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ORIGINAL RESEARCH

Acute myeloid leukemia patient with *FLT3-ITD* and *NPM1* double mutation should undergo allogeneic hematopoietic stem cell transplantation in CR1 for better prognosis

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Background: According to the recent National Comprehensive Cancer Network (NCCN) guidelines, the risk level in acute myeloid leukemia (AML) patients with *FLT3-ITD* and *NPM1* double mutation (AML$^{FLT3\text{-}ITD+/NPM1+}$) depends on the allelic ratio of *FLT3-ITD*. But despite a low or high allelic ratio of *FLT3-ITD*, AML$^{FLT3\text{-}ITD+/NPM1+}$ patients belong to the favorable or intermediate risk, for whom allogeneic stem cell transplantation is not obligated. However, some latest studies pointing out that *NPM1* and *FLT3-ITD* double mutation patients showed an inferior prognosis, which have raised concern about the risk categorization and more effective treatment of AML$^{FLT3\text{-}ITD+/NPM1+}$ patients.

Methods: A total of 76 patients were selected for coexisting *FLT3* and *NPM1* mutations with normal cytogenetics. The prognostic risk factors were analyzed, and treatment strategies including allogeneic stem cell transplantati1on and chemotherapy were compared.

Results: In 76 AML$^{FLT3\text{-}ITD+/NPM1+}$ patients, 36.8% of patients had hyperleukocytosis (HL) and *DNMT3A R882* mutation was the most common concomitant gene (23.7%). For 53 patients in the complete remission (CR), 22 had received allogeneic hematopoietic stem cell transplantation (allo-HSCT) on first complete remission (CR1). Patients in transplantation group had better overall survival (OS) and disease-free survival (DFS) than chemotherapy only (P=0.002 and 0.001, respectively). In multivariable Cox model analyses, HL and *DNMT3A R882* mutation were independent adverse prognostic factors (all P<0.05) for AML$^{FLT3\text{-}ITD+/NPM1+}$ patients. Nevertheless, allo-HSCT was an independent good factor of OS and DFS (P=0.001 and 0.000; HR =0.173 and 0.138; 95% CI were 0.062–0.483 and 0.049–0.389). And allo-HSCT could moderately improve the poor prognosis of AML $^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882+}$.

Conclusion: Although, AML$^{FLT3\text{-}ITD+/NPM1+}$ patients are categorized as favorable or intermediate risk levels according to recent NCCN and ELN guidelines, these patients should receive allo-HSCT in CR1 for a longer survival. AML$^{FLT3\text{-}ITD+/NPM1+}$ patients with *DNMT3A R882* mutation had a very poor prognosis, and allo-HSCT could moderately improve their survival.

Keywords: *FLT3-ITD*, *NPM1*, *DNMT3A R882*, allo-HSCT

Introduction

FMS-like tyrosine kinase 3 (*FLT3*) belongs to the receptor tyrosine kinase class III, and is specifically expressed on hematopoietic progenitor cells. *FLT3* plays a role in cell survival, proliferation and differentiation of hematopoietic progenitor cells.[1]

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FLT3 gene is one of the most frequently mutated genes in acute myeloid leukemia (AML), and is reported in 25–30% of AML patients.[2,3] There are two types of *FLT3* mutation, internal tandem duplication of *FLT3* (*FLT3-ITD*) and tyrosine kinase domain of *FLT3* (*FLT3-TKD*). *FLT3-ITD* is the major type and reported among 20–30% AML patients,[4] while *FLT3-TKD* in only found in about 7% AML patients.[5–7] *FLT3-ITD* is associated with adverse disease features, including high initial peripheral white blood cell (WBC) count, high early recurrence rate and a low overall survival (OS) rate.[3,5,8,9] According to NCCN and ELN, *FLT3-ITD* mutation with normal cytogenetics has a poor risk prognosis. *NPM1* is a gene for expression of nucleophosmin, which belongs to nucleophosmin/nucleoplasmin family of proteins.[10] *NPM1* mutations happen in 45–64% adult AML cases.[11–14] With normal cytogenetics profile, AML with *NPM1* mutation (AML^{NPM1+}) has a favorable prognosis, but when coexisted with *FLT3-ITD*, the risk level of AML depends on the allelic ratio (AR) of FLT3-ITD. *NPM1* mutation with low AR of *FLT3-ITD* was considered as favorable-risk group, but when combined with high AR was classified as intermediate-risk group.[15] Despite a low or high AR of *FLT3-ITD*, $AML^{FLT3\text{-}ITD+/NPM1+}$ patients belong to the favorable or intermediate risk according to the recent NCCN guidelines. These group of patients are not obligated to receive allo-HSCT. However, this risk classification on *FLT3-ITD* and *NPM1* double mutated AML was not accepted by some clinicians, and several studies provided evidence that this type of AML is with unfavorable risks.[16–21] What is the optimal treatment for $AML^{FLT3\text{-}ITD+/NPM1+}$ patients is also under investigation. In this study, we retrospectively analyzed the clinical features and risk factors of $AML^{FLT3\text{-}ITD+/NPM1+}$, and discussed whether hematopoietic stem cell transplantation is necessary after complete remission (CR).

Material and methods

Patients

We performed an individual patient data-based retrospective analysis of 76 patients evaluated at our hospital between July 2009 and March 2018, who were diagnosed as AML with positive mutation in *FLT3-ITD* and *NPM1*. Patients with acute promyelocytic leukemia were excluded. Written informed consent was obtained from all patients. This study was approved by the Human Research Ethics Committee of Zhejiang University. Details, such as patient age and sex, WBC counts at diagnosis, percentage of blast cells in bone marrow, AML French-American-British classification subtypes, karyotype, recurrent fusion genes such as *PML-RARa; ETO; CBF$_\beta$* and combined mutation genes as *FLT3-ITD, NPM1, DNMT3A R882□CEBPA, KIT, IDH1/IDH2, TET2*; treatment regimens, and response to therapy were reviewed. Hyperleukocytosis (HL) is defined as the peripheral WBC counts is above 100*109/L at diagnosing.

Part of the patients' data is listed in Table 1.

