Table I. WT1 mRNA expression levels in PB and BM from patients with different MDS subtypes and AML-MDS according to FAB classification.

		WT1 mRNA expression level			
		Peripheral blood		Bone marrow	
Disease	No. of patients	Log (mean ± SD)	Geometric mean (copies/μg RNA)	Log (mean ± SD)	Geometric mean (copies/μg RNA)
MDS	115	2.56 ± 1.05	360	3.35 ± 0.87	2240
AML-MDS	11	4.10 ± 0.96	12 600	4.52 ± 0.77	33 100
AML-MDS (CR)	2	1.89 ± 0.20	80	2.98 ± 0.39	1000
CMML	3	2.17 ± 0.54	150	3.04 ± 0.54	1100
CLL	1	1.92	80	3.33	2140
Atypical CML	1	—	<50	1.95	90
AA	8	—	<50	2.64 ± 0.37	440
ICUS	3	—	<50	2.16 ± 0.36	140
ITP	1	—	<50	2.13	130
PNH	1	—	<50	2.8	630
PRCA	2	—	<50	2.17 ± 0.12	150
Erythroid hypoplasia	1	—	<50	1.94	90
Unclassified	23	2.14 ± 0.56	140	2.96 ± 0.61	910
Total	172	2.50 ± 1.05	320	3.27 ± 0.90	1860

PB, peripheral blood; BM, bone marrow; MDS, myelodysplastic syndromes; AML-MDS, acute myeloid leukemia-evolved MDS; FAB, French-American-British; CR, complete remission; CMML, chronic myelomonocytic leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; AA, aplastic anemia; ICUS, idiopathic cytopenia of unknown significance; ITP, idiopathic thrombocytopenic purpura; PNH, paroxysmal nocturnal hemoglobinuria; PRCA, pure red-cell aplasia.

stage progressed. Significant differences in both PB and BM expression were seen between RA and RAEB, RA and RAEB-t, refractory anemia with ringed sideroblasts (RARS) and RAEB, and RARS and RAEB-t ($p < 0.05$).

WT1 mRNA expression in PB and BM for each IPSS risk group

WT1 mRNA expression levels in PB and BM for each IPSS risk group were compared in the 115 patients with MDS. A tendency for WT1 mRNA expression to increase in both PB and BM was observed in each IPSS risk group as the risk of transformation to AML increased from low to high. Significant differences ($p < 0.05$) in WT1 mRNA expression were observed in risk groups between low and intermediate-2, low and high, intermediate-1 and intermediate-2, and intermediate-1 and high in PB samples; and between low and intermediate-1, low and intermediate-2, low and high, intermediate-1 and intermediate-2, and intermediate-1 and higher in BM samples [Figure 3(b)]. The correlation between IPSS score and WT1 mRNA expression was evaluated, and a correlation of $r = 0.57$ was found for both PB and BM samples.

Next, the WT1 mRNA expression levels in PB and BM between IPSS risk groups were compared in the 69 patients with RA [Figure 3(c)]. As the risk increased from low to intermediate-2, the level of WT1 mRNA expression in both PB and BM increased. Moreover, when the distribution of WT1 mRNA expression between each risk group was evaluated, a significant difference ($p < 0.05$) was found in PB between low and intermediate-2; in BM, significant differences were found between low and intermediate-1, and low and intermediate-2.

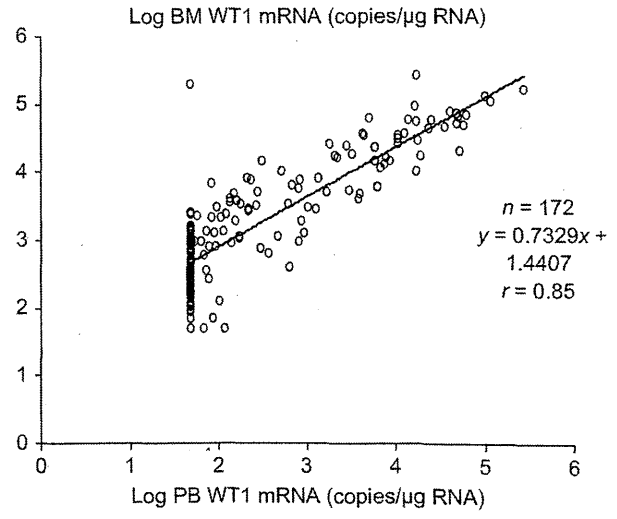

Figure 2. Correlation of WT1 mRNA expression in PB and WT1 mRNA expression in BM.

Correlation between IPSS karyotype and WT1 mRNA expression

A total of 114 patients with MDS were categorized into the three prognostic groups of good, intermediate and poor in accordance with their IPSS karyotype, and the levels of WT1 mRNA expression in their PB and BM samples were compared. One patient with MDS was excluded from this analysis because chromosome testing was not performed. The WT1 mRNA expression level increased in both PB and BM samples as the karyotype indicated a poorer prognosis. Among karyotypes, significant differences ($p < 0.05$) in WT1 mRNA expression were found between the good and intermediate and between the good and poor groups [Figure 3(d)].

Correlation between WT1 mRNA expression and percentage of blasts in BM

The correlation between blast ratio and WT1 mRNA expression in PB and BM was investigated in 114 patients with MDS (excluding one patient in whom the blast ratio could not be measured). The correlation between blast ratio and PB WT1 mRNA expression was $r = 0.51$, and the correlation between blast ratio and BM WT1 mRNA expression was $r = 0.48$.

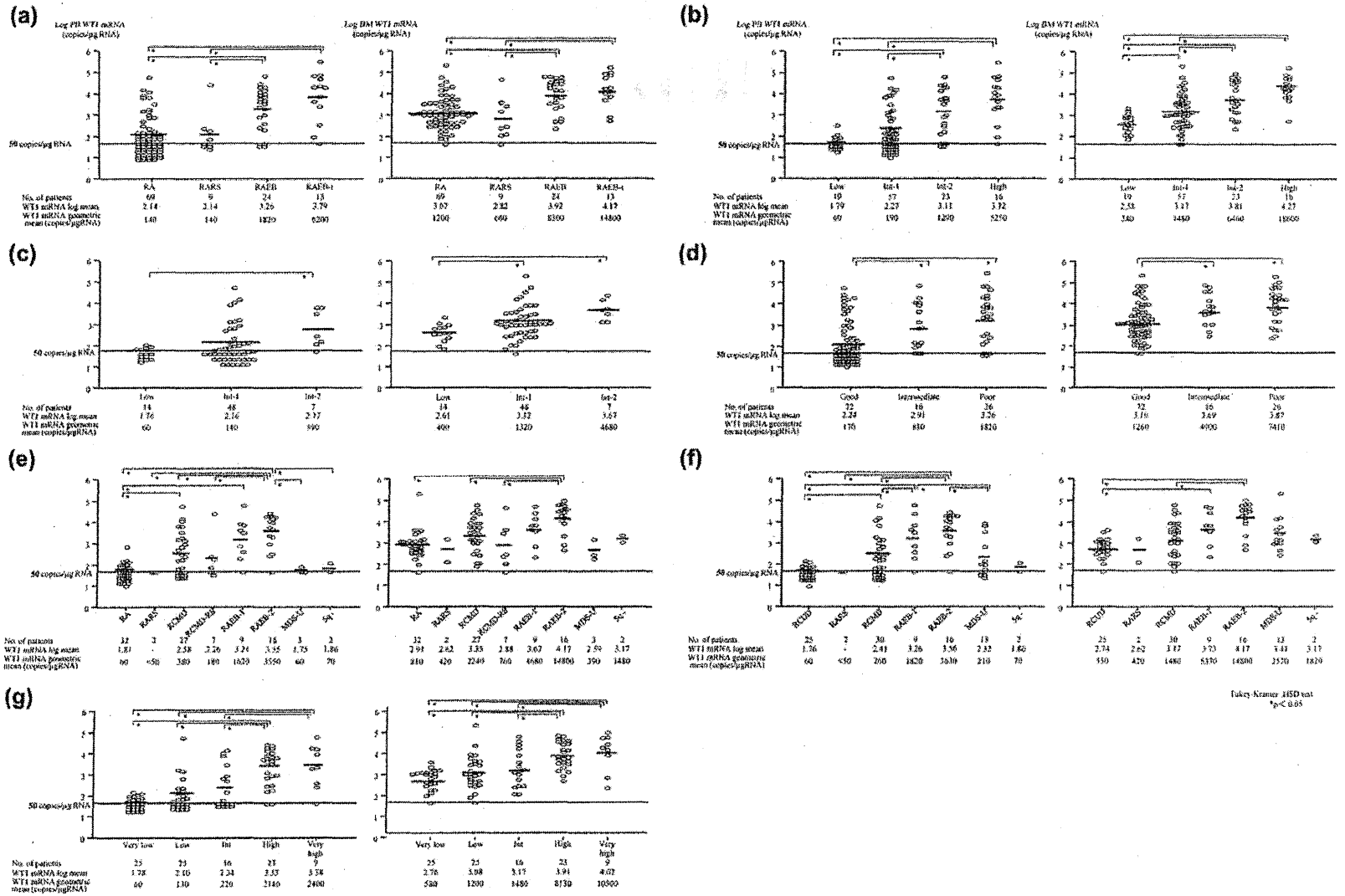

Figure 3. WT1 mRNA expression levels in PB and BM from patients with MDS (a) according to FAB classification, (b) according to IPSS category, (c) patients with RA according to IPSS category, (d) according to chromosomal karyotype, (e) according to WHO 2001 classification, (f) according to WHO 2008 classification, (g) according to WPSS category. In intergroup comparison of WT1 mRNA expression, the Tukey-Kramer HSD test was performed using log-transformed values of WT1 mRNA expression with a level of significance of $p < 0.05$. Bold lines represent mean WT1 mRNA expression after log transformation. Fine lines represent lower limit of detection of WT1 mRNA (50 copies/μg RNA).

Analytical results based on 2001 WHO classification

WT1 mRNA expression in PB and BM for each MDS disease stage based on 2001 WHO classification

Figure 3(e) shows the assay results for WT1 mRNA expression in PB and BM in 98 patients in various MDS disease stages categorized on the basis of the 2001 WHO classification. The WT1 mRNA expression levels in both PB and BM tended to increase with the progression to each MDS subtype. When the levels of WT1 mRNA expression in each disease stage were investigated, significant differences ($p < 0.05$) were found in PB between RA and refractory cytopenia with multilineage dysplasia (RCMD), RA and RAEB-1, RA and RAEB-2, RARS and RAEB-2, RCMD and RAEB-2, RCMD with ringed sideroblasts (RCMD-RS) and RAEB-2, RAEB-2 and unclassified MDS (MDS-U), and RAEB-2 and 5q− syndrome; in BM, significant differences were found between RA and RAEB-2, RCMD and RAEB-2, and RCMD-RS and RAEB-2.

Correlation between WT1 mRNA expression and percentage of blasts in BM based on 2001 WHO Classification

The correlation between the blast ratio and WT1 mRNA expression in PB and BM was investigated in 97 patients with MDS (excluding one patient in whom the blast ratio could not be measured). The correlations between the blast ratio and WT1 mRNA expression were $r = 0.50$ in PB and $r = 0.46$ in BM.

