as an increased alveolar to arterial oxygen gradient or new restrictive lung findings [3]. Despite intensive supportive care, a considerable percentage of patients die usually within 3 weeks of diagnosis. According to previous studies, the incidence of IPS is reported to be 3–15%, and the mortality of IPS is reported to be 50–80% [4–8]. Although the pathogenesis of IPS remains unknown, several risk factors including the development of acute graft versus host disease (GVHD), unrelated donor, conditioning regimen without total body irradiation (TBI), and umbilical cord blood transplantation, have been reported [9–12]. However, most studies on IPS are based on adult patients. In this retrospective study, we report the incidence, clinical features, and risk factors of IPS in 251 children who underwent HSCT at our center.

METHODS

Patients and Transplantations

We reviewed database records of 251 consecutive patients who received HSCT at the Department of Pediatrics, Nagoya University Hospital, between January 1990 and July 2009. Patient characteristics are summarized in Table I. The subjects consisted of 140 males and 111 females between 0.3 and 22.7 years of age with a median age of 7.0 years. Underlying diseases included hematological malignancies (n = 98), malignant solid tumors (n = 62), non-malignant hematological diseases (n = 60), immunological diseases (n = 25), and metabolic diseases (n = 6). Hematological malignancies and malignant solid tumors were defined as malignant diseases (n = 160), and the others as benign (n = 91). Malignant diseases in the first or second remission and all benign diseases were defined as the standard risk group (n = 149), and malignant diseases in the third or more remission or not in remission were defined as the high-risk group (n = 102).

Sixty-two patients underwent autologous HSCT, and the other 189 patients underwent allogeneic HSCT. Among the allogeneic group, 89 recipients received their graft from unrelated donors with 48 of these donors matched at the allele level of human leukocyte antigen (HLA) A, B, Cw, and DRB1, and 100 recipients received their graft from related donors of which 52 were HLA-matched donors at the allele level. Twenty-three patients underwent allogeneic cord blood transplantation. One hundred thirty-one patients received a myeloablative-conditioning (MAC) regimen including a TBI-based regimen (n = 89). The busulfan (BU)-containing regimen (n = 68) consisted of two standard BU doses; one was 16 mg/kg (range, 16–18.7 mg/kg) for MAC regimen, and the other was 8 mg/kg (range, 6.4–9.6 mg/kg) for non-MAC or TBI-based MAC regimen. From November 2007, the administration route of BU was switched from oral to intravenous. Fifty-nine recipients received oral BU, and the other nine recipients received intravenous BU. In the allogeneic setting, prophylaxis against GVHD comprised tacrolimus (continuous intravenous infusion of 0.02 mg/kg/day starting on day −1, with dose adjustments to maintain blood levels of 5–15 ng/ml) or cyclosporine A (intravenous infusion of 3 mg/kg/day starting on day −1, aiming for a trough level of 100–250 ng/ml) with short-term methotrexate (intravenous infusion of 15 mg/m^2 on

Additional Supporting Information may be found in the online version of this article.

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Conflict of interest: Nothing to declare.

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TABLE I. Patient Characteristics

		N	Incidence of IPS	P-value
Total		251	20	
Gender	Male / Female	140 / 111	11 / 9	NS
Age (y)	<2 / 2–10 / 10<	40 / 119 / 92	4 / 12 / 4	NS
Year	1990–1999 / 2000–2009	84 / 167	4 / 16	NS
Diagnosis	Malignancy / Benign	160 / 91	17 / 3	0.04
Risk of underlying disease	Standard risk / High risk	149 / 102	7 / 13	0.01
Prior HSCT	No / Yes	232 / 19	18 / 2	NS
Graft	Auto / Allo	62 / 189	2 / 18	0.10
Donor	Auto / Related / Unrelated	62 / 100 / 89	2 / 10 / 8	NS
HLA disparity	Auto / Match / Mismatch	62 / 118 / 71	2 / 11 / 7	NS
Stem cell source	BM or PB / CB	228 / 23	17 / 3	NS
Conditioning intensity	MAC / RIC	131 / 120	16 / 4	NS
TBI-containing regimen	>8 / <8 (Gy)	89 / 162	7 / 13	NS
BU-containing regimen	Yes / No	68 / 183	11 / 9	<0.01
Acute GVHD (grade 2–4)	Yes / No / Auto	50 / 139 / 62	7 / 11 / 2	0.01
CMV antigenemia	Positive / Negative	73 / 178	8 / 12	NS

BM, bone marrow; BU, busulfan; CB, cord blood; CMV, cytomegalovirus; GVHD, graft versus host disease; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplantation; IPS, idiopathic pulmonary syndrome; MAC, myeloablative conditioning; NS, not significant; PB, peripheral blood; RIC, reduced intensity conditioning; TBI, total body irradiation; y, year

day +1 and 10 mg/m^2 on days +3, +6, and +11). The administration route of calcineurin inhibitor was switched to oral after the patients recovered from gastrointestinal toxicity. Acute and chronic GVHD was diagnosed and graded according to the established criteria [13,14]. The institutional ethics committee of Nagoya University Graduate School of Medicine approved the review of patient records and data collection for analyses.

Supportive Care

Platelet concentrates and red blood cell concentrates were transfused to patients whose platelet levels declined below approximately 20 × 10^9/L and whose hemoglobin levels declined to below 8 g/dl, respectively. Engraftment day was defined as the first of the 3 consecutive days in which the patient had an absolute neutrophil count greater than 0.5 × 10^9/L. Failure to engraft by day +30 was considered as primary graft failure. All patients received trimetoprim-sulfamethoxazole orally or inhaled pentamidine as prophylaxis against *Pneumocystis jirovecii*. Patients received a standard dose of oral amphotericin B and acyclovir as fungal and viral prophylaxis. Peripheral blood was obtained weekly from engraftment to discharge to test for cytomegalovirus (CMV) antigenemia. Patients received pre-emptive therapy with ganciclovir when the test became positive. From 1997, weekly viral studies using real-time polymerase chain reaction for CMV, Epstein–Barr virus (EBV) and human herpesvirus 6 (HHV6) were conducted up until 90 days post-transplant [15].

Diagnosis and Management of IPS

IPS was defined as the presence of multilobar infiltrates by chest X-ray or CT scan, clinical signs of pneumonia with abnormal pulmonary physiology including the need for supplemental oxygenation with declining pulse oximetry values, the absence of active lower respiratory tract infection determined by bronchoalveolar lavage (BAL), and lung biopsy or autopsy [3]. Microscopic analysis of the smears of pelleted cells from BAL fluid was performed after staining with Gram, Giemsa, Papanicolaou, and Ziehl–Neelsen methods. BAL fluid was also cultured for bacteria and fungal species. Patients with symptoms of fluid overload who had responded to diuretics were not categorized as having IPS. Patients without BAL that responded quickly to anti-microbial agents were also not considered to have IPS.

The day of onset of IPS was defined as the day in which the symptoms of shortness of breath and hypoxemia were first recognized. Patients with the diagnosis of IPS were given an oxygen supplement by positive airway pressure or mechanical ventilation as clinically indicated, and received steroid therapy (1–2 mg/kg/day of methylprednisolone; mPSL). Steroid dose was increased for the patients whose symptoms deteriorated. Good response (GR) was defined as the ability to completely discontinue all supplemental oxygen support within 28 days from onset of IPS. Patients who failed to discontinue oxygen support within the 28-day period, those who died from IPS or from any causes within the 28-day period, were considered as having persistent or progressive disease (PD).

