Identification of Molecular Predictors of Response in a Study of Tipifarnib Treatment in Relapsed and Refractory Acute Myelogenous Leukemia

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Abstract **Purpose:** Microarray technology was used to identify gene expression markers that predict response to the orally available farnesyltransferase inhibitor tipifarnib (Zarnestra, R115777) in acute myelogenous leukemia (AML).

Experimental Design: Gene expression profiles from 58 bone marrow samples from a cohort of relapsed and refractory AML patients were analyzed on the Affymetrix U133A gene chip that contains ~22,000 genes.

Results: Supervised statistical analysis identified eight gene expression markers that could predict patient response to tipifarnib. The most robust gene was the lymphoid blast crisis oncogene (*AKAP13*), which predicted response with an overall accuracy of 63%. This gene provided a negative predictive value of 93% and a positive predictive value of 31% (increased from 18%). *AKAP13* was overexpressed in patients who were resistant to tipifarnib. When overexpressed in the HL60 and THP1 cell lines, *AKAP13* increased the resistance to tipifarnib by approximately 5- to 7-fold.

Conclusion: Diagnostic gene expression signatures may be used to select a group of AML patients that might respond to tipifarnib.

Tipifarnib (Zarnestra, R115777) is an orally available non-peptidomimetic competitive farnesyltransferase inhibitor (FTI) that has been shown to inhibit the proliferation of a variety of human tumor cell lines both *in vitro* and *in vivo* (1, 2). A phase 1 clinical study of tipifarnib showed a 32% response rate in patients with refractory or relapsed acute myelogenous leukemia (AML; ref. 3). Activity has also been seen in early clinical studies for myelodysplastic syndrome (4), multiple myeloma (5), and chronic myelogenous leukemia (6).

The farnesyltransferase enzyme mediates the covalent attachment of a 15-carbon farnesyl moiety to the COOH-terminal CAAX (C, cysteine; A, aliphatic residue; X, any amino acid) recognition motif (7). This farnesylation is further processed by cleavage of the three terminal amino acids (AAX) and methylation of the COOH-terminal isoprenylcysteine. The inhibition of protein farnesylation abrogates the correct subcellular localization required for protein function. Originally, the oncogenic Ras protein was thought to be the target for the antiproliferative effects of FTIs in cancer biology (8). However, it has since been shown that inhibition of Ras farnesylation does not account for all actions of tipifarnib. For example, FTIs do not always require the presence of mutant Ras protein to produce antitumor effects (3). Indeed, although early clinical studies were designed around populations with a high frequency of *ras* mutations, such as advanced colorectal and pancreatic cancer (both with high incidence of K-*ras* mutations), no significant difference in response rates was seen when compared with placebo (9, 10).

Several other farnesylated proteins have been implicated as candidate targets that may mediate the antitumorigenic effects of FTIs, including the small GTPase proteins RhoB, the centromere proteins CENP-E and CENP-F, the protein tyrosine phosphatase PTP-CAAX, and the nuclear membrane structural lamins A and B. The inhibition of farnesylation of these proteins may lead to the antiproliferative effect of FTIs and also

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indirectly modulate several important signaling molecules, including transforming growth factor-β type II receptor (11), mitogen-activated protein kinase/extracellular signal-regulated kinase (12), phosphatidylinositol 3-kinase/AKT2 (13), Fas (CD95), nuclear factor-κB (14, 15), and vascular endothelial growth factor (16). Regulation of these signaling pathways leads to the modulation of cell growth, proliferation, and apoptosis. Thus, FTIs may have complex inhibitory effects on several cellular events and pathways.

Although it is clear that FTIs function by inhibiting protein farnesylation, it is still not known what genes are implicated in the antitumor effects of tipifarnib in hematopoietic malignancies. Microarray technology allows for the measurement of the steady-state mRNA level of thousands of genes simultaneously, thereby representing a powerful tool for identifying genes and gene pathways that correlate with FTI action. Global gene expression monitoring was therefore used in a phase 2 clinical study of tipifarnib in relapsed and refractory AML to identify genes that predict response to this FTI in hematologic malignancies.

