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Gene Mutation Patterns in Patients with Minimally Differentiated Acute Myeloid Leukemia

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Abstract

Minimally differentiated acute myeloid leukemia (AML-M0) is a rare subtype of AML with poor prognosis. Although genetic alterations are increasingly reported in AML, the gene mutations have not been comprehensively studied in AML-M0. We aimed to examine a wide spectrum of gene mutations in patients with AML-M0 to determine their clinical relevance. Twenty gene mutations including class I, class II, class III of epigenetic regulators (*IDH1*, *IDH2*, *TET2*, *DNMT3A*, *MLL*-PTD, *ASXL1*, and *EZH2*), and class IV (tumor suppressor genes) were analyzed in 67 patients with AML-M0. Mutational analysis was performed with polymerase chain reaction–based assays followed by direct sequencing. The most frequent gene mutations from our data were *FLT3*-ITD/*FLT3*-TKD (28.4%), followed by mutations in *IDH1*/*IDH2* (28.8%), *RUNX1* (23.9%), N-*RAS*/K-*RAS* (12.3%), *TET2* (8.2%), *DNMT3A* (8.1%), *MLL*-PTD (7.8%), and *ASXL1* (6.3%). Seventy-nine percent (53/67) of patients had at least one gene mutation. Class I genes (49.3%) were the most common mutated genes, which were mutually exclusive. Class III genes of epigenetic regulators were also frequent (43.9%). In multivariate analysis, old age [hazard ratio (HR) 1.029, 95% confidence interval (CI) 1.013-1.044, $P = .001$) was the independent adverse factor for overall survival, and *RUNX1* mutation (HR 2.326, 95% CI 0.978-5.533, $P = .056$) had a trend toward inferior survival. In conclusion, our study showed a high frequency of *FLT3*, *RUNX1*, and *IDH* mutations in AML-M0, suggesting that these mutations played a role in the pathogenesis and served as potential therapeutic targets in this rare and unfavorable subtype of AML.

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Introduction

Minimally differentiated acute myeloid leukemia (AML-M0) accounts for approximately 2% to 5% of all AMLs according to the French-American-British classification [1]. It frequently occurs in elderly patients and confers a poor prognosis [2]. Morphologically, the leukemic cells are large and agranular blasts mimicking lymphoblasts and negative for cytochemical reactions of myeloperoxidase (MPO), Sudan Black B, or nonspecific esterase [1]. The immunophenotypic characteristics of AML-M0 blasts are low expression of MPO, positive for at least one myeloid antigen (CD13, CD33, CD15, or CD11b), frequent expression of stem cell–associated antigens (CD34, HLA-DR, CD117), TdT, and occasional coexpression of lymphoid-associated antigens (CD7 or CD19) [1,3]. As for cytogenetic abnormalities, despite that the incidence of abnormal, complex, or unbalanced chromosomal changes has been reported to be more frequent, there are no recurrent or specific cytogenetic abnormalities in AML-M0 [3]. In AML, gene mutations not only have an implication in molecular

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pathogenesis but also provide a prognostic relevance in addition to the cytogenetic subtypes [4].

Previous studies have focused on class I and class II mutations in AML-M0 [5–8]. The development of AML was oftentimes caused by at least two-hit process mostly by class I and class II mutations. The class I mutation is defined by activating mutations of receptor tyrosine kinases and RAS signaling pathways, and the class II mutation is loss-of-function mutations of hematopoietic transcription factors [9]. *RUNX1* mutation was the most common gene mutation described in AML-M0 [5]. *FLT3* mutation was also reported as a recurrent gene mutation, whereas *RAS* and *PTPN11* mutations were less frequent in AML-M0 [6–8]. Other gene mutations with prognostic relevance have not been studied comprehensively in AML-M0, including mutated genes of epigenetic regulators, such as *IDH1*, *IDH2*, *TET2*, *DNMT3A*, *ASXL1*, and *EZH2* genes [10–13].

We thus examined a wide spectrum of gene mutations, including class I genes of activated signaling pathways (*FLT3*-ITD, *FLT3*-TKD, *C-FMS*, *KIT*, N-*RAS*, K-*RAS*, *PTPN11*, and *JAK2*^{V617F}), class II genes affecting hematopoietic transcription and differentiation (*RUNX1*, *NPM1*, and *CEBPα*), class III genes of epigenetic regulators (*IDH1*, *IDH2*, *TET2*, *DNMT3A*, *MLL*-PTD, *ASXL1*, and *EZH2*), and class IV genes of tumor suppressors (*WT1* and *TP53*) from the bone marrow cells of patients with AML-M0 at the initial diagnosis. The status of gene mutations was also correlated with the clinicohematological features to determine their clinical relevance in patients with AML-M0.