Statistical Analysis

The incidence of IPS was analyzed by cumulative incidence method. Then, we statistically analyzed risk factors associated with the development of IPS. The variables included age, gender, underlying disease, conditioning regimen (myeloablative or not, BU-containing or not, and TBI-containing or not), disease status at transplantation, donor type, HLA disparity, development of acute GVHD and CMV serology. Acute GVHD was analyzed as a time-dependent variable. Only acute GVHD diagnosed prior to IPS was considered as the factor for the analyses.

Risk factors for developing IPS were evaluated by univariate and multivariate analysis using the Cox regression model. A multivariate model was constructed with forward stepwise methods using threshold P-values of 0.10 for removal or addition to the model. Values of $P < 0.05$ were considered statistically significant. Measures of association were expressed as hazard ratios (HR) with

95% CI. Survival was estimated using the Kaplan–Meier method and differences were assessed using the log-rank test. All analyses were performed using Statview 5.0 (SAS, Inc., Cary, NC) and Prism 5.0a (GraphPad Software, Inc., San Diego, CA).

RESULTS

Incidence and Clinical Features of IPS

Twenty of 251 patients developed IPS. The cumulative incidence of IPS at 2 years after HSCT was 8.0% (95% CI, 5.1–12.4%; Fig. 1). The median duration from HSCT to a diagnosis of IPS was 67 days (range, 12–486 days). All patients had significant hypoxia and needed supplemental oxygen; 19 patients received steroid therapy, 9 patients required mechanical ventilation, 2 patients were administered with infliximab, and 1 patient was administered with basiliximab.

Characteristics of the patients with IPS are summarized in Supplemental Table I. The median age was 6.6 years (range 0.9–15.2 years); 11 were male and 9 were female. Underlying disease consisted of hematological malignancies (n = 13), malignant solid tumors (n = 4), and benign diseases (n = 3). Twelve patients were classified as the high-risk group, and the other eight patients as the standard risk group. Their conditioning regimens included TBI-containing regimens (n = 7) and BU-containing regimens (n = 12). Of the 20 patients, 2 patients underwent autologous HSCT, 10 patients received bone marrow transplantations from related donors (7 HLA matched donors and 3 mismatched donors), 5 patients received bone marrow transplantations from unrelated donors (3 HLA matched donors and 1 mismatched donors), and 3 patients received unrelated cord blood transplantations (3 HLA mismatched donors).

Seven patients showed grade II–IV of acute GVHD prior to onset of IPS. All of them had lesions only in the classical target organs such as the skin, gut and liver, and none of them showed pulmonary complications at the onset of acute GVHD. Median duration from diagnosis of acute GVHD to onset of IPS was 119 days, and the range was 8–168 days. Two of the patients were diagnosed with IPS within 20 days after onset of acute GVHD. On the other hand, 2 of the 20 patients showed extended chronic GVHD before developing IPS.

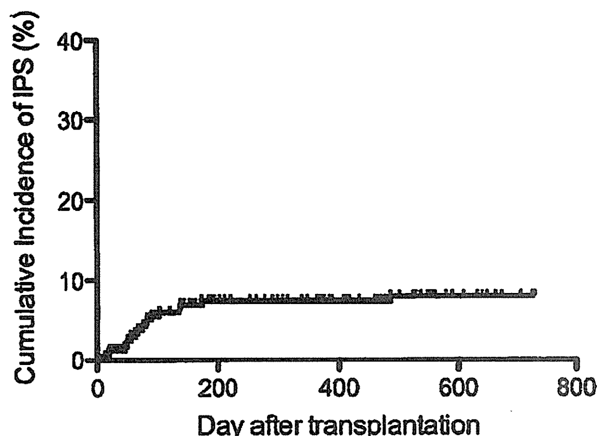

Fig. 1. The probability of developing IPS was 8.0% in 251 children who underwent HSCT at our center.

Of the 20 patients with IPS, 16 patients had complete resolution of respiratory symptoms. On the other hand, 3 patients died from IPS, and the median time from the onset of IPS to their death was 26 days (range, 0–31 days). Within 5 years after HSCT, 11 of 20 patients with IPS died because of disease relapse (n = 4), IPS (n = 3), diffuse alveolar hemorrhage (n = 1), chronic GVHD (n = 1), cerebral infarction (n = 1), or invasive fungal infection (n = 1). Patients developing IPS had significantly higher 5-year transplant-related mortality compared to the patients without IPS (52% (95% CI, 19–77%) vs. 13% (95% CI, 5–25%), $P < 0.001$, shown in Fig. 2). The probability of 5-year overall survival in patients with IPS was significantly lower than in patients without IPS (42% (95% CI, 25–64%) vs. 68% (95% CI, 59–76%), $P = 0.01$, shown in Fig. 3).

Risk Factors for the Incidence of IPS

Univariate analysis showed that malignant diseases, high-risk disease, BU-containing regimen, and grade II to IV acute GVHD were significant risk factors for developing IPS (Table I, Supplemental Tables II and III). Multivariate analysis confirmed that the high-risk group (HR, 2.5; 95% CI, 1.0–6.7; $P = 0.05$) and receiving the BU-containing regimen (HR, 3.5; 95% CI, 1.3–9.9; $P < 0.01$) were the significant risk factors (Table II). The administration route of BU had no impact on the incidence of IPS (oral, 20% (95% CI, 5–42%); intravenous, 17% (95% CI, 0–77%); $P = 0.87$).

DISCUSSION

We retrospectively reviewed the incidence, risk factors, and clinical features of IPS following HSCT in 251 children transplanted between January 1990 and July 2009 in a single center. The incidence of IPS (8.0%) in our cohort was similar to that in previous reports (3–15%) [4–8].

Patients with IPS had poor prognosis despite intensive supportive care that included mechanical ventilation. In our study, three patients died of IPS, and the median time to their death from

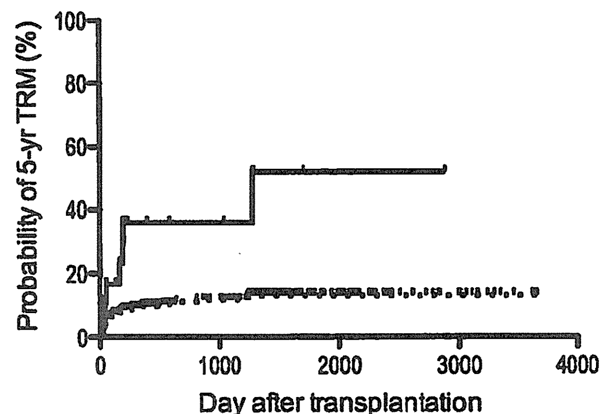

Fig. 2. The probability of 5-year transplant-related mortality (5yr TRM) in the patients with IPS (n = 20, solid line) was 52%, by contrast that in the patients without IPS (n = 231, broken line) was 13%. There was significant difference between two cohorts ($P < 0.001$) by Log-rank test.

