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Original Article

Hematopoietic stem cell microtransplantation in patients aged over 70 with acute myeloid leukemia: a multicenter study

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Abstract: In the era of molecular targeted drugs, elderly patients with acute myeloid leukemia (AML) are still very difficult to treat, especially those older than 70 years. The decline in immune function leads to serious infection and disease recurrence. The microtransplant treatment regimen (MST) chemotherapy combined with allogeneic hematopoietic stem cell infusion is a new cell therapy regimen. The aim of this MST study was to improve the survival of elderly patients by graft versus leukemia action and improving T-cell immune function. From May 2012 to July 2020, one hundred and eleven patients aged 70 to 88 years with de novo AML were analyzed retrospectively. After induction chemotherapy, patients whom complete remission (CR) was achieved were given another 2 cycles of postremission therapy. The MST groups were given allogeneic stem cell infusion after each chemotherapy cycle. CR, leukemia-free survival, and overall survival (OS) were compared between groups. Additionally, the immune function and the T cell receptor (TCR) library of T cells were detected and analyzed. The MST group exhibited an encouragingly high CR rate (63.8%), even in high-risk patients (54%), and this rate was significantly higher than that in the chemotherapy alone group. The 1-year OS of MST patients was 57.7%, and it was 55.9% in the high-risk group. It was only 37.3% in the chemotherapy alone group. Higher numbers of naive T cells were found in the MST population than in the chemotherapy alone group. More updated T-cell clones were observed in MST patients by T-cell receptor repertoire analysis with a next-generation sequencing methodology. These results suggest that MST is a safe and practical regimen conducive to longer-term survival in patients of a highly advanced age with AML. Furthermore, it has broad clinical value in the recovery of immune function in elderly patients.

Keywords: Acute myeloid leukemia, hematopoietic stem cell, micro-transplantation, elderly patients, T cell receptor repertoire

Introduction

The median age of patients with AML at diagnosis is 69 years, with approximately one-third of patients aged 75 years or above. The treatment outcomes of patients with AML continuously deteriorate with progressively increasing age [1]. Age is of significant clinical relevance, in that it exerts a profound prognostic impact on treatment outcome. Compared with younger patients, older patients are more prone to experience high-risk cytogenetic and hematologic disorders, secondary AML, or higher expression of genes leading to drug resistance [2, 3]. Currently, the rate of complete response (CR) is reportedly between 30% and 50% in elderly patients with AML, with a mean survival ranging from just 8 to 12 months. Data from the European Oncology Working Group for more than 6000 patients showed that the 1-year overall survival (OS) was approximately 50% and 30% for patients with AML aged 55-65 and 65-75 years, respectively. The 3-year OS was only approximately 26.8% and 12.8% for these patients [4]. Additionally, the benefits of receiving first-line therapy in patients over 70 years

with AML remain controversial [5, 6]. Elderly patients with AML (EAML) generally have immune dysfunction and decline, which in turn results in severe infection complications and disappointing relapse outcomes. Although allogeneic hematopoietic stem cell transplantation (allo-HSCT) conditioned by a reduced intensity can be applied to EAML, side effects such as high transplant-related mortality (TRM) and graft-versus-host disease (GVHD) have considerably limited the application of allo-SCT in such patients. Recent developments in immunotherapy and molecular targeted therapy, such as FLT3, IDH1/2, and BCL-2 inhibitors combined with the demethylating agents decitabine or azacytidine, have improved the remission and survival rates of EAML. Nevertheless, the mean OS of these patients is only 14 to 18 months, with even worse results for patients aged over 70 [7-9]. The treatment regimens of chemotherapy, stem cell transplantation and targeted drugs each have their own advantages and disadvantages for elderly patients, and the aim of updating treatment plan is to maintain the advantages of the above regimen while minimizing the disadvantages.

Recently, we designed an infusion of HLA-mismatched donor granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood stem cells (GPBSCs) combined with chemotherapy (micro-transplantation, [MST]) that increased the CR rate, improved survival and precluded GVHD in patients with AML [10, 11]. However, studies involving patients aged over 70 years treated with MST are scarce and the specific mechanisms by which MST functions are unclear. In this study, we focused on patients aged 70 to 88 years with AML undergoing HLA-mismatched MST.

Materials and methods

Patients and donors

Patients aged 70 to 88 years with de novo AML diagnosed from May 2012 to July 2020 were identified. The protocol for this retrospective cohort study was approved by the Human Ethics Committee at each center. The diagnoses were defined according to the French, American, British and World Health Organization-criteria, and the prognostic risk groups were defined according to the 2018 European Leukemia Net classification [12].

