Recently, a novel oral iron chelator, deferasirox (Exjade®), has been introduced in more than 60 countries, including Japan. The introduction of deferasirox may improve compliance with iron chelation therapy [4]. Under these circumstances, the National Research Group on Idiopathic Bone Marrow Failure Syndromes in Japan drew up Japanese guidelines for the treatment of transfusion-induced iron overload. Herein, we describe the current status of iron overload in transfusion-dependent patients in Japan, and development of the proposed guidelines for the treatment of transfusion-induced iron overload.

2 Current status of transfusion-induced iron overload in Japan

In 2005, the first nationwide survey on iron overload in transfusion-dependent patients in Japan was carried out [3]. This retrospective survey investigated the outcomes of iron overload-related morbidity and mortality from August 2001 to December 2005. A questionnaire was sent to hematology departments in hospitals all over Japan, and 43 hospitals responded by returning data on 292 patients.

Demographic data showed that MDS and AA accounted for about 80% of the underlying diseases: MDS, 52.1%; AA, 30.8%; pure red cell aplasia (PRCA), 5.1%; and myelofibrosis (MF), 4.5%. Serum ferritin levels were significantly correlated with the lifetime total number of RBC transfusion units received. Figure 1 shows the relationship between the number of RBC units and mean ferritin level, indicating the percentage of patients with an abnormal ferritin level (≥1,000 ng/mL) for any total number of RBC units received as analyzed by a logistics model. The goodness-of-fit of this model between theoretical and actual values was assessed by Pearson chi-squared test, and the estimated number of RBC units required to raise ferritin to ≥1,000 ng/mL in 50 and 75% of patients was calculated as 21.5 and 43.4 units, respectively.

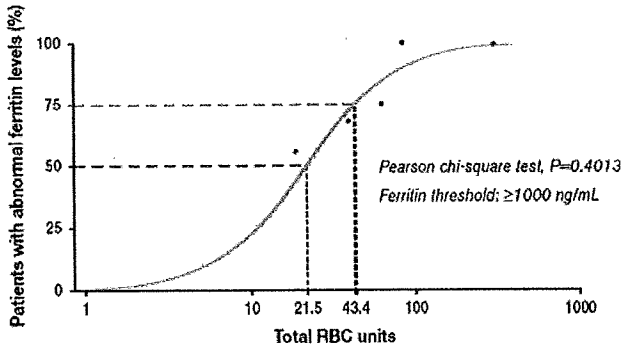

Fig. 1 Relationship between serum ferritin and total number of red blood cell units. [3] Modified with permission from Takatoku et al. *Eur J Haematol.* 2007;78:487–494. ©2007 Blackwell Publishing

Serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) abnormalities were significantly correlated with transfusion frequency and increased ferritin levels; there was a significantly ($P < 0.0001$) higher prevalence of SGOT and SGPT abnormality in patients with high serum ferritin than in those whose serum ferritin was <1,000 ng/mL (Fig. 2). Moreover, among patients in whom cardiac function was evaluated, abnormalities were found in 21.9%, and cardiac abnormality was weakly correlated with serum ferritin levels. These data indicate that ferritin levels can be a useful predictor of hepatic and cardiac dysfunction. Fasting blood sugar (FBS) abnormality was also correlated with transfusion frequency.

In the survey, 75 deaths were reported, most of which were caused by infection and leukemia. However, cardiac and hepatic failure was noted in 24% and 6.7% of cases, respectively. Patients who died from cardiac or hepatic failure had received more transfusions than those who died from other causes, and among 38 patients in whom serum ferritin levels were available, 37 patients died with serum ferritin levels ≥1,000 ng/mL; the majority of patients (24 patients) had serum ferritin levels >5,000 ng/mL. These data indicate that multiple transfusion therapy is associated with a high risk of fatal complications caused by iron overload. Recently, similar analyses have been reported describing that transfusion-dependent MDS patients show significantly shorter survival than those who do not require transfusions and that transfusion-induced iron overload significantly affects survival [5].

3 Iron chelation therapy

As phlebotomy is not an option because of underlying bone marrow failure, the only way to treat iron overload is with iron chelation therapy. Until recently, the only available iron chelating agent in Japan was DFO. Because of the limited absorption from the gastrointestinal tract and short biological half-life of the agent, the drug must be administered by parenteral injections at least 5–7 times a week, or continuously for optimal effectiveness [6]. In the survey, 43.2% of patients received DFO, but only 8.6% received DFO daily or continuously; most of the patients were administered the drug intermittently (average once per 1.9 weeks) or concurrently with transfusion [3]. While improvements in serum ferritin, SGOT, SGPT and FBS were noted in the patients who received DFO daily or continuously, these data did not improve, and rather worsened, in those without optimal administration (Table 1). This indicates that appropriate administration of the chelating agent is needed for sufficient therapeutic results.

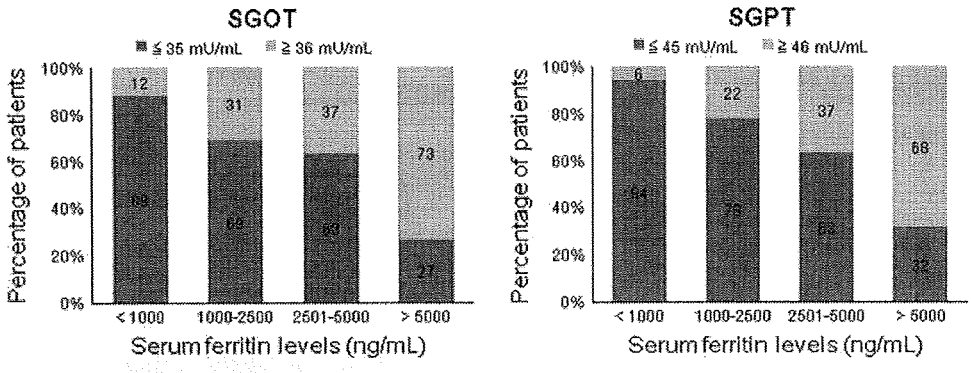

Fig. 2 Relationship between serum transaminase abnormality and serum ferritin levels. [3] Modified with permission from Takatoku et al. *Eur J Haematol.* 2007;78:487–494. ©2007 Blackwell Publishing

Table 1 Average changes in laboratory values during the period of transfusion dependence in patients receiving deferoxamine treatment

Parameter	Intermittent (once/1.9 week)	Concurrent with transfusion	Daily/continuous
Serum ferritin[a,b] (ng/mL)	+2222.8 ($n = 36$)	+2204.8 ($n = 19$)	−1135.2 ($n = 9$)
SGOT[a,c] (mU/mL)	+28.0 ($n = 53$)	+40.0 ($n = 30$)	−9.2 ($n = 10$)
SGPT (mU/mL)	+28.6 ($n = 53$)	+10.3 ($n = 30$)	−28.8 ($n = 10$)
FBS (mg/dL)	+31.2 ($n = 31$)	+8.2 ($n = 12$)	−4.8 ($n = 5$)

[3] Modified with permission from Takatoku et al. *Eur J Haematol.* 2007;78:487–494. ©2007 Blackwell Publishing

[a] Intermittent versus continuous, $P < 0.05$

[b] Continuous versus concurrent, $P < 0.01$

[c] Continuous versus concurrent, $P < 0.05$

Moreover, it has also been reported that iron chelation not only reduced iron burden and improved organ dysfunction, but also ameliorated the hemoglobin levels of iron-overloaded patients [7, 8]. Although the biological mechanism of the hematopoietic recovery remains to be elucidated, this fact indicates that iron itself negatively impacts on hematopoiesis, and in some conditions removal of iron burden from the hematopoietic environment can restore normal hematopoiesis.

Deferasirox is easily absorbed in the gastrointestinal tract and has an elimination half-life of 8–16 h, which means that deferasirox is continuously present in the plasma with once-daily dosing [9]. In a large Phase III trial, deferasirox was comparable with DFO at decreasing iron burden in β-thalassemic patients [10]. Deferasirox also reduced iron burden in patients with various anemias including MDS [11]. These findings indicate that oral iron chelators can improve patients' quality of life by ameliorating organ dysfunction and preventing iron damage, even improving hematopoiesis itself. Oral iron chelators are expected to prolong survival of transfusion-dependent patients.

4 Japanese guidelines for the treatment of iron overload in transfusion-dependent patients

The clinical significance of iron chelation is undeniable and requires attention. With the availability of deferasirox in Japan, the frequency of continuous treatment may be strengthened and many more patients can benefit from chelation therapy. To help optimal iron chelation therapy, the National Research Group on Idiopathic Bone Marrow Failure Syndromes drew up the Japanese guidelines for the treatment of transfusion-induced iron overload. To date, guidelines for iron overload have been developed in several countries [6, 12–14], and the Japanese guidelines were designed to align with the international guidelines (see the paper by Dr. Gattermann in this issue). The essential features of the Japanese guidelines are depicted in Fig. 3 and Table 2.