Materials and Methods

Patients and Materials

From 1991 to 2010, a total of 67 patients fulfilling the diagnostic criteria of *de novo* AML-M0 at Chang Gung Memorial Hospital and Mackay Memorial Hospital was enrolled. The diagnosis of AML-M0 was made according to the French-American-British criteria: >30% blasts in bone marrow, <3% of blasts positive for MPO or Sudan Black B, and expression of at least one myeloid antigen [1]. Patients with leukemia blasts expressing specific lymphoid markers (cytoCD3, cytoCD79a, or cytoCD22) were excluded in this study. G-banding method was used for karyotypic analysis, and results were interpreted according to the International System for Human Cytogenetic Nomenclature. Cytogenetic categorization of favorable-, intermediate-, and adverse-risk groups was accorded to the criteria recommended by European LeukemiaNet (ELN) Guidelines [4]. A panel of monoclonal antibodies including myeloid-associated antigens (CD13, CD33, CD11b, CD14, CD15, and/or CD41a), lymphoid-associated antigens (CD7, CD19, cytoCD3, and cytoCD22 or cytoCD79a if necessary), as well as lineage-nonspecific antigens (CD34, CD117, HLA-DR, TdT, or CD56) was used to determine the immunophenotypes of leukemia cells. The study was approved by the Institutional Review Boards of Chang Gung Memorial Hospital and Mackay Memorial Hospital.

Cell Fractionation

The mononuclear cells were obtained from bone marrow samples by Ficoll-Hypaque density gradient centrifugation (1.077 g/ml; Amersham Pharmacia, Buckinghamshire, United Kingdom). The mononuclear cells were then cryopreserved in medium containing 10% DMSO and 20% FBS at −70°C or in liquid nitrogen until test.

DNA, RNA Extraction, and cDNA Preparation

Genomic DNA was extracted from frozen mononuclear cells of bone marrow samples by using the QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. The TRIzol Reagent (Life Technology, Carlsbad, CA) was used to extract RNA that was reversely transcribed to cDNA with the Superscript II RNase H2 reverse transcriptase kit (Invitrogen Corporation, Carlsbad, CA).

Detection of Gene Mutations

FLT3-ITD, *FLT3*-TKD, *KIT*, *C-FMS*, K-*RAS*, N-*RAS*, *JAK*^{V617F}, *PTPN11*, *RUNX1*, *CEBPα*, *NPM1*, *TP53*, *WT1*, *IDH1*, *IDH2*, *TET2*, *DNMT3A*, *MLL*-PTD, *ASXL1*, and *EZH2* mutations were analyzed. The genomic DNA–polymerase chain reaction or reverse transcription–polymerase chain reaction assays followed by direct sequencing were used to detect *FLT3*-ITD [14], point mutations at tyrosine kinase domain of *FLT3* (*FLT3*-TKD) [15], *KIT* and C-*FMS* mutations [16], point mutations at codons 12, 13, and 61 in exons 1 and 2 of N-*RAS* and K-*RAS* genes [17], exons 3 to 8 of *RUNX1* mutations [18], *CEBPα* mutations [19], *MLL*-PTD [20], *PTPN11*, *TET2*, *IDH1*, *IDH2*, *DNMT3A*, and *ASXL1* [21] as previously described. The detection of mutated genes in *TP53*, exons 1 to 3 and exons 7 to 9 of *WT-1*, *JAK2*^{V617F}, and *NPM1* was performed according to the previously reported methods of other investigators with some modification [22–25]. The detection of *EZH2* mutation was carried out using a self-designed and/or previously reported method, which was described in detail in the Supplementary Materials (Tables W1–W3 and Figure W1).

Statistical Analysis

Fisher exact test, χ^2 analysis, and Wilcoxon rank-sum test were used whenever appropriate to make comparisons between groups. Estimates of survival were calculated according to the Kaplan-Meier method. Comparisons of estimated survival curves were analyzed by the log-rank test. For multivariate analysis, a Cox regression model was used to identify prognostic variables. Variables with *P* values of .2 or less in univariate analysis were included in the model. In all analysis, the *P* values were two-sided and considered statistically significant when values were lower than .05. Statistical analysis was carried out by SPSS version 17.0 (SPSS Inc, Chicago, IL).

