Gene mutation profile and risk stratification in *AML1-ETO*-positive acute myeloid leukemia based on next-generation sequencing

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Abstract. Gene mutations play an important role in the development and progression of *AML1-ETO*-positive acute myeloid leukemia (AE-AML). Nevertheless, the gene mutation profile in this subtype of leukemia remains unclear. In addition, the clinical and prognostic effects of different mutant genes may be underestimated. In the present study, gene sequencing was conducted at diagnosis and relapse with next-generation sequencing (NGS) in 64 patients with newly diagnosed AE-AML, and 44/64 (68.8%) patients were found to present with a median of 2 (1-10) recurrent mutations at diagnosis and 6/11 (54.5%) cases were found to present with genetic alterations at relapse. c-*KIT* mutation was the most common in this cohort, with an incidence of 27/64 (42.2%) at diagnosis, followed by *ASXL1* (n=10, 15.6%), *MET* (n=8, 12.5%), *MLH1* (n=6, 9.4%), *TET2* (n=5, 7.8%), and *FBXW7*, *TP53* and *DNMT3A* (n=5, 7.8%). Survival analysis showed that *c-KIT* (exon 8, 17) but not exon 10 adversely affected survival. In addition, *ASXL1* and *TP53* were poor impact factors for recurrence-free survival (RFS) (P<0.05), and *ASXL1*, *MET*, *FBXW7* and *TP53* had a negative impact on overall survival (OS) (P<0.05). Multivariate analysis showed that *c-KIT* (exon 8, 17) [RFS: hazard ratio (HR) 3.36, 95% confidence interval (CI) 1.54-7.34, P=0.002; OS: HR 2.84, 95% CI 1.20-6.71, P=0.018] and *ASXL1* mutations (RFS: HR 3.13, 95% CI 1.34-7.32, P=0.009; OS: HR 3.94, 95% CI 1.62-9.61, P=0.003) were independent adverse factors for survival. Further, co-mutation of these two genes showed even worse effect on disease outcome. Collectively, additional gene mutations play critical role in AE-AML. *C-KIT* and *ASXL1* mutations are the two most common mutations in this subtype of leukemia. *C-KIT* (exon 8, 17) but not exon 10, and also the *ASXL1* mutation poorly affect the disease outcome of this disease.

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Introduction

T(8; 21)/*AML1-ETO*-positive acute myeloid leukemia (abbreviated as AE-AML), accounting for 5-10% of all cases of AML (1), is classified as a favorable leukemia subtype according to the World Health Organization (WHO) classification of AML (2). Nevertheless, numerous studies show a high incidence of extramedullary leukemia (EML) and additional cytogenetic abnormalities (ACAs). Moreover, the long term survival ranges from 28 to 70%, indicating the clinical heterogeneity of this subtype of leukemia (1,3,4).

In addition, gene mutations frequently occur and critically play a role in the development and progression of AE-AML. Krauth *et al* reported that up to 49.6% AE-AML patients present with additional gene mutations at diagnosis, and 66.7% undergo genetic alterations at the time of relapse (4). The *c-KIT* mutation is known to be the most common molecular event in this subtype of leukemia, present in up to 12.8-48% of AE-AML patients, adversely affecting the disease outcome (1,4). Several studies have shown that the *AML1/ETO* fusion gene is unable to singly induce leukemia, but requires additional molecular events. The *c-KIT* mutation has been proven to cooperate with the *AML1-ETO* fusion to induce leukemia (3,5). Still there are other gene mutations, including *WT1*, *FLT3-ITD* and *PDGFR* mutations, that have been reported to be involved in the progression of this subtype of leukemia (6-8), further supporting an oncogenic cooperation in leukemogenesis between *RUNX1-RUNX1T1* and additional molecular alterations. Therefore, detailed detection of the gene mutation profile at diagnosis and relapse could deepen the understanding of leukemia development and progression. There is a current demand for large-scale mutation screening in the clinical

setting with a limited amount of clinical sample and reasonable turnaround time. Currently, next-generation sequencing (NGS), known as torrent Ion personal genome sequencing (PGM) platform, provides the advantages of parallel sequencing and high throughput multiplexing ability, facilitating routine and simultaneous parallel and targeted sequencing of all genes. This has been widely used in mutation profile detection and study (9-11). In our recent study (12), we designed a panel for AML gene mutation detection, which targets 67 genes covering the full coding sequence of 17 genes (Table SI) and exonic hot spot for 50 genes (Table SII). We used this panel to test 27 patients with chromosome normal (CN)-AML with NGS, and certified the result with Sanger sequencing at the same time. We found 100% sensitivity and specificity of the new platform when compared with Sanger sequencing. Meanwhile, the NGS with high-throughput had superiority in gene mutation profile detection. In this series, we used the same panel to detect the gene mutation profile at diagnosis and relapse in 64 patients with newly diagnosed AE-AML and further studied the role of different mutations on the clinical characteristics and survival. This was an observational clinical study without any interventions in regards to patient treatment, which was decided by their doctors. In this study, we enrolled 64 patients to demonstrate the common phenomenon of gene mutations at disease diagnosis and molecular alteration at leukemia relapse, and also the impact of gene mutations on clinical characteristics and disease outcome. The study aimed to further support the importance of molecular events in leukemia development and progression in AE-AML. The molecular meaning of the oncogenic pathway of *AML1-ETO* fusion and associated translocations are not the main point of this study, thus the study does not investigate this area in depth.

Patients and methods

Patients. Patients with newly diagnosed AE-AML, according to the WHO 2008 Classification of AML (2), and admitted to Nanfang Hospital from April 2006 to December 2013, were enrolled into this study. The characteristics of the patients are documented in Table I. All samples and clinical data were obtained upon approval of the Nanfang Hospital, Southern Medical College of Medicine Institutional Review Boards. Written informed consent was obtained from all patients and/or guardians except those admitted to Nanfang Hospital from the year 2006 to 2011, since we had obtained ethical approval with an exemption of informed consent for those patients in 2012. According to the time that we received the ethical approval, the patients admitted to the hospital before 2012 were defined as retrospectively assigned participants, and those admitted post 2012 were defined as prospectively assigned cases.

Gene mutation sequencing. In our previous study (12), we designed a gene mutation panel for 67 AML-targeted genes covering full coding sequence of 17 genes commonly mutated in AML according to previous reports and exonic hot spot for 50 genes from the Ion AmpliSeq Cancer Hotspot Panel V2 kit (Thermo Fisher Scientific, Inc.) (detailed information is shown in Tables SI and SII). Primers for multiplex PCR of 67 target genes were designed by Ion AmpliSeq Designer (www.ampliseq.com). DNA (10 ng) (quantified by Qubit™ Fluorometer; Life Technologies Corporation, Carlsbad, CA, USA) was used and the customized Ion AmpliSeq panel was processed using the Ion AmpliSeq Library kit 2.0 (Thermo Fisher Scientific, Inc.). Workflows related to digestion of the primers, phosphorylation of the amplicons, emulsion PCR, enrichment template-positive and sequencing on Ion 316/318 chips were performed according to the manufacturer's instructions. The raw signal data were analyzed using Torrent Suite v.4.0.2 (Life Technologies). The pipeline included signaling processing, base calling, quality score assignment, adapter trimming, read alignment to human genome GRCh37 references, mapping quality control, and coverage analysis. Several filtering steps were used to generate final variant calling.

In the present study, to further verify the results of the NGS, the detection of *FLT3-ITD*, *c-KIT (exon 8* and *17)*, *NPM1*, *JAK2 (V617F)* and *DNMT3A (R882)* mutations were performed by Sanger sequencing at the same time.

MRD monitoring. *AML1-ETO* transcript levels, assayed by quantitative real-time polymerase chain reaction (qPCR) as described in our previous research (13,14), presented an *AML1-ETO/ABL* ratio, which was used to monitor minimal residual disease (MRD) after induction therapy and every consolidation chemotherapy, and then at 3-month intervals for the first 2 years of follow up, and at relapse. Major molecular remission (MMR) was defined as a >3-log reduction in *AML1-ETO* mRNA transcript levels when compared with the pre-treatment (15).