The contents of the guidelines are as follows:

Patients who may benefit from chelation therapy: The guidelines are applicable to transfusion-dependent patients with primary (MDS, AA, PRCA, MF, etc.) and secondary (chemotherapy-induced, etc.) bone marrow failure. Transfusion-dependent patients are defined as those receiving >2 RBC units/month for ≥6 months. Because organ dysfunction becomes symptomatic after a certain period of time, it is suggested that iron chelation therapy is offered to patients with an expected survival of more than 1 year. The international guidelines for MDS patients also recommend that they should have a life expectancy of ≥1 year.

Diagnosis of iron overload: After patients become transfusion dependent, regular examination of serum ferritin is required to monitor iron burden at least once every 3 months. For early diagnosis of organ dysfunction,

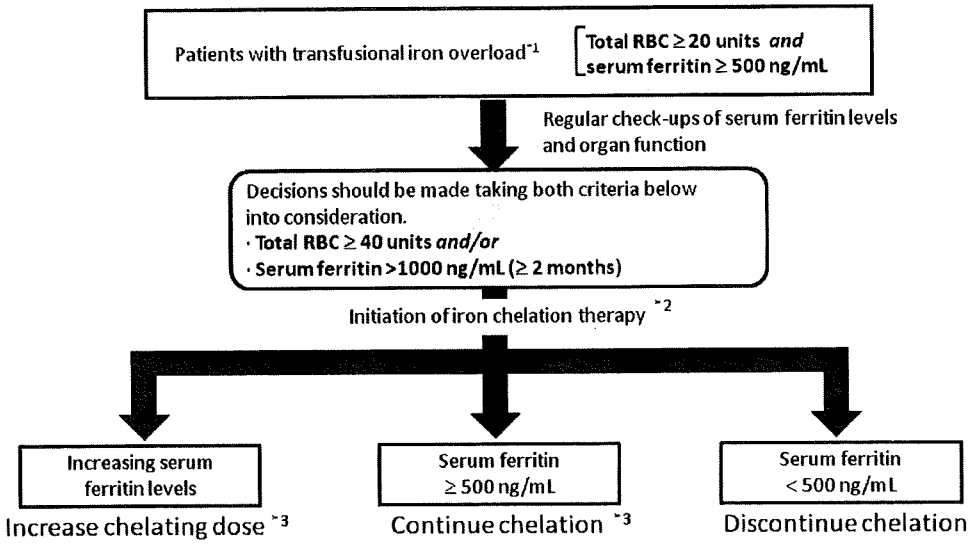

Fig. 3 A flow chart for the treatment of transfusion-dependent iron overload

*1 Patients who are transfusion dependent (≥ 2 RBC units/month for ≥ 6 months) and are expected to survive for >1 year.
*2 Monitoring serum ferritin levels at least once in 3 months is required.
*3 Regular check-ups of renal and hepatic function, and annual eye and hearing tests are necessary.

periodic check-ups of cardiac, hepatic and pancreatic endocrine functions are recommended.

Patients can be said to be iron overloaded when their serum ferritin levels reach >500 ng/mL and when they have received >20 Japanese RBC units (in pediatric patients, >50 mL/kg body weight). Severity of iron overload is determined by serum ferritin levels and organ dysfunction (Table 2, lower part).

Initiating iron chelation therapy: Administration of an iron chelator is the only recommended treatment for iron overload in patients with bone marrow failure. To initiate iron chelation therapy, confirmation of serum ferritin levels >1,000 ng/mL for more than 2 months, at least in two successive examinations, is recommended. The nationwide survey reported that more than 90% of patients who suffered from organ dysfunction had serum ferritin levels >1,000 ng/mL, and prevalence of hepatic dysfunction increases in parallel with ferritin levels [3] (Fig. 2). Therefore, a serum ferritin level >1,000 ng/mL is considered the appropriate point to initiate iron chelation. However, serum ferritin levels are not reliable in patients with inflammatory conditions such as Still's disease and hemophagocytic syndrome, or in those with malignancies. In these cases, transfusion history should be taken into account. Therefore, receiving a total of more than 40 Japanese RBC transfusion units (in pediatric patients, >100 mL/kg body weight) was included as another recommended criterion. As mentioned previously, about 75% of patients who received >40 RBC units have serum ferritin levels >1,000 ng/mL, indicating that 40 units of RBC transfusion can be a good indicator of transfusion-induced hyperferritinemia. However, transfusion history alone is also not reliable, because serum ferritin levels may not increase in patients with chronic bleeding and hemolysis. Furthermore, patients who have already discontinued transfusion therapy with successful treatment may not require iron chelation therapy. If neither of these two criteria is applicable, chelation therapy should not be started.

Target ferritin maintenance levels and adverse effects of iron chelators: During chelation therapy, monitoring of iron burden and organ functions should be continued. After initiating chelation therapy, serum ferritin levels should decrease, but if they continue to increase, even 3–6 months after starting treatment, an increase in dose is necessary. When patients are minimally transfusion dependent (<2 RBC units/month) or already free of transfusions, dose adjustment must be determined carefully.

It is recommended that serum ferritin levels are maintained at 500–1,000 ng/mL, and when ferritin levels are below 500 ng/mL at two successive examinations, chelators should be discontinued. As an excessive reduction in iron burden is harmful, the guidelines have determined this target value (500–1,000 ng/mL) with a safety margin.

As iron chelating agents can induce adverse effects on the kidney, liver and sensory organs [10], regular examination of renal and hepatic functions, and periodical (prior to treatment and annually after initiation) ophthalmologic examinations and hearing tests, are recommended. If an abnormal increase in serum creatinine level is noticed, the drug should be decreased or discontinued. In patients with a high risk of renal dysfunction, weekly monitoring of creatinine level is recommended, at least during the first

Table 2 Japanese guidelines for transfusional iron overload (main points)

Patients	Transfusion-dependent patients with bone marrow failure syndromes who are likely to survive for >1 year
Diagnosis of iron overload	1. Total RBC >20 units[a] (in pediatric patients, RBCs >50 mL/kg body weight) *and* 2. Serum ferritin >500 ng/mL
Criteria for initiating chelation therapy	1. Total RBC >40 units[a] (in pediatric patients, RBCs >100 mL/kg body weight) *and/or* 2. Serum ferritin >1,000 ng/mL Decisions should be made taking both criteria into consideration, especially for patients: –with chronic bleeding or hemolysis; –who no longer need RBC transfusions; –with complications that chronically raise serum ferritin levels independently of transfusion; e.g., Still's disease, hemophagocytic syndrome and malignancies
Target serum ferritin maintenance level	Serum ferritin 500–1,000 ng/mL

Classified severity of iron overload

Serum ferritin (ng/mL)	With normal organ function	With organ dysfunction
>500	Stage 1A	Stage 1B
>1,000	Stage 2A	Stage 2B
>2,500	Stage 3A	Stage 3B
>5,000	Stage 4A	Stage 4B

The severity of iron overload is defined by serum ferritin level and organ dysfunction (cardiac, liver and pancreatic endocrine dysfunction). The dysfunction must be considered to be related to iron overload; i.e., the organ dysfunction progresses as serum ferritin or transfusion burden increase

The criteria for specific organ dysfunction are as follows

–Cardiac dysfunction: LVEF <50%

–Hepatic dysfunction: abnormal transaminase levels, fibrosis and cirrhosis of the liver

–Pancreatic endocrine dysfunction: impaired glucose tolerance

[a] 20 and 40 units of the Japanese RBC transfusion correspond to 10 and 20 Western RBC units, respectively

month. Furthermore, if drug-induced hepatic injury is suspected, withdrawal of the drug with appropriate treatments is needed. It has been reported that iron chelators can cause hearing loss and cataracts. Therefore, if any signs of dysfunction are noticed a dose reduction or discontinuation of the drug is necessary and prompt consultation by an ophthalmologist or otorhinolaryngologist is required. In pediatric patients, annual monitoring of height, weight and state of secondary sex characteristics are needed for an early diagnosis of abnormal development.

5 Conclusions

The retrospective survey of transfusion-dependent patients revealed that the mortality rate is raised in heavily iron-overloaded patients, with liver and cardiac dysfunction being the primary cause of death [3]. Daily or continuous chelation therapy is effective in reducing iron burden and improving organ function, but practically, daily or continuous administration through parenteral injection is difficult. In Japan, a novel oral chelator, deferasirox, has recently been approved. Oral iron chelators can improve compliance of treatment and many more patients who need iron chelation may benefit from a reduction in iron burden and improvement of organ function, which ultimately may lead to the improvement of patients' prognosis and quality of life.

