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RESEARCH

Integrating *ex vivo* drug sensitivity and genetic mutation analysis improves prediction of chemotherapy response in acute myeloid leukemia

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Abstract

Acute myeloid leukemia (AML) is a heterogeneous hematological malignancy characterized by extensive genomic alterations and molecular diversity, posing significant therapeutic challenges. Current treatment strategies lack precise predictive biomarkers for chemotherapy response, highlighting the need for tools to guide precision therapy. In this study, we evaluated *in vitro* chemotherapy sensitivity in bone marrow samples from 98 AML patients using the PharmaFlow platform. Sensitivity to 10 commonly used chemotherapeutic agents (including venetoclax) and 20 combination regimens was assessed. Patients were stratified into three groups based on their PharmaFlow sensitivity profiles: multi-sensitive, intermediate-resistant, and multi-resistant. Analysis of these groups revealed that the rate of complete remission (CR) after one cycle of induction therapy decreased with increasing resistance. Additionally, integration of *in vitro* chemotherapy sensitivity data with mutational profiles indicated that *DNMT3A* mutations were linked to greater resistance to venetoclax, whereas *CEBPA* mutations in the bZIP region were linked to increased sensitivity. External validation in an independent cohort of 56 patients treated with "7+3" plus venetoclax confirmed a significantly lower CR rate in *DNMT3A*-mutant cases and a trend toward higher CR rates in *CEBPA*-mutant cases. Furthermore, multivariate Cox regression analysis identified multi-resistance in PharmaFlow stratification as an independent risk factor for overall survival (OS). High-throughput *ex vivo* drug sensitivity testing can help predict induction chemotherapy outcomes in AML. When combined with genetic mutation analysis, it helps identify mutation signatures that respond better to specific treatments, supporting the development of therapeutic strategies.

Keywords Acute myeloid leukemia · *Ex vivo* chemosensitivity test · Genetic biomarker · Venetoclax · PharmaFlow

Introduction

Acute myeloid leukemia (AML), the most common acute leukemia in adults, is a heterogeneous hematopoietic malignancies originating from hematopoietic stem/hematopoietic progenitor cells (HSCs/HPCs) (Khwaja et al. 2016; Short et al. 2018). Cellular and molecular genetic abnormalities are the pathogenesis of AML. The dysregulation of gene expression and function leads to HSCs/HPCs differentiation block, apoptosis disorder and malignant proliferation, which leads to the onset of leukemia. The complete response rate of the "7+3" regimen, which consists of 7 days of cytarabine and 3 days of an anthracycline is still not ideal. Identify more sensitive chemotherapy regimens for patients can help improve the clinical response rate of patients (Burnett et al. 2015; Forsberg

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and Konopleva 2024; Löwenberg et al. 2009; Yates et al. 1973). Patients with different molecular genetic abnormalities respond differently to the same treatment regimen (Kantarjian et al. 2025). However, the number of biochemical and genetic biomarkers associated with drug sensitivity is very limited, so that the prediction of individual patient response before treatment delivery remains challenging. Investigating the correlations between genetic biomarkers and chemotherapy sensitivity could facilitate the selection of more suitable induction chemotherapy regimens for AML patients.

Venetoclax functions as a selective inhibitor of the BCL-2 protein (Souers et al. 2013). As a BH3-mimetic, venetoclax specifically targets the anti-apoptotic BCL-2 protein in susceptible cells, facilitating the displacement of the pro-apoptotic protein BIM from BCL-2. This displacement subsequently activates the effector proteins BAX or BAK, leading to apoptosis via the release of cytochrome c (Chen et al. 2005; Kluck et al. 1997; Souers et al. 2013; Yang et al. 1997). BCL-2 is highly expressed in AML cells, including leukemia stem cells, and thus, in both older and younger patients, venetoclax showed significant activity in combination with hypomethylating agent or chemotherapy (DiNardo et al. 2020; Pan et al. 2014; Pratz et al. 2022; Wang et al. 2022).

The PharmaFlow platform is a novel automated flow cytometry-based platform that enables rapid data acquisition, analysis, and reporting of results through proprietary analysis software. Automated PharmaFlow enables high-throughput measurement of the *ex vivo* pharmacology of single drugs and drug combinations in malignant cells from peripheral blood (PB) or bone marrow (BM) samples (Hernández et al. 2017). Bone marrow samples from leukemia patients were added to the drug screening orifice. After incubation, the killing efficiency of each chemotherapy drug and its combination on leukemia cells was detected by flow cytometry. Then, the adaptive data of various drugs and combinations to patients were calculated by using the data model database, which provided the basis for clinicians to make individualized treatment plans and implement precision medicine (Bennett et al. 2014). In this study, we evaluated the clinical relevance of gene mutations and the *ex vivo* chemotherapy sensitivity of 10 drugs commonly used for AML induction therapy in 98 patients. Meanwhile, we assessed the consistency of *ex vivo* sensitivity with actual clinical treatment results.

Materials and methods

Patients

The study enrolled 98 patients diagnosed with AML between September 2017 and August 2023. BM samples were collected in heparinized tubes from patients. Patients with acute promyelocytic leukemia (APL) or with AML secondary to myelodysplastic syndrome were excluded. The diagnosis and classification were based on the 2022 World Health Organization (WHO) and 2022 European Leukemia Net (ELN) criteria (Arber et al. 2022; Döhner et al. 2022). The study was approved by the Research Ethics Committee of Qilu Hospital. All patients provided informed consent to participate in the study.

PharmaFlow chemosensitivity profiling

The *ex vivo* chemosensitivity profiling was performed by the PharmaFlow platform in the laboratories of the Hosea Precision Medicine. The patient's fresh bone marrow sample was stored in a heparinized tube at −4 °C. PharmaFlow chemosensitivity profiling was performed within 24h after sampling. The bone marrow samples were incubated with the corresponding drug for 48–72h in a simulated *in vivo* environment. After drug incubation, leukemic cells were labeled with fluorescently coupled antibodies and detected by flow cytometry. The response effect of drug was assessed by counting the remaining live pathological cells (LPCs) at different concentrations after a set incubation time, which was used to create dose-response curves (Onecha et al. 2020). Previous publications have detailed the PharmaFlow sampling and analysis methods(Bennett et al. 2014; Hernández et al. 2017; Martínez-Cuadrón et al. 2019). Ten drugs were screened for PharmaFlow chemosensitivity profiling: cytarabine (Ara-C), aclarubicin (ACLA), cladribine (CLA), daunorubicin (DNR), etoposide (ETO), fludarabine (FLU), idarubicin (IDA), homoharringtonine (HHT), mitoxantrone (MIT) and venetoclax (VEN). The patient's sensitivity to these drugs was quantified on a scale ranging from 0 to 100% (PharmaFlow score), representing a continuum of increasing sensitivity. These drugs made up 20 common combined chemotherapy regimens that were also tested by PharmaFlow chemosensitivity profiling (Supplementary Table 1). PharmaFlow assessed patient's sensitivity to these 20 regimens, and divided the results into five grades: grade 1 (0-19.99%, resistant), grade 2 (20%-39.99%, relatively resistant), grade 3 (40%-59.99%, moderately resistant), grade 4 (60%-79.99%, relatively sensitive), and grade 5 (80%-100%, sensitive).