Results

Patient Characteristics

The baseline characteristics of the 67 patients with AML-M0 are listed in the Supplemental Materials (Table W4). The median age was 49.0 years (range 0.3-97.9 years). Twenty-two of the 67 (32.8%) patients were female. The estimated median overall survival (OS) was 5.1 months (Figure W2*A*). Thirty-nine patients received standard AML induction protocol (60 mg/m^2 daunomycin for 3 days and 150 mg/m^2 cytarabine for 7 days for adults and TPOG-AML97A for children with an age younger than 18 years) [26]. Six patients (age 64.3-81.2 years) received low dose therapy including low dose cytarabine, hydroxyurea, or melphalan. Seventeen patients with a median age of 73.1 years old received supportive care only. Five (7.5%) patients were treated as acute lymphoblastic leukemia. Of the 39 patients who received standard AML therapy, 7 had early death during the induction therapy and 29 achieved complete remission yet with a relapse rate of 72.4% (21/29). Three patients from those who

relapsed underwent hematopoietic stem cell transplantation (HSCT) after second complete remission. The median OS of the 39 patients who received chemotherapy was 11.1 months (Figure W2*B*). The median follow-up time for this cohort was 4.8 months (range 0-176.6 months). Fifty-nine patients died and eight were still alive.

Frequency and Types of Mutations in AML-M0

Of the 67 patients with *de novo* AML-M0, 79.1% (53/67) patients were found to have at least one gene mutation among the genes examined. The diagram of the gene mutation status in patients with AML-M0 is illustrated in Figure 1. The pairwise cooperativeness between gene mutations is depicted in Figure 2.

Class I mutations. *FLT3*-ITD mutations were detected in 22.4% (15/67) and *FLT3*-TKD in 6.0% (4/67) patients. *RAS* mutations were present in 12.3% (8/65) patients, with three K-*RAS* and five N-*RAS* mutations. The frequency of *PTPN11*, *C-FMS*, and *JAK2*^{V617F} mutations were 6.2% (4/65), 1.8% (1/56), and 1.6% (1/64), respectively. None had *KIT* mutation.

Class II mutation. A total of 17 *RUNX1* mutations was detected in 23.9% (16/67) patients, including two nonsense mutations, seven frameshift, seven missense mutations, and one insertion mutation. One patient had *CEBPα* and *NPM1* mutations each.

Class III mutations. *IDH* mutations were present in 28.8% (19/66) patients. All *IDH* mutations were missense mutations, with *IDH1*-R132 in five, *IDH2*-R140 in five, and *IDH2*-R172 in nine patients. *TET2*, *DNMT3A*, *MLL*-PTD, and *ASXL1* mutations were detected less frequently, occurring in 8.2% (5/61), 8.1% (5/62), 7.8% (5/64), and 6.3% (4/64) patients, respectively. *EZH2* mutation was detected in only 1 of 60 patients examined.

Class IV mutations. *TP53* and *WT1* occurred in 7.8% (5/64) and 4.8% (3/63) patients, respectively.

On the basis of the functional class of gene groups, 49.3% (33/67) patients had class I mutations that were mutually exclusive, 26.9% (18/67) had class II, 43.9% (29/66) had class III, and 12.5% (8/64) had class IV mutations. Twenty-four patients had gene mutations within a single class, with 12 in class I, 4 in class II, 7 in class III, and 1 in class IV.

Karyotypes and the Association with Gene Mutations

Cytogenetic data were available for 49 patients. Twenty-nine (59.2%) patients had normal karyotypes, 7 (14.3%) patients had a single abnormality, 3 (6.1%) patients had two abnormalities, and 10 (20.4%) patients had complex cytogenetic aberrations. Monosomal karyotypes were found in eight (16.3%) patients. Trisomy 21, trisomy 8, monosomy 8, and del(5q) were found in four, three, three, and two patients, respectively. Only one patient with isolated trisomy 13 had concomitant *RUNX1* mutation. Thirty-eight patients (77.6%) were in the intermediate-risk group, 11 (22.4%) patients in the unfavorable-risk group, and none had favorable cytogenetics by the ELN criteria. All gene mutations examined were not associated with specific ELN cytogenetic risk groups or normal karyotypes.