Analytical results based on 2008 WHO classification

WT1 mRNA expression in PB and BM for each MDS disease stage based on 2008 WHO classification

Figure 3(f) shows the assay results for WT1 mRNA expression in PB and BM in a total of 97 patients in various MDS disease stages categorized on the basis of the 2008 WHO classification. WT1 mRNA expression in both PB and BM tended to increase with the progression to each MDS subtype.

When the distribution of WT1 mRNA expression for each disease stage was examined, significant differences ($p < 0.05$) were found in PB between refractory cytopenia with unilineage dysplasia (RCUD) and RCMD, RCUD and RAEB-1, RCUD and RAEB-2, RARS and RAEB-2, RCMD and RAEB-1, RCMD and RAEB-2, RAEB-1 and MDS-U, and RAEB-2 and MDS-U; in BM, significant differences were found between RCUD and RAEB-1, RCUD and RAEB-2, and RCMD and RAEB-2.

Correlation between WT1 mRNA expression and percentage of blasts in BM based on 2008 WHO classification

The correlations between blast ratio and WT1 mRNA expression in 96 patients (excluding one patient with MDS whose blast ratio could not be measured) were $r = 0.50$ in PB and $r = 0.46$ in BM.

WT1 mRNA expression in PB and BM for each WPSS risk group

WT1 mRNA expression in PB and BM was compared in 98 patients with MDS classified according to WPSS risk group [Figure 3(g)]. As the risk increased from very low to very high, WT1 mRNA expression in both PB and BM also tended to rise. When the distribution of WT1 mRNA for each risk group was evaluated, significant differences ($p < 0.05$) were found in both PB and BM between very low and high, very low and very high, low and high, low and very high, intermediate and high, and intermediate and very high. Moreover, when the correlation between the WPSS score and WT1 mRNA expression was investigated, the values were $r = 0.61$ in PB and $r = 0.55$ in BM.

Differential diagnosis between RA and AA

Differential diagnosis based on WT1 mRNA expression in PB samples

The WT1 mRNA expression level in PB was less than 50 copies/μg RNA in all eight patients with AA, whereas it was less than 50 copies/μg RNA in 34 patients with RA and 50–52 100 copies/μg RNA in 35 of 69 patients with RA. The statistical analysis by Wilcoxon rank-sum test revealed a statistical difference between eight patients with AA and 65 patients with RA ($p = 0.01$). Sixty-nine patients with RA were further categorized into three groups by bone marrow findings: hypoplastic RA ($n = 20$), hyperplastic RA ($n = 15$) and normoplastic RA ($n = 30$), excluding the non-categorized RA ($n = 4$). Significant differences were observed between AA and each of hypoplastic ($p = 0.04$) or normoplastic RA ($p = 0.02$), whereas no difference was shown between the AA and hyperplastic RA group ($p = 0.10$) by Steel test (Figure 4). From these findings, a differential diagnostic cut-off value between RA and AA of 50 copies/μg RNA for WT1 mRNA expression in PB is considered appropriate, for which the sensitivity was 50.7% (35/69) and the specificity was 100% (8/8).

Differential diagnosis based on WT1 mRNA expression in BM samples

The WT1 mRNA expression level in BM was 251–2600 copies/μg RNA in eight patients with AA, whereas it was less than 50 copies/μg RNA in one of 69 patients with RA and 69–196 000 copies/μg RNA in the others. The statistical analysis by Wilcoxon rank-sum test revealed no statistical difference between eight patients with AA and 65 patients with RA. Sixty-nine patients with RA were similarly categorized into three groups: hypoplastic, hyperplastic and normoplastic RA, excluding the non-categorized RA. Statistical analysis by Steel test revealed a significant difference between AA and normoplastic RA groups ($p = 0.04$), whereas there were no significant differences between the AA and each of hypoplastic RA and hyperplastic RA groups (Figure 4).

When receiver operating characteristic (ROC) analysis was performed to evaluate the performance of BM WT1 mRNA expression as an indicator to differentiate between RA and AA, the area under the curve was 0.713, and the Youden index [18] showed 432 copies/μg RNA. Moreover, the sensitivity was 69.6% (48/69), and the specificity was 75.0% (6/8) (Supplementary Figure to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2012.745074).

When the PB cut-off value of 50 copies/μg RNA was inserted into the regression line formula obtained

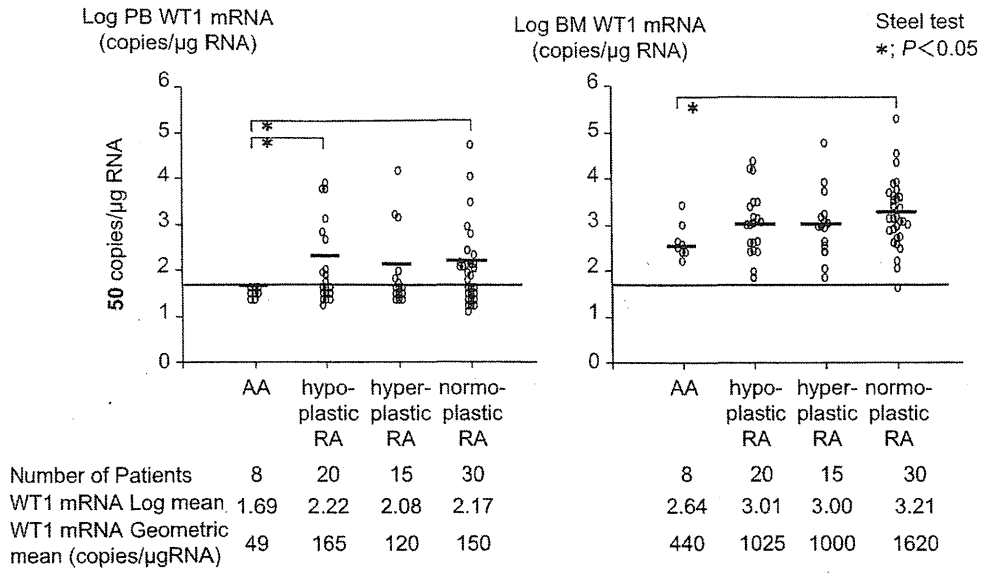

Figure 4. Comparison of WT1 mRNA expression between AA and RA groups (hypoplastic, hyperplastic and normoplastic RA). In intergroup comparison of WT1 mRNA expression, Steel test was performed using log-transformed values of WT1 mRNA expression with a level of significance of $p < 0.05$. Bold lines represent mean WT1 mRNA expression after log transformation. Fine lines represent lower limit of detection of WT1 mRNA (50 copies/μg RNA).

from the correlation between WT1 mRNA expression in PB and BM (Figure 2), BM WT1 mRNA expression became 480 copies/μg RNA. When 500 copies/μg was evaluated as the cut-off value for BM WT1 mRNA expression, the sensitivity was 68.1% (47/69) and the specificity was 75.0% (6/8). Based on these results, 500 copies/μg RNA was considered to be an appropriate cut-off value for the differential diagnosis between RA and AA using WT1 mRNA expression in BM.

Comprehensive analysis using cut-off values

The PB and BM samples in each disease and MDS subtype were further evaluated for their WT1-positive rates, using the WT1 mRNA expression cut-off values determined above (PB: 50 copies/μg RNA; BM: 500 copies/μg RNA) (Table II). For AML-MDS (11 patients), the WT1 mRNA-positive rates were a high 100% (11/11) for PB and 90.9% (10/11) for BM, and in MDS (115 patients), the WT1 mRNA-positive rates were 61.7% (71/115) for PB and 73.0% (84/115) for BM, which were the second highest after AML-MDS. In contrast, all patients with AA, ICUS, ITP, PNH, PRCA and erythroid hypoplasia had low positive rates of 0% for PB and 18.8% (3/16) for BM. The WT1 mRNA-positive rates for PB and BM increased with MDS disease stage progression (Table II).

Table II. WT1 mRNA-positive rate in PB and BM from patients with different MDS subtypes and AML-MDS according to FAB classification.

Subtype	No. of patients	WT1 mRNA-positive rate (%)	
		Peripheral blood	Bone marrow
RA	69	50.7 (35/69)	68.1 (47/69)
RARS	9	44.4 (4/9)	44.4 (4/9)
RAEB	24	83.3 (20/24)	87.5 (21/24)
RAEB-t	13	92.3 (12/13)	92.3 (12/13)
AML-MDS	11	100.0 (11/11)	90.9 (10/11)
Total	126	65.1 (82/126)	74.6 (94/126)

PB, peripheral blood; BM, bone marrow; MDS, myelodysplastic syndromes; AML-MDS, acute myeloid leukemia-evolved MDS; FAB, French-American-British; RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess of blasts; RAEB-t, refractory anemia with excess of blasts in transformation.

Discussion

In this study, the clinical usefulness of the measurement of WT1 mRNA expression in risk assessment of MDS was evaluated using a WT1 assay kit. Recently, a steady stream of reports has indicated the usefulness of WT1 mRNA measurement. The group of Cilloni [6] confirmed that WT1 mRNA expression potentially fulfills all the requirements for an additional marker for risk assessment in MDS, compared with the conventional methods. The measurement of WT1 can be effective, particularly in cases in which BM aspiration and/or cytogenetic analysis fail or are not informative [6].

Furthermore, in their findings in a long-term prospective study, Tamura *et al.* [19] reported that a significant correlation ($p = 0.0186$) was seen between WT1 mRNA expression and survival time when WT1 mRNA expression in PB was categorized into three groups of less than 10^2, 10^2–10^4, and greater than 10^4 copies/μg RNA, that the median survival time for each group was 62.7 months, 29.9 months and 11.6 months, respectively; and that the time until transformation to leukemia was the shortest in the group with the highest WT1 mRNA expression. In addition, they reported that in univariate analysis, WT1 mRNA expression was a predictive parameter for transformation to leukemia, and in multivariate analysis, it was a significant predictive parameter along with the IPSS score [19]. As described above, Tamaki *et al.* reported similar findings [4].

This study was conducted using not only the FAB classification system but also the 2001 and 2008 WHO classification systems. It was confirmed that in all three classification systems, WT1 mRNA expression in both PB and BM increases significantly in MDS subtypes with disease stage

progression. In addition, both PB and BM WT1 mRNA expression increased significantly as the risk of transformation to AML rose in the IPSS and WPSS risk groups. Furthermore, a correlation of $r = 0.57$ between the IPSS score and WT1 mRNA expression was seen in both PB and BM. The correlations between the WPSS score and WT1 mRNA expression were $r = 0.61$ in PB and $r = 0.55$ in BM. In comparison with the IPSS, the WPSS allows the assessment of survival time and progression of leukemic transformation at all time periods during the clinical course, leading to continued prognostic evaluation while reviewing the risk. WT1 mRNA expression correlates with the WPSS prognosis, and despite the single-point quantitation, the results in this study indicate that WT1 mRNA is useful as a time-course prognostic marker in the same manner as the WPSS.