Treatment. All patients received the '3+7' regimens consisting of daunorubicin (DNR) at a daily dose of 40-60 mg/m^2 or idarubicin (IDA) at a daily dose of 8-10 mg/m^2 or other anthracyclines for 3 days and cytarabine (Ara-C) at 100-150 mg/m^2 per day for 7 days as induction chemotherapy for 1-2 cycles. Patient who did not achieve complete remission (CR) received salvage chemotherapy. After CR, they were mainly treated with a standard-dose Ara-C-based (SDAC-based) regimen, defined as the conventional induction regimens mentioned as before with or without 1-2 cycles of Ara-C 1-2 g/m^2 q12h (every 12 h) x 6-8 times, before the year 2010; after that year mainly with an intermedium-dose Ara-C-based (IDAC-based) regimen, defined as receiving ≥3 cycles of Ara-C 1-2 g/m^2 q12h x 6-8 times with/without autologous hematopoietic stem cell transplantation (auto-HSCT). Salvage chemotherapy included ICE (idarubicin, cytarabine, etoposide), CAG (aclarubicin, cytarabine, granulocyte-colony stimulating factor), FLAG (fludarabine, cytarabine, granulocyte-colony stimulating factor) and CLARA (cladribine, cytarabine). Allogeneic-HSCT was recommended for all eligible patients with *c-KIT* mutation, continuously positive MRD, and in CR2. Detailed treatment protocol is shown in Fig. S1.

Survival and statistical analyses. Disease-free survival (DFS) was defined as the time between CR1 to the first relapse, censored at the date of the last follow-up or death. Overall survival (OS) was defined as the time between diagnosis and death or the date of the last follow up.

Numerical data are represented in the form of median (range) and were compared using Mann-Whitney nonparametric U test. A nonparametric test was performed using

Table I. Clinical characteristics of the 64 patients with *AML1-ETO*-positive AML.

Characteristics	Total	SDAC-based	IDAC-based	P-value
Patients (n)	64	31	31	
Median age (years) (range)	27.5 (2-65)	27 (5-65)	28 (2-60)	0.978
Sex, male/female (ratio)	39/25 (1.6)	19/12 (1.6)	19/12 (1.6)	1.000
Blood counts, median values (range)				
WBC count ($x10^9$/l)	20.3 (2.3-72.4)	21.4 (3.1-70.3)	19.0 (2.3-72.4)	0.163
Hemoglobin level (g/dl)	75.5 (39.0-127.0)	70.0 (39.0-123.0)	79.0(41.0-127.0)	0.023
PLT count ($x10^9$/l)	23.5 (5.0-137.0)	21.0 (5.0-93.0)	24.0 (9.0-137.0)	0.468
EML (rate, %)	20 (31.3)	12 (38.7)	7 (22.6)	0.168
Marrow blasts (range) (%)	35.0 (3.0-94.0)	35.0 (3.0-94.0)	35.0 (7.0-93.0)	0.418
CD56-positive (n=63) (rate, %)	41 (65.1)	20 (64.5)	21 (70.0)	0.648
Cytogenetic aberrations in addition to t(8;21)(q22;q22) (n=52)				
Sole t(8;21)/ACAs	28/24	13/10	15/14	0.730
Loss of sex chromosomes (rate, %)	19 (36.5)	7 (30.4)	12 (41.8)	0.416
Molecular mutations in addition to RUNX1-RUNX1T1 (%)				
c-KIT (exon 8; 17)	20 (31.3)	14 (45.2)	6 (19.4)	0.030
ASXL1	10 (15.6)	8 (25.8)	2 (6.5)	0.038
MET	8 (12.5)	5 (16.1)	3 (9.7)	0.449
MLH1	6 (9.4)	4 (21.9)	2 (6.5)	0.390
TET2	5 (7.8)	3 (9.7)	2 (6.5)	0.641
FBXW7	5 (7.8)	2 (6.5)	3 (9.7)	0.641
TP53	5 (7.8)	4 (21.9)	1 (3.2)	0.162
DNMT3A	5 (7.8)	3 (9.7)	2 (6.5)	0.641
Allo-HSCT (rate, %)	13/62 (21.0)	7 (22.6)	6 (19.4)	0.755

AML, acute myeloid leukemia; SDAC, standard-dose Ara-C; IDAC, median-dose Ara-C; Ara-C, arabinosylcytosine; WBC, white blood cell; PLT, platelet; EML, extramedullary leukemia; ACA, additional chromosomal abnormality; Allo-HSCT, allogenic hematopoietic stem cell transplantation; c-KIT, KIT proto-oncogene, receptor tyrosine kinase; ASXL1, ASXL transcriptional regulator 1; MET, MET proto-oncogene, receptor tyrosine kinase; MLH1, MutL homolog 1; TET2, Tet methylcytosine dioxygenase 2; FBXW7, F-box and WD repeat domain containing 7; TP53, tumor protein P53; DNMT3A, DNA methyltransferase 3α.

the Chi-square test and survival analyses were calculated by Kaplan-Meier survival curves and the log-rank test. Univariate analysis with the log-rank test and multivariate analyses on categorized data were performed using Cox regression. SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. A P-value <0.05 was considered statistically significant.

Results

Clinical characteristics of the patients with AE-AML. Of all the 79 newly diagnosed AE-AML patients, only 64 cases were tested for gene mutations with NGS and are involved in this study. The characteristics of the 64 patients are documented in Table I. The median age of onset was 27.5 (range 2-65) years; 20 (31.3%) patients had EML, 25/52 (48.12%) patients presented with ACAs, and 19/52 (36.5%) cases with sex chromosomal defects.

The frequency of additional gene mutations at diagnosis. First we verified the sensitivity and specificity of the NGS. The result showed 100% consistency in the positive and negative detection of *FLT3-ITD*, *c-KIT (exon 8 and 17)*, *NPM1*, *JAK2 (V617F)* and *DNMT3A (R882)* mutations between the NGS and Sanger sequencing (Fig. S2).

Among the 64 patients being tested for gene sequencing with NGS, a median 2 (1-10) types of additional molecular mutations were detected in 44 (68.8%) patients, including 19 (29.7%) cases with one mutation, 16 (25.0%) with two and 9 (14.1%) with three or more mutations (Fig. 1C). The most common recurrent mutations occurred in *c-KIT* (n=27, 42.2%), including 12 (18.8%) D816, 7 (10.9%) M541, 5 (7.8%) N822, 1 (1.5%) R815_D816insVI, 1 (1.5%) Y418del and 1 N822_M541 co-mutation; *ASXL1* (n=10, 15.6%, including 1 double mutation), *MET* (n=8, 12.5%), *MLH1* (n=6, 9.4%), *TET2* (n=5, 7.8%, including 1 double mutation) and *FBXW7*, *TP53* and *DNMT3A* (n=5, 7.8%). This was followed by 4 (6.3%) *PAX5* and *CEBPA*, 3 (4.7%) *KMT2A*, *ATM*, *FLT3* and *NRAS*, 2 (3.1%) *DNMT3L*, *PDGFRA*, *APC*, *HRAS* and *RUNX1*, 1 (3.1%) *SH2B3*, *SMAD4*, *KRAS* and *NPM1* (Fig. 1A and D). The nature of these mutations is shown in Table SIII. When genes were categorized into functional groups, the most common mutations were those involved in the tyrosine kinase pathway (n=34, 53.1%), chromatin modification (n=16,