Clinical data collection and study endpoints

Clinical data were collected since diagnosis, including the following parameters: age, gender, white blood cells (WBC), blast cells, French-American-British (FAB) classification, ELN risk stratification and induction treatment. Refractory AML is defined as failure to achieve

complete remission (CR) after two cycles of standard induction therapy.

The endpoints were CR and overall survival (OS). OS is defined as the time from the first treatment to death. Of the 98 patients, nine were lost to follow-up due to changes in contact information, which were excluded from survival analysis.

Statistical analysis

For statistical analysis, SPSS Statistics version 26 and GraphPad Prime 10.0 were utilized. $P<0.05$ (*), 0.01 (**), 0.001 (***), and 0.0001 (****) represent the significance. To determine if the data were normally distributed, the Shapiro-Wilk test was applied. If the data deviated from normality, the median was utilized, and nonparametric methods were employed. Conversely, if the data satisfied the normality assumption, the mean was reported, and parametric analysis was conducted. The two-tailed Student's t-test was utilized to compare the two groups, while comparisons among multiple groups were conducted using a one-way ANOVA. Categorical parameters were compared by Chi square test or Fisher's exact test (according to their application range). Hierarchical clustering and Z-score normalization of rows or columns of data matrix were performed to draw heatmaps according to analysis requirements. The hierarchical clustering of drug sensitivity profiles was performed using Euclidean distance as the distance metric and the complete linkage method. The resulting heatmaps were then sorted based on the mean value of the assigned clusters for clearer visualization of patient groups. The classification of each group within the PharmaFlow category was determined by the primary clusters derived from the clustering tree used for grouping. Spearman's rank correlation coefficient was used to evaluate the strength and direction of the association between the two variables. The Kaplan-Meier survival plot was selected to analyze survival time and the differences in the survival curves were compared by the log-rank test. Variables with P-values below 0.05 in the univariate Cox regression analyses were incorporated into the multivariate Cox regression models. Multivariate Cox proportional hazards models were utilized to estimate hazard ratios (HR).

Results

Patient characteristics

A total of 98 patients with AML (non-M3 type) were included, of whom 85 (86.7%) were newly diagnosed (ND), 13 (13.3%) were relapse/refractory (R/R). The median age of patients was 47 years (14–87 years). The 2022 ELN AML risk stratification showed that 40.8% (40/98), 39.8% (39/98), and 19.4% (19/98) of patients were stratified into favorable, intermediate, and adverse risk, respectively.

In total, 23.5% (23/98) patients were treated with DA (daunorubicin + cytarabine) regimen, 41.8% (41/98) with IA (idarubicin + cytarabine), 7.1% (7/98) with IAV (idarubicin + cytarabine + venetoclax), and 27.6% (27/98) with the other regimens in the induction. In addition, 8.2% (8/98) received allogeneic hematopoietic stem cell transplantation. Nine of these patients were lost to follow-up due to changes in contact information and were excluded from all survival analyses in this study. CR was observed in 83.1% of ND patients (64.9% in the first cycle) and 25% of R/R patients. The detailed clinical data of WBC and blast cell counts, and AML FAB classification were summarized in Table 1.

The most prevalent cellular and molecular genetic abnormalities identified in the patient cohort were as follows: FMS-like tyrosine kinase 3 (*FLT3*) mutations were present in 26.5% of patients, with 84.6% of these exhibiting *FLT3*-ITD mutations and 15.4% displaying *FLT3*-TKD mutations. CCAAT enhancer binding protein A (*CEBPA*) mutations were observed in 23.5% of patients, with 56.5% of these mutations occurring in the basic leucine zipper (bZIP) region and 43.5% occurring outside the bZIP region. Additionally, mutations in Nucleophosmin 1 (*NPM1*) were found in 21.4% of patients, DNA methyltransferase 3 alpha (*DNMT3A*) mutations in 19.4%, Tet methylcytosine dioxygenase 2 (*TET2*) mutations in 14.3%, Neuroblastoma RAS Viral Oncogene Homolog (*NRAS*) mutations in 12.2%, and *CBFB::MYH11* fusion in 11.2% of the cohort (Supplementary Table 2).

The PharmaFlow categories can predict the treatment response and prognosis of AML patients

Clinical follow-up data of 89 patients after induction therapy were available in this study. We divided these patients into two groups: newly diagnosed patients who achieved CR after induction therapy ($n=64$) were included in one group (CR patients), newly diagnosed patients who were NR after 2 induction attempts ($n=13$) and R/R patients ($n=12$) were included in the other group (NR + R/R patients). The stratification of ELN risk stratification was significantly better in the CR patients than in the NR + R/R patients ($P=0.0121$, Fig. 1A).

We drew a heatmap of these patients' drug sensitivity results (Fig. 1B). CR patients and NR + R/R patients were shown in green and yellow respectively in the heatmap. Blue indicated that the patient was sensitive to the corresponding drug combination, while red indicated resistant. Utilizing PharmaFlow cluster analysis, patients were

Table 1 General characteristics of 98 AML patients

Characteristics	AML patients ($n=98$)
Gender (female/male), n	53/45
Age, median (range), years	47 (14–87)
WBC, median (range), 10^9/L	17.24 (0.15-479.73)
Hb, median (range), g/L	79.5 (31–131)
PLT, median (range), 10^9/L	35 (1-249)
Blasts in the bone marrow, median (range), %	76 (21–96)
AML status at entry, n (%)	
Newly diagnosed	85 (86.7%)
Relapse & Refractory	13 (13.3%)
FAB classification, *n* (%)	
AML-M1	2 (2.0%)
AML-M2	4 (4.1%)
AML-M4	26 (26.5%)
AML-M5	54 (55.1%)
others	12 (12.2%)
2022 ELN risk stratification, *n* (%)	
Favorable	40 (40.8%)
Intermediate	39 (39.8%)
Adverse	19 (19.4%)
Induction chemotherapy regimen, *n* (%)	
IA	41 (41.8%)
DA	23 (23.5%)
HA	1 (1.0%)
IAV	7 (7.1%)
Azacitidine	11 (11.2%)
Decitabine	10 (10.2%)
Others	5 (5.1%)
Allo-HSCT, *n* (%)	8 (8.2%)
Follow-up time, median (range), months	13.1 (0.03–73.4)
Response to induction therapy	
Newly diagnosed	($n=77$)
CR, *n* (%)	64 (83.1%)
CR after 1 cycle, *n* (%)	50 (64.9%)
NR, *n* (%)	13 (16.9%)
Relapse & Refractory	($n=12$)
CR, *n* (%)	3 (25%)
NR, *n* (%)	9 (75%)

AML acute myeloid leukemia, *WBC* white blood cells, *Hb* hemoglobin, *PLT* platelet count, *ELN* European leukemia Net, *CR* complete remission, *NR* non-remission, *HSCT* hematopoietic stem cell transplantation, *IA* idarubicin + cytarabine, *DA* daunorubicin + cytarabine, *HA* homoharringtonine + cytarabine, *IAV* idarubicin + cytarabine + venetoclax

stratified into three distinct categories: multi-sensitive (MS), intermediate-resistant (IR), and multi-resistant (MR). Notably, the proportion of patients achieving CR after one cycle of induction therapy was 73.68% in MS, 59.38% in IR and 31.58% in MR ($P=0.0095$, Fig. 1C). Whereas, there was no significant difference in CR rates between the adverse-risk and the favorable & intermediate-risk patients