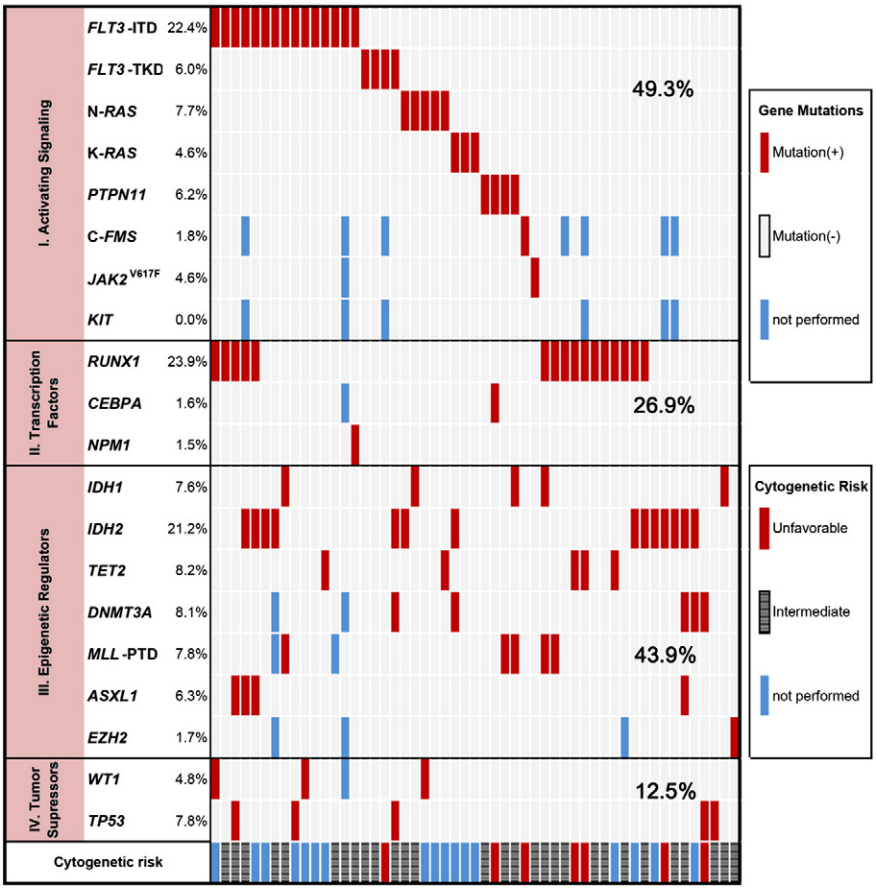

Figure 1. Diagram of AML-M0 patients with gene mutations. The gene mutation status, cooperating mutations, and cytogenetic risk groups in AML-M0 patients at the initial diagnosis are illustrated.