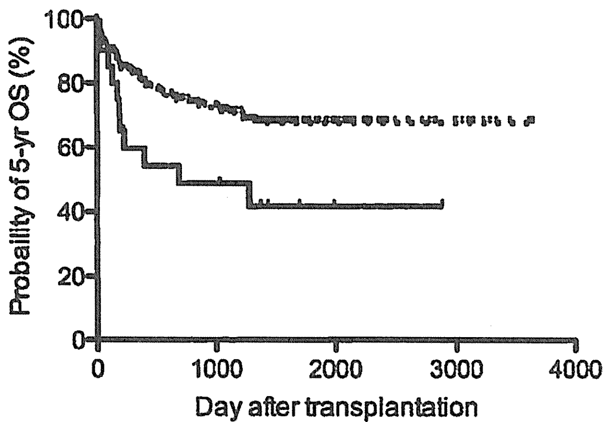

Fig. 3. The probability of 5-year overall survival (5yr OS) in the patients with IPS (n = 20, solid line) was 42%, by contrast that in the patients without IPS (n = 231, broken line) was 68%. There was significant difference between two cohorts ($P = 0.01$) by Log-rank test.

onset of IPS was 26 days (range, 0–31 days). All of them received steroid therapy but did not respond to it. Patients with IPS had significantly higher 5-year transplant-related mortality as compared to the patients without IPS.

We aimed to identify the risk factors for IPS. By multivariate analysis, the high-risk group and the conditioning regimen with BU were identified as the independent risk factors for IPS. Recipients in the high-risk group received a greater amount of cumulative chemical agents and irradiation before HSCT compared to recipients in the standard risk group, and this increased their risk of lung injury. Recent reports have described that cellular senescence mediated by BU can induce secretion of proinflammatory cytokines, matrix metalloproteases, and epithelial-growth factors that are known to participate in the pathogenesis of pulmonary fibrosis and tissue injury [16–18]. High exposure to BU has been linked to the occurrence of veno-occlusive disease of the liver [19]. Other complications including lung toxicity may be caused by high exposure to BU [20–24]. Area under the curve (AUC) is the most reliable to predict these complications, but it is highly variable among patients, and unpredictable systemic exposure is related to its pharmacokinetic properties, especially in children. Intravenous (IV) BU is a relative new administration method [25–28], and so we were interested in addressing whether the administration route of BU was responsible for increasing the incidence of IPS in the current study. However, the administration route had no impact on the incidence of IPS. The optimal method of administrating BU requires further assessment.

TABLE II. Risk Factors Associated With Idiopathic Pneumonia Syndrome by Multivariate Analysis

Variables		HR	95% CI	P-value
Diagnosis	Malignancy / Benign	1.2	0.5–8.3	0.85
Risk of underlying disease	High / Standard	2.5	1.0–6.7	0.05
BU-containing regimen	Yes / No	3.5	1.3–9.9	<0.01
Acute GVHD (grade 2–4)	Yes / No	1.5	0.6–3.9	0.41

BU, busulfan; CI, confidence intervals; GVHD, graft versus host disease; HR, hazard ratio.

The median time of onset was initially reported to be between 42 and 49 days after HSCT, but several recent studies have reported earlier onset. According to reports of the pediatric series from Vanderbilt University, IPS developed at a median of 17 days after transplant. All patients had associated acute and hyperacute GVHD that occurred simultaneously or within 48 hours preceding the onset of IPS [10]. Another study from a Seattle group showed a significant relationship between acute GVHD and the incidence of IPS, in which the median time to onset of IPS was 22 days after receiving a fully myeloablative regimen and 16 days after receiving a non-myeloablative regimen [11]. In the present study, acute GVHD did not show statistical power for increased risk of IPS. Moreover, none of the patients developed acute GVHD within 7 days preceding the onset of IPS. The median onset of IPS in our cohort was 67 days, which is much later than in the two studies mentioned above [10,11]. This difference might be due to whether acute GVHD was a significant risk factor for IPS or not. Acute GVHD has not been identified consistently as a risk factor for IPS in previous studies. These findings suggest that the causes of IPS may vary among patients, and that lung injury associated with acute GVHD is distinct from other causes such as BU-related IPS.

In our cohort, all of the 20 patients had significant hypoxia at the onset of IPS and needed an oxygen supplement; 19 patients received steroid therapy, 9 patients required mechanical ventilation, 2 patients were administered with infliximab, and 1 patient was administered with basiliximab. After these therapies, 16 patients had complete resolution of their respiratory symptoms. The patients who received infliximab and/or basiliximab had complete resolution of respiratory symptoms, however, they died later because of relapse of the disease and invasive fungal infection. Recent reports have described that systemic release of proinflammatory cytokines is related to developing IPS, and neutralization of these proinflammatory cytokines is a promising treatment option for IPS [29–33]. They showed that ten of 15 patients with IPS had complete response to the administration of etanercept, an inhibitor of soluble tumor necrosis factor alpha receptor. A prospective phase 3 trial is warranted to confirm these results.

In conclusion, 8.0% of the 251 pediatric patients developed IPS after HSCT and their prognosis were extremely poor. By the multivariate analysis, the high-risk group and BU based conditioning regimen were identified as the independent risk factors for developing IPS. We have to be careful for developing IPS and manage to prevent GVHD in patients with these risk factors. The active prophylactic strategy for IPS is warranted.

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bjh research paper

Chromosome abnormalities in advanced stage T-cell lymphoblastic lymphoma of children and adolescents: a report from Japanese Paediatric Leukaemia/Lymphoma Study Group (JPLSG) and review of the literature

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Summary

T-cell acute lymphoblastic leukaemia (T-ALL) and T-cell lymphoblastic lymphoma (T-LBL) are combined into one category as T lymphoblastic leukaemia/lymphoma in the current World Health Organization (WHO) classification. However, there is still ongoing discussion on whether T-ALL and T-LBL are two separate entities or represent two variant phenotypes of the same disease. Cytogenetic analysis has been used to identify the molecular background of haematological malignancies. To compare the distribution of chromosomal abnormalities of T-ALL and T-LBL, large series of cytogenetic data are required, but are absent in T-LBL in contrast to the abundant data in T-ALL. Among 111 T-LBL cases in our clinical trial, we obtained complete cytogenetic data from 56 patients. The comparison between our cytogenetic findings and those from three published T-LBL studies revealed no significant difference. However, meta-analysis showed that translocations involving chromosome region 9q34 were significantly more common in T-LBL than in T-ALL. In particular, four out of the 92 T-LBL cases, but none of the 523 paediatric T-ALL cases, showed translocation t(9;17)(q34;q22–23) (P = 0·0004). Further studies are needed for the possible linkage between abnormal expression of genes located at 9q34 and/or 17q22–23 and the unique 'lymphoma phenotype' of T-LBL.

Keywords: T-cell lymphoma, child, non-Hodgkin lymphoma, cancer cytogenetics, leukaemia.

In children and adolescents, precursor T lymphoblastic neoplasms have been classified into two diseases: T-cell acute lymphoblastic leukaemia (T-ALL) and T-cell lymphoblastic lymphoma (T-LBL). Although the current World Health Organization (WHO) classification designates both malignancies as T lymphoblastic leukaemia/lymphoma (Borowitz & Chan, 2008), there is continuing discussion on whether T-ALL and T-LBL are two separate entities or whether they represent two different clinical presentations of the same disease. They show overlapping clinical, pathological and immunophenotypic features. In general, the word 'lymphoma' is used if there is a bulky mass in the mediastinum or elsewhere, with less peripheral blood and bone marrow (BM) involvement. Most study groups distinguish between leukaemia and lymphoma on the basis of the extent of BM involvement: patients with <25% lymphoblasts in the BM are diagnosed with lymphoblastic lymphoma; in cases

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of 25% or more BM blasts, the diagnosis is leukaemia. While this distinction may appear somewhat arbitrary, a notable observation is that T-LBL patients with large mediastinal masses frequently exhibit little, if any, evidence of tumour dissemination and BM involvement, but the molecular background for this difference is unknown.