Materials and Methods

Clinical evaluation and response definitions. The current study was part of an open-label, multicenter, noncomparative phase 2 clinical study in which patients with relapsed or refractory AML (17) were treated with tipifarnib at a starting oral dose of 600 mg twice daily for the first 21 consecutive days of each 28-day cycle. Patients were enrolled into two cohorts: those with relapsed AML and those with refractory AML. A total of 252 patients (135 relapsed and 117 refractory) was treated.[7] Eighty patients chose to provide bone marrow samples for RNA microarray analysis, for which a separate informed consent was required. The appropriate ethics committee or institutional review board, in accordance with the regulations of each participating country, approved the protocol and informed consent documents. The overall response rate was relatively low in this study. Therefore, for the purposes of the gene expression profiling, response to tipifarnib was defined as patients who had an objective response (complete remission, complete remission with incomplete platelet recovery, or partial remission) or a hematologic response (decrease of >50% of leukemic blast cells in bone marrow) as determined either by central review or by the clinical site. Stable disease was defined as no hematologic response but no progression of the disease. For the purposes of marker discovery, patients with stable disease were considered neither responders nor nonresponders and, therefore, were removed from supervised analysis. Complete remission was defined as <5% bone marrow blasts with a neutrophil count >1,000/μL, a platelet count <100,000/μL, and no extramedullary disease. Complete remission with incomplete platelet recovery was defined similarly, except for a platelet count <100,000/μL sufficient to ensure transfusion independence. Partial remission was defined as at least a 50% decrease in bone marrow blasts with partial neutrophil (>500/μL) and platelet count (>50,000/μL) recovery as well as patients who achieved a complete remission without confirmation 4 weeks later. Response had to be confirmed at least 4 weeks after first documentation.

Sample collection and microarray processing. Bone marrow samples were collected from patients before treatment with tipifarnib, diluted with PBS (pH range, 7.2-7.6; KCl, 0.2 g/L; NaCl, 8.0 g/L; KH_2PO_4, 0.2 g/L; Na_2HPO_4, 1.15 g/L), and centrifuged with Ficoll-diatrizoate (1.077 g/mL). WBCs were washed twice with PBS, resuspended in fetal bovine serum with 10% DMSO, and immediately stored at −80°C. Cells were thawed and total RNA was extracted from cell samples using the RNeasy kit (Qiagen, Santa Clarita, CA). RNA quality was checked using the Agilent Bioanalyzer (Santa Clara, CA). Synthesis of cDNA and cRNA was done according to Affymetrix (Santa Clara, CA) protocols (Supplementary Material 1). The microarray data have been deposited in National Center for Biotechnology Information Gene Expression Omnibus[8] and are accessible through Gene Expression Omnibus Series accession number GSE5122.

ras mutation analysis. Activating N-*ras* and K-*ras* mutations were identified by restriction endonuclease-mediated selective PCR and RFLP analysis as described previously (3). Exons 1 and 2 of both *ras* genes were simultaneously amplified in a single multiplex reaction, and an aliquot was used for a second round of PCR. Resistance to cleavage at natural or primer-induced restriction enzyme sites in second-round amplicons indicated the presence of a mutation that had abolished the site at the loci being analyzed. Restriction enzymes for the analysis of specific loci were *Bst*NI (K-*ras* codon 12), *Bsl*I (K-*ras* codon 13, N-*ras* codons 12 and 13), *Msc*I (N-*ras* codon 61, positions 1 and 2), *Hae*III (K-*ras* codon 61, position 1), *Bfa*I (N-*ras* codon 61, position 3), and *Tru*9I (K-*ras* codon 61, positions 2 and 3). Reactions were digested overnight, and PCR products were analyzed on an Agilent Bioanalyzer.