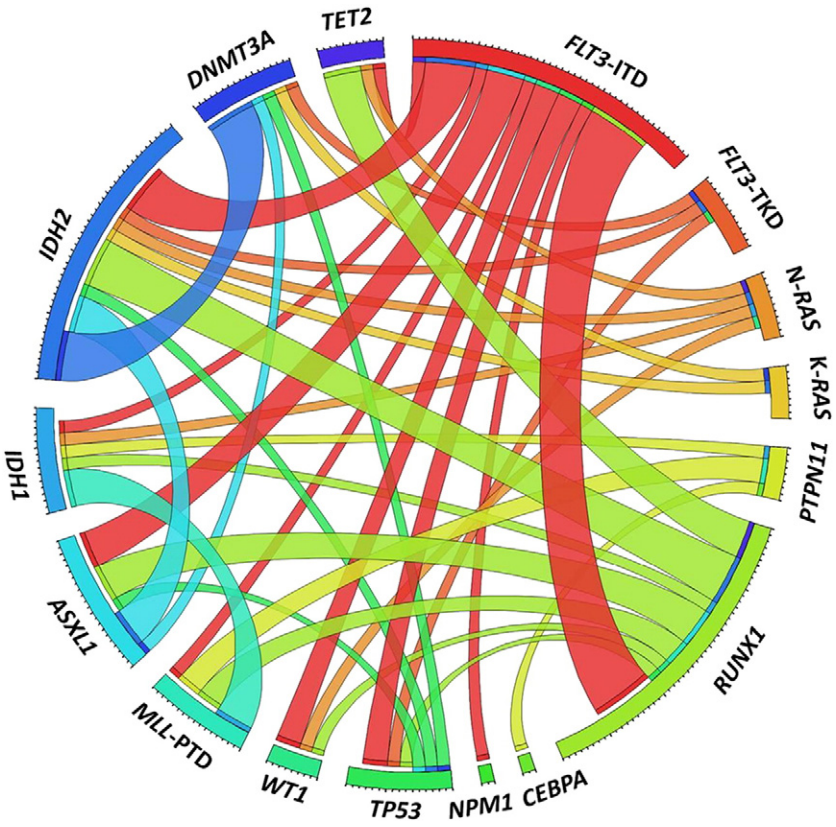

Figure 2. Pairwise cooperativeness between gene mutations in AML-M0 patients. A circus diagram depicts the pairwise cooperativeness of gene mutation in AML-M0 patients. The length of the arc represents the number of mutations in the first gene, and the width of the ribbon represents the number of patients with a mutation in the second gene.

Correlations of Gene Mutations with Clinicohematological Features of Patients with AML-M0

We correlated gene mutations with clinical parameters, including age, sex, hemoglobin level, platelet counts, white blood cell counts, percentages of circulating blasts and marrow blasts, and cytogenetic risk groups (Table 1). *RUNX1* and *IDH* mutations were significantly associated with older age (median 68.0 *vs* 46.7 years for *RUNX1*, $P = .011$; 66.0 *vs* 44.1 for *IDH*, $P = .016$). Patients with *MLL*-PTD were younger than those without *MLL*-PTD (37.0 *vs* 54.0, $P = .044$). *ASXL1* and *DNMT3A* mutations were associated with lower WBC counts (2.1×10^9/l *vs* 15.6×10^9/l for *ASXL1*-mutated and wild-type, $P = .021$; 2.3×10^9/l *vs* 15.9×10^9/l for *DNMT3A*-mutated and wild-

Table 1. Clinical Characteristics of AML-M0 Patients with *RUNX1*, *FLT3*, *IDH*, and *RAS* Mutations.

	RUNX1 ($n = 67$)			*FLT3*-ITD or *FLT3*-TKD ($n = 67$)			*IDH1*/*IDH2* ($n = 66$)			N-*RAS*/K-*RAS* ($n = 65$)		
	Mutation	Wild type	*P*	Mutation	Wild type	*P*	Mutation	Wild type	*P*	Mutation	Wild type	*P*
Number of patients	16	51		19	48		19	47		8	57	
Age (years)	68.0 (5.5-97.9)	46.7 (0.3-84.2)	.011	46.7 (11.0-75.8)	54.3 (0.3-97.9)	.461	66.0 (5.5-84.2)	44.1 (0.3-97.9)	.016	49.1 (16.0-73.5)	49.0 (0.3-97.9)	.905
Sex (male/female)	4/12	18/33	.550	7/12	15/33	.660	7/12	15/32	.701	3/5	19/38	1.000
Hemoglobin (g/l)	77 (40-110)	74 (38-124)	.600	68 (40-117)	77 (38-124)	.519	75 (40-121)	75 (38-124)	.756	89 (38-124)	75 (40-127)	.340
Platelet ($\times 10^9$/l)	4.5 (0.8-13.9)	3.2 (0.1-59.8)	.394	4.4 (0.8-13.5)	3.0 (0.2-59.8)	.681	3.3 (0.1-19.9)	3.0 (0.2-59.8)	.851	2.8 (.2-59.8)	3.3 (0.1-23.6)	.761
WBC ($\times 10^9$/l)	22.9 (1.1-187.2)	10.7 (0.6-397.8)	.595	15.9 (0.6-204.6)	13.0 (0.7-379.2)	.761	5.4 (0.6-63.6)	15.2 (0.7-379.2)	.184	31.5 (5.4-156.6)	10.7 (0.6-379.2)	.123
Circulating blasts (%)	61.8 (0-98.2)	53.0 (0-99.3)	.899	63 (1-98.0)	53.5 (0-99.3)	.882	46.0(0-98.0)	55.0 (0-99.3)	.817	88.0 (30.0-98.5)	49.3 (0-99.3)	.012
Marrow blasts (%)	88.4 (76.0-99.0)	90.1 (29.4-98.8)	.857	91.8 (72.2-99.0)	88.5 (29.4-98.4)	.557	88.2 (43.5-95.3)	89.4 (29.4-99.0)	.954	94.6 (69.2-96.0)	88.9 (29.4-99.0)	.104
Karyotype			.708			.252			.246			1.000
Favorable	0	0		0	0		0	0		0	0	
Intermediate	10	28		11	27		12	26		3	35	
Poor	2	9		1	10		1	10		1	9	
Trisomy 13	1	0		0	1		0	1		0	1	
Monosomy	1	7		2	6		1	7		0	8	
EFS[†]	1.9 (0-5.2)	2.2 (0-5.5)	.133	5.3 (4.1-6.5)	1.9 (0.8-3.0)	.207	1.0 (0.5-1.5)	3.0 (0-6.1)	.863	1.0 (0.7-1.3)	3.0 (0-6.0)	.139
OS[†]	2.6 (0-6.2)	5.3 (0-12.8)	.168	15.2 (6.8-23.6)	3.0 (0.2-5.8)	.171	5.1 (0.4-9.8)	5.3 (0-13.2)	.897	1.0 (0-3.1)	6.3 (0.8-11.8)	.096