Chromosomal analysis has been widely used as a primary step that is required to narrow down the responsible genes that define a disease entity. For instance, discovery of Ph chromosome led to the identification of the chimeric *BCR/ABL1* gene, which is responsible for and defines chronic myeloid leukaemia. Compared with T-ALL, chromosomal abnormalities in T-LBL are not well defined. Reports in the literature and current textbooks claim that the typical chromosomal aberrations reported in T-ALL can also be found in T-LBL (Borowitz & Chan, 2008). However, there are no large series of cytogenetic data on T-LBL (Burkhardt, 2010).

This study aimed to fill the gap regarding cytogenetic data in T-LBL and compare the cytogenetic findings of T-ALL and T-LBL, which may lead to identification of the molecular background behind phenotypical differences between the two disease entities.

Study patients

From November 2004 to October 2010, 154 eligible children (aged 1–18 years) with newly diagnosed advanced stage LBL (Murphy stages III and IV) (Murphy, 1980) were entered in the Japanese Paediatric Leukaemia/Lymphoma Study Group (JPLSG) ALB-NHL03 study (UMIN000002212, http://www.umin.ac.jp/ctr/index-j.htm). Patients with primary immunodeficiencies, Down syndrome and T-cell diseases as second malignancies were excluded. The ethics committee of each participating institute approved the study protocol.

Cytogenetic analysis

Cytogenetic analysis was performed on cell suspensions obtained from 31 tumour/lymph nodes, 19 pleural effusions and six bone marrow samples. The methods of chromosome preparation for cytogenetic analysis are described elsewhere (Sanger *et al*, 1987; Horsman *et al*, 2001). Karyotypes are described according to the International System for Human Cytogenetic Nomenclature (ISCN) (Shaffer & Tommerup, 2005). Only those cases with abnormal cytogenetic study results, defined as two or more cells with the same structural abnormality or the same numerical gain, three or more cells with the same numerical loss or isolated cells with disease-associated abnormalities, were eligible for inclusion in this study.

Statistical methods

Two-tailed Fisher's exact test was used to analyse the patients' characteristics and the frequency of each chromosome abnormality. Significant differences in the analysis of he frequency of each chromosome abnormality were determined by the two-tailed Fisher's exact test with Bonferroni correction comparison. The P value threshold for inclusion of a new variable was chosen to be $P < 0{\cdot}003$ in this analysis (0·05/17, after Bonferroni correction). A review of T-LBL and T-ALL karyotypes reported in the literature was obtained from a PubMed search and information on chromosome abnormalities and gene fusions was obtained from Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer (http://cgap.nci.nih.gov/Chromosomes/Mitelman).

Results

Patient characteristics

A total of 154 children were enrolled on JPLSG ALB-NHL03 protocols; 111 cases were T-LBL. Among 111 T-LBL cases, the study population for the current analysis included 56 patients for whom complete cytogenetic data were obtained. With respect to presenting features, patients with reviewed and accepted cytogenetic data were similar to both those without accepted cytogenetic data and the entire cohort of concurrently enrolled T-lineage LBL patients (Table S1).

Frequency of chromosomal abnormalities

Multiple chromosome abnormalities were identified in 31 patients (45%). Structural chromosome abnormalities were identified in 29 patients (52%), and numerical chromosome abnormalities were identified in 18 patients (32%). Ploidy results included pseudodiploid in 14 patients (25%), hypodiploid in three patients (5%), hyperdiploid with 47–50 chromosomes in 10 patients (18%), hyperdiploid with more than 50 chromosomes in four patients (7%) and diploid in 25 patients (45%) (Table S2).

All of the hypodiploid cases had 43–45 chromosomes; none had a near-haploid karyotype. Of the four cases with more than 50 chromosomes, two had near-tetraploid karyotypes. The frequencies of ploidy groups in this series are compared with those reported in other series of karyotyped T-LBL patients and paediatric T-ALL (Table S2). Structural chromosome abnormalities were identified in 29 patients (52%). In the current study, seven patients (13% of those with abnormal karyotypes) exhibited a rearrangement at one or more of the chromosome bands (7p15, 7q32–36 and/or 14q11–13) that are the locations of T-cell receptor chain genes. Rearrangements in the 14q11–13 region, in which the T-cell receptor α/δ chain genes are located, were present in three patients (5%) of the karyotypically abnormal cases in this series (Table S2). Structural abnormalities involving chromosome region 9q34 were identified in nine patients (16%). Translocations involving chromosome region 9q34 were identified in three patients (5%) (t(9;17)(q34;q22), t(7;9)(q34;q34) and t(2;9)(q23;q34)). In comparison between cytogenetic findings in the current data and combined data of three published reports (Burkhardt

et al, 2006; Lones *et al*, 2007; Uyttebroeck *et al*, 2007; Table S1), the frequencies of numerical and structural cytogenetic abnormalities in T-LBL and T-ALL had no significant difference (Table S2).

We compared the cytogenetic findings in the current study with the published reports from the three largest-scale studies on T-LBL (Burkhardt *et al*, 2006; Lones *et al*, 2007; Uyttebroeck *et al*, 2007; Table S3) and those from the two largest-scale studies on T-ALL combined (Heerema *et al*, 1998; Schneider *et al*, 2000; Table S3) (Table I). The frequencies of almost all of the cytogenetic abnormalities in T-LBL and T-ALL had no significant difference, but translocation involving chromosome region 9q34 was significantly more common in T-LBL than in T-ALL (P = 0·0004, Table S3) and translocation t(9;17) was also more common in T-LBL (4%, 4/92) than in T-ALL (0%, 0/523, P = 0·0004) (Table I).

The current study included a patient with translocation t(9;17)(q34;q22). As far as we could tell from the consulted published reports, all T-LBL patients with translocation t(9;17) presented with a mediastinal mass and without any bone marrow involvement (Kaneko *et al*, 1988; Shikano *et al*, 1992) (Table II).

Table I. Comparison of cytogenetic findings between T-LBL and T-ALL.

	T-LBL		T-ALL		
	n	%	*n*	%	*P* value
Total	92		523		
Normal karyotype†	36	39	219	42	0·6478
Abnormal karyotype	56	61	304	58	0·6478
Hypodiploid	4	4	20	4	0·9999
Pseudodiploid	30	33	204	39	0·2000
Hyperdiploid(47–50)	18	20	64	12	0·0328
Hyperdiploid(>50)	4	4	16	3	0·5217
Any translocation	26	28	177	34	0·3367
Any del chrome.	19	21	160	31	0·0328
Any der chrome.	4	4	58	11	0·0583
del(6q)	6	7	69	13	0·0833
Loss of 9p	10	11	44	8	0·5487
Any 14q11–13 abnormality	10	11	72	14	0·5100
Any 7q32–36 abnormality	7	8	35	7	0·8220
Any translocation including 9q34	8	9	7	1	0·0004*
t(7;10)	1	1	2	0	0·3855
t(10;11)	1	1	8	2	0·9999
t(9;17)	4	4	0	0	0·0004*

†Includes one Klinefelter syndrome, and one inv(9) without other abnormality in current report.

The P value threshold for inclusion of a new variable was chosen to be 0·003 (0·05/17, after Bonferroni correction). *P < 0·003.