The inclusion criteria were as follows: (1) age 70-88 years, male or female sex, and any race or ethnicity; (2) no prior anti-acute leukemia treatment (including hypomethylators for leukemia or MDS) with the exception that prior hydroxyurea and/or leukapheresis were permitted; (3) confirmed acute myeloid leukemia based on the bone marrow morphology, histochemical and immunophenotype assessments; (4) Karnofsky score ≥60%, Eastern Cooperative Oncology Group (ECOG) performance status ≤2; (5) MST group patients have one donor who is HLA matched at 0-7/10 loci. In addition, and who voluntarily donates hematopoietic stem cells and signs the consent form; (6) LVEF measured by echocardiogram or multiple gated acquisition scan (MUGA) is within the normal range (LVEF>50%); (7) the Informed Consent Form (ICF) signed by each subject (or his r her legal representatives), indicating that he or she understands the purpose and procedures of the research, and is willing to participate in the research; and (8) donor inclusion criteria: namely, that the donor meets the institution's criteria for related peripheral blood hematopoietic stem cell donors and must be able to tolerate the cell separation and collection process; and sign the informed consent form. The exclusion criteria were as follows: (1) any major surgery within 4 weeks before selection; (2) diagnosis of malignancy other than AML currently receiving or recently requiring medical treatment, except if the malignancy was adequately treated and had been in remission for more than three years prior to selection; (3) acute promyelocytic leukemia, myeloid sarcoma, chronic myeloid leukemia accelerated phase and blast crisis; (4) intracranial hemorrhage within 6 months prior to selection; (5) cardiovascular disease with clinical significance, such as uncontrolled or highly symptomatic cardiac arrhythmias, congestive heart failure, or myocardial infarction within 6 months prior to screening, or New York Heart association (NYHA) function class 3 (moderate) or class 4 (severe) heart disease; (6) active hepatitis B (positive PCR) or hepatitis C (positive PCR) or HIV; (7) life-threatening illness other than AML; (8) illicit drug dependence; (9) psychiatric disorder or cognitive impairment that in the researcher's judgment would make the subject unlikely to adhere to the protocol requirements.

A total of 111 elderly patients were enrolled, of whom 80 selected MST, 15 conventional che-

motherapy, and 16 support therapy. The 80 patients treated with MST were further divided into the high risk group (n=50) and standard risk group (n=30) based on the presence of high-risk prognostic factors other than age [13, 14].

The primary objectives of this study were to examine the CR rate, leukemia-free survival (LFS), and OS. In addition, the number of T cells recovered, recovery of T-cell receptor (TCR) clones, relapse rate, NRM, and toxicity were also evaluated. Furthermore, differences in treatment outcomes between elderly patients with high-risk AML and those with standard-risk AML undergoing MST regardless of age were observed. Additionally, the efficacy of and complications associated with conventional chemotherapy and MST in elderly patients were analyzed and compared. Variables that showed significant differences across the MST and chemotherapy alone groups were investigated further in multivariable logistic regression models using the forward LR method to identify significant risk factors for OS.

Treatment design

Induction therapy: Induction therapy consisted mainly of daunorubicin (45-60 mg/m^2), mitoxantrone (8-10 mg/m^2) or idarubicin (10-12 mg/m^2) for 3 days along with cytarabine 100-150 mg/m^2 for 5 days (DA or MA or IA), followed by an infusion of GPBSCs 24 h (Day 0) after the completion of cytarabine in the MST group. Other induction therapies included decitabine 10 mg/m^2 for 5 days, cytarabine 10 mg/m^2 every 12 h for 14 days, aclarubicin 14 mg/m^2 for 4 days and additional G-CSF 200 μg/m^2 for 14 days (DAAG), followed by an infusion of GPBSCs 24 h (Day 0) after the completion of cytarabine. If CR was not achieved after the first cycle of induction therapy, a second cycle of the same induction therapy was given. Patients in the routine chemotherapy group received the same chemotherapy regimen but without a donor cell infusion. Patients in the support treatment group were given only oral hydroxyurea, blood transfusion, anti-infection treatment, and nutrition supplementation.

Postremission therapy: Patients in whom CR was achieved two courses of MST as postremission therapy, which consisted of intermediate-dose cytarabine (1.0 g/m^2 for 6 doses) or DAAG chemotherapy, followed by an infusion of GPBSCs after cytarabine chemotherapy with up to 10-week intervals between the courses in the MST group. Supplementary MST consolidation treatments were administered if minimum residual disease (MRD) remained positive. None of the patients received any GVHD prophylaxis or further maintenance therapy. The recommended dose adjustment was as follows: for patients aged over 75, the dose of cytarabine was reduced to 500 mg/m^2 for 6 doses during postremission therapy. Patients in the conventional chemotherapy group with CR were given the same consolidation treatment regimen as patients in the MST group.

Mobilization and apheresis of donor peripheral mononuclear cells

Apheresis and mobilization of HLA-mismatched donor peripheral mononuclear cells were performed as described previously. After apheresis, the donor cells were aliquoted and cryopreserved in liquid nitrogen; however, fresh donor cells were used in the first course of treatment. The cell infusion was performed 24 hours following the chemotherapy course. The median numbers (range) of mononuclear, CD34$^+$, CD3$^+$, and natural killer (NK) cells infused per course were, respectively, 3.6 (2.7-4.5) × 10^8, 2.2 (1.6-3.3) × 10^6, 1.1 (0.8-1.6) × 10^8, and 0.5 (0.2-0.8) × 10^8 cells per kilogram, respectively.

Detection of donor chimerism

Peripheral-blood cells or bone marrow cells from all patients were tested for hematopoietic donor chimerism by standard cytogenetic analysis and a semiquantitative PCR-based analysis of the short tandem repeats as previously described.

Response criteria and outcome evaluation

Patient responses, including CR, LFS, OS, and NRM, were determined according to the revised recommendations of the International Working Group for Diagnosis in Acute Myeloid Leukemia. LFS was measured as the length of time from CR to relapse or death from any cause. OS was defined as the time from diagnosis to death from any cause or to the latest date of follow-up until July 2020. Acute GVHD and chronic GVHD were defined according to published criteria. Death within 4 weeks after the initiation of induction therapy was defined as early death.