Values are expressed as medians (range).

[†] Values are expressed as medians (95% CI).

type, $P = .032$). *RAS* mutations were associated with higher percentage of circuiting blasts (88.0% *vs* 49.3%, $P = .012$). Other gene mutations, including *FLT3* mutations, had no correlation with any clinical features.

Outcome Analysis

We assessed the impact of cytogenetic risk group and *FLT3*, *IDH*, *RUNX1*, *RAS*, *ASXL1*, *DNMT3A*, *TET2*, and *MLL*-PTD mutations on OS and event-free survival (EFS; Table 2). Less prevalent gene mutations were excluded from statistical analysis. Cytogenetics had prognostic significance, with a median OS of 13.8 months [95% confidence interval (CI) 3.2-24.4 months] and 4.4 months (95% CI 1.6-7.2 months) for intermediate- and unfavorable-risk groups, respectively ($P = .043$). The mutational status of *FLT3* ($P = .171$), *IDH* ($P = .897$), *RUNX1* ($P = .168$), *RAS* ($P = .096$), *ASXL1* ($P = .760$), *TET2* ($P = .076$), *DNMT3A* ($P = .996$), or *MLL*-PTD ($P = 0.247$) did not have significant influence on OS or EFS by the log rank test (Figure W3). When the gene mutations were categorized according to their functional class groups, no impact on OS or EFS was observed with regard to the functional class groups.

To assess whether the gene mutations had independent prognostic values in patients with AML-M0 in context of other clinical and molecular parameters, we performed multivariate analysis for *FLT3*, *RUNX1*, *RAS*, *TP53*, and *TET2* mutations adjusting for age, platelet counts, percentage of marrow blasts, and/or ELN cytogenetic risk groups (Table 2). The result showed that age [hazard ratio (HR) 1.029, 95% CI 1.013-1.044, $P = .001$] was the most significant adverse factor for OS, and patients with *RUNX1* mutation (HR 2.326, 95% CI 0.978-5.533, $P = .056$) had a trend toward an inferior survival. For EFS, age (HR 1.016, 95% CI 1.000-1.033, $P = .055$) was the independent unfavorable risk factor after adjusting for cytogenetic risk group, platelet counts, *RAS*, *MLL*-PTD, *TET2*, and *TP53* gene mutation status.

Discussion

Previous studies reporting the clinical features or gene mutation patterns in patients with AML-M0 were mainly from the western population and a few from the Japanese population [6]. In this study, we examined 20 gene mutations in 67 patients with AML-M0 from the Taiwanese ethnicity, and it showed a wider range of gene mutations in AML-M0 compared to the western and other Asian population. It has been described that *RUNX1* mutations were common in AML-M0 with reported frequencies ranging from 12.7% to 46% [5,6,27,28] and up to 65.4% in those with normal karyotypes [29]. Another common mutation in patients with AML-M0 was *FLT3*-ITD, with a frequency of 22% to 29% [6,7]. We found that *FLT3*-ITD, *RUNX1*, *IDH1*, *IDH2*, and *RAS* were the recurrent mutations with frequencies between 10% and 30% in our series, and our results of *FLT3-ITD* and *RUNX1* mutations were mostly in line with previous studies. However, the frequency of *RUNX1* mutation seemed to be slightly higher in our cohort compared to the Japanese population (23.9% *vs* 15.7%) [6]. The frequency of *RAS* (12.3%) and *PTPN11* (6.2 %) mutations was slightly higher in our series compared with the study of Roumier et al. [7], which showed that *RAS* and *PTPN11* mutations occurred less frequently in patients with AML-M0. Of note, we found a high occurrence of *IDH2* (21.2%), which has not been previously described. Other gene mutations of epigenetic regulators including *TET2*, *DNMT3A*, *MLL*-PTD, and *ASXL1* mutations occurred less frequently and *EZH2* mutation was rare. If we took the gene functional groups into consideration, approximately half of the patients with AML-M0 had class I gene mutations (49.3%) and class II gene mutations involving hematopoietic differentiation occurred in one fourth of patients, suggesting that the receptor tyrosine kinase/RAS signaling was the most important pathway involved in AML-M0, having *FLT3* mutations being the main causal factor. Mutations of epigenetic regulator genes (class III) detected in about 40% of patients also played an important role in the leukemogenesis of AML-M0.

