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Original Article

Mutations of Epigenetic Modifier Genes as a Poor Prognostic Factor in Acute Promyelocytic Leukemia Under Treatment With All-Trans Retinoic Acid and Arsenic Trioxide

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ABSTRACT

Background: Acute promyelocytic leukemia (APL) is a model for synergistic target cancer therapy using all-trans retinoic acid (ATRA) and arsenic trioxide (ATO), which yields a very high 5-year overall survival (OS) rate of 85 to 90%. Nevertheless, about 15% of APL patients still get early death or relapse. We performed this study to address the possible impact of additional gene mutations on the outcome of APL.
Methods: We included a consecutive series of 266 cases as training group, and then validated the results in a testing group of 269 patients to investigate the potential prognostic gene mutations, including *FLT3*-ITD or -TKD, *N-RAS*, *C-KIT*, *NPM1*, *CEPBA*, *WT1*, *ASXL1*, *DNMT3A*, *MLL* (fusions and PTD), *IDH1*, *IDH2* and *TET2*.
Results: More high-risk patients (50.4%) carried additional mutations, as compared with intermediate- and low-risk ones. The mutations of epigenetic modifier genes were associated with poor prognosis in terms of disease-free survival in both training (HR = 6.761, 95% CI 2.179–20.984; P = 0.001) and validation (HR = 4.026, 95% CI 1.089–14.878; P = 0.037) groups. Sanz risk stratification was associated with CR induction and OS.
Conclusion: In an era of ATRA/ATO treatment, both molecular markers and clinical parameter based stratification systems should be used as prognostic factors for APL.
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1. Introduction

Acute promyelocytic leukemia (APL) is a distinct subtype of acute myeloid leukemia with typical chromosomal translocation t(15; 17), which results in the formation of leukemogenic chimerical gene *PML–RARα*. The PML–RARα oncoproteins exert a dominant-negative effect on their normal counterparts, PML and RARα, which are involved in the regulation of the physiological processes of myeloid differentiation, apoptosis and DNA replication/repair (Chen et al., 1994; Degos et al., 1995; Fenaux et al., 1994; Huang et al., 1988; Niu et al., 1999; Villa et al., 2007; Wang et al., 2010; Zhu et al., 2007). Bleeding tendency caused by either hypofibrinogenemia or disseminated intravascular coagulation (DIC) and accumulation of promyelocytes in the bone marrow (BM) are common clinical features (Chen et al., 1994; Degos et al., 1995; de la Serna et al., 2008; Tallman and Altman, 2009).

The revolutionary introduction of all-trans retinoic acid (ATRA) has significantly changed the treatment paradigm of APL (Huang et al., 1988; Muindi et al., 1992; Sanz and Lo-Coco, 2011). The terminal differentiation of APL cells upon effect of ATRA, instead of cytolysis by conventional chemotherapy, ameliorates the coagulopathy of the disease and increases the complete remission (CR) rate of induction (Tallman and Altman, 2009; Muindi et al., 1992; Sanz and Lo-Coco, 2011; Zhu et al., 1999). Currently, ATRA based treatment has become the standard care of the APL which significantly prolongs disease-free survival (DFS) and overall survival (OS) (Fenaux et al., 1994; Avvisati et al., 1996; De Botton et al., 1998; Estey et al., 1999, 2005; Hu et al., 2009; Shen et al., 2004). Since the 1990s, arsenic trioxide (ATO) has yielded breakthroughs in treating both relapsed and newly diagnosed APL patients and has been shown to induce differentiation and apoptosis of APL cells. Mechanistic exploration proved that ATRA and ATO exert direct

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effects on distinct functional domains of the same PML–RARα oncoprotein. While ATRA targets the ligand-binding domain (LBD) of RARα moiety, arsenic binds to adjacent cysteine residues located within the RING, B Boxes and coiled coil (RBCC) domain of PML. Both agents induce degradation of the fusion protein. Interestingly, arsenic binding results in an oligomerization of PML–RARα and increases its interaction with the small ubiquitin-like protein modifier (SUMO)-conjugating enzyme UBC9, leading to enhance SUMOylation. The sumoylated fusion protein on its turn recruits the ubiquitin E3 ligase RNF4 and undergoes proteasome-mediated degradation (Chen et al., 1996; Jeanne et al., 2010; Rajendra et al., 2012; Zhang et al., 2010; Zhu et al., 1997). In addition, leukemia-initiating cells (LICs), which are responsible for relapse of leukemia, could be cleared by ATRA/ATO based treatment in murine models, providing further rationale of combined use of the two agents (Au et al., 2001; Ito et al., 2008; Nasr et al., 2008, 2010; Zhou et al., 2010). The potential synergic effect of ATRA and ATO owing to different modes of action represents thus a strong impetus for the trial of combination of ATRA and ATO in the newly diagnosed APL. Indeed, ATRA/ATO combination as first line induction therapy with chemotherapy as consolidation has not only brought a shortened time to achieve CR but more importantly reduced disease burden and substantially enhanced survival as compared to patients treated with each of the two agents in an initial randomized trial at the Shanghai Institute of Hematology (SIH) (Hu et al., 2009; Shen et al., 2004). Then in the extended arm of ATRA/ATO/chemotherapy combination for 85 APL patients, remarkable 5-year event-free survival (EFS) and OS rates of 89.2% ± 3.4% and 91.7% ± 3.0%, respectively, were achieved (Hu et al., 2009). The results were then confirmed by several hematology/oncology centers in Western countries. Lo-Coco et al., 2013 recently reported that by using ATRA and ATO alone in the treatment of newly diagnosed low-to-intermediate-risk patients, the CR rate could be as high as 100% with a 2 year EFS of 97% (Lo-Coco et al., 2013).