Analysis of T-cells subsets by flow cytometry

Peripheral blood mononuclear cell (PBMC) samples were collected from patients after MST or at the CR stage after chemotherapy alone and from a normal elderly population aged over 70. In preparation for analysis, PBMCs were thawed and rested overnight. Cell staining was performed essentially as previously described [15]. Data were acquired on a CantoII flow cytometer (BD) and analyzed using FACSDiva (BD, version 6.1). T-cell subsets were defined as follows: total T lymphocytes $CD3^+CD19^-$, NK cells $CD3^-CD(16+56)^+$, NKT cells $CD3^+CD(16+56)^+$, regulatory T cells $CD4^+CD25^+CD127^{dim}$, helper T cells $CD3^+CD4^+CD8^-$, killer T cells $CD3^+CD4^-CD8^+$, Y/δ T cells $CD3^+CD4^-CD8^-$, activated T cells $CD3^+HLA\text{-}DR^+$, Th1 cells $CD3^+CD4^+IFN\text{-}r^+IL\text{-}4^-$, Th2 cells $CD3^+CD4^+IFN\text{-}r\text{-}IL\text{-}4^+$, Th0 cells $CD3^+CD4^+IFN\text{-}r^+IL\text{-}4^+$, naïveCD4 cells $CD3^+CD4^+D45RA^+$, naïveCD8 cells $CD3^+CD8^+CD45RA^+$, memoryCD4 cells $CD3^+CD4^+CD45RO^+$, and memoryCD8 cells $CD3^+CD8^+CD45RO^+$. Except for Th1, Th2 and Th0 cells, which were the proportions of $CD3^+CD4^+$ cells, the remaining subpopulation cells were the proportions of total T cells.

T-cell receptor (TCR) repertoire analysis a CDR3 next-generation sequencing (NGS) methodology

The V, D, J genes were designated according to the nomenclature provided by the international Im Muno Gene Tics information system (IMGT). The purified products were sequenced on the Illumina high-throughput sequencing platform, with a paired-end sequencing of 150 bp and a sequencing data volume of 2G. Length distribution analysis, known as CDR3 spectrum typing, was performed in accordance with the addition of nontemplate nucleotides in the V-(D)-J region, and applied to evaluate T-cell clonality and diversity. Clonality values were calculated from the entropy of the TCR Vβ CDR3 frequency distribution and subsequently normalized by logarithm (the number of unique TCR Vβ CDR3).

With respect to bioinformatics analysis, BCL2FASTQ software was adopted to convert NGS offline data into FASTQ format, while the numerical quality was filtered and counted with the aid of FASTP software. R software (version 3.6) was used to draw the length distribution map of CDR3 and the rectangular stacked charts [16-19]. TCR dynamic monitoring was performed in 4 patients in the MST and chemotherapy alone groups, and their TCR clone recovery was analyzed and compared.

Statistical analyses

SPSS 26 software was used for all statistical analyses. Variables related to clinical characteristics between age groups were compared using the chi-square (χ^2) test or Fisher's exact test. Survival data were analyzed by means of the log-rank test and survival curves were generated by the Kaplan-Meier method. A t-test or Wilcoxon-test was used to assess the probability of significant differences in survival. Cox proportional hazards regression models were applied to evaluate the relative factors for OS. Statistical significance was defined as a 2-sided $P<0.05$.

Results

Characteristics of patients and donors

The median age of the patients was 74 (range, 70-88) years. In total, 32% of the patients were above 75 years of age in the high-risk MST group and, in the standard-risk MST group, 43.3% of patients were over 75 years of age. In the high-risk MST group, 6% of the patients were above 80 years of age, and in the standard MST group, 20% of the patients were above 80 years of age. In the chemotherapy alone group, 20% of the patients were above 75 years of age, and in the support group, 43.8% of the patients were above 75 years of age. There was no difference in the sex ratio or FAB subtyping classification among the four groups. In the high-risk MST group, 34% of the patients had a history of MDS. Of the 80 patient/donor pairs, 6 were matched at 0 of 10 HLA loci, 12 were matched at 1-4 of 10 loci, 54 were matched at 5 of 10 loci, and 8 were matched at 6-7 of 10 HLA loci. In terms of patient/donor relationships, of the 80 patient/donor pairs, 32 donors were the sons of patients, 38 donors were the daughters of patients, 3 donors were the grandchildren of patients, 4 donors were otherwise related to patients by blood, and 3 donors were unrelated to the patients by blood (**Table 1**).

Response to induction chemotherapy

The overall CR rate was 63.8% in the two MST groups, which was significantly higher than that

Table 1. Characteristics of study group

Group (n)	MST (80)		non-MST (31)	
	high risk	standard risk	chemo	support care
Number (%)	50 (100%)	30 (100%)	15	16
midian age (range)	73 (70-82)	74 (70-88)	73 (70-85)	75
age over 75 years	16 (32%)	13 (43.3%)	3 (20%)	7 (43.8%)
age over 80 years	3 (6%)	6 (20%)	1 (6%)	0
sex				
female	27 (54%)	9 (30%)	7 (46.7%)	4 (25%)
male	23 (46%)	21 (70%)	8 (63.3%)	12 (75%)
FAB subtype				
M1	2 (4%)	1 (3.3%)	0	0
M2	12 (24%)	10 (33.3%)	2 (13.3%)	2 (12.5%)
M4	10 (20%)	14 (46.7%)	5 (33.3%)	6 (37.5%)
M5	10 (20%)	5 (16.7%)	3 (20%)	4 (25%)
M6	2 (4%)	0	2 (13.3%)	0
T-AML	5 (10%)	0	1 (6%)	0
MDS-AML	17 (34%)	0	3 (20%)	4 (25%)
Cytogenetics				
Good [inv(16), t(8;21), t(16;16)]	0	3 (10%)	0	0
intermediate (nomal, +8, others)	37 (74%)	28 (93.3%)	11 (73.3%)	13 (81.3%)
poor (complex, -5, -7, 5q-, 7q-, 11q23, et al.)	12 (24%)	0	4 (26.7%)	3 (18.7%)
Donor				
daughter/son/grandchild/other relative/unrelated	20/24/2/2/2	12/14/1/2/1	0	0

Abbreviations: MST, microtransplantation; T-AML, transformed acute myeloid leukemia; MDS-AML, AML with a myelodysplastic syndrome.

in the chemotherapy group (63.8% vs. 46.7%; P=0.213). A significant differences in the CR rate was also observed between the two MST groups (54% vs. 80%; P=0.019) (**Table 2**).