In univariate analysis, age was the most significant adverse factor for OS and EFS. Unfavorable cytogenetic subgroup also conferred a poor risk factor for OS. Most of the genes with high occurrence in the present series have been reported to be poor prognostic molecular markers in patients with AML, especially in the cytogenetically normal group, including *FLT3*-ITD [30,31], *IDH2* [12,32], and

Table 2. Univariate and Multivariate Analyses with Respect to EFS and OS.

	EFS						OS					
	Univariate			Multivariate			Univariate			Multivariate		
	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
Age	1.015	1.005-1.024	.003	1.016	1.000-1.033	.055	1.019	1.008-1.029	<.001	1.029	1.013-1.044	.001
Karyotype risk	1.613	0.799-3.253	.182	1.250	0.515-3.036	.622	2.059	1.004-4.224	.049	1.735	0.712-4.227	.225
Platelet counts	0.976	0.941-1.012	.191	0.967	0.894-1.047	.413	0.969	0.930-1.009	.124	0.923	0.840-1.014	.093
Marrow blasts (%)	1.007	0.991-1.024	.392				1.015	0.997-1.033	.108	1.001	0.980-1.022	.956
FLT3 mutation	0.693	0.389-1.234	.213				0.665	0.369-1.201	.176	0.414	0.163-1.055	.065
RUNX1 mutation	1.549	0.867-2.768	.140	2.365	0.946-5.915	.066	1.508	0.835-2.724	.173	2.326	0.978-5.533	.056
IDH mutation	0.950	0.531-1.702	.864				0.963	0.540-1.716	.897			
RAS mutation	1.767	0.816-3.824	.149	2.405	0.679-8.518	.174	1.893	0.877-4.087	.104	1.728	0.500-5.975	.388
MLL-PTD	0.492	0.177-1.366	.173	0.525	0.143-1.921	.300	0.553	0.199-1.536	.256			
ASXL1 mutation	0.898	0.279-2.888	.857				0.835	0.260-2.681	.762			
DNMT3A mutation	1.375	0.425-4.442	.595				1.002	0.399-2.518	.996			
P53 mutation	2.106	0.747-5.936	.159	0.942	0.263-3.373	.927	1.762	0.693-4.481	.234			
PTPN11 mutation	0.61	0.190-1.960	.407				0.707	0.221-2.265	.559			
TET2 mutation	0.445	0.174-1.142	.092	1.183	0.297-4.716	.812	0.437	0.169-1.128	.087	1.308	0.314-5.441	.712
Class I mutation	0.797	0.481-1.320	.378				0.714	0.426-1.196	.200			
Class II mutation	1.492	0.850-2.617	.163				1.447	0.819-2.557	.204			
Class III mutation	1.063	0.639-1.769	.813				1.150	0.686-1.927	.595			
Class IV mutation	1.985	0.884-4.456	.097				1.712	0.804-3.645	.163			

RUNX1 mutations [29,33]. However, we did not observe such correlation in our patients. *NPM1* mutation occurred in about 50% of AML patients with normal karyotypes and was associated with a favorable survival [34,35]. About 60% of the patients had normal karyotypes, but only one patient had *NPM1* mutation in our study. The poor outcome of our AML-M0 patients might be attributed partly to the near absence of *NPM1* mutation along with the high occurrence of *FLT3*-ITD, which is a poor prognostic factor in normal karyotype AML. The lack of prognostic impact of *FLT3* mutations in the present study might be attributed to the limited number of AML-M0 patients with general short survival, which avert a confirmed conclusion. *IDH2* had a higher occurrence than *IDH1* in AML, but the location of *IDH2* mutation was more frequent on R140 than on R172 in the previous studies, in which M0 accounted for less than 5% of the studied population [12,32,36–38]. Interestingly, we observed that *IDH2*-R172 occurred more frequently than *IDH2*-R140 or *IDH1*-R132 in the present AML-M0 series. *IDH2* mutation on R172 has been reported to confer a poorer outcome compared to *IDH2*-R140 or *IDH1*-R132 in AML [36,38,39]. The number of each subtype of *IDH* mutants was very small to make a meaningful statistic analysis. Findings from the multivariate analysis showed consistency with the previous study on that *RUNX1* mutation was the poor prognostic factor for OS and EFS [29].