Laboratory and clinical evidence strongly suggests that mutations of LBD of RARα and B box2 of PML in *PML–RARα* are responsible for resistance to ATRA and ATO, respectively (Jeanne et al., 2010; Zhang et al., 2010; Goto et al., 2011). However, these mutations generally occur at the time of relapse. Previously we and others observed that at disease presentation, some other molecular events could co-exist with *PML–RARα* such as mutations of *NRAS*, *KRAS*, FMS-like tyrosine kinase 3 [with internal tandem duplication (*FLT3*-ITD) or *FLT3* point mutations (*FLT3*-TKD)] and *JAK1*. In APL patients treated with ATRA and chemotherapy, these mutations might exert potential impact on the biological behavior, clinical manifestation and treatment outcome (Chan et al., 2006; Rampal and Levine, 2011; Shen et al., 2011). More recently, when new genetic markers such as *DNMT3A* and *IDH1*/*2* were used for mutation screening in APL and genomic data of some APL cases were available (Shen et al., 2011; Wang et al., 2013), it was revealed that still other gene mutations could exist and be related to disease outcome.

In this study, we have investigated the gene mutational status of APL in addition to *PML–RARα* to clarify their potential biological and clinical impact among patients treated with ATRA/ATO based protocol in an attempt to further improve the stratification system of APL.

2. Methods

2.1. Patients and Treatment

A total of 535 newly diagnosed APL patients were enrolled from February 2001 to February 2014, whose samples were sent to SIH for molecular assessment. The training group consisted of 266 patients who were treated by Shanghai APL regimen with ATRA/ATO based protocol in SIH affiliated centers (Hu et al., 2009), during February 2001 and March 2008. The validation group consisted of 269 patients in three multi-center clinical trials from SIH, Zhejiang Institute of Hematology (ZIH) and Peking University Institute of Hematology (Zhu et al., 2013). All cases in these three groups were treated with the same ATRA/ATO based protocol as in the training group. The detailed treatment protocols are seen in Supplementary Fig. 1.

The diagnosis of the disease was established according to the nomenclature of WHO for leukemia. Cytogenetic and molecular examinations of t(15;17) and/or *PML–RARα* were performed as previously described. Based on the levels of initial WBC and platelets by Sanz risk stratification, the patients could be classified into 3 groups: high-risk, WBC above 10×10^9/L; low-risk, WBC below 10×10^9/L and platelet more than 40×10^9/L, and intermediate-risk, all the remaining cases, respectively (Sanz et al., 2000).

This study was approved by the Institutional Review Board (IRB) of all participating hospitals. All patients were given informed consent for both treatment and cryopreservation of bone marrow (BM) and peripheral blood (PB) according to the Declaration of Helsinki.

2.2. Molecular Genetic Analysis

Genomic DNA and total RNA were extracted as previously reported. Regular RT-PCR of 3 distinct [long (L), short (S), and variant (V)] types of *PML–RARα* transcripts were analyzed as described previously (Dicker et al., 2011).

Gene mutational status analysis was performed in all patients. A series of gene markers were chosen based on our previous study on a group of 388 APL patients (Shen et al., 2011) and on detailed analysis of the genomic data in the literature (Cancer Genome Atlas Research N, 2013). Mutations including *FLT3*-ITD or -TKD, *N-RAS*, *C-KIT*, *NPM1*, *CEPBA*, *WT1*, *ASXL1*, *DNMT3A*, *MLL*, *IDH1*, *IDH2* and *TET2* genes were assessed by distinct approaches. A chip-based matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS) analysis system (iPLEXTM, Sequenom, San Diego, CA, USA) was used to analyze the mutational status of *FLT3*-TKD, *N-RAS*, *NPM1*, and whole gene sequencing was applied to investigate the mutations of *FLT3*-ITD, *C-KIT*, *CEPBA*, *WT1*, *ASXL1*, *DNMT3A*, *IDH1*, *IDH2* and *TET2* genes (Shen et al., 2011; Bashey et al., 1992; Bowen et al., 2005; Damm et al., 2011). Six *MLL*-related common fusion genes including *MLL-AF9*, *MLL-AF10*, *MLL-AF6*, *MLL-ELL*, *MLL-ENL* and *MLL-AF17* were detected via a multiplex RT-PCR strategy (Dicker et al., 2011). The mutational status of *MLL*-PTD was determined by RT-PCR technique.

A RT-PCR was performed to detect mutations of *PML–RARα* in relapsed patients. 1 μg of total RNA was reversely transcribed to cDNA using M-MLV reverse transcriptase kit (Invitrogen). Then r-Taq polymerase kit (Takara) was used to perform PCR and amplified PCR fragments were subjected to Sanger sequencing. Since recent studies had revealed the enrichment of *PML* mutations in the RBCC B2 domain and of *RARα* mutations in LBD domain (Goto et al., 2011), the primers for PCR amplification of these two domains were designed as follows: PML-RBCC-Forward 5′-CAGATTGTGGATGCGCAGGC and PML-RBCC-Reverse 5′-CAGCCGACTGGCCATCTCCT;RARA-LBD-Forward5′-GAAGAA GAAGGAGGTGCCCAandRARA-LBD-Reverse 5′-CATCTTCAGCGTGATCAC CC. The PCR was carried out in the reaction mixtures according to the provided protocol of rTaq and amplification was under the following conditions: 95 °C for 5 min of one cycle, followed by 35 cycles of 30 s at 95 °C, 40 s at 58 °C and 50 s at 72 °C, and a final stage of 5 min at 72 °C. The resulting data of Sanger sequencing were analyzed by software Chromas lite (Technelysium).

2.3. Statistical Analyses

Fisher's exact P test was used to compare the difference of CR rates which was defined according to the criteria of International Working Group (Cheson et al., 2003). One way Anova test was used to compare the age, WBC count and BM blasts at diagnosis in different groups. OS was measured from the date of disease diagnosis to death (failure) or alive at the last follow-up (censored). DFS was defined as the duration from the documentation of CR to treatment failure such as relapse, refractory disease, death, or alive in CR at the last follow-up (censored)

Table 1
Clinical characteristics of the patients.