OS and LFS

Of the 80 patients in the MST groups, 48 (60%) finished three courses of MST, namely, 28 (56%) patients in the high-risk MST group and 20 (66.7%) patients in the standard MST group. Compared with MST patients, only 6 (40%) of the 15 patients in the chemotherapy group finished 3 courses of chemotherapy because of a lower CR rate and higher complication rate. The mean LFS time of all patients was 10±1.1 months and 5±2.8 months in both MST groups and the chemotherapy group, respectively. The mean OS times were 13±0.7 months and 6±2.6 months in both MST groups and the chemotherapy group, respectively. No statistical difference was found in the 1-year probability of LFS between the MST and the chemotherapy groups (37.8% vs. 16.7%; P=0.365). Furthermore, there was no significant difference in the 1-year probability of LFS between the high-risk MST group and the standard MST group (36.1% vs. 40.1%; P=0.784). The 1-year probability of OS in the MST groups was higher than that in the chemotherapy group (57.7% vs. 37.3%; P=0.043), and the OS values were 55.9% and 68.6% in the high-risk MST group and the standard MST group, respectively (P=0.227). The 2-year OS was 22%, 35.9% and 18.7% in the high-risk MST, standard MST, and chemotherapy groups, respectively (**Figure 1**). Multiparametric Cox model analysis was used with the survival time as an indicator, and prognostic factors including age, sex, prognostic classification of disease, and treatment grouping with MST or chemotherapy alone. A total of 94 patients were analyzed, not including the palliative, supportive care group, due to more abandonment and early complications in the support group. The results showed that age and MST treatment were independent prognostic

Table 2. Response data by the modified IWG criteria

	total	high risk with MST	standard risk with MST	*p*1	chemo	support care	*p*2
Number (%)	80 (100%)	50 (100%)	30 (100%)		15	16	NA
treated over 3 courses	48 (60%)	28 (56%)	20 (66.7%)	0.346	6 (40%)	0	0.151
treated 2 course	17 (21.3%)	9 (18%)	8 (26.7%)	0.359	3 (20%)	0	0.913
treated 1 course	15 (18.8%)	11 (22%)	4 (13.3%)	0.336	6 (40%)	0	0.069
CR, no. %	51 (63.8%)	27 (54%)	24 (80%)	0.019#	7 (46.7%)	0	0.213
partial remission	3 (3.8%)	3 (6%)	1 (3.3%)	0.596	0	0	NA
relapse	23/51 (45.1%)	13/27 (48.1%)	10/24 (41.7%)	0.642	6/7 (85.7%)	0	0.044#
no response	23 (28.8%)	20/50 (40%)	3/30 (10%)	0.004#	3 (20%)	0	0.485
NRM	11/80 (13.8%)	5/50 (10%)	6/30 (20%)	0.209	4/15 (26.7%)	16	0.208
early death	10/80 (12.6%)	7/50 (14%)	3/30 (10%)	0.600	8/15 (53.3%)	NA	0.000#
12 months LFS	37.80%	36.10%	40.1%	0.784	16.70%		0.365
12 months OS	57.70%	55.90%	68.60%	0.227	37.30%		0.043#
24 months LFS	24.50%	24.00%	25.1%	0.784	NA		0.365
24 months OS	22.80%	22.00%	35.90%	0.227	18.70%		0.043#
36 months LFS	12.20%	12.00%	25.10%	0.784	NA		
36 months OS	18.30%	14.70%	23.90%	0.227	NA		
12 months RL	57.10%	58.10%	56.00%	0.845	83.30%		0.222
24 months RL	70.50%	71.40%	68.60%	0.845	NA		NA
36 months RL	85.30%	85.70%	68.60%	0.845	NA		NA
granulocyte recovery	12	12	11	0.812	12		NA
platelet recovery	14	14	12	0.759	16		NA

Abbreviatons: chemo chemotherapy alone group: CR, complete remission; NRM, nonrelapse mortality; LFS, Leukemia free survival; OS, overall survival; RL, relapse; early death, os <3 months; granulocyte recovery, median days to granulocyte recovery to 1 × 10^9/L; platelet recovery, median days to platelet recovery to 50 × 10^9/L; p1, high risk MST VS. standard risk MST; p2, totall MST VS. Chemotherapy; #, P<0.05.

factors (P=0.032, RR 1.065, 95% CI 1.006-1.028; P=0.040, RR 1.957, 95% CI 1.031-3.715).

NRM and relapse

The relapse rates were 48.1%, 41.7%, and 85.7% in the high-risk MST, standard MST, and chemotherapy groups, respectively, indicating a significant difference between the MST and the chemotherapy groups (P=0.044). The mean relapse times were 10±1.7, 11±3.1 and 6±2.9 months in the three groups. The 1-year cumulative incidence of relapse was 58.1%, 56%, and 83.3% in the three groups, respectively (P=0.222). The NRM was 10%, 20%, and 26.7% in the three groups (P=0.208).

