Heterogeneity in refractory acute myeloid leukemia

Sachi Horibata[a,1,2], Gege Gui[b,1], Justin Lack[c,d,1], Christin B. DeStefano[b,e], Michael M. Gottesman[a,2], and Christopher S. Hourigan[b,2]

[a]Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; [b]Laboratory of Myeloid Malignancies, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20814; [c]NIAID Collaborative Bioinformatics Resource, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; [d]Advanced Biomedical Computational Science, Frederick National Laboratory for Cancer Research sponsored by the National Cancer Institute, Frederick, MD 21702; and [e]Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD 20814

Contributed by Michael M. Gottesman, March 21, 2019 (sent for review February 11, 2019; reviewed by Edward Sausville and Joshua F. Zeidner)

Successful clinical remission to therapy for acute myeloid leukemia (AML) is required for long-term survival to be achieved. Despite trends in improved survival due to better supportive care, up to 40% of patients will have refractory disease, which has a poorly understood biology and carries a dismal prognosis. The development of effective treatment strategies has been hindered by a general lack of knowledge about mechanisms of chemotherapy resistance. Here, through transcriptomic analysis of 154 cases of treatment-naive AML, three chemorefractory patient groups with distinct expression profiles are identified. A classifier, four key refractory gene signatures (RG4), trained based on the expression profile of the highest risk refractory patients, validated in an independent cohort ($n = 131$), was prognostic for overall survival (OS) and refined an established 17-gene stemness score. Refractory subpopulations have differential expression in pathways involved in cell cycle, transcription, translation, metabolism, and/or stem cell properties. Ex vivo drug sensitivity to 122 small-molecule inhibitors revealed effective group-specific targeting of pathways among these three refractory groups. Gene expression profiling by RNA sequencing had a suboptimal ability to correctly predict those individuals resistant to conventional cytotoxic induction therapy, but could risk-stratify for OS and identify subjects most likely to have superior responses to a specific alternative therapy. Such personalized therapy may be studied prospectively in clinical trials.

acute myeloid leukemia | cancer heterogeneity | drug resistance

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by abnormal clonal hematopoietic progenitors. For the past several decades, standard intensive induction therapy has involved a combination of cytarabine- and anthracycline-based cytotoxic chemotherapy, for example, "7 + 3" (1). Achievement of complete remission is required for long-term survival and cure (2). Despite general trends toward improvements in overall survival (OS) due to better supportive care, up to 30–40% of patients will have chemorefractory disease, defined as failure to achieve a morphological complete response (CR) after one to two cycles of induction therapy (3–6). These patients face a particularly dismal prognosis, with a median survival of less than 1 y; hence, a better understanding of the disease biology and identification of successful treatment approaches are critical to improve patient outcomes (7, 8). Understanding the heterogeneity of refractory AMLs and distinct cellular properties within each refractory group is critical to decipher underlying resistance mechanisms to induction therapy.

Among known independent risk factors associated with primary refractoriness are leukemia biology variables such as cytogenetic risk, Fms-related tyrosine kinase 3 internal tandem duplication (*FLT3*-ITD), and nucleophosmin (*NPM1*) mutation status (9–11). The presence of unfavorable cytogenetic markers such as a complex or monosomal karyotype, inv (3)/t(3;3), or *TP53* mutation predicts a poor response to intensive induction chemotherapy, with only a minority of such patients achieving remission after induction chemotherapy (12). In addition to cytogenetic and molecular factors, other risk factors for primary refractory disease include clinical variables such as older age and an antecedent hematological neoplasm (13–15). Recent studies have suggested that primary treatment failures might also be the result of AML clones harboring intrinsic properties of hematopoietic stem cells and quiescence, and gene expression signatures may be predictive of outcome (11, 16–19).

To better characterize the mechanisms of resistance to conventional cytotoxic induction therapy in AML patients, a transcriptomic analysis of pretreatment samples from adult patients with newly diagnosed untreated AML was performed. Among those with refractory disease to 7 + 3 induction, signature pathways and gene sets differentially expressed relative to complete responders were identified, unveiling heterogeneity in intrinsic resistance mechanisms and allowing a classifier prognostic for OS to be established. In addition, ex vivo drug sensitivity data from cells derived from the same patients were analyzed, allowing for validation of pathway enrichment results and providing insight into possible effective treatment strategies.

Results

Clinical Responses. Data from 154 patients treated at one of six US academic medical centers met the eligibility criteria of having received one cycle of 7 + 3 induction chemotherapy for a first diagnosis of previously untreated AML with a postinduction clinical restaging result recorded and having RNA sequencing performed by a central laboratory on a pretreatment sample (20). Patients had a mean age of 54 (range 21–77) y, 52% were male, and 83% were white, with 32%, 29%, and 35% classified as

Significance

Acute myeloid leukemia (AML) is the name given to a diverse set of highly fatal blood cancers. The typical initial treatment is combination cytotoxic chemotherapy with at least two drugs: 7 days of cytarabine with 3 days of an anthracycline. As treatment at initial diagnosis is often given emergently, there is a great need for pretreatment markers capable of identifying patients for whom standard treatment is likely to be suboptimal. This work uses RNA-sequencing analysis of a diverse cohort of highly annotated AML patient samples to demonstrate heterogeneity among those patients not responding to standard chemotherapy, then uses this information both to develop a prognostic classifier for survival and to suggest potentially superior therapeutic approaches for those at highest risk.

Author contributions: S.H., M.M.G., and C.S.H. designed research; S.H., G.G., J.L., and C.B.D. performed research; S.H., G.G., J.L., and C.B.D. analyzed data; S.H., G.G., J.L., C.B.D., and C.S.H. wrote the paper; and M.M.G. supervised the study.

Reviewers: E.S., University of Maryland; and J.F.Z., University of North Carolina School of Medicine.

Conflict of interest statement: C.S.H. receives laboratory research funding from the Merck Sharp & Dohme and SELLAS Life Sciences Group AG.

[1]S.H., G.G., and J.L. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: sachi.horibata@nih.gov, mgottesman@nih.gov, or hourigan@nih.gov.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1902375116/-/DCSupplemental.

Published online May 7, 2019.

favorable, intermediate, and adverse risk, respectively, based on 2017 European LeukemiaNet (ELN) risk stratification by genetics (21) (Table 1 and *SI Appendix*, Table S1). One hundred eleven patients (72%) were reported as having achieved a CR after one cycle of 7 + 3 induction chemotherapy. Survival data were available for 142 (92%) of the cohort. Those achieving an initial CR had longer survival (median OS of 33 mo) than refractory (Ref) patients (median OS of 13 mo) (Fig. 1*A*). ELN genetic risk group was associated with the likelihood of achieving a CR and with OS (Fig. 1*B* and *SI Appendix*, Fig. S1*A*). Overall, 84% of patients had at least one somatic mutation detected, 19% had adverse cytogenetics, and 90% had a somatic mutation and/or adverse cytogenetics (Fig. 1*C*).

Genome-Wide Expression Profiling of Refractory AML Patients. Pairwise differential gene expression of pretreatment samples from all Ref patients ($n = 43$) was compared with CR ($n = 111$) (Fig. 2*A*). The top 100 differentially expressed genes are shown in Fig. 2*B*. Many of the genes significantly differentially expressed at a false discovery rate (FDR) < 0.05 have previously been implicated in drug resistance across multiple cancers, including *BAK1*, *PPP1R13L*, and *NFKBIE* (19, 22–24).

We next tested how well the 17-gene stemness (LSC17) score (16) and the 29-gene predictive score with 2010 UK Medical Research Council (MRC) risk group (PS29MRC) (19) predicted the patient treatment response. While a high LSC17 score was often observed in Ref patients, it is difficult to predict individual patient responses due to considerable overlap with the range of LSC17 scores observed in those achieving a CR (Fig. 3 *A* and *B*). PS29MRC signatures for Ref patients were uninformative in this cohort (*SI Appendix*, Fig. S1*B*). To access whether LSC17 score can predict the OS within our patient cohorts, we investigated the OS of patients with LSC17 scores greater than the median ($LSC17^{hi}$) and those with LSC17 scores lower than the median ($LSC17^{lo}$) before treatment. We found that LSC17 score was able to stratify the survival probability within those achieving a CR to an initial cycle of 7 + 3 induction ($P = 0.017$) (Fig. 3*C*), but not in those who were refractory ($P = 0.52$) (Fig. 3*D*).