Statistical analysis. To identify genes that predict response with high sensitivity and high negative predictive value, a percentile analysis was used. Genes that were up-regulated or down-regulated in 100% of responders compared with at least 40% of nonresponders were identified. A mean 2-fold difference in expression that was statistically significant ($P < 0.05$) was also required of the selected genes. Specificity cutoffs higher than 40% were also tested with a fixed sensitivity of 100%. The χ^2 test and Student's t test were then used to test the significance of the correlations between patient response and patient covariates, including *ras* mutation status and gene expression. Unsupervised k-means and hierarchical clustering were done in OmniViz. The predictive value of the selected genes was then analyzed by leave-one-out and leave-five-out cross-validation methods. Here, one (or five) sample(s) was (were) removed from the data set and the marker was reselected from 11,723 genes. The predictive value of this gene was then tested on the left-out sample(s) using a linear discriminant analysis. Sensitivity was calculated as the number of true positives detected by the test divided by the sum of true positives plus false negatives. Specificity was calculated as the number of true negatives detected by the test divided by the sum of true negatives and false positives. Positive predictive value was calculated as the number of true positives divided by the number of true positives and false positives. Negative predictive value was calculated as the number of true negatives divided by the number of true negatives and false negatives. The positive likelihood ratio of a patient responding to treatment is sensitivity divided by 1 minus specificity. Receiver operator curves were used to choose appropriate thresholds for each classifier, requiring a sensitivity of 100%. The receiver operator curve diagnostic calculates the sensitivity and specificity for each parameter.

Real-time reverse transcription-PCR validation. Taqman real-time reverse transcription-PCR was used to verify the microarray results of the *AHR* and *AKAP13* genes (Supplementary Material 2).

Cell line culture and AKAP13 overexpression assay. The AKAP13 vectors oncoLBC and protoLBC and vector control (pSRα-neo) were obtained from Dr. Deniz Toksoz (Tufts University School of Medicine, Boston, MA; ref. 18). The HL60 and THP1 cell lines were obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 with 10% fetal bovine serum. Cells were transiently transfected with each vector using the Nucleofector kit (Amaxa, Gaithersburg, MD) according to the manufacturer's instructions and kept under G418 (300-600 μg/mL). Tipifarnib or doxorubicin was then added in various concentrations to triplicate cultures (1.5×10^5

[7] J-L. Harousseau et al., accepted for publication.

[8] http://www.ncbi.nlm.nih.gov/geo/

Table 1. Comparison of profiled and nonprofiled patients

Covariate	Subset of 58 patients	Remaining 194 patients
Response, *n* (%)	10 (17.2)	28 (14.4)
Male, *n* (%)	28 (48.3)	119 (61.3)
Average age (y)	60	60
Relapsed disease, *n* (%)	31 (53.4)	56 (28.9)
Cytogenic risk, *n* (%)	34 (58.6)	104 (53.6)
Average blasts (%)	55	50

cells/mL). Cells were counted at day 4 after transfection. Cell counts were normalized to cultures with the lowest concentration of drug to give a percentage of viable control cells.

Results

Expression profiling of relapsed and refractory AML. FTIs were originally designed to specifically inhibit farnesyltransferase activity, thereby blocking the oncogenic Ras pathways. Therefore, we initially analyzed DNA from the bone marrow of 80 patients with relapsed or refractory AML for activating *ras* mutations and investigated the possible correlation between *ras* mutation and the response to tipifarnib. Analysis was successful in 78 samples, with 23% and 5% of patients having N-*ras* and K-*ras* mutations, respectively (Supplementary Table S2); however, the mutation status did not correlate with objective response or overall survival.

We therefore did gene expression profiling to identify novel signatures that could be used to predict response to the FTI tipifarnib. Bone marrow samples were obtained for gene expression analysis from 80 patients before treatment with tipifarnib. Fifty-eight of the 80 samples passed quality control measures, including RNA quality and chip performance. The 58 patients and the remainder of the clinical study population (n = 194) were similar with regard to age, sex, AML class (relapsed or refractory), cytogenic risk factors, baseline blast counts, response, and overall survival (Table 1). The gene expression data were integrated with the clinical information, and retrospective analyses were done to identify genes that could separate responders from nonresponders with a high level of sensitivity.