Hematopoietic recovery

The median time needed for neutrophil recovery in the high-risk MST, standard MST and chemotherapy groups was 12, 11 and 12 days, respectively, and the median time necessary for platelet recovery was 14, 12 and 16 days after induction therapy, respectively, which means there was no significant difference between these groups.

Donor chimerism

Of the 80 patients treated by MST, none demonstrated stable full donor chimerism. Approximately 40% of the patients had transient fever within 24 h after cell infusion, but the body temperature returned to normal after symptomatic treatment.

Enumeration of T-cell subsets

The results showed that the total T cell ratio in patients after MST treatment and patients with CR after chemotherapy alone was higher than the proportion of T cells in normal elderly patients. The results were statistically different. The proportion of total T cells was higher in the MST group than in the chemotherapy alone group and was also statistically different. The ratio of CD4 T cells was higher in the MST-CR and chemotherapy groups than in the normal population, and CD8 T cells were higher in MST-CR or NCR patients than in patients after che-

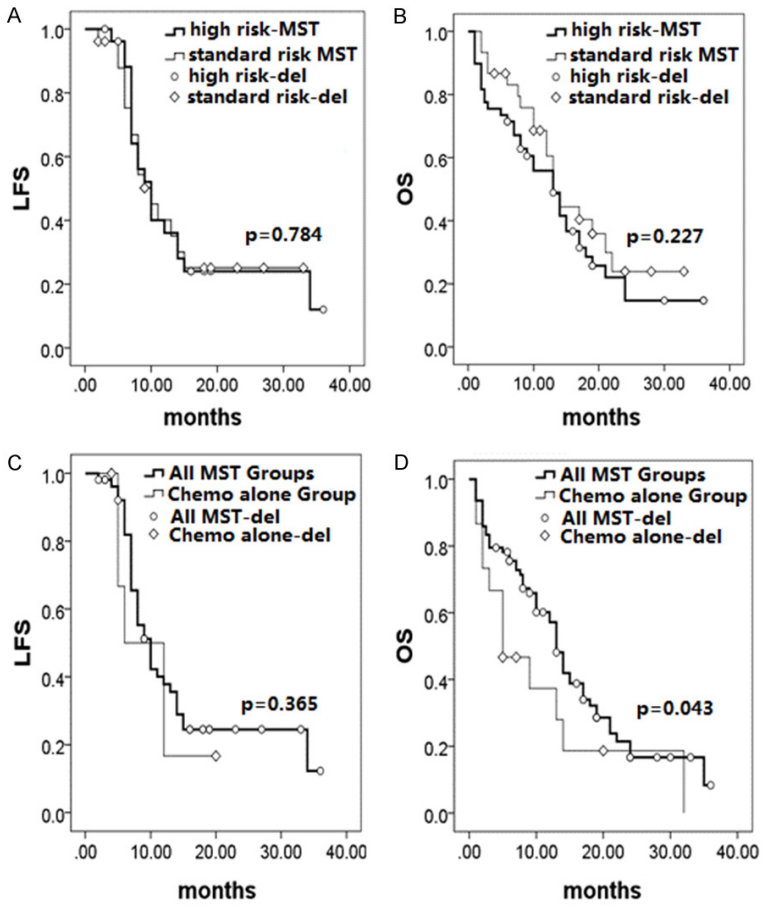

Figure 1. A, B. The probabilities of LFS and OS in the high-risk MST group compared with the standard MST group. The mean months: LFS, 10±1.2 vs. 10±1.5 months, P=0.784; OS, 13±2 months vs. 13±1.2 months, P=0.227. C, D. The probabilities of LFS and OS in all of the MST patients compared with the chemotherapy alone patients. The mean months: LFS, 10±1.1 vs. 5±2.8 months, P=0.365; OS, 13±0.7 months vs. 6±2.6 months, P=0.043.

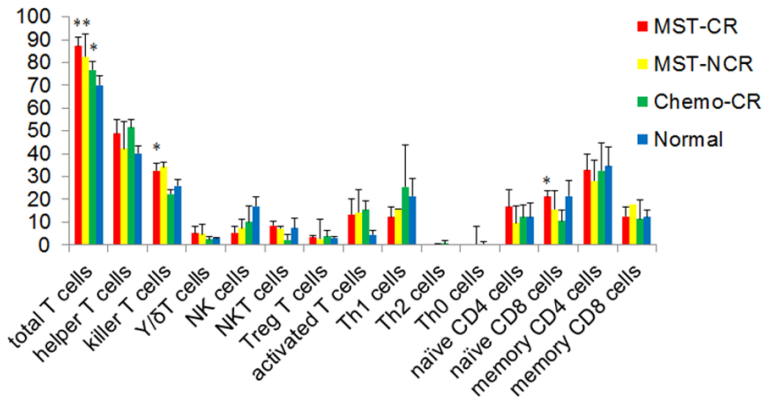

Figure 2. Enumeration of T-cell subsets by flow cytometry. MST-CR, patients with complete remission (CR) after MST; MST-NCR, patients with non-CR after MST; chemo-CR, patients with CR after chemotherapy alone; normal, normal population aged over 70. * indicates a statistically significant different (*P*<0.05).

motherapy alone. The proportion of Y/δT cells was higher in the MST group than in the chemotherapy alone group and in the normal population. The proportion of activated T cells was higher in patients after both MST and chemotherapy than in the normal population. Both naive CD4 and CD8 T cell levels were higher in the MST-CR group patients than in the chemotherapy group patients, and the levels of naive CD8 T cells were statistically different in the two groups (**Figure 2**).