	Training group (n = 266)	Validation group (n = 269)
Age (years)		
Mean	36.8 ± 16.0	39.6 ± 13.2
Range	14–77	14–76
Gender		
Male	136 (51.2)	140 (52.0)
Female	130 (48.8)	129 (48.0)
WBC count, $\times 10^9$/L		
Median	2.8	2.5
Range	0.3–205.7	0.3–122.2
Risk stratification		
High	57 (21.4)	74 (27.5)
Intermediate	131 (49.2)	117 (43.5)
Low	78 (29.3)	78 (20.0)
FLT3		
Mutated	34 (12.8)	50 (18.9)
ITD	22 (64.7)	35 (70.0)
TKD	12 (35.3)	15 (30.0)
Not mutated	232 (87.2)	215 (81.1)
Failed	0	4
RAS		
Mutated	14 (5.4)	9 (3.9)
Not mutated	244 (94.6)	242 (96.1)
Failed	8	18
WT1		
Mutated	11 (4.4)	13 (4.9)
Not mutated	238 (95.6)	253 (95.1)
Failed	17	3
EMG (%)		
Mutated	14 (5.3)	21 (7.8)
Not mutated	224 (94.7)	219 (92.2)
Failed	28	29

WBC, white blood cell; BM, bone marrow; and n, number of patients.
"Failed" refers to the samples without detection results due to quality problems.
EMG refers to a group of epigenetic modifier genes investigated in this work.

(Cheson et al., 2003; Creutzig and Kaspers, 2004). Kaplan–Meier and hazard ratio analyses were used to calculate and compare the OS and DFS, respectively. Cox model was used for the multivariate analysis of associations of survival and potential prognostic factors. A limited backward selection procedure was used to exclude redundant variates (Gill, 1992). The above statistical procedures were all performed with the SPSS statistical software package, version 16.0.

3. Results

3.1. Characteristics of Patients

The demographic data of 535 APL patients in both training (266 cases) and validation (269 cases) groups were depicted in Table 1. The clinical variables including age, gender, and median WBC count were similar between the two cohorts. The validation group seemed to contain more *FLT3* mutation cases and high-risk patients. However, the frequency of ITD and TKD in training and validation groups (P = 0.641) was similar.

3.2. Molecular Aspects of the Patients

In the pooled data of 535 patients, *FLT3*-ITD or -TKD (84/531, 15.8%), *N-RAS* (23/509, 4.5%) and *WT-1* (24/515, 4.7%) mutations were identified as the three most common additional gene mutations. Notably, mutations of epigenetic modifier genes (EMGs), such as *DNMT3A* (0.3%), *TET2* (4.5%), *IDH1* (0.4%), *IDH2* (0.2%) and *ASXL1* (1.6%), could co-exist with *PML–RARα* fusion, whereas no PTD mutation or fusion of *MLL* was noted in any cases, suggesting a mutual exclusivity between *MLL* abnormalities and the *PML–RARα* characteristic of APL. Put together, 35 (6.5%) out of 535 patients harbored EMG mutations.

As shown in Table 2, the *FLT3*-ITD/TKD mutations and *N-RAS* mutations were associated with a phenotype of high WBC count (P < 0.001 and P = 0.001, respectively), whereas *FLT3* mutations also showed a male tendency (P = 0.012). When the possible relationships between distinct gene mutations were analyzed, 164 patients (30.7%) carried at least one of the above mentioned mutations, while 18 patients (3.4%) carried double or triple additional mutations. EMG mutations tended to co-exist with other mutations. Among 18 patients with two or three gene mutations, 11 (61.1%) cases contained EMG abnormalities (P < 0.001).

The correlations between additional mutations and clinical risk groups were addressed (Fig. 1). Of note, over half of patients in high-risk group carried these mutations (66/131, 50.4%), significantly higher than those in the intermediate- (62/248, 25.0%) and low-risk group (36/156, 23.1%) (P < 0.001, respectively). High-risk patients tended to carry more signaling molecule gene mutations, such as *FLT3* (P < 0.001), as compared with low-intermediate risk ones. The distributions of other mutations were similar among different groups as shown in Table 2.

Table 2
Gene mutations and clinical aspects.

Gene mutations		Gender No. of the patients		Mean age (yrs)	Median WBC count, $\times 10^9$/L (range)	Sanz risk stratification	
		Male	Female			High	Low and Intermediate
FLT3-ITD or -TKD (Failed = 4)	Mutated	54	30	37.2 ± 15.3	8.85 (0.5–205.7)	39	45
	Not mutated	220	227	38.3 ± 14.5	2.3 (0.3–122.2)	91	356
		P = 0.012		P = 0.577	P < 0.001	P < 0.001	
N-RAS (Failed = 26)	Mutated	14	9	35.1 ± 12.4	8.0 (0.54–78.9)	9	14
	Not mutated	252	234	38.2 ± 14.8	2.5 (0.3–205.7)	115	371
		P = 0.523		P = 0.317	P = 0.001	P = 0.132	
WT-1 (Failed = 20)	Mutated	15	9	35.9 ± 16.0	2.7 (0.6–72.0)	9	15
	Not mutated	251	240	38.3 ± 14.6	2.7 (0.3–205.7)	122	369
		P = 0.403		P = 0.440	P = 0.832	P = 0.148	
EMG (Failed = 57)[a]	Mutated	23	12	40.1 ± 15.5	2.4 (0.8–58.4)	11	24
	Not mutated	222	221	37.9 ± 14.5	2.8 (0.3–205.7)	112	331
		P = 0.081		P = 0.395	P = 0.866	P = 0.425	

WBC, white blood cell; BM, bone marrow; and no., number.
"Failed" refers to the samples without detection results due to quality problems.
EMG refers to a group of epigenetic modifier genes investigated in this work.
One patient with *IDH1* mutation but failed in *TET2* analysis was considered as EMG mutated.
[a] Failed number: *MLL* fusion gene/PKD mutations (n = 1), *DNMT3A* (n = 13), *IDH1* (n = 26), *IDH2* (n = 24), *ASXL1*(n = 31), and *TET2* (n = 27).