The data went through several filtering steps before identification of differentially expressed genes. First, genes that were not expressed in at least 10% of the samples were removed. This reduced the number of genes from approximately 22,000 to 11,723 genes. For unsupervised analyses, genes that showed little variation in expression across the data set (coefficient of variance of <45% across all the samples) were also excluded and quantile normalization was applied to the remaining 5,728 genes. At this stage, an unsupervised k-means clustering analysis was done to identify any differences between patients based on their global gene expression profiles. Six main clusters of patients were identified using this technique. No separation between responders and nonresponders was observed (Supplementary Fig. S1). This is not unexpected because only a handful of genes may be associated with the antitumor effect of FTIs. For example, it is possible that the differential expression of a single gene that is involved in FTI biology affects clinical responses and this would be masked by the noise introduced from the other ∼5,700 genes.

Identification of genes that are differentially expressed between responders and nonresponders. We next did supervised analysis of the gene expression data to identify genes that would predict response with a high sensitivity and high negative predictive value. This is important for an oncology therapy with a low response profile because it would be undesirable to withhold therapy from potential responders. To that end, we used selection criteria to identify genes that were differentially expressed between 100% of responders compared with at least 40% of nonresponders. The selected genes also had to show at least a mean 2-fold difference in expression that was statistically significant (P < 0.05). Assuming no perfect predictive biomarker exists, fixing the sensitivity at 100% generally means that the specificity of an analyte will suffer. As such, we found that higher specificity cutoffs (>40%) identified too few genes for further analysis of multiple gene classifiers. Four patients were removed from the analysis because they were classified as having stable disease, and these patients cannot be clearly defined as either responders or nonresponders. Inclusion of stable disease patients may bias the analysis for selecting genes associated with prognosis irrespective of drug treatment. This resulted in comparing 10 responders with 44 nonresponders. From 11,723 genes, a total of 8 genes was identified that could stratify responders and nonresponders (Table 2) and that gave significant P values in a t test (P < 0.05). The genes included those involved in signal transduction, apoptosis, cell proliferation, oncogenesis, and, potentially, FTI biology.

Table 2. List of top eight genes that predict response to tipifarnib

Probe set ID	Gene title	Gene symbol	AUC*	Fold change (R/NR)
208325_s_at	A kinase (PRKA) anchor protein 13	*AKAP13*	0.830	0.491
202820_at	Aryl hydrocarbon receptor	*AHR*	0.807	0.446
204362_at	SRC family associated phosphoprotein 2	*SCAP2*	0.777	0.431
213479_at	Neuronal pentraxin II	*NPTX2*	0.738	0.115
212384_at	HLA-B–associated transcript 1	*BAT1*	0.725	0.458
206148_at	Interleukin-3 receptor, α (low affinity)	*IL3RA*	0.705	0.375
210666_at	Iduronate 2-sulfatase (Hunter syndrome)	*IDS*	0.645	0.395
206637_at	Purinergic receptor P2Y, G protein coupled, 14	*P2RY14*	0.627	0.369

Abbreviations: ID, identification; AUC, area under the curve; R, responders; NR, nonresponders.
*Area under the curve was calculated from receiver operator characteristic analysis. This is an indication of the overall diagnostic accuracy.