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ORIGINAL ARTICLE

Diagnosis of acute myeloid leukemia according to the WHO classification in the Japan Adult Leukemia Study Group AML-97 protocol

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Abstract We reviewed and categorized 638 of 809 patients who were registered in the Japan Adult Leukemia Study Group acute myeloid leukemia (AML)-97 protocol using morphological means. Patients with the M3 subtype were excluded from the study group. According to the WHO classification, 171 patients (26.8%) had AML with recurrent genetic abnormalities, 133 (20.8%) had AML with multilineage dysplasia (MLD), 331 (51.9%) had AML not otherwise categorized, and 3 (0.5%) had acute leukemia of ambiguous lineage. The platelet count was higher and the rate of myeloperoxidase (MPO)-positive blasts was lower in AML with MLD than in the other WHO categories. The outcome was significantly better in patients with high (≥50%) than with low (<50%) ratios of MPO-positive blasts ($P < 0.01$). The 5-year survival rates for patients with favorable, intermediate, and adverse karyotypes were 63.4, 39.1, and 0.0%, respectively, and 35.5% for those with 11q23 abnormalities ($P < 0.0001$). Overall survival (OS) did not significantly differ between nine patients with $t(9;11)$ and 23 with other 11q23 abnormalities ($P = 0.22$). Our results confirmed that the cytogenetic profile, MLD phenotype, and MPO-positivity of blasts are associated with survival in patients with AML, and showed that each category had the characteristics of the WHO classification such as incidence, clinical features, and OS.

Keywords AML · WHO classification · Myeloperoxidase · Multilineage dyplasia · 11q23 abnormalities

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1 Introduction

The French-American-British (FAB) classification of acute myeloid leukemia (AML), based on morphological and cytochemical findings, was established in 1976 and has since become the standard classification [1, 2]. However, specific chromosomal and genetic abnormalities that have been extracted from analyses of prognostic factors for AML are recognized as important in selecting treatment strategies and are reflected in the AML classification as

factors that are required to establish the disease entity [3]. The 1999 World Health Organization (WHO) classification includes morphological, immunological, cytogenetic, genetic, and clinical features [4–6]. The WHO and FAB classifications differ in several aspects. The blast threshold required for a diagnosis of AML was reduced from 30 to 20%, and new AML categories have been added for cytogenetic abnormalities, the presence of multilineage dysplasia (MLD), as well as a history of chemotherapy and subtypes for acute basophilic leukemia, acute panmyelosis with myelofibrosis, and myeloid sarcoma. The WHO classification comprises more subtypes and is more comprehensive than the FAB classification.

Cytogenetic features are important prognostic factors in AML [3, 7–12]. However, 11q23 abnormalities have not yet been established as a cytogenetic risk classification. Over 30 partner genes with 11q23 abnormalities have been described, and some reports indicate that patients with $t(9;11)$ have a relatively more favorable prognosis than those with other partner chromosomes/partner genes [13–16].

In the present study, we reviewed stained smears of blood and bone marrow from patients who were registered in the Japan Adult Leukemia Study Group (JALSG) AML-97 trial, and classified them into FAB subtypes and WHO categories. We also evaluated their survival on the basis of the WHO classification, the myeloperoxidase (MPO)-positivity of blasts, and cytogenetic findings including 11q23 abnormalities.

2 Patients and methods

2.1 Patients

Between December 1997 and July 2001, 809 patients aged from 15 to 66 years with untreated AML (excluding M3) were registered from 103 institutions in the AML-97 trial of the JALSG. The patients were diagnosed with AML according to the FAB criteria at each institution. Patients with a history of MDS, hematological abnormalities before the diagnosis of AML, or a history of chemotherapy were not eligible for the AML-97 trial.

2.2 Treatment strategies

Details of the JALSG AML-97 treatment protocol are described elsewhere [17]. In brief, all patients underwent induction therapy consisting of idarubicin (3 days) and Ara-C (7 days). Patients who achieved complete remission were randomized into one of two arms of consolidation chemotherapy alone or in combination with maintenance chemotherapy. Patients who were placed into intermediate/poor risk groups according to the JALSG scoring system [17] and who had an HLA-identical sibling ($\leq$50 years old) were simultaneously assigned to receive allogeneic hematopoietic stem cell transplantation during their first remission.

2.3 Morphologic and cytochemical analyses

Peripheral blood and bone marrow smears from registered patients were sent to Nagasaki University for staining with May-Giemsa, MPO, and esterase, and the diagnosis was then reevaluated by the Central Review Committee for Morphological Diagnosis. Patients were subsequently categorized according to the FAB and WHO classifications. Dyserythropoietic features were defined as >50% dysplastic features in at least 25 erythroblasts and dysgranulopoietic features including $\geq$3 neutrophils with hyposegmented nuclei (pseudo-Pelger–Heut anomaly), and hypogranular or agranular neutrophils (>50% of $\geq$10 neutrophils). Dysmegakaryopoietic features were defined as $\geq$3 megakaryocytes that were micronuclear, multiseparate nuclear, or large mononuclear [18].

We assessed the ratios (%) of MPO-positive blasts on MPO-stained bone marrow smears using the diaminobenzidine method [19].

2.4 Cytogenetic analysis

Cytogenetic analysis was performed at either laboratories in participating hospitals or authorized commercial laboratories. The karyotypes of leukemic cells were collected through the JALSG AML-97 case report forms and reviewed by the Central Review Committee for Karyotyping. The patients were classified into favorable, intermediate, or adverse risk groups based on karyotypes according to results of the Medical Research Council (MRC) AML 10 trial [3]. The favorable risk group included patients with $t(8;21)$ and inv(16), whether alone or in combination with other abnormalities. The intermediate risk group included those with a normal karyotype and other abnormalities that were not classified as either favorable or adverse. The adverse risk group included patients with a complex karyotype with four or more numerical or structural aberrations, -5, deletion (5q), and -7, whether alone or in combination with intermediate risk or other adverse risk abnormalities.

2.5 Statistical analysis

The overall survival (OS) for all patients was defined as the interval from the date of diagnosis to that of death. We applied the Kaplan–Meier method to estimate OS and

Table 1 Patient characteristics

Age (year)	45 (15–66)
Male/female	390/248
WBC count ($\times 10^9$/l)	13.7 (0.4–709)
Hemoglobin (g/dl)	8.3 (3.8–17.2)
Platelet count ($\times 10^9$/l)	52 (0–890)
Bone marrow blasts (%)	56 (6–99)

Values are presented as the median (range)

WBC white blood cell

5-year survival. We compared survival rates between groups using the log-rank test (Stat View J 5.0). Differences were examined by the Chi-square test using Excel software. All *P*-values are two-sided, and values <0.05 were considered significant.

3 Results

3.1 Patient characteristics

Of the 809 registered patients, 638 were consistent with the WHO classification. Data were incomplete for 10 of the 638 patients. Table 1 lists the characteristics of the patients. The median age of all 638 patients (390 males and 248 females) was 45 years (range 15–66 years). The median values of WBC, hemoglobin (Hb), platelets, and the ratio of blasts in the bone marrow were 13.7 $\times$ 10^9/l, 8.3 g/dl, 52.0 $\times$ 10^9/l, and 56.0%, respectively.

3.2 FAB classification

Table 2 shows the FAB classification of the 638 patients. Most were classified as M2 (n = 261; 40.9%), followed by M4 (n = 148; 23.2%), and M1 (n = 109; 17.1%) with M0, M4Eo, M5a, M5b, M6, M7, and acute leukemia of ambiguous lineage comprising the remainder in that order.

3.3 WHO classification and clinical characteristics

Table 3 shows the patients categorized according to the WHO classification. The first category of AML with recurrent genetic abnormalities accounted for 171 patients (26.8%), 133 (20.8%) were in the second category of AML with MLD, 331 (51.9%) were in the fourth category of AML not otherwise categorized, and 3 (0.5%) were categorized as having acute leukemia of ambiguous lineage. Most patients in the second category were identical to those with a de novo MLD phenotype. We found that 144 patients diagnosed with the MLD phenotype comprised 133 (92.4%) in the second category, 10 (7.0%) with 11q23 abnormalities, and 1 (0.7%) with acute leukemia of ambiguous lineage. Figure 1 shows the OS of each category. The 5-year survival rates of the first, second, and fourth categories were 58.2, 22.5, and 40.9% (P < 0.0001), respectively.

Table 2 Number of patients according to the FAB classification

Subtype	Description	No. of patients	%
M0	Minimally differentiated acute myeloid leukemia (AML)	30	4.7
M1	AML without maturation	109	17.1
M2	AML with maturation	261	40.9
M4	Acute myelomonocytic leukemia (AMMoL)	148	23.2
M4Eo	AMMoL with eosinophils	23	3.6
M5a	Acute monoblastic leukemia	19	3.0
M5b	Acute monocytic leukemia	24	3.8
M6	Acute erythroleukemia	16	2.5
M7	Acute megakaryoblastic leukemia	5	0.8
Acute leukemia of ambiguous lineage		3	0.5
Total		638	100

Table 3 Number of patients according to the WHO classification

Category and subtype	No. of patients	%
I. AML with recurrent genetic abnormalities	171	26.8
t(8;21)(q22;q22);(AML1/ETO)	113	17.7
inv(16)(p13;q22) or t(16;16)(p13;q22);(CBFβ/MYH11)	26	4.1
t(15;17)(q22;q12)(PML/RARα)	–	–
11q23(MLL)abnormalities	32	5.0
II. AML with multilineage dysplasia	133	20.8
Following MDS	–	–
Without antecedent MDS	133	20.8
III. AML and MDS, therapy-related	–	–
Alkylating agent-related	–	–
Topoisomerase type II inhibitor-related	–	–
Other types	–	–
IV. AML not otherwise categorized	331	51.9
AML, minimally differentiated	25	3.9
AML without maturation	99	15.5
AML with maturation	108	16.9
Acute myelomonocytic leukemia (AMMoL)	63	9.9
AMMoL with eosinophilia	5	0.8
Acute monoblastic leukemia	8	1.3
Acute monocytic leukemia	16	2.5
Acute erythroid leukemia	6	0.9
Acute megakaryoblastic leukemia	1	0.2
Acute leukemia of ambiguous lineage	3	0.5
Total	638	100