At present, allogeneic hematopoietic stem cell transplant is the only curative treatment for MDS. However, determination of the timing of allogeneic transplant is very difficult because many patients are older, treatment-related deaths frequently occur, and there are large individual differences in the rate of disease progression. Allogeneic transplant is selected as the therapeutic regimen for MDS when no increase in blast cells is confirmed, taking into consideration the development of transfusion dependency and frequency of infections [20]. In addition, allogeneic transplant is selected when a future increase in blast cells is predicted by karyotypic analysis even though no increase is currently observed. It is recommended that transplant be performed before the progression to cytopenia caused by an increase in blast cell clones and before the progression to acute leukemia, although induction chemotherapy may be required when an increase in blast cells is observed [21]. On the other hand, another study suggested that delaying transplant until the advanced stage of disease results in a longer survival time for low and intermediate-1 IPSS risk groups, while early transplant was recommended for the intermediate-2 and high groups [22]. The period after CR is achieved is considered to be the standard timing to perform transplant for acute leukemia, but determining CR is extremely challenging. Our results revealed that periodic monitoring of WT1 mRNA expression in patients with MDS provided useful information for predicting the timing of transplant.

RA, a subtype in the early MDS disease stage, is often difficult to differentiate from AA [23]. In a previous study by Iwasaki *et al.*, no difference in WT1 mRNA expression was observed between RA and AA [9]. However, our data revealed the possibility of WT1 expression level to differentiate AA and RA groups using both peripheral blood and bone marrow samples (Figure 4). In the present statistical analysis, significant differences were observed between AA and hypoplastic RA ($p = 0.04$) in PB. The number of subjects was limited, and further trial is required for more detailed analysis. Moreover, tentative cut-off values for WT1 mRNA expression were set at 50 copies/μg RNA in PB and 500 copies/μg RNA in BM. Although the number of patients was small, the results showed that the level of WT1 mRNA expression could differentiate between RA and AA, with specificity in PB and BM of 100% (8/8) and 75.0% (6/8), respectively. This provides evidence that the measurement of WT1 mRNA expression can play a role in the differential diagnosis of RA and AA.

The WT1 assay kit is used clinically in Japan as a marker to monitor MRD in patients wth AML. In MDS, a clonal disorder of pluripotent hematopoietic stem cells, WT1 mRNA expression increases depending on the MDS subtype and disease stage. In contrast, the mechanism by which WT1 mRNA expression increases in MDS is not considered to correlate simply with the fluctuation in leukemic clones, as seen in AML. In normal hematopoiesis, WT1 mRNA is expressed mainly in CD34-positive cells. In contrast, in patients with MDS, WT1 mRNA is also expressed in CD34-negative cells, particularly in lineages exhibiting abnormalities [24]. In our study, the level of WT1 mRNA expression within the RA group was shown to increase with the increase in IPSS risk [Figure 3(c)]. Moreover, a similar trend of increasing WT1 expression was found in the RCUD and RCMD groups according to the 2008 WHO classification, although no significant increase in blast cells in BM was observed in these groups. Taken together, these findings indicate that the increase in WT1 mRNA expression in patients with MDS may reflect the divergence of MDS clones from normal clones and preleukemic changes.

In patients with MDS, evaluating the changes in WT1 mRNA levels simultaneously in PB and BM samples provides useful information on disease stage progression or risk assessment in individual patients. In addition, the WT1 mRNA-positive rate in each subtype of MDS was high (50–90%) in both PB and BM in this study, suggesting that a single measurement of WT1 mRNA is sufficient for MDS diagnosis, particularly for differentiating RA from AA.

Overall, this study provides evidence that the measurement of the level of WT1 mRNA expression in PB and BM serves as a supplemental marker for MDS diagnosis and prognostic assessment. This assay has great potential to contribute to more appropriate diagnoses and therapeutic decisions in patients with MDS and to evaluate the timing of allogeneic transplant.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

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Supplementary material available online

Supplementary figure showing ROC analysis of WT1 mRNA expression in BM in RA and AA groups

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ORIGINAL ARTICLE

Normal karyotype acute myeloid leukemia with the CD7+ CD15+ CD34+ HLA-DR + immunophenotype is a clinically distinct entity with a favorable outcome

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Abstract Recently, the presence of *CEBPA* mutation was identified as an important prognostic factor for normal karyotype (NK) acute myeloid leukemia (AML). Because AML with *CEBPA* mutation is closely associated with CD7, CD15, CD34, and HLA-DR expression, we investigated the prognostic implications of CD7+ CD15+ CD34+ HLA-DR + immunophenotype in NK-AML. We analyzed the immunophenotype of 329 patients with NK-AML from the Japan Adult Leukemia Study Group (JALSG) AML97 population. NK-AML with the CD7+ CD15+ CD34+ HLA-DR + immunophenotype was classified as the CEBPA type, and NK-AML that did not meet this criterion was considered as

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the non-CEBPA type. The influence of the CEBPA status on event-free survival (EFS) and overall survival (OS) was assessed using log-rank test and a multivariate Cox proportional hazard regression model. Furthermore, the surface antigen expression profile in AML according to the *CEBPA* mutation status (monoallelic or biallelic) was also investigated. Of the 329 NK-AML patients that were studied, 39 and 243 were classified as having CEBPA and non-CEBPA type NK-AML, respectively. Patients with CEBPA type NK-AML had significantly better EFS and OS than those with non-CEBPA type NK-AML. Multivariate analysis showed that the CEBPA type and white blood cell (WBC) counts of >20 × 10^9/L were independent prognostic factors for EFS and OS. Moreover, NK-AML with the biallelic *CEBPA* mutation was more closely associated with CD34 positivity than that with the monoallelic *CEBPA* mutation. NK-AML with the CD7+ CD15+ CD34+ HLA-DR + immunophenotype is a clinically discrete entity, and this may have a possible role in risk stratification.

Keywords Normal karyotype acute myeloid leukemia · CD7 · CD15 · CD34 · HLA-DR · CEBPA · Prognostic factor

Introduction

In recent years, immunophenotyping of hematologic neoplasms has become standard practice to establish a diagnosis and define the origin of the malignant cell lineage. Patients with acute myeloid leukemia (AML) often show aberrant cellular antigen expression as well as chromosomal abnormalities. The clinical significance of surface antigen expression has been studied for more than 20 years, but thus far, it has yielded inconsistent results [1]. Nevertheless, if the evaluation of antigen expression is limited to a subtype of AML, we would be more likely to find a significant relationship between surface antigen expression and prognosis. For example, the significance of CD56 expression as an adverse prognostic factor in both acute promyelocytic leukemia (APL) and AML with t(8;21) is widely accepted [2, 3].

Normal karyotype (NK)-AML is the most common subtype of AML, accounting for 40–50 % of cases [4-6]. Patients with this subtype are considered to have an intermediate risk, and upfront hematopoietic stem cell transplantation (HSCT) is commonly recommended [7-9]. However, even though treatment strategies seem promising, the prognosis of NK-AML is variable when molecular evaluation of the *FLT3*, *NPM1*, and *CEBPA* mutations is taken into account [10-14]. Schlenk et al. [10] reported that NK-AML patients with the *CEBPA* mutation or *NPM1* mutation, but without the *FLT3* mutation, had a favorable prognosis and that upfront HSCT in these patients did not contribute to the overall survival (OS). Although the detection of subgroups is necessary for decisions on the most appropriate treatment strategy, routine molecular diagnoses are often difficult in clinical practice.

AML with the *CEBPA* mutation has a homologous surface antigen expression that is closely associated with CD7, CD15, CD34, and HLA-DR positivity [15, 16]. We speculated that immunophenotyping for CD7, CD15, CD34, and HLA-DR in patients with NK-AML could identify a distinct subtype of AML that clinically mimics AML with the *CEBPA* mutation. In the Japan Adult Leukemia Study Group (JALSG) AML97 study, almost 42 % of the patients with AML were diagnosed with NK-AML. For this study, results of surface antigen expression were obtained at the time of enrollment. Further, we used data from the JALSG AML97 study to investigate the clinical significance of these surface antigens for the prognosis of patients with NK-AML.

Patients and methods

Patients

We conducted a retrospective review of patient data from the multicenter JALSG AML97 study. Detailed information of this study and its results has previously been reported [17, 18]. Briefly, between December 1997 and July 2001, patients aged 15–64 years, with newly diagnosed de novo AML, excluding those with APL, were consecutively enrolled to the JALSG AML97 study. In total, 789 of the 809 AML patients were eligible for the study, and informed consent was obtained from all patients or their guardians before enrollment. The study protocol was approved by the research ethics boards of all participating institutions, and the study was conducted in accordance with the Declaration of Helsinki.

Cytogenetic studies

The results from the cytogenetic studies, which were performed at each of the institutions, were reported to the JALSG Statistical Center. Routinely, 20 metaphases were counted for each patient and analyzed according to the recommendations of the International System for Human Cytogenetic Nomenclature.

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Flow cytometry

Immunophenotyping was performed at each institution, primarily on freshly collected bone marrow or peripheral blood samples that were collected at the time of diagnosis. Leukemic cell analysis was performed at local or reference laboratories by standard immunofluorescence methods using monoclonal antibodies directed against the CD2, CD3, CD4, CD5, CD7, CD8, CD11b, CD13, CD15, CD19, CD33, CD34, CD41a, CD56, and HLA-DR surface antigens. Samples were considered positive if at least 20 % of blasts expressed the antigen.

Treatment regimen used in the JALSG AML97 study

Induction therapy consisted of Ara-C at a dose of 100 mg/m^2 per day as a continuous infusion on days 1–7 and idarubicin (IDR) at a dose of 12 mg/m^2 per day as a 30-min infusion on days 1–3. Patients who did not achieve remission after the first induction cycle were given the same therapy again. Patients who obtained complete remission (CR) within two courses of induction therapy were randomly assigned to a group that received either four courses of standard dose consolidation therapy without maintenance (arm A) or three courses of standard dose consolidation along with six courses of maintenance therapy (arm B). In the JALSG AML97 study, the 5-year overall survival rate and the 5-year disease-free survival (DFS) rate between the arms were not statistical different [17].

Surface antigen expression profile according to the CEBPA mutant pattern

We also investigated the surface antigen expression profiles according to *CEBPA* mutant pattern in 318 AML patients based on the data records of AML patients enrolled at the Kumamoto and Nagasaki Universities. High molecular weight genomic DNA was extracted from the bone marrow or peripheral blood samples after Ficoll separation of mononuclear cells. Mutations of the *CEBPA* gene was detected by genomic DNA PCR, and direct sequencing was performed at each institution, as described previously [19, 20].

Statistical analysis

OS for all patients was defined as the period from the date of diagnosis to the date of death. Event-free survival (EFS) was defined as the period from the date of diagnosis to the date of the first recurrence after CR or any cause of death. All patients who underwent HSCT were censored from the EFS analysis on the date of HSCT treatment. The Kaplan-Meier method was used to estimate the EFS and OS. The log-rank test was used to compare the EFS or OS of the two groups. Factors that could potentially affect clinical outcome, including age, sex, WBC count, performance status at diagnosis, and the expression of each surface antigen were analyzed by the multivariate Cox proportional hazard regression model. Fisher's exact test and Student's *t* test were used to compare factor differences between the two groups. Statistical analysis was performed with the JMP software version 8.0.1 (SAS Institute Inc., Cray, NC, USA).

Results

Definition of the CEBPA type

For this study, CEBPA type NK-AML was defined as NK-AML that showed the CD7+ CD15+ CD34+ HLA-DR + immunophenotype because these antigens are commonly expressed in AML with the *CEBPA* mutation [15, 16]. Non-CEBPA type NK-AML was defined as NK-AML that did not have the CD7+ CD15+ CD34+ HLA-DR + immunophenotype.