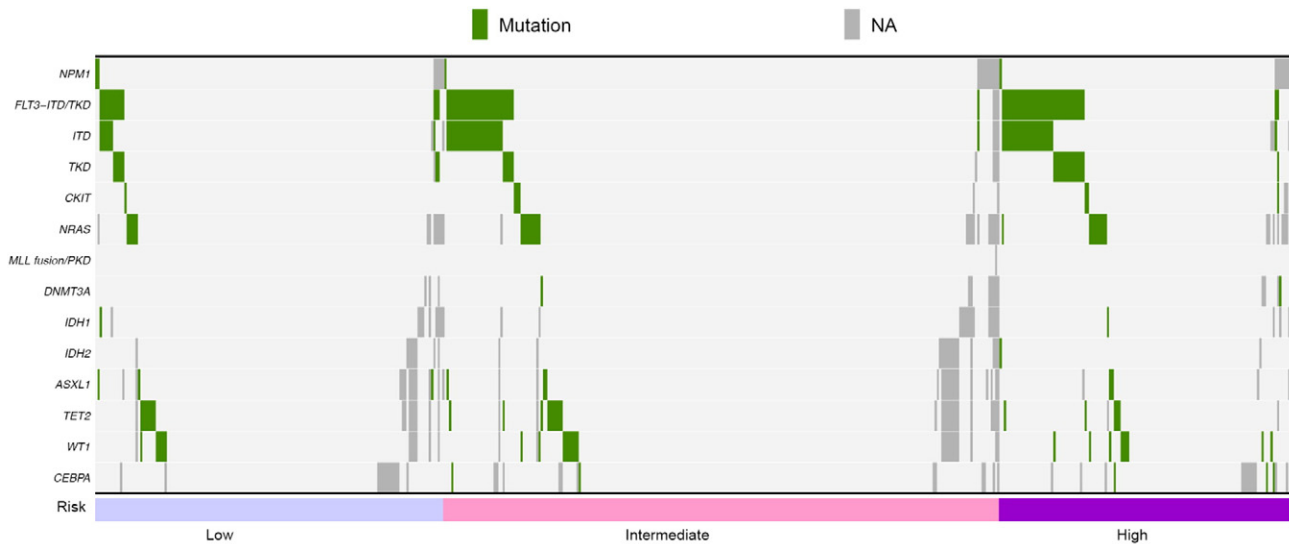

Fig. 1. Correlation between gene mutations and clinical risk groups. High-risk patients contained more mutations additional to *PML–RARα* (50.4%, 66/131) as compared with intermediate- (25.0%, 62/248, P < 0.001) and low-risk (23.1%, 36/156, P < 0.001) groups.

3.3. Treatment Response

In the training group, 251 out of 266 (94.4%) APL cases treated with ATRA/ATO combination therapy in SIH-related centers during 2001 to 2008 achieved CR. Among 269 cases treated with the same regimen but in a multi-center trial setting as validation group (Table 3), 253 out of 269 patients (94.1%) reached to CR. In univariate analysis for potential prognostic factor on induction outcome, Sanz low-risk patients could achieve a higher CR rate than the intermediate-high-risk patients in both training and validation groups (P = 0.007 for both training and validation groups, Table 3). No adverse effects of *FLT3*-ITD/TKD, *N-RAS*, *WT1* and EMG mutations were observed on CR induction.

A multivariate analysis was performed in 467 pooled patients of training and validation groups, who were with complete molecular results of *FLT3*, *N-RAS*, *WT1*, and all EMG mutations. Sanz risk stratification (P = 0.001, OR 2.815 [1.566–5.059]) and *WT1* (P = 0.048, OR 0.297 [0.089–0.988]) were correlated with the results of CR induction. The complete set of covariates that entered into multivariate model was shown in Table 5.

Table 3
The relationship between Sanz risk stratification or gene mutational status and CR rate.

	Training group		Validation group	
	CR no. (%)	P value	CR No. (%)	P value
Sanz risk stratification				
High	52/57 (91.2)	0.007[a]	65/74 (87.8)	0.007[a]
Intermediate	121/131 (92.3)		110/117 (94.0)	
Low	78/78 (100)		78/78 (100)	
FLT3-ITD or -TKD				
Mutated	32/34 (94.1)	0.697	47/50 (94.0)	1.000
Not mutated	219/232 (94.4)		202/215 (94.0)	
N-RAS				
Mutated	13/14 (92.9)	0.552	9/9 (100)	1.000
Not mutated	231/244 (94.7)		227/242 (93.8)	
WT1				
Mutated	9/11 (81.8)	0.136	11/13 (84.6)	0.179
Not mutated	225/238 (94.5)		239/253 (94.5)	
EMG				
Mutated	13/14 (92.9)	0.217	19/21 (90.5)	0.068
Not mutated	221/224 (98.7)		205/209 (98.1)	
Double or triple mutations				
Yes	8/9 (88.9)	0.412	8/9 (88.9)	0.429
No	243/257 (94.6)		245/260 (94.2)	

[a] Low risk vs. intermediate-risk or high-risk.

Early death (ED) constituted the main reason of induction failure. Among the total 31 ED cases, the main causes of death were intracranial hemorrhage (22 patients), followed by differentiation syndrome (6 patients) and cerebral/cardiac infarction (3 patients).

3.4. Survival Analysis

In univariate analysis, Sanz risk stratification was strongly associated with the prognosis in terms of OS (HR = 0.402, 95% CI 0.223–0.725; P = 0.002) in training group, which was confirmed by validation group OS (HR = 0.323, 95% CI 0.165–0.632; P = 0.001) (Fig. 2A–D). Interestingly, EMG mutations suggested an inferior treatment outcome in terms of OS (HR = 3.335, 95% CI 1.139–9.759; P = 0.028) and DFS (HR = 6.761, 95% CI 2.179–20.984; P = 0.001) in training group, with a median follow-up of 84 months (Fig. 3A and B). We then addressed the survival impact of clinical and molecular factors in 269 cases of validation group, with a median follow-up of 38 months. Although EMG mutations did not have a statistically significant effect on OS (P = 0.119, Fig. 3C), they profoundly affected the DFS (HR = 4.026, 95% CI 1.089–14.878; P = 0.037, Fig. 3D). No prognostic effects of *FLT3*-ITD/TKD on OS and DFS were observed in either training or validation groups (Fig. 4A–D). Similarly, no significant effects of *WT1* and *NRAS* mutations were noted on treatment outcomes in the two groups (Supplementary Table 1). Although double or triple mutations suggested poor prognosis in training group, they were not proved in validation group (Supplementary Fig. 2).

In the pooled set of 535 patients, the median follow-up time was 48 months. The estimated 5-year survival rates for different clinical and gene mutational status were shown in Table 4. In the multivariate analysis of 467 patients, the independent factors indicating poor prognosis included Sanz risk stratification for OS (OR = 0.386, 95% CI 0.247–0.605, P < 0.001) and EMG mutations for OS (OR = 2.721, 95% CI 1.212–6.105, P = 0.015) and DFS (OR = 4.702, 95% CI 1.895–11.671, P = 0.001), respectively (Table 5).

