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Kinase Pathway Dependence in Primary Human Leukemias Determined by Rapid Inhibitor Screening

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Abstract

Kinases are dysregulated in most cancers, but the frequency of specific kinase mutations is low, indicating a complex etiology in kinase dysregulation. Here, we report a strategy to rapidly identify functionally important kinase targets, irrespective of the etiology of kinase pathway dysregulation, ultimately enabling a correlation of patient genetic profiles to clinically effective kinase inhibitors. Our methodology assessed the sensitivity of primary leukemia patient samples to a panel of 66 small-molecule kinase inhibitors over 3 days. Screening of 151 leukemia patient samples revealed a wide diversity of drug sensitivities, with 70% of the clinical specimens exhibiting hypersensitivity to one or more drugs. From this data set, we developed an algorithm to predict kinase pathway dependence based on analysis of inhibitor sensitivity patterns. Applying this algorithm correctly identified pathway dependence in proof-of-principle specimens with known oncogenes, including a rare FLT3 mutation outside regions covered by standard molecular diagnostic tests. Interrogation of all 151 patient specimens with this algorithm identified a diversity of kinase targets and signaling pathways that could aid prioritization of deep sequencing data sets, permitting a cumulative analysis to understand kinase pathway dependence within leukemia subsets. In a proof-of-principle case, we showed that *in vitro* drug sensitivity could predict both a clinical response and the development of drug resistance. Taken together, our results suggested that drug target scores derived from a comprehensive kinase inhibitor panel could predict pathway dependence in cancer cells while simultaneously identifying potential therapeutic options. *Cancer Res; 73(1); 285–96. ©2012 AACR.*

Introduction

Gene-targeted cancer therapies have achieved remarkable clinical outcomes in recent years (1–4). In particular, cell-permeable small molecules that exhibit inhibitory activity against tyrosine kinases have generated great interest. Tyrosine kinases represent a gene family widely implicated in cancer pathogenesis (5, 6), and dysregulation of specific tyrosine kinases has been observed in most hematologic malignancies, including chronic myeloid leukemia (CML; ref. 7), chronic myelomonocytic leukemia (CMML; refs. 8–11), other myeloproliferative neoplasms (MPN; refs. 12–15), acute myeloid leukemia (AML; refs. 9, 16–20), acute lymphoblastic leukemia (ALL; refs. 21–26), and chronic lymphocytic leukemia (CLL; refs. 27–30). Although a minority of patients with hematologic malignancies are successfully treated with kinase inhibitors, most patients remain ineligible for this form of targeted therapy due to lack of knowledge of the specific kinase pathways involved.

Many strategies exist to better understand kinase dysregulation in cancer including the recent development of deep sequencing techniques, which are accelerating our understanding of cancer genetics. Thus far, however, many studies of malignancies with predicted kinase pathway dependence have not found frequent mutations in kinase genes (31–34). These findings suggest that kinase pathway dependence in malignant cells often occurs due to complex genetic mechanisms. Hence, although deep sequencing represents an immensely powerful technique, it may not independently allow for prediction of kinase targets and kinase inhibitor therapies. Instead, understanding of the best kinase inhibitor therapies for patients will likely require the combination of deep sequencing with complementary studies that can define kinase

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targets regardless of mutational status. These functionally important kinase pathways can then be correlated with genetic profiles that have been revealed by deep sequencing.

To better define the use of kinase inhibitor therapies in hematologic malignancies, we have developed a small-molecule kinase inhibitor panel designed to identify kinase pathway dependence in primary leukemia samples. To analyze kinase pathway dependence based on this functional data, we have developed an accompanying bioinformatics approach to predict the kinase targets underlying inhibitor sensitivity profiles. This algorithm takes advantage of our knowledge of the gene products that are targeted by each drug as well as the fact that these target profiles are partially overlapping. Using the overlap of effective drugs and eliminating targets of ineffective drugs, we are able to predict critical kinase targets and signaling pathways for individual patient samples. These kinase target predictions represent a manner by which functional data from drug screening could be integrated with genomics data such as deep sequencing to aid in prioritization of sequence variants and, thus, accelerate our understanding of molecular etiologies of cancer as well as application of individualized therapeutic approaches for patients.

Materials and Methods

Kinase inhibitors

Kinase inhibitors were purchased from or were generously provided by the sources outlined in Supplementary Table S7.