In total, 329 patients were diagnosed with NK-AML. The expression of CD7, CD15, CD34, and HLA-DR was examined in 303, 201, 306, and 302 patients, respectively. Of the

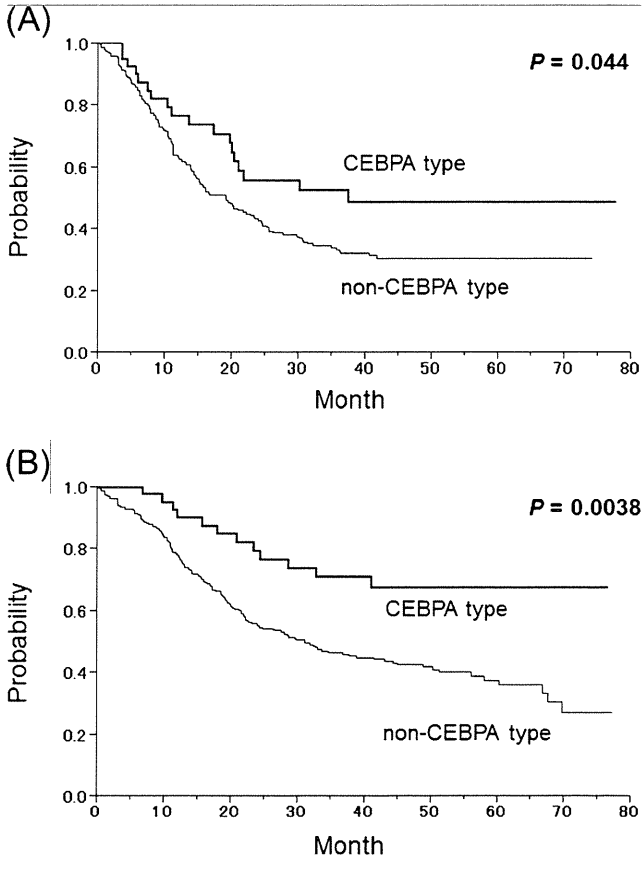

Fig. 1 Kaplan-Meier curves show event-free survival (EFS) and overall survival (OS) according to the CEBPA status. EFS and OS for each group are shown in **a** and **b**, respectively. Log-rank test revealed the 5-year EFS to be 48.5 and 30.5 % for patients with CEBPA and non-CEBPA type NK-AML, respectively, which was significantly different (P=0.044). The 5-year OS of patients with CEBPA and non-CEBPA type NK-AML was also significant (67.4 and 35.7 %, respectively; P=0.0038)

Table 1 Analysis of prognostic factors for event-free survival in the NK-AML population (n=329)

Factors	Number (positive/negative)	Univariate analysis		Multivariate analysis	
		HR (95 % CI)	P value	HR (95 % CI)	P value
Age > 50 years	131/198	1.04 (0.77–1.39)	0.812		
Female sex	141/188	0.81 (0.60–1.09)	0.168		
WBC count of $>20\times10^9$/L	165/164	1.74 (1.30–2.34)	0.0002	1.56 (1.14–2.14)	0.0052
Performance status ≥2	29/300	1.21 (0.72–1.91)	0.461		
CD7	108/195	0.96 (0.70–1.31)	0.808		
CD15	113/88	0.77 (0.52–1.13)	0.174		
CD34	166/140	1.26 (0.94–1.71)	0.127		
HLA-DR	255/47	1.16 (0.77–1.82)	0.483		
CEBPA type	39/243	0.61 (0.36–0.96)	0.034	0.59 (0.35–0.94)	0.026

329 NK-AML patients, 39 were classified as having the CEBPA type and 243 were classified as having the non-CEBPA type NK-AML. We excluded 47 patients whose of immunophenotype could not be determined.

EFS and OS

The 5-year EFS rate for patients with CEBPA type NK-AML was 48.5 %, which was significantly higher than the 30.5 % for patients with non-CEBPA type NK-AML (P=0.044, Fig. 1a). Furthermore, the 5-year OS rate for patients with CEBPA type NK-AML was also significantly higher than that for patients with non-CEBPA type NK-AML (67.4 vs 35.7 %, P=0.0038, Fig. 1b).

Univariate analysis showed that the outcome of patients with increased WBC counts at diagnosis was significantly worse (Tables 1 and 2), in agreement with previous reports [1]. Furthermore, CEBPA type was also a significant factor for better EFS and OS (Tables 1 and 2). Multivariate analysis showed CEBPA type and increased WBC counts to be independent prognostic factors (Tables 1 and 2). Other factors such as age, performance status, sex, or the expression of each of the single surface antigens did not affect the EFS and OS rates.

Our study included 12 patients with CEPBA type NK-AML and 77 patients with non-CEBPA type NK-AML who received HSCT. There was no significant difference among patients who received HSCT in these two groups (Table 3). The 2-year OS after HSCT in these groups were 61 and 41 %, respectively, which did not reach significance (P=0.467).

Clinical profiles in CEBPA type

The CEBPA type was identified as an independent prognostic factor for EFS and OS. Therefore, we analyzed the characteristics of CEBPA type (Table 3). Sex, WBC count, or performance status was not associated with CEBPA or non-CEBPA type NK-AML. In contrast, compared with non-CEBPA type NK-AML, CEBPA type NK-AML was associated with younger age, higher myeloperoxidase (MPO)-positive rates, frequent presentation with Auer rods, and a French-American-British (FAB) classification of M1 or M2.

Table 2 Analysis of prognostic factors for overall survival in the NK-AML population (n=329)

Factors	Number (positive/negative)	Univariate analysis		Multivariate analysis	
		HR (95 % CI)	P value	HR (95 % CI)	P value
Age > 50 years	131/198	1.19 (0.89–1.60)	0.240		
Female sex	141/188	0.80 (0.59–1.08)	0.144		
WBC count of $>20\times10^9$/L	165/164	1.51 (1.13–2.03)	0.0059	1.44 (1.05–1.97)	0.023
Performance status ≥2	29/300	1.23 (0.72–1.96)	0.437		
CD7	108/195	0.79 (0.57–1.09)	0.150		
CD15	113/88	0.72 (0.49–1.07)	0.101		
CD34	166/140	1.10 (0.81–1.50)	0.526		
HLA-DR	255/47	0.95 (0.64–1.47)	0.807		
CEBPA type	39/243	0.41 (0.22–0.71)	0.0008	0.40 (0.21–0.69)	0.0005

Table 3 Correlations of the clinical profiles and treatments in patients with CEBPA and non-CEBPA type NK-AML

Factors	CEBPA type (n=39)	Non-CEBPA type (n=243)	P value
Clinical profile			
Age (year), median (range)	38 (16–64)	48 (15–64)	0.018
Sex (male/female)	27:12	138:105	0.164
WBC count ($\times 10^9$/L), median (range)	21.8 (3.0–449.5)	19.1 (0.6–709.0)	0.301
Auer rod: positive/negative	31/7	101/139	<0.0001
FAB M1 or M2/others	37/2	122/121	<0.0001
MPO positivity of >50 %/≤50 %	30/6	95/105	<0.0001
Performance status ≥2/0–1	4/35	19/224	0.538
HSCT	12	77	1.000
CR1	4	33	0.798
CR2	5	14	0.157
Others	3	30	0.592

MPO myeloperoxidase, *FAB* French-American-British classification, *HSCT* hematopoietic stem cell transplantation, *CR* complete remission

Surface antigen profiles according to *CEBPA* mutant pattern

We investigated the expression of surface antigens according to monoallelic or biallelic *CEBPA* mutation in patients with AML. Of the 318 AML patients, 41 were diagnosed with the *CEBPA* mutation, which included 29 with biallelic and 12 with monoallelic mutations. The presence of the *CEBPA* mutation was common in intermediate risk AML, including NK-AML. In contrast, the mutation was uncommon in core-binding factor (CBF)-AML and adverse risk AML.

To investigate the association between surface antigen expression and *CEBPA* mutant pattern in NK-AML patients, the expressions of CD7, CD34, and HLA-DR were examined (Table 4). There was a significantly higher frequency of CD34 expression in AML patients with biallelic *CEBPA* mutation than in those with monoallelic mutation.

Discussion

The results of this study show that the CD7+ CD15+ CD34+ HLA-DR + immunophenotype is a significant predictor of OS in patients with NK-AML. We were able to analyze data from a well-designed, uniform, prospective study. In addition, we observed that CEBPA type NK-AML was a discrete clinical entity, which is closely associated with high MPO positivity rates, Auer rod positivity, FAB classification of M1 or M2, and a younger age. These characteristics are very similar to the characteristics of AML with *CEBPA* mutation as previously reported: AML with the *CEBPA* mutation also had high MPO rates [19], FAB classification of M1 or M2 [21], and better OS [10, 11]. In addition, the prognosis of CEBPA type NK-AML was almost similar to that in the favorable risk group of AML as indicated by the JALSG scoring system [17] and that in AML patients with the *CEBPA* mutation [10, 11], as previously reported. Moreover, of 282 patients with NK-AML, 14 % had CEBPA type NK-AML, which is similar to that observed in previous reports (i.e., 10–18 % of patients with NK-AML had the *CEBPA* mutation) [22].

Table 4 Surface antigen expression in AML patients according to monoallelic or biallelic *CEBPA* mutation

Factors	Monoallelic *CEBPA* mutation Positive/negative (%)	Biallelic *CEBPA* mutation Positive/negative (%)	P value
CD7	7/3 (70)	23/4 (85)	0.360
CD34	5/5 (50)	25/2 (93)	0.009
HLA-DR	11/0 (100)	23/2 (92)	1.000

It is also known that the expressions of CD7, CD34, and HLA-DR are associated with poor clinical outcomes in AML [1]. In contrast to previous reports, our study, which was limited to NK-AML patients, demonstrated that the expression of single surface antigens including CD7, CD15, CD34, and HLA-DR did not have prognostic significance. Moreover, the CEBPA immunophenotype demonstrated a favorable OS even though the population positively expressed CD7, CD34, and HLA-DR, which have previously been considered poor prognostic factors.

It was previously reported that CD7 expression is associated with the *FLT3* mutation [23], *CEBPA* mutation [15, 16], and adverse risk cytogenetics [2, 24]. Furthermore, CD34 positivity is frequently observed in patients with adverse risk cytogenetics and t(8;21) AML [2], and it is negatively associated with *NPM1* mutations [25]. Another surface antigen, the B-cell marker CD19, which is observed in t(8;21) AML and that is associated with negativity for *KIT* mutation, favorably affects CR in AML patients with t(8;21) [2, 26]. Taken together, these results represent the difficulty in analyzing the heterogeneous population of AML as a total group because the impact of chromosomal and/or molecular abnormalities on prognosis makes it difficult to conclusively interpret the significance of surface antigen expression.

On the other hand, the population, which we detected with this combination of surface antigens, is evidently a distinct subtype of NK-AML with a discrete clinical profile (Table 3). Our findings show that specific subtypes of AML such as NK-AML with the *CEBPA* mutation potentially exist, and it suggests that the population of patients who have a favorable prognosis may well be identified through an analysis of the surface antigens that are expressed. Therefore, if the molecular evaluation is unavailable, an analysis of surface antigens may help in identifying patients with a favorable prognosis.