T-LBL: current study (JPLSG ALB-NHL03) combined with three published reports(Burkhardt *et al*, 2006; Lones *et al*, 2007; Uyttebroeck *et al*, 2007).

T-ALL: combined two published reports (Heerema *et al*, 1998; Schneider *et al*, 2000).

Discussion

This is the largest study involving cytogenetic analysis of T-LBL and the first study to directly compare cytogenetic findings of T-LBL and T-ALL. The frequencies of almost all of the cytogenetic abnormalities in both entities were found to have no significant difference, but translocation involving chromosome region 9q34 was significantly more common in T-LBL than in T-ALL. The current study included a patient with unique translocation t(9;17)(q34;q22). Interestingly, four out of the 92 T-LBL cases, but none of the 523 paediatric T-ALL cases, showed this translocation (P = 0·0004) (Table I). Translocation t(9;17) has been reported in several haematological diseases, such as precursor B-cell ALL (Coyaud *et al*, 2010), acute myeloid leukaemia (Mrózek *et al*, 2001), chronic myeloid leukaemia (DeAngelo *et al*, 2004), chronic lymphocytic leukaemia (Michaux *et al*, 2005), diffuse large B-cell lymphoma (Hammond *et al*, 1992) and follicular lymphoma (Aamot *et al*, 2007), but these breakpoints, 9q34 and 17q22–23, are limited in the cases of T-LBL (http://cgap.nci.nih.gov/Chromosomes/Mitelman). These results imply a linkage between abnormal expression of genes located at 9q34 and/or 17q22–23 and the unique phenotypes of the T-LBL mentioned above.

Cytogenetic analysis has been used to identify the molecular background of haematological malignancies. To compare the distribution of chromosomal abnormalities of T-ALL and T-LBL, large series of cytogenetic data are required, but are absent in T-LBL in contrast to the abundant data in T-ALL. Three recent series of cytogenetic data on paediatric T-LBL have been published, reporting the cytogenetic findings in 13, 11 and 12 paediatric T-LBL cases (Burkhardt *et al*, 2006; Lones *et al*, 2007; Uyttebroeck *et al*, 2007). Thus, this study can play a role to fill the gap of cytogenetic data on T-LBL.

Translocation involving chromosome region 9q34 was found to be significantly more common in T-LBL than in T-ALL (Table I). Among genes located in the 9q34 region, *SET, PKN3, ABL1, NUP214* and *NOTCH1* have previously been implicated in malignancy, with *SET, ABL1, NUP214* and *NOTCH1* being implicated in leukemogenesis (Ellisen *et al*, 1991; van Vlierberghe *et al*, 2008; Hagemeijer & Graux, 2010).

An oncogenic *SET-NUP214* fusion gene has been reported in a case of acute undifferentiated leukaemia with a reciprocal translocation t(9;9)(q34; q34) (von Lindern *et al*, 1992) and NK adult acute myeloid leukaemia as a result of a cryptic deletion of 9q34 (Rosati *et al*, 2007). van Vlierberghe *et al* (2008) identified the *SET-NUP214* fusion gene in three patient samples out of 92 paediatric cases of T-cell leukaemia. *SET-NUP214* may contribute to T-ALL pathogenesis by inhibition of T-cell maturation through the transcriptional activation of the *HOXA* genes (van Vlierberghe *et al*, 2008). However, the frequency of this mutation in T-LBL is unknown.

NOTCH1, previously termed *TAN1*, was discovered as a partner gene in T-ALL with a translocation t(7;9)(q34;q34.3), and was found in <1% of T-ALLs (Ellisen *et al*, 1991). Several

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Table II. Clinical characteristics and detailed karyotype data in T-LBL patients with t(9;17).

	Age (years)	Sex	Tumour site	Stage	BM blast %	Karyotype
Kaneko *et al* (1988)	14	F	Mediastinum	III	0	46,XX,t(9;17)(q34;q23)
	15	M	Mediastinum	III	0	46,XY,-9,del(6)(q13q21),t(9;17)(q34;q23),+der(9)t(9;17)(q34;q23)
	10	M	Mediastinum	III	0	47,XY,+19,t(9;17)(q34;q23)
Shikano *et al* (1992)	14	F	Mediastinum	III	0	46,XX,t(9;17)(q34;q23)
	7	M	Mediastinum	III	0	49,XY-l,+der(l)t(l;?)(p36;?),t(9;17)(q34;q23),+14,+marl,+mar2
	5	F	Mediastinum	III	0	47,XX,t(9;17)(q34;q23),+der(17)t(9;17)(q34;q23)
Burkhardt *et al* (2006)	ND	ND	ND	ND	ND	46,XX,del(6)(q1?2q1?6),t(9;17)(q34;q22)
	ND	ND	ND	ND	ND	47,XX,t(9;17)(q34;q22),+20
Lones *et al* (2007)	8	M	Mediastinum	III	0	47,XY,t(9;17)(q3?4;q2?3),+20
Current study	7	M	Mediastinum	III	0	46,XY,t(9;17)(q34;q22)

ND, no data available.

study groups reported *NOTCH1* mutations in 31–62% of T-ALL patients (Weng *et al*, 2004; Breit *et al*, 2006; van Grotel *et al*, 2006; Zhu *et al*, 2006; Malyukova *et al*, 2007; Asnafi *et al*, 2009; Gedman *et al*, 2009; Park *et al*, 2009). In contrast, only two studies reported *NOTCH1* mutation analyses in T-LBL: Park *et al* (2009) reported *NOTCH1* mutations in six out of 14 paediatric T-LBL patients (43%), and Baleydier *et al* (2008) reported mutations in six out of nine paediatric T-LBL (66%), with 32 adult patients with *NOTCH1* mutations in 16 cases (54% in all patients) (Baleydier *et al*, 2008). According to these reports, the frequencies of *NOTCH1* mutation were not significantly different between T-LBL and T-ALL.

ABL1 fusion genes have been identified that provide proliferation and survival advantage to lymphoblasts. *NUP214-ABL1, EML1-ABL1, BCR-ABL1* and *ETV6-ABL1* chimeric genes have been reported. The most frequent one in T-ALL is the *NUP214-ABL*1 fusion gene, which has been identified in 6% of cases, in both children and adults (Graux *et al*, 2009). In addition, using an oligonucleotide microarray, *ABL1* overexpression was identified in 8% of cases in T-ALL (Chiaretti *et al*, 2007). Our review of these published reports indicated that the frequency of ABL1 mutation in T-LBL is unknown.

Raetz *et al* (2006) analysed the gene expression profiles of ten T-ALL BM samples and nine T-LBL samples using a microarray. They identified 133 genes for which the expression levels differed between T-LBL and T-ALL. *ZNF79* (encoding zinc finger protein 79) and *ABL1*, both located in chromosome region 9q34, were included in these genes and showed at least twofold higher overexpression in T-LBL than that in T-ALL. Additionally, *MED13* (previously termed *THRAP1*), which is located in 17q22-q23, also showed at least twofold higher overexpression in T-LBL than that in T-ALL (Raetz *et al*, 2006). Taking these findings together, it is possible that *ZNF79, ABL1* or *THRAP1* as well as other genes at 9q34 and 17q22–23 are involved in the 'lymphoma phenotype' such as a bulky mass in the mediastinum and minimal BM involvement. These findings need further study to determine if this linkage constitutes a unique 'lymphoma phenotype'.