3.5. Point Mutations in Coding Sequences of PML–RARα in Relapsed Patients With EMG Mutations

To investigate the possible association of point mutations in *PML–RARα* to ATRA/ATO sensitivity, we examined the coding sequences of *PML–RARα* in five pairs of samples at initial diagnosis and the first relapse in patients with EMG mutations. *PML* mutation was identified in

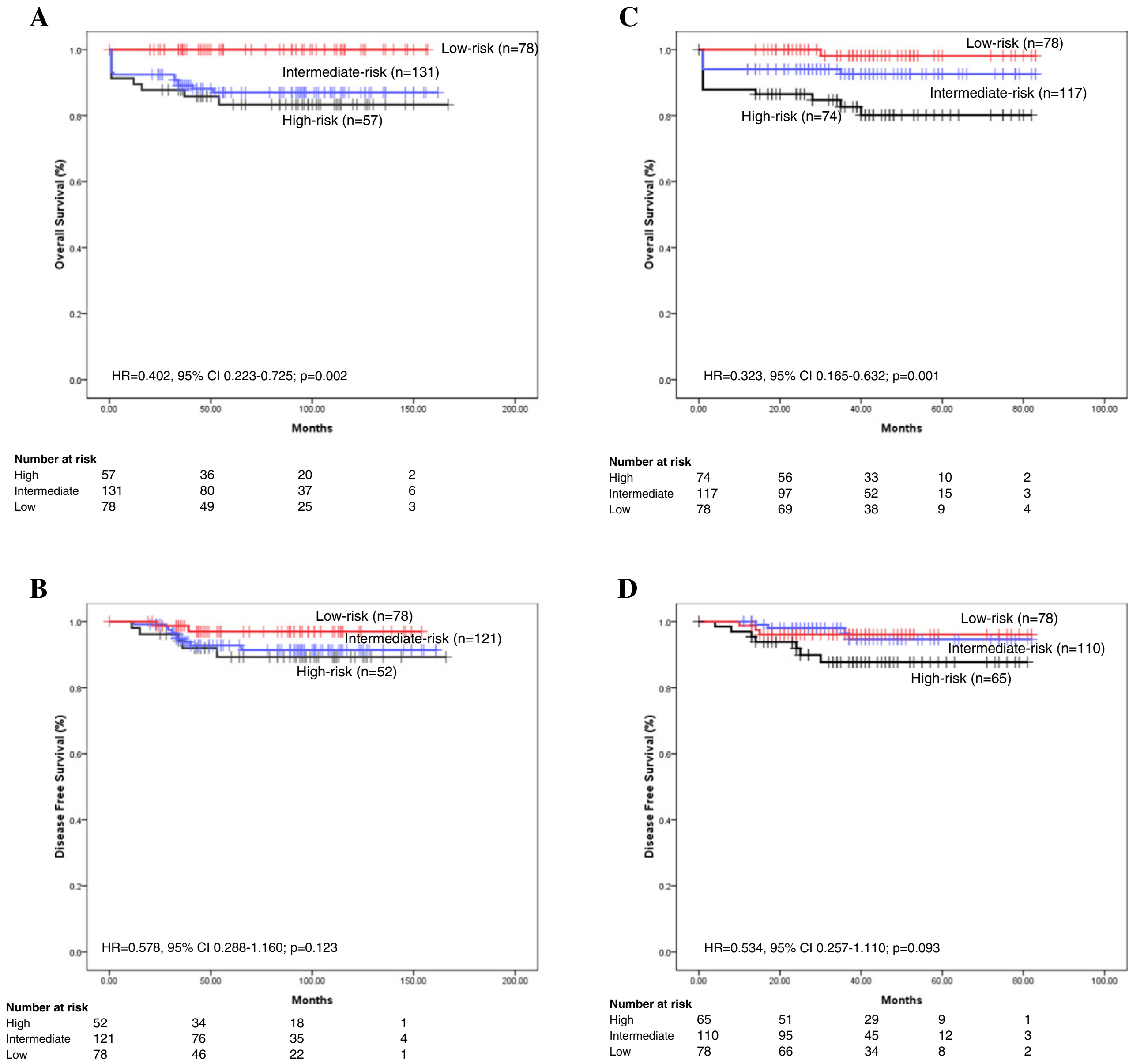

Fig. 2. Kaplan–Meier curves for overall survival (OS) and disease-free survival (DFS) according to Sanz's risk stratification in univariate analysis. (A, B) OS and DFS of different risk groups in training group. (C, D) OS and DFS of different risk groups in validation group.

one patient in the B2 box (L218H) of the RBCC domain at relapse (Supplementary Fig. 3), which was associated with drug resistance and cause of death. No mutations were identified in the *RARα* portion of the fusion transcripts in all five pairs of samples. In the four patients without any mutations of *PML–RARα*, three were still sensitive to ATRA/ATO while the other case with a *DNMT3A* mutation at initial diagnosis developed a CNS leukemia and died soon.

4. Discussion

ATRA plus chemotherapy (anthracyclines alone or with Ara-C) has been used as routine practice for treatment of APL in hematology/oncology centers worldwide (Degos et al., 1995; Avvisati et al., 1996; De Botton et al., 1998; Estey et al., 2005; Lo-Coco et al., 2013; Sanz et al., 2000). ATO was tested in re-induction in relapsed patients with a CR rate more than 80% (Tallman and Altman, 2009; Shen et al., 2004, 2001). We initially proposed simultaneous use of ATRA and ATO in newly diagnosed patients based on several lines of evidence. First, ATO and ATRA target, respectively, the N- and C-terminals of PML–RARα and modulate key pathways involved in apoptosis/differentiation. Hence a combined use of the two agents may bring synergistic effect (Wang et al., 2010; Zhang et al., 2010). Second, evidence from preclinical study showed that ATRA/ATO combination could significantly reduce the tumor burden or even eliminate the disease in experimental APL animal models (Lallemand-Breitenbach et al., 1999). Third, transcriptome/proteome analysis of APL cells upon the joint effect of the two agents showed enhanced induction of differentiation/apoptosis associated pathways in the absence of obvious induction of pathways related to cell toxicity (Zheng et al., 2005). Fourth, the ATO-induced second or third CR in patients relapsed after treatment with ATRA/chemotherapy usually didn't last long.