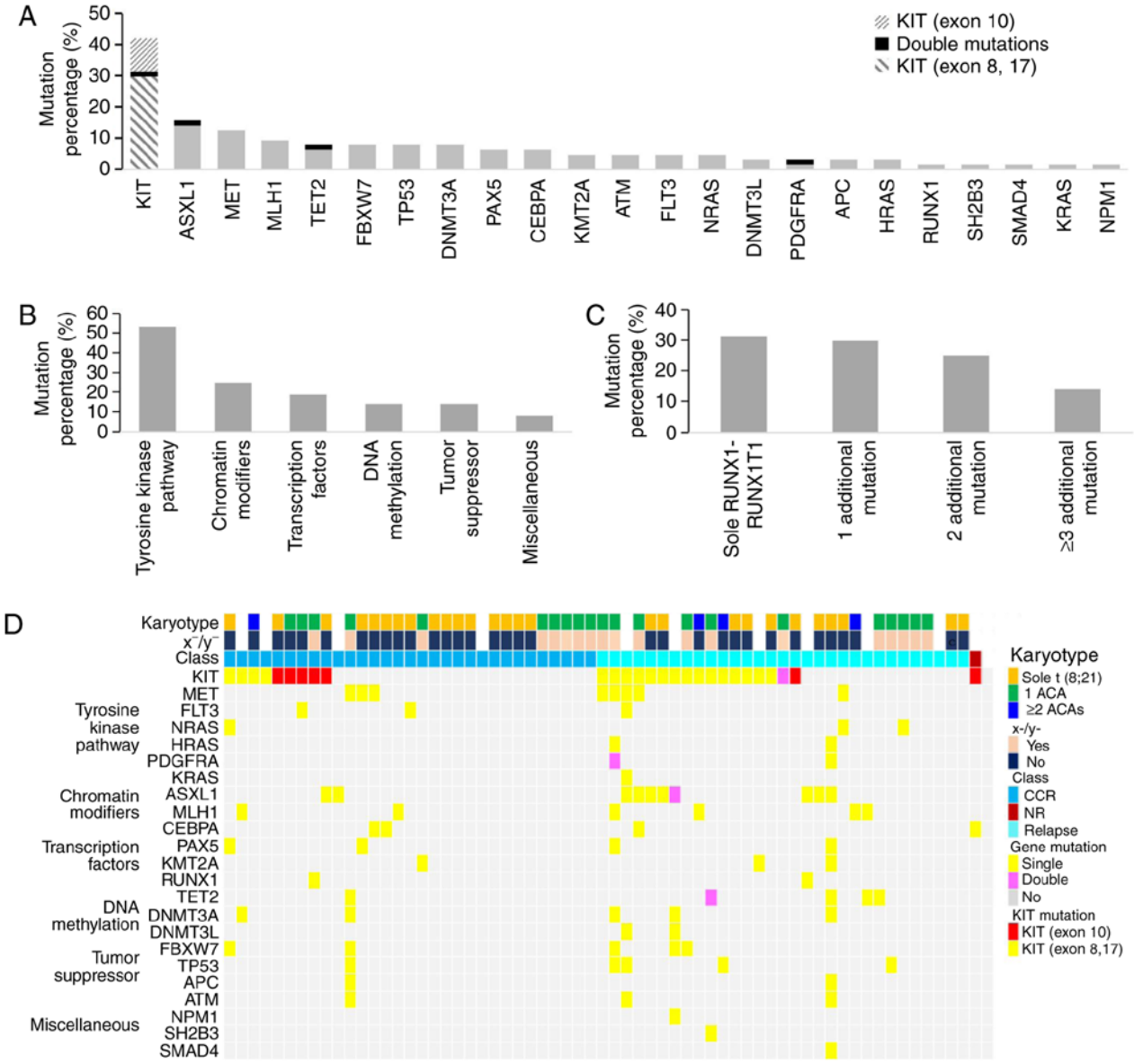

Figure 1. Gene mutation profile in newly diagnosed *AML1-ETO*-positive AML patients. Gene mutation sequencing was performed in 64 patients with NGS. (A) A total of 27 (42.2%) patients presented with *c-KIT* mutation, including 19 (29.7%) mutated at exon 8 or 17, 7 (10.9%) at exon 10 and one (1.6%) with double mutations. Ten (15.6%) cases presented with an *ASXL1* mutation, including 9 (14.1%) with a single mutation and one (1.6%) with double mutations. Eight (12.5%) patients presented with the *MET* mutation. Six (9.4%) cases harbored an MLH1 mutation. *TET2*, *FBXW7*, *TP53* and *DNMT3A* genes were respectively mutated in 5 (7.8%) patients, including one patient with a double mutation in the *TET2* gene. Four (6.3%) patients presented with *PAX5* and *CEBPA* mutations, respectively. Three (4.7%) harbored *KMT2A*, *ATM*, *FLT3* and *NRAS* mutations, respectively. Two (3.1%) had a *DNMT3L*, *PDGFRA*, *APC* and *HRAS* mutation, respectively, including one with double mutations in the gene. One (1.6%) case harbored the *RUNX1*, *SH3B3*, *SMAD4*, *KRAS* and *NPM1* mutations, respectively. (B and D) All the mutant genes were classified into different groups according to their function. A total of 34 (53.1%) patients presented with a tyrosine kinase pathway gene mutation, 16 (25.0%) showed a chromatin modification gene mutation, 12 (18.8%) harbored a transcription gene mutation, 9 (14.1%) presented with DNA methylation and tumor-suppression gene mutations, respectively, and 5 (7.8%) showed miscellaneous function gene mutation. (C) Among those patients with gene mutations, there were 19 (29.7%) harboring one mutation, 16 (25.0%) with 2 mutations and 9 (14.1%) with ≥3 mutations. (D) Mutation profile according to clinical features included karyotype, loss of sex chromosome and treatment response. All mutant genes detected are shown. Some mutations co-occurred or were exclusive and some were associated with higher relapse. AML, acute myeloid leukemia; NGS, next-generation sequencing. AML1, acute myeloid leukemia 1 protein [also known as RUNX family transcription factor 1 (RUNX1)]; ETO, eight twenty one protein [also known as RUNX1 partner transcriptional co-repressor 1 (RUNX1T1)]; c-KIT, KIT proto-oncogene, receptor tyrosine kinase; ASXL1, ASXL transcriptional regulator 1; MET, MET proto-oncogene, receptor tyrosine kinase; MLH1, MutL homolog 1; TET2, Tet methylcytosine dioxygenase 2; FBXW7, F-box and WD repeat domain containing 7; TP53, tumor protein P53; DNMT3A, DNA methyltransferase 3α; PAX5, paired box 5; CEBPA, CCAAT enhancer binding protein α; KMT2A, lysine methyltransferase 2A; ATM, ATM serine/threonine kinase; FLT3, Fms related tyrosine kinase 3; NRAS, NRAS proto-oncogene, GTPase; DNMT3L, DNA methyltransferase 3 like; PDGFRA, platelet derived growth factor receptor α; APC, APC regulator of WNT signaling pathway; HRAS, HRas proto-oncogene, GTPase; PDGFA, platelet derived growth factor subunit A; SH2B3, SH2B adaptor protein 3; SMAD4, SMAD family member 4; KRAS, KRAS proto-oncogene, GTPase; NPM1, nucleophosmin 1.

25.0%), transcription (n=12, 18.8%), DNA methylation (n=9, 14.1%), tumor suppression (n=9, 14.1%) and miscellaneous function (n=5, 7.8%) (Fig. 1B and D).

Clinical implications of gene mutations and gene co-mutation. We analyzed the clinical influence of the most common recurrent mutations including *c-KIT*, *ASXL1*, *MET*,

Table II. Influence of the gene mutations on RFS and OS.