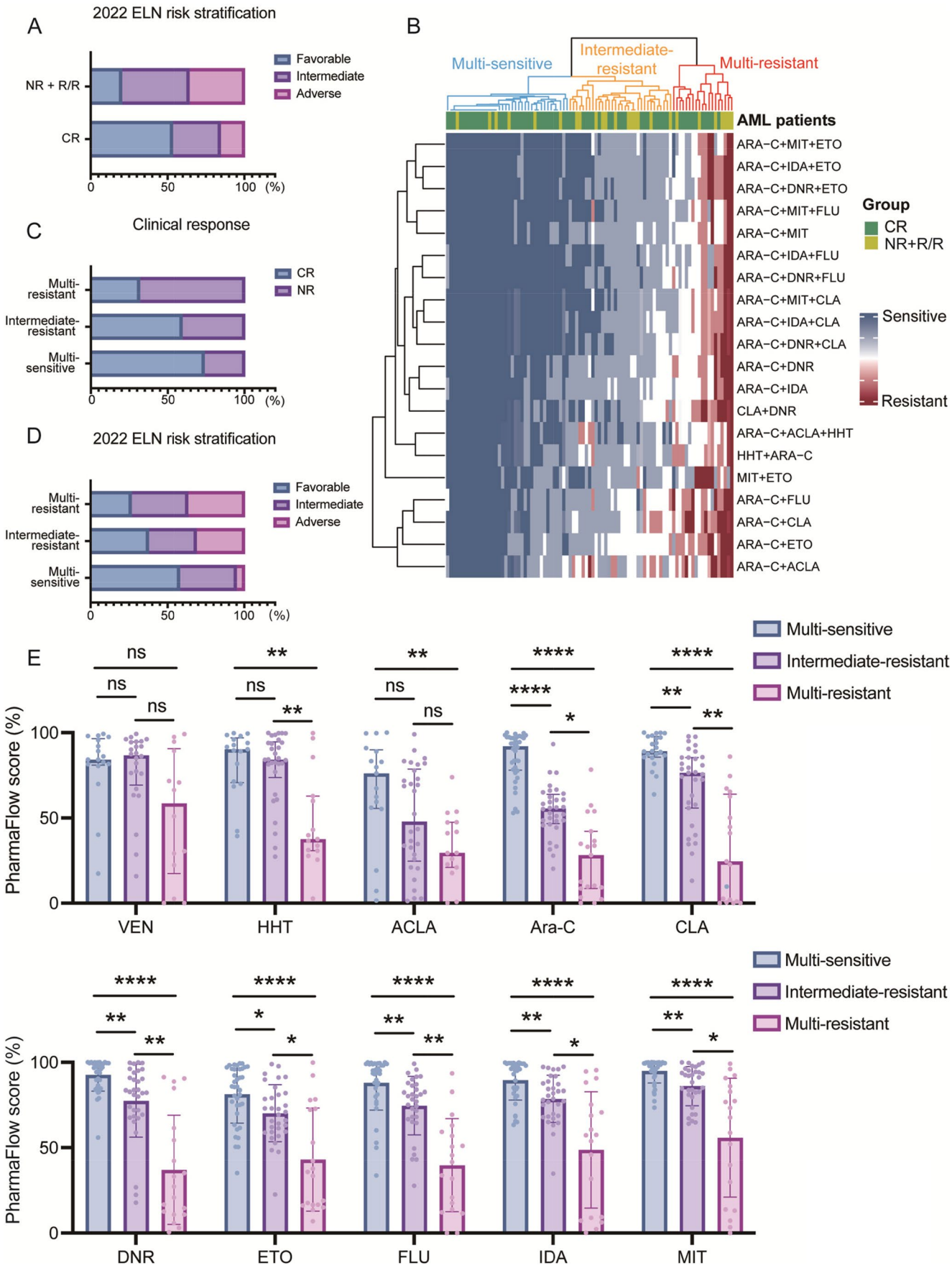
Fig. 1 Chemotherapy sensitivities of AML patients based on PharmaFlow categories. (**A**) The 2022 ELN risk stratification of AML patients. CR (complete remission), $n=64$; NR (non-remission), $n=13$; R/R (Relapse & Refractory), $n=12$; $P=0.0121$ (P value is based on a Chi-square test). (**B**) The heatmap illustrates the drug sensitivities of 89 patients with AML. Each column represents an individual patient, with CR patients highlighted in green and the NR + R/R patients in yellow. Each row corresponds to a specific chemotherapy regimen. In heatmap, the color variations reflect the patient's sensitivity to the respective chemotherapy regimen, where blue indicates drug sensitivity and red signifies drug resistance. Utilizing PharmaFlow cluster analysis, patients were stratified into three distinct categories: multi-sensitive (MS, $n=38$), intermediate-resistant (IR, $n=32$), and multi-resistant (MR, $n=19$). (**C**) The complete remission rate of each PharmaFlow category after 1 cycle induction therapy (73.68% in MS, 59.38% in IR and 31.58% in MR, $P=0.0095$, Chi-square test). (**D**) The ELN risk stratification of each PharmaFlow category (favorable-risk: 57.90% in MS, 37.50% in IR and 26.32% in MR, $P=0.0194$, Chi-square test). (**E**) *Ex vivo* sensitivities of individual drugs in each PharmaFlow category. The higher the PharmaFlow score, the more sensitive the patient is to the drug. Data are shown as median with interquartile range; $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$ (Kruskal-Wallis test)

stratified by ELN stratification (Supplementary Fig. 1). The proportion of patients with favorable-risk according to the ELN stratification was 57.90% in MS, 37.50% in IR and 26.32% in MR ($P=0.0194$, Fig. 1D). Additionally, all patients ≥ 65ys were classified into IR + MR ($P=0.0289$, Supplementary Fig. 2). This result suggested the PharmaFlow categories can predict the response to treatment and prognosis of patients very well.

We then compared the *ex vivo* sensitivity of single drugs in the three categories. The higher the PharmaFlow score, the more sensitive the patient was to the drug. It was found that the PharmaFlow score of a single drug decreased in the MR category compared with MS and IR categories for all drugs except venetoclax. There was no difference in the PharmaFlow scores of venetoclax and homoharringtonine between MS and IR categories, suggesting that patients in the IR category may benefit from the use of these chemotherapeutic agents (Fig. 1E).

Patients in the three PharmaFlow categories have different genetic characteristics

Patients in different PharmaFlow categories demonstrated distinct enrichment of genetic abnormalities as shown in Fig. 2A. A higher proportion of patients with *CEBPA* bZIP in-frame mutations (*CEBPA*^{mu} in bZIP) was found in the MS category (26.32% in MS vs. 5.88% in IR + MR, $P=0.0069$, Fig. 2B). Patients harboring *FLT3-ITD* mutations were predominantly found in the MS category (39.47% in MS vs. 9.80% in IR + MR, $P=0.0009$, Fig. 2C). Conversely, patients with *IDH1* mutations were more frequently observed in the IR + MR category (13.73% in IR + MR vs. 0.00% in MS, $P=0.0476$, Fig. 2D). Similarly, all patients with *SRSF2* ($P=0.0780$, Fig. 2E), *ASXL1*