It is known that biallelic *CEBPA* mutations, but not monoallelic mutations, have a favorable prognosis [27]. Our study, which included all cases with the *CEBPA* mutation, showed that 93 % of cases with biallelic mutation were positive for CD34, while only 50 % of cases with monoallelic mutation were positive (Table 4), suggesting that CD34 is an important factor for distinguishing the mutant pattern. Because low activity of *CEBPA* is thought to be a critical factor for sustaining the immature character of AML cells [28], we hypothesized that a more potent inactivation of *CEBPA* by biallelic mutation results in the frequent incidence of CD34 positivity.

In this study, immunophenotypic analyses were not performed at a central facility. Another limitation of our study is the possible selection bias; not all the institutes performed cytometric analysis of all AML97 study antigens at the time of patient enrollment. This resulted in a reduction in sample size, because CEBPA status was not detected in 14 % of patients with NK-AML. JALSG has recently investigated whether molecular evaluations, including that of the *CEBPA* mutation, affect patient prognosis [29]. In the future, we hope to clarify the relationships among surface antigen expression, cytogenetics, molecular evaluation results, and clinical features of AML. In conclusion, we found that the CD7+ CD15+ CD34+ HLA-DR + immunophenotype has a potential role in risk stratification for patients with NK-AML.

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Conflict of interest There are no relevant conflicts of interest to disclose.

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ORIGINAL ARTICLE

Comprehensive analysis of genetic alterations and their prognostic impacts in adult acute myeloid leukemia patients

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To clarify the cooperative roles of recurrently identified mutations and to establish a more precise risk classification system in acute myeloid leukemia (AML), we comprehensively analyzed mutations in 51 genes, as well as cytogenetics and 11 chimeric transcripts, in 197 adult patients with *de novo* AML who were registered in the Japan Adult Leukemia Study Group AML201 study. We identified a total of 505 mutations in 44 genes, while only five genes, *FLT3, NPM1, CEBPA, DNMT3A* and *KIT*, were mutated in more than 10% of the patients. Although several cooperative and exclusive mutation patterns were observed, the accumulated mutation number was higher in cytogenetically normal AML and lower in AML with *RUNX1-RUNX1T1* and *CBFB-MYH11*, indicating a strong potential of these translocations for the initiation of AML. Furthermore, we evaluated the prognostic impacts of each sole mutation and the combinations of mutations and/or cytogenetics, and demonstrated that AML patients could be clearly stratified into five risk groups for overall survival by including the mutation status of *DNMT3A*, *MLL*-PTD and *TP53* genes in the risk classification system of the European LeukemiaNet. These results indicate that the prognosis of AML could be stratified by the major mutation status in combination with cytogenetics.

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Keywords: acute myeloid leukemia; gene mutations; prognosis; risk factor

INTRODUCTION

Acute myeloid leukemia (AML) is a clinically and genetically heterogeneous disease.[1,2] Although about 80% of younger adults with AML achieve complete remission (CR) with induction chemotherapy, more than half of the CR patients relapse, even if they receive intensive consolidation therapies. Allogeneic hematopoietic stem cell transplantation (allo-SCT) is applied to the patients who have risk factors for relapse, and it has been demonstrated by meta-analysis that allo-SCT at the first CR improves the long-term prognosis of the cytogenetically intermediate- and adverse-risk groups.[3] Cytogenetic-risk classification for AML is well established and commonly used as criteria for the application of allo-SCT at the first CR, whereas there is clinical heterogeneity in the intermediate-risk group, particularly cytogenetically normal (CN)-AML.[4] Recent advances and the accumulation of information on the prognostic relevance of recurrent genetic alterations have made more detailed risk stratification possible in AML patients.[5–19] The European LeukemiaNet (ELN) has recommended a novel risk classification system on the basis of the cytogenetic and genetic status.[2] In this system, CN-AML is stratified into two risk groups according to the mutation status of *FLT3*, *NPM1* and *CEBPA*: patients with *NPM1* mutation but not *FLT3*-ITD and those with *CEBPA* mutation are included in the favorable-risk (FR) group, and patients with *FLT3*-ITD and those with neither *NPM1* mutation nor *FLT3*-ITD are categorized into the intermediate-I-risk (IR-I) group. Long-term prognosis according to the ELN classification system was retrospectively evaluated in well-established cohorts, and it has

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been demonstrated that the ELN system is useful for further risk stratification of younger adult patients with CN-AML.[20,21] However, it has been reported that another genetic status, such as mutations in epigenetic modifier-encoding genes, could more precisely distinguish the prognosis in each ELN-risk group.[5,22] In addition, the prognostic impacts of recently identified mutations in spliceosome and cohesin complex genes on AML remain unclear.

The Japan Adult Leukemia Study Group (JALSG) conducted six phase III trials for adult *de novo* AML from 1987 (AML87, AML89, AML92, AML95, AML97 and AML201).[23] In the JALSG AML201 study, we prospectively compared a standard dose of idarubicin (IDR) with a higher dose of daunorubicin (HiDNR) in combination with cytarabine (Ara-C) as induction therapy, and three courses of high-dose Ara-C (HiDAC) with four courses of conventional standard-dose multiagents as consolidation therapy in CR patients.[24,25] We demonstrated that HiDNR was equivalent to IDR, as induction therapy and HiDAC was of benefit only to patients with core-binding factor (CBF)-AML as consolidation therapy. Although the CR rate remained at 75–80% during the six JALSG studies, 7-year overall survival (OS) was improved to 48% in the AML201 study from 29% in the AML87 study. Allo-SCT was conducted in only 7.1% of registered patients in the AML87 study, whereas 45.8% of registered patients received allo-SCT not only at the first CR but also after relapse or primary induction failure in the AML201 study, indicating that active application of allo-SCT even after relapse or primary induction failure might contribute to the improvement of OS. These results collectively suggested that a novel risk stratification system for decision making of allo-SCT at the first CR is required.

In this study, we comprehensively analyzed mutations in 51 genes that have been recurrently identified in myeloid neoplasm as well as cytogenetics, and evaluated the association of genetic status with prognostic and clinical features in patients who were registered in the AML201 study.

PATIENTS AND METHODS

Patients and samples

The study population included 197 newly diagnosed *de novo* AML patients, except for those with acute promyelocytic leukemia, who were registered in the JALSG AML201 study (UMIN Clinical Trials Registry C000000157, http://www.umin.ac.jp/ctrj/). The diagnosis of AML was on the basis of the French–American–British (FAB) classification.[26] Median follow-up time was 32.5 months. The age distribution is presented in Table 1. In the AML201 study, patients were randomly assigned to receive either IDR or HiDNR for induction therapy, and those who achieved CR were again randomized to receive either four courses of conventional consolidation therapy or three courses of HiDAC therapy.[24,25] Of the 197 patients, 98 and 99 patients were assigned to IDR and HiDNR arms for induction therapy, respectively. CR was achieved in 161 of 197 (81.7%) patients, and 80 and 77 patients were assigned to HiDAC and conventional consolidation therapies, respectively (Table 1).

High molecular weight DNA and total RNA were extracted from bone marrow samples using standard methods.[27–29]

Cytogenetic G-banding analysis was performed by standard methods. We also examined 11 chimeric gene transcripts (Major: *BCR-ABL1*, Minor: *BCR-ABL1*, *PML-RARA*, *RUNX1-RUNX1T1*, *CBFB-MYH11*, *DEK-NUP214*, *NUP98-HOXA9*, *MLL-MLLT1*, *MLL-MLLT2*, *MLL-MLLT3*and *MLL-MLLT4*) by reverse transcriptase-mediated quantitative PCR as previously reported.[30]

Morphological diagnosis, the FAB classification and karyotypes were reviewed and confirmed by the central review committees of the JALSG using the BM samples obtained at diagnosis.

We obtained informed consent from all patients to use their samples for banking and molecular analysis, and approval was obtained from the ethics committees of the participating institutes.

Screening for mutations in 51 genes

A custom-made oligonucleotide probe library was designed to capture the exons of 51 genes that have been recurrently identified in myeloid neoplasm (Supplementary Table 1). Captured and enriched exons were subjected to sequencing on an Illumina HiSeq (Illumina, San Diego, CA, USA).[31–33] Sequence variation annotation was performed using known polymorphism databases, followed by mutation characterization. Each predicted variant sequence was confirmed by Sanger sequencing. Internal tandem duplication of the *FLT3* gene (*FLT3*-ITD) and partial tandem duplication of the *MLL* gene (*MLL*-PTD) were examined as previously reported.[28,34]

Table 1. Characteristics of the 197 patients

Characteristics	*Number*	*(%)*
Age (year)		
15–19	6	3.0
20–29	32	16.2
30–39	35	17.8
40–49	33	16.8
50–59	69	35.0
60–64	22	11.2
FAB subtype		
M0	7	3.6
M1	36	18.3
M2	89	45.2
M4	34	17.3
M4Eo	9	4.6
M5	21	10.7
M6	1	0.51
Cytogenetic-risk group		
Favorable	55	27.9
RUNX1-RUNX1T1	41	20.8
CBFB-MYH11	14	7.1
Intermediate	100	50.8
Normal cytogenetics	72	36.5
Unfavorable	23	11.7
Complex karyotype	16	8.1
t(11q23) excluding t(9;11) and t(11;19)	3	1.5
t(9;22)	2	1.0
−7	1	0.5
Not determined	19	9.6
Induction therapy		
IDR + Ara-C	98	49.7
DNR + Ara-C	99	50.3
Achieving CR	161	81.7
Consolidation therapy		
High-dose Ara-C	80	51.0
Multiagent CT	77	49.0

Abbreviation: IDR, idarubicin. The study population included 197 newly diagnosed *de novo* AML patients except for acute promyelocytic leukemia, and equally assigned to induction and consolidation arms. Nine patients showed the M4Eo FAB type, and all of them harbored the *CBFB-MYH11* transcript.