CDR3 sequence analysis of TCR

The TCR of patients showed polyclonal expression and relatively uniform expression at half a year and one year following MST treatment, indicating a rapid recovery of immune function. The subcloning data of the top 50 VJ genes showed that the VJ gene clustering was close half a year following and before treatment in the chemotherapy group, while the VJ gene in the MST group one year and half a year after the treatment was contrastingly closer, demonstrating a greater number of neonatal VJ clones in the MST group. Moreover, as is visible from the left cluster lines, the expression of VJ gene cloning varied dramatically between the MST group and chemotherapy alone group, indicating that the infusion of donor cells may affect the expression of VJ cloning of the recipients (**Figure 3A**). The results from the diagram of subclone update frequency showed that at 90.4% and 92.3% of the subclones of the MST-treated groups were updated within

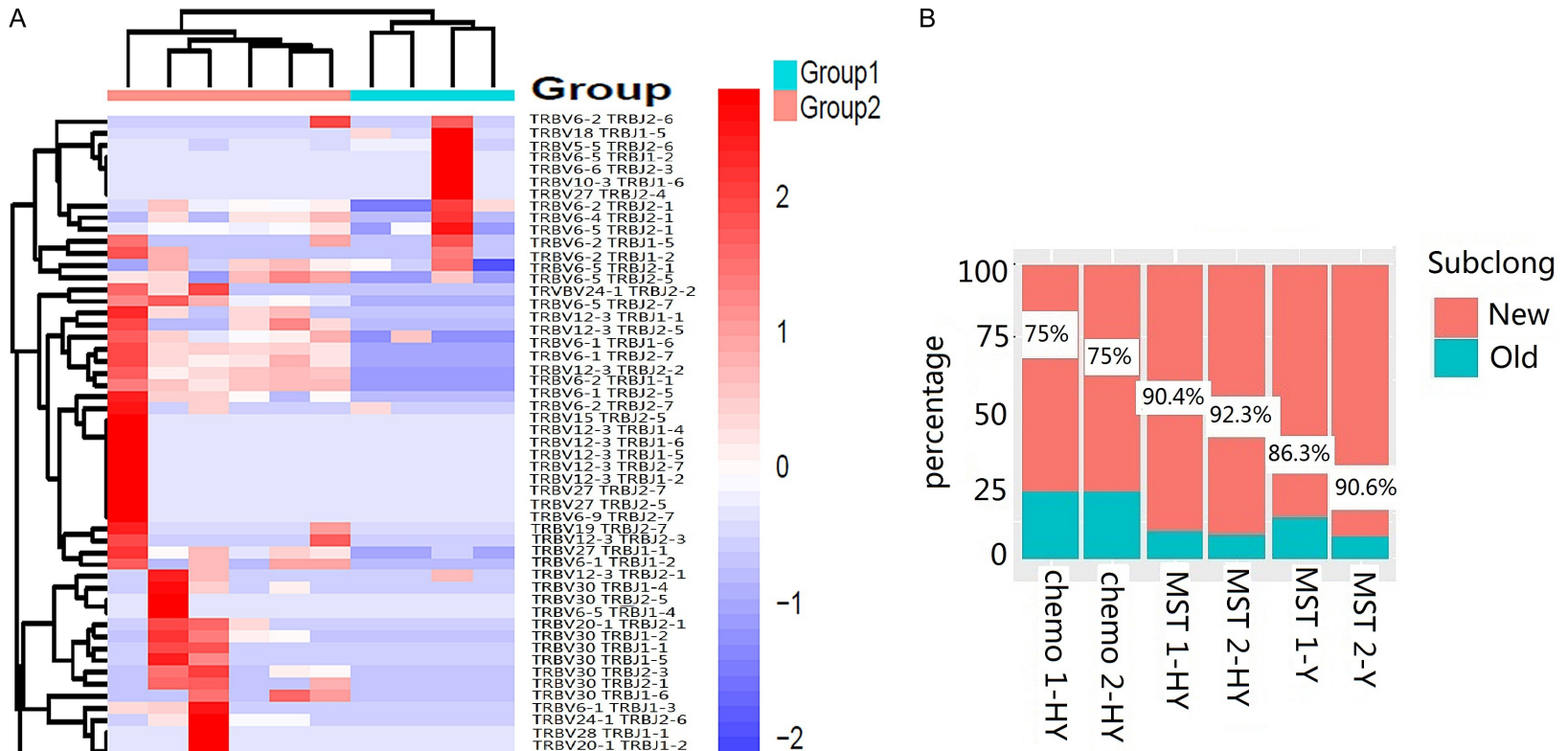

Figure 3. A. Comparative diagram of subcloning of the top 50 VJ genes of samples. Group 1 was the chemotherapy alone group, and group 2 was the MST group. The changes in color from blue to red indicate increased expression of subclones. The left and upper cluster lines indicate the closeness of different subclones. MST 1, MST 2, chemo 1 and chemo 2 mean 4 cases by MST or chemotherapy alone. The Pre means results before treatment; Y, one year; HY, half a year. B. Comparison diagram of subclone update frequency. Results showed that 90.4% and 92.3% subclones of two patients were updated half a year following the treatment, compared with the number before MST. Moreover, 80.6% and 90.3% of the clones continued updating 1 year following the treatment, a sharp contrast with the result half a year after treatment. For the two patients in the chemotherapy alone group, only 75% of the clones were updated half a year after the treatment, compared with the number before the treatment. A significant difference was detected between the two groups, P=0.004.

