

## RESEARCH ARTICLE

# Associations Between Age, Cytogenetics, FLT3-ITD, and Marrow Leukemia Cells Identified by Flow Cytometry

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### Abstract

**Objectives:** To explore the relationships between age, cytogenetic subgroups, molecular markers, and cells with leukemic aberrant immunophenotype in patients with acute myeloid leukemia (AML). **Methods:** In this study, we evaluated the correlations between age, cytogenetic subgroups (normal, balanced and unbalanced karyotype), molecular mutations (NPM1, FLT3-ITD, and CEBPA mutations) and marrow leukemia cells (LC) identified by flow cytometry in 256 patients with de novo AML. **Results:** From age group 10-19 years to age group  $\geq 60$  years, the percentage of LC decreased from  $67.0 \pm 18.4\%$  to  $49.0 \pm 25.1\%$  ( $F = 2.353$ ,  $P = 0.041$ ). LC percentage was higher in patients with balanced karyotypes ( $65.7 \pm 22.4\%$ ), than those with unbalanced karyotypes ( $46.0 \pm 26.6\%$ ) ( $u = 3.444$ ,  $P = 0.001$ ) or a normal karyotype ( $49.9 \pm 22.1\%$ ) ( $u = 5.093$ ,  $P < 0.001$ ). Patients with FLT3-ITD ( $64.3 \pm 19.5\%$ ) had higher LC percentages compared with those without ( $54.2 \pm 24.3\%$ ) ( $u = 2.794$ ,  $P = 0.007$ ). **Conclusions:** Associations between age, cytogenetics, molecular markers, and marrow leukemia cells may offer beneficial information to understand the biology and pathogenesis of AML.

**Keywords:** Acute myeloid leukemia - leukemia cells - age - cytogenetics - molecular mutations

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### Introduction

Acute myeloid leukemia (AML) at diagnosis can show highly heterogeneous patterns, as it can be characterized by rapid blast proliferation and sudden onset of clinical symptoms, while in other cases, clinical courses may be determined by leukocytopenia and, eventually, by an oligo- or asymptomatic clinical prophase (Haferlach et al., 2012). Peripheral white blood cells and marrow blasts could be affected by age (Appelbaum et al., 2006), cytogenetics (Su et al., 2013), and molecular mutations (Schnittger et al., 2002; Thiede et al., 2002; Schnittger et al., 2005; Haferlach et al., 2012) in patients with AML. There was a trend toward a higher peripheral white blood cell counts and percentage of peripheral blasts among patients younger than age 56 years old (Appelbaum et al., 2006). We found that patients with balanced karyotypes showed lower peripheral leukocytes compared with those of normal and unbalanced karyotypes (Su et al., 2013). NPM1 mutations and FLT3-ITD were also reported to be linked with higher peripheral leukocytes and higher marrow blast percentage (Schnittger et al., 2002; Thiede et al., 2002; Schnittger et al., 2005; Haferlach et al., 2012). However, no data is available in AML about the associations between age, cytogenetic subgroups, molecular markers, and cells with leukemic immunophenotype. Furthermore, the correlations between cytogenetic subgroups, molecular markers and age in AML have been reported by previous studies (Schneider et al., 2012; Su et al., 2013). Thus, this study was designed to evaluate the integrated effect

of age, cytogenetic subgroups, and molecular markers on proliferation of cells with leukemia-associated aberrant immunophenotype (LAIP) identified by flow cytometry in patients with AML.

### Materials and Methods

#### Patients

From January 1 st, 2009, to October 31 st, 2012, 256 patients with de novo AML were enrolled in this study, who were residents of the northeast region of China, including the Jilin, Hei Longjiang, and Liaoning provinces. All of the participating patients gave informed consent prior to enrolment in the study, and this study was approved by the ethics committee of Jilin University and conducted in accordance with the Declaration of Helsinki.