	RFS			OS		
	HR	95% CI	P-value	HR	95% CI	P-value
Univariate[a,b]						
c-KIT (exon 8,17 vs. exon 10, WT)	3.31	1.60-6.81	0.001	3.25	1.48-7.18	0.003
ASXL1 (mutated vs. WT)	2.94	1.30-6.66	0.010	3.65	1.57-8.49	0.003
MET (mutated vs. WT)	2.36	0.89-6.28	0.085	3.44	1.36-8.71	0.009
TET2 (mutated vs. WT)	2.00	0.69-5.79	0.202	2.29	0.78-6.71	0.130
MLH1 (mutated vs. WT)	1.61	0.56-4.65	0.380	2.25	0.77-6.58	0.141
FBXW7 (mutated vs. WT)	2.66	0.77-9.19	0.122	4.13	1.35-12.62	0.013
TP53 (mutated vs. WT)	3.05	1.04-8.91	0.042	4.20	1.56-11.28	0.004
DNMT3A (mutated vs. WT)	1.34	0.40-4.45	0.636	2.58	0.88-7.54	0.083
KMT2A (mutated vs. WT)	1.06	0.25-4.50	0.940	1.56	0.37-6.63	0.549
CEBPA (mutated vs. WT)	1.06	0.14-7.85	0.955	1.11	0.15-8.25	0.918
PAX5 (mutated vs. WT)	1.15	0.27-4.86	0.852	1.40	0.33-5.97	0.650
Multivariate[c]						
c-KIT (exon 8,17 vs. exon 10, WT)	3.36	1.54-7.34	0.002	2.84	1.20-6.71	0.018
ASXL1 (mutated vs. WT)	3.13	1.34-7.32	0.009	3.94	1.62-9.61	0.003

[a]Factors with P<0.10 in the univariate analyses were subjected to multivariate analysis afterwards. [b]Patients with an unknown variable were included in the analysis using a dummy variable indicating missing data. [c]Backward stepwise Cox proportional-hazard modeling was used in multivariate analysis of risk factors. RFS, recurrence-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; WT, wild-type; c-KIT, KIT proto-oncogene, receptor tyrosine kinase; ASXL1, ASXL transcriptional regulator 1; MET, MET proto-oncogene, receptor tyrosine kinase; MLH1, MutL homolog 1; TET2, Tet methylcytosine dioxygenase 2; FBXW7, F-box and WD repeat domain containing 7; TP53, tumor protein P53; DNMT3A, DNA methyltransferase 3α; KMT2A, lysine methyltransferase 2A; CEBPA, CCAAT enhancer binding protein α; PAX5, paired box 5.

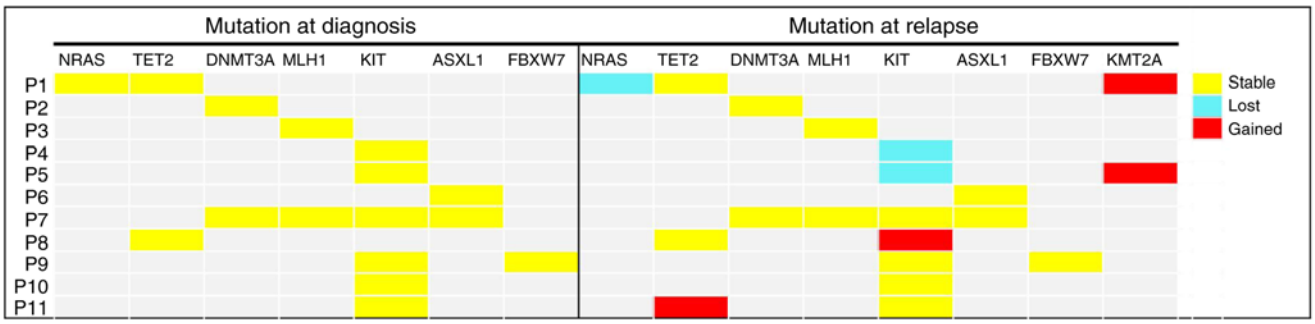

Figure 2. Pattern of gained and/or lost molecular mutations in *AML1-ETO*-positive AML at diagnosis and in the case of relapse. AML, acute myeloid leukemia.

MLH1, *TET2*, *FBXW7*, *TP53* and *DNMT3A*, and found no significant difference in the clinical characteristics between the gene mutation and wild-type (WT) groups, except for a higher incidence of ACAs (P=0.025) in the *TP53* mutation group as compared with the *TP53* WT (Table SIV). According to gene function, we found significantly higher bone marrow blast in the transcription and miscellaneous mutation group, as well as higher bone marrow blast and loss of sex chromosome in DNA methylation mutation group as compared with the WT group, respectively (Table SV).

Associations between molecular mutations were also assayed among those eight recurrent mutations. The *DNMT3A* mutation was found to be mutually exclusive of *MLH1* (P=0.014), *TET2* (P=0.005), *FBXW7* (P<0.001) and *TP53* (P=0.005), and also a trend of association with *MET* mutations (P=0.053) was noted. Apart from the *DNMT3A* mutation, the *FBXW7* mutation was mutually exclusive of *c-KIT (exon 8 and 17)* (P=0.014) and *TP53* (P=0.005), and a trend of association with the *MET* mutation (P=0.053) was noted. In addition to the *DNMT3A* and *FBXW7* mutations, *TP53* was also mutually exclusive of *MET* (P=0.001) (Fig. 1D and Table SIV).

Genetic alterations at relapse. Among the 31 relapsed patients, 11 had a mutation detection with NGS at relapse. In 6/11 (54.5%) patients, the initial molecular mutation pattern was altered at relapse; 4 (36.3%) patients gained new mutations and 3 (27.3%) experienced loss of one initial mutation.