Gene mutation analyses

Bone marrow mononuclear cells were isolated and the DNA extracted using a DNA Extraction kit (Invitrogen, Shanghai, People'sRepublic of China). The forward primer of *FLT3-ITD* was 5′-GCAATTTAG-GTATGAAAGCCAGC −3′, the reverse primer is 5′-CTTTCAGCATTTTGACGGCAACC-3′. The forward primer of *NPM1* gene is 5′-TGTCTATGAAGTGTTGTGGTTCC-3′, the reverse primer is 5′-GGACAGCCAGATATCAACTG-3′. The forward primer of *DNMT3A* gene is 5′-GTAAAACGACGGCCAGT CCTCTCTCCCACCTTTCCTC-3′, the reverse primer was 5′-CAGGAAACAGCTATGACCCTGAGTGCCGGGTTGT TTAT-3′. All PCR primers were linked with M13F/R universal primer. The total volume of the PCR reaction system was 20 μL, including 200 ng DNA, 20 pmol PCR primers, 25 mmol/L $MgCl_2$, 2.5 mmol/L dNTP, 2 μL 10× PCR buffer, 0.2 μL HotTaq DNA polymerase (Qiagen, Shanghai, People'sRepublic of China). Reaction conditions: denaturation at 94°C for 5 mins, 94°C for 30 s, 58°C for 40 s and 72°C for 1 min for 35 cycles. PCR products were then sequenced by ABI 3500 Genetic Analyzer (Applied Biosystems). *CEBPA, KIT, IDH1*/*IDH2* and *TET2* mutations were analyzed by next-generation sequencing technology (San Valley Diagnostics).

Treatments

All 76 patients adopted the IA scheme (idarubicin and cytarabine; 62cases) or HAA scheme (harringtonine, aclacinomycin and cytarabine; 14cases) for induced chemotherapy. After achieving CR, they were then treated with another course of IA or HAA, and then all patients were treated with intermediate-dose cytarabine (2.0/m^2) for 2–3 courses, coupled with standard dose chemotherapies composed with aclacinomycin, cytarabine, etoposide, harringtonine, idarubicin and mitoxantrone as consolidation chemotherapies. When relapsed, patients were treated with FLAG (fludarabine/cytarabine/granulocyte colony-stimulating factor) or the

Table 1 Patients' characteristics

Variable	All patients (n =76)	Allo-HSCT (n=22)	Chemotherapy (n=31)	*P*-value	*DNMT3A R882* mutated (n=18)	*DNMT3A R882* unmutated (n=43)	*P*-value	HL (n=28)	Non-HL (n=48)	*P*-value
Age, years (%)				0.007			0.197			0.024
<60	58(76.3)	22(100.0)	22(71.0)		16(88.9)	31(72.1)		17(60.7)	41(85.4)	
≥60	18(23.7)	0	9(29.0)		2(11.1)	12(27.9)		11(39.3)	7(14.6)	
Gender (%)				0.574			0.404			1.000
Male	36(47.4)	11(50.0)	12(38.7)		6(33.3)	20(46.5)		13(46.4)	23(47.9)	
Female	40(52.6)	11(50.0)	9(61.3)		12(66.7)	23(53.5)		15(53.6)	25(52.1)	
HL (%)				1.000			0.559	/	/	/
Yes	28(36.8)	6(27.3)	9(29.0)		7(38.9)	13(30.2)		/	/	/
No	48(63.2)	16(72.7)	22(71.0)		11(61.1)	30(69.8)		/	/	/
BM blast (%)				0.275			0.597			0.021
Median(range)	79.5(22.0–97.0)	72.5(22.0–97.0)	79.0(41.0–93.5)		79.5(51.0–95.0)	80.0(22.0–97.0)		85.0 (43.5–95.0)	76.5(22.0–97.0)	
FAB type				1.000			0.001			0.296
M0	2(2.6)	1(4.5)	1(3.2)		1(5.6)	1(2.3)		1(3.6)	1(2.1)	
M1	12(15.8)	4(18.2)	5(16.1)		1(5.6)	9(20.9)		3(10.7)	9(18.8)	
M2	36(47.4)	10(45.5)	15(48.4)		4(22.2)	25(58.1)		11(39.3)	25(52.1)	
M4	1(1.3)	0	1(3.2)		0	1(2.3)		1(3.6)	0	
M5	25(32.9)	7(31.8)	9(29.0)		12(66.7)	7(16.3)		12(42.9)	13(27.1)	
Induction regimen				0.431			1.000			0.760
IA	62(81.6)	18(81.8)	28(90.3)		16(88.9)	37(86.0)		22(78.6)	40(83.3)	
HAA	14(18.4)	4(18.2)	3(9.7)		2(11.1)	6(14.0)		6(21.4)	8(16.7)	
DNMT3A R882(%)				0.755	/	/	/			0.259
Unmutated*	43(56.6)	16(72.7)	20(64.5)		/	/	/	13(46.4)	30(62.5)	
Mutated	18(23.7)	5(22.7)	8(25.8)		/	/	/	7(25.0)	11(22.9)	
Unknow	15(19.7)	1(4.5)	3(9.7)		/	/	/	8(28.6)	7(14.6)	
KIT (%)				1.000			1.000			1.000
Wild type	75(98.7)	22(100.0)	30(96.8)		0	1(2.3)		28(100.0)	47(97.9)	
Mutated type	1(0.3)	0	1(3.2)		18	42(97.7)		0	1(2.1)	

(*Continued*)

Table 1 (Continued).

Variable	All patients (n =76)	Allo-HSCT (n=22)	Chemotherapy (n=31)	*P*-value	*DNMT3A R882* mutated (n=18)	*DNMT3A R882* unmutated (n=43)	*P*-value	HL (n=28)	Non-HL (n=48)	*P*-value
IDH1(%)				0.633			0.309			1.000
Wild type	71(93.4)	21(95.5)	28(90.3)		18(100.0)	38(88.4)		26(92.9)	45(93.8)	
Mutated type	5(6.6)	1(7.4)	3(9.7)		0	5(11.6)		2(7.1)	3(6.3)	
IDH2(%)				0.683			1.000			0.042
Wild type	69(90.8)	19(86.4)	28(90.3)		16(88.9)	38(88.4)		28(100.0)	41(85.4)	
Mutated type	7(9.2)	3(13.6)	3(9.7)		2(11.1)	5(11.6)		0	7(14.6)	
TET2				0.563			0.073			0.623
Wild type	72(94.7)	20(90.9)	30(96.8)		15(83.3)	42(97.3)		26(92.9)	46(95.8)	
Mutated type	4(5.3)	2((9.1)	1(3.2)		3(16.7)	1(2.3)		2(7.1)	2(4.2)	
Therapy (%)		/	/	/			0.564			0.306
Chemotherapy	0	/	/	/	13(72.2)	27(62.8)		22(78.6)	32(66.7)	
Allo-HSCT	22(100.0)	/	/	/	5(27.8)	16(37.2)		6(21.4)	16(33.3)	
CR (%)							0.319			0.005
Yes	53(69.8)	/	/	/	13(72.2)	36(83.7)		15(53.6)	38(79.2)	
No	15(19.7)	/	/	/	4(22.2)	4(9.3)		11(39.3)	4(8.3)	
Unknown	8(10.5)	/	/	/	1(5.6)	3(7.0)		2(7.1)	6(12.5)	

Abbreviations: HL, hyperleukocytosis; BM, bone marrow; Allo-HSCT, allogeneic hematopoietic stem cell transplantation; CR, complete remission.
Note: **DNMT3A R882* unmutated includes *DNMT3A* wild type and *DNMT3A non-R882* mutation.