Indeed, with the ATRA/ATO combination therapy, the clinical outcome of APL has dramatically changed and the disease has been transformed from highly fatal to highly curable in a great majority of patients (Chen et al., 1994; Fenaux et al., 1994; Huang et al., 1988; Niu et al., 1999; Tallman and Altman, 2009; Hu et al., 2009; Shen et al.,

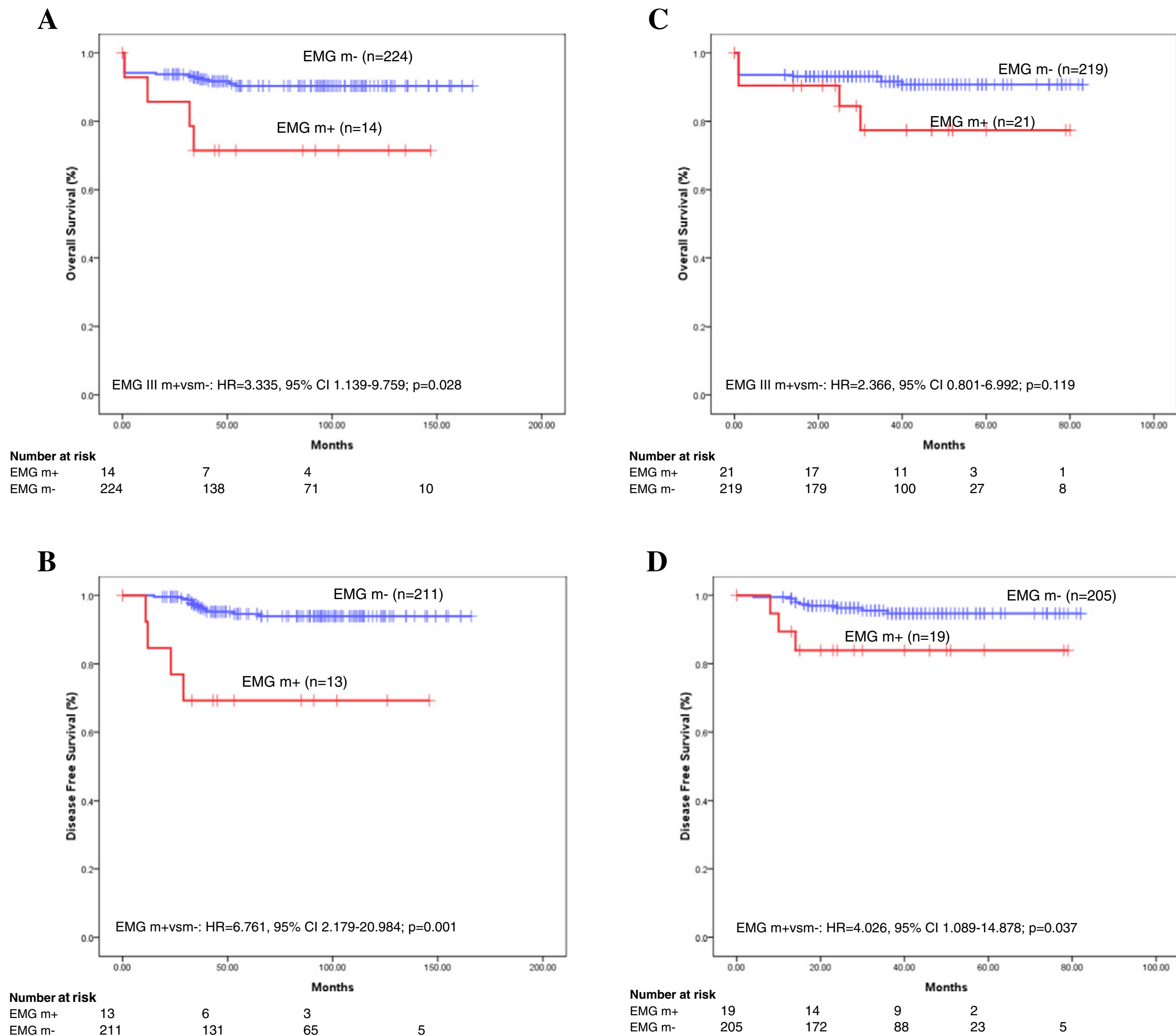

Fig. 3. Kaplan–Meier curves for overall survival (OS) and disease free survival (DFS) according to EMG mutational status in univariate analysis. (A, B) OS and DFS of patients with EMG mutations in training group. (C, D) OS and DFS of patients with EMG mutations in validation group.

2004; Zhang et al., 2010; Zhu et al., 2013, 1997; Ito et al., 2008; Zhou et al., 2010; Lo-Coco et al., 2013; Shen et al., 2001). Recent evidence from Huang et al. also proved that ATRA in combination of an oral arsenic compound, As_4S_4, is as effective as ATRA/ATO (Zhu et al., 2013). A comparison of the results between a historic control group of 97 cases treated with ATO as rescue for relapse after ATRA/chemotherapy regimen (ATRA → ATO group) yielded an outcome significantly inferior to that achieved by simultaneous use of the two medicines in 535 cases in this series (ATRA/ATO group). With a median follow-up time of 60 months, the 5 year OS and DFS rates were respectively 68.3 $\pm$ 4.8% and 76.4 $\pm$ 5.4% in ATRA → ATO group as compared to 89.9% $\pm$ 1.4% and 92.9 $\pm$ 1.3% in ATRA/ATO group ($P < 0.001$ for both OS and DFS, Supplementary Fig. 4A, B). However, APL is not a homogenous disease and can be stratified into low-, intermediate- and high-risk groups according to clinical parameters, such as WBC and platelet (Sanz and Lo-Coco, 2011; Sanz et al., 2000). Even with ATRA/ATO treatment, a sizable portion of high-risk patients (17.6% in this study) still experienced difficult induction intercourse, disease recurrence and short survival. A low percentage of cases in low- and intermediate-risk groups still got relapse (3.2% and 5.2%, respectively). Therefore, it is important to identify more sensitive markers to have a better stratification system for APL (Sanz and Lo-Coco, 2011).

