Supplemental Material
High efficacy of Azacitidine plus HAG in acute myeloid leukemia: an open-label, single-arm, multi-center, phase 2 study

Jun Li1*, Qi Han1*, Yanqing Huang1*, Yanhui Wei1, Jie Zi1, Lidong Zhao2, Zhimei Cai2, Xuzhang Lu2, Rong Xiao3, Yanming Zhang4, Xiaotian Yang4, Hao Xu5, Naitong Sun6, Wanchuan Zhuang7, Zhengdong Wu8, Yuan Xia9, Yanli Xu9, Bin He10, Wei Zhu11, Fengling Min12, Yongchun Chen12, Banghe Ding13, Peimin Shi14, Jing Xie14, Hua Tang15, Zefa Liu15, Bingzong Li16, Yu Sun16, Hongxia Qiu17, Limin Duan17, Elanora Dovat18, Chunhua Song18, 19#, Laszlo SzeKely20, Sinisa Dovat18, Zheng Ge18#

1Department of Hematology, Zhongda Hospital, School of Medicine, Southeast University, Institute of Hematology Southeast University, Nanjing, China. 2Department of Hematology, The First People's Hospital of Lianyungang, Lianyungang, China. 3Department of Hematology, Changzhou No.2 People's Hospital, Changzhou, China. 4Department of Hematology, Huai'an Second People's Hospital, Huai'an, China. 5Department of Hematology, Yancheng No.1 People's Hospital, Yancheng, China. 6Department of Hematology, Yancheng Third People's Hospital, Yancheng, China. 7Department of Hematology, The Second People's Hospital of Lianyungang, Lianyungang, China. 8Department of Hematology, Jiangsu Taizhou People's Hospital, Taizhou, China. 9Department of Hematology, Nanjing First Hospital, Nanjing Medical University, Nanjing, China. 10Department of Hematology, Northern Jiangsu People's Hospital, Yangzhou, China. 11Department of Hematology, Xuzhou No. 1 People's Hospital, Xuzhou, China. 12Department of Hematology, Affiliated Hospital of Yangzhou University, Yangzhou, China. 13Department of Hematology, Taixing People's Hospital, Taizhou, China. 14Department of Hematology, Xinghua City People's Hospital, Xinghua, China. 15Department of Hematology, The Second Affiliated Hospital of Soochow University, Suzhou, China. 16Department of Geriatric Hematology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China. 17Hershey Medical Center, Pennsylvania State University College of Medicine, Hershey, USA. 18Division of Hematology, The Ohio State University Wexner Medical Center, the James Cancer Hospital, Columbus, USA. 19Department of Clinical Pathology and Cancer Diagnostics, Karolinska University Hospital, Department of Laboratory Medicine, Division of Pathology, Karolinska Institute, Sweden.

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*These authors contributed equally to the work.

#Co-correspondence to:
Zheng Ge, M.D., Ph.D.
Department of Hematology
Zhongda Hospital, School of Medicine, Southeast University
Institute of Hematology Southeast University
87 Dingjiaqiao Street, Nanjing 210009, China.
Telephone: +86 25-83262468
Fax: +86 25-83262471
E-mail: zhengge@seu.edu.cn
ORCID: orcid.org/0000-0001-8028-1612

Chunhua Song, M.D., Ph.D.
Hershey Medical Center,
Pennsylvania State University Medical College
The James Comprehensive Cancer Center
Ohio State University, Division of Hematology
536 Biomedical Research Tower, 460 W. 12th Ave.
Columbus, OH 43210, USA
Telephone: 614-292-8715, FAX: 614-293-7526
E-mail: chunhua.song@oscmc.edu
ORCID: 0000-0002-4081-2543
Supplemental Methods

Clinical endpoint and assessments

The primary endpoint was the composite complete remission (complete remission [CR] or complete remission with incomplete hematologic recovery [CRi]). Secondary endpoints were overall survival (OS) defined as the time from study entry to death from any cause), relapse-free survival (RFS) is defined as the time from achieving a CR until disease recurrence or death), adverse events (AE) (including hematological and non-hematological AE defined as any unfavorable and unintended signs including an abnormal laboratory finding, symptom, or disease). Treatment failure was defined as not achieving CR or CRi after two cycles of induction therapy.

Prespecified correlative assessments included targeted gene panel sequencing to assess associations between somatic mutation patterns and therapeutic responses as well as disease progression.