CLAG (cladribine/cytarabine/granulocyte colony-stimulating factor), or decitabine+CAG (cytarabine, aclacinomycin and granulocyte colony-stimulating factor) for re-induction therapy. Twenty-two patients received allogeneic hematopoietic stem cell transplantation (allo-HSCT) at CR1. We adopted a myeloablative pretreatment scheme based on busulfan, cyclophosphamide before transplantation, and used mycophenolate combined with cyclosporine A plus methotrexate to prevent graft-versus-host disease (GVHD). For those patients who received HLA-haploidentical allo-HSCT, antithymocyte globulin was added to prevent GVHD.

Statistical analyses

SPSS Statistics (Version 23.0. Armonk, NY: IBM Corp.) was used for statistical analyses. We used a chi-square test for comparisons between sample rates including clinical characteristics, protocol and CR rate. OS and disease-free survival (DFS) were analyzed by the Kaplan–Meier method, risk factor analysis was analyzed by Cox Regression method, and the log-rank test was adopted to compare differences between groups. The P-value <0.05 was considered to be significantly different.

Results

Biological and clinical characteristics

We identified 76 AML patients coexistent with *FLT3-ITD* and *NPM1* mutations. All patients were with normal conventional cytogenetics. The median age was 50 years (range, 14–71 years) with elderly patients (≥60 years) accounted for 23.7%. The male/female ratio was 0.9. The median percentage of bone marrow blast was 79.5% (range, 22.0–97.0%). WBC counts ranged from 1.6 to 229.6×10^9/L with a median of 62.2×10^9/L, and 36.8% of patients were hyper-leukocytosis at diagnosis. 18/76 (23.7%) detected *DNMT3A R882* mutation, and 15/76 (19.7%) patients were not detected or unknown. In order to remove the effects from CEBPA, patients with *CEBPA* double mutation were excluded. *KIT, IDH1, IDH2* and *TET2* mutations were also detected and the incidence rates were of no differences between transplantation and chemotherapy groups.

OS and DFS

After induction chemotherapy, 53/76 (69.8%) of patients obtained CR, 15/76 (19.7%) did not reach CR and 8/76 (10.5%) were unknown. Twenty-two patients underwent allo-HSCT at CR1 (Table 1). The median follow-up time was 20 months for all patients. At the end of the follow-up, 42 (55.3%) patients died and 19/53 (35.8%) relapsed. The median DFS time for all AML$^{FLT3\text{-}ITD+/NPM1+}$ patients was 8.1 months (range, 0–87.4 months), the median OS time was 12.2 months (range, 0.2–89.2 months) (Figure 1). To clarify the better treatment for these AML$^{FLT3\text{-}ITD+/NPM1+}$patients, allo-HSCT group (n=22) vs chemotherapy group (n=31) were compared. Transplantation group has better DFS and OS than chemotherapy group (medium OS: not reach vs 14.5 months, P=0.002; medium DFS: not reach vs 9.3 months, P=0.001) (Figure 2A and B). Among 44 patients received CR who were younger than 60 years. Transplant still significantly improved the prognosis (medium OS: not reach vs 12.3 months, P=0.004; medium DFS: not reach *vs* 8.1months, P=0.002) (Figure 2C and D).

Our study also showed that several AML-related gene mutations were co-existed in AML$^{FLT3\text{-}ITD+/NPM1+}$ patients, including *DNMT3A R882* (18/23.7%), *IDH2* (7/9.2%), *IDH1* (5/6.6%), *TET2* (4/5.3%) and *KIT* (1/0.3%). The incidence rates of all above gene mutations were equal in chemotherapy and transplantation groups (all P>0.05).

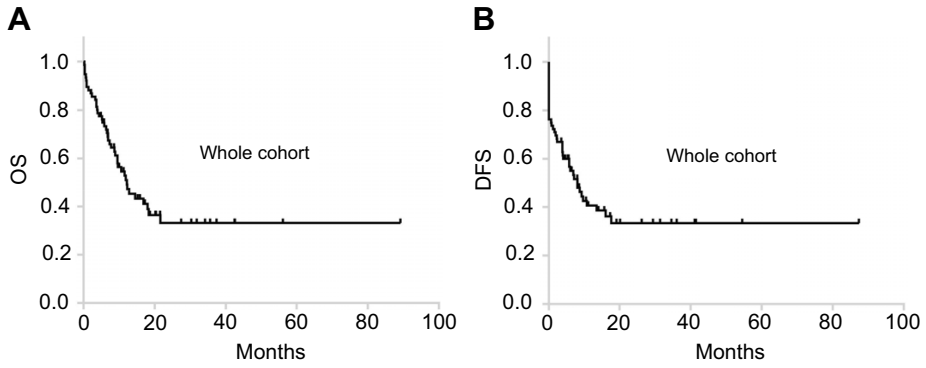

Figure 1 Prognostic analysis of all 76 patients. (**A**) Overall survival of all 76 AML$^{FLT3\text{-}ITD+/NPM1+}$ patients. (**B**) Disease-free survival of all 76 AML$^{FLT3\text{-}ITD+/NPM1+}$ patients.
Abbreviations: OS, overall survival; DFS, disease-free survival.