A
2022 ELN risk stratification
NR + R/R
CR
Favorable
Intermediate
Adverse
0
50
100 (%)
B
Multi-sensitive
Intermediate-resistant
Multi-resistant
AML patients
ARA−C+MIT+ETO
ARA−C+IDA+ETO
ARA−C+DNR+ETO
ARA−C+MIT+FLU
ARA−C+MIT
ARA−C+IDA+FLU
ARA−C+DNR+FLU
ARA−C+MIT+CLA
ARA−C+IDA+CLA
ARA−C+DNR+CLA
ARA−C+DNR
ARA−C+IDA
CLA+DNR
ARA−C+ACLA+HHT
HHT+ARA−C
MIT+ETO
ARA−C+FLU
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ARA−C+ACLA
Group
CR
NR+R/R
Sensitive
Resistant
C
Clinical response
Multi-resistant
Intermediate-resistant
Multi-sensitive
CR
NR
0
50
100 (%)
D
2022 ELN risk stratification
Multi-resistant
Intermediate-resistant
Multi-sensitive
Favorable
Intermediate
Adverse
0
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100 (%)
E
Multi-sensitive
Intermediate-resistant
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PharmaFlow score (%)
0
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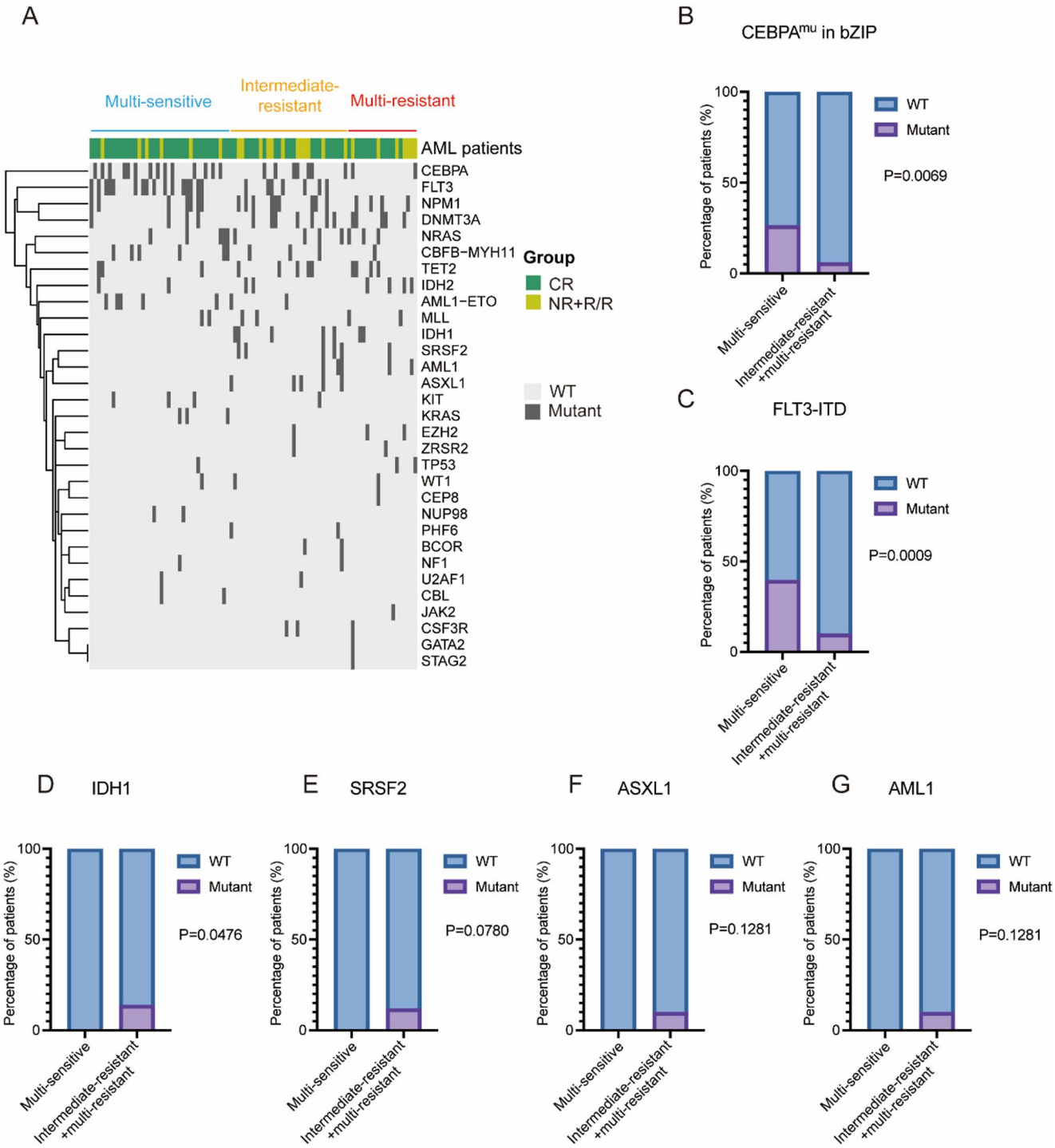


Fig. 2 Genetic abnormality profiles of AML patients in Pharma-Flow categories. (**A**) Heatmap of genetic abnormalities of 89 AML patients. Each column corresponds to an individual patient, with the CR patients shown in green and the NR + R/R patients in yellow. Each row denotes a specific genetic mutation. The heatmap illustrates the mutation characteristics observed in the patients involved in this study, with gray patches indicating the presence of mutations. WT, wild-type. (**B**-**G**) Distribution of patients with mutations in the multi-sensitive (MS, $n = 38$) and the intermediate-resistant + multi-resistant (IR + MR, $n = 51$) categories. P value of *CEBPA*^{mu} in bZIP, *FLT3-ITD* are based on Chi-square tests. P value of *IDH1*, *SRSF2*, *ASXL1* and *AML1* are based on Chi-square tests with Yates' correction

($P=0.1281$, Fig. 2F) or *AML1* ($P=0.1281$, Fig. 2G) mutations were found within the IR + MR category. These results indicated patients carrying favorable gene mutations were more likely to be classified into the better PharmaFlow category, and vice versa.

Different genotypes of patients have different sensitivity to chemotherapy drugs

We drew a heatmap illustrating the correlation between genetic biomarkers and *ex vivo* drug sensitivity of 10 chemotherapy drugs (Fig. 3A). We evaluated the reliability of the correlations and trends in the Fig. 3A heatmap by comparing the single drug sensitivities of patients with specific gene mutations to those without mutations.

Certain genetic abnormalities were linked to resistance to chemotherapeutic agents. For instance, the presence of the *CEBPA* mutation in the bZIP region demonstrated increased sensitivity to venetoclax, ACLA, Ara-C, CLA, FLU, and MIT (Fig. 3B). The presence of the *DNMT3A* mutation was associated with increased resistance to venetoclax and Ara-C (Fig. 3C). Additionally, the *AML1* mutation conferred resistance to Ara-C and etoposide (Fig. 3D). A total of 7 patients in the enrolled patient cohort received the IAV (idarubicin + cytarabine + venetoclax) induction regimen, and their CR rate after 1 cycle induction was significantly higher than that of patients receiving the "7 + 3" induction regimen (100% vs. 55.6%, $P=0.0367$, Fig. 3E). To validate the association between these gene mutations and venetoclax sensitivity, we analyzed data from an independent cohort of 56 AML patients treated with "7 + 3" plus venetoclax regimens. In this cohort, the CR rate was 100% for patients with *CEBPA* mutations compared to 82.6% for those without *CEBPA* mutations ($P>0.05$, Supplementary Fig. 3E). By contrast, *DNMT3A*-mutant patients had a lower CR rate of 60.0%, compared with 91.3% in wild-type patients ($P=0.0389$, Supplementary Fig. 3F). These findings help to select personalized venetoclax-based therapeutic strategies for patients with specific gene mutations. The associations for the other tested genes and *in vitro* drug sensitivity are summarized in Supplementary Fig. 3.