Table 3. Most frequent adverse events of MST treatment and chemotherapy alone

adverse event	No. of patients					
	high risk with MST (n=50)	Total %	standard risk with MST (n=30)	Total %	Chemo (n=15)	Total %
Cardic	3	6%	3	10%	6	40%
rash	3	5%	2	6.70%	0	0
Hemorrhage/bleeding	2	4%	2	6.70%	3	20%
Hepatic	2	4%	0	0	0	0
Neurologic	0	0	0	0	0	0
Renal	0	0	2	6.70%	1	6.60%
intestinal infection	5	10%	3	10%	5	33.30%
respiratory infection	13	26%	8	26.70%	7	21.70%
septicemia	8	16%	5	16.70%	6	46.70%
thrombosis	0	0	0	0	0	0
Graft versus host disease	0	0	0	0	0	0
cytomegalovirus/EB virus	0	0	0	0	0	0
multiple organ failure	5	10%	3	10%	6	40%

Abbreviation: EB, Epstein Barr.

half a year following the treatment, which was remarkably higher than that before MST. Moreover, in contrast to half a year following the treatment, 80.6% and 90.3% of the clones kept updating 1 year after the treatment, and 75% of the clones in the chemotherapy group were updated half a year following the treatment. Thus, there was a significant difference between the MST and chemotherapy alone groups, P=0.004 (**Figure 3B**).

GVHD and severe adverse events

No definite clinical acute or chronic GVHD was observed in any patient. Five patients developed transient rash, two exhibited renal insufficiency, 6 patients had heart disease, 8 had intestinal infections, 21 had lung infections, 13 had sepsis, 2 had cerebral hemorrhage, and 2 had hepatic diseases, with no CMV or EB infections during MST treatment. The overall rate of severe infection was approximately 16% in the MST group, which was markedly lower than that in the chemotherapy group (46.7%). Eight patients (10%) with advanced disease developed multiple organ failure (MOF) in the MST groups, whereas the chemotherapy group demonstrated a higher MOF rate (**Table 3**).

Discussion

Counter to the recommended guidelines, appropriately intensive chemotherapy regimens compared with palliative and low-dose chemotherapy have been shown to improve survival in elderly patients with AML based on a few small-sample clinical trials [1, 5]. However, most medical centers are reluctant to administer intensive chemotherapy to patients aged 70 and above, because it may leads to hematopoietic depression, severe complications, and even a higher risk of death. Instead, most of these centers prefer to prescribe low-dose demethylated drugs, such as decitabine, azacytidine, venetoclax, or a half-dose DCAG regimen, even though the results regarding the CR rate and OS are far below expectations due to their insufficient capability for killing the leukemia clones [6], regardless of their instrumentality in reducing tumor cell load. The age range of the 111 elderly patients in this study was from 70 to 88 years, with an average of 74. The kappa score was greater than 60 points, and none of patients had severe cardiopulmonary diseases. The data showed that the mean OS of the patients treated with MST was longer than that of the elderly patients receiving chemotherapy alone [4]. The 1-year and 2-year OS of these patients were 57.7% and 22.8%, respectively, which were roughly the same as the findings from publications on patients aged 55 to 65 treated with chemotherapy alone.

Several key factors are involved in improving the survival of elderly patients with MST. First, CR is the primary observation index, because

only by increasing CR is it possible to prolong disease-free survival. However, the occurrence of malignant genes and clonal leukemia mutations clearly increases because of advancement in age, to the extent that maintaining an appropriate intensity of chemotherapy is necessary for developing sufficient killing capability against leukemia clones to obtain a reasonably high CR rate. The induction regimen in this study was a chemotherapy regimen, for instance, anthracycline combined with cytarabine. For patients above 75 years of age, the anthracycline drug dose was correspondingly reduced. Additionally, the GPBSCs used by MST also played an important role in the induction protocol. The input cells contained numerous allogeneic lymphocytes, including CD4, CD8, NK, and DC cells, where the number of CD3 cells in a single infusion was close to 1×10^8/kg, and the total number of multiple CD3 cell transfusions was 3-5 $\times 10^8$/kg. In contrast, the number of CD3 cells in other published lymphocyte immunotherapies is generally 10^5/kg [20, 21]. As a result, a synergistic effect derived from chemotherapy and allogeneic hematopoietic stem cell infusion is capable of both maximizing the killing effect on leukemia cells and ensuring safety. In this study, the overall CR rate was above 50%, close to that for much younger patients.

Intensive chemotherapy administered to elderly patients results in a lengthy period of hematopoietic depression and serious infection complications, which is another side effect that is difficult to tackle. The cells used in the MST protocol were peripheral hematopoietic stem cells activated by G-CSF. These cells contain many hematopoietic stem progenitor cells, such as CD34 and CD38 cells. Although these cells are unstably implanted after infusion, they release many hematopoietic differentiation-related stimulating factors, promoting the rapid recovery of the recipient's own hematopoiesis [22, 23]. The specific mechanism has been investigated and expounded in published articles [20]. In this study, leukocytes and platelets generally recovered in approximately 2 weeks, which applied even to patients over 70 years of age. Another important facilitator for promoting hematopoietic recovery is a sufficient intensity of chemotherapy to remove malignant cells to achieve complete remission, which in turn accelerates the recovery of normal hematopoietic stem cells. The prevention and management of severe infection are also inseparably connected with the two abovementioned factors. The cells used in MST contain many allogeneic lymphocytes, including CD4, CD8, NK, and DC cells, by which the incidence of severe infection can be significantly reduced. Our center has repeatedly used such cells along with antibiotics for the rescue of patients with highly refractory, panresistant bacterial infections, achieving results better than expected [24]. Although nearly 36% of the patients in this study experienced various infection-related complications during treatment, most of the patients were effectively cured, and mortality was predictably attributable to various complications following leukemia relapse.