Response to therapy was monitored by analysis of blood and bone marrow aspirates. Response assessment was done at the end of cycles 1 and 2 (no CR/CRI after cycle 1), and then after every 2 cycles of consolidation and every 3 months during maintenance to confirm ongoing response. Responses were categorized based on the revised International Working Group criteria for AML[1, 2].

Minimal residual disease (MRD) assessment by multicolor flow cytometry (MFC) was done on pretreatment bone marrow and all subsequent bone marrow examinations with the assay sensitivity of 0.01% as previously described[3]. Bone marrow samples were obtained at diagnosis and evaluated for the presence of cytogenetic and molecular aberrations. For documentation of mutations, the entire coding sequences of 58 genes known to be frequently mutated in myeloid malignancies were sequenced with a targeted leukemia exome-seq panel (Table S5) as described below and supplemental methods.

AE and laboratory values, graded according to the Common Terminology Criteria for Adverse Events version 4.0, were evaluated at least once every cycle during induction and consolidation and then at least every 3 months during the maintenance.
**Sample size estimation**

In this trial, the optimal Simon’s two-stage design method was used to determine the sample size. A composite complete remission rate ≤ 40% (p0) would be considered a null hypothesis (the outcome was unacceptable). While a composite complete remission rate of higher than 60% (p1) would grant the regimen for further exploration. An estimation power of 95%, with a significance level of 2.5% was used to test the hypothesis.

Accordingly, 35 participants in the 1st stage with additional 54 participants in the 2nd stage where needed. Aza+HAG regimen would be discontinued if 15 or fewer achieved CR/CRi in the 1st stage. Patients’ enrollment in the 2nd stage would be held until the outcome of the 1st stage interim analysis. If the Aza+HAG regimen continued, the activity of treatment would be considered a null hypothesis if less than 44 out of 90 enrolled cases achieved CR/CRi. Considering a 20% dropout rate, the estimated sample size was 112 patients.

**HAG regimen control**

A total of 14 ND AML patients with HAG induction regimen (homoharringtonine, cytarabine, G-CSF) from Jan 2016 to Aug 2019 in Zhongda Hospital (Nanjing, China) were enrolled as the control. The 14 patients include 12 de novo and 2 secondary AML.

HAG regimen consisted of HHT 1mg/m²/d on days 1-14 intravenous over 3 hours, cytarabine 10mg/m² every 12 hours on days 1-14 subcutaneous, and G-CSF 200µg/m²/d subcutaneous from day 1 until WBC>10×10⁹/L(14-day HAG schedule) (4/14 patients); HHT 1mg/m²/d on days 1-7 intravenous over 3 hours, cytarabine 10mg/m² every 12 hours on days 1-7 subcutaneous, and G-CSF 200µg/m²/d subcutaneous from day 1 until WBC>10×10⁹/L(7-day HAG schedule) (10/14 patients).

Baseline demographic and disease characteristics were generally balanced between 40 ND AML patients with Aza+ HAG regimen versus 14 ND AML patients with HAG regimen in the same center (Zhongda Hospital) (Table S2).

Comparison of CR/CRi rate between Aza+HAG and HAG group was performed using the
Chi-square test or Fisher exact test (when the sample size is small and with less degree of freedom). The comparison of OS and RFS between the two groups was performed using Kaplan-Meier estimates with the log-rank test. A threshold P-value < 0.05 was considered a statistically significant difference.

**Targeted exome-seq panel for gene mutation screening in AML patients**

A leukemia targeted-exome-seq panel including 58 genes was used for screening the gene mutations in 103 enrolled patients by next-generation sequencing (NGS) before and after the induction therapy[4, 5]. The target genes in the panel are listed in Table S5.

Agilent SureSelect Human All Exon V4+UTRs (Agilent) was used for the coding exons plus UTRs of target genes. Probes for each exon of each target gene are designed on NCBI (https://www.ncbi.nlm.nih.gov/). The targeted exome-seq method is performed as reported[6]. Briefly, the genomic DNA was isolated from bone marrow samples with the genomic DNA isolation kit (Qiagen, Hilden, Germany). All DNA samples were sheared with a Covaris E220 instrument generating approximately 260 bp DNA fragments. The fragmented DNA was processed into Illumina-compatible sequencing libraries using Kapa Hyper Prep Kit (Illumina, San Diego, CA, USA). Each library was uniquely barcoded and captured by the leukemia panel probes, followed by PCR amplification and sequencing on a HiSeq 2500 (Illumina) with 2x100 bp reads. The sequencing reads were aligned to the human genome by following Broad Institute’s GATK best-practice pipeline to call germline short variants (SNPs and Indels). Called variants were annotated using ANNOVAR (version 2.3). Exonic variants with exonic, nonsynonymous, stop-gain, or stop-loss, novel SNPs, and with predicted deleterious/damaging functions were manually surveyed by IGV to confirm.