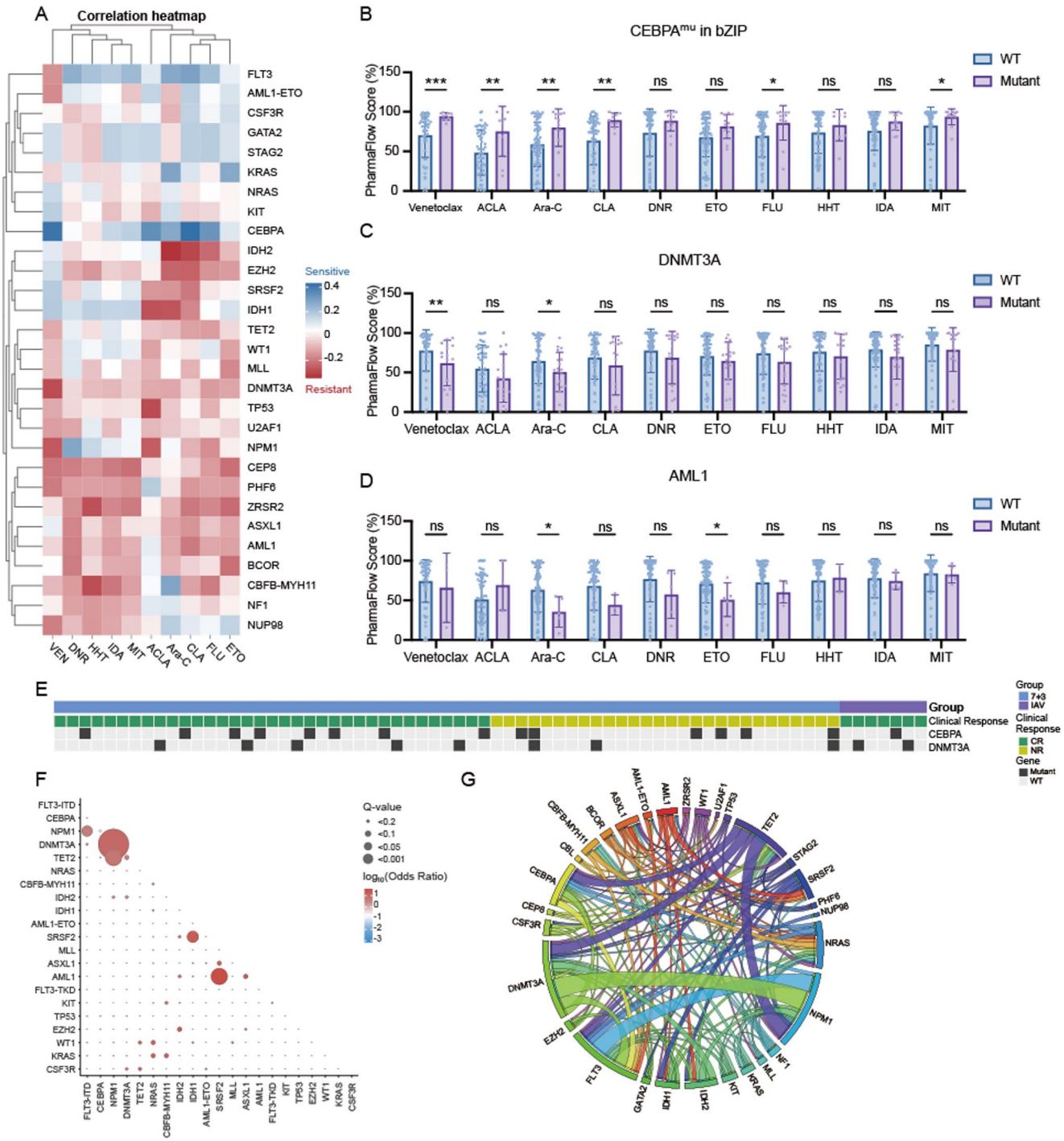
In this study, we also analyzed the influence of genetic co-mutation patterns on drug sensitivities. In our cohort, the *NPM1* gene frequently co-mutated with other genes, including *DNMT3A*, *FLT3-ITD*, and *TET2* (Fig. 3F & G).

PharmaFlow profiling assists in choosing effective combination drug regimens for AML patients

In the heatmaps of drug combinations and gene mutations (Fig. 4A), in most cases, the sensitivity trend of drug combinations was the same as that of the corresponding single drug. But the heatmaps still offered some interesting speculations. For example, patients harboring *EZH2* mutations exhibited resistance to all drug combinations (Fig. 4A), while it showed sensitivity to venetoclax (Fig. 3A), suggesting that the incorporation of venetoclax into their induction regimen may be advantageous. Conversely, patients with *GATA2* or *STAG2* mutations demonstrated sensitivity to various single drugs but exhibited resistance to all tested drug combinations (Figs. 3A and 4A). Consequently, there is a need to develop novel chemotherapy regimens specifically tailored for patients with *GATA2* or *STAG2* mutations. Patients harboring *AML1* mutations exhibited increased resistance to the Ara-C + IDA, Ara-C + IDA + FLU, and Ara-C + DNR (Fig. 4B-C and E) regimens. However, no difference in drug resistance was observed for the Ara-C + IDA + CLA regimen, irrespective of *AML1* mutation status (Fig. 4D). Additionally, patients with *DNMT3A* mutations were more resistant to the Ara-C + IDA and Ara-C + IDA + FLU (Fig. 4B and C) regimens. In contrast, there was no significant difference in *ex vivo* resistance to the Ara-C + IDA + CLA and Ara-C + DNR regimen between patients with and without *DNMT3A* mutations (Fig. 4D and E). Therefore, for patients with *AML1* or *DNMT3A* mutations, the Ara-C + IDA + CLA regimen may be the preferred induction therapy. In comparison, patients with CEBPA bZIP mutations showed enhanced sensitivity to the Ara-C + IDA, Ara-C + IDA + FLU, Ara-C + IDA + CLA, and Ara-C + DNR (Fig. 4B-E) regimens. Cross-sectional analysis of *in vitro* drug sensitivity data holds potential to identify applicable patient populations that respond to specific drug combinations, thereby aiding the development of novel induction regimens.

The PharmaFlow stratification can predict overall survival of AML patients

In this study, AML patients in the multi-resistant category exhibited a significantly reduced overall survival (OS) compared to those in the multi-sensitive category ($P=0.0191$, Fig. 5A). Notably, significant correlations were identified between specific genetic mutations and OS. Patients harboring mutations in *TP53* ($P<0.0001$, Fig. 5B) and *DNMT3A* ($P=0.0159$, Fig. 5C) experienced markedly shorter OS compared to those without such mutations. In contrast, individuals with the *CBFB::MYH11* rearrangement demonstrated improved OS ($P=0.0203$, Fig. 5D). Univariate Cox regression analysis revealed that WBC count, PLT count, Hb, multi-resistant category in PharmaFlow stratification, and ELN risk stratification were significant prognostic factors ($P<0.05$, Table 2). Furthermore, multivariate



Cox regression analysis indicated that, after adjusting for WBC count, PLT count, Hb and ELN risk classification, as independent prognostic factors, patients with multi-resistant category had a significantly decreased survival compared to those with multi-sensitive category (HR = 3.852, 95% CI 1.448 to 10.100, $P = 0.007$, Fig. 5E-F, and Table 2).