Statistical analysis

Differences in continuous variables were analyzed by the unpaired *t*-test or the Mann–Whitney *U*-test for distribution between two groups. Analysis of frequencies was performed using Fisher's exact test for 2×2 tables or Pearson's χ^2 test for larger tables. A multivariate analysis to identify risk factors for achieving CR was performed by the logistic regression model. Survival probabilities were estimated by the Kaplan–Meier method, and differences in the survival distributions were evaluated using the log-rank test. OS was defined as the time from the date of entry into the AML201 study to death due to any cause or last follow-up. Disease-free survival (DFS) was defined as the time from the day of achieving CR to relapse, death due to any cause or last follow-up. Patients undergoing SCT were not censored at the time of transplantation. The prognostic significance of the clinical variables was assessed using the Cox proportional hazards model. These statistical analyses were performed with Prism 5 (GraphPad Software, La Jolla, CA, USA) and JMP Pro10 (SAS Institute Japan,

Tokyo, Japan). For all analyses, the *P*-values were two-tailed, and a *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Cytogenetic analysis

Cytogenetic analysis revealed a normal karyotype in 72 (36.5%) patients and an abnormal karyotype in 106 (53.8%) patients, including 41 AML with t(8;21) (q22;q22); *RUNX1-RUNX1T1* and 14 AML with inv(16) (p13q22); *CBFB-MYH11*. However, karyotypes could not be determined in 19 (9.6%) patients because we could not obtain sufficient mitotic cells. On the basis of the G-banding karyotype and chimeric transcript analyses, patients were assigned to favorable- ($n=55$, 27.9%), intermediate- ($n=100$, 50.8%) and adverse-risk ($n=23$, 11.7%) groups according to the refined MRC criteria (Table 1).[4]

Frequencies of mutations

We identified mutations in 44 of 51 genes analyzed in the 197 AML patients. However, there were only five genes (*FLT3, NPM1, CEBPA, DNMT3A* and *KIT*) that were mutated in more than 10% of the patients (Figure 1a and Supplementary Table 1). Each position and type of mutation is presented in Supplementary Figure 1. As germ-line controls were available in a limited number of patients, we could not completely confirm that all identified mutations were somatic mutations. Therefore, there is a possibility that a part of identified mutations might be rare SNPs. *FLT3* mutation was the most frequently identified (50 patients, 25.4%), followed by *NPM1* (38 patients, 19.2%), *DNMT3A* (32 patients, 16.2%), *CEBPA* (31 patients, 15.7%) and *KIT* mutations (28 patients, 14.2%). Of the 50 patients with *FLT3* mutations, 36 (18.3%) and 17 (8.6%) patients harbored *FLT3*-ITD and *FLT3*-KDM, respectively, and three patients harbored both mutations. Of the 31 patients with *CEBPA* mutations, 19 (9.6%) and 12 (6.1%) patients harbored double *CEBPA* (*CEBPA*-D) mutations and a single *CEBPA* (*CEBPA*-S) mutation, respectively. Of the 28 patients with *KIT* mutations, 4, 2 and 23 patients harbored mutations in exon 8, exons 10–11 and exon 17 of the *KIT* gene, respectively, and one patient harbored mutations in both exons 10–11 and exon 17.

Although mutations in the 51 analyzed genes were not identified in 14 (7.1%) patients, 183 (92.9%) patients harbored one or more mutations; one mutation in 36, two mutations in 56, three mutations in 40, four mutations in 27, five mutations in 17, six mutations in five and seven mutations in two patients. The mean mutation number per patient was 2.56 ± 0.11 in all patients, whereas it was significantly higher in patients with a normal karyotype (3.18 ± 0.16) than in those with an aberrant karyotype (2.10 ± 0.15) ($P<0.0001$). Furthermore, mean mutation numbers per patient in AML with *RUNX1-RUNX1T1* (1.68 ± 0.17) and *CBFB-MYH11* (1.57 ± 0.20) were significantly lower than that in all samples ($P=0.0008$ and 0.0123, respectively) (Figure 1b).

The mean mutation number per patient aged 60–64 years (3.18 ± 0.41) tended to be higher, although there was no significant difference between the mean mutation number and age (Supplementary Figure 2).

Genetic alterations found in AML have been conceptually grouped into class I mutation, which causes constitutive activation of intracellular signals that contribute to the growth and survival, and class II mutation that blocks differentiation and/or enhance self-renewal by altered transcription factors.[35–37] Recently, it has been suggested that mutations that modify the epigenetic status generate a new class because of their overlap mutations both with class I and class II mutations.[13,38] In this study, Class II mutations (*NPM1, CEBPA, RUNX1* and *GATA2* mutations, and *RUNX1-RUNX1T1* and *CBFB-MYH11*) were the most frequently identified (138/197; 70.1%), followed by Class I mutations (*FLT3, KIT, N/KRAS, PTPN11, JAK1/3* and *TP53* mutations) (116/197; 58.9%) and mutations that modify the epigenetic status (*ASXL1, ATRX, EZH2, TET2, PBRM1, DNMT3A, IDH1/2, KDM6A, MLL* and *DOT1L* mutations) (91/197; 46.2%). Furthermore, mutations of NOTCH family genes (*NOTCH1 and NOTCH2*), cohesin complex genes (*STAG2, SMC1A, SMC3* and *RAD21*), BCOR family genes (*BCOR* and *BCORL1*), NCOR family genes (*NCOR1, NCOR2* and *DIS3*) and spliceosome genes (*SF3B1, U2AF1, SRSF2* and *ZRSR2*) were identified in 19 (9.6%), 22 (11.2%), 17 (8.6%), 22 (11.2%) and 9 patients (4.6%), respectively (Figure 1c).

Association between gene mutations and cytogenetics

The prevalence of each gene mutation differed among the cytogenetic-risk groups. *KIT* mutations were preferentially identified in the favorable cytogenetic-risk group. *FLT3*-ITD, *NPM1, CEBPA* and *DNMT3A* mutations were preferentially identified in the intermediate-risk group, particularly in patients with a normal karyotype. *BCORL1* and *TP53* mutations were preferentially identified in the poor-risk group; in particular, *TP53* mutations were frequent in patients with a complex karyotype. In addition, *PHF6* mutations were also frequently identified in patients with a complex karyotype (Figure 2 and Supplementary Table 2).

Overlap mutations

Several patterns of overlap mutations were identified in this comprehensive mutation analysis (Supplementary Figures 3 and 4). Significantly overlapped mutations were observed between *FLT3* mutations and *NPM1, DNMT3A* and *MLL*-PTD mutations; *NPM1* mutations and *DNMT3A, IDH1* and *IDH2* mutations; *CEBPA* mutations and *TET2* mutations; *ASXL1* mutations and spliceosome gene mutations; *DIS3* mutations and *MLL* mutations; *DNMT3A* mutations and *PTPN11* mutations; *GATA2* mutations and *CEBPA*-D mutations; *K/NRAS* mutations and *WT1* mutations and *BCOR/BCORL1* mutations; *RUNX1* mutations and *U2AF1, MLL-PTD, BCOR/BCORL1* and *PHF6* mutations; *SF3B1* mutations and *NRAS* mutations; and *TET2* mutations and *STAG2* mutations. In contrast, mutually exclusive mutations were observed between *FLT3* mutations and *KIT, K/NRAS* and *CEBPA*-D mutations; *NPM1* mutations and *CEBPA*-D and *RUNX1* mutations; and *CEBPA* mutations and *IDH1/2* mutations.

According to the conceptual classification of the mutated genes, overlap mutations between Class I, Class II and epigenetic modifying gene mutations were frequently observed. However, these major mutations widely coexisted with other family gene mutations, such as the cohesin complex, BCOR family and spliceosome gene mutations (Figure 1d). Although biological functions of mutated genes have not been fully clarified, we also present frequencies and associations of mutated genes according to the provisional gene function in the Supplementary Figure 5.

Association of gene mutations with clinical characteristics

Several associations between mutations and clinical characteristics were observed. *DNMT3A* mutations and *MLL*-PTD were more frequently identified in patients over 50 years old than in those less than 50 years old ($P=0.0064$ and $P=0.0121$, respectively), whereas the other mutations were not significantly associated with age (Supplementary Table 3).

Several mutations were associated with the white blood cell count at diagnosis. *FLT3-ITD, NPM1, DNMT3A* and *NOTCH1* mutations were significantly associated with the high white blood cell count. In contrast, *ASXL1* and *IDH1* mutations were associated with a lower white blood cell count (Supplementary Table 4).

Association of gene mutations with the CR rate

We analyzed the association of mutations with the CR rate. By Fisher's exact test, *RUNX1-RUNX1T1* or *CBFB-MYH11, KIT, NPM1* and *CEBPA*-D mutations were identified as favorable factors for

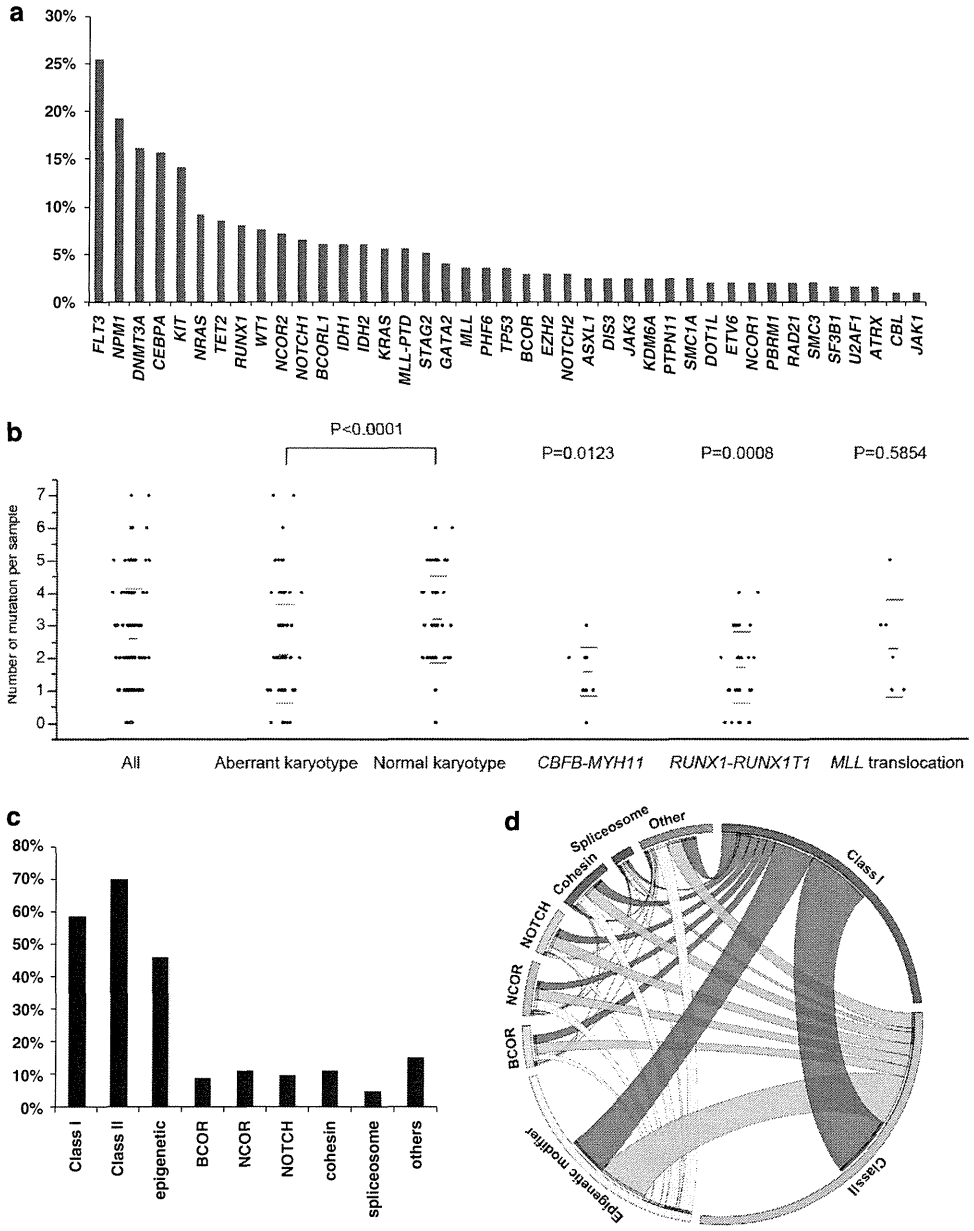

Figure 1. Frequencies and associations of mutated genes. (**a**) Frequencies of analyzed gene mutations. Frequency of each mutated gene is shown. Mutations were identified in 44 genes of 51 genes analyzed in 197 AML patients. Only five genes (*FLT3, NPM1, CEBPA, DNMT3A* and *KIT*) were mutated in more than 10% of the patients. (**b**) Mutated gene numbers according to the cytogenetics. Mean mutation number ± s.d. is indicated by horizontal lines. Mean mutation number per one patient in patients with normal karyotype (3.18 ± 0.16) was significantly higher than in that with aberrant karyotype (2.10 ± 0.15) ($P<0.0001$). Those in AML with *RUNX1-RUNX1T1* (1.68 ± 0.17) and *CBFB-MYH11* (1.57 ± 0.20) were significantly lower than that in all samples ($P=0.0008$ and $P=0.0123$, respectively). That in AML with *MLL*-translocation tended to be lower but not statistically significant (2.28 ± 0.57, $P=0.5854$). (**c**) Frequencies of mutations according to the conceptual classification. Mutations in Class I, Class II and epigenetic modifying genes were frequently identified. (**d**) Association of mutated genes according to the conceptual classification. Circos plot of mutated genes according to the function is shown.[45] Overlap mutations between Class I, Class II and epigenetic modifying genes mutations were frequently observed. These major mutations were widely coexistent with another family genes, such as cohesin complex, BCOR family and spliceosome genes.