The association of gene mutations with clinical response, relapse, and risk status was analyzed with R 4.0.1 software and depicted as a waterfall figure. The association of the gene mutations with OS and RFS was also evaluated by the Kaplan-Meier method[7-9].

**Meta-analysis of HAG regimen in treating elderly AML patients**

To evaluate the clinical response of the HAG regimen in treating an elderly patient with AML,
we conducted a meta-analysis of the HAG regimen by carefully screening MEDLINE, PubMed, EMBASE, and CNKI (Chinese) databases. Inclusion criteria were: 1) included unfit AML (previously untreated) patients (age over 60 years or ineligible for receiving standard chemotherapy) who received HAG regimen; 2) reported the clinical responses (CR or CRi).

A total of 453 patients from 17 studies [10-26] were finally included in this study, meta-analysis was conducted on the R 4.0.1 platform (meta-package). Funnel plot was routinely used to detect publication bias. To make the included data normalized, we used the Shapiro-Wilk normality test to choose the best transformation method (arcsine conversion, free-man tukey conversion, logistic conversion, logarithmic conversion). The fixed-effect model will be applied if the heterogeneity is less than 25% ($I^2$). Otherwise, a random-effect model will be applied.

**Statistical analysis**

The distribution of survival was estimated with the use of the Kaplan-Meier method. The lower limit and upper limit of 95% confidence interval were calculated by the Wilson method. The student t-test was used to identify differences between groups. Categorical parameters were compared with the chi-squared test or Fisher’s exact test. Statistical analysis was performed on STATA 16.0 software.

**Data sharing statement**

The patient datasets for the current study are not publicly accessible following local health research ethics protocols; however, they may be available from the corresponding author.

De-identified individual-level data and the data dictionary will be made available to qualified researchers who present study protocols, which will require approval by the institute health research ethics committee and principal investigator. These data will only be made available from study sites at which the institution and ethics review board allow such release.

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Supplemental Tables

Table S1 Baseline characteristics of patients enrolled in Aza+ HAG regimen

| Characteristics(n=112) | Participants N (%) | Median [IQR] |
|------------------------|--------------------|--------------|
| **Sex**                |                    |              |
| Male                   | 57 (50.9)          |              |
| Female                 | 55 (49.1)          |              |
| **Race or ethnicity**  |                    |              |
| Asian                  | 112 (100)          |              |
| White                  | 0                  |              |
| Black                  | 0                  |              |
| other                  | 0                  |              |
| **Age**                |                    |              |
| Median                 | 65 [57.3-70.8]     |              |
| <60y                   | 33 (29.5)          |              |
| ≥60y                   | 79 (70.5)          |              |
| **Blood cell counting**|                    |              |
| Median WBC (10^9/L)    | 5.2 [2.20-21.8]    |              |
| Median Hemoglobin (g/L)| 73 [62.0-90.5]     |              |
| Median PLT (10^9/L)    | 50 [24.5-96.8]     |              |
| **FAB classification** |                    |              |
| M0                     | 1 (0.893)          |              |
| M1                     | 9 (8.04)           |              |
| M2                     | 61 (54.5)          |              |
| M3                     | 0                  |              |
| M4                     | 7 (6.25)           |              |
| M5                     | 27 (24.1)          |              |
| M7                     | 1 (0.893)          |              |
| Unclassified           | 6 (5.36)           |              |
| **Diagnosis**          |                    |              |
| Newly diagnosed        | 72 (64.3)          |              |
| De novo                | 56 (50.0)          |              |
| Secondary              | 16 (14.3)          |              |
| Favorable*             | 19 (17.0)          |              |
| Intermediate*          | 31 (27.7)          |              |
| Poor*                  | 22 (19.6)          |              |
| Relapsed/refractory    | 40 (35.7)          |              |
| **Mutation**           |                    |              |
| DNMT3A                 | 25 (24.3)          |              |
| IDH1/2                 | 23 (22.3)          |              |
| TET2                   | 20 (19.4)          |              |
| NPM1                   | 18 (17.5)          |              |
| FLT3                   | 15 (14.6)          |              |
| ASXL1                  | 15 (14.6)          |              |
| CEBPA                  | 11 (10.7)          |              |
| RUNX1                  | 11 (10.7)          |              |
| NRAS                   | 10 (9.71)          |              |
| TP53                   | 9 (8.74)           |              |
| BCOR                   | 8 (7.77)           |              |
| KIT                    | 7 (6.80)           |              |

Data are n (%), unless otherwise stated.