Adverse effects of gene mutations additional to *PML–RARα* fusion, especially *FLT3*-ITD/TKD, on the prognosis of APL drew attentions in recent years; however, the results were controversial among different research groups (Beitinjaneh et al., 2010). Previous prognostic studies had often been limited by the diversity of the treatment protocols, and the absence of ATO in the front-line treatment (Avvisati et al., 1996; Shen et al., 2004; Zhou et al., 2010; Lo-Coco et al., 2013; Zhu et al., 2013; Lou et al, 2013.; Raffoux et al., 2003). In this work, we examined the possible effects of additional gene mutations in two large cohorts of APL patients with homogenous ATRA/ATO treatment. It was found that a number of gene point mutations or insertion events could occur in APL in the presence of fusion gene, with the mutations of signaling molecules being the most frequent ones. Mutations of *FLT3* and *N-RAS* were significantly correlated with high initial WBC count at diagnosis. With regard to risk groups, we discovered that nearly half of the high-risk patients (50.4%) carried additional mutations, mostly those of *FLT3* and *N-RAS*, which could reflect the molecular complexity in this group as compared with intermediate- and low-risk ones. A large body of evidence

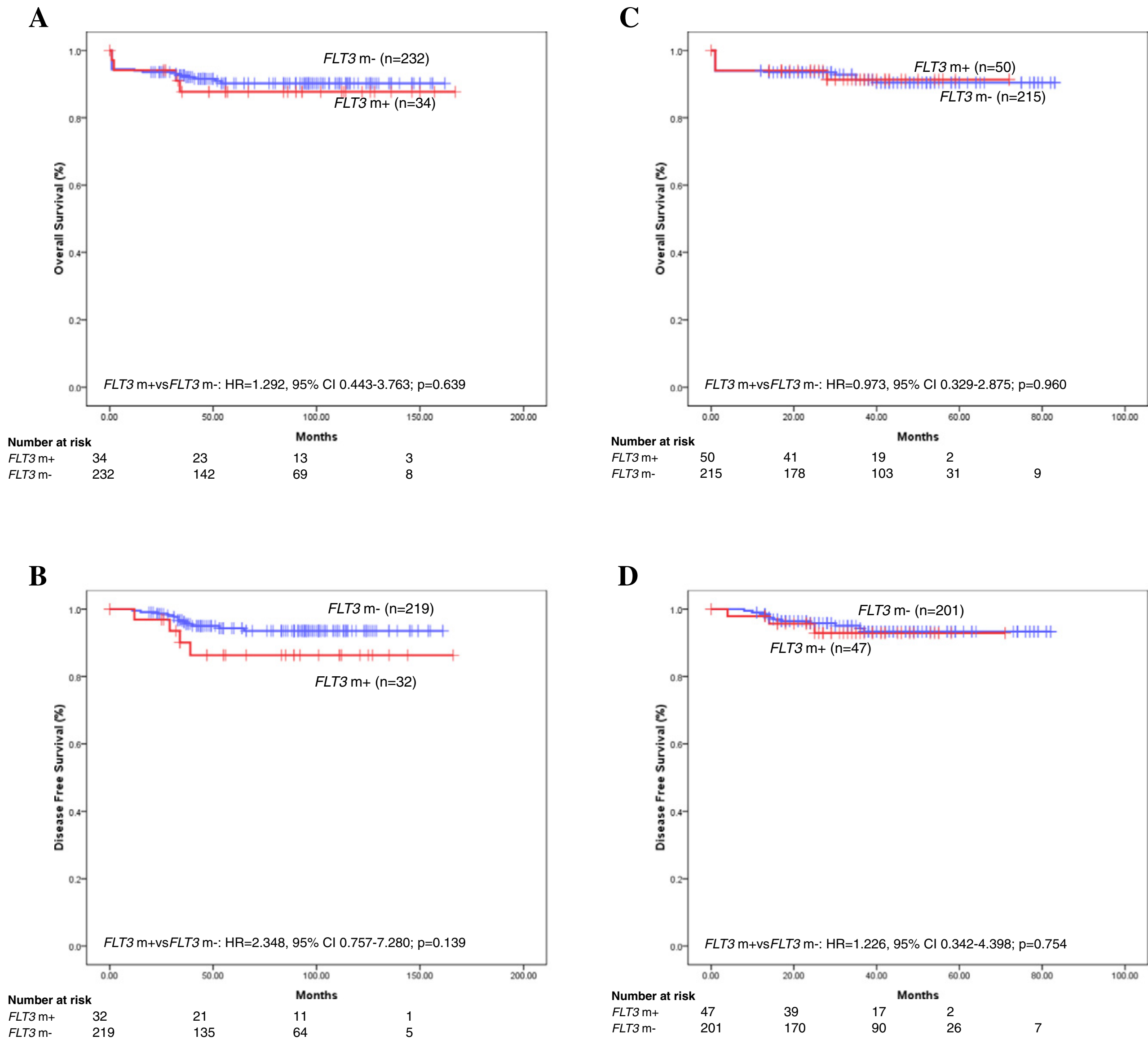

Fig. 4. Kaplan–Meier curves for overall survival (OS) and disease free survival (DFS) according to *FLT3* mutational status in univariate analysis. (A, B) OS and DFS of patients with *FLT3* mutations in training group. (C, D) OS and DFS of patients with *FLT3* mutations in validation group.