achieving CR, and *TP53* mutation was an unfavorable factor; however, multivariate logistic regression analysis including all analyzed mutations showed that only *NPM1* (Hazard ratio (HR): 96.206, 95% Confidence interval (CI): 2.247–411.9, $P=0.0172$) and *TP53* (HR: 22.222, 95% CI: 1.597–333.3, $P=0.0172$) mutations were identified as favorable and unfavorable factors for achieving CR, respectively (Table 2 and Supplementary Table 5).

Importantly, *KIT* mutations were closely associated with *RUNX1-RUNX1T1* or *CBFB-MYH11*, whereas the other mutations that confer the achievement of CR were mutually exclusive (Supplementary Figures 3 and 6). In the patients with *RUNX1-RUNX1T1* or *CBFB-MYH11, NPM1* and *CEBPA*-D mutations, the CR rate (106/112; 94.6%) was significantly higher than for those with the other genotypes (55/85; 64.7%) ($P<0.0001$).

Prognostic impacts of mutations

We next analyzed the prognostic impact of each mutation. By univariate analysis, *FLT3*-ITD (HR: 1.805, 95% CI: 1.130–2.885, $P=0.0135$), *DNMT3A* (HR: 1.696, 95% CI: 1.055–2.725, $P=0.0291$),

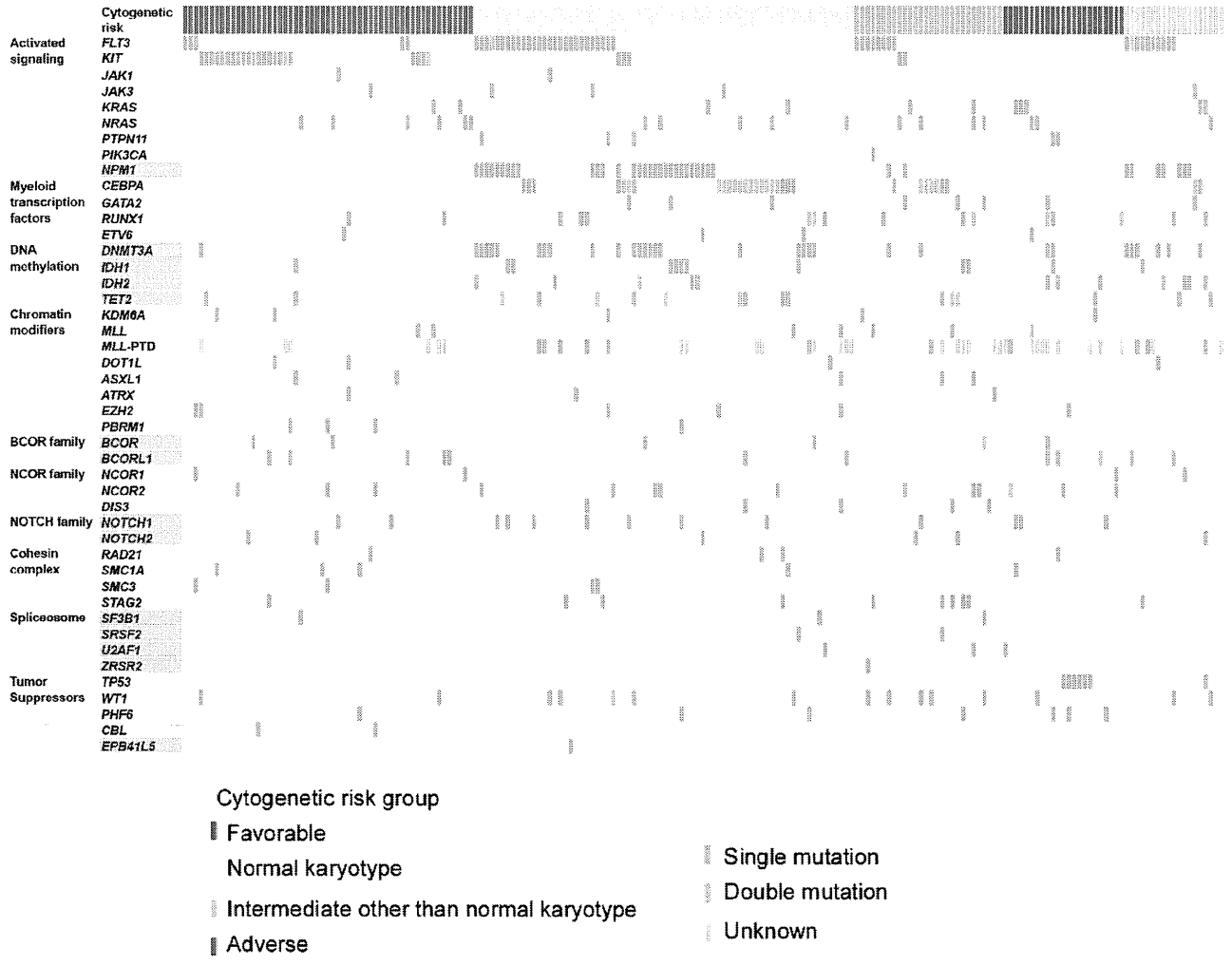

Figure 2. Mutation status according to the cytogetics-risk groups. Identified mutations in analyzed AML patients are shown according to the cytogenetic-risk groups. Pink boxes indicate single mutations and orange boxes indicate double mutations.

Table 2. Gene mutations affecting the CR achievement

Mutations	*CR rate (%)*		P-*value*
	Positive	*Negative*	
Fisher's exact test			
NPM1	97	78	0.0041
CEBPA-D-Mt.	100	80	0.0273
KIT	96	79	0.0326
RUNX1-RUNX1T1 or *CBFB-MYH11*	91	78	0.0409
TP53	14	84	0.0002

Mutations	*HR (95% CI)*	P-*value*
Multivariate analysis		
Wild-*NPM1*	96.206 (2.247–411.9)	<0.0001
TP53 mutation	22.222 (1.597–333.3)	0.0172

Abbreviations: CI, confidence interval; CR, complete remission; HR, hazard ratio. By the Fisher's exact test, *RUNX1-RUNX1T1* or *CBFB-MYH11*, *KIT*, *NPM1* and *CEBPA*-D mutations were identified as the favorable factor for achieving CR, and *TP53* mutation was for the unfavorable factor. The multivariate logistic regression analysis including all analyzed mutations showed that only wild-*NPM1* and *TP53* mutation were identified as unfavorable factors for achieving CR.

TP53 (HR: 15.167, 95% CI: 6.555–35.094, $P<0.001$), *MLL*-PTD (HR: 3.782, 95% CI: 1.948–7.346, $P<0.001$) and *RUNX1* (HR: 2.301, 95% CI: 1.278–4.146, $P=0.0055$) mutations and the karyotypes other than *RUNX1-RUNX1T1* or *CBFB-MYH11* (HR: 2.786, 95% CI: 1.608–4.831, $P=0.0003$) were identified as unfavorable prognostic factors for OS (Table 3 and Supplementary Figure 7). Multivariate Cox regression analysis with stepwise selection showed that *TP53* (HR: 14.803, 95% CI: 6.259–35.009, $P<0.001$), *MLL*-PTD (HR: 2.853, 95% CI: 1.401–5.810, $P=0.0039$) and *RUNX1* (HR: 1.965, 95% CI: 1.054–3.663, $P=0.0336$) mutations and the karyotypes other than *RUNX1-RUNX1T1* or *CBFB-MYH11* (HR: 2.353, 95% CI: 1.342–4.132, $P=0.0028$) were independent poor prognostic factors for OS (Table 3).

In this cohort, mutations of NOTCH family, the cohesin complex, BCOR family and spliceosome genes were frequently identified. NOTCH family and BCOR family genes were not associated with the CR rate, OS and DFS. Although mutations of cohesin complex genes were not associated with the CR rate and DFS, the patients harboring those mutations revealed better OS than those without mutations ($P=0.0274$) (Figure 3). The CR rate and DFS of patients with spliceosome gene mutations tended to be lower than for those without mutations, although both differences were not statistically significant: the CR was achieved in five of the nine (55.6%) and 156 of the 188 (83.0%) patients ($P=0.0601$), and 3-year DFS were 0% and 38.9% ($P=0.1117$) in those with and

without mutations, respectively (Supplementary Table 3 and Supplementary Figure 8).

When the patients were stratified into the risk groups recommended by ELN, that is, FR, IR-I, IR-II and AR groups included 92, 35, 42 and 28 patients, respectively. The ELN system well stratified the long-term prognosis of adult AML patients, whereas the OSs of IR-I and AR groups were the same in the present cohort (Supplementary Figure 9). Therefore, we analyzed whether another mutations could further stratify the prognosis in each ELN-risk group. *MLL*-PTD, *GATA2* and *TP53* mutations were identified as further poor prognostic factors in IR-I, IR-II and AR groups, respectively. Furthermore, we identified that the *DNMT3A* mutation was a poor prognostic factor in the FR group except for the AML with t(8;21) (q22;q22); *RUNX1-RUNX1T1* or inv(16) (p13q22); *CBFB-MYH11* (CBF-AML) (Figure 4).

Table 3. Unfavorable prognostic factors for overall survival (OS)

Mutations	*HR (95% CI)*	*P-value*
Univariate analysis		
TP53	15.167 (6.555–35.094)	<0.0001
MLL-PTD	3.782 (1948–7.346)	<0.0001
Non CBF	2.786 (1.608–4.831)	0.0003
RUNX1	2.301 (1.278–4.146)	0.0055
FLT3-ITD	1.805 (2.247–4119)	0.0135
DNMT3A	1.696 (1.055–2.725)	0.0291
Multivariate analysis		
TP53	14.803 (6.259–35.009)	<0.0001
MLL-PTD	2.853 (1.4017–5.810)	0.0039
Non CBF	2.353 (1.342–4.132)	0.0028
RUNX1	1.965 (1.054–3.663)	0.0336

Abbreviations: CI, confidence interval; HR, hazard ratio. By the univariate analysis, *FLT3*-ITD, *DNMT3A*, *TP53*, *MLL*-PTD and *RUNX1* mutations and the karyotypes other than *RUNX1-RUNX1T1* or *CBFB-MYH11* were identified as adverse prognostic factors for OS. Multivariate Cox regression analysis with stepwise selection showed that *TP53*, *MLL*-PTD and *RUNX1* mutations and the karyotypes other than *RUNX1-RUNX1T1* or *CBFB-MYH11* were independent poor prognostic factors for OS.