*Risk classification was evaluated by ELN2017 risk category.

Abbreviations: FAB classification=French–American–British classification; WBC: white blood cell; PLT: platelet.
Table S2 The baseline characteristics between the patients treated with Aza+HAG and HAG regimen

|                                | Aza+HAG | HAG     | P      |
|--------------------------------|---------|---------|--------|
| **Mean age (SD; years)**       | 40(100) | 14(100) | 0.4672 |
| **Disease type**               |         |         | 0.311  |
| De novo AML                   | 28(70.0)| 12(85.7)|        |
| Secondary AML*                | 12(30.0)| 2(14.3) |        |
| **Sex**                       |         |         | 0.535  |
| Male                          | 21(52.5)| 6(42.9) |        |
| Female                        | 19(47.5)| 8(57.1) |        |
| **Median WBC counting (10^9/L; IQR)** | 6.0 (1.95-19.8) | 7.38 (2.49-15.6) | 0.4937 |
| **Median Plt counting (10^9/L; IQR)** | 66.0 (37.3-103.5) | 53 (18.5-124) | 0.9403 |
| **Median Hemoglobin (g/L; IQR)** | 73.5 (62.0-91.8) | 82 (72.8-97.8) | 0.2032 |
| **ELN2017 risk category**     |         |         | 0.1077 |
| Favorable                     | 12(30.0)| 1(7.14) |        |
| Intermediate                  | 13(32.5)| 4(28.6) |        |
| Poor                          | 15(37.5)| 8(57.1) |        |
| Unclassified                  | 0       | 1(7.14) |        |
| **FAB classification**        |         |         | 0.290  |
| M0                            | 0       | 1(7.14) |        |
| M1                            | 4(10)   | 0       |        |
| M2                            | 24(60)  | 10(71.4)|        |
| M3                            | 0       | 0       |        |
| M4                            | 0       | 0       |        |
| M5                            | 11(27.5)| 3(21.4) |        |
| M6                            | 0       | 0       |        |
| M7                            | 1(2.5)  | 0       |        |
| Not established               | 0       | 0       |        |
| **Mutated genes**             |         |         | 0.999  |
| Mutation of FLT3              | 7(17.5) | 2(14.3) |        |
| Mutation of NPM1              | 14(35.0)| 1(7.14) | 0.10   |
| Mutation of TP53              | 6(15.0) | 1(7.14) | 0.662  |
| Mutation of KIT               | 3(7.50) | 1(7.14) | 0.999  |

Data are n (%), unless otherwise stated.
*secondary AML: AML arising from preexisting myeloid neoplasms, including myelodysplastic syndrome, myeloproliferative neoplasms, or exposure to potentially leukemogenic agents.
### Table S3 Adverse events of Aza+ HAG regimen in enrolled patients

| AEs (adverse events) | All grades | Grades ≥3 |
|----------------------|------------|-----------|
| Constipation         | 7/6 (6.25) | 0         |
| Diarrhea             | 6/5 (36)  | 10/8 (8.93) |
| Vomiting             | 10/8 (9.3) | 4/3 (5.7) |
| Hypokalemia          | 10/8 (9.3) | 4/3 (5.7) |
| Peripheral edema     | 0          | 0         |
| Fatigue              | 23/20 (5)  | 6/5 (36)  |
| Hemorrhage           | 24/21 (4)  | 11/9 (82) |
| Cardiac arrhythmia   | 3/2 (68)   | 2/1 (79)  |
| Infection            | 65/58 (0)  | 38/3 (39) |
| Nausea               | 21/18 (8)  | 0         |
| Alanine/aspartate transaminase elevation | 10/8 (9.3) | 10/8 (9.3) |
| Fever                | 38/33 (9)  | 2/1 (79)  |

**Non-Hematologic AEs**

**Early mortality**
- Died within 4 weeks: 2 (1.79)

**Hematologic AEs**

**Median duration of neutropenia (IQR; days)**
- Aza + 7-day HAG: 11 (7-19)
- Aza + 14-day HAG: 16 (11-25)

**Median duration of thrombocytopenia (IQR; days)**
- Aza + 7-day HAG: 10 (6.25-18)
- Aza + 14-day HAG: 17 (12-27)

Data are n (%), unless otherwise stated.