Discussion

AML is highly heterogeneous, so the prognosis of different patients varies greatly: a 5-year overall survival rate of 40%-45% in the young and a 5-year survival rate of less than 10% in the elderly (>60 years of age). Such a grim

Fig. 3 Correlation between *in vitro* drug sensitivities and gene mutations alongside cytogenetic profiles. (**A**) Heatmap of drug sensitivities and gene mutations of 97 AML patients. Each row of the heatmap represents a specific genetic mutation, while each column corresponds to a chemotherapy drug. Ara-C, cytarabine; ACLA, aclarubicin; CLA, cladribine; DNR, daunorubicin; ETO, etoposide; FLU, fludarabine; IDA, idarubicin; HHT, homoharringtonine; MIT, mitoxantrone; VEN, venetoclax. (**B-D**) Comparison of drug sensitivities in AML patients with wild-type (WT) and mutant genes. The higher the PharmaFlow score, the more sensitive the patient is to the drug. (**B**) Patients with *CEBPA bZIP* in-frame mutations exhibit increased sensitivity to venetoclax (WT: $n=55$, Mutant: $n=9$, P=0.0002), ACLA (WT: $n=62$, Mutant: $n=9$, P=0.0092), Ara-C (WT: $n=85$, Mutant: $n=12$, P=0.0071), CLA (WT: $n=70$, Mutant: $n=10$, P=0.0028), FLU (WT: $n=84$, Mutant: $n=13$, $P=0.0101$), and MIT (WT: $n=84$, Mutant: $n=13$, P=0.0205). (**C**) Patients with *DNMT3A* mutations show enhanced resistance to venetoclax (WT: $n=48$, Mutant: $n=16$, $P=0.0067$) and Ara-C (WT: $n=78$, Mutant: $n=19$, P=0.0304) (**D**) Patients with *AML1* mutations demonstrate greater resistance to Ara-C (WT: $n=92$, Mutant: $n=5$, $P=0.0236$) and ETO (WT: $n=92$, Mutant: $n=5$, $P=0.0430$). Data are shown as median with interquartile range; *$P<0.05$; **$P<0.01$; ***P<0.001; P values of Fig. 3B-3D are based on Mann-Whitney tests. (**E**) CR rate of patients after 1 cycle induction therapy with the "IAV" regimen ($n=7$) and "7+3" regimen ($n=63$) (100% vs. 55.6%; $P=0.0367$). (**F**) Bubble plot illustrating gene co-mutations in AML patients. (**G**) A Circos plot visually representing the relationships among various genes exhibiting co-mutations

prognosis underscores the necessity for further research to explore more effective treatment strategies for each patient (Hassan et al. 2017; Stölzel et al. 2016).

As an *in vitro* drug sensitivity screening technology, PharmaFlow test can accurately predict the clinical response of patients to different treatment strategies (Zhang et al. 2021). We divided patients into multi-sensitive, intermediate-resistant and multi-resistant categories based on the PharmaFlow cluster analysis of the 20 chemotherapy regimens. The CR rate after 1 cycle induction therapy in the intermediate- and multi-resistant group was significantly lower than that of multi-sensitive group (73.68% in MS, 59.38% in IR, 31.58% in MR, $P=0.0095$). Besides, multi-resistant PharmaFlow stratification was significantly correlated with reduced OS ($P=0.0191$) and served as an independent adverse prognostic factor for OS. Our results showed that the PharmaFlow test can predict patients' sensitivity to chemotherapy drugs. Previous studies have also shown that PharmaFlow test has great clinical utility in predicting both sensitive and resistant patients (Lin et al. 2020; Megías-Vericat et al. 2019).

On this basis, we analyzed the correlation between *ex vivo* sensitivity of chemotherapeutic drugs and different gene mutations. Patients with $CEBPA^{mu}$ in bZIP showed increased *ex vivo* sensitivity to venetoclax, aclarubicin, cytarabine, cladribine, fludarabine, and mitoxantrone. Conversely, reduced *ex vivo* sensitivity was observed in patients with *DNMT3A* mutation in venetoclax and cytarabine, *AML1* mutation in cytarabine and etoposide. At the same time, many new phenomena discovered in this study need to be further verified in independent cohorts.

Recent studies have demonstrated a significant association between *CEBPA* mutations in the bZIP domain and favorable prognostic outcomes, whereas *CEBPA* mutations outside the bZIP domain do not exhibit this association (Taube et al. 2022). Specifically, patients harboring *CEBPA* mutations in bZIP exhibited higher CR rates, better OS, and a lower risk of recurrence (Wakita et al. 2022). Notably, this study's cluster analysis revealed that patients with $CEBPA^{mu}$ in bZIP displayed a tendency toward multi-sensitivity, whereas those with *CEBPA* mutations out of bZIP did not exhibit this characteristic.

The dysregulation of DNA methylation is a key event for AML initiation and progression (Yang et al. 2019). DNA methyltransferases (DNMTs) and Ten-eleven translocations (TET) are required to regulate DNA methylation (Greenberg and Bourc'his 2019). Several gene mutations associated with DNA methylation are often observed in AML, including *TET2*, *DNMT3A*, *IDH1* and *IDH2* (Prado et al. 2021). The loss of function mutations of *TET2* and *DNMT3A* are common injuries in AML (Figueroa et al. 2010; Ley et al. 2010; Russler-Germain et al. 2014). *TET2* and *DNMT3A* may be associated with venetoclax resistance as observed in the Fig. 3A heat map. In contrast, we observed that *IDH1* and *IDH2* mutations may contribute to venetoclax sensitivity in AML. Simultaneously, the gain of function mutations of *IDH1* or *IDH2* often occur in AML, which impaired *TET2* catalytic function in AML cells (Figueroa et al. 2010; Xu et al. 2011). Within these genes, we found that patients with *DNMT3A* mutations may be more resistant to venetoclax ($P=0.0067$). This observation is consistent with previous studies reporting that *DNMT3A* mutations are associated with poor response and shorter survival in AML patients receiving venetoclax-based regimens (Wang et al. 2020) (Yu et al. 2025). Wang R et al. showed that in young adult AML patients eligible for intensive chemotherapy, DAV regimens induced high CR rates, deep MRD-negative CRc rates and extended overall survival after one cycle of induction therapy, suggesting venetoclax as an effective complement to traditional intensive chemotherapy (Wang et al. 2024). Yet our clinical cohorts showed that patients with a mutation in *DNMT3A* have lower rate of complete remission to "7+3" & venetoclax regimen (60.0% vs. 91.3%, $P=0.0389$) in respect to *DNMT3A* wild type patients. Many studies have shown that venetoclax improves its efficacy when used in combination with DNA methyltransferase inhibitors (DNMTi) (Jonas and Pollyea 2019). The combination of treatment strategies for venetoclax in patients with *DNMT3A* mutations remains to be explored.

