Supplemental Appendix

Contents

Supplementary methods

NPM1 gene sequencing

PCR was performed using DNA from the samples as the template and primers (sense: 5′-GGTCTCTGTTCTTT, CTGTTGATTTCC-3′ and antisense: 5′-CAACACATT CTTGGCAATAGAACCT-3′). The 50-μl PCR mixture contained 25 μl of 2× Universal PCR Master mix (TianGen Biotech, Beijing, China), 900 nM primers, and 300 ng DNA. The PCR protocol was as followed: pre-denaturing at 95°C for 5 min, followed by 30 cycles at 95°C for 40 s, 55°C for 40 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. The purified PCR products were subjected to sequence analysis with an ABI3700 DNA sequencer. The newly screened mutants were subcloned into pMD18-T vectors, followed by transformation, screening, and further sequencing.

Screening of the ITD mutations in the *FLT3* gene

The presence of an *FLT3/ITD* was determined by amplifying a region spanning exons 14 and 15 by using the primers 14F (5′-CAA TTT AGG TAT GAA AGC C-3′) and 15R (5′- GTA CCT TTC AGC ATT TTG AC-3′) followed by 8% polyacrylamide gel electrophoresis as described previously [1].

Conditioning regimen

The conditioning protocol was as follows: cytarabine, busulfan (3.2 mg kg^{-1} d^{-1}, intravenously from days –8 to –6, day 0 being the first day of donor cell infusion), cyclophosphamide (1.8 g m^{-2} d^{-1} from days –5 to –4), and semustine (250 mg m^{-2} d^{-1} on day –3). Cytarabine was administered at 4 $g.m^{-2}$. d^{-1} (days –10 to –9) in the HID group, 2 $g.m^{-2}$. d^{-1} (days –10 to –9) in URD group, and at 2 $g.m^{-2}$. d^{-1} (day -9) in the ISD group.

Protocol for graft-versus-host disease (GVHD) prophylaxis

All the haploidentical related donor (HID) HSCT recipients received rabbit antithymocyte globulin (ATG, thymoglobulin, 2.5 mg/kg/day, days −5 to −2; Sanofi, France), cyclosporine A (CsA), mycophenolate mofetil (MMF), and short-term methotrexate (MTX) for GVHD prophylaxis. CsA (2.5 mg/kg, q12h, intravenous [i.v.]) was used from day −3, of which the trough concentration was adjusted to 150–250 ng/mL. It was switched to oral administration when the patient's bowel function returned to normal. From day −3, 0.25–0.5 g of MMF was administered orally every 12 h, then it was discontinued when neutrophil engraftment was achieved. Following graft infusion, a dose of 15 mg/m^2 of MTX was administered i.v. on day +1, as well as a dose of 10 mg/m^2 on days +3, +5, and +11 for HID and unrelated donor (URD) HSCT recipients. For identical sibling donor (ISD) HSCT recipients, a dose of 15 mg/m^2 of MTX was administered i.v. on day +1, as well as a dose of 10 mg/m^2 on days +3, and +6. Particularly, patients with maternal donors or collateral relative donors could receive two doses of 14.5 mg/kg cyclophosphamide on days +3 and +4 post-HSCT based on ATG.[2]

MFC detection of MRD

Eight-color MFC was performed in all patients as a routine clinical test on bone marrow aspirate samples that were obtained as part of baseline assessment before and after HSCT according to previous studies.[3-6] A panel of eight antibody combinations that recognize CD7, CD11b, CD13, CD14, CD16, CD19, CD33, CD34, CD38, CD41, CD45, CD56, CD61, CD64, CD71, CD117, CD123, and HLA-DR was used for MRD detection, and 0.2–1 million events per tube were acquired on a FACS Cant II. The isotype control monoclonal antibodies were used. Positive MRD was considered when a cluster of more than 25 cells with leukemia-associated immunophenotypes (LAIP) and SSC characteristics identified in all plots of interest and carrying at least two LAIP markers identified at diagnosis was observed. For those without LAIP markers at diagnosis, MRD was identified as a cell population showing deviation from the normal patterns of antigen expression seen on specific cell lineages at specific stages of maturation compared

with either normal or regenerating marrow.[7] A lower limit of detection (LOD) of 0.01% was targeted. When abnormal cells were identified, the cells were quantified as a percentage of the total CD45+ white cell events. Any measurable level of MRD was considered positive. The standardized assays and quality controls were performed according to previous reports.[8, 9]