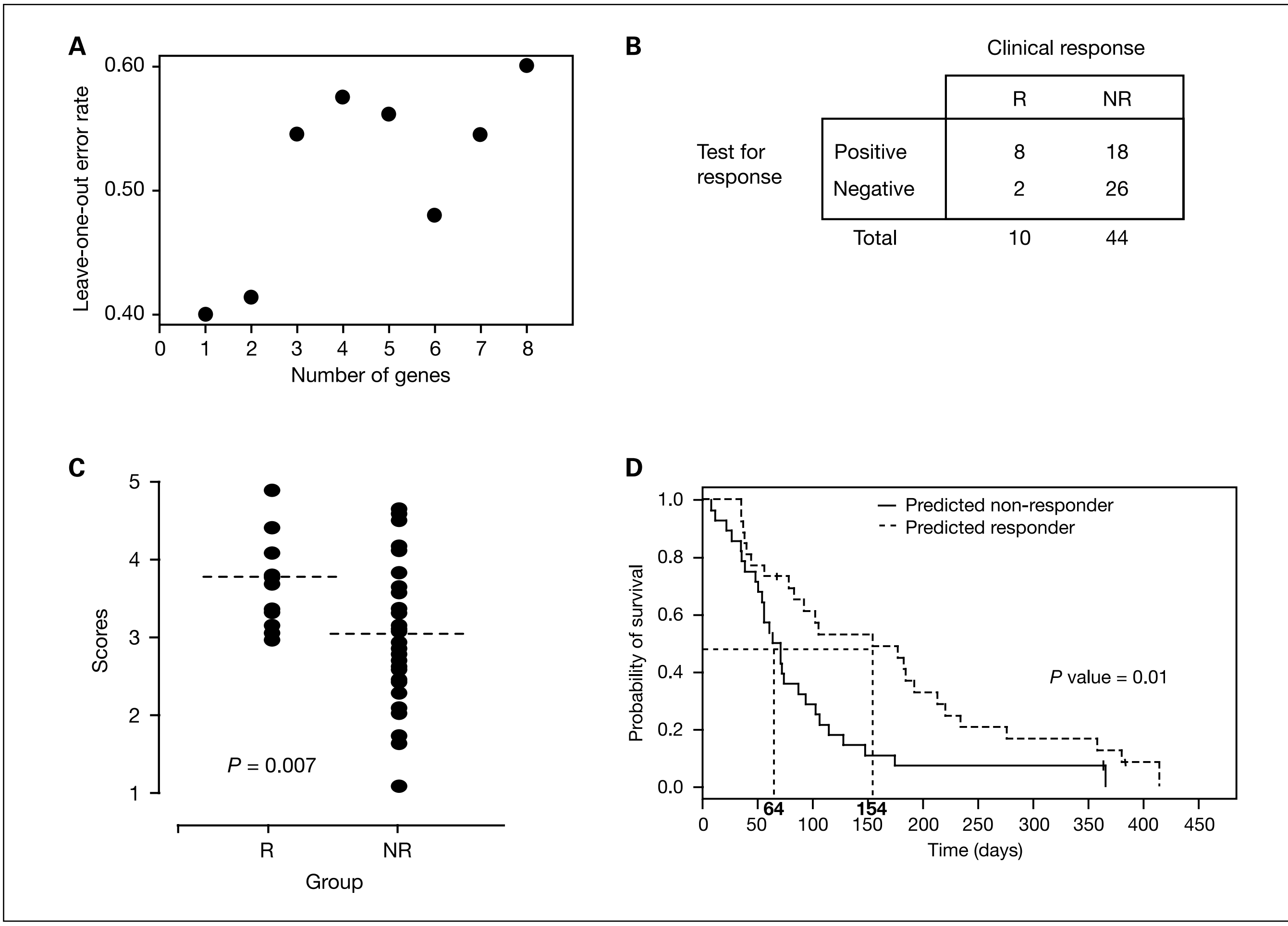

Fig. 1. Identification of a minimal set of predictive markers. *A,* a leave-one-out cross-validation was done selecting genes with a sensitivity of 100%, specificity of 40%, and fold change >2. Independent classifiers were tested that contained from one to eight genes ranked by area under the curve. The resulting error rate is plotted. *B,* a 2 × 2 table generated from a leave-one-out cross-validation done using AKAP13 as a classifier on the responders (*R*) and nonresponders (*NR*). *C,* gene expression values of AKAP13. The *P* value indicates a significant difference in the gene expression between the response groups. *D,* Kaplan-Meier curves generated from patients classified by AKAP13 as being responders and nonresponders. Median survival times.

Real-time reverse transcription-PCR validation of gene markers. To verify the microarray gene expression data, Taqman real-time reverse transcription-PCR was done on cDNA that was used for generating the labeled target cRNA for microarray hybridization. Two genes were selected to verify the gene expression data. The *AHR* and *AKAP13* genes were chosen because the use of these genes resulted in the highest level of specificity for responders. The correlation coefficient was 0.74 for *AHR* and 0.94 for *AKAP13*, indicating that the microarray gene expression data could be validated by PCR (Supplementary Fig. S2).