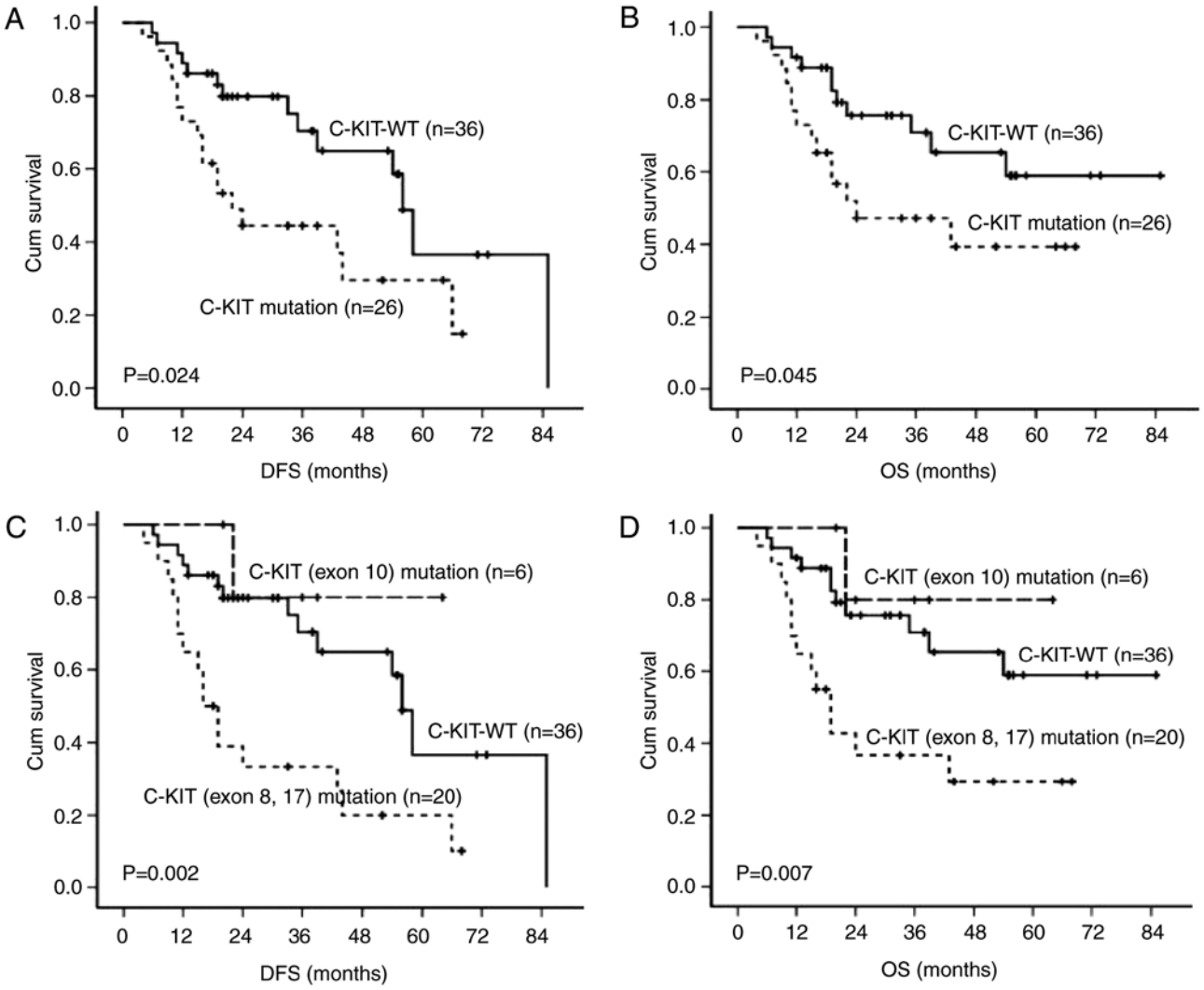

Figure 3. *c-KIT (exon 8, 17)* mutation confers an adverse effect on DFS and OS. *c-KIT*-WT vs. *c-KIT* mutation: (A) DFS: 55.0±6.1 vs. 34.2±5.0%, P=0.024; (B) OS: 61.1±5.8 vs. 37.6±5.4%, P=0.045. *c-KIT*-WT vs. *c-KIT (exon 8, 17)* mutation vs. *c-KIT (exon 10)* mutation: (C) DFS: 55.0±6.1 vs. 28.2±5.1 vs. 55.6±7.5%, P=0.002; (D) OS: 61.1±5.8 vs. 31.6±5.9 vs. 55.6±7.5%, P=0.007. *c-KIT*, KIT proto-oncogene, receptor tyrosine kinase; DFS, disease-free survival; OS, overall survival; WT, wild-type.

Mutations commonly gained at relapse were *KMT2A* (2/11, 18.2%), followed by *c-KIT* and *TET2* (each n=1, 9.1%). Loss of initial mutation at relapse was observed in *c-KIT* (2/11, 18.2%), as well as *NRAS* (1/11, 9.11%). The *c-KIT* mutation was the most common molecular event in the relapsed patients; 7/11 (63.6%) were associated with *c-KIT* mutation at relapse (1 gained, 2 lost and 4 stable) (Fig. 2).

Patient survival and the influence of gene mutations on survival. Among the 64 patients, 1 patient declined further chemotherapy and was excluded from the outcome analysis; 63 patients completed one to two courses of induction chemotherapy, and 59 (93.7%) gained CR. Among the 4 patients with no remission, 1 patient declined further treatment and was excluded from the survival analysis, and the other 3 patients received salvage therapy (two with CAG regimen and one with FLAG) and all obtained CR. After acquiring CR, 31 patients received SDAC-based consolidation; the other 31 received IDAC-based regimens; 13 cases underwent allo-HSCT, of which 7 were in the SDAC-based group and 6 were in the IDAC-based group. Between the two groups, except for a lower hemoglobin level, and a higher incidence of *c-KIT (exon 8, 17)* and *ASXL1* mutation in the SDAC-based group, there was no significant difference in the clinical characteristics (Table I). With a median follow-up of 23.5 (4-85) months, cumulative 18/55 (32.7%) patients obtained MMR after two courses of consideration, 31 (50.0%) patients relapsed, and 25 (40.3%) cases succumbed to the disease. The 3-year DFS and OS percentages were 50.0±7.0 and 55.0±7.0%, respectively. Two patients died of treatment-related diseases, the 23 died of leukemia progression. Survival analysis showed that the patients with *c-KIT* mutation had a lower rate of DFS (P=0.024) and OS (P=0.045) when compared with the DFS and OR of those with *c-KIT*-WT (Fig. 3A and B). When *c-KIT* mutations were sub-grouped into exon 8, 17 and exon 10, DFS and OS analysis showed no obvious difference in the patients with *c-KIT (exon 10)* mutation and WT (P>0.05), yet DFS and OS were prolonged in both these groups when compared with the DFS and OS in the *c-KIT (exon 8, 17)* mutation group (P<0.05) (Fig. 3C and D), suggesting that *c-KIT (exon 8, 17)*