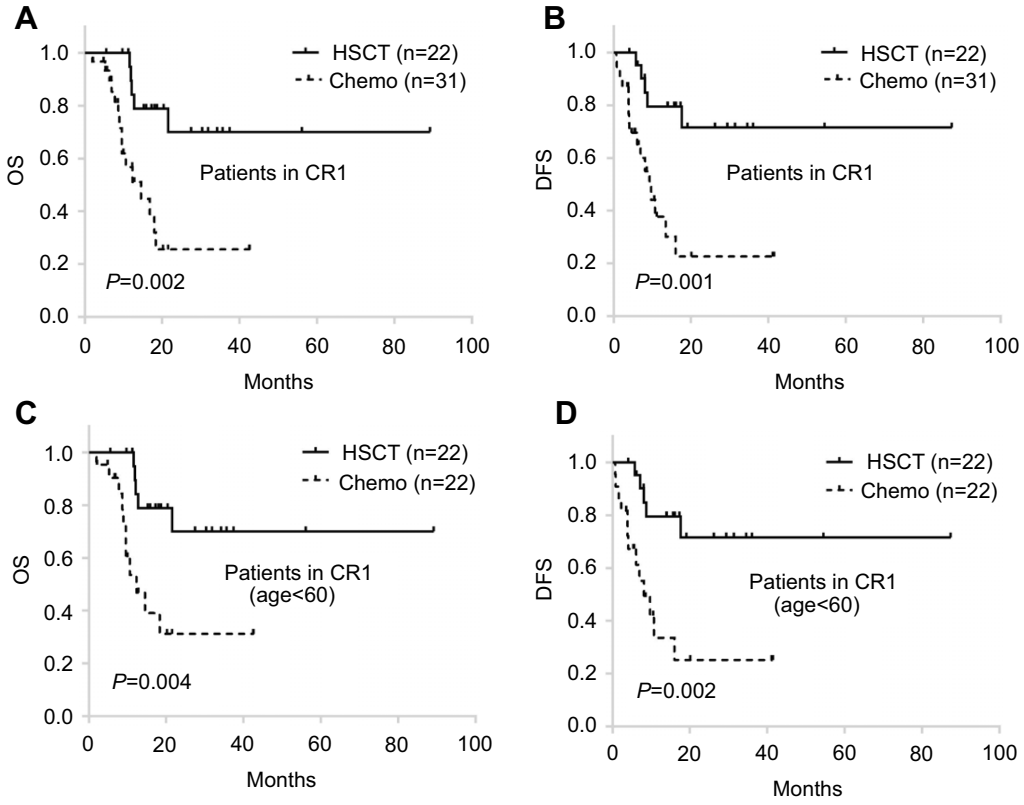

Figure 2 Comparison of chemotherapy and allo-HSCT in patients received CR1. (**A, B**): OS and DFS of the 53 AML$^{FLT3\text{-}ITD+/NPM1+}$ patients received CR1. (**C**, **D**) OS and DFS of the 44 AML$^{FLT3ITD+/NPM1+}$patients (age<60 years) received CR1.
Abbreviations: OS, overall survival; DFS, disease-free survival; HSCT, hematopoietic stem cell transplantation; chemo, chemotherapy; CR1, first complete remission.

Thus, the result that allo-HSCT had better prognosis than chemotherapy alone for AML$^{FLT3\text{-}ITD+/NPM1+}$ patients was not interfered by concomitant mutations.

In 76 AML$^{FLT3\text{-}ITD+/NPM1+}$ patients, 18 patients were also detected as *DNMT3A R882* mutation positive, with a percentage of 23.7%, which is similar to reports which is about 20% in all AML patients.[22] Depending on having *DNMT3A R882* mutation or not, AML$^{FLT3\text{-}ITD+/NPM1+}$ patients were divided into AML$^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882+}$group (n=18) and AML$^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882-}$group (n=43). AML$^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882+}$ patients had worse OS and DFS than AML$^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882-}$ patients (medium OS: 9.5 months vs not reach, *P*=0.007; medium DFS: 6.1 months vs not reach, *P*=0.002) (Figure 3A and B). There was no statistical difference in CR rate between *DNMT3A R882* mutated group and *DNMT3A R882* unmutated group (72.2% vs 83.7%, *P*=0.319, Table 1), but the cumulative incidence (CI) of relapse in the *DNMT3A R882* mutated group was significantly higher than the unmutated group (*P*=0.009) (Figure 5A). This indicates that *DNMT3A R882* mutation can increase the relapse rate of AML$^{FLT3\text{-}ITD+/NPM1+}$ patients, and the reduced survival of AML$^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882+}$group was caused by the high relapse rate but not the poor CR rate. To further investigate how to improve the poor prognosis of AML$^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882+}$ patients, we compared the OS and DFS in allo-HSCT and chemotherapy alone therapies. Results showed that allo-HSCT can significantly improve the OS and DFS both in AML$^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882+}$group and in AML$^{FLT3\text{-}ITD+/NPM1+/DNMT3\ R882-}$group (both *P*<0.001, Figure 3C–F). However, in AML$^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882+}$patients, the advantage in allo-HSCT group was not obvious, the 1-year OS rate was still <30%.

We also noticed that 28/76 patients had HL. The prognosis of HL group (n=28) vs. non-HL group (n=48) were analyzed, which showed that the HL group had worse OS and DFS (medium OS: 6.9 months vs 18.0 months, *P*=0.008; medium DFS: 3.8 months vs 13.5 months, *P*=0.009, Figure 4A and B). Patients with HL had significantly worse CR rate than those with non-HL (53.6% *vs* 79.2%, *P*=0.005) (Table 1), and there was no significant difference in CI of relapse rates between these two groups (*P*=0.371) (Figure 5B). So that the poor survival in HL