AKAP13 is the most robust marker. A minimal set of genes was identified that would provide the best diagnostic accuracy from the eight selected genes. Classifiers were built with an increasing number of genes based on the area under the curve values from receiver operator characteristic analysis, and the error rate of these classifiers was calculated using leave-one-out cross-validation while keeping the sensitivity of predicting response at 100% (Fig. 1A). The *AKAP13* gene could predict response with the lowest error rate of <40% (Fig. 1A). The error rate increased to >50% when more than two genes were used in the classifier. For the *AKAP13* gene, the leave-one-out cross-validation showed a negative predictive value of 93% and a positive predictive value of 31%, with an overall diagnostic accuracy of 63% and positive likelihood ratio of 2.0 (Fig. 1B). The expression value for *AKAP13* in each patient is shown in Fig. 1C. Therefore, for the group of patients with low expression of AKAP13, the response rate to tipifarnib was 31% (8 of 26) compared with 18% (10 of 54) in the current patient population.

Using the *AKAP13* gene, Kaplan-Meier analysis showed a significant difference in survival between the predicted responder group and the nonresponder group (Fig. 1D). There were 18 clinically defined nonresponders who were classified as predicted responders as measured by AKAP13 expression. Interestingly, these patients had better overall survival compared with the 26 patients correctly classified as nonresponders (Supplementary Fig. S3). This could indicate that *AKAP13* gene expression predicts a level of response to therapy that cannot be predicted by using conventional clinical response criteria; however, a greater number of patients will need to be analyzed to validate this hypothesis.

Overexpression of AKAP13 increases resistance to tipifarnib in AML. The *AKAP13* gene was the most robust marker of resistance to tipifarnib. We therefore investigated its involvement in FTI biology by overexpressing the oncoLBC and protoLBC variants of this gene in the HL60 and THP1 cell lines and testing for sensitivity to tipifarnib. Increased expression of the AKAP13 variants was confirmed by quantitative reverse transcription-PCR (data not shown). Overexpression of both AKAP13 variants in these AML cell line models led to an approximately 5- to 7-fold increase in resistance to tipifarnib compared with control cells (Fig. 2A and C). Both the LBC oncogene and proto-oncogene increased the resistance to tipifarnib to the same extent as seen by a parallel rightward shift of the kill curves by more than one log unit compared with control. When the cells overexpressing the AKAP13 variants were treated with the non-FTI chemotherapeutic doxorubicin, there was no significant increase in resistance (Fig. 2B and D).

Discussion

Targeted therapies recently developed for cancer suggest that clinical benefit should correlate with specific receptors, enzymes, or intracellular machinery [e.g., human epidermal growth factor receptor 2 (19), estrogen receptor (20), and BCR-ABL (21)]. Using pharmacogenomics to isolate a predictive set of genes that a priori may not be known to correlate with sensitivity to therapy is an advance that has been gaining additional attention. Expression profiles have been found that predict response to anticancer compounds, including standard chemotherapeutics (22–24) and novel selective anticancer agents (21, 25). The recent discovery of pharmacogenetic profiles that may predict response to the tyrosine kinase inhibitor gefitinib (Iressa) in a subset of patients with non–small cell lung cancer has prompted additional investigation in a prospective study (26–28). This study extends this area of investigation by identifying pharmacogenetic profiles that may predict response to the FTI tipifarnib in AML patients who have limited therapeutic options.

In a phase 2 study of relapsed and refractory AML patients, we have identified gene expression profiles that predict response to tipifarnib, a novel FTI. This class of compounds is showing promise in the treatment of hematologic malignancies (3–5) and solid tumors, such as breast cancer (29) and recurrent glioma (30). However, although clinical responses are being shown, there is a growing need to tailor therapy by identifying patients who are most likely to respond to the drug