Protocol for preemptive donor lymphocyte infusion

Granulocyte colony-stimulating factor mobilized peripheral blood stem cells were administered instead of the more common unstimulated donor blood lymphocytes. The initial dose of mononucleated cells (MNCs) for DLI and the dose of cells for repetitive infusion were $1\text{-}2\times10^8$ MNCs/kg. DLI doses were also defined as $CD3^+$ cells per kilogram of recipient weight (1.0×10^7/kg -10.0×10^7/kg). Patients could also receive anti-leukemic chemotherapy 48–72 hours before DLI. Patients received immunosuppressive drugs such as CSA to prevent GVHD after DLI for 6–8 weeks at the discretion of the attending physicians (and usually depending on the patient's GVHD status after DLI). The starting dosage of CSA was 2.5 $mg\cdot kg^{-1}\cdot day^{-1}$, which was adjusted to maintain a plasma concentration > 100 ng/mL.[10, 11]

Protocol for preemptive interferon-α treatment

Recombinant human IFN-α-2b injections (Anferon; Tianjin Hualida Biotechnology Co., Ltd., Tianjin, China) were administered subcutaneously for 6 cycles (twice weekly in every 4 weeks cycle) at dosages of 3 million units for patients older than 16 years, and at 3 million units per square meter for those younger than 16 years (capped by 3 million units). Prolonged treatment with IFN-α was permitted at the request of patients.[12]

Supplementary Table 1. Univariable analysis of clinical outcomes following HSCT in intermediate-risk triple-muted AML patients

Outcomes	HR (95% CI)	*P*-value
Relapse		
Age		
18–54 years	1	
≥ 55 years	0.90 (0.11–7.69)	0.921
Sex		
Male	1	
Female	3.83 (0.45–32.83)	0.220
Courses of induction chemotherapy before first CR		
1	1	
> 1	2.25 (0.41–12.34)	0.349
Disease status before allo-HSCT		
CR1	1	
> CR1	0.05 (0–3.27*10^7)	0.792
HCT-CI scores before allo-HSCT		
0 (low risk)	1	
1–2 (intermediate risk)	0.67 (0.08–5.73)	0.714
≥ 3 (high risk)	0	0.993
MRD status before allo-HSCT		
Negativity	1	
Positivity	0.51 (0.06–4.34)	0.535
Donor type		
Matched sibling donor	1	
Haploidentical related donor	37.18 (0.02–7.02*10^4)	0.348
Unrelated donor	1.00 (0–7.04*10^{11})	1.000
Donor/recipient gender matching		
Others	1	
Female donor/male recipient combination	0.04 (0–1209.67)	0.541
Blood group disparity		
Matched	1	
Minor mismatched	0.85 (0.10–7.59)	0.882
Major mismatched or minor and major mismatched	0.44 (0.05–3.90)	0.457
Conditioning regimen		
Chemotherapy-based regimen	1	
TBI-based regimen	0.05 (0–1.40*10^6)	0.727
Graft type		
BM + PB	1	
PB	1.08 (0.20–5.92)	0.928
Treatment failure as defined by event-free survival		
Age		
18–54 years	1	
≥ 55 years	0.29 (0.04–2.20)	0.230
Sex		
Male	1	