Gene Expression Reveals Heterogeneity Between Refractory AML Patients. To explain the limited applicability of LSC17 scores among those cases refractory to initial treatment, we performed consensus clustering to evaluate the extent of heterogeneity among these 43 refractory cases (25). The consensus cumulative distribution function (CDF) and the delta area under the curve (AUC) for CDF indicate that $k = 3$ best describes the 43 refractory samples [Fig. 4*A* and *SI Appendix*, Fig. S1*C*; henceforth referred to as refractory group 1 (Ref1, $n = 21$), refractory group 2 (Ref2, $n = 11$), and refractory group 3 (Ref3, $n = 11$)]. Relative to the complete responders, Ref1, Ref2, and Ref3 had unique gene expression profiles based on a differential expression analysis. All refractory groups exhibit higher LSC17 scores compared with the complete responders (Fig. 4*B*), and Ref3 has the highest LSC17 score. PS29MRC signatures were uninformative in these subgroups (*SI Appendix*, Fig. S1*D*). The three clusters of Ref patients based on the gene expression profiles described above had significantly different survivals, with the worst survival seen in the Ref3 subset (median OS of only 10 mo in Ref3 vs. 13 mo in Ref1, 15 mo in Ref2, and 33 mo in CR; log-rank test: $P = 0.0018$; Fig. 4*C*). Pairwise differential gene expression of pretreatment samples from Ref1 ($n = 21$), Ref2 ($n = 11$), Ref3 ($n = 11$), and CR ($n = 111$) were compared with each other. The top 100 differentially expressed genes reveal unique gene expressions in each refractory subgroup (Fig. 4*D*; enlarged figures are illustrated in *SI Appendix*, Figs. S2–S4).

The comparison of OS within Ref patients indicated that the gene expression signature of Ref3 patients could be of potential significance for survival prediction. A list of genes was generated by considering the FDR and fold change (FC) of genes that were differentially expressed between Ref3 and Ref1/Ref2 subgroups. The least absolute shrinkage and selection operator (LASSO) (26, 27) was used to further reduce the number of genes, and the final binary group classification was based on the support vector machine (SVM) results. We identified *GUSB*, *ALDH3B1*, *AMOT*, and *RAB32* as four key refractory gene signatures (RG4) that can better predict patient survival. Patients that had gene signatures similar to those of Ref3 patients were labeled as $RG4^{pos}$ (refractory gene model-positive), while the other patients were in the $RG4^{neg}$ (refractory gene model-negative) group. Those within the $RG4^{pos}$ group ($n = 25$, 18%, median OS of 10 mo) had significantly shorter survival than those who were in the $RG4^{neg}$ group ($n = 117$, 82%, median OS of 33 mo) (log-rank test: $P = 0.007$; Fig. 4*E*). This classification could also stratify for survival within the Ref patient cohort or LSC^{hi} patient

Table 1. Patient demographics

Characteristic	Complete responders ($n = 111$)	Refractory disease ($n = 43$)	Risk ratio	*P*
Mean age at diagnosis	54 (±14)	52 (±16)		0.56
Race/ethnicity				0.28
White	95 (86%)	33 (77%)	0.92	
Other	16 (14%)	10 (23%)	1.37	
Gender				0.95
Male	57 (51%)	23 (53%)	1.03	
Female	54 (49%)	20 (47%)	0.97	
Molecular alterations				<0.01
FLT3 wild type/*NPM1* wild type	59 (53%)	32 (74%)	1.26	
FLT3-ITD/*NPM1* wild type	8 (7%)	6 (14%)	1.53	
FLT3 wild type/*NPM1* mutated	28 (25%)	1 (2%)	0.12	
FLT3-ITD/*NPM1* mutated	16 (15%)	4 (10%)	0.72	
2017 ELN risk stratification				<0.01
Favorable	46 (41%)	4 (9%)	0.29	
Intermediate	30 (27%)	15 (35%)	1.19	
Adverse	32 (29%)	22 (51%)	1.46	
Unknown	3 (3%)	2 (5%)	1.43	

Risk ratio = r/r_0. $r_0 = 0.28$ (percentage of total Ref patients); *r*, probability of being in the refractory group given each category.

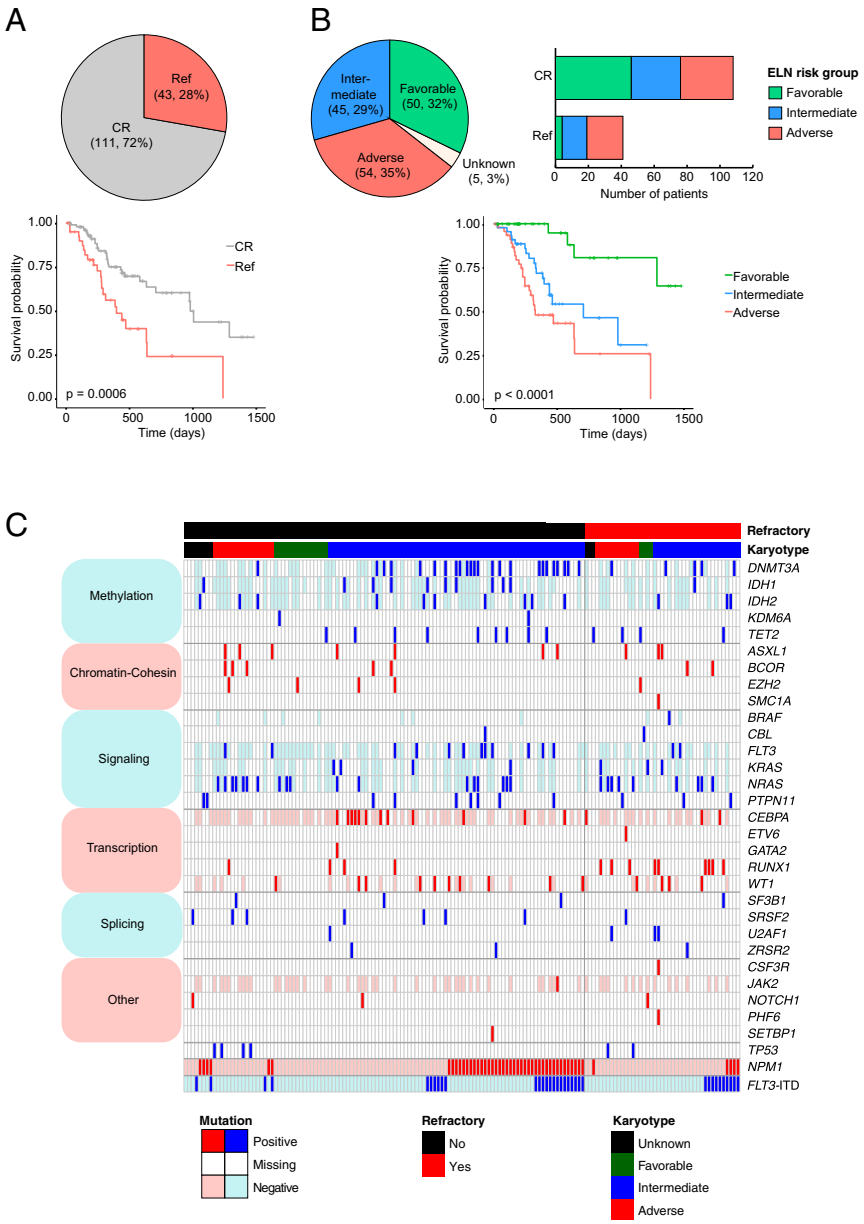


Fig. 1. OS and somatic mutation summary for the 154 cases of newly diagnosed AML patients. (*A*) Two groups of patients that had a CR (n = 111) or refractory (Ref, n = 43) response upon induction (cytarabine- and anthracycline-based) chemotherapy. The pie chart shows the proportion of patients in each group, with corresponding Kaplan–Meier (K-M) survival curves with log-rank test P values. (*B*) The 2017 ELN risk stratification summary. The pie chart and the stacked bar chart show the overall percentage and the percentage within the CR or Ref group, followed by K-M curves of three risk groups. (*C*) Heat map of all somatic mutations with cytogenetic information. Somatic mutations are grouped by gene functions/class (68). *NPM1* and *FLT3*-ITD results were based on results of clinical testing, with other mutations based on exome and/or targeted DNA-sequencing panel results (20).

cohort (*SI Appendix*, Fig. S5 *A* and *B*), and showed a higher level of significance when combined with LSC17 groups (log-rank t test: $P = 0.0008$; Fig. 4*E*) than the use of LSC17 groups alone.