CEBPA encodes a transcription factor that plays a critical role in granulocyte differentiation and is one of the common genetic alterations observed in AML. Mutations in bZIP domain of *CEBPA* are strongly associated with

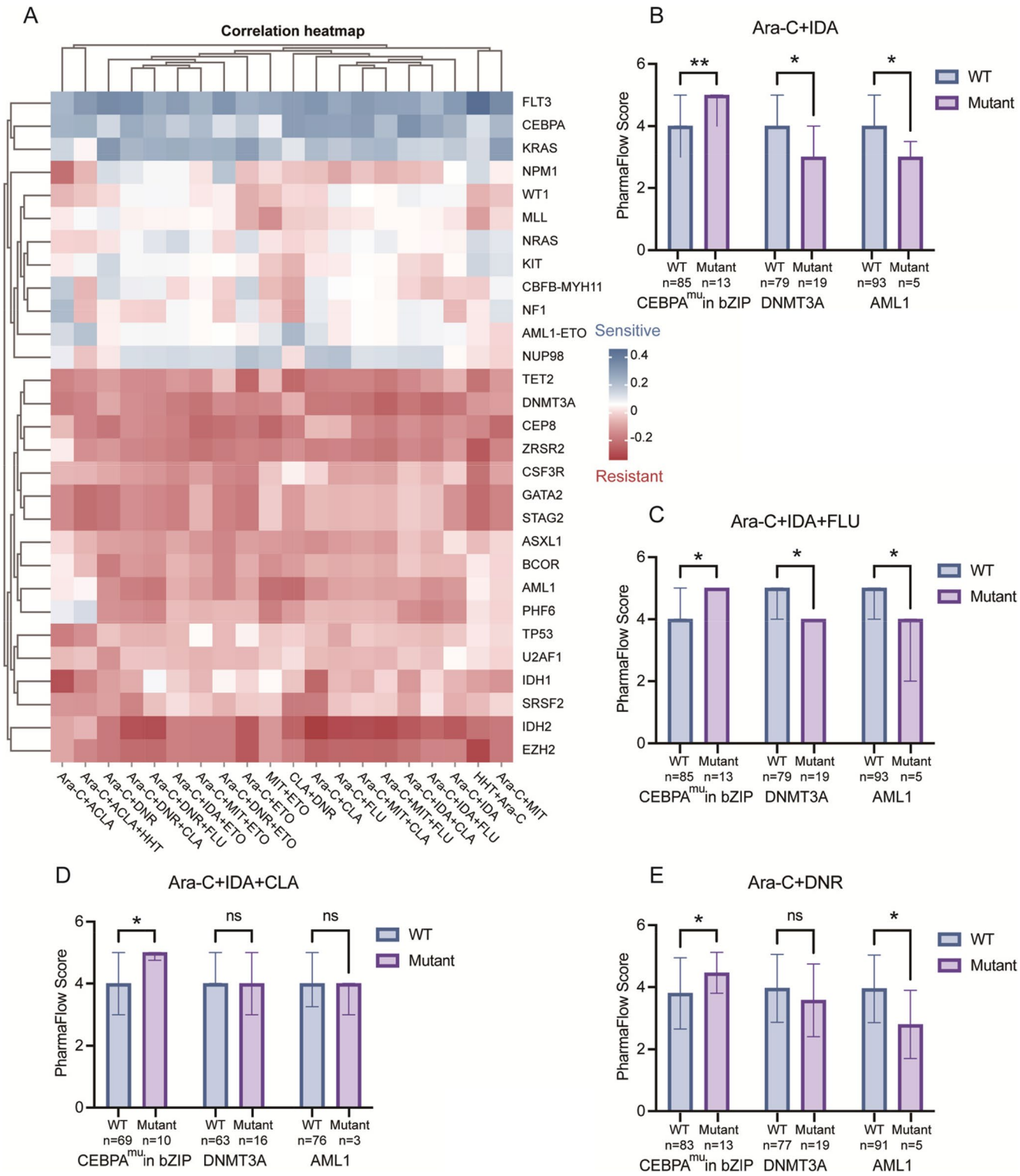


Fig. 4 Correlation between *in vitro* drug combination sensitivities and gene mutations alongside cytogenetic profiles. (**A**) Heatmap of drug combination sensitivities and gene mutations of 98 AML patients. Each row represents a genetic mutation, and each column signifies a drug combination regimen. (**B**-**E**) Comparison of drug combination sensitivities in AML patients with wild-type (WT) and mutant genes. The higher the PharmaFlow score, the more sensitive the patient is to the drug combination regimen. Data are shown as median with interquartile range; *$P<0.05$; **$P<0.01$. P values of Fig. 5B and E are based on Mann-Whitney tests