#### Cytogenetic, molecular mutation analyses and leukemia cell identification

Standard culturing and banding techniques were used to analyze the chromosome karyotype and the clonal abnormalities were defined and described according to the International System for Human Cytogenetic Nomenclature (Shaffer et al., 2009). All of the patients were classified into 1 of the 3 following karyotype groups based on the type of primary abnormality identified: (1) normal karyotype group (no abnormality detected); (2) balanced karyotype group (primary balanced translocation or inversion), and (3) unbalanced karyotype group (genetic material gains or losses without a primary or known

**Table 1. General Information of De Novo AML Patients**

	n	Percent (%)
Median (range)	43 (7 ~ 82)	
Gender		
Male	128	50.0 (128/256)
Female	128	50.0 (128/256)
FAB classification		
M0 ~ M1	4	1.56 (4/256)
M2	107	41.8 (107/256)
APL	63	24.6 (63/256)
M4	35	13.7 (35/256)
M5	42	16.4 (42/256)
M6	5	1.95 (5/256)

balanced abnormality) (Su et al., 2013). The mutational status of molecular markers NPM1 (n = 256), FLT3-ITD (n = 253), and CEBPA (n = 57) were analyzed and polymerase chain reactions (PCR) were performed as described previously (Hollink et al., 2009; Wouters et al., 2009; Park et al., 2011). Flow cytometry was employed to identify the population of leukemia cells (LC) with leukemia-associated aberrant immunophenotype (LAIP), in each patient from the initial BM aspirate as described previously (Kaleem et al., 2003).

*Statistics*

The Statistics Package for Social Sciences (SPSS) software (Version 16.0) (SPSS Inc., Chicago, IL, USA) was used to calculate statistical differences. Independent sample t-test or Mann-Whitney U test were used to compare between different groups. For comparison between three or more groups, Analysis of Variance (ANOVA) or Kruskal-Wallis H test were employed. Receiver operating curve (ROC) was performed to calculate the correlations between LC percentage and FLT3-ITD. Partial correlation analysis was used to avoid the influence from other variables. A *P* < 0.05 was considered significant.

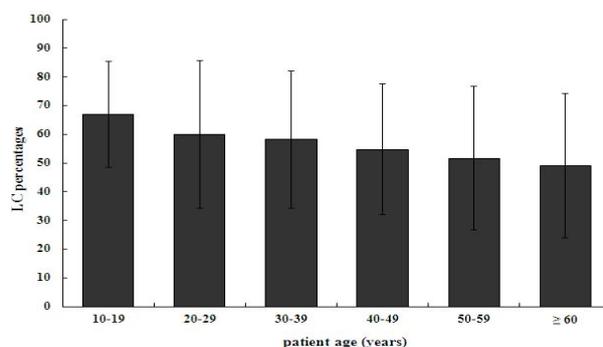
**Results**

*General characteristics*

General patient characteristics were summarized in Table 1. The most common subtype in the present cohort was M2 (41.8%, n = 107), followed by acute promyelocytic leukemia (APL) (24.6%, n = 63).

*The frequencies of cytogenetic subgroups and molecular markers*

Of 256 patients with de novo AML, 225 cases (87.9%) had successful cytogenetic results. Based on their



**Figure 1. Leukemia Cell Percentages of Different Age Groups in Patients with AML**

cytogenetic classification, the patients were divided into 3 large groups, as follows: 50.7% (114/225) had normal karyotypes, 39.1% (88/225) had balanced karyotypes and 10.2% (23/225) had unbalanced karyotypes. Of the balanced karyotypes, 53 cases (60.2%) were t(15;17)(q22;q21), 28 cases (31.8%) were t(8;21)(q22;q22), 3 cases (3.41%) were inv(16)(p13q22), and the remaining 4 cases (4.55%) were other balanced karyotypes.

The frequencies of NPM1, FLT3-ITD, and CEPBA mutations were 15.2% (39/256), 14.6% (37/253), and 26.3% (15/57), respectively. While in those with normal karyotype, 27.2% (31/114), 16.8% (19/113), and 36.1% (13/36) of the patients carried NPM1, FLT3-ITD, and CEPBA mutations, respectively.