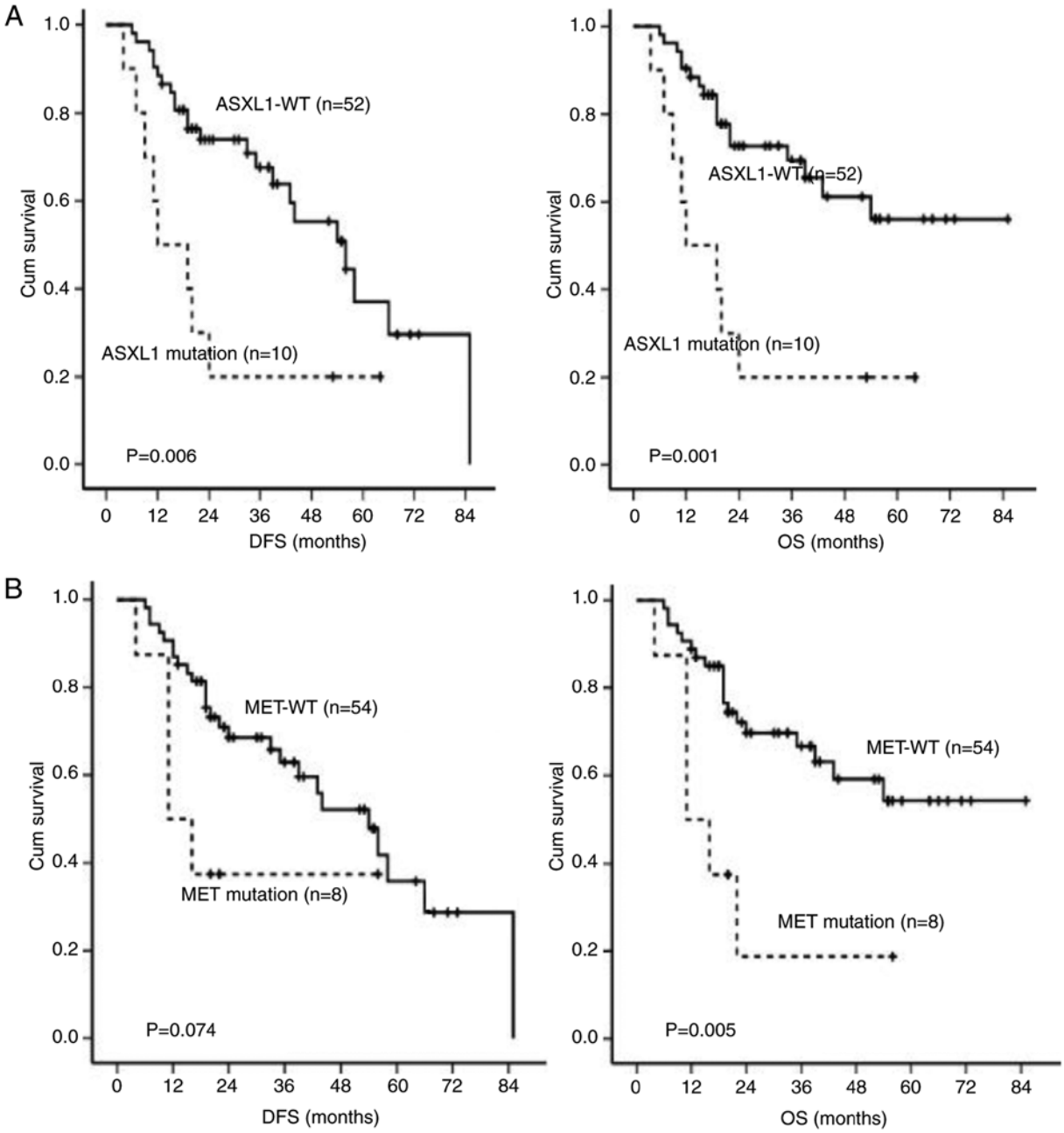

Figure 4. *ASXL1*, *MET*, *TP53* and *FBXW7* mutations displayed a poor prognostic effect. (A) *ASXL1*-WT vs. *ASXL1* mutation: DFS: 51.3±4.8 vs. 23.4±6.7%, P=0.006; OS: 59.1±5.0 vs. 23.4±6.7%, P=0.001. (B) *MET*-WT vs. *MET* mutation: DFS: 49.4±4.7 vs. 27.6±7.8%, P=0.074; OS: 57.5±4.9 vs. 21.3±6.7%, P=0.005. *ASXL1*, ASXL transcriptional regulator 1; *MET*, MET proto-oncogene, receptor tyrosine kinase; *TP53*, tumor protein P53; *FBXW7*, F-box and WD repeat domain containing 7; DFS, disease-free survival; OS, overall survival; WT, wild-type.

but not exon 10 mutation adversely affects patient survival. Univariate analysis showed that *ASXL1* and *TP53* mutations were adverse factors for DFS (P<0.05), and *ASXL1*, *MET*, *FBXW7* and *TP53* mutations had a poor effect on OS (P<0.05) (Fig. 4 and Table II). When the number of gene mutation was taken into account, increasingly adverse RFS and OS were observed with increasing number (Fig. 5).

Multivariate analysis data revealed that *c-KIT (exon 8, 17)* (RFS: HR 3.36, 95% CI 1.54-7.34, P=0.002; OS: HR 2.84, 95% CI 1.20-6.71, P=0.018) and *ASXL1* mutations (RFS: HR 3.13, 95% CI 1.34-7.32, P=0.009; OS: HR 3.94, 95% CI 1.62-9.61, P=0.003) were independent adverse factors for both RFS and OS (Table II). When consolidation regimens were taken into consideration, the same result was shown. Among the patients with IDAC-based regimens, the cohort with *c-KIT (exon 8, 17)* mutation had significantly poorer DFS and OS; also among those with SDAC-based regimens, patients with the mutation had apparently, although not significantly, worse DFS and OS. Meanwhile, significantly poorer DFS and OS were observed in the *ASXL1*-mutant patients with SDAC-based regimens (Fig. S3). Furthermore, according to *c-KIT (exon 8, 17)* and *ASXL1* mutation or not, we divided all the 62 patients into three groups, as no mutation (n=37), one mutation (n=20) and two mutation groups (n=5), and found that the patients with two mutations had the worst DFS and OS, followed by those with one mutation; no mutation patients had the best DFS

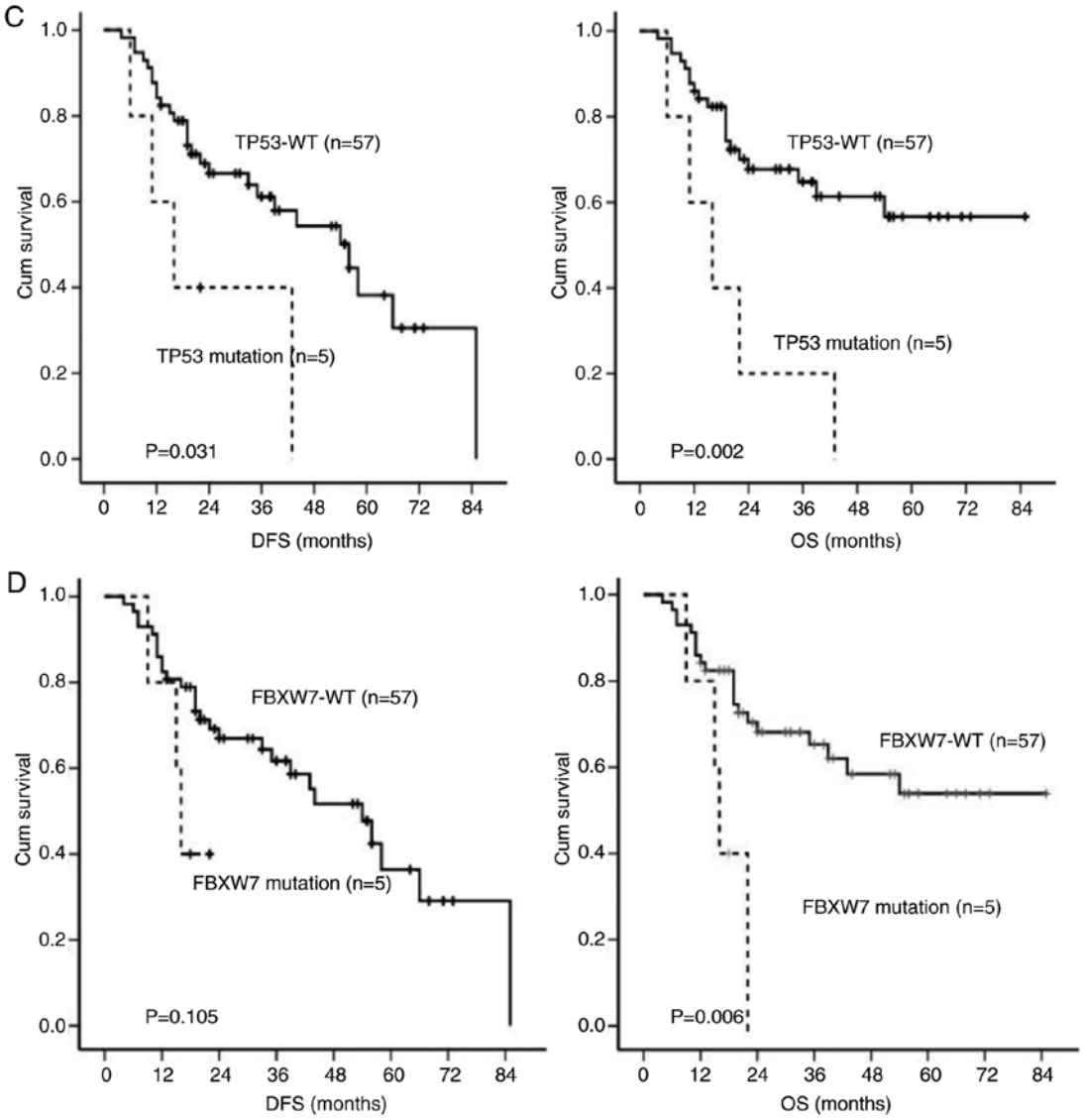

Figure 4. Continued. *ASXL1*, *MET*, *TP53* and *FBXW7* mutations displayed a poor prognostic effect. (C) *TP53*-WT vs. *TP53* mutation: DFS: 49.6±4.7 vs. 23.8±8.3%, P=0.031; OS: 57.8±4.8 vs. 19.6±6.4%, P=0.002. (D) *FBXW7*-WT vs. *FBXW7* mutation: DFS: 48.7±4.6 vs. 16.8±2.2%, P=0.105; OS: 56.6±4.8 vs. 16.8±2.5%, P=0.006. *ASXL1*, ASXL transcriptional regulator 1; *MET*, MET proto-oncogene, receptor tyrosine kinase; *TP53*, tumor protein P53; *FBXW7*, F-box and WD repeat domain containing 7; DFS, disease-free survival; OS, overall survival; WT, wild-type.

and OS (Fig. 6A and B), indicating that co-mutation of *c-KIT (exon 8, 17)* and *ASXL1* had a worse effect on DFS and OS than a single mutation. When the therapeutic regimens were taken into account, the same result was observed in either SDAC-based or IDAC-based group, although the difference was not significant in the IDAC-based group (Fig. 6C-F).