This finding was then validated in an independent AML cohort from The Cancer Genome Atlas (TCGA) (28) (Fig. 4*F* and *SI Appendix*, Fig. S5*C*). Among 131 patients from TCGA, 95 were predicted to be in the $RG4^{neg}$ group and 36 in the $RG4^{pos}$ group. The survival of the $RG4^{neg}$ group was significantly longer than that of the $RG4^{pos}$ group, with a median OS of 27 mo vs. 12 mo (log-rank test: $P = 0.0049$). Combined with the LSC17 scores, the patients in both the $RG4^{pos}$ group and the LSC^{hi} group (n = 14, 11%) had inferior OS to those in the $RG4^{neg}$ group and LSC^{lo} group (n = 52, 40%), with a median OS of 12 mo vs. 47 mo (log-rank test: $P = 0.0035$).

Refractory Subgroups Express Distinct Biological Pathways. To further explore the biology of these three Ref patient clusters, gene set enrichment analysis (GSEA) using both the canonical pathways (cp; Fig. 5*A*) and the chemical and genetic perturbations (cgp; *SI Appendix*, Fig. S6) collections from the Molecular Signatures Database (MSigDB) was conducted. GSEA revealed both distinctions and similarities among each of the three refractory clusters in terms of the most differentially activated pathways, with Ref1 largely distinct from Ref2 and Ref3 in terms of the most significantly enriched pathways (Fig. 5*A*). For Ref1, the dominant enrichment signal indicated strongly increased expression of pathways involved in the cell cycle and DNA replication/repair, distinct from Ref2 and Ref3 (Fig. 5 *B*–*E*). Pathways involved in translation were significantly down-regulated for both Ref1 and

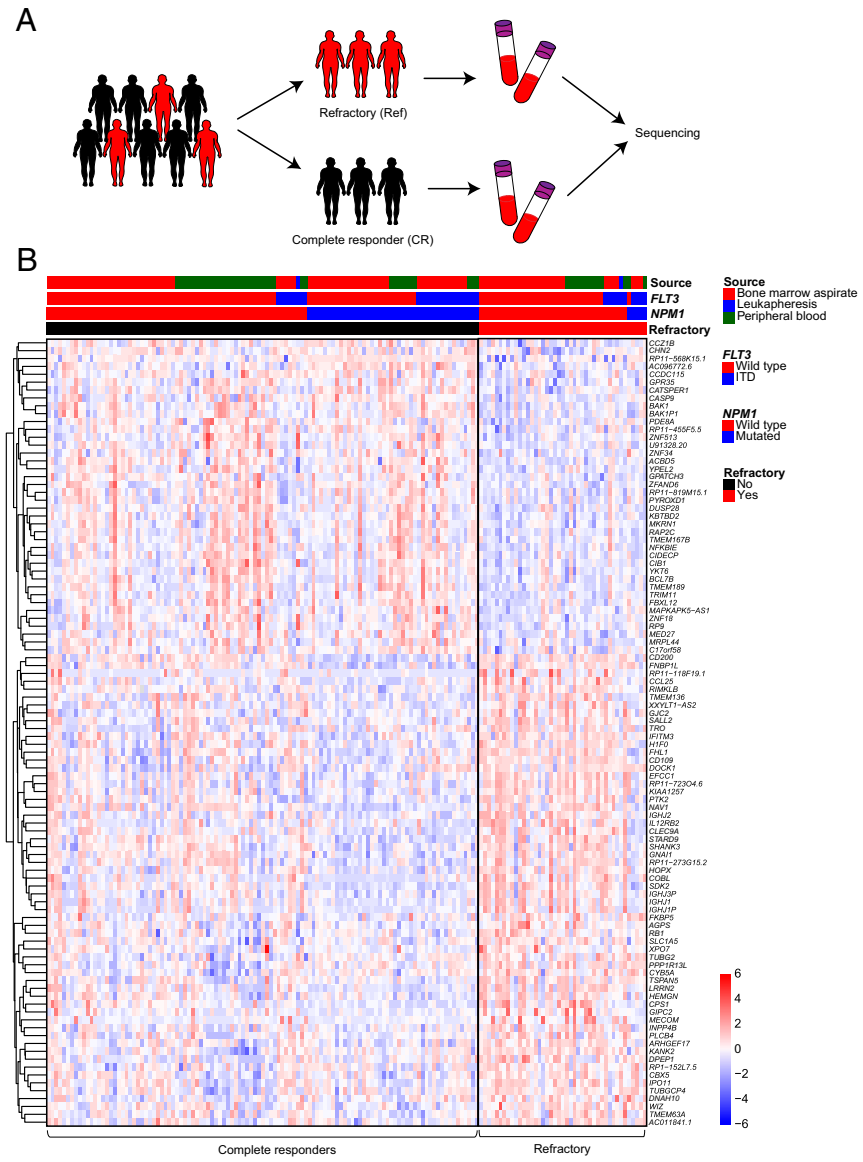


Fig. 2. Genome-wide gene expression profile of pretreatment samples from patients. (*A*) Schematic illustration of study design. (*B*) Top 100 differentially expressed genes based on adjusted *P* value (FDR). Sample level expression values are mean-centered log2 counts per million with trimmed mean of M values (TMM) normalization.

Ref3 (Fig. 5 *B*–*E*). For both Ref2 and Ref3, metabolic pathways are significantly down-regulated, while showing an indication of up-regulation in Ref1 (Fig. 5 *B*–*E*). For Ref3, the Reactome generic transcription pathway was highly significantly up-regulated, as were ATP-binding cassette (ABC) transporters (Fig. 5*F* and *SI Appendix*, Fig. S7).

For the cgp collection (*SI Appendix*, Fig. S6), results were generally in agreement with the cp collection (Fig. 5 *A*–*D*), with Ref1 being generally distinct from the other two clusters and highly enriched for cell cycle and DNA repair gene sets. Stem cell signatures previously identified as being predictive of outcomes and relapse in AML (16, 18) exhibit varying levels of activity across our refractory subgroups (Fig. 4*B* and *SI Appendix*, Fig. S6). The Ref3 cluster shows very strong up-regulation of many hematopoietic and lymphoid stem cell gene sets. The GSEA results are in agreement with the LSC17 (16) distributions observed for each of these refractory clusters, with Ref3 having the highest LSC17 score (Fig. 4*B*). Together, transcriptome analysis revealed three refractory groups with distinct cellular properties (Fig. 5*E*).

***NPM1* Mutational Status.** In addition to patient responses, information regarding the source of the specimens, karyotype, and presence of somatic mutations in genes recurrently mutated in AML such as *NPM1* and *FLT3*-ITD was incorporated into our analysis. Mutations in *NPM1* and *FLT3*-ITD were identified in 32% (49 of 154) and 22% (34 of 154) of the samples, respectively, as depicted in Table 1 and in concordance with previous findings (9, 10, 29). There was a significantly higher fraction of *NPM1*-mutated/*FLT3* wild-type cases in the complete responders compared with the refractory groups (28 cases vs. one case; $P = 0.0014$), in accordance with previous observations (10, 30–32).

To examine the functional differences between resistant and responsive *NPM1* mutants, we conducted pairwise differential expression analysis between these two groups (*SI Appendix*, Fig. S8*A*). Likely due to the small number of Ref patients who had an *NPM1* mutation (only five samples), we detected no significantly differentially expressed genes at an FDR < 0.1. GSEA indicated that the refractory *NPM1*-mutant patients were most similar to the

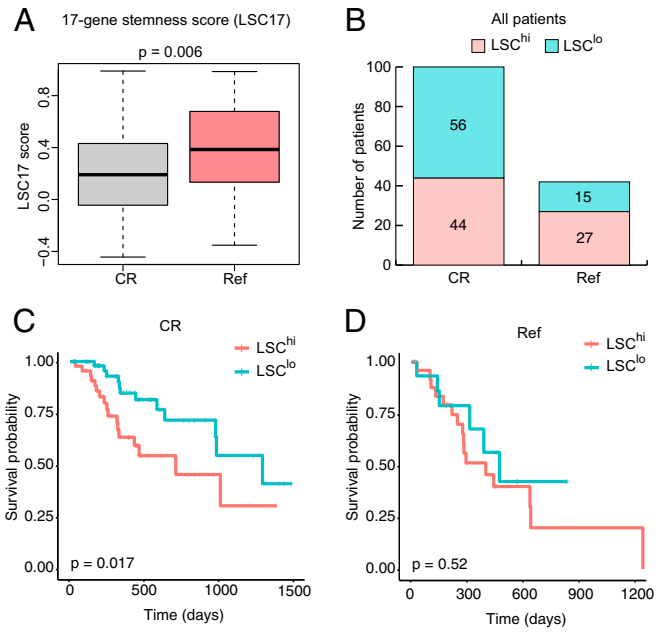


Fig. 3. LSC17 score and related survival. Patients are separated by the median score and labeled as LSC^{hi} (patients with scores greater than median) and LSC^{lo} (patients with scores lower than median). (*A*) LSC17 scores of AML patients in CR and refractory (Ref) groups, with *t* test *P* values. (*B*) Bar chart of LSC^{hi} and LSC^{lo} patient number in CR and Ref groups. (*C*) Kaplan–Meier (K-M) curves of LSC^{hi} and LSC^{lo} groups for CR patients. (*D*) K-M curves of LSC^{hi} and LSC^{lo} groups for Ref patients.