In addition, we also evaluated two recently reported risk stratification systems on the basis of genetic status in our cohort.[5,39] Patel *et al.*[5] reported a risk stratification system on the basis of cytogenetics and genetic status According to their system, our patients were clearly stratified into three risk groups, although the patients in the intermediate cytogenetic risk with a favorable mutational risk profile and those in the favorable cytogenetic-risk profile showed the same probability of OS (Supplementary Figure 10a). Grossmann *et al.*[39] reported a prognostic model solely on the basis of molecular mutations. Although our cohort did not include AML with *PML-RARA*, our patients were clearly stratified into four risk groups. However, the patients in the very favorable group and those in the favorable group showed the same probability of OS (Supplementary Figure 10b).

On the other hand, *KIT* mutations were frequently identified in CBF-AML, while they were not a poor prognostic factor for either OS or DFS (Supplementary Figure 11a). According to the types of *KIT* mutations, CBF-AML patients harboring mutations in exon 17 of the *KIT* gene showed worse prognosis than those harboring the other types of *KIT* mutation, although this was not statistically significant (Supplementary Figure 11b). Notably, in the CBF-AML patients harboring *KIT* mutations, OS and DFS of those treated with three courses of HiDAC consolidation therapy tended to be better than those treated with four courses of conventional standard-dose multiagent therapy (Supplementary Figure 11c).

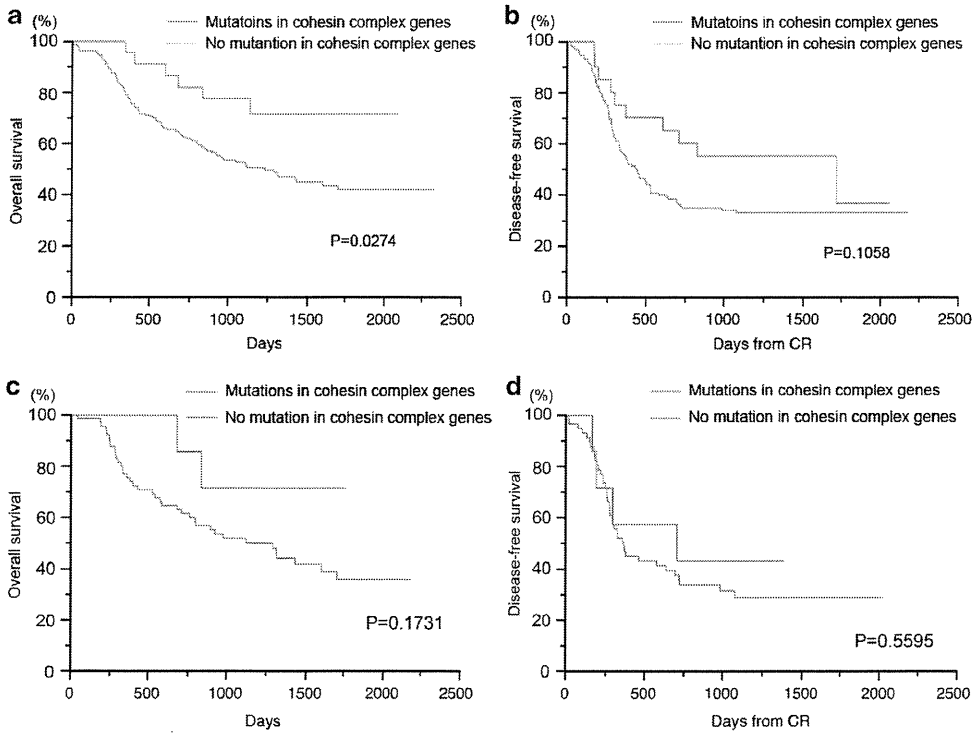

Figure 3. Prognostic impact of mutations in cohesin complex genes. Kaplan–Meier curves for OS and DFS according to the mutations in cohesin complex genes are shown. (**a**) OS in the total patients, (**b**) DFS in the total patient, (**c**) OS in the patients with normal karyotype, (**d**) DFS in the patients with normal karyotype. Although mutations of cohesin complex genes were not associated with the CR rate and DFS, the patients harboring those mutations revealed better OS than those without mutations ($P = 0.0274$). In the patients with normal karyotype, OS of the mutated patients tended to be better that that of unmutated patients, though statistical significance was not observed ($P = 0.1731$).

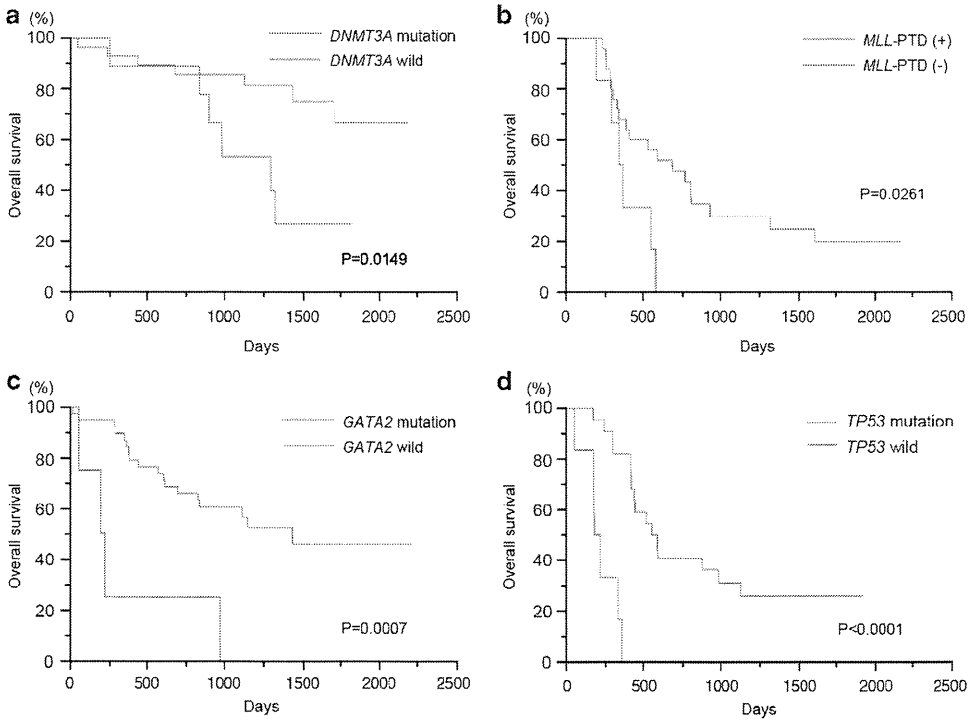

Figure 4. Mutations that could further stratify the ELN-risk groups into two risk groups. (**a**) *DNMT3A* mutation was a poor prognostic factor in the FR group except for the AML with t(8;21) (q22;q22); *RUNX1-RUNX1T1* or inv(16) (p13q22); *CBFB-MYH11* (CBF-AML). (**b**) *MLL*-PTD was a poor prognostic factor for the OS in the ELN IR-I group. (**c**) *GATA2* mutation was a poor prognostic factor for the OS in the ELN IR-II group. (**d**) *TP53* mutation was a poor prognostic factor for the OS in the ELN AR group.

In CBF-AML, the accumulated mutation number was lower than in the other types of AML, although many kinds of mutation were identified (Supplementary Figure 12). However, we could not identify a gene that affects the prognosis of CBF-AML.

Taking these results together, we tried to modify the genetic criteria for the ELN stratification system. When the CN-AML patients with *DNMT3A* mutations of the FR group and the patients with *MLL*-PTD of the IR-I group were included in the IR-I and the AR group, respectively, we could more clearly stratified the patients into four risk groups for OS than the original ELN system (Figures 5a and b). Furthermore, as the prognosis of the patients with *TP53* mutations were very unfavorable as previously reported,[39] we could more clearly stratify the patients into five risk groups for OS by classifying the *TP53*-mutated patients as the very adverse-risk group (Figures 5c and d).

DISCUSSION

In this study, we comprehensively analyzed mutations of 51 genes by the targeting sequence, and identified a total of 505 mutations in 44 genes in 197 adult *de novo* AML patients except for APL. The whole-genome and -exome analysis demonstrated recurrent mutations in a total of 260 genes in 200 AML patients, suggesting that another mutations might be accumulated in the presently analyzed AML cells.[40] However, frequencies of most mutated genes were reportedly less than 10%. In consistence, only five genes (*FLT3, NPM1, CEBPA, DNMT3A* and *KIT*) were mutated in more than 10% patients in our study, and each mutation frequency was almost the same as previous reports.[5,17,19,39] The frequency of *KIT* mutation was relatively higher in our study than previous reports,[5,40] while this is caused by the higher frequency of CBF-AML (28.0%) in the Japanese patients, in which *KIT* mutations are frequently identified. Our study, therefore, essentially includes major genetic regions, which may affect the pathogenesis and prognosis of AML. However, mutation analyses were not thoroughly performed in all subtypes of AML, such as acute erythroid leukemia and acute megakaryoblastic leukemia because of their lower frequencies. Further analyses are required to fully clarify the genetic alterations in AML.

The whole-genome and -exome analysis demonstrated that an average of mutated genes in coding regions per sample was 5.24.[40] Of note was that there were significant differences in mutated gene numbers among the types of cytogenetics and mutations: the mean mutation numbers were higher in AML with *RUNX1-RUX1T1*, and are lower in that with *PML-RARA* and *MLL* translocations than that of all samples. As analyzed gene numbers were limited, mean mutated gene number per sample (2.56 ± 0.11) was low in our study; however, there were different features from the previous report. In our study, higher mutation number was observed in CN-AML (3.18 ± 0.16), and lower was in CBF-AML. Furthermore, the mutation number in AML with *MLL*-translocation tended to be lower (2.28 ± 0.57, $P = 0.5854$). These results collectively suggested that recurrent cytogenetic abnormalities, such as *RUNX1-RUNX1T1, CBFB-MYH11* and *MLL*-translocation, have a strong potential for the initiation of AML, and that most of accumulated mutations in AML with these cytogenetics might be passenger mutations.

It has been reported that common mutations in AML, such as *DNMT3A, NPM1, CEBPA, IDH1/2* and *RUNX1*, were mutually exclusive of the transcription-factor fusions, indicating the high potential for leukemia initiation.[10,11,13,40,41] Consistently, *DNMT3A, NPM1* and *CEBPA* mutations were not identified in CBF-AML, but frequent in CN-AML. In addition, we identified that *MLL*-PTD mutation was also exclusive of CBF-AML. In CBF-AML, *KIT* mutations were preferentially identified, whereas several types of mutations were also accumulated, suggesting that many mutations could act as a driver mutation for the clonal expansion of the initiating clone with *RUNX1-RUNX1T1* and