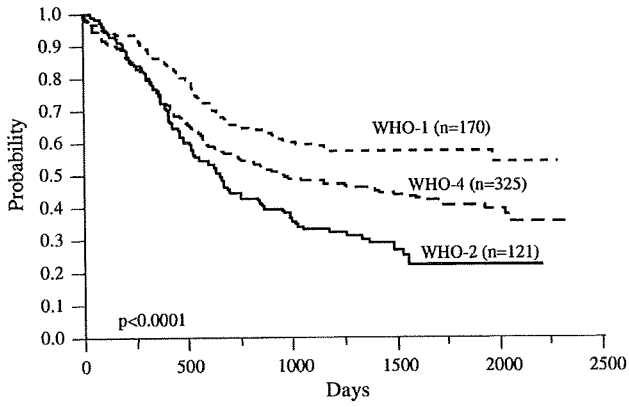

Fig. 1 Overall survival of patients categorized according to the WHO classification

Table 4 compares the clinical features among the WHO categories. The mean values of platelets, WBC, Hb, and the ratio (%) of blasts in bone marrow and of MPO-positive blasts significantly differed, whereas age did not significantly differ. Patients in the second category had a higher platelet count (111.0×10^9/l), whereas those with 11q23 abnormalities had a lower count (38.3×10^9/l) compared with those of other subtypes.

The WBC count of patients with $t(8;21)$ was 1.4×10^9/l and lower than in other subtypes. The MPO-positive rate of blasts among patients with $t(8;21)$ was higher (93.3%) and that of patients in the second category was lower (34.0%), than in other subtypes. All patients were grouped as high- or low-MPO according to ≥50% or <50% of MPO-positive blasts, respectively. A total of 339 patients (53.1%) were classified as high-MPO, 268 (42.0%) as low-MPO, and the MPO status of blasts could not be assessed in 31 (4.9%). Figure 2 shows the OS of patients with high- or low-MPO. The 5-year survival rate for patients with high or low-MPO was 50.7 and 29.6%, respectively ($P < 0.0001$).

3.4 Cytogenetics

All 638 patients were classified into favorable ($n = 139$; 21.8%), intermediate ($n = 413$; 64.7%), and adverse ($n = 54$; 8.5%) cytogenetic risk groups (Table 5). Figure 3 shows the OS according to this stratification. The 5-year survival rates were 63.4, 39.3, and 0.0% in the favorable, intermediate (except for those with 11q23 abnormalities), and adverse risk groups, respectively, and 35.5% in the group with 11q23 abnormalities ($P < 0.0001$).

The numbers of patients with or without MLD and high- or low-MPO in each cytogenetic risk group are listed in Table 6. None of those with the MLD phenotype were classified into the favorable risk group, while 129 (89.6%) and 15 (10.4%) of 144 patients with MLD were classified into intermediate or adverse risk groups, respectively. Only 15 patients (4.4%) in the high-MPO group were classified as having an adverse risk, while 11 (4.1%) in the low-MPO group were included in the favorable risk group.

The 32 patients with 11q23 abnormalities comprised 11 (34.4%) with $t(11;19)$, 9 (28.1%) with $t(9;11)$, 5 (15.6%) with del(11)(q23), 4 (12.5%) with $t(6;11)$, and 3 (9.4%) with $t(11;17)$. Figure 4 shows the OS of the intermediate risk group. The 5-year survival rate was 44.0% in patients with a normal karyotype, 35.5% in those with 11q23 abnormalities, and 30.6% in other patients including those with $t(7;11)$, $t(6;9)$, and Ph(+) abnormalities, respectively ($P = 0.033$).

Table 7 shows the relationship between $t(9;11)$ ($n = 9$) and other 11q23 abnormalities ($n = 23$). More patients with low-MPO, without MLD, or with the FAB M5 subtype were found in the group with $t(9;11)$ than with other 11q23 abnormalities. The survival rates between the two groups did not significantly differ ($P = 0.22$, data not shown).

4 Discussion

We attempted to classify selected patients who were reviewed morphologically and had available chromosomal data according to the WHO system. However, our series had some limitations in terms of analysis and patient selection. Although we obtained chromosomal data, genetic data were not available. Patients who were diagnosed with AML M3 or who had $t(15;17)$, a history of MDS, or preceding hematological abnormalities, or who had previously undergone chemotherapy, were not eligible for the present study. However, multicenter trials might have some advantages in diagnosing AML according to the WHO classification, because morphological diagnoses and karyotypes are reviewed by the corresponding institutional committees.

The incidence of each category of the WHO classification was similar to those in several reports when patients with $t(15;17)$ and therapy-related AML were excluded [20–22]. We and several others have shown that approximately 30% of patients have recurrent genetic abnormalities. Multiplex reverse transcriptase-polymerase chain reaction (RT-PCR) assays have recently been applied to analyze cytogenetic abnormalities [21, 23, 24]. This method might cause the frequency of the first WHO category to increase. Thus, the multiplex RT-PCR assay might have to be incorporated into the WHO system. The JALSG has started a cohort study in which all AML patients in participating hospitals are registered and analyzed according to the WHO classification. That study should clarify the real ratios of the AML subtypes in the WHO classification.

Table 4 Comparison of clinical findings of patients diagnosed according to the WHO classification

Category		Platelets ($\times 10^9$/l ± SE)	WBC ($\times 10^9$/l ± SE)	Hb (g/dl ± SE)	Age (year ± SE)	Blasts in bone marrow (%±SE)	MPO positivity of blasts (%±SE)
I	*t*(8;21)	76.7 ± 56.43 (113)[a]	1.4 ± 0.6 (113)	7.8 ± 0.2 (113)	41.6 ± 1.3 (113)	49.9 ± 2.0 (113)	93.3 ± 3.3 (108)
	inv(16)	57.8 ± 52.03 (26)	6.6 ± 1.2 (26)	9.2 ± 0.5 (26)	44.5 ± 2.6 (26)	50.5 ± 4.1 (26)	66.9 ± 6.7 (26)
	11q23	38.3 ± 30.8 (32)	4.3 ± 1.1 (32)	8.9 ± 0.4 (32)	41.6 ± 2.4 (32)	56.3 ± 3.7 (32)	43.6 ± 6.1 (32)
II		111.0 ± 121.5 (133)	3.0 ± 0.5 (133)	8.3 ± 0.2 (133)	44.2 ± 1.2 (133)	48.0 ± 1.8 (133)	34.0 ± 3.1 (126)
IV		72.8 ± 91.7 (330)	5.1 ± 0.3 (331)	8.8 ± 0.1 (330)	43.8 ± 0.7 (331)	65.7 ± 1.2 (328)	53.7 ± 1.9 (312)
		$P < 0.0001$	$P < 0.0001$	$P = 0.0004$	$P = 0.4077$	$P < 0.0001$	$P < 0.0001$

SE standard error, *WBC* white blood cell, *MPO* myeloperoxidase, *Hb* hemoglobin

[a] Number of patients

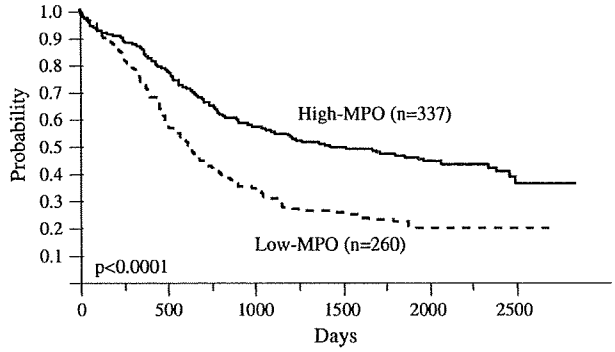

Fig. 2 Overall survival of patients with high or low MPO-positive blasts

Table 5 Distribution of patients classified by cytogenetic risk

Cytogenetic risk group	No. of patients	%
Favorable	139	21.8
t(8;21)	113	17.7
inv(16)	26	4.1
Intermediate	413	64.7
Normal karyotype	267	41.8
11q23	32	5.0
Ph(+)	7	1.1
t(7;11)(p15;p15)	4	0.6
t(6;9)	4	0.6
Other	131	20.5
Adverse	54	8.5
Complex	41	6.4
−7	2	0.3
abn3	5	0.8
del5q	2	0.3
−5	1	0.2
Other	3	0.5
Total	638	100.0