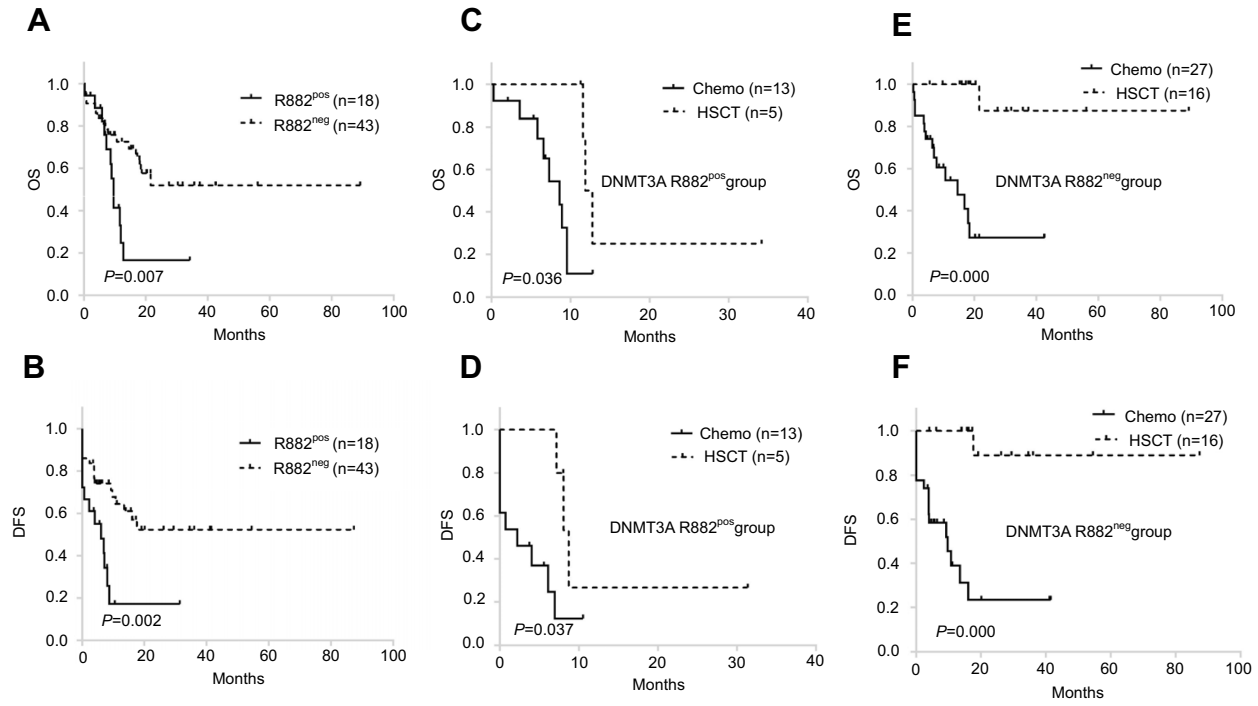

Figure 3 Comparison of outcome in different mutation groups. (**A, B**) OS and DFS of AML$^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882+}$ vs. AML$^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882-}$in 61 patients. (**C**, **D**) Comparison of the survival of chemotherapy group and allo-HSCT group in 18 AML$^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882+}$ patients. (**E**, **F**) Comparison of the survival of chemotherapy group and allo-HSCT group in 43 AML$^{FLT3ITD+/NPM1+/DNMT3A\ R882-}$patients.

Abbreviations: OS, overall survival; DFS, disease-free survival; pos, positive; neg, negative; HSCT, hematopoietic stem cell transplantation; chemo, chemotherapy.

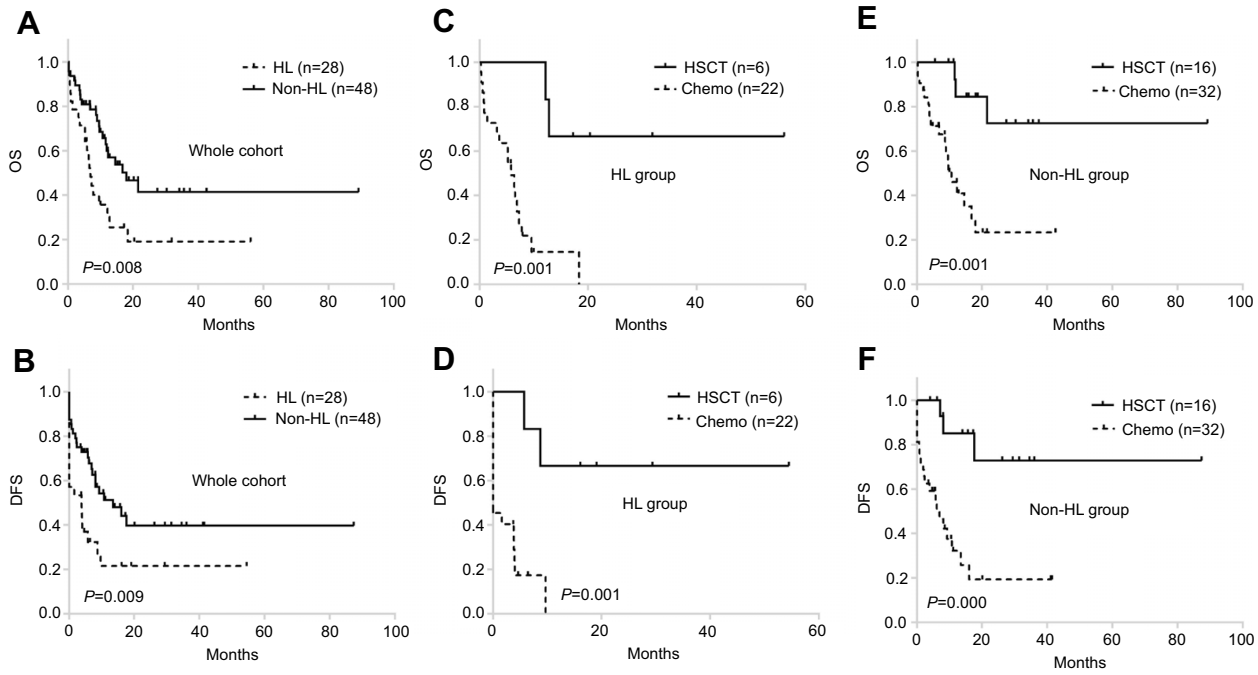

Figure 4 Comparison of outcome in different clinical groups associated with HL. (**A, B**) OS and DFS of HL vs. non-HL in all 76 patients. (**C**, **D**) Comparison the survival of chemotherapy group and allo-HSCT group in 28 patients with HL. (**E, F**) Comparison the survival of chemotherapy group and allo-HSCT group in 48 patients with non-HL.

Abbreviations: OS, overall survival; DFS, disease-free survival; HSCT, hematopoietic stem cell transplantation; chemo, chemotherapy; HL, hyperleukocytosis.

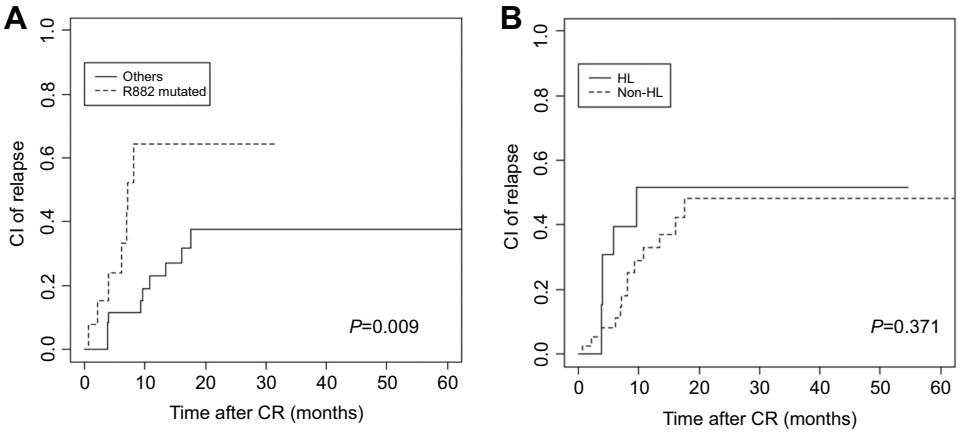

Figure 5 Comparison of relapse rate in different biological and clinical groups. (**A**) CI of relapse between AML$^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882+}$and AML$^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882-}$ in 49 patients received CR (*P*=0.009). (**B**) CI of relapse between HL and Non-HL in 53 patients received CR (*P*=0.371).
Abbreviations: CI, cumulative incidence; HL, hyperleukocytosis; CR, complete remission.

group is majorly caused by the inferior CR rate but not a high relapse rate. Allo-HSCT could prolong OS and DFS, no matter whether patients are with HL. (all $P<0.05$) (Figure 4C–F). There is no significant difference ($P=0.306$) on the ratio of HL patients between allo-HSCT and chemotherapy groups, thus the better prognosis of AML$^{FLT3\text{-}ITD+/NPM1+}$ patients achieved in allo-HSCT group is not caused by a lower ratio of HL patients.