Discussion

The *AML1-ETO* fusion gene cannot singly induce leukemia but instead cooperates with secondary mutations to induce leukemia, presenting a '2-hit model' in leukemia development (3,16,17). Moreover, data have revealed a multitude of genetic and epigenetic aberrations in leukemogenesis (18,19). In accordance with these, the present study showed that up to 44/64 (68.8%) patients presented a median of 2 (1-10) recurrent mutations at diagnosis and 6/11 (54.5%) cases underwent genetic alterations at relapse. The most common mutations at diagnosis occurred in tyrosine kinase, followed by chromatin modification, transcription, DNA methylation, tumor suppression and miscellaneous function. Among those mutations, tyrosine kinase, DNA methylation and tumor suppression were found to be commonly co-mutated, shown as mutual exclusion of *c-KIT (exon 8, 17)* or *MET* with *DNMT3A* or *FBXW7* or *TP53*, and *DNMT3A* with *FBXW7* or *TP53*, supporting multi-oncogenic cooperation in leukemogenesis.

In line with previous studies (4,19), the *c-KIT* mutation was the most common molecular event in this study, with an incidence of 27/64 (42.2%) at diagnosis, and 7/11 being associated with molecular alteration at relapse, further supporting, from the clinical point of view, that *c-KIT* mutation plays a critical role in the development and progression of *AML1-ETO*-positive acute myeloid leukemia (AE-AML). As the *c-KIT* mutation is well accepted as a poor factor in this subtype of leukemia (1,11,20), to further clarify which exon mutation performs the key role, according to the mutant

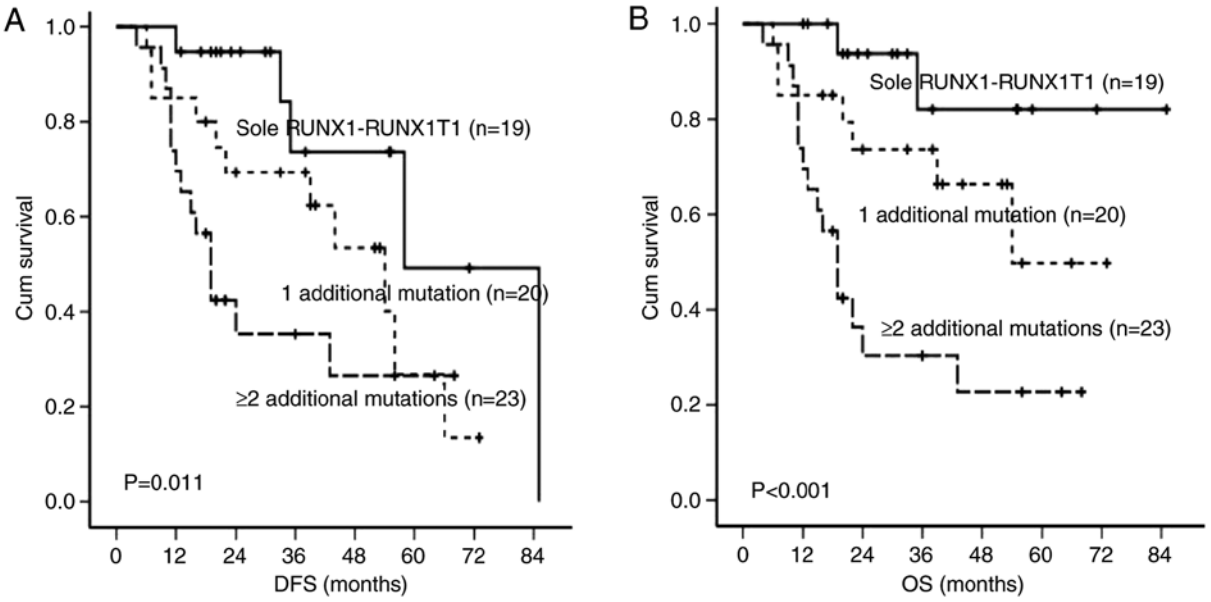

Figure 5. Increasing mutation number confers an increasingly adverse effect on patient prognosis. Sole *RUNX1-RUNX1T1* vs. 1 additional mutation vs. ≥2 additional mutations: (A) DFS: 63.8±9.2 vs. 44.0±5.5 vs. 31.1±5.4%, P=0.011; (B) OS: 75.0±6.6 vs. 51.5±6.3 vs. 29.0±5.1%, P<0.001. *RUNX1*, RUNX family transcription factor 1 [also known as acute myeloid leukemia 1 protein (AML1)]; *RUNX1T1*, RUNX1 partner transcriptional co-repressor 1 [also known as eight twenty one protein (ETO)]; DFS, disease-free survival; OS, overall survival.

exon, we divided the patients with *c-KIT* mutation into exon 8, 17 and exon 10 groups, and found that, agreeing with previous studies (4,21), *c-KIT (exon 8, 17)* but not exon 10 had an adverse effect on disease-free survival (DFS) (exon 8, 17 vs. exon 10 vs. WT: 28.2±5.1% vs. 55.6±7.5% vs. 55.0±6.1%, P=0.002) and overall survival (OS) (31.6±5.9% vs. 55.6±7.5% vs. 61.1±5.8%, P=0.007). Furthermore, univariate analysis supported the independent adverse effect of *c-KIT (exon 8, 17)* mutation on survival [DFS: HR 3.36 (1.54-7.34), P=0.002; OS: HR 2.84 (1.20-6.71), P=0.018]. Also the same result was observed when treatment regimens were taken into consideration.

The *ASXL1* mutation has also been reported to occur frequently in AE-AML and is associated with a poor disease outcome. Krauth *et al* (4) reported that 11.5% patients with AE-AML presented with the *ASXL1* mutation, and compared with WT, *ASXL1*-mutated patients had poorer RFS (*de novo* AML: 20 vs. 59.1%, P=0.011; total cohort: 28.6 vs. 56.7%, P=0.021). Micol *et al* (22) found that 11/110 (10.0%) patients with AE-AML presented with an *ASXL1* mutation, and those with this mutation had a trend for increasing risk for relapse (54.6 vs. 25%, P=0.226). In the present study, *ASXL1* mutation was the second most common mutation with an incidence of 15.6%, and was found to adversely impact RFS (51.3±4.8% vs. 23.4±6.7%, P=0.006) and OS (59.1±5.0% vs. 23.4±6.7%, P=0.001). In addition, univariate analysis data showed that the *ASXL1* mutation was an independent adverse factor for survival [DFS: HR 3.13 (1.34-7.32), P=0.009; OS: HR 3.94 (1.62-9.61), P=0.003]. Also the same result was observed when the treatment regimens were taken into account, suggesting that the role of the *ASXL1* mutation in AE-AML warrants further attention.

The *MET* (*c-MET*) and *TP53* mutations were found to be two more important adverse factor for DFS and OS in the present study. It is known that *MET* plays an important role in cell proliferation and differentiation, and its aberrant expression, which could be activated by gene mutation, is found to be associated with promotion of tumorigenesis (23). The *MET* mutation has been reported to be involved in the development and metastasis of various solid cancers, and adversely affects the disease outcome (24,25). Autocrine activation of the MET signaling pathway was found in almost 40% of patients with AML (26,27), and increased *MET* activation was associated with leukemia relapse, especially in the t(15;17) and t(8;21) cytogenetic subtypes (28). However, the *MET* mutation in AML is rarely reported. To our interest, in this series, 8 out of 64 (12.5%) patients harbored *MET* mutation and presented a poor outcome, suggesting that MET aberrant activation in AE-AML may be associated with *MET* mutation; furthermore, MET mutation may act as a poor prognostic factor in AE-AML. *TP53* is a central tumor-suppressor gene, which is involved in cell cycle regulation and apoptosis induction (29). The tumor-suppressor activity of the protein is typically abolished and reverts to having a negative impact on survival when *TP53* mutates (30-32), of which the most frequent type in tumors is missense and deletion mutations, although gain-of-function mutations have also been described (33). It has been reported that AML1-ETO activates the p53 pathway and then sensitizes leukemia cells to DNA damage (34), while loss of the p53 response pathway is associated with disease progression. However, the incidence and biological effect of the *TP53* mutation in AE-AML remains unclear. In this series, 5 (7.8%) patients were detected to harbor *TP53* mutations; 2 presented with missense mutations and 3 with deletions, and these patients presented with poor DFS and OS.