Few reports have included clinical data with the WHO classification. We found that the platelet count was higher among patients in the second category than in other categories. This supports our previous finding that the platelet count is higher in patients with AML accompanied by the MLD phenotype [25]. Among patients with MLD, none were in the favorable risk group, whereas the intermediate or adverse risk ratios among these patients were 89.6 and 10.4%, respectively. These differences might influence the finding that OS was better among patients without than with MLD ($P = 0.0002$, data not shown). Previous studies have also associated the MLD phenotype with a poorer outcome, although MLD is not significantly prognostic on multivariate analysis [18, 26], and a German group showed that dysplastic features correlate with adverse karyotypes

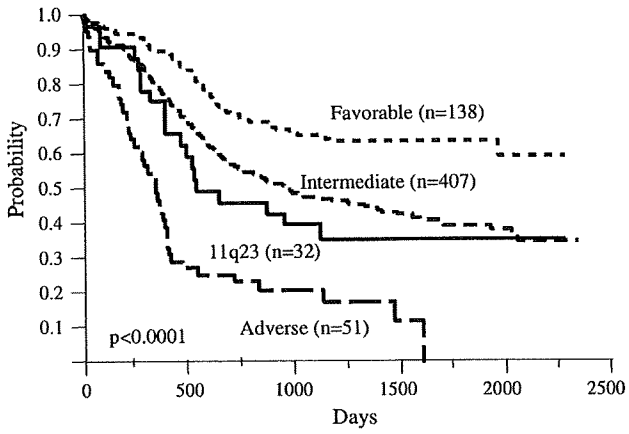

Fig. 3 Overall survival of patients stratified according to cytogenetic risk groups. Significant differences were observed between patients with a favorable, intermediate (except 11q23), and adverse karyotype ($P < 0.0001$)

Table 6 Relationship between cytogenetic risk groups and MLD phenotype or MPO-positive rates of blasts

	Favorable $n = 139$	Intermediate $n = 445$	Adverse $n = 54$	Total
MLD				
+	0	129 (89.5%)	15 (10.4%)	144
–	138 (28.2%)	292 (59.6%)	38 (7.8%)	490
Unknown	1	2	1	4
MPO				
High	123 (36.3%)	201 (59.3%)	15 (4.4%)	339
Low	11 (4.1%)	221 (82.5%)	36 (13.4%)	268
Unknown	5	23	3	31

High- and low-MPO indicates a percentage of myeloperoxidase positive blasts ≥50 or <50%, respectively

MLD multilineage dysplasia

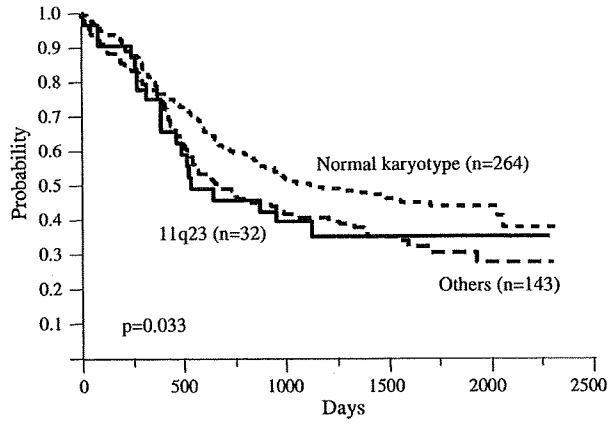

Fig. 4 Overall survival of patients with subtypes of intermediate cytogenetic risk. Significant differences were observed between patients with a normal karyotype and those with 11q23 abnormalities ($P = 0.033$)

[26]. Furthermore, patients in the second category had a lower MPO-positive rate of blasts, whereas those with $t(8;21)$ had a higher rate. Patients with high- and low-MPO were more frequently observed in the favorable and adverse risk groups, respectively. Multivariate analysis has shown that MPO is a significant factor affecting OS [19]. We did not assess prognostic factors by multivariate analysis here because the main theme of this study was to categorize patients according to the WHO classification, and we have already examined these in a previous series [18, 19].

Several studies have demonstrated the impact of specific cytogenetic abnormalities on survival in AML [3, 7–12, 20–22]. The cytogenetic risk groups stratified the AML patients in the present study according to the MRC system, as in these reports [3]. Therefore, we confirmed the clinical usefulness of cytogenetics as the first category of the WHO classification. We found that 32 patients had 11q23 abnormalities. The MRC system revealed that de novo and secondary AML patients with 11q23 abnormalities had an intermediate outcome with an OS rate of 45% at 5 years ($n = 60$; median age, 17 years) in a younger cohort [3] and an OS rate of 0% at 5 years ($n = 11$; median age 64 years) in an elderly cohort [7]. In contrast, SWOG/ECOG trials including adult de novo AML patients (age, 16–55 years) assigned those with 11q abnormalities to the unfavorable cytogenetic subgroup [8]. Our data showed that patients with 11q23 abnormalities have an intermediate rather than adverse outcome. The prognostic effect of 11q23 abnormalities might depend on the partner gene. Several studies have shown that 11q23 abnormalities with $t(6;11)$ and $t(10;11)$ are associated with a poor prognosis, whereas $t(9;11)$ is associated with a superior OS and such patients might respond well to intensive treatment, especially when the chemotherapy regimen includes high-dose cytarabine [15, 27–30]. The CALGB study has shown that the median OS of 13.2 months among 23 patients with $t(9;11)$ was significantly longer than the 7.7 months among 24 patients with other 11q23 rearrangements ($P = 0.009$) [30]. In a recent CALGB series of 54 patients with 11q23 abnormalities, 27 patients with $t(9;11)$ had an intermediate outcome and a median OS of 13.2 months, whereas those with $t(6;11)$ or $t(11;19)$ had a poor outcome of 7.2 or 8.4 months [15]. Conversely, Schoch et al. showed that 14 patients with $t(9;11)$ had a median OS of 10.0 months compared with the 12.8 months of 26 patients with other MLL rearrangements, and that the two cytogenetic groups did not significantly differ [13]. Our data showed that nine patients with $t(9;11)$ were more frequently involved in M5. The MPO and MLD features significantly differed between patients with $t(9;11)$ and those with other 11q23 abnormalities. However, the CALGB study found no significant differences in myelodysplastic features between the two

Table 7 Comparison of *t*(9;11) and other 11q23 abnormalities

	No. of patients	Auer		MPO*		MLD*		FAB						Median age (year)	Median survival (day)
		+	−	High	Low	+	−	M1	M2	M4	M4Eo	M5a**	M5b		
t(9;11)	9	0	9	1	8	0	9	0	0	3	0	6	0	39	1031.00
Other 11q23	23	5	18	13	10	10	13	1	3	13	1	2	3	48	520.00
Total	32	5	27	14	18	10	22	1	3	16	1	8	3	44.5	531.5

High- and low-MPO indicates a percentage of myeloperoxidase-positive blasts ≥50 or <50%, respectively

MLD multilineage dysplasia

* $P < 0.05$, ** $P < 0.01$

cytogenetic groups [30]. In terms of OS, our results showed no significant differences between patients with *t*(9;11) and those with other 11q23 abnormalities ($P = 0.22$). Some problems are associated with the analyses of 11q23 abnormalities. We had few patients with these abnormalities, particularly individual translocations, and genetic analysis was not performed. Thus, the prognostic risk of 11q23 abnormalities cannot be concluded from the present study. Nonetheless, these abnormalities were never associated with a favorable risk. To classify 11q23 abnormalities into each prognostic risk group, further investigations and genetic analyses of a large number of patients with 11q23 abnormalities are required.

The fourth WHO category, which is not otherwise categorized, accounted for 52% of patients in the present study. Most of them were classified into the intermediate risk group, and no prognostic subdivisions were valuable. Using cytogenetic features as a prognostic factor in groups with a normal karyotype has limitations, and such patients accounted for 64.6% of the intermediate risk group (data not shown). Additional factors are required to stratify these patients. We and several others suggested that differences could be based on molecular genetic analysis [22, 31–35]. For example, FLT3 mutations are important biomarkers of a normal karyotype and might be valuable for stratifying the intermediate risk group. Further follow-up studies might also shed light on the roles of FLT3 ITD mutations in the development of AML and aid their use as novel molecular targeting agents against AML [22, 32]. Bienz et al. identified CEBPA mutations, FLT3-ITD, and differing levels of BAALC expression as having independent prognostic significance in patients with a normal karyotype [33]. If these genetic markers can be confirmed as being of clinical significance, genetic analyses will probably be incorporated into the WHO classification.

In summary, our results confirmed those of previous studies showing the prognostic significance of cytogenetics, MLD, and MPO-positivity of blasts in AML. Furthermore, we categorized patients with de novo AML according to the WHO classification and showed the clinical characteristics and OS of each category.

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EML4-ALK Fusion Is Linked to Histological Characteristics in a Subset of Lung Cancers

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Introduction: Very recently, we have found a novel fusion product between the echinoderm microtubule-associated protein-like4 (EML4) and the anaplastic lymphoma kinase (ALK) in non-small cell lung cancers (NSCLCs). Tumors featuring EML4-ALK fusion constitute one subtype of NSCLC that might be highly sensitive to ALK inhibitors. Herein, we present results of a first large scale study of EML4-ALK fusion in lung cancers.