Risk factors for OS and DFS

Risk factors as age, gender, WBC (HL or Non-HL), treatment (allo-HSCT or chemotherapy), combined mutated genes including *DNMT3A R882, IDH1, IDH2, TET2* and *KIT* were evaluated with univariate analysis (Table 2). Only factors with a *P*-value of <0.2 in the univariate analysis were included in the multivariate analysis model. Multivariate Cox model analysis (Table 3) showed that age ≥60, with HL, and *DNMT3A R882* mutation were independent risk factors for OS of AML $^{FLT3\text{-}ITD+/NPM1+}$ patients ($P=0.005$, 0.042 and 0.001; HR=3.035, 1.994 and 4.339; 95% CI were 1.395–6.601,1.027–3.872 and 1.798–10.474, respectively). HL and *DNMT3A R882* mutation also were independent risk factors for DFS ($P=0.015$ and 0.003; HR =2.327 and 3.435; 95% CI were 1.177–4.603 and 1.514–7.793, respectively). Allo-HSCT was an independent benefit factor of both OS and DFS ($P=0.001$ and 0.000; HR=0.173 and 0.138; 95% CI were 0.062–0.483 and 0.049–0.389, respectively). None of the other factors, including sex, *IDH1, IDH2, TET2* and *KIT* mutation were found significantly associated with OS and/or DFS in multivariate analysis.

A major concern was then raised, how about the survival of AML $^{FLT3\text{-}ITD+/NPM1+}$ patients without three risk factors

Table 2 Analysis of risk factors of *FLT3-ITD* and *NPM1* double mutated AML

Factor	OS (months)		DFS (months)	
	Log rank X^2 test	*P*-value	Log rank X^2 test	*P*-value
Age (≥60 vs<60 years)	10.554	0.001	5.458	0.019
WBC (HL vs Non-HL)	6.978	0.008	6.808	0.009
Therapy (HSCT vs Chemo)	21.708	0.000	22.794	0.000
DNMT3A R882 (Mutated vs Unmutated*)	28.900	0.000	32.870	0.000
Gender (Female vs Male)	0.001	0.982	0.064	0.801
IDH1 (Mutated vs Wild)	1.349	0.245	1.879	0.170
IDH2 (Mutated vs Wild)	1.051	0.305	1.545	0.214
TET2 (Mutated vs Wild)	0.153	0.696	0.112	0.738
KIT (Mutated vs Wild)	1.124	0.289	1.143	0.285

Note: **DNMT3A R882* unmutated includes *DNMT3A* wild type and *DNMT3A non-R882* mutation
Abbreviations: OS, overall survival; DFS, disease-free survival; WBC, white blood cell; Chemo, chemotherapy; HL, hyperleukocytosis; HSCT, hematopoietic stem cell transplantation.

Table 3 Multivariate Cox model analysis of *FLT3-ITD* and *NPM1* double mutated AML

Factor	OS(months)			DFS (months)		
	P-value	HR	95%CI	*P*-value	HR	95%CI
Age						
≥60 versus <60 years	0.005	3.035	1.395-6.601	0.32	1.436	0.694–2.968
WBC count				9		
HL versus Non-HL					2.327	1.177–4.603
Therapy	0.042	1.994	1.027-3.872	0.01		
HSCT versus				5	0.138	0.049–0.389
Chemotherapy						
DNMT3A R882	0.001	0.173	0.062-0.483	0.00	3.435	1.514–7.793
Mutated versus				0		
Unmutated*					0.396	0.052–3.020
IDH1	0.001	4.339	1.798-10.474	0.00		
Mutated versus				3		
Wild						
	/	/	/	0.372		

Note: **DNMT3A R882* unmutated includes *DNMT3A* wild type and *DNMT3A non-R882* mutation.
Abbreviations: OS, overall survival; DFS, disease-free survival; WBC, white blood cell; HL, hyperleukocytosis; HSCT, hematopoietic stem cell transplantation.

including age ≥60, HL and *DNMT3A R882* mutation? With statistical analysis on 24 AML $^{FLT3\text{-}ITD+/NPM1+}$ patients without either of three risk factors, the Kaplan–Meier curve showed that allo-HSCT had a trend of better OS than chemotherapy without significant difference (*P*=0.054), but the DFS could be significantly prolonged after allo-HSCT (*P*=0.032). This indicates that allo-HSCT can be recommended for all the AML $^{FLT3\text{-}ITD+/NPM1+}$ patients, no matter they show above three risk factors or not (Figure 6).

Discussion

According to NCCN 2018 and ELN 2017 guidelines, the risk level in AML$^{FLT3\text{-}ITD+/NPM1+}$ dependents on the AR of *FLT3-ITD* mutation. Below 0.5 is defined as low AR. When *FLT3-ITD*'s AR is low, AML$^{FLT3\text{-}ITD+/NPM1+}$ falls to low risk, when *FLT3-ITD*'s AR is high, this type of AML falls to intermediate risk. In this study, we did not detect the AR of *FLT3-ITD*, because this laboratory examination is not a regular item in our hospital and its cost is high. But according to guidelines, with either low or high *FLT3-ITD* AR, patients with *FLT3-ITD* and *NPM1* double mutations fall in low or intermediate risk levels, for them allo-HSCT is not obligated.

However, some clinicians view the NCCN and ELN recommendation with skepticism. In two published validation studies,[16,17] when comparing low *FLT3-ITD* AR patients with high AR, no significant differences of survival were found in AML^{NPM1+} patients. Moreover, in a