*The relationships between age, cytogenetics, molecular markers, and leukemia cells*

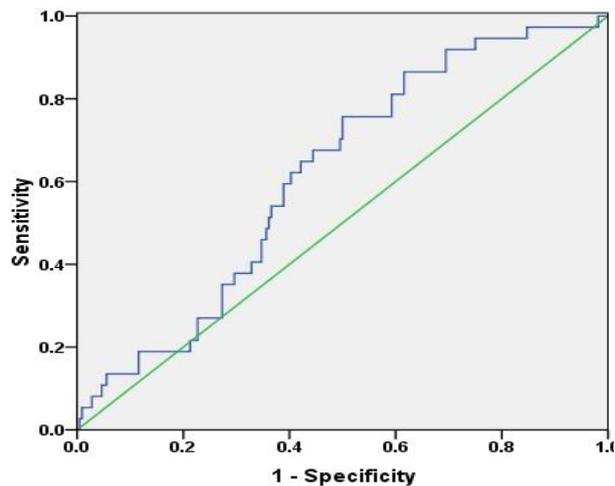
From age group 10-19 years to age group ≥ 60 years, the percentage of LC decreased from 67.0 ± 18.4% to 49.0 ± 25.1% (*F* = 2.353, *P* = 0.041) (Figure 1).

LC percentage was higher in patients with balanced karyotypes (65.7 ± 22.4%), than those with unbalanced karyotypes (45.9 ± 26.6%) (*u* = 3.444, *P* = 0.001), and normal karyotype (49.9 ± 22.1%) (*u* = 5.093, *P* < 0.001). Patients with t(15;17)(q22;q21) had higher LC percentage (78.7 ± 12.1%) compared with those with t(8;21)(q22;q22) (45.9 ± 18.5%) (*u* = 6.792, *P* < 0.001), normal karyotype (49.9 ± 22.1%) (*u* = 8.140, *P* < 0.001), and other karyotypes (46.3 ± 25.9%) (*u* = 5.505, *P* < 0.001). However, LC percentage was not significantly different between patients with t(8;21)(q22;q22), normal karyotype, and other karyotypes (all *P* > 0.05).

The relationships between LC percentage and molecular mutations were listed in Table 2. Both NPM1 and CEBPA mutations had no influence on LC percentage. However, patients with FLT3-ITD had higher LC percentage compared with those without. ROC analysis

**Table 2. The Relationship Between Leukemia Cell Percentage and Molecular Mutations**

	Leukemia cell percentage (%)					
	All	Statistical value	<i>P</i>	Normal karyotype	Statistical value	<i>P</i>
NPM1 mutations	52.5 ± 24.0	<i>t</i> = 0.955	0.341	54.5 ± 23.5	<i>t</i> = 1.367	0.174
NPM1 negative	56.5 ± 24.0			48.2 ± 21.5		
FLT3-ITD	64.3 ± 19.5	<i>u</i> = 2.794	0.007	66.3 ± 11.6	<i>u</i> = 3.90	<0.001
FLT3-ITD negative	54.2 ± 24.3			48.2 ± 21.5		
CEBPA mutations	56.3 ± 24.7	<i>t</i> = 0.581	0.563	50.9 ± 20.4	<i>t</i> = 0.262	0.795
CEBPA negative	52.2 ± 20.2			53.0 ± 24.4		



**Figure 2. Correlations Between Leukemia Cell Percentages and FLT3-ITD**

showed that the patients had significant higher frequency of FLT3-ITD when their LC percentage was higher than 56.0% (Area under curve = 0.616, Sensitivity = 75.5%, Specificity = 50.0%,  $P = 0.024$ ) (Figure 2).