Ref3 group, however, with down-regulation of metabolism and translation (*SI Appendix*, Fig. S8*B*).

Heterogeneous Drug Sensitivity of Refractory AML Subgroups. Distinct combinations of differentially expressed genes and pathways appear to be contributing to the refractory phenotype of each refractory subgroup. In an attempt to validate the gene- and pathway-level heterogeneity described above and also to translate these results to the patient bedside, we tested whether these refractory groups exhibit differential sensitivities to small-molecule inhibitors with diverse modes of action. For 103 of the 154 patients described above (29 Ref patients and 74 responsive patients), the Beat AML working group conducted an ex vivo drug sensitivity assay on freshly isolated mononuclear cells of AML patients exposed to 122 small-molecule inhibitors (20) (Fig. 6*A*). A reduced AUC relative to that of the complete responders indicates that cell viability in the presence of the drug was reduced relative to the complete responders, while a relatively higher AUC indicates elevated cell viability relative to complete responders. To test the validity of this analysis, we used quizartinib, a drug known to significantly improve the OS of *FLT3*-ITD–mutated relapsed/refractory AML patients, as a positive control (33). In support of previous findings, we found samples from *FLT3*-ITD–positive AML patients were killed more efficiently with quizartinib compared with those from *FLT3*-ITD–negative AML patients (Fig. 6*B*) as shown by their lower AUC. The receiver operating characteristic curve indicates that patients with a positive *FLT3*-ITD mutation will have lower drug sensitivity values than patients with a negative *FLT3*-ITD mutation 86.2% of the time. We used a *t* test and one-way ANOVA to test for unequal mean AUC among the refractory group and the complete responder group for each drug to identify those that significantly affected cell viability of one or more subgroups. These analyses identified nine drugs with significant effects (with both *P* values for a *t* test and ANOVA < 0.05; *SI Appendix*, Table S2).

Interestingly, drug sensitivity was not uniform between refractory subgroups, as shown using the examples of GW-2580, a cFMS kinase inhibitor, and venetoclax, a *Bcl-2* inhibitor recently approved by the US Food and Drug Administration for AML (34) (Fig. 6*C*), which showed significantly different efficacy between Ref subgroups and compared with samples from patients achieving CR to conventional therapy. Overall, Ref3 was the most resistant subgroup to all drugs tested.

Sensitivity to Flavopiridol Across All Three Refractory Subgroups. Flavopiridol (Alvocidib), a potent cell cycle inhibitor of CDK9 (35) and other targets (36), had the most significant and the strongest cytotoxic effect on refractory AML patient samples compared with complete responders (Fig. 6*D*). Specifically, it had the strongest inhibitory effect for the Ref1 cluster, which pathway analysis and GSEA identified as having significantly elevated cell cycle signaling (Fig. 5 *A* and *B*). Using the data available from AML patients with drug sensitivity ($n = 103$), we found that the adverse ($n = 33$, 32%) ELN risk group had higher sensitivity against flavopiridol compared with the favorable ($n = 36$, 35%) ELN risk group (*t* test: $P = 0.028$; Fig. 6*E*). We further evaluated the gene expression difference between AML patients who are sensitive or resistant to flavopiridol and found distinct alterations in their gene expression (Fig. 6*F*). Interestingly, Ref patients were more sensitive to flavopiridol compared with the complete responders (nine Ref patients are flavopiridol-sensitive, while only one Ref patient is flavopiridol-resistant) (Fig. 6F). Together, these data suggest that a biomarker-driven approach may be able to identify those likely to benefit from flavopiridol, so that this promising drug candidate may be tested not only for proven refractory AML patients but also in those as yet untreated but likely to have a poor treatment response to conventional cytotoxic induction therapy.

Discussion

Despite advances in supportive care and recent drug approvals for the treatment of AML, the prognosis is still often poor. Those with chemorefractory disease, encompassing up to 30–40% of all adults with de novo AML, have a particularly dismal prognosis, with a median survival of less than a year and less than 10% achieving long-term survival (8, 37). Although some patients can be salvaged with further intensive chemotherapy, no regimen has been shown to be superior and benefit is lacking in a large majority of cases (38).

Previously described predictive gene expression-based signatures (16, 19) performed suboptimally in identifying, at the level of an individual AML patient, resistance to initial 7 + 3 cytotoxic induction therapy in this "real-world" cohort. We show, using both transcriptome and ex vivo drug sensitivity analysis, biological heterogeneity in cases refractory to an initial cycle of induction chemotherapy. RNA-sequencing expression analysis clustered samples from Ref patients in this cohort into three different groups, Ref1, Ref2, and Ref3, each having distinct cellular properties promoting intrinsic treatment resistance. Based on GSEA, Ref1 was the most common refractory group and was characterized by significantly up-regulated cell cycle, proliferation, and DNA replication/repair gene sets and by down-regulated translation. Ref2 was enriched with translation genes, while metabolic pathways were significantly down-regulated. Ref3 was characterized by significant down-regulation of pathways involving translation and metabolism as well as strong up-regulation of many hematopoietic and lymphoid stem cell gene sets, including ABC transporters (Fig. 5*E* and *SI Appendix*, Fig. S7).

These results help to shed light on the current controversy about the role of ABC transporters, such as ABCG2, as predictors and contributors to treatment failure in AML. Multiple reports indicate ABC transporters are associated with poor-prognosis AML, as well as with development of resistance to chemotherapy.