### Table S4 Clinical responses in patients with different gene mutations

| Gene | Total | Newly diagnosed |
|------|-------|----------------|
|      | CR/Cri| PR  | NR  | Total | CR/Cri rate | PR  | NR  | Total | CR/Cri rate |
| **BCOR** | 8     | 0   | 0   | 8     | 100%        | 7   | 0   | 0     | 7     | 100%        |
| **NPM1** | 16    | 1   | 1   | 18    | 88.9%       | 14  | 0   | 1     | 15    | 93.3%       |
| **KIT**  | 6     | 0   | 1   | 7     | 85.7%       | 3   | 0   | 0     | 3     | 100%        |
| **IDH1** | 8     | 1   | 2   | 11    | 72.7%       | 7   | 0   | 0     | 7     | 100%        |
| **CEBPA** | 8    | 1   | 2   | 11    | 72.7%       | 7   | 0   | 0     | 7     | 100%        |
| **DNMT3A** | 16   | 3   | 6   | 25    | 64.0%       | 16  | 1   | 3     | 20    | 80.0%       |
| **RUNX1** | 7     | 3   | 1   | 11    | 63.6%       | 6   | 1   | 1     | 8     | 75.0%       |
| **TET2** | 12    | 3   | 5   | 20    | 60.0%       | 10  | 1   | 3     | 14    | 71.4%       |
| **ASXL1** | 9     | 5   | 1   | 15    | 60.0%       | 8   | 1   | 0     | 9     | 88.9%       |
| **FLT3** | 8     | 3   | 4   | 15    | 53.3%       | 5   | 1   | 1     | 7     | 71.4%       |
| **IDH2** | 6     | 4   | 2   | 12    | 50.0%       | 6   | 2   | 1     | 9     | 66.7%       |
| **NRAS** | 4     | 2   | 4   | 10    | 40.0%       | 4   | 1   | 2     | 7     | 57.1%       |
| **TP53** | 1     | 3   | 5   | 9     | 11.1%       | 1   | 2   | 3     | 6     | 16.7%       |
Table S5 The Leukemia Panel for next generation sequencing

| Gene  | Gene   | Gene  | Gene  | Gene  |
|-------|--------|-------|-------|-------|
| ABL1  | BRAF  | CEBPA | ETV6  | HRAS  |
| ANKRD26 | CALR | CSF3R | EZH2  | IDH1  |
| ASXL1 | CBL  | CUX1  | FLT3  | IDH2  |
| ATRX  | CBLB | DDX41 | GATA1 | IKZF1 |
| BCOR  | CBLC  | DNMT3A | GATA2 | JAK2  |
| BCSR1 | CDKN2A | ETKN1 | GNAS  | JAK3  |
| KDM6A | NPM1  | PTEN  | SMC1A | TP53  |
| KIT   | NRAS  | PTPN11 | SMC3  | U2AF1 |
| KMT2A | PDGFRA | RAD21 | SRSF2 | WT1   |
| KRAS  | PHF6  | RUNX1 | STAG1 | ZRSR2 |
| MPL   | PIGA  | SETBP1 | STAG2 |       |
| NF1   | PPM1D | SF3B1 | TET2  |       |
Fig S1 Clinical procedure of Aza +HAG regimen in this trial (A), a total of 115 AML patients were screened, 3 of them were not stratified according to the inclusion criteria. Finally, 112 patients were enrolled in this trial. Patients withdrawn from the cohort were followed for survival; OS (B) and RFS (C) curve of newly diagnosed AML patients versus secondary AML patients (arising from preexisting myeloid neoplasms).
Fig S2 Meta-analysis result of HAG regimen (homoharringtonine, cytarabine, G-CSF) in treating old/unfit newly diagnosed AML patients. (A) A total of 453 old/unfit AML patients (ineligible to receive intensive chemotherapy) from 17 studies were included, the CR/CRi rate of the HAG regimen was 47.0% (random-effects model, 95%CI, 41.0% to 53.0%) ; (B) Funnel plot of included 17 studies, no obvious bias was observed (linear regression test: t=-0.77, p=0.4559).
Fig S3 Survival of enrolled AML patients with the indicated gene mutants. OS (A) and RFS (B) in patients with FLT3 mutation (ITD or TKD) versus FLT3 wild type. OS (C) and RFS (D) in patients with ASXL1 mutation versus ASXL1 wild type. OS (E) and RFS (F) in patients with IDH1 mutation versus IDH1 wild type. *P<0.05.