Collection of patient samples and cell culture

All clinical samples were obtained with informed consent with approval by the Institutional Review Boards of Stanford University (Stanford, CA), Oregon Health & Science University (Portland, OR), the Children's Oncology Group, and Erasmus Medical Center/Sophia Children's Hospital (Rotterdom, the Netherlands). Blood or bone marrow from patients was separated on a Ficoll gradient and mononuclear cells were treated with ammonium-chloride-potassium (ACK) lysis buffer. The only exceptions to this procedure were cases of atypical CML or chronic neutrophilic leukemia, where samples were only processed with ACK lysis buffer to preserve the neoplastic granulocytes that would otherwise be lost on the Ficoll gradient. Cells from myeloid leukemia samples were cultured in R10 [RPMI-1640 medium supplemented with 10% FBS (Atlanta Biologicals), L-glutamine, penicillin/streptomycin (Invitrogen), and fungizone (Invitrogen)] supplemented with 10^{-4} M 2-mercaptoethanol (Sigma). Cells from lymphoid leukemia samples were cultured in R20 [RPMI-1640 medium supplemented with 20% FBS (Atlanta Biologicals), L-glutamine, penicillin/streptomycin (Invitrogen), and fungizone (Invitrogen)] supplemented with 10^{-4} M 2-mercaptoethanol (Sigma) insulin-transferrin-sodium selenite (Invitrogen).

Kinase inhibitor screen

Kinase inhibitors were stored at 10 to 100 mmol/L in dimethyl sulfoxide (DMSO; stock concentration was 1,000 times the final concentration of the highest tested dose). Drugs were used at final concentrations shown in Supplementary Table S4. For creation of replicate plates of the library, each drug concentration was diluted to twice the final concentration and 50 μL were plated into 96-well plates using a Hydra 96-channel automated pipettor (Matrix Technologies). Plates were sealed with adhesive lids (Bio-Rad; microplate seal B), wrapped in aluminum foil, and stored at −20°C until use. Upon receipt of a patient sample, plates were thawed at 37°C, 5% CO_2 for 1 hour and centrifuged at 800 × *g* before removal of adhesive lids. Subsequently, patient samples were suspended into culture media at a concentration of 1,000,000 cells per mL, such that addition of 50 μL to each well would deliver 50,000 cells to that well (this also dilutes the drugs to their final, desired concentration). Cells were incubated for 3 days at 37°C, 5% CO_2 and subjected to a CellTiter 96 AQ_{ueous} One solution cell proliferation assay (Promega). Each plate contained 7 wells without any drug. The average absorbance value of these 7 wells was used for data normalization and the kill curve of each drug gradient was assessed relative to this average no-drug point.

Quantification of patient response and effective drug targets

An algorithm was designed and implemented using Excel and Visual Basic to provide automated IC_{50} calculation and therapeutic target identification. IC_{50} values were calculated using second-degree polynomial regression curves fit through 5 data points (average of no drug wells and 4 serial dilution points). All curves were manually inspected and a small number of IC_{50}s were corrected in 2 circumstances: (1) The curve fit intersected the IC_{50} at 2 distinct points —the lower concentration intersect was used in these instances; (2) the polynomial curve fit yielded an artificial IC_{50} not reflected in the data points (generally due to increasing cell viability over the course of the drug titration). For a given sample, drug IC_{50} values were considered effective if they were less than or equal to 5-fold below the median IC_{50} for all samples tested. Where an IC_{50} was not achieved for a given drug, an IC_{50} value equal to the highest drug concentration used was arbitrarily assigned. After effective and ineffective drugs were determined for each sample, a drug target score was assigned by the program for each potential therapeutic target.

The drug target score is based on the IC_{50} measured effectiveness of panel drugs against a given therapeutic target for a given patient. Each drug is associated with a "tiered" ranking of target kinases for which the drug has been shown to biochemically associate (35, 37–43). Weights are used to give a stronger quantitative emphasis to targets with a higher ranking (Figs. 4A and 4C). Scores are determined empirically for a given sample by assigning positive weighted scores to targets of effective drugs and negative weighted scores to targets of ineffective drugs. Drug effectiveness threshold and tiered ranking weights were determined empirically using patient samples and cell lines with known kinase signaling abnormalities. The algorithm generates a cumulative drug target score for each target according to the following equations:

$$\text{Effective drug target score} = \sum_{i=1}^{n} \text{Weight}_{Tier}(i)$$

Where $\text{Weight}_{Tier}(i)$ is a given drug target's tier ranking for drug i and n is the number of effective drugs.

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And

$$\text{Ineffective drug target score} = \sum_{j=1}^{m} \text{Weight}_{Tier}(j)$$

Where $\text{Weight}_{Tier}(j)$ is a given drug target's tier ranking for drug j and m is the number of ineffective drugs.

$n + m = 66$.

Final drug target score = effective drug target score + ineffective drug target score

Hierarchical clustering was conducted using GenePattern software (Broad Institute). Sample clustering and 2-way clustering by row (drug) and column (patient sample) were conducted using Pearson correlation distance shown in Fig. 6 and Supplementary Figs. S2 and S3.