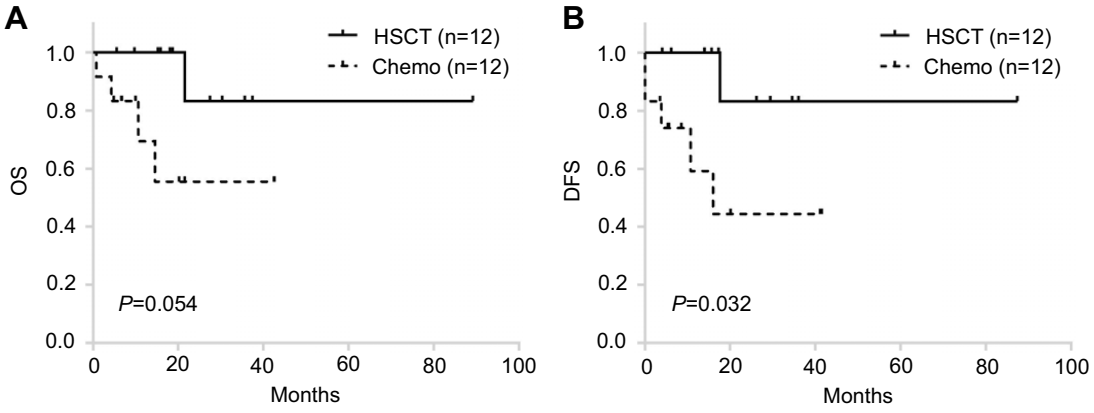

Figure 6 Comparison of chemotherapy and allo-HSCT in AML$^{FLT3\text{-}ITD+/NPM1+}$ patients without three risk factors including age ≥60, HL and *DNMT3A R882* mutation. (**A, B**) OS and DFS of 24 AML$^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882-}$ patients younger than 60 years old and without HL.
Abbreviations: OS, overall survival; DFS, disease-free survival; HSCT, hematopoietic stem cell transplantation; chemo, chemotherapy.

large group of AML patients,[18] low *FLT3-ITD* AR with *NPM1* mutated AML, which was classified in NCCN as favorable risk level, should be considered as intermediate-risk group. And the similar conclusion was also reported by Liu et al[19]. Besides, some studies indicated that allo-HSCT improves the prognosis in *NPM1* mutated AML with *FLT3-ITD* low AR.[19–21] Moreover, Patel et al[23] reported that high variant allele frequency of *NPM1* predict poor outcomes in *de novo* AML, even after undergoing hematopoietic stem cell transplantation. And the effect of high *NPM1* variant allele frequency on prognosis was not affected by the level of *FLT-ITD* AR. These findings directly challenge the prognostic risk stratification of *FLT3-ITD* and *NPM1* double-mutated AML. What is the optimal treatment for this type of AML is obscure too.

In order to know more about the *FLT3-ITD* and *NPM1* mutation double positive AML, we studied 76 AML $^{FLT3\text{-}ITD+/NPM1+}$ patients. Our result did not support that AML$^{FLT3\text{-}ITD+/NPM1+}$ patients have favorable prognosis: the median DFS time for all AML$^{FLT3\text{-}ITD+/NPM1+}$ patients was 8.1 months (range, 0–87.4 months), the median OS time was 12.2 months (range, 0.2–89.2 months). Allo-HSCT group had significantly prolonged OS and DFS of AML $^{FLT3\text{-}ITD+/NPM1+}$ patients than chemotherapy alone. Here, as age was not balanced between transplantation and chemotherapy groups, we analyzed 44 patients in chemotherapy group who were younger than 60 years with all the 22 patients in transplantation group who are younger than 60. And the results also showed that allo-HSCT can significantly improve the prognosis in AML $^{FLT3\text{-}ITD+/NPM1+}$ patients. (Figure S1) We further analyzed the impact of induction regimen on prognosis. Among 76 patients, 62 patients received IA scheme and 14 adopted homoharringtonine-based induction regimens (HAA). After remission, all patients were treated with intermediate-dose cytarabine (2.0/m^2) for 2–3 courses and coupled with standard dose chemotherapies such as AAE, IAE, AA and MAE. Previous reports showed the assessment of intermediate-dose cytarabine monotherapy vs. *intermediate-dose* cytarabine combination treatment of standard dose chemotherapies did not present a significant difference with respect to RFS and OS.[24] Thus, no matter which standard dose chemotherapies were used, all patients can be considered as to be received with consolidation treatments equally. The only difference is the induction chemotherapy. Thus, we divided patients of chemotherapy group into two sub-groups: IA group (62 cases) and HAA group (14 cases) (Table S1). According to the K-M survival analysis, we found that the IA group achieved a better prognosis than the HAA group (data not shown). Then, we compared consolidation chemotherapy with transplantation in IA group, the median OS for chemotherapy group is 9.5 months, median DFS is 4months, while transplantation group was not reached for both OS and DFS (both P=0.000) (Figure S1A and B). In order to balance the age, 38 patients aged younger than 60 years old were analyzed, and the result still supported that allo-HSCT can improve patients' OS and DFS than consolidation chemotherapy did (P=0.006 and 0.002, respectively) (Figure S1C and D). There were only four patients adopted SCT in HAA group. As SCT had better OS and DFS in IA group, we could say transplantation improved survival in HAA group. Thus, transplantation group should have better survival than chemotherapy group in a whole. Also, after excluded three risk factors including age ≥60, HL and *DNMT3A R882*, allo-HSCT still showed better survival than chemotherapy. Thus, our result supported that AML $^{FLT3\text{-}ITD+/NPM1+}$ patients, with either low or high *FLT3-ITD* AR, accept allo-HSCT at CR1 to improve their survival.

HL is defined as the WBC count above 100,000/mm^3 in peripheral blood at the initial diagnosis. In this study, the proportion of patients with HL at initial diagnosis of AML$^{FLT3\text{-}ITD+/NPM1+}$ patients accounted for 36.8%, which is higher than the ratio in de novo AML patients (5–20%) reported in the previous literature.[25–28] Moreover, HL was found to be an independent risk factor for AML$^{FLT3\text{-}ITD+/NPM1+}$. Patients with HL suffered shorter OS and DFS than non-HL. The high ratio of HL may be one of the causes of poor survival of AML$^{FLT3\text{-}ITD+/NPM1+}$ patients. It is generally believed that AML patients presented with HL have a particularly dismal prognosis because of 1) A higher risk of early death resulting from HL complications, including disseminated intravascular coagulation, tumor lysis syndrome, and leukostasis; 2) a higher probability of relapse and death in the long run.[25,29–31] In this study, we found that the CI of relapse rate of HL did not differ from that of non-HL. But patients with HL had significantly worse CR rate than with non-HL. So the poor OS and DFS of HL group is not related to the higher relapse rate, but could the lower CR rate.