In addition, in agreement with previous research (4), the number of gene mutations was important in regards to the prognostic effect in our study; an increasingly poor RFS and OS with an increase in the mutation number was observed in the whole cohort. Also, co-mutation of *c-KIT (exon 8, 17)* and

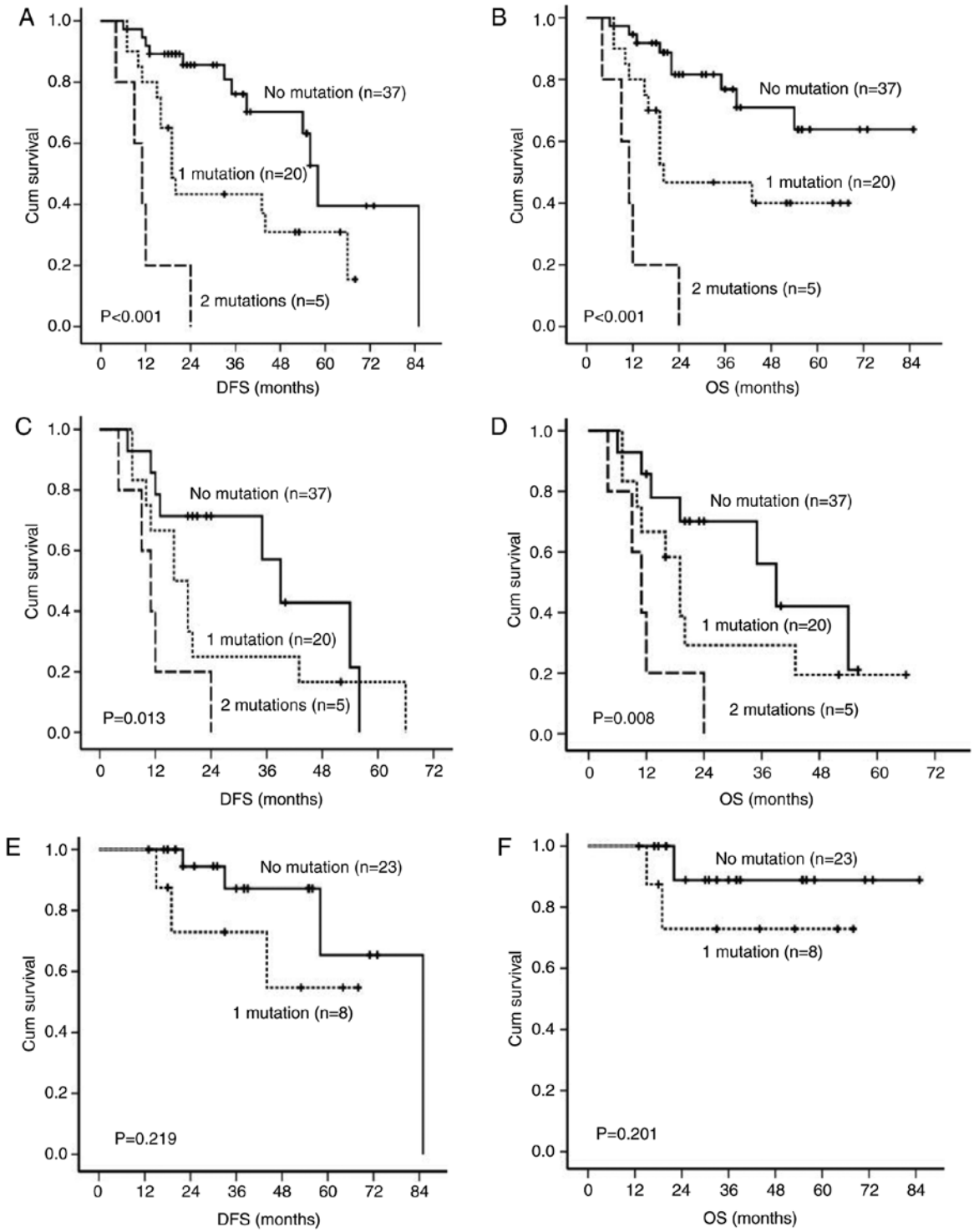

Figure 6. Co-mutation of *c-KIT (exon 8, 17)* and *ASXL1* mutation exhibited a worse effect on survival than the single mutation. *c-KIT (exon 8, 17)* and *ASXL1* mutations were multi-variately analyzed to be independent adverse factors for both DFS and OS. According to the absence or presence of the mutants of these two genes, all patients were divided into three groups: no mutation vs. 1 mutation vs. 2 mutations: (A) DFS: 58.4±6.1 vs. 34.4±5.5 vs. 12.0±3.3%, P<0.001; (B) OS: 65.0±5.6 vs. 37.9±6.0 vs. 12.0±3.3%, P<0.001. Among the patients receiving SDAC-based regimens for consolidation therapy, there was a significant difference in the DFS and OS among these groups. No mutation vs. 1 mutation vs. 2 mutations: (C) DFS: 37.1±5.7 vs. 25.0±6.2 vs. 12.0±3.3%, P=0.013; (D) OS: 37.2±5.7 vs. 26.9±6.5 vs. 12.0±3.3%, P=0.008. Among those receiving IDAC-based regimens, an apparent, though not significant, difference in the DFS and OS was observed between the patients with no mutation and 1 mutation. No mutation vs. 1 mutation: (E) DFS:71.8±7.7 vs. 49.9±8.3%, P=0.219; (F) OS: 78.0±4.7 vs. 54.2±84%, P=0.201. c-KIT, KIT proto-oncogene, receptor tyrosine kinase; ASXL1, ASXL transcriptional regulator 1; DFS, disease-free survival; OS, overall survival; SDAC-based, standard-dose Ara-C-based regimen; IDAC-based, intermedium-dose Ara-C-based regimen.

ASXL1 mutations conferred a worse effect on RFS and OS, as compared with a single mutation, further supporting the multi-oncogenic cooperation in cancer progression.

In summary, additional gene mutations play a critical role in AE-AML. This was demonstrated by the fact that 44/64 (68.8%) patients presented with recurrent mutations at

diagnosis and 6/11 (54.5%) cases underwent genetic alterations at relapse. The *c-KIT* mutation appears to be the most common molecular event in this subtype of leukemia, followed by *ASXL1*. *c-KIT (exon 8, 17)* but not exon 10, and also the *ASXL1* mutation have a negative impact on survival; co-mutation of these two gene shows an even worse effect. The prognostic effect of *MET* and *TP53* mutations in AE-AML warrants further study.

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Trial registration website: chictr.org.cn: Registration number: ChiCTR1900024337. Registered date: August 21, 2019. This clinical trial was retrospectively registered.

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Availability of data and materials

The corresponding author can be contacted for all requests for data.

Authors' contributions

GY and CY performed investigations, gene mutation and expression detection, analyzed data and wrote the paper. FW performed the gene mutation analysis and analyzed the data. LJ and ZZ analyzed the data. DX, XJ, JZ and QL performed the investigations. FM designed the study and wrote the paper. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All samples and clinical data were obtained upon approval of the Nanfang Hospital, Southern Medical College of Medicine Institutional Review Boards. The reference number is [2012] ethical review (115). All patients and/or guardians provided written informed consent. The content of the informed consent included the agreement of patient clinical data being published in any form.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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