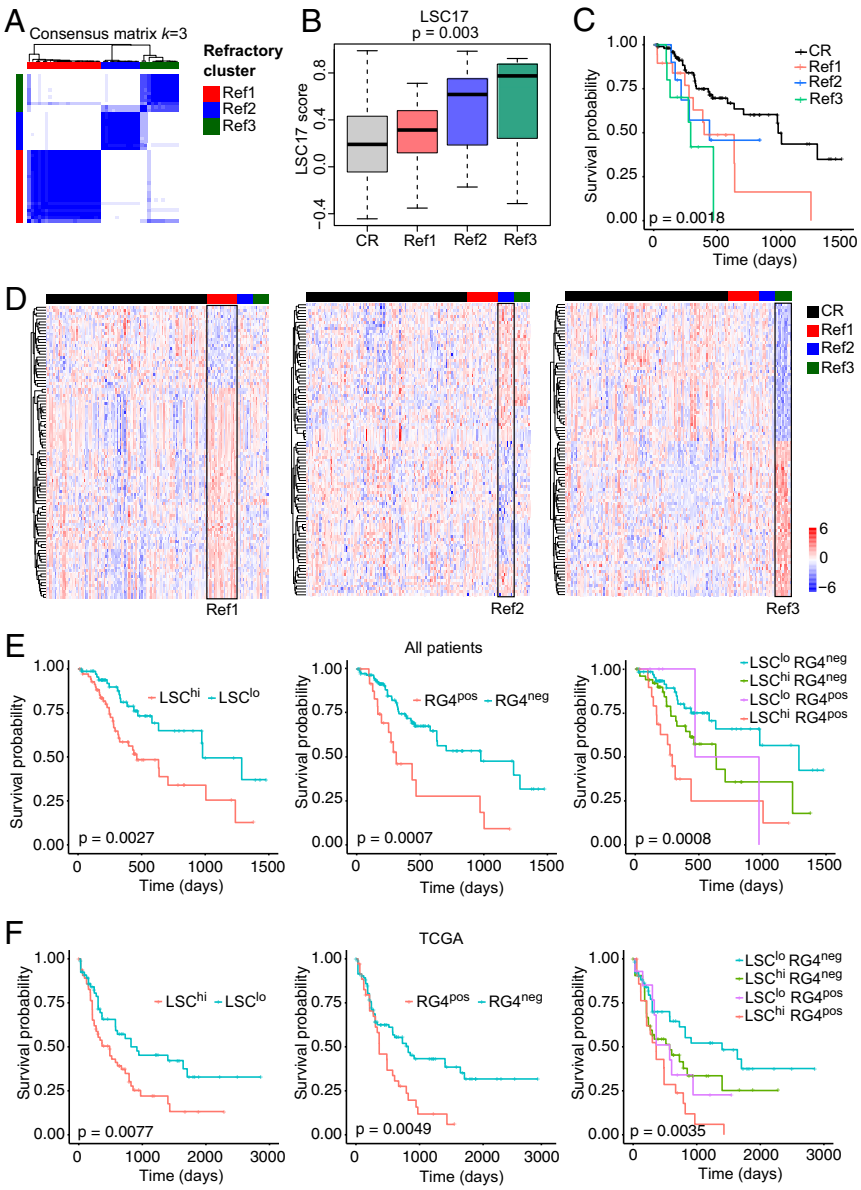
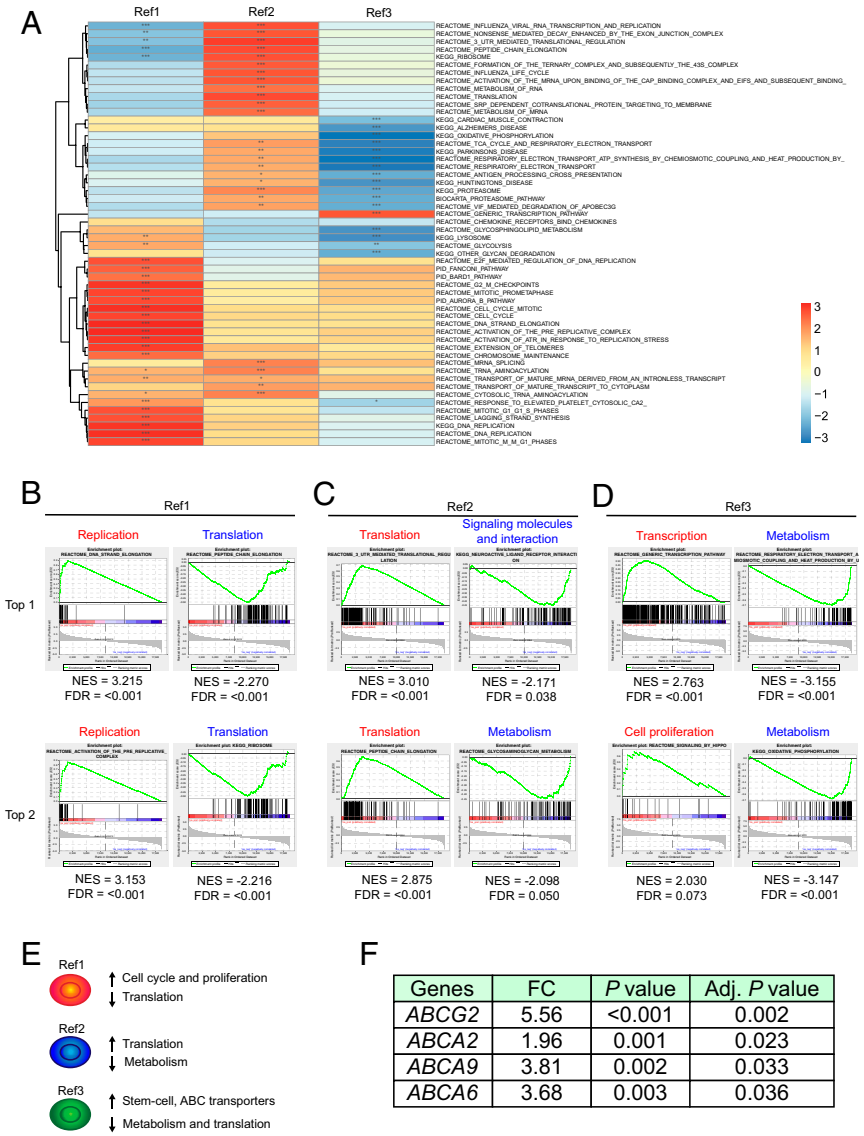


Fig. 4. Expression profile of pretreatment samples from newly diagnosed AML patients reveals distinct subpopulations of refractory AML. (*A*) Hierarchical clustering of refractory groups reveals three distinct subpopulations (Ref1, Ref2, and Ref3). (*B*) LSC17 scores of complete responders (CR), Ref1, Ref2, and Ref3. (*C*) OS of CR, Ref1, Ref2, and Ref3 ($P = 0.0018$). (*D*) Top 100 differentially expressed genes in Ref1, Ref2, and Ref3 based on adjusted *P* value (FDR). Sample level expression values are mean-centered log2 counts per million with trimmed mean of M values (TMM) normalization. (*E*) OS of patients. The Kaplan–Meier (K-M) curves are plotted for all patients separated by LSC17 scores, by expression profile similar to Ref3 (patients similar to Ref3 are labeled as $RG4^{pos}$, with the others labeled as $RG4^{neg}$), and combined. (*F*) OS of TCGA cohort. The K-M curves are plotted for all patients separated by LSC17 scores, by expression profile similar to Ref3, and combined.

However, the failure of the Eastern Cooperative Oncology Group (ECOG) 3999 trial to demonstrate that the potent third-generation ABCB1 inhibitor zosuquidar improves the response to chemotherapy in AML (39) led to an editorial comment that it is time to give up on the use of ABC transporter inhibitors in the treatment of AML (40). Owing to the likely inadequacy of the zosuquidar treatment in this ECOG study, the specificity of its inhibition of only one of several ABC transporters, and the need to stratify AML populations to target those patients with poor-prognosis leukemia whose cells express ABC transporters (41), we suggested that it was too soon to give up on ABC inhibitors (42) and that further studies were needed to address these issues. The current work shows that it is only a small subset (Ref3) of intrinsically resistant AMLs that express significant amounts of ABC multidrug transporters (*ABCG2*, *ABCA2*, *ABCA9*, and *ABCA6*); therefore, any future efforts to inhibit these transporters should be targeted to this subpopulation.

The distinct gene/pathway signatures of each refractory group suggest that targeted agents may have variable but predictable therapeutic benefits. To directly address this possibility, an ex vivo drug sensitivity analysis was performed, revealing nine drugs (*t* test and ANOVA: $P < 0.05$) with cytotoxic activity on at least one of the refractory groups. Flavopiridol was identified as a particularly strong candidate for refractory AML therapy. Flavopiridol targets cell cycle regulation and had significant cytotoxicity for the Ref1 group, which is enriched for up-regulated cell cycle gene sets. It shows broad efficacy within refractory subgroups, however, consistent with the claim that it has the ability to kill, by



F

Genes	FC	*P* value	Adj. *P* value
ABCG2	5.56	<0.001	0.002
ABCA2	1.96	0.001	0.023
ABCA9	3.81	0.002	0.033
ABCA6	3.68	0.003	0.036

Fig. 5. Characterization of enriched cp in refractory subgroups. (*A*) GSEA (MSigDB, C2 cp) of refractory subgroups (Ref1, Ref2, and Ref3) compared with the complete responder group. Top 20 gene sets with an FDR < 0.05 in at least one comparison are plotted in the heat map (***$P < 0.01$, **$P < 0.05$, *$P < 0.1$). Red and blue correspond to up-regulated and down-regulated pathways, respectively. GSEA of the top two pathways up-regulated (red) and down-regulated (blue) in Ref1 (*B*), Ref2 (*C*), and Ref3 (*D*) is illustrated. The normalized enrichment score (NES) and FDR are indicated. (*E*) Characteristic altered cellular processes in refractory AML. Red, blue, and green cells indicate Ref1, Ref2, and Ref3, respectively. (*F*) List of significantly up-regulated ABC transporters in Ref3. The FC (compared with the complete responders) is shown. Adj., adjusted.

Bcl-2–independent apoptosis, tumor cells resistant to other chemotherapy agents (43). This drug has already successfully been used in combination for the treatment of both newly diagnosed high-risk and Ref/relapsed AML patients (44–47), with complete remission rates often superior to 7 + 3. Biomarker-based clinical trials of this combination are now ongoing (48).