Methods: Using reverse transcription-polymerase chain reaction for EML4-ALK fusion mRNA, we investigated 149 lung adenocarcinomas, 48 squamous cell carcinomas, 3 large-cell neuroendocrine carcinomas, and 21 small-cell carcinomas. For EML4-ALK-positive cancers, we further investigated the presence of ALK fusion proteins by immunohistochemistry.

Results: Five of 149 adenocarcinomas (3.4%) showed EML4-ALK fusion mRNA, this being totally lacking in carcinomas of other types (0/72). In all the fusion-positive cases, ALK fusion protein could be detected in the cytoplasm immunohistochemically. The five fusion cases featured two EML4-ALK variant 1 fusions and three variant 2 fusions. Histologically, both variant 1 cases were mixed type adenocarcinomas, showing papillary with bronchioloalveolar components. Interestingly, all three variant 2 cases were acinar adenocarcinomas, the link being statistically significant ($p = 0.00018$). None of the five fusion-positive cases demonstrated any mutations of EGFR or KRAS, pointing to a mutually exclusive relationship ($p = 0.014$). There was no association with smoking habits.

Conclusions: In the present first investigation of EML4-ALK fusion in a large study of lung cancers (5/221), we found an interesting histotype-genotype relationship. Furthermore, we could detect the fusion protein by immunohistochemistry, pointing to possible clinical applications.

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Lung cancer is the leading cause of cancer death in men and women worldwide. Identification of activating mutations of the epidermal growth factor receptor (EGFR) is one of the most intriguing recent discoveries in the field of lung cancer research.[1,2] EGFR mutations are present in one subtype of lung adenocarcinoma, and tumors with this mutation have been shown to be highly sensitive to gefitinib (Iressa). The subtype is prevalent in women, and in patients of Japanese and other Asian ethnicity, especially in nonsmokers.[1,3,4] With the move to personalized cancer therapy, we need to understand oncologic biology at the molecular level in individual lesions to be able to treat cancers with specific molecular-targeting strategies.

Very recently, we have found a novel transforming fusion gene resulting from linkage between the echinoderm microtubule-associated protein-like4 (EML4) and the anaplastic lymphoma kinase (ALK) genes in non-small cell lung cancers (NSCLCs).[5] Tumors featuring EML4-ALK fusion constitute one subtype of NSCLC which might be highly sensitive to ALK inhibitors. The fusion gene is formed by a small inversion within chromosome 2p. EML4 on chromosome 2p21 belongs to the family of echinoderm microtubule-associated protein-like proteins, localized in the cytoplasm, and is necessary for correct microtubule formation.[6,7] ALK on chromosome 2p23 codes for a receptor tyrosine kinase and was first identified as a fusion partner of nucleophosmin (NPM) in anaplastic large-cell lymphomas (ALCLs) with a t(2;5) chromosome rearrangement.[8,9] NPM is an RNA-binding protein that transports ribonucleoproteins between the nucleus and cytoplasm and contributes a nuclear localization signal to the NPM-ALK fusion protein.[10] Other chromosome translocations involving the ALK locus have been identified in ALCLs[11,12] as well as in inflammatory myofibroblastic tumors (IMTs).[13] The fusion point of ALK is conserved among all these chimeric tyrosine kinases including EML4-ALK, resulting in fusion of the entire intracellular kinase domain of ALK to the different partners.[14]

Herein, we present a first large scale study of EML4-ALK fusion in lung cancers, including SCLCs. Furthermore, we detail clinicopathologic and genetic features of fusion-positive lung cancers.

PATIENTS AND METHODS

Clinical Samples and RNA Extraction

This study was conducted with clinical samples from 149 lung adenocarcinomas, 48 squamous cell carcinomas, 3 large cell neuroendocrine carcinomas, and 21 SCLCs. Many of these samples were previously examined and reported.[15–21] For example, most adenocarcinomas were examined as to their mRNA levels of PTEN[19]or HOXB2,[21] and some adenocarcinomas were examined as to their let-7 microRNA levels.[20] All were collected with ethical committee approval and informed consent from patients undergoing surgery at the Cancer Institute Hospital, Tokyo, Japan, between May 1995 and August 2004. Histologic diagnosis was according to World Health Organization classifications[22] as well as to differentiation-grading criteria for adenocarcinomas of the Japanese Lung Cancer Society.[23,24] All lesions were grossly dissected and snap-frozen in liquid nitrogen within 20 minutes of removal and stored at −80°C until total RNA extraction and purification using an RNeasy Mini Kit (QIAGEN, Valencia, CA). RNA quality and absence of genomic DNA contamination were checked by formaldehyde agarose gel electrophoresis.

Reverse Transcription-Polymerase Chain Reaction and Sequencing Analysis

Total RNAs were reverse transcribed with random primers and SuperScript III reverse transcription (Invitrogen, Carlsbad, CA). To detect fusion transcripts derived from EML4 and ALK, reverse transcription-polymerase chain reaction (RT-PCR) experiments were carried out with primers Fusion-RT-S (5′-GTGCAGTGTTTAGCATTCTTGGGG-3′) and Fusion-RT-AS (5′-TCTTGCCAGCAAAGCAGTAGTTGG-3′). We used PCR primers 5′-GTCAGTGGTGGACCTGACCT-3′ and 5′-TGAGCTTGACAAAGTGGTCG for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. For PCR of the fusion transcripts, after initial denaturation at 94°C for 10 minutes, 32 cycles each consisting of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and strand elongation at 72°C for 1 minute were performed, followed by a final elongation at 72°C for 10 minutes. For GAPDH, amplification was performed for 35 cycles with denaturation for 1 minute at 94°C, primer annealing for 30 seconds at 58°C, and elongation for 30 seconds at 72°C. PCR was performed using AmpliTaq Gold (Applied Biosystems, Foster City, CA) and amplified fragments were subjected to direct sequence analysis.

Immunohistochemical Analysis

A representative tissue block from each lesion was selected, and 4-μm tissue sections were routinely deparaffinized in xylene and rehydrated through graded ethanols. Immunohistochemical staining was performed using the EnVision + DAB system (DAKO, Carpinteria, CA) and a mouse monoclonal anti-ALK antibody (ALK1, DAKO, 1:20).

DNA Extraction and Mutation Analysis of EGFR and KRAS

Of 149 patients with adenocarcinomas, both EGFR and KRAS data were available for 62 and EGFR data alone for a further 12. DNA extraction and mutation analysis of EGFR and KRAS were performed as described previously.[19]

Analysis of Clinicopathologic Parameters

Survival data were analyzed by the log-rank test using cancer death-specific survival data. We analyzed statistical correlations for the other clinicopathologic features using the Student t test, Fisher exact test, or χ^2 test as appropriate. The two-sided significance level was set at $p < 0.05$.

RESULTS

Using RT-PCR for EML4-ALK fusion mRNA, we investigated the presence of the EML4-ALK translocation in 221 lung cancers (Table 1). Five of 149 adenocarcinomas (3.4%) featured EML4-ALK fusion mRNA, whereas other types of carcinoma were all negative (0/72) (Figure 1). Of the five fusion-positive cases, two had EML4-ALK variant 1 and three had variant 2.[5] The fusions were confirmed by direct sequencing.

Histologically, both the variant 1 cases were mixed type adenocarcinomas, papillary with bronchioloalveolar components (Figure 2*A*). Interestingly, all three variant 2 cases were acinar adenocarcinomas, moderately or poorly differentiated (Figure 2*B*). The link between variant 2 and acinar morphology was statistically significant ($p = 0.00018$, Fisher exact test).

Immunohistochemically, all the five fusion-positive cases showed ALK fusion protein in the cytoplasm (Figure 3) in line with the absence of any nuclear localization signal in the EML4 gene. We cannot rule out the possibility of detecting endogenous ALK protein.

Table 2 summarizes details for clinicopathologic and genetic features of the fusion-positive lung cancers. Genetically, all lacked mutations of EGFR or KRAS ($p = 0.014$ as

TABLE 1. EML4-ALK Fusion and Histology

Histology	Total	EML4-ALK(+)	EML4-ALK(−)
Adenocarcinoma	149	5 (3.4%)	144 (97%)
Subtype			
Adenocarcinoma with mixed subtype	89	2[a] (2.3%)	87 (98%)
Papillary adenocarcinoma	35	0 (0%)	35 (100%)
Acinar adenocarcinoma	18	3[b] (17%)	15 (83%)
Solid adenocarcinoma with mucin	4	0 (0%)	4 (100%)
Bronchioloalveolar carcinoma	3	0 (0%)	3 (100%)
Squamous cell carcinoma	48	0 (0%)	48 (100%)
Large cell neuroendocrine carcinoma	3	0 (0%)	3 (100%)
Small cell carcinoma	21	0 (0%)	21 (100%)

[a] Variant 1; Fisher exact test, $p = 0.66$ (Adenocarcinoma with mixed subtype vs. the other adenocarcinomas).

[b] Variant 2; Fisher exact test, $p = 0.00018$ (Acinar adenocarcinoma vs. the other adenocarcinomas).