Female	1.57 (0.54–4.59)	0.411
Courses of induction chemotherapy before first CR		
1	1	
> 1	4.32 (1.56–11.97)	0.005
Disease status before allo-HSCT		
CR1	1	
> CR1	4.90 (1.10–21.88)	0.038
HCT-CI scores before allo-HSCT		
0 (low risk)	1	
1–2 (intermediate risk)	2.23 (0.75–6.67)	0.955
≥ 3 (high risk)	1.86 (0.23–14.87)	0.557
MRD status before allo-HSCT		
Negativity	1	
Positivity	2.62 (0.95–7.24)	0.064
Donor type		
Matched sibling donor	1	
Haploidentical related donor	6.08 (0.80–46.52)	0.082
Unrelated donor	18.41 (1.07–317.90)	0.045
Donor/recipient gender matching		
Others	1	
Female donor/male recipient combination	0.91 (0.21–4.02)	0.897
Blood group disparity		
Matched	1	
Minor mismatched	0.93 (0.20–4.36)	0.921
Major mismatched or minor and major mismatched	1.14 (0.37–3.50)	0.821
Conditioning regimen		
Chemotherapy-based regimen	1	
TBI-based regimen	2.34 (0.30–18.51)	0.420
Graft type		
BM + PB	1	
PB	1.05 (0.36–3.06)	0.934
Treatment failure as defined by leukemia-free survival		
Age		
18–54 years	1	
≥ 55 years	0.29 (0.04–2.20)	0.230
Sex		
Male	1	
Female	1.57 (0.54–4.59)	0.411
Courses of induction chemotherapy before first CR		
1	1	
> 1	4.32 (1.56–11.97)	0.005
Disease status before allo-HSCT		
CR1	1	
> CR1	4.90 (1.10–21.88)	0.038
HCT-CI scores before allo-HSCT		

0 (low risk)	1	
1–2 (intermediate risk)	2.23 (0.75–6.67)	0.151
≥ 3 (high risk)	1.86 (0.23–14.87)	0.557
MRD status before allo-HSCT		
Negativity	1	
Positivity	2.62 (0.95–7.24)	0.064
Donor type		
Matched sibling donor	1	
Haploidentical related donor	6.08 (0.80–46.52)	0.082
Unrelated donor	18.41 (1.07–317.90)	0.045
Donor/recipient gender matching		
Others	1	
Female donor/male recipient combination	0.91 (0.21–4.02)	0.897
Blood group disparity		
Matched	1	
Minor mismatched	0.93 (0.20–4.36)	0.921
Major mismatched or minor and major mismatched	1.14 (0.37–3.50)	0.821
Conditioning regimen		
Chemotherapy-based regimen	1	
TBI-based regimen	1.44 (0.19–10.97)	0.725
Graft type		
BM + PB	1	
PB	1.05 (0.36–3.06)	0.934
Treatment failure as defined by overall survival		
Age		
18–54 years	1	
≥ 55 years	0.53 (0.07–4.18)	0.547
Sex		
Male	1	
Female	1.80 (0.46–6.95)	0.397
Courses of induction chemotherapy before first CR		
1	1	
> 1	4.15 (1.19–14.40)	0.025
Disease status before allo-HSCT		
CR1	1	
> CR1	8.78 (1.84–41.97)	0.007
HCT-CI scores before allo-HSCT		
0 (low risk)	1	
1–2 (intermediate risk)	0.93 (0.19–4.50)	0.933
≥ 3 (high risk)	3.15 (0.38–26.21)	0.289
MRD status before allo-HSCT		
Negativity	1	
Positivity	1.11 (0.29–4.31)	0.877
Donor type		
Matched sibling donor	1	
Haploidentical related donor	3.71 (0.47–29.25)	