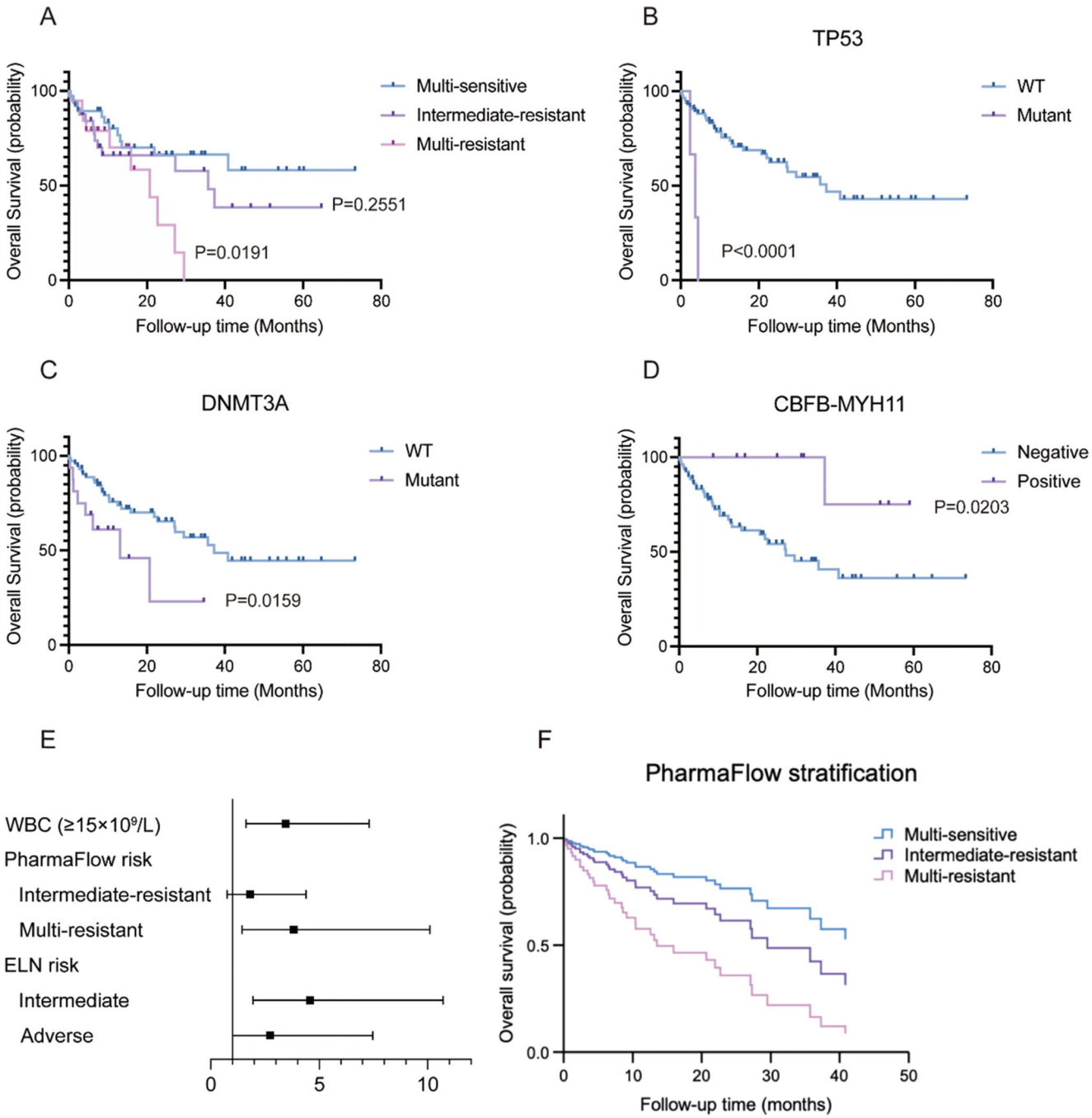


Fig. 5 Kaplan-Meier estimates for overall survival and Cox proportional hazard models. (**A**) Kaplan-Meier survival analysis for PharmaFlow stratification. Multi-sensitive, $n=38$; intermediate-resistant, $n=32$; multi-resistant, $n=19$. (**B**) Overall survival (OS) of AML patients categorized by *TP53* mutation status. WT, $n=86$; Mutant, $n=3$. (**C**) Kaplan-Meier analysis of OS concerning *DNMT3A* mutation status. WT, $n=73$; Mutant, $n=16$. (**D**) The results of Kaplan-Meier analyses for OS in patients with *CBFB::MYH11* rearrangement. WT, $n=79$; Mutant, $n=10$. (**E**) The forest plot demonstrates the multivariate analysis of factors influencing OS among 89 AML patients. (**F**) Kaplan-Meier survival analysis of AML patients with multi-resistance in PharmaFlow, stratified by independent prognostic factors

a favorable prognosis (Wakita et al. 2022). Chen X et al. demonstrated that *CEBPA* interacts with *DNMT3A* on specific DNA sequences, thereby preventing *DNMT3A* from catalyzing CpG methylation. Mutations in the bZIP domain of *CEBPA* that disrupt its DNA binding capacity alleviate *DNMT3A* inhibition, resulting in hypermethylation of PRC2 target genes (Chen et al. 2022). Consequently, *CEBPA* can inhibit *DNMT3A* activity. This study identified that in-frame mutations in the *CEBPA* bZIP domain may be associated with increased sensitivity to venetoclax

Table 2 Univariate and multivariate Cox proportional hazard models for overall survival

Clinical features	Univariate analysis		Multivariate analysis	
	P value	HR (95% CI) *	*P* value	HR (95% CI) *
WBC (≥15×10^9/L)	0.019	2.144 (1.091-4.210)	0.001	3.448 (1.627-7.308)
PLT (<10×10^9/L)	0.038	2.738 (1.059–7.079)	0.133	/
Hb (<50 g/L)	0.049	2.287 (1.146–4.582)	0.055	/
PharmaFlow stratification	0.080		0.031	
Intermediate-resistant	0.224	1.630 (0.741–3.582)	0.185	1.816 (0.752–4.388)
Multi-resistant	0.025	2.700 (1.135–6.427)	0.007	3.825 (1.448–10.100)
ELN risk stratification	0.005		0.001	
Intermediate	0.002	3.771 (1.662–8.560)	<0.001	4.562 (1.941–10.719)
Adverse	0.013	3.354 (1.296–8.682)	<0.05	2.736 (1.002–7.476)

* *CI* confidence interval, *HR* hazard ratio

in AML. Additionally, a recent large-scale retrospective study reported that among newly diagnosed AML patients with *CEBPA* mutations, those receiving first-line venetoclax plus a hypomethylating agent achieved a significantly higher composite complete remission (CR/CRi) rate than those treated with liposomal daunorubicin-cytarabine (88% vs. 50%, $P=0.03$) (Fathima et al. 2026). However, no studies have yet explored whether *CEBPA* mutations in the bZIP domain affect venetoclax chemotherapy sensitivity in AML patients. We propose that further clinical data are required to clarify these observations, and basic experimental research should be conducted to investigate the underlying mechanisms.

To enhance the efficacy of the "7 + 3" regimen, numerous clinical studies have investigated the incorporation of a third agent, including additional chemotherapeutic or targeted agents (Estey et al. 1999; Holowiecki et al. 2004; Nazha et al. 2013). The integration of purine nucleoside analogues, such as cladribine, clofarabine and fludarabine, into induction therapy for patients newly diagnosed with AML has yielded improved outcomes (Kadia et al. 2023). Research indicates that the inclusion of cladribine in the standard "7 + 3" regimen, as opposed to fludarabine, results in a higher response rate and significantly enhances overall survival in these patients (Holowiecki et al. 2012). Our study demonstrates that, compared to fludarabine, the addition of cladribine to the IA regimen effectively mitigates *in vitro* resistance induced by *AML1* or *DNMT3A* mutations, corroborating findings from previous clinical studies.

This study, however, has several limitations. First, the cohort size ($n=98$) is relatively small and derived from a single center, which may limit the statistical power for detecting all clinically relevant associations, particularly in subgroup analyses involving rare genetic alterations. This also necessitates caution in generalizing the findings to the broader AML population, pending validation in larger, multi-center cohorts. Second, the induction regimens received by patients in this cohort were not uniform, which may have introduced bias in the prognostic analysis. Third, while PharmaFlow incubates bone marrow samples with the drug and thereby preserves much of the native microenvironment, it does not fully capture the influence of bone marrow stromal cells and other complex *in vivo* factors. This may limit the platform's ability to predict relapse and long-term clinical outcomes. Fourth, although prior reports suggest that PharmaFlow-guided induction therapy can increase remission rates (Zhang et al. 2021), the present study did not further validate this potential benefit. The clinical utility of PharmaFlow therefore requires confirmation through broader prospective clinical trials. Fifth, while the platform can assess *ex vivo* sensitivity to a range of commonly used chemotherapeutic agents, predicting response to certain drugs with distinct mechanisms, such as azacitidine, remains challenging. Finally, validation in the independent cohort was restricted to venetoclax-based regimens and involved a relatively small sample size. Broader validation across multiple cohorts and diverse treatment settings are needed to confirm the generalizability of the findings. Moreover, its translation into routine clinical practice will require further validation through larger, multi-center prospective studies.

Conclusions

The PharmaFlow assay is a high-throughput *in vitro* drug sensitivity testing technique that shows potential for predicting clinical responses to induction chemotherapy in patients with AML. In this retrospective study, resistance determined by PharmaFlow stratification was associated with poorer induction treatment outcomes and higher prognostic risk. When integrated with patient-specific genetic mutation analysis, PharmaFlow may help identify genetic profiles associated with sensitivity to particular treatment regimens. Furthermore, distinct patterns of gene co-mutations may contribute to interpatient variability in *in vitro* drug sensitivity. These findings suggest that combined analysis of drug sensitivity profiling and genetic characteristics could

inform treatment selection, although prospective validation is needed to establish clinical utility.

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Data availability The data of this study are available within the article and in the Supplementary Information, or from the corresponding authors upon request.

Declarations

Ethics approval The study was conducted in accordance with the Declaration of Helsinki, and approved by the Research Ethics Committee of Qilu Hospital with the reference number KYLL-2020(KS)-633.

Consent for publication Not applicable.

Patient consent Informed consent was obtained from all subjects involved in the study.

Clinical trial number Not applicable.

Competing interests The authors declare no competing interests.

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