suggests that molecular markers are not only correlated to clinical features at disease onset, but also potentially associated with drug response and prognosis of AML. Nevertheless, the impact of certain biomarkers can be deeply affected by treatment factors. To this end, it may be noteworthy that although *FLT3*-ITD or -TKD mutations exerted negative impact on the OS and DFS in historic control of 97 patients in ATRA → ATO group (Supplementary Fig. 5A, B), the introduction of ATO into the first line treatment in ATRA/ATO group abrogated this negative impact in both training and validation groups (Fig. 4A–D). In contrast, the adverse effects of EMG abnormalities were not influenced by ATRA/ATO combination, especially for DFS. The mechanism underlying negative prognostic effect of EMG mutations remains unclear. It is assumed that several factors may contribute to its effect. First, by altering DNA methylation and/or histone modification patterns, EMG mutations on their own can profoundly affect the gene expression profiles in favor of the acquisition of growth and survival advantages of hematopoietic stem/progenitor cells (Shen et al., 2011; Jasielec et al., 2014; Jeong et al., 2014; Yan et al., 2011) and some of them have been shown to be leukemogenic in animal models (Chen et al., 2009, 2013). Hence, the presence of these mutations could render APL stem/progenitor cells even more resistant to current therapies. Second, EMG mutations tended to co-exist with other mutations (in this series of APL, 11/35, $P < 0.001$), which may add complexities to leukemogenesis. Although the negative effect of multiple mutations could not be proved in validation group, longer follow-up is warranted. Third, gene mutations associated with epigenetic regulations are involved in DNA repair (Schar and Fritsch, 2011), whose abnormalities could make APL stem/progenitor cells prone to mutational events especially under the selective pressure of ATRA/ATO, such as those of *PML–RARα* gene, which potentially leads to the relapse of the disease. Of course, more clinical and experimental evidence should be accumulated to support the above view points.

Sanz risk stratification is still useful in identifying patient cohorts with different treatment outcomes, especially in terms of early CR induction, which was the only independent factor in multivariate analysis in this study. In both training and validation groups, 78/78 (100%) and 78/78 (100%) patients of low-risk groups achieved CR, and ED tended to occur in the high-risk group. In addition, Sanz risk stratification was significant in predicting OS (Fig. 2, Table 5). However, the effect of Sanz's

Table 4
The estimated 5 year OS and DFS rates in different clinical and gene mutation groups.

	Estimated 5 year OS rate (%)	Estimated 5 year DFS rate (%)
Sanz risk stratification		
High	80.5 ± 3.8	87.8 ± 3.4
Intermediate	89.1 ± 2.2	93.4 ± 1.9
Low	99.2 ± 0.8	96.3 ± 1.7
P, HR (95% CI)	<0.001, 0.355 (0.229–0.550)	0.022, 0.554 (0.335–0.919)
FLT3		
Mutated	89.9 ± 1.6	89.1 ± 4.0
Not mutated	89.4 ± 3.6	93.6 ± 1.4
P, HR (95% CI)	0.818, 1.093 (0.512–2.336)	0.188, 1.765 (0.757–4.115)
WT-1		
Mutated	83.3 ± 7.6	NA*
Not mutated	89.7 ± 1.5	92.2 ± 1.4
P, HR (95% CI)	0.241, 1.845 (0.663–5.135)	0.422, 0.046 (0.000–84.431)
N-RAS		
Mutated	87.0 ± 9.1	85.9 ± 9.5
Not mutated	90.2 ± 1.5	93.3 ± 1.3
P, HR (95% CI)	0.954, 0.959 (0.232–3.958)	0.617, 1.443 (0.343–6.069)
EMGs		
Mutated	74.3 ± 8.0	76.7 ± 7.8
Not mutated	90.2 ± 1.6	93.9 ± 1.4
P, HR (95% CI)	0.008, 2.797 (1.305–5.998)	<0.001, 5.238 (2.225–12.332)
Double or triple mutations		
Yes	68.8 ± 11.7	78.3 ± 11.1
No	90.6 ± 1.4	93.4 ± 1.3
P, HR (95% CI)	0.008, 3.471 (1.374–8.763)	0.035, 3.6000 (1.092–11.872)

NA, not available *no relapse due to small sample size.

score in predicting the recurrence of the disease was not strong. Therefore, albeit the introduction of ATRA/ATO into front-line treatment, risk factors adapted treatment strategy may be reconsidered. Given the excellent results of low-risk patients (5 year OS and DFS of 99.2 ± 0.8 and 96.3 ± 1.7%, respectively), less toxic regimens such as ATRA/ATO without chemotherapy may be used as already reported by Lo-Coco et al., 2013. As for intermediate-risk patients, whose prognosis was relatively poor compared to low-risk ones in our series, randomized clinical trial is warranted to compare the results of ATRA/ATO with chemotherapy and those of ATRA/ATO without chemotherapy. New consolidation strategies containing ATO, Ara-C and other potential new agents might be the choice for high-risk group. Of note, cases with EMG mutations may need further enhanced therapy such as high dose anthracyclines and regulatory agents for DNA methylation and/or histone modification to improve their clinical outcome.

In conclusion, in an era of ATRA/ATO treatment, both molecular markers, EMG in particular, and clinical parameter based stratification systems should be used as potential prognostic factors for APL. More refined protocols considering the complexity of molecular pathogenesis of APL such as additional mutations should be designed, especially in high- and intermediate-risk groups, in an attempt to cure all APL patients.

Author Contributions

S.-J.C., Z.C., and J.-M.L. were the principal investigators who conceived the study. S.-J.C., Z.C., J.-M.L., Z.-Y.W., Z.-X.S., and Y.S. coordinated and oversaw the study. Y.S., Y.-K. F, and Y.-M.Z., performed most of the experiments. Z.-H.G. was responsible for bioinformatics investigation. B.C., J.-Y.S., C.C., Y.-J.L., J.J. and H.-H.Z. participated in sample bank management. J.H., W.-L.Z., J.-Q.M., L.C., and H.-M.Z. gathered detailed clinical information for the study and helped to perform clinical analysis. Z.C., S.-J.C. and Y.S. wrote the manuscript. All the authors declared that no relevant financial conflict of interest was involved in this work.

Conflict of Interest

We declare that we have no conflict of interest.

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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.ebiom.2015.04.006.

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Table 5
Multivariate analysis of clinical and molecular variables for CR, OS and DFS in the pooled group.

Variables	CR		OS		DFS	
	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)
Age	NS	–	NS	–	NS	–
WBC count	NS	–	NS	–	NS	–
FLT3 mutations	NS	–	NS	–	NS	–
WT1 mutations	0.048	0.297 (0.089–0.988)	NS	–	NS	–
N-RAS mutation	NS	–	NS	–	NS	–
EMG mutations	NS	–	0.015	2.721 (1.212–6.105)	0.001	4.702 (1.895–11.671)
Double or triple mutations	NS	–	NS	–	NS	–
Sanz risk stratification	0.001	2.815 (1.566–5.059)	<0.001	0.386 (0.247–0.605)	0.09	0.638 (0.380–1.072)

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