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Authorship

MS designed the study, prepared the data file, performed the analysis, interpreted data and wrote the manuscript. SS is a lead principal investigator for the JPLSG ALB-NHL03 study. AN contributed to pathological diagnosis. YH contributed to chromosome analysis. YO is a principal investigator contributing a patient to this study. AMS contributed to statistical analysis. KH received a research grant from the Ministry of Health, Labour and Welfare of Japan. MT is a chairperson of JPLSG. TM is a chairperson of JPLSG lymphoma committee. SS, KH, MT and TM were primarily responsible for the study design, data analysis and interpretation of the data. All authors approved the final manuscript.

Disclosure

The authors declare no competing financial interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Respective clinical characteristics with and without karyotype data in 111 T-LBL patients in the current study.

Table S2. Comparison of cytogenetic findings in T-LBL between current study and combined data of three published reports.

Table S3. Published data of cytogenetic findings in T-LBL and T-ALL.

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bjh research paper

Prospective study of a therapeutic regimen with all-*trans* retinoic acid and anthracyclines in combination of cytarabine in children with acute promyelocytic leukaemia: the Japanese childhood acute myeloid leukaemia cooperative study

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Summary

In childhood acute promyelocytic leukaemia (APL), the efficacy of therapy combining cytarabine with all-*trans* retinoic acid (ATRA) and anthracyclines remains unclear in terms of long-term prognosis. Between August 1997 and March 2004, 58 children with APL (median age: 11 years) were enrolled into an acute myeloid leukaemia (AML) study (AML99-M3) and followed up for a median time of 86 months. The regimen included ATRA and anthracyclines combined with cytarabine in both induction and consolidation. In induction, two patients died of haemorrhage and four patients developed retinoic acid syndrome. Of 58 patients, 56 (96·6%) achieved complete remission, two of whom relapsed in the bone marrow after 15 and 19 months respectively. Sepsis was a major complication, with an incidence of 5·6–10·9% in the consolidation blocks, from which all but one of patients recovered. Consequently, 7-year overall and event-free survival rates were 93·1% and 91·4% respectively, and cumulative incidence of relapse plateaued at 3·6% after 2 years. Follow-up survey of 54 patients revealed no patients with late cardiotoxicity or secondary malignancy, except one with asymptomatic prolongation of QTc interval. This study suggests that the combination of cytarabine with ATRA and anthracycline-based therapy may have useful implications in the perspective of long-term prognosis and late adverse effects for childhood APL.

Keywords: childhood acute promyelocytic leukaemia, all-*trans* retinoic acid, anthracyclines, cytarabine, long-term prognosis.

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The prognosis of patients with acute promyelocytic leukaemia (APL), a distinct subtype of acute myeloid leukaemia (AML) (Grignani *et al*, 1994), has been improved dramatically by the introduction of differentiation induction therapy with all-*trans* retinoic acid (ATRA) (Fenaux *et al*,1999; Tallman *et al*, 2002; Sanz *et al*, 2004). However, recent clinical trials with ATRA and anthracycline-based chemotherapy found that recurrent disease posed a major problem, especially for high-risk patients. (Sanz *et al*, 2000, 2009).

Childhood APL, which consists of only 7–10% of all patients, is often associated with risk factors such as hyperleucocytosis (Guglielmi *et al*,1998; Mann *et al*, 2001); however, few studies of paediatric patients have specifically examined its long-term prognosis. In those studies, the complete remission (CR) and overall survival (OS) rates have been improved to >80%, but event-free survival (EFS) remains at around 70–80% because of increased cumulative incidence of relapse (CIR). (de Botton *et al*, 2004; Ortega *et al*, 2005; Testi *et al*, 2005) In addition to frequent relapse in the bone marrow, extramedullary (EM) relapse involving mostly the central nervous system (CNS) occurs at incidence of 1–5%. (Ko *et al*, 1999; de Botton *et al*, 2006; Chow & Feusner, 2009) The therapeutic effectiveness of cytarabine added to anthracycline-based consolidation therapy has been reported for high-risk adult patients(Adès *et al*, 2006, 2008), but the efficacy of cytarabine in addition to the combination of ATRA and anthracyclines in consolidation remains unknown for paediatric patients.

More recently, there has been increasing concern regarding long-term adverse effects, including cardiotoxicity and secondary malignancy, for children with leukaemia. The cumulative dosage of anthracyclines may be related to the risk of late cardiotoxicity as well as therapy-related myelodysplastic syndrome (t-MDS)/AML for childhood malignancies (Nysom *et al*,1998; Le Deley *et al*, 2003). Although such effects of anthracyclines are yet undetermined for APL, the cumulative dosage of anthracyclines may be an important perspective of the long-term prognosis of children with APL.

This report describes the outcome of a prospective study for childhood APL, AML99-M3, in which patients received therapy with cytarabine in addition to ATRA and anthracyclines. The improved outcome of this study suggests that the combination of cytarabine, ATRA and anthracyclines may have useful implications in the perspective of long-term prognosis and late adverse effects for childhood APL.

Patients and methods

Patients

Between August 1997 and March 2004, 58 children with *de novo* APL (31 males and 27 females; median age of 11 years [range:11 months – 16 years] were enrolled in the AML99-M3 study of the Japanese Childhood AML Cooperative Study Group, and a follow-up survey was performed in May 2010 (Table I). Three patients with APL were not recruited to this study: two had already started another chemotherapeutic regimen for AML when APL was diagnosed; the other died of intracranial haemorrhage (ICH) at diagnosis. The relevant institutional review board approved the protocol. Written informed consent was obtained from the parents of all patients. APL was diagnosed according to the French–American–British (FAB) criteria (Bennett *et al*, 1982); the involvement of t(15;17) translocation was examined cytogenetically. APL patients with t(15;17) translocation or *PML-RARA* chimaeric gene confirmed through examinations with fluorescence *in situ* hybridization (FISH) or reverse transcription–polymerase chain reaction (RT-PCR) were registered to this study.

Treatment protocol

In remission induction therapy, ATRA was initiated (45 mg/m^2, until CR) and then daunorubicin (45 mg/m^2 per d, days 6–8) and cytarabine (200 mg/m^2, days 6–12) were added (Fig 1). For patients with a white blood cell (WBC) count >10 × 10^9/l at diagnosis or after the initiation of ATRA therapy, chemotherapy was started before day 6. Consolidation

Table I. Characteristics of patients with APL (N = 58).