Ref3 shows a poor response to all of the drugs, possibly because Ref3 has more down-regulated pathways and a number of up-regulated ABC transporters, which are well-characterized mechanisms of resistance to a broad range of anticancer drugs (42, 49), diminishing this group's response to drug treatment. Flavopiridol is an ABCG2 substrate. This may explain why flavopiridol is less effective in the Ref3 group, which expresses *ABCG2*. Although flavopiridol is still effective in Ref3, its effect may be enhanced with the help of ABC inhibitors. Ref3 also shows down-regulation of translation and conventional metabolic pathways consistent with recent reports regarding leukemic stem cells (50, 51). Thus, further evaluation of other small molecules tailored against enriched pathways in each refractory AML group may produce more effective treatment outcomes. Finally, it is clear that the Ref3 group is associated with inferior survival (Fig. 4*C*). We established a binary classifier based on the gene expression signature of Ref3 with four selected genes, RG4, which was validated in an independent AML cohort as both prognostic and also having the ability to further improve LSC17-based stratification.

This study has several strengths and limitations. It provides information on the heterogeneity of refractory AML and mechanisms of intrinsic resistance. The findings herein suggest that small-molecule inhibitors targeting aberrant biological pathways can produce antiproliferative effects in otherwise refractory leukemia cells. Drugs with apparently low efficacy using this ex vivo methodology, such as venetoclax and lenalidomide, may be effective clinically, however, as combination treatment or in specific

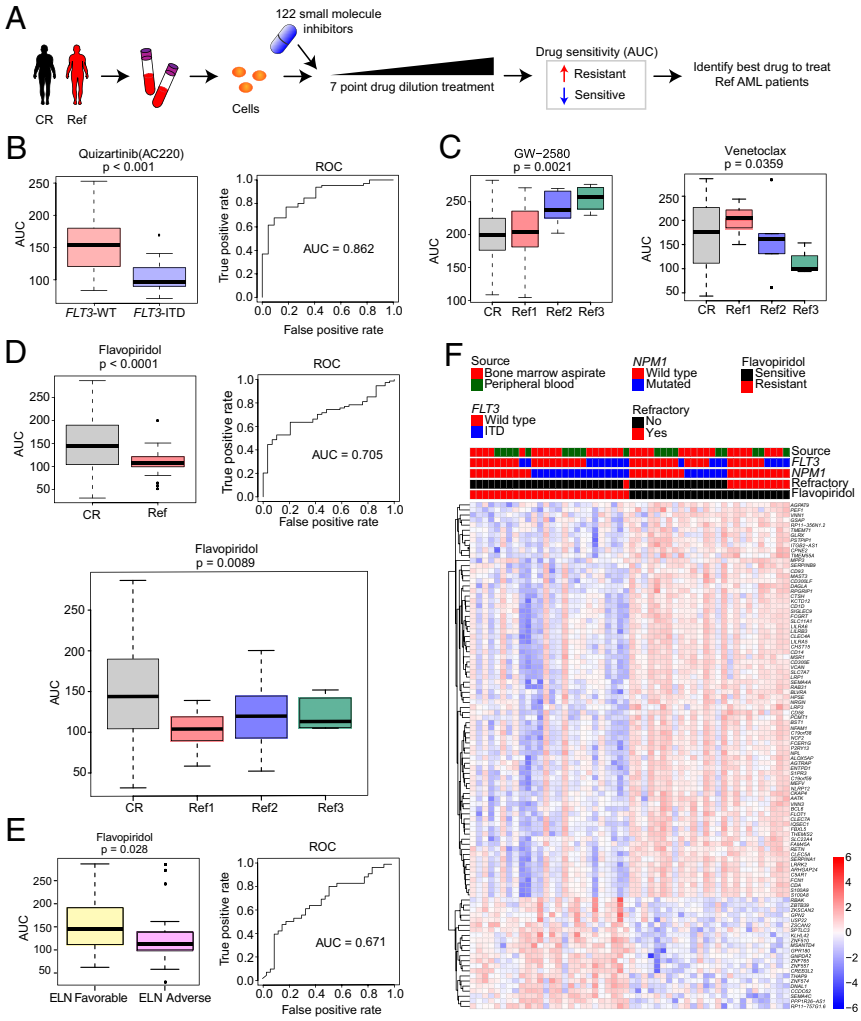


Fig. 6. Drug sensitivity landscape of refractory AML. (*A*) Schematic illustration of study design. A total of 103 pretreatment samples from newly diagnosed cases of AML that had either a CR ($n = 74$) or refractory (Ref) response ($n = 29$) upon induction chemotherapy were used in this study. The freshly isolated mononuclear cells of AML patients were exposed to seven-point drug dilution treatment against 122 small-molecule inhibitors. The drug sensitivity of these patient-isolated cells was examined. (*B*) Box plot illustrates treatment response of samples from *FLT3*-ITD and *FLT3* wild-type (WT) AML patients against quizartinib (AC220), a drug known to improve the OS of *FLT3*-ITD–mutated AML patients. The receiver operating characteristic (ROC) curve indicates the significance of the quizartinib effect on *FLT3*-ITD patient samples. (*C*) Box plot illustrates treatment response between CR, Ref1, Ref2, and Ref3 patient samples against GW-2580 (*P* value: ANOVA between four groups) and venetoclax (*P* value: ANOVA between refractory subgroups). (*D*) Box plot illustrates treatment response between CR and refractory (Ref) or CR, Ref1, Ref2, and Ref3 patient samples against flavopiridol. ROC curves indicate the significance of the flavopiridol effect on patient samples. (*E*) Box plot illustrates treatment response of patient samples with an adverse or favorable ELN classification against flavopiridol. The ROC curve indicates the significance of the flavopiridol effect on samples from patients with an adverse or favorable ELN classification. (*F*) Top 100 differentially expressed genes of AML patient samples based on their sensitivity to flavopiridol (all with an adjusted $P < 0.02$). Sample level expression values are mean-centered log2 counts per million with trimmed mean of M values (TMM) normalization. Sensitivity to flavopiridol was defined based on the top 25% AUC and bottom 25% AUC. The resistant group has an AUC $\geq$ 164.6 (26 patients composed of 25 CR and 1 Ref patients) with a significant binomial test ($P = 0.0015$). The sensitive group has an AUC $\leq$ 100.88 (26 patients composed of 16 CR and 10 Ref patients) with no significant binomial test ($P = 0.615$).

disease subsets (34, 52). An assay with viability as the read-out may underestimate the efficacy of drugs with noncytotoxic mechanisms of action. We focused on complete remission to initial cytotoxic induction therapy in patients treated with 7 + 3 chemotherapy; those refractory to other approaches may have different profiles. Similarly, we determined refractory status on the basis of response after receipt of a single cycle of 7 + 3, while many such Ref patients achieve a CR only after a second cycle of induction (53). Unfortunately, subsequent responses were not available for evaluation in this dataset. Patients were treated at US academic medical centers according to local standards of care. While variations in 7 + 3 chemotherapy dosing exist and may have differential benefit in particular AML subgroups, information regarding potentially predictive factors from these patients would typically not be available to the prescribing physician at the time of initial treatment. Certainly, the 72% overall CR rate suggests these patients received high-quality induction therapy. We also allowed inclusion of patients treated with adjunctive therapy in addition to 7 + 3, as CR rates for such combinations have not been demonstrated to be statistically superior (54). Complete remission is only a surrogate for OS, and while we dichotomized remission responses here, we understand that remissions associated with incomplete count recovery may have a different prognosis (55, 56) and that reductions in disease burden can be quantified with higher sensitivity (57, 58). The training dataset of refractory cases used to establish the RG4 classifier was small, and the result may

not be applicable to those treated with induction therapies other than 7 + 3. While the results presented here are informative and, in principle, point to the potential of personalized therapy for refractory AML, their applicability to the clinical setting must await clinical trials to establish the safety and efficacy of such approaches (20, 47, 59–64). The biological heterogeneity described here explains the limited efficacy of current therapeutic approaches to treat refractory AML and the suboptimal ability of pretreatment disease biology, clinical factors, and gene expression-based signatures to correctly identify, on an individual patient level, the likelihood of achieving complete remission after conventional cytotoxic induction therapy.

Materials and Methods

Patient Cohort Selection. Our cohort was selected from the Beat AML dataset (20) based on the following criteria. Adult patients (age > 21 y) with samples coded as being from the specimen group "initial acute leukemia diagnosis" with a diagnosis of "AML and related precursor neoplasms" at the time of specimen collection were included. Patients with a *PML-RARA* fusion, t(15;17) karyotype, and/or a diagnosis of acute promyelocytic leukemia were excluded. Only patients treated with 7 + 3 (cytarabine and an anthracycline) with a documented response to induction therapy were eligible. Only patients with analysis of RNA sequencing from a preinduction therapy sample were eligible.