Approximately half of the patients had multiple cooperating gene mutations. More than 80% of patients harboring mutations of epigenetic regulator genes had other coexisted gene mutations. Although *ASXL1* mutations occurred in only four patients with AML-M0, remarkably, three of them were associated with both *RUNX1* and *FLT3*-ITD mutations. Likewise, all of the five patients with *MLL*-PTD carried other mutated genes. Our findings supported a multiple-hit model of leukemogenesis in AML [40]. Previous studies have shown that *RUNX1* mutations frequently coexisted with *FLT3*-ITD or *FLT3*-TKD [6], trisomy 13 (the locus of the *FLT3* gene), or *FLT3* overexpression [27–29]. We found only one patient harboring both *RUNX1* mutation and trisomy 13 and five patients with coexisting *RUNX1* and *FLT3*-ITD mutations. The underlying mechanism of their cooperating roles in leukemogenesis merits further investigation.

Most AML-M0 patients succumb to the disease despite current standard treatment. For an aggressive AML subtype as AML-M0, having HSCT early in the course of the disease had improved the outcome in some small series [41,42]. We did not observe a significantly prolonged survival in AML-M0 patients who received HSCT in this study. The small number of patients who received HSCT and the delayed timing for HSCT in our cohort may effectuate the lack of significant benefit of HSCT in our patients. Complete gene mutation profiling for AML-M0 patients at the diagnosis may further help in identifying biologic risk factors and potential therapeutic targets. The relevance of gene mutations in predicting therapeutic response of myeloid malignancies to targeted agents has not been well established. Flt3 antagonists combined with chemotherapy or hypomethylating agents have been reported to have impact on response rates in a phase II study [43]. Potential links between gene mutations and the treatment responses to hypomethylating agents have been reported in a few small series. Itzykson et al. reported that patients with mutated *TET2* and favorable cytogenetics had a higher response rate to azacitidine compared to patients with wild-type *TET2* in myelodysplastic syndromes and low blast count AML [44]. Metzeler et al. reported a higher complete remission rate in AML patients with *DNMT3A* mutations treated with decitabine [45]. *TET2* and/or *DNMT3A* mutations were found to be independent response predictor to methyltransferase inhibitors in patients with myelodysplastic syndrome or its related disorders [46]. However, these results have not been confirmed in randomized clinical trials. IDH also serves as a potential therapeutic target in AML. Chaturvedi et al. recently reported that a small molecular inhibitor targeting mutant IDH1 induced apoptosis and decreased colony formation in methylcellulose of IDH1-mutant human primary bone marrow cells [47]. Our study reveals the complex and heterogeneous molecular aberrations in AML-M0. As coexistence of gene mutations occurs frequently, combined therapy through multiple targeting might be the reasonable approach in future studies.

In summary, we analyzed a broad spectrum of known mutated genes involved in myeloid neoplasms from 67 patients with *de novo* AML-M0 at diagnosis. To the best of our knowledge, this is the most comprehensive study regarding the gene mutation patterns in AML-M0. We found that AML-M0 was characterized by high frequency of *FLT3*-ITD, *RUNX1*, and *IDH* mutations. In addition to the signaling pathway, we further demonstrated that AML-M0 was frequently associated with mutations of epigenetic regulator genes, occurring in more than 40% of patients. Other than old age, *RUNX1* mutation was associated with a trend of inferior survival by multivariate analysis, while *FLT3*, *IDH*, and other gene mutations did not have impact on the outcome. Cooperation of multiple mutations in different classes of genes was common. Our findings suggested that AML-M0 is a complex and heterogeneous subtype of AML in terms of molecular aberrations. The high frequency of gene mutations in epigenetic modifiers implies that epigenetic deregulation, which frequently cooperates with other gene mutations, may play an important role in the pathogenesis of AML-M0. Further studies on how epigenetic regulator mutations interact with mutated genes affecting cell proliferation and/or differentiation in AML-M0 are warranted. Our findings also suggest the potential implication of combined therapies with targeted agents in this rare and unfavorable subtype of AML.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2014.06.002.

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