Characteristics	Median	Range	No. (%)
Age, years	11	0·9–16	
<5			9 (16)
5 to 10			14 (24)
>10			35 (60)
Sex			
Male			31 (53)
Female			27 (47)
WBC, ×10^9/l	4·3	0·7–171	
<10			36 (62)
≥10			22 (38)
Haemoglobin, g/l	91	37–131	
<10			44 (76)
≥10			14 (24)
Platelets, ×10^9/l	2·3	5–233	
<40			48 (83)
≥40			10 (17)
FAB subtype			
Typical			53 (91)
Variant			5 (9)
Cytogenetics			
t(15;17)			47 (81)
t(15;17) + others			9 (15)
Normal			1 (2)
Unknown			1 (2)
PML-RARA			
Examined			47 (81)
Long isoform (bcr1)			21
Short isoform (bcr3)			8
bcr not determined			18
Not examined			11 (19)

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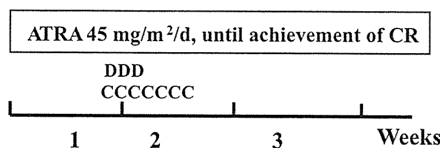
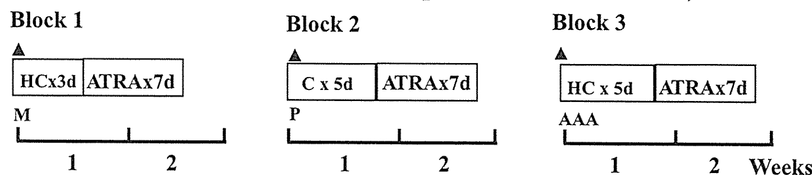
(1) Remission induction phase



(2) Consolidation phase (serial twice repeat of the same blocks)



(3) Maintenance phase (every 3 months, 4 times for 1 year)

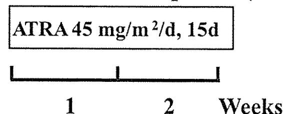


Fig 1. Scheme of AML99-M3 protocol. In remission induction, oral administration of ATRA (45 mg/m^2 per d) was combined with daunorubicin (D) (45 mg/m^2) and cytarabine (C) (200 mg/m^2). In consolidation, administration of ATRA (45 mg/m^2 per d for 7 d) was combined with mitoxantrone (M) (10 mg/m^2 per d, day 1) and high-dose cytarabine (HC) (3 g/m^2 × 2/d, days 1–3) in Block 1, with pirarubicin (P) (45 mg/m^2 per d, day 1) and cytaratibine (C) (200 mg/m^2 per d, days 1–5) in Block 2, and with aclarubicin (A) (30 mg/m^2 per d, days 1–3) and high-dose cytarabine (HC) (3 g/m^2 per d, days 1–5) in Block 3. For CNS prophylaxis, intrathecal injection (▲) of methotrexate, cyatarbine and hydrocortisone at day 1 of every consolidation block in age-adjusted doses as described in Methods. In maintenance therapy, ATRA (45 mg/m^2 per d, days 1–15) was administrated every 3 months for 1 year.

therapy consisted of six courses of block treatment (Blocks 1, 2 and 3), in which each block was performed every month and the same block was repeated serially twice. In respective blocks, chemotherapy with cytarabine and each of the different anthracycline agents was administrated respectively and then ATRA (45 mg/m^2 per d, 7 d) was administrated consecutively. Block 1 consisted of mitoxantrone (10 mg/m^2 per d × day 1) and cytarabine (3 g/m^2 × 2/d × days 1–3), Block 2 of pirarubicin (45 mg/m^2 per d × day 1) and cytarabine (200 mg/m^2 per d × days 1–5), and Block 3 of aclarubicin (30 mg/m^2 per d × days 1–3) and cytarabine (3 g/m^2 per d × days 1–5). On the first day of each consolidation block, patients received intrathecal (IT) therapy with methotrexate (3 mg for <3 months; 6 mg for 3 months to <1 year; 7·5 mg for 1 year; 10 mg for 2 years; 12·5 mg for 3 years or older), cytarabine (6 mg for <3 months; 12 mg for 3 months to <1 year; 15 mg for 1 year; 20 mg for 2 years; 25 mg for 3 years or older) and hydrocortisone (10 mg for <3 months; 10 mg for 3 months to <1 year; 15 mg for 1 year; 20 mg for 2 years; 25 mg for 3 years or older). In maintenance therapy, ATRA alone (45 mg/m^2 per d) for 15 consecutive days was given every 3 months, for a total of four times during 1 year.

Adverse effects

Retinoic acid (RA) syndrome was diagnosed based on clinical signs, including fever, respiratory distress, pulmonary infiltration, pleural and pericardial effusion and renal failure. (Ko *et al*, 1999) When RA syndrome was diagnosed or strongly suspected, ATRA therapy was stopped and the patients received administration of dexamethasone (8 mg/m^2 per d, i.v. in two doses) unless they clinically improved. Disseminated intravascular coagulopathy (DIC), bacterial infections, and other adverse effects were summarized in each phase of treatment. Long-term adverse effects, including cardiotoxicity and secondary malignancy, were surveyed through followe-up analysis. For evaluation of the potential risk of cardiotoxicity, cumulative doses of anthracyclines were converted to equivalent doses of daunorubicin using ratios in 1:3–1:5 for idarubicin/mitoxantrone, 1:1·6 for pirarubicin, and 1:0·2 for aclarubicin (Warrell, 1986; Lenk *et al*, 1990; Sakata-Yanagimoto *et al*, 2004).

Minimal residual disease (MRD) monitoring

For MDR monitoring, the *PML-RARA* chimaeric mRNA in marrow samples was detected using RT-PCR as described (Suzuki *et al*, 2001). Serial evaluation of MRD monitoring was performed every 3 months for 17 patients whose bone marrow samples were sent to the reference laboratory.

Statistical analysis

The OS and EFS were calculated from the beginning date of induction therapy to the date of events; failure to achieve CR, relapse or death of any cause. The OS and EFS were analyzed

using the Kaplan–Meier method. Statistical analyses used the Statistical Package for the Social Sciences (SPSS) software, version 16 (SPSS Japan Inc., Tokyo, Japan), estimated by the log-rank test and considered to be significant when a P value is $<0{\cdot}05$. For patients who achieved CR, cumulative incidence functions of relapse as well as death without relapse were calculated using the competing risk method with the cmprsk software package (http://biowww.dfci.harvard.edu/~gray), ver.2.1-5 on R ver.2.10.1.

Results

Patient characteristics

The median follow-up period of 58 patients was 86 months (range: 16 d–12·1 years) (Table I). The median age of patients was 11 years (range: 11 months–16 years); 35 (60%) patients were over 10 years old; 31 patients were male and 27 were female. The WBC counts at diagnosis were $0{\cdot}9$–171×10^9/l (median: $4{\cdot}3 \times 10^9$/l) and 22 patients (38%) had WBC counts $>10 \times 10^9$/l. The proportion of these high-risk patients was comparable to that (35–48%) reported by other studies for childhood APL (de Botton *et al*, 2004; Ortega *et al* 2005; Testi *et al*, 2005). Haemoglobin levels were 37–131 g/l (median: 91 g/l). Platelet counts were 5–233×10^9/l (median: 23×10^9/l) and 48 patients (83%) had a platelet count $<40 \times 10^9$/l at diagnosis. Haematological examination identified FAB:M3 morphology in 53 patients and five others exhibited the microgranular FAB: M3v morphology. No patient showed leukaemic infiltration in the cerebrospinal fluid obtained by lumbar puncture performed for CNS prophylaxis at the beginning of consolidation therapy.

Cytogenetic examination revealed that 47 patients had t(15;17) translocation abnormality alone, nine had t(15;17) with additional chromosomal abnormalities, one with normal karyotype, and one with no result. In the latter two patients, the involvement of *PML-RARA* chimaeric gene was confirmed using RT-PCR. Examinations for *PML-RARA* were performed in 47 patients. RT-PCR detected *PML-RARA* in 29 patients, 21 of whom showed the long type (bcr1) isoform; eight showed the short type (bcr3) isoform. Eighteen patients had *PML-RARA* detected by FISH analysis without differentiation of the isoform types. No patient had ATRA-insensitive fusion genes, such as the *ZBTB16-RARA* caused by the t(11;17) chromosomal translocation.