Patient Sample Normalization. The statistical analyses were performed using R, version 3.5.0 (65). Raw counts of RNA-sequencing data from pretreatment samples from 154 patients were obtained from the Beat AML working group (20). Only genes expressed at >1 counts per million in at least 10 samples were carried forward, and the count matrix was normalized using limma voom quantile normalization (66).

Differential Gene Expression Analysis of Refractory Versus Complete Responders. The normalized data were used for differential expression analysis by the "lmFit" and "eBayes" functions in limma comparing the CR and refractory groups. There were 49 significant genes with an FDR < 0.05 and 139 significant genes with an FDR < 0.1. The top 100 genes were selected for the heat map. For differential expression analysis between refractory subgroups and the CR group, the numbers of significant genes are 1, 0, 1,839 (FDR < 0.05) and 12, 0, 3,140 (FDR < 0.1) for the Ref1, Ref2, and Ref3 clusters, respectively.

Sample Clustering of Refractory AML Patients. Samples were clustered using ConsensusClusterPlus, v1.42.026 (34) at $k = 2$–10 and using the top 1,500 genes based on median absolute deviation. These genes were then log2-transformed and median-centered, and ConsensusClusterPlus was run for $k = 2$–10 with 80% resampling for 1,000 iterations of a hierarchical clustering based on Pearson correlation distance. The optimal number of clusters was chosen based on the consensus CDF and the relative change in area under the CDF curve. When there were three clusters, the CDF reached its approximate maximum, while there was no appreciable increase when clustering number increased.

Survival Analysis. Survival analysis was performed using the R "survival" and "survminer" packages. Kaplan–Meier survival curves were plotted for groups of interest with the log-rank test *P* value. Median OS in each group was also calculated for comparison.

Refractory Group Gene Signature Modeling. Differential gene expression analysis within three refractory subgroups was conducted using the same method as the comparison between CR and Ref patients. Genes were selected by choosing an FDR < 0.05 and an absolute value of logFC > 1. The genes were annotated using the Ensembl database, and those within a protein-coding transcript type remained in the list. The LASSO was used for further variable reduction with cross-validation. Four genes (*GUSB*, *ALDH3B1*, *AMOT*, and *RAB32*) remained to fit an SVM for classification with a Gaussian kernel. All of the count values were log2-transformed.

Enrichment Analysis. GSEAs were conducted using preranked GSEA (67) and the MSigDB, v6.2. The input data for the preranked GSEA are the t-statistics from the differential gene expression analysis for all genes after filtering (18,514 genes). The Entrez gene IDs were transformed to gene symbols, and duplications were removed. Two gene set databases were used for the analysis: c2.cp.v6.2.symbols.gmt (cp) and c2.cgp.v6.2.symbols.gmt (cgp).

Drug Sensitivity Analysis. AUC data for 122 small-molecule inhibitors were obtained from the Beat AML working group (20). Inhibitors and patients with more than 50% of missingness were removed from the analysis, leaving 105 inhibitors and 103 patients. A *t* test was used to test for significant mean differences between samples from the CR group and refractory groups. Single-factor ANOVA was used to test for significant mean differences between the CR group and refractory subgroups, and Dunnett's post hoc test and pairwise *t* test were used to calculate *P* values.

Clinical Information. For clinical information, we compared the distribution of age, gender, race, ELN risk stratification, and molecular alterations between two groups. Different statistical tests were applied based on the types of variables (categorical/continuous) and the number of observations in each category (*t* test, Fisher exact test, and χ^2 test).

Data Availability. Genomic datasets used for this study had previously been deposited in the database of Genotypes and Phenotypes (dbGaP) and Genomic Data Commons (20). The dbGaP study ID is 30641 and the accession ID is phs001657.v1.p1. TCGA data were downloaded using the Genomic Data Commons (GDC) Data Portal.

ACKNOWLEDGMENTS. We thank the Beat AML consortium for creating this publicly available dataset. We thank C. Tognon, J. Tyner, and R. P. Gale for their expertise and input on an earlier version of this typescript. We also thank M. Cam, P. Jailwala, and M. Hall for their technical assistance and G. Leiman for editorial assistance. This work was supported by the Intramural Research Program of the National Cancer Institute and the National Heart, Lung, and Blood Institute of the NIH. The opinions and assertions expressed herein are those of the author(s) and do not necessarily reflect the official policy or position of the Uniformed Services University or the Department of Defense.

1. Yates JW, Wallace HJ, Jr, Ellison RR, Holland JF (1973) Cytosine arabinoside (NSC-63878) and daunorubicin (NSC-83142) therapy in acute nonlymphocytic leukemia. *Cancer Chemother Rep* 57:485–488.
2. Walter RB, et al. (2010) Effect of complete remission and responses less than complete remission on survival in acute myeloid leukemia: A combined Eastern Cooperative Oncology Group, Southwest Oncology Group, and M. D. Anderson Cancer Center Study. *J Clin Oncol* 28:1766–1771.
3. Burnett AK, et al. (2012) Addition of gemtuzumab ozogamicin to induction chemotherapy improves survival in older patients with acute myeloid leukemia. *J Clin Oncol* 30:3924–3931.
4. Thol F, Schlenk RF, Heuser M, Ganser A (2015) How I treat refractory and early relapsed acute myeloid leukemia. *Blood* 126:319–327.
5. Ravandi F (2011) Primary refractory acute myeloid leukaemia–In search of better definitions and therapies. *Br J Haematol* 155:413–419.
6. Kantarjian H (2016) Acute myeloid leukemia–Major progress over four decades and glimpses into the future. *Am J Hematol* 91:131–145.
7. Estey E (2015) Why is progress in acute myeloid leukemia so slow? *Semin Hematol* 52: 243–248.
8. Kavanagh S, et al. (2018) AML refractory to primary induction with Ida-FLAG has a poor clinical outcome. *Leuk Res* 68:22–28.
9. Papaemmanuil E, et al. (2016) Genomic classification and prognosis in acute myeloid leukemia. *N Engl J Med* 374:2209–2221.
10. Stirewalt DL, et al. (2006) Size of FLT3 internal tandem duplication has prognostic significance in patients with acute myeloid leukemia. *Blood* 107:3724–3726.
11. MacPherson L, Dawson MA (2017) Survival of the fittest: Darwinian selection underpins chemotherapy resistance in AML. *Cell Stem Cell* 21:291–292.
12. Schlenk RF, Döhner H (2013) Genomic applications in the clinic: Use in treatment paradigm of acute myeloid leukemia. *Hematology (Am Soc Hematol Educ Program)* 2013:324–330.
13. Walter RB, et al. (2015) Resistance prediction in AML: Analysis of 4601 patients from MRC/NCRI, HOVON/SAKK, SWOG and MD Anderson Cancer Center. *Leukemia* 29: 312–320.
14. Ravandi F, et al. (2010) Characteristics and outcome of patients with acute myeloid leukemia refractory to 1 cycle of high-dose cytarabine-based induction chemotherapy. *Blood* 116:5818–5823, quiz 6153.
15. Lindsley RC, et al. (2015) Acute myeloid leukemia ontogeny is defined by distinct somatic mutations. *Blood* 125:1367–1376.
16. Ng SW, et al. (2016) A 17-gene stemness score for rapid determination of risk in acute leukaemia. *Nature* 540:433–437.
17. Shlush LI, et al. (2017) Tracing the origins of relapse in acute myeloid leukaemia to stem cells. *Nature* 547:104–108.
18. Valk PJ, et al. (2004) Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med* 350:1617–1628.
19. Herold T, et al. (2018) A 29-gene and cytogenetic score for the prediction of resistance to induction treatment in acute myeloid leukemia. *Haematologica* 103:456–465.
20. Tyner JW, et al. (2018) Functional genomic landscape of acute myeloid leukaemia. *Nature* 562:526–531.
21. Döhner H, et al. (2017) Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood* 129:424–447.