Taking the relationships between age, cytogenetics and molecular mutations into account, we using partial correlation analysis to explore if the associations between age, t(15;17)(q22;q21), FLT3-ITD, and LC percentage were independent. Patient age ( $r = -0.133$ ,  $P = 0.044$ ), t(15;17)(q22;q21) ( $r = 0.551$ ,  $P < 0.001$ ), and FLT3-ITD ( $r = 0.243$ ,  $P < 0.001$ ) were all found to be correlated with LC percentage with partial correlation analysis.

## Discussion

To the best of our knowledge, this was the first study to evaluate the integrated effect of age, cytogenetic subgroups, and molecular markers on proliferation of leukemia cells identified by flow cytometry in detail. Unlike white blood cell counts and marrow blasts (histochemical staining) used in previous studies, we identified the population of cells with flow cytometry, which was more direct, objective, and accurate to determine the percentage of leukemia cells. We found that patient age, cytogenetics, and molecular markers were all associated with LC percentage in patients with de novo AML.

Genetic alterations are the hallmarks of cancer, which are associated with enhanced proliferative or anti-apoptotic capacities of leukemia cells. The oncogenic fusion protein, PML-RAR $\alpha$ , generated by reciprocal chromosomal rearrangement t(15;17), blocks the differentiation hindering progenitors to reach the postproliferative stage and to undergo programmed cell death, leading to overproduction of immature myeloid cells in the bone marrow (Puccetti & Ruthardt, 2004). Many studies have documented the functions of AML1-ETO fusion protein created by t(8;21), including inhibiting differentiation and subsequent apoptosis, and activating signals for cell proliferation (Gardini et al., 2008; Steffen et al., 2011). Transgenic mice expressing PML-RAR $\alpha$  under the control of the myeloid-specific promoter, cathepsin G, develop myeloid hyperplasia, with 15% to

30% of mice progressing to leukemia (Grisolano et al., 1997). However, the fusion gene, AML1-ETO, alone is not sufficient for inducing leukemia and additional genetic abnormalities are probably involved (Wang et al., 2011; Li et al., 2013). In this study, we found that patients with t(15;17)(q22;q21) presented with higher LC percentage compared with those with t(8;21)(q22;q22) or other karyotypes, which also supported the critical role of PML-RAR $\alpha$  in inducing leukemogenesis.

Molecular mutations, such as NPM1, FLT3-ITD, or CEBPA mutations were reported to be correlated with higher peripheral white blood cell counts and/or higher marrow blast percentage (Schnittger et al., 2002; Thiede et al., 2002; Schnittger et al., 2005; Haferlach et al., 2012). However, in this study, only FLT3-ITD was the molecular mutation found to be associated with higher marrow LC percentage. The stimulating impact of FLT3-ITD on cell proliferation is well established (Hayakawa et al., 2000; Mizuki et al., 2000; Gilliland & Grifn, 2002; Haferlach et al., 2012). FLT3-ITD leads to constitutive activation of FLT3 kinase (Gilliland & Grifn, 2002; Haferlach et al., 2012). Transfecting of IL-3 dependent cell lines with mutant FLT3-transfected cells resulted in autonomous cell growth (Hayakawa et al., 2000) and presence of the FLT3-ITD has been associated with strong constitutive activation of the STAT pathway (Mizuki et al., 2000; Haferlach et al., 2012). In mice, FLT3-ITD could induce a myeloproliferative disorder characterized by leukocytosis and splenomegaly (Kelly et al., 2002; Haferlach et al., 2012). ROC analysis was also adopted for the first time to evaluate the correlation between FLT3-ITD and LC percentage. The patients tended to have significant higher frequency of FLT3-ITD when their LC percentage was higher than 56.0%.

Previous study has already demonstrated that young patients with AML tended to have higher white blood cell counts and blast percentage (Appelbaum et al., 2006). We also observed that LC percentage decreased with ages, which was consistent with the hypothesis that leukemia stem cells become senescent with ages (Rajaraman et al., 2006).

In conclusion, the associations of age, cytogenetics, molecular markers, and marrow leukemia cells may offer beneficial information to understand the biology and pathogenesis of AML.

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