This study also revealed that 23.7% of AML$^{FLT3\text{-}ITD+/NPM1+}$ patients were also positive for *DNMT3A R882* mutation. Among the six combined mutational genes including *DNMT3A R882, IDH1, IDH2,TET2*

and *KIT*, only *DNMT3A R882* was the independent risk factor for OS and DFS of AML $^{FLT3\text{-}ITD+/NPM1+}$ patients. Ley et al's study showed that in the de novo AML patients, the co-occurrence between mutations in *FLT3, DNMT3A*, and *NPM1* was the most prominent,[32] and this triple-mutation represent a novel subtype of AML for the distinct molecular characteristics. Loghavi et al found that 20% of de novo AML have *DNMT3A, NPM1* and *FLT3* mutation coexistence.[33] A large number of studies reported that *DNMT3A* mutation predicts poor outcome.[32–37] Kumar et al found that *DNMT3A R882* mutation plays an important role in normal chromosome AML patients' prognosis and clinical outcomes in the presence of *NPM1* and FLT3 mutations.[38] Although various *DNMT3A* mutations have been identified in AML, *R882* is the most frequent, accounting for 70–80% of all DNMT3A mutations.[39] *DNMT3A R882* mutation was widely accepted as a poor prognostic factor in AML patients.[40–42] The effect of *non-R882* mutation was not very clear, some studies showed that both *R882* and *non-R882* mutations of *DNMT3A* appeared to be associated with a negative prognostic impact on OS.[22,43] Here, we only present the data with *DNMT3A R882 mutation*. Our results showed that AML$^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882+}$ patients had significant worse outcomes than AML$^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882-}$ patients. Although the survival was only moderately increased, allo-HSCT can give better OS and DFS in AML$^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882+}$ patients. We also found that there was no difference of CR rate between AML $^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882+}$ and AML $^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882-}$ patients, but AML $^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882+}$ patients had a higher relapse rate. Thus, the poor survival of AML $^{FLT3\text{-}ITD+/NPM1+/DNMT3A\ R882+}$ AML patients may be due to the higher relapse rate.

We concluded that AML$^{FLT3\text{-}ITD+/NPM1+}$ is associated with an unfavorable survival. Age ≥60, with HL at diagnosing, and *DNMT3A R882* mutation were independent risk factors for *FLT3-ITD* and *NPM1* double mutated AML. Allo-HSCT can improve the survival of AML$^{FLT3\text{-}ITD+/NPM1+}$ patients. Thus, although AML$^{FLT3\text{-}ITD+/NPM1+}$ patients were considered with favorable to intermediate risk, they should undergo allo-HSCT at CR1. Further studies need to be done to know better of this type of AML.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

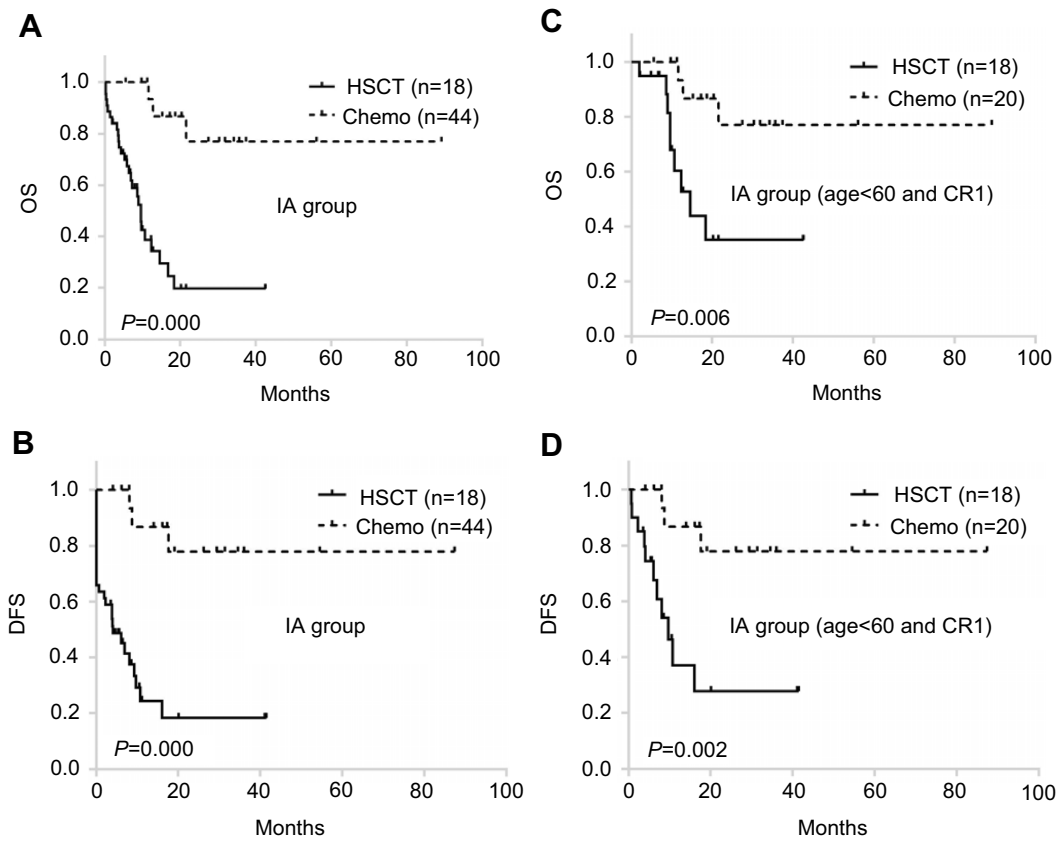

Figure S1 Comparison of chemotherapy and allo-HSCT in AML FLT3-ITD+/NPM1+ patients received IA as induction regimen. (**A, B**) OS and DFS of all AML FLT3-ITD +/NPM1+ patients. (**C**,**D**) OS and DFS of the AML FLT3-ITD+/NPM1+ patients with age<60 years.

Abbreviations: IA, idarubicin and cytarabine; OS, overall survival; DFS, disease-free survival; HSCT, hematopoietic stem cell transplantation; chemo, chemotherapy;

Table S1 Biological and clinical characteristics

Variable	IA group (n=62)	HAA group (n=14)	*P*-value
Age, years (%)			0.730
<60	48 (77.4)	10 (71.4)	
≥60	14 (22.6)	4 (28.6)	
Gender (%)			1.000
Male	29 (46.8)	7 (50.0)	
Female	33 (53.2)	7 (50.0)	
HL (%)			0.760
Yes	40 (64.5)	8 (57.1)	
No	22 (35.5)	6 (42.9)	
BM blast (%)			0.707
Median (range)	80.0 (22.0–97.0)	74.3 (35.0–93.0)	
FAB type			0.821
M0	2 (3.2)	0	
M1	11 (17.1)	1 (7.1)	
M2	28 (45.2)	8 (57.1)	
M4	1 (1.6)	0	
M5	20 (32.3)	5 (35.7)	
DNMT3A R882(%)			0.081
Unmutated*	37 (59.7)	6 (42.9)	
Mutated	16 (25.8)	2 (14.3)	
Unknow	9 (14.5)	6 (42.9)	
Therapy (%)			1.000
Chemotherapy	44 (71.0)	10 (71.4)	
Allo-HSCT	18 (29.0)	4 (28.6)	
CR (%)			0.113
Yes	46 (74.2)	7 (50.0)	
No	11 (17.7)	4 (28.6)	
Unknow	5 (8.1)	3 (21.4)	