Clinical course and statistical analysis

In induction therapy, two patients (3·4%) died from ICH and pulmonary bleeding after 16 and 24 d respectively. CR was achieved in 56 patients (96·6%), two of whom exhibited relapse at bone marrow; one relapsed at 15 months and died of ICH, the other relapsed at 19 months and remains in second CR after treatment with marrow transplantation. For patients who achieved CR, the period of ATRA administration in induction was a median of 29 d (range 14–60 d), during which 13 patients temporarily discontinued the administration of ATRA for a median 4 d (range 1–31 d). Overall, four patients died: two of DIC with haemorrhage during induction, one of sepsis and meningitis in remission, and one of ICH after relapse. Consequently, the OS and EFS rates at 7 years were respectively, 93·1% (95% confidence interval [CI], 86·5–99·7%) and 91·4% (95% CI, 84·0–98·4%) (Fig 2A). No significant difference was found in the OS and EFS rates between patients with or without haematological risk factors, such as WBC count $>10 \times 10^9$/l or platelet count $<40 \times 10^9$/l. (Sanz *et al*, 2000) (Figs 2B, C) The CIR was 3·6% (95%CI: 0–8·5%) at 7 years, while the cumulative incidence of death without relapse, one of the competing events, was 1·8% (95%CI: 0–5·3%) at 7 years (Fig 2D).

Adverse effects and events

Table II presents the incidence of adverse effects and the duration of neutropenia. In induction therapy, DIC was observed in 10 patients (17%) and four of these patients (7%) showed haemorrhagic complications including retinal haemorrhage in two patients and ICH and/or pulmonary haemorrhages in the other two who died. RA syndrome, which occurred in 7% of cases, was resolved with cessation of ATRA and administration of dexamethasone, the incidence of which was comparable to those (7–19%) reported by other studies of childhood APL (de Botton *et al*, 2004; Ortega *et al* 2005; Testi *et al*, 2005).

Bacterial infection was the major adverse effect in induction and consolidation, and sepsis with documented microbes was determined at a higher incidence during consolidation than induction. Although one patient died in remission of pseudomonas sepsis and meningitis after Block 2 consolidation, all other patients recovered from sepsis with treatment. A proportional relationship was apparent between the periods of neutropenia ($<0{\cdot}1 \times 10^9$/l) and the incidence of whole infections at any sites, including gingivitis, stomatitis, bronchopneumonia, enteritis, or cellulites during neutropenia, and herpes zoster only in maintenance. Other complications included impaired consciousness or convulsion associated with pseudotumour cerebri and aclarubicin-related dysuria in consolidation Block 3. Severe headache/nausea associated with ATRA therapy was experienced at an incidence of 8–22% throughout treatment.

Table III shows the characteristics of five patients with early death or relapse, two of whom exhibited at least one of the following: WBC count $>10 \times 10^9$/l, M3v morphology, *PML-RARA* bcr3 isoform. The proportion of these patients was not significantly different from that of the whole population of 58 patients. Because of adverse effects, Block 3 consolidation was omitted or reduced in dosage at the physician's discretion in five patients, including two with WBC count $>10 \times 10^9$/l, of whom all remained in remission for 4·9–8·9 years.

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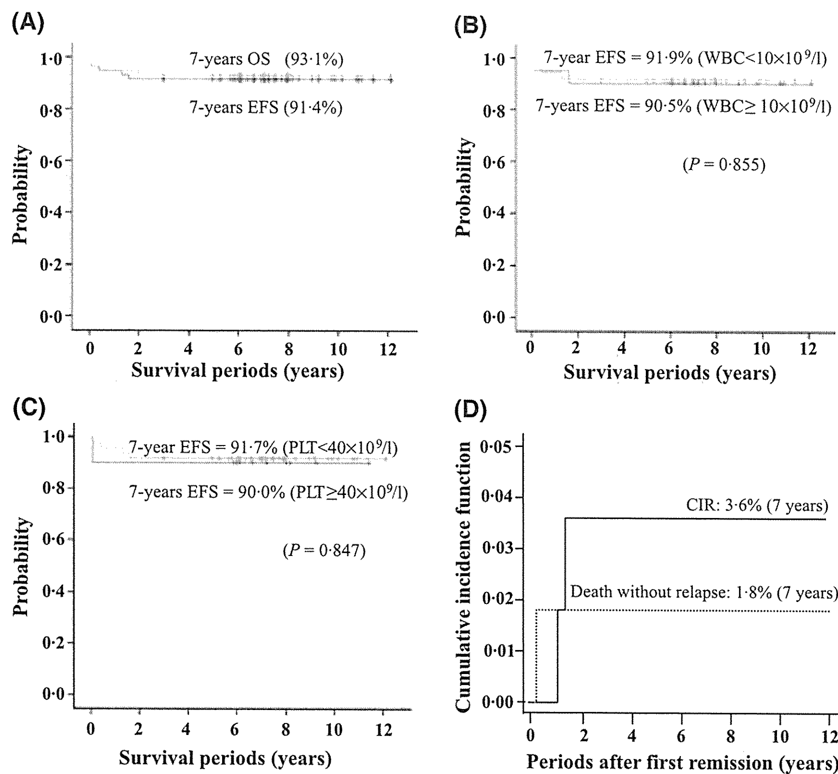

Fig 2. Analysis of the rates of OS, EFS and cumulative incidence functions of CIR and death without relapse in patients treated with the AML99-M3 protocol. (A) OS and EFS rates of total patients; (B) EFS rates of patients with WBC count > 10 × 10^9/l or <10 × 10^9/l at diagnosis; (C) EFS rates of patients with a platelet (PLT) count <40 × 10^9/l or >40 × 10^9/l at diagnosis. No significant difference was found in the EFS rates of patients with and without these risk factors. (D) the cumulative incidence functions of CIR (solid line) and death without relapse (dotted line). [Correction added on 1 October 2010, after first online publication: The data in Figure 2B was amended.]

Table II. Incidence of adverse effects and periods of neutropenia.

	Induction	Consolidation			Maintenance
		Block 1	Block 2	Block 3	
No of assessed patients	55	54	54	53	49
Deterioration of DIC with serious haemorrhages, %	7·2	0	0	0	0
Sepsis, %	1·8	9·2	10·9	5·6	0
Infection of any site, %	10·8	14·5	14·8	15·9	10·2
RA syndrome, %	7	0	0	0	0
Consciousness impairment and/or convulsion, %	3·6	1·8	0	0	0
Severe headache or nausea, %	23·6	11·1	12·9	13·2	8·1
Dysuria, %	0	0	0	3·7	0
Duration of ANC < 0·5, days	17·2	14·3	16·1	16·1	0
Duration of ANC < 0·1, days	6·3	10	10·3	10·9	0

DIC, disseminated intravascular coagulopathy, RA syndrome, retinoic acid syndrome; ANC, absolute neutrophil count, ×10^9/l.

In the evaluation of late cardiotoxicity, echocardiography and electrocardiogram were performed in 18 patients, of whom one patient showed asymptomatic prolongation of the QTc interval in the electrocardiogram. Except for this patient, no clinical symptoms of late cardiotoxicity was seen in other patients including those who did not receive examinations. As of May 2010, no patient had developed t-MDS/AML.

MRD monitoring

In 17 patients, including six with WBC count > 10 × 10^9/l, MRD monitoring was performed at the initial onset and subsequently every 3 months; the monitoring period was an average of 13·6 months. As a result, MRD levels became undetectable (lower than 10^{-3}–10^{-4}) after consolidation Block 1 in 16 patients (94%) and another PCR-positive patient