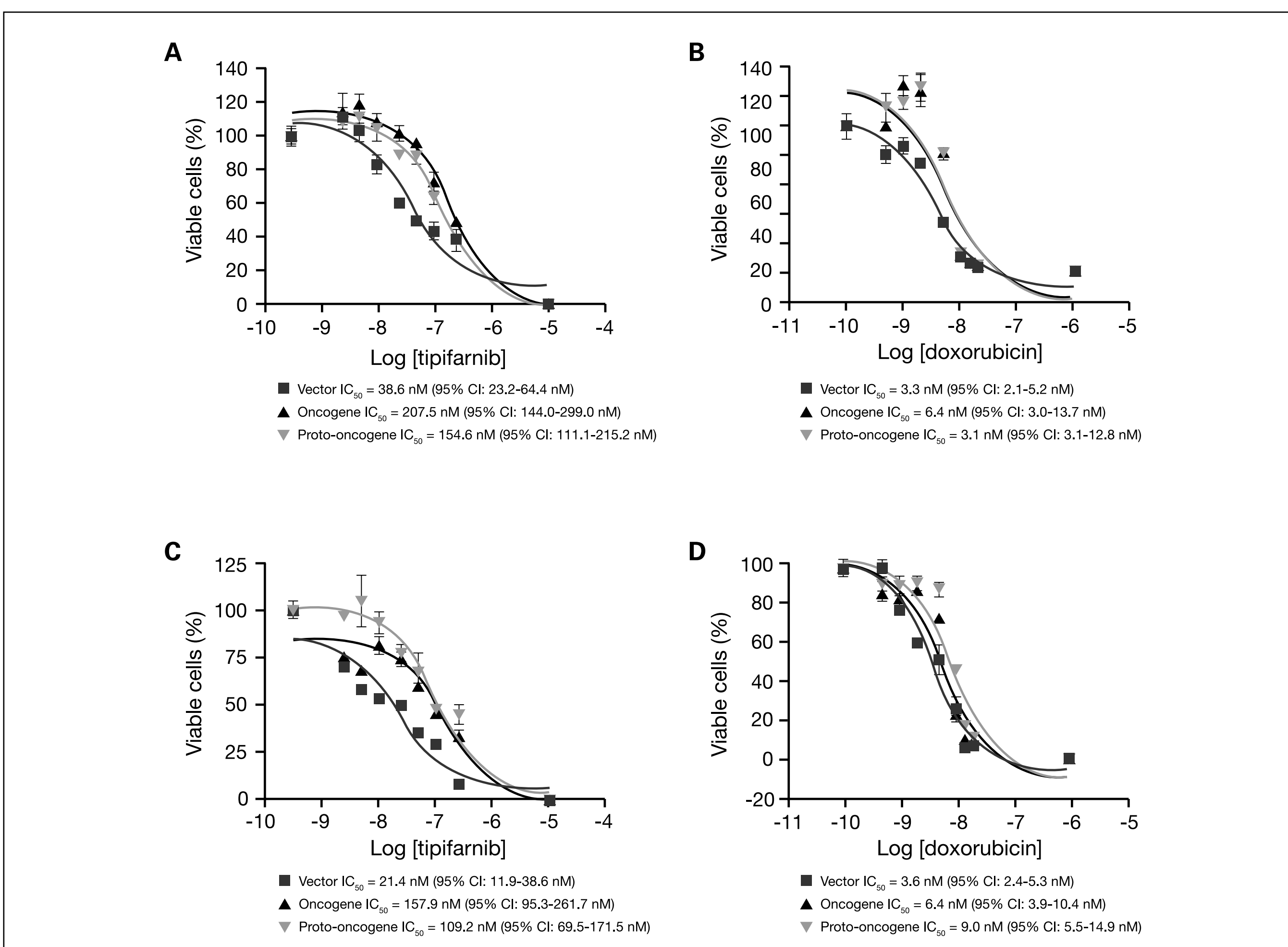

Fig. 2. Overexpression of AKAP13 in AML cell lines. *A,* tipifarnib kill curves of THP1 cells transfected with AKAP13 variants. *B,* doxorubicin kill curves of THP1 cells transfected with AKAP13 variants. *C,* tipifarnib kill curves of HL60 cells transfected with AKAP13 variants. *D,* doxorubicin kill curves of HL60 cells transfected with AKAP13 variants. Cell counts were normalized to cultures with no drug to give a percentage of control. Points, mean; bars, SE.

and are, therefore, the best candidates for treatment. Furthermore, although Ras was considered to be a primary target of this class of drugs, several clinical studies have shown that they are not necessarily effective in populations with a high frequency of *ras* mutations (9, 10). The lack of response seen in advanced colorectal and pancreatic cancer may be due to the alternative prenylation pathway available to K-ras proteins following inhibition of farnesylation (31, 32). However, N-ras can also be alternatively geranylgeranylated and yet patients with AML have been responsive to tipifarnib regardless of the N-*ras* mutational status of their tumors (3). There are several other farnesylated proteins that are involved in important signaling and proliferation pathways. Therefore, other genes may have an effect on patient sensitivity to this class of antitumorigenic compounds. We therefore hypothesized that, through a genome-wide screening approach, novel markers that predict response to FTIs could be identified.