22. Notari M, et al. (2011) Inhibitor of apoptosis-stimulating protein of p53 (iASPP) prevents senescence and is required for epithelial stratification. *Proc Natl Acad Sci USA* 108:16645–16650.
23. Ge W, et al. (2017) iASPP is an antioxidative factor and drives cancer growth and drug resistance by competing with Nrf2 for Keap1 binding. *Cancer Cell* 32:561–573.e6.
24. Mansouri L, et al. (2016) Frequent NFKBIE deletions are associated with poor outcome in primary mediastinal B-cell lymphoma. *Blood* 128:2666–2670.
25. Wilkerson MD, Hayes DN (2010) ConsensusClusterPlus: A class discovery tool with confidence assessments and item tracking. *Bioinformatics* 26:1572–1573.
26. Friedman J, Hastie T, Tibshirani R (2010) Regularization paths for generalized linear models via coordinate descent. *J Stat Softw* 33:1–22.
27. Choi SW, Gibbons LE, Crane PK (2011) lordif: An R package for detecting differential item functioning using iterative hybrid ordinal logistic regression/item response theory and Monte Carlo simulations. *J Stat Softw* 39:1–30.
28. Ley TJ, et al.; Cancer Genome Atlas Research Network (2013) Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med* 368: 2059–2074.
29. Ley TJ, et al. (2010) DNMT3A mutations in acute myeloid leukemia. *N Engl J Med* 363: 2424–2433.
30. Heath EM, et al. (2017) Biological and clinical consequences of NPM1 mutations in AML. *Leukemia* 31:798–807.
31. Schnittger S, et al. (2005) Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. *Blood* 106: 3733–3739.
32. Thiede C, et al. (2006) Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). *Blood* 107:4011–4020.
33. Cortes JEK, et al. (2018) Efficacy and safety of single-agent quizartinib (Q), a potent and selective FLT3 inhibitor (FLT3i), in patients (pts) with FLT3-internal tandem duplication (FLT3-ITD)-mutated relapsed/refractory (R/R) acute myeloid leukemia (AML) enrolled in the global, phase 3, randomized controlled quantum-R trial. *Blood* 132:563.
34. Konopleva M, et al. (2016) Efficacy and biological correlates of response in a phase II study of venetoclax monotherapy in patients with acute myelogenous leukemia. *Cancer Discov* 6:1106–1117.
35. Chao SH, et al. (2000) Flavopiridol inhibits P-TEFb and blocks HIV-1 replication. *J Biol Chem* 275:28345–28348.
36. Sausville EA, et al. (1999) Cyclin-dependent kinases: Initial approaches to exploit a novel therapeutic target. *Pharmacol Ther* 82:285–292.
37. Ferguson P, et al.; UK NCRI AML Working Group (2016) An operational definition of primary refractory acute myeloid leukemia allowing early identification of patients who may benefit from allogeneic stem cell transplantation. *Haematologica* 101: 1351–1358.
38. Ramos NR, Mo CC, Karp JE, Hourigan CS (2015) Current approaches in the treatment of relapsed and refractory acute myeloid leukemia. *J Clin Med* 4:665–695.
39. Cripe LD, et al. (2010) Zosuquidar, a novel modulator of P-glycoprotein, does not improve the outcome of older patients with newly diagnosed acute myeloid leukemia: A randomized, placebo-controlled trial of the Eastern Cooperative Oncology Group 3999. *Blood* 116:4077–4085.
40. Libby E, Hromas R (2010) Dismounting the MDR horse. *Blood* 116:4037–4038.
41. Shaffer BC, et al. (2012) Drug resistance: Still a daunting challenge to the successful treatment of AML. *Drug Resist Updat* 15:62–69.
42. Robey RW, et al. (2018) Revisiting the role of ABC transporters in multidrug-resistant cancer. *Nat Rev Cancer* 18:452–464.
43. Achenbach TV, Müller R, Slater EP (2000) Bcl-2 independence of flavopiridol-induced apoptosis. Mitochondrial depolarization in the absence of cytochrome c release. *J Biol Chem* 275:32089–32097.
44. Zeidner JF, et al. (2015) Randomized multicenter phase II study of flavopiridol (alvocidib), cytarabine, and mitoxantrone (FLAM) versus cytarabine/daunorubicin (7+3) in newly diagnosed acute myeloid leukemia. *Haematologica* 100:1172–1179.
45. Zeidner JF, et al. (2018) Final results of a randomized multicenter phase II study of alvocidib, cytarabine, and mitoxantrone versus cytarabine and daunorubicin (7 + 3) in newly diagnosed high-risk acute myeloid leukemia (AML). *Leuk Res* 72:92–95.
46. Karp JE, et al. (2012) Randomized phase II study of two schedules of flavopiridol given as timed sequential therapy with cytosine arabinoside and mitoxantrone for adults with newly diagnosed, poor-risk acute myelogenous leukemia. *Haematologica* 97: 1736–1742.
47. Litzow MR, et al. (2019) A randomized trial of three novel regimens for recurrent acute myeloid leukemia demonstrates the continuing challenge of treating this difficult disease. *Am J Hematol* 94:111–117.
48. Zeidner JDL, et al. (2018) Zella 201: A biomarker-guided phase II study of alvocidib followed by cytarabine and mitoxantrone in MCL-1 dependent relapsed/refractory acute myeloid leukemia (AML). *Blood* 132:30.
49. Gottesman MM, Fojo T, Bates SE (2002) Multidrug resistance in cancer: Role of ATP-dependent transporters. *Nat Rev Cancer* 2:48–58.
50. Jones CL, et al. (2018) Inhibition of amino acid metabolism selectively targets human leukemia stem cells. *Cancer Cell* 34:724–740.e4, and erratum (2019) 35:333–335.
51. Pollyea DA, et al. (2018) Venetoclax with azacitidine disrupts energy metabolism and targets leukemia stem cells in patients with acute myeloid leukemia. *Nat Med* 24: 1859–1866.
52. Zeidan AM, et al. (2017) A phase 2 trial of high dose lenalidomide in patients with relapsed/refractory higher-risk myelodysplastic syndromes and acute myeloid leukaemia with trilineage dysplasia. *Br J Haematol* 176:241–247.
53. Kern W, et al. (2003) Early blast clearance by remission induction therapy is a major independent prognostic factor for both achievement of complete remission and long-term outcome in acute myeloid leukemia: Data from the German AML Cooperative Group (AMLCG) 1992 trial. *Blood* 101:64–70.
54. Stone RM, et al. (2017) Midostaurin plus chemotherapy for acute myeloid leukemia with a FLT3 mutation. *N Engl J Med* 377:454–464.
55. Przepiorka D, Deisseroth A, Farrell AT (2015) Acute myeloid leukemia response measures other than complete remission. *J Clin Oncol* 33:3675–3676.
56. Chen X, Xie H, Estey EH (2015) Reply to D. Przepiorka et al. *J Clin Oncol* 33:3676–3677.
57. Schuurhuis GJ, et al. (2018) Minimal/measurable residual disease in AML: A consensus document from the European LeukemiaNet MRD Working Party. *Blood* 131: 1275–1291.
58. Hourigan CS, Gale RP, Gormley NJ, Ossenkoppele GJ, Walter RB (2017) Measurable residual disease testing in acute myeloid leukaemia. *Leukemia* 31:1482–1490.
59. Kurtz SE, et al. (2017) Molecularly targeted drug combinations demonstrate selective effectiveness for myeloid- and lymphoid-derived hematologic malignancies. *Proc Natl Acad Sci USA* 114:E7554–E7563.
60. Pemovska T, et al. (2013) Individualized systems medicine strategy to tailor treatments for patients with chemorefractory acute myeloid leukemia. *Cancer Discov* 3: 1416–1429.
61. Casado P, et al. (2018) Proteomic and genomic integration identifies kinase and differentiation determinants of kinase inhibitor sensitivity in leukemia cells. *Leukemia* 32:1818–1822.
62. Swords RT, et al. (2018) Ex-vivo sensitivity profiling to guide clinical decision making in acute myeloid leukemia: A pilot study. *Leuk Res* 64:34–41.
63. Snijder B, et al. (2017) Image-based ex-vivo drug screening for patients with aggressive haematological malignancies: Interim results from a single-arm, open-label, pilot study. *Lancet Haematol* 4:e595–e606.
64. Tyner JW, et al. (2013) Kinase pathway dependence in primary human leukemias determined by rapid inhibitor screening. *Cancer Res* 73:285–296.
65. R Core Team (2018) R: A Language and Environment for Statistical Computing (R Foundation for Statistical Computing, Vienna), Version 3.5.0.
66. Ritchie ME, et al. (2015) Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 43:e47.
67. Subramanian A, et al. (2005) Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 102: 15545–15550.
68. Wong HY, et al. (2019) Molecular measurable residual disease testing of blood during AML cytotoxic therapy for early prediction of clinical response. *Front Oncol* 8:669.