How to prolong the disease-free survival of elderly patients is another area of interest. In this study, after improving the CR rate in elderly patients, 2 courses of MST were given as consolidation and intensive therapy, which was composed of moderate-dose chemotherapy and multiple stem cell infusions. The consolidation MST proved to be effective in further reducing the leukemia load and decreasing MRD values. Considering the physical tolerance of chemotherapy in elderly patients, 2 courses of intensive treatment were administered. An analysis of a larger number of cases is needed to ascertain whether 3 or more doses of chemotherapy are more effective than 2 doses.

GPBSCs are generally associated with two functions in consolidation and intensive therapy. First, repeated infusions of GPBSCs provide allogeneic lymphocytes that can constantly produce GVL effects [21, 22, 25]. Second, repeated infusion of heterologous lymphocytes continuously stimulates and activates the recipient's immune system, and upregulates the recipient's immune status, resulting in the killing of leukemia cells [23]. TCR results showed that nearly 90% of the new clones were produced at half a year and one year after MST treatment according to a next-generation sequencing methodology. These clones were different not only from those of patients before treatment but also from the existing clones of donors. It would be of clinical significance to further determine whether those were previously unexpressed clones in the TCR bank of patients or newborn ones carrying over infor-

mation from both the recipient and donor. Regardless of the sources of the clones, the conclusion that can be reached here is that these changes are the equivalent of immune T-cell rejuvenation in older patients with leukemia at an average age of 75, suggesting that MST is of significant clinical value to the recovery of immune function in elderly patients.

In this study, the impacts of high-risk factors on CR and OS were compared. The data show that a high CR rate was maintained in patients treated with MST, which was true even for high-risk patients, despite a higher relapse rate of leukemia in the high-risk MST group. Therefore, it is advisable that newly diagnosed patients with high-risk factors adopt a positive attitude toward treatment-prolonged survival time. Alternatively, the therapeutic efficacy was limited in patients with high-risk factors, where MST could only delay the recurrence of the disease, but not produce a permanent solution to the problem. Further studies are advised to focus on the ways in which MST is combined with more effective targeted drugs, as well as improvement of techniques of modified stem cells for elderly patients to prolong survival [26, 27].

A large number of allogeneic hematopoietic stem cells were repeatedly infused, and no immunosuppressants were used before and after MST treatment. Therefore, it is particularly important to observe and identify GVHD, although most of the patients did not exhibit a stable and high percentage of chimerism in donor cells. These procedures are a prerequisite for screening the donor and recipient: an evaluation of immune function in the recipients, including tests of humoral immunity and T-cell function; identification of the patients' previous immune disorder-related diseases, such as rheumatic immune system diseases or severe liver diseases; confirmation of previous long-term use of immune-damaging drugs, especially recent use of fludarabine; and HLA typing of the donor and recipient. The results of HLA typing of the donor and recipient are critically important and may contraindicate MST in cases where donors or patients have more than 2 homozygous HLA loci.

It is difficult to determine whether GVHD or severe infection is responsible for symptoms such as persistent fever in the initial stage following MST, which are accompanied by skin congestion, rash, an elevated liver enzyme profile and occasionally mild diarrhea. Although the presence of donor chimerism is likely to make pinpointing the causes much easier, in the early stage of cell transfusion, the above symptoms appear within 2 weeks, which are particularly noticeable in donors having a low proportion of mixed chimerism. As a result, differential diagnosis needs to be considered in view of changes in inflammatory factors and the liver and myocardial enzyme spectrum [25].

The allogeneic hematopoietic stem cells collected after G-CSF mobilization in MST are fundamentally different from those used by previous cellular therapies, such as donor lymphocyte infusion and umbilical cord blood transfusion. First, peripheral blood cells after G-CSF mobilization contain not only many hematopoietic stem and progenitor cells, but also immune-related cell subsets and some pre-T cells still in the developmental stage [28]. Second, MST uses cells from healthy HLA-incompatible allogeneic donors, whereas classical donor lymphocyte infusion uses mature and terminal-stage lymphocytes or lymphocytes from patients with autoimmune disorders. These differences may account for the unique role played by MST in treating elderly patients with AML.

Conclusion

The results from this study show that MST is safe and effective and promotes the expansion of T cells and the recovery of immune function in elderly patients, even those above the age of 70.

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Disclosure of conflict of interest

None.

Abbreviations

allo-HSCT, Although allogeneic hematopoietic stem cell transplantation; AML, acute myeloid leukemia; CR, complete response; DA or MA or IA, daunorubicin idarubicin mitoxantrone cytarabine; DAAG, decitabine cytarabine aclarubicin G-CSF; EAML, Elderly patients with AML; G-CSF, granulocyte colony-stimulating factor; GPBSCs, granulocyte colony-stimulating factor-mobilized peripheral blood stem cells; GVHD, graft-versus-host disease; IMGT, international Im Muno Gene Tics information system; LFS, leukemia-free survival; MOF, multiple organ failure; MST, HLA-mismatched hematopoietic stem cell micro-transplantation; NGS, Next Generation Sequencing; NK, natural killer; OS, overall survival; PBMC, Peripheral blood mononuclear cell; TCR, T cell receptor; TRM, transplant-related mortality.

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