Using microarray analysis, eight gene markers were identified that have the potential to predict response to tipifarnib. A subset of these markers was both predictive of drug response and also thought to have the potential to be involved in FTI biology. The top candidate discovered from the microarray studies was the lymphoid blast crisis oncogene (*oncoLBC* or *AKAP13*). Although *AKAP13* was originally identified from a patient with chronic myelogenous leukemia, its overexpression has not before been documented in AML. This gene functions as a guanine nucleotide exchange factor for the Rho proteins (10, 18) and as a protein kinase A anchoring protein (33). *AKAP13* contains a region that is homologous to an α-helical domain that is known to interact with lamin B (34). This association could lead to lamin B activation via protein kinase A. Both RhoB and lamin B are farnesylated and are candidate targets of FTIs. AKAP13 is also a proto-oncogene because loss of its three-prime end causes cellular transformation (35).

Rho proteins are potentially important antitumorigenic targets for FTIs (36, 37). RhoB, RhoA, and RhoC have been found to be overexpressed in multiple cancer types (37). Although most of these Rho proteins are geranylgeranylated, they interact closely with each other and the farnesylated ras, RhoE, and RhoB small GTPases (37, 38). Furthermore, it has been shown that RhoH, RhoB, and RhoE can act in an antagonistic fashion to the transforming abilities of RhoA and RhoG (39). The activity of RhoA, and possibly other related small GTPases, is increased by the guanine nucleotide exchange factor lymphoid blast crisis oncogene (*AKAP13*; refs. 35, 40). In addition, *AKAP13* may increase mitotic activity by activating lamin B via protein kinase A (34). Therefore, the increased activity of *AKAP13* could lead to an increased cellular profile of transformation. This might allow for the leukemic blast cell to overcome the antitumorigenic effects of FTIs through compensatory pathways (41). In contrast, when *AKAP13* is underexpressed, FTIs may be more effective in blocking these pathways. We also showed that overexpression of *AKAP13* (both oncoLBC and protoLBC variants) increased the IC_{50} of the HL60 and THP1 AML cell lines by approximately 5- to 7-fold, thus recapitulating what was observed in patients who did not respond to tipifarnib. This increase in resistance was not seen when cells were treated with the non-FTI chemotherapeutic doxorubicin. This indicates that overexpression of *AKAP13* is a relevant marker of resistance to FTIs. As such, it may also be a useful alternative drug target for patients who are resistant to tipifarnib.

AKAP13 gene expression predicts a level of response to therapy that cannot be predicted by using conventional clinical response criteria. Alternatively, this raises the question of whether the gene signature for predicting response to FTI treatment also has prognostic value irrespective of FTI therapy. Although our *in vitro* data showed that cells overexpressing AKAP13 variants did not increase resistance to non-FTI chemotherapy, this issue is also being addressed by evaluating the signature in AML patients treated in a randomized study of tipifarnib versus best supportive care. In addition, we evaluated an independent prognostic signature identified in newly diagnosed AML (42). Although this signature significantly stratified good- and poor-outcome patients, it did not identify patients specifically responding to tipifarnib.[9]

The technology used in this study can also be applied in other pathologic conditions. Pharmacogenomics can be used to predict and identify patients who might respond better to a specific targeted therapy. Separating those patients who should respond from those who are likely not to respond to rationally designed targeted therapies will help ensure that the appropriate patients are receiving the therapy, which should result in better patient care and ultimately improve response rates and survival. In addition, such studies may help to elucidate mechanisms of action or resistance and serve to identify potential new targets for antineoplastic therapy.

[9] M. Raponi et al., unpublished results.

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