Unrelated donor	0	0.214
Donor/recipient gender matching		0.989
Others	1	
Female donor/male recipient combination	1.62 (0.34–7.62)	0.543
Blood group disparity		
Matched	1	
Minor mismatched	0.52 (0.06–4.19)	0.535
Major mismatched or minor and major mismatched	0.52 (0.11–2.51)	0.416
Conditioning regimen		
Chemotherapy-based regimen	1	
TBI-based regimen	2.25 (0.28–17.76)	0.443
Graft type		
BM + PB	1	
PB	1.28 (0.33–4.95)	0.724
Non-relapse mortality		
Age		
18–54 years	1	
≥ 55 years	0.04 (0–2118.35)	0.554
Sex		
Male	1	
Female	0.75 (0.11–5.30)	0.769
Courses of induction chemotherapy before first CR		
1	1	
> 1	11.2 (1.16–107.91)	0.037
Disease status before allo-HSCT		
CR1	1	
> CR1	30.33 (4.09–224.81)	0.001
HCT-CI scores before allo-HSCT		
0 (low risk)	1	
1–2 (intermediate risk)	1.67 (0.15–18.38)	0.677
≥ 3 (high risk)	7.68 (0.67–87.82)	0.101
MRD status before allo-HSCT		
Negativity	1	
Positivity	2.52 (0.36–17.89)	0.355
Donor type		
Matched sibling donor	1	
Haploidentical related donor	1.22 (0.13–11.73)	0.864
Unrelated donor	0	0.992
Donor/recipient gender matching		
Others	1	
Female donor/male recipient combination	6.29 (0.89–44.65)	0.066
Blood group disparity		
Matched	1	
Minor mismatched	0	0.988
Major mismatched or minor and major mismatched	0.62 (0.06–5.45)	0.623
Conditioning regimen		

Chemotherapy-based regimen	1	
TBI-based regimen	7.01 (0.73–67.78)	0.092
Graft type		
BM + PB	1	
PB	1.52 (0.16–14.60)	0.718

BM, bone marrow; HCT–CI, hematopoietic cell transplantation–specific comorbidity index; HSCT, hematopoietic stem cell transplantation; PB, peripheral blood.

Supplementary Table 2. Distribution of other co-mutant molecular abnormalities in AML patients with triple mutation

Mutation	*n* (%)
TET	3 (5.7)
WT1	5 (9.4)
IDH2	7 (13.2)
CEBPA	3 (5.7)
KRAS	1 (1.9)
NARS	3 (5.7)
MLL-PTD	1 (1.9)
KMT2D	1 (1.9)
JAK2	1 (1.9)
c-KIT	2 (3.8)
Others*	30 (56.6)

*Others included RAD21, RELN, PRPF8, SMC1A, SMC3, SETBP1, BRCA2, NF1, PTPN11, LYST, NEAS, MECOM

Supplementary Table 3 The 1.5-year clinical outcomes in triple mutated AML patients with positive and negative MRD before allo-HSCT

	MRD positivity (n = 15)		MRD negativity (n = 38)	
	Cumulative Incidence (%)	95% CI (%)	Cumulative Incidence (%)	95% CI (%)
1.5-year relapse	6.7	0–19.8	14.1	2.4–25.9
1.5-year leukemia free survival	79.0	60.4–100.0	80.4	6.7–68.4
1.5-year overall survival	79.4	61.2–100.0	82.9	71.4–96.4
1.5-year non-relapse mortality	13.9	0–32.5	5.4	0–12.8

$^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001

Supplementary Table 4 The 1.5-year clinical outcomes after allo-HSCT in triple-mutated AML patients with R882 and non-R882 mutations

	R882 mutation (n = 32)		Non-R882 mutation (n = 21)	
	Cumulative Incidence (%)	95% CI (%)	Cumulative Incidence (%)	95% CI (%)
1.5-year relapse	6.7	0–16.0	19.3	1.8–36.9
1.5-year leukemia free survival	86.8	75.5–99.8	70.8	53.6–93.5
1.5-year overall survival	90.0	80.0–100.0	70.8	53.6–93.5
1.5-year non-relapse mortality	6.5	0–15.3	9.8	0–23.1

$^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001

Supplementary Table 5 The 1.5-year clinical outcomes after first remission in triple-mutated AML patients with allo-HSCT and those with consolidation chemotherapy alone

	Patients with allo-HSCT (n = 53)	Patients with chemotherapy alone (*n* = 6)
1.5-year relapse, *n* (%)	6 (11.3)	5 (83.3)
1.5-year leukemia free survival, *n* (%)	44 (83.0)	1 (16.7)
1.5-year overall survival, *n* (%)	44 (83.0)	2 (33.3)
1.5-year non-relapse mortality, *n* (%)	4 (7.5)	0

Allo-HSCT, allogeneic hematopoietic stem cell transplantation

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