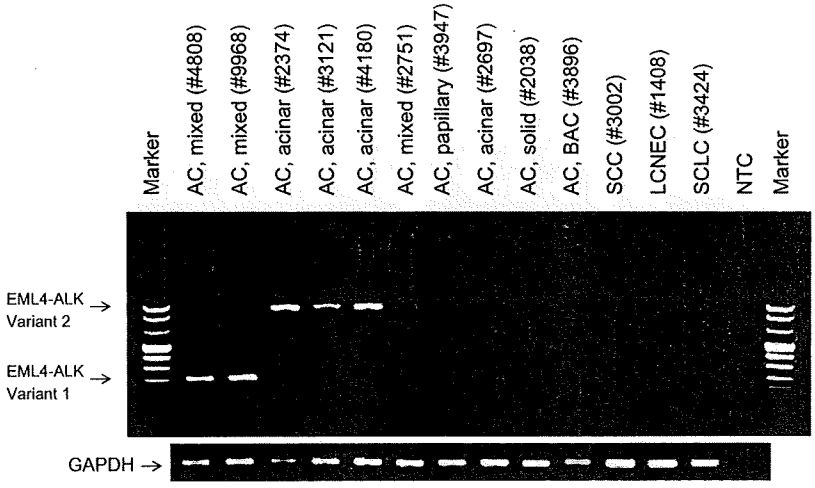

FIGURE 1. RT-PCR for EML4-ALK fusion mRNA. All the 5 fusion-positive cases and fusion negative cases of all the histologic subtypes examined are shown. RT-PCR results for GAPDH mRNA are also included as an internal control. AC, adenocarcinoma; mixed, adenocarcinoma with mixed subtype; papillary, papillary adenocarcinoma; acinar, acinar adenocarcinoma; solid, solid adenocarcinoma with mucin; BAC, bronchioloalveolar carcinoma; SCC, squamous cell carcinoma; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; NTC, no template control.

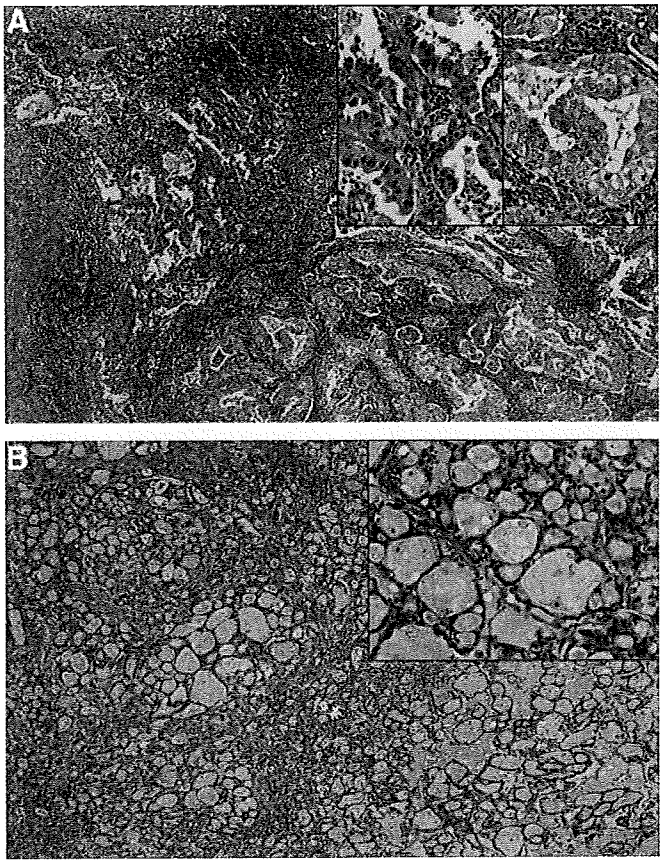

FIGURE 2. Representative examples of histologic features. Both the 2 variant 1 cases were mixed type adenocarcinomas, with papillary and bronchioloalveolar components (*A*). All 3 variant 2 cases were acinar adenocarcinomas (*B*).

compared with expectation). Patients with EML4-ALK fusion-positive tumors were younger than those without by 4 years, though this was not statistically significant. One patient was 43 years old and died 4 months after surgery (variant 2, acinar adenocarcinoma, poorly differentiated, clinical stage-IV, cerebellar metastasis). The other four patients were 58 to 66 years old and are alive now. There was no association between EML4-ALK fusion and smoking habits, although the sample numbers were small ($p = 0.77$).

We analyzed the survival data statistically with the log rank test, but there was no prognostic significance of EML-ALK fusion ($p = 0.84$).

DISCUSSION

In the present first large scale study of a novel EML4-ALK fusion in 221 lung cancers including 21 SCLCs, 5 of 149 adenocarcinomas (3.4%) proved positive for fusion mRNA and fusion protein. Interesting histotype-genotype relationships were observed. Although both variant 1 cases were papillary adenocarcinomas with bronchioloalveolar components, all the three variant 2 cases were of acinar type. Furthermore, none of these lesions had mutations in EGFR or KRAS, pointing to a mutually exclusive relationship.

The ALK gene encodes a transmembrane receptor tyrosine kinase that belongs to the insulin receptor superfamily and is most similar to leukocyte tyrosine kinase.[25] Postnatal ALK expression is normally restricted to a few scattered cells in the nervous system,[26] but chromosomal translocations involving the ALK are characteristic of ALCLs and IMTs. An increasing number of translocation patterns have been identified and other neoplasms with similar changes have been identified, such as large B-cell lymphomas.[27] Very recently, we described a novel subpopulation of NSCLCs with ALK translocations,[5] and it is very likely that other examples will be identified by further searches. ALK-positive ACLCs predominantly affect younger patients and, if treated with chemotherapy, have a more favorable prognosis than their negative counterparts.[28] Similarly ALK-positive IMTs primarily affect younger patients.[13] In this study, ALK-positive lung adenocarcinomas were also found in younger patients when compared with ALK-negative tumors. Especially, one of the five patients was 43 years old, very young for lung adenocarcinomas. Although one example is insufficient for discussion, ALK-positive adenocarcinomas might include younger patients. Although survival data analysis demonstrated no significant difference between ALK-positive

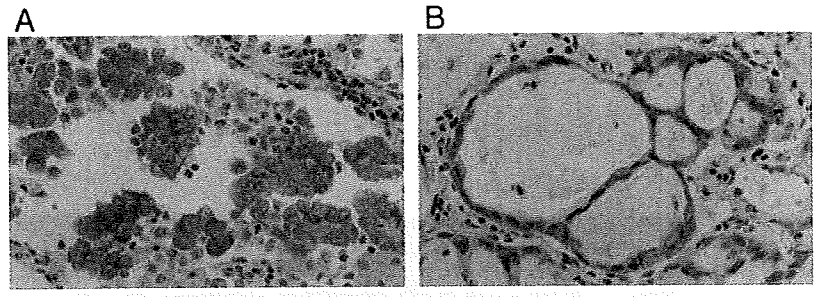

FIGURE 3. Representative examples of immunohistochemical features. Both adenocarcinomas with mixed subtype (*A*) with the variant 1 EML4-ALK fusion and acinar adenocarcinomas (*B*) with the variant 2 show ALK fusion protein in their cytoplasm.

TABLE 2. Relationship between EML4-ALK Fusion and Clinicopathologic and Genetic Features in Lung Adenocarcinomas

		EML4-ALK fusion		
Variables category	**No. samples (%)**	**(+) (n = 5)**	**(−) (n = 144)**	p
Age (yr; mean ± SD)	149	59.4 ± 9.7	63.4 ± 8.7	0.31[a]
Sex				0.87[b]
Male	80 (54%)	2 (40%)	78 (54%)	
Female	69 (46%)	3 (60%)	66 (46%)	
Smoking habit				0.77[b]
Never	65 (44%)	3 (60%)	62 (43%)	
Smoker	84 (56%)	2 (40%)	82 (57%)	
Tumor size				0.40[b]
<30mm	77 (52%)	4 (80%)	73 (51%)	
30 mm ≤	72 (48%)	1 (20%)	71 (49%)	
Differentiation				0.73[c]
Well	48 (32%)	1 (20%)	47 (33%)	
Moderate	62 (42%)	2 (40%)	60 (42%)	
Poor	39 (26%)	2 (40%)	37 (26%)	
EGFR				0.034[b]
Mutation (+)	41 (55%)	0 (0%)	41 (59%)	
Mutation (−)	33 (45%)	5 (100%)	28 (41%)	
KRAS				0.92[b]
Mutation (+)	7 (11%)	0 (0%)	7 (12%)	
Mutation (−)	55 (89%)	5 (100%)	50 (88%)	
EGFR or KRAS				0.014[b]
Mutation (+)	38 (61%)	0 (0%)	38 (67%)	
Mutation (−)	24 (39%)	5 (100%)	19 (33%)	
p-Stage				0.73[b]
I	63 (43%)	2 (40%)	61 (43%)	
II–IV	85 (57%)	3 (60%)	82 (57%)	

Percentages may not total 100, because of rounding.
[a] Student t test.
[b] Fisher exact test.
[c] Yates χ^2 test.

and negative adenocarcinomas, this might be due to the small number of positive cases. Whatever is the cause, for ALK-positive tumors, molecular targeted therapies including ALK inhibitors may be used.

ALK1 antibody, used in the immunohistochemical analysis, detects the cytoplasmic region of the ALK protein and also detects the full-length endogenous ALK protein. When we detect the positive staining of ALK1, three possibilities are considerable: (i) EML4-ALK fusion protein, (ii) endogenous full-length ALK protein, or (iii) ALK fusion protein with another partner. The five EML4-ALK fusion cases immunostained positive for ALK with variable intensity. Endogenous full-length ALK protein might, however, be also detected by immunohistochemistry. Therefore, EML4-ALK fusion should be confirmed by RT-PCR practically, although the immunohistochemistry can be used for the screening purpose.

In conclusion, we here found a minor subpopulation of lung adenocarcinomas featuring EML4-ALK fusion with evidence of histotype-genotype relationships. Furthermore, we could detect the fusion protein by immunohistochemistry, pointing to clinical applications.

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Identification of a constitutively active mutant of JAK3 by retroviral expression screening

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Abstract

To identify transforming genes in acute myeloid leukemia (AML) we here constructed a retroviral cDNA expression library from an AML patient, and then used this library to infect a mouse cell line 32Dcl3-mCAT. cDNA inserts of the cell clones which proliferated in the presence of granulocyte colony-stimulating factor were derived from *JAK3* encoding a JAK3 mutant with a valine-to-alanine substitution at codon 674 and two additional amino acid substitutions. The transforming activity of JAK3(V674A) was confirmed by its introduction into 32Dcl3-mCAT. Sequencing of the original *JAK3* cDNA derived from the patient, however, failed to detect the V674A mutation.

Keywords: JAK3; Retrovirus; Acute myeloid leukemia; cDNA expression library

1. Introduction

Acute myeloid leukemia (AML) is a clonal disorder of immature progenitor cells in the hematopoietic system. Chromosomal translocations are present in the AML blasts of 20–30% of individuals with this condition, and the fusion genes generated by such translocations have been shown to possess transforming activity [1,2]. Even with current technology, however, chromosomal abnormalities are not detectable in the blasts of almost half of AML patients [3]. Furthermore, although point mutations in a variety of genes implicated in cell growth or differentiation, such as *RAS*, *FLT3*, *KIT*, and *RUNX1*, are detectable in some such blasts, many cases of AML are not associated with a detectable gene anomaly [4]. Clarification of the transforming events in such AML cases would thus be expected to provide a basis for the development of effective treatments.

Functional screening based on transforming activity is one potential approach to the efficient isolation of tumor-promoting genes in AML. Focus formation assays with mouse 3T3 fibroblasts have indeed proved successful for the identification of oncogenes in human cancer [5]. In such screening assays, genomic DNA isolated from cancer specimens is used to transfect 3T3 cells and the formation of transformed cell foci is then evaluated. There are, however, substantial drawbacks to such screening for AML. First, expression of the exogenous genes in conventional 3T3 screening is driven by the associated promoters or enhancers, so that oncogenes are able to exert transforming effects in 3T3 cells only if their regulatory regions are active in fibroblasts, which is not guaranteed. Furthermore, given that the transcriptome and proteome would be expected to differ markedly between fibroblasts and leukemic blood cells, active oncogenes in the latter cells may not function properly in the former cells even if they are adequately expressed.

We reasoned that these concerns may be overcome through expression of test cDNAs under the control of an ectopic

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promoter and with the use of myeloid cells, instead of 3T3 cells, for the assay. We have now constructed a retroviral cDNA expression library from a purified $CD133^+$ stem cell fraction isolated from a patient with AML and used this library to infect the mouse myeloid cell line 32Dcl3 [6]. We then screened for transforming genes that override the differentiation program of 32Dcl3 cells triggered by granulocyte colony-stimulating factor (G-CSF). Furthermore, in preparation of the cDNA library, we took advantage of a polymerase chain reaction (PCR)-based system that preferentially amplifies full-length cDNAs [7]. Through this screening, we isolated a cDNA encoding a constitutively active form of the protein kinase JAK3. However, investigation of *JAK3* cDNA sequence in the original RNA failed to detect the activating mutation, indicating that the mutation arose probably from an experimental artifact.

2. Materials and methods

2.1. Cell culture and clinical samples

32Dcl3 cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and mouse interleukin (IL) – 3 (20 U/mL; Sigma, St. Louis, MO). To increase the efficiency of retroviral infection, we generated 32D-mCAT cells, which overexpress a receptor (mCAT) for ecotropic retrovirus [8], through infection of 32Dcl3 cells with a recombinant retrovirus containing both mCAT and blasticidin-S resistance genes. For induction of granulocyte differentiation, 32D-mCAT cells were cultured with mouse G-CSF (0.5 ng/mL, Sigma) instead of IL-3. The BOSC23 packaging cell line [9] was maintained in Dulbecco's modified Eagle's medium – F12 (Invitrogen) supplemented with 10% FBS and 2 mM L-glutamine.

$CD133^+$ cells were purified with anti-CD133 MicroBeads and a Mini-MACS magnetic cell separation column (Miltenyi Biotec, Auburn, CA), as described previously [10], from mononuclear cells of bone marrow from a patient with AML, who provided written informed consent.

2.2. Construction of a retroviral library

A retroviral plasmid library was constructed as described previously [7]. In brief, total RNA was extracted from the purified $CD133^+$ cells with the use of an RNeasy Mini column and RNase-free DNase (Qiagen, Valencia, CA), and first-strand cDNAs were then synthesized with PowerScript reverse transcriptase, the SMART IIA oligonucleotide, and CDS primer IIA (all from Clontech, Palo Alto, CA). The resulting cDNAs were then amplified for 20 cycles with 5′-PCR primer IIA and LA Taq polymerase (Takara Bio, Shiga, Japan). The PCR products were ligated to a BstXI adapter (Invitrogen) and then incorporated into the pMX retroviral plasmid [11]. The pMX-cDNA plasmids were introduced into ElecroMax DH10B cells (Invitrogen) by electroporation.

2.3. 32D-mCAT transformation assay

BOSC23 cells (1.8×10^6) were seeded in a 6-cm culture plate and transfected with a mixture comprising 2 μg of retroviral plasmids, 0.5 μg of pGP plasmid (to express *gag-pol* proteins, Takara Bio), 0.5 μg of pE-eco plasmid (to express ecotropic *env* protein, Takara Bio), and 18 μL of Lipofectamine reagent (Invitrogen). Two days after transfection, the culture supernatant was collected and incubated with 32D-mCAT cells (5×10^5) for 24 h in the presence of Retronectin (Takara Bio). The 32D-mCAT cells were then incubated for an additional 24 h with mouse IL-3, washed with cytokine-free medium, and cultured at an initial density of 1×10^4 to 4×10^4 cells/mL in the presence of mouse G-CSF (0.5 ng/mL).

2.4. Recovery of cDNAs from 32D-mCAT cells

Genomic DNA was isolated from 32D-mCAT cells that grew without differentiation (determined by the presence of polymorphonuclear neutrophils as assessed by cytospin preparations stained with the Wright Giemsa solutions) in the presence of G-CSF and was subjected to PCR with 5′-PCR primer IIA and LA Taq polymerase for 50 cycles of 98 °C for 20 s and 68 °C for 6 min. Amplified DNA fragments were purified by gel electrophoresis and ligated into the pT7Blue-2 vector (EMD Biosciences, San Diego, CA) for nucleotide sequencing. To confirm the transforming activity of the isolated cDNAs, they were again isolated by PCR with 5′-PCR primer IIA and PfuUltra High-Fidelity DNA polymerase (Stratagene, La Jolla, CA) for 30 cycles of 95 °C fro 30 s, 60 °C for 30 s and 72 °C for 4 min. The cDNAs were then individually ligated into the pMX plasmid and used to generate recombinant retroviruses. 32D-mCAT cells were then infected with the resulting viruses and cultured with G-CSF.

2.5. Protein analysis

Wild-type human *JAK3* cDNA (a kind gift of Dr. James N. Ihle) was used to generate mutant cDNAs with the use of a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Oligonucleotides used for mutagenesis were 5′-GGGATGGGGGGCTGTACGTAGATGGGGTGGC-3′ and 5′-GCCACCCCATCTACGTACAGCCCCCCATCCC-3′ for JAK3(H463Y), 5′-GAGTGACCCTGGGGCCAGCCCCGCTGTGTTAAGCC-3′ and 5′-GGCTTAACACAGCGGGGCTGGCCCCAGGGTCACTC-3′ for JAK3(V674A), and 5′-GATGGGATGTGAGCGGGGTGTCCCCGCCCTCTG-3′ and 5′-CAGAGGGCGGGGACACCCCGCTCACATCCCATC-3′ for JAK3(D1043G). The wild-type or mutant cDNAs were individually ligated into the pMX-ires-neo retroviral vector in order to generate recombinant retroviruses encoding both JAK and the neomycin resistance gene. 32D-mCAT cells were infected with the resulting viruses and cultured for >2 weeks in the presence of IL-3 and