Results

Development of a kinase inhibitor panel for analysis of primary leukemia specimens

The ubiquitous role of tyrosine kinases in regulating critical cellular processes leading to malignancy suggests that a large percentage of leukemia (and other malignancy) patient samples would exhibit sensitivity to inhibition of one or more kinase pathways. To test this hypothesis, we compiled a library of 66 small-molecule kinase inhibitors with collective activity against two-thirds of the tyrosine kinome (Supplementary Table S1). Because many nontyrosine kinases are also critical regulators of cellular growth/survival, we also included drugs with activity against select families of nontyrosine kinases including phosphoinositide-3 kinase (PI3K)/AKT, PKC, PKA, IκK, RAF/MEK/ERK, c-*jun*-NH2-kinase (JNK), p38, AMPK, aurora kinases, and cyclin-dependent kinases (Supplementary Table S1). Each inhibitor was plated at 4 graded concentrations that bracket the predicted on-target IC_{50} value. Primary patient samples were incubated with this panel of drugs for 3 days at which point a tetrazolium-based cell viability assay (MTS) was conducted for assessment of cell viability. All values were normalized to cells incubated in the absence of drug (Supplementary Fig. S1).

Analysis of 151 leukemia patient samples with small-molecule kinase inhibitor panel

Over a 2-year period, we accrued and tested 151 fresh, primary leukemia patient samples against this panel of kinase inhibitors. The cohort was comprised of 34 AML, 42 ALL, 31 MPN, and 44 CLL patients. Detailed clinical and demographic information about this patient cohort can be found in Supplementary Table S2. Assessment of kinase inhibitor hypersensitivity profiles of these 151 leukemia patient samples revealed a wide diversity of responses to kinase inhibitors, even when patients were grouped according to diagnostic subsets and kinase inhibitors grouped according to predicted kinase target spectra (Fig. 1). Despite this heterogeneity of responses, certain trends emerged, such as more frequent sensitivity to PI3K/AKT inhibitors in lymphoid samples. In addition, select cases could be identified with universal sensitivity to whole families of kinase inhibitors. For example, AML case 07335 exhibited universal sensitivity to all ERBB family inhibitors on the panel, suggesting involvement of an ERBB family member in maintenance of the viability of malignant cells from that patient (Fig. 1). Overall, 70% of patients exhibited hypersensitivity to one or more kinase inhibitors (IC_{50} data for each drug for each patient specimen listed in Supplementary Table S3; raw data points used to generate these IC_{50}s listed in Supplementary Table S4). Nine of the drugs on our panel are currently approved by the U.S. Food and Drug Administration (FDA; imatinib, nilotinib, dasatinib, sunitinib, erlotinib, gefitinib, lapatinib, sorafenib, and pazopanib), and approximately 40% of samples exhibit hypersensitivity to one or more of these 9 drugs. Hypersensitivity to a drug was determined by comparison of the response of each individual sample with the response of all other samples (patient IC_{50} compared with whole cohort median IC_{50}). In this way, we could define outlier samples that were truly hypersensitive to a given drug versus responses at higher concentrations that might occur due to off-target toxicity of the compound. Rank ordering of patient IC_{50}s for each drug helps illustrate this point (Supplementary Fig. S2). To better distinguish inhibitor sensitivity profiles that were similar from patient to patient, we applied one-way Pearson correlation for hierarchical clustering of the data (Supplementary Fig. S3). Notably, although drug responses are clearly not uniform among diagnostic subsets, there were large groups of patients diagnosed with the same type of leukemia [notably ALL (shaded red) and CLL (shaded yellow); Supplementary Fig. S3] with similar responses to these kinase inhibitors. However, the segregation of sample responses by cell type was far from complete with a number of isolated lymphoid samples clustering amidst myeloid cases and vice versa. In addition, 2-way hierarchical clustering revealed drugs with similar activity profiles across patients (Supplementary Fig. S4). Many of these drugs clustered in groups that would be predicted on the basis of known target profiles of the compounds such as BIRB-796 and VX-745 (both p38 inhibitors), flavopiridol and BMS-387032 (both CDK inhibitors), and EKB-569 and CI-1033 (both ERBB-family inhibitors).

Logical prediction of oncogenic signaling pathways using inhibitor sensitivity profiles

Clinical and research interest in the application of kinase inhibitors has led to a concerted effort to develop techniques that characterize the targets to which each compound can effectively bind (35–38). Because a majority of the kinase inhibitors on our panel have been characterized in this manner, we realized that this information could be used to predict the critical kinase targets and signaling pathways that underlie the observed kinase inhibitor sensitivity patterns. Development of this bioinformatics approach relies on the fact that all kinase inhibitors on our panel bind multiple targets and the target spectra for these drugs are partially overlapping. Thus, if a sample exhibits sensitivity to 2 different drugs, the kinase targets that are commonly inhibited by both drugs are implicated as most likely to explain this sensitivity pattern (unless there are 2 different pathways operational in that sample). A second step can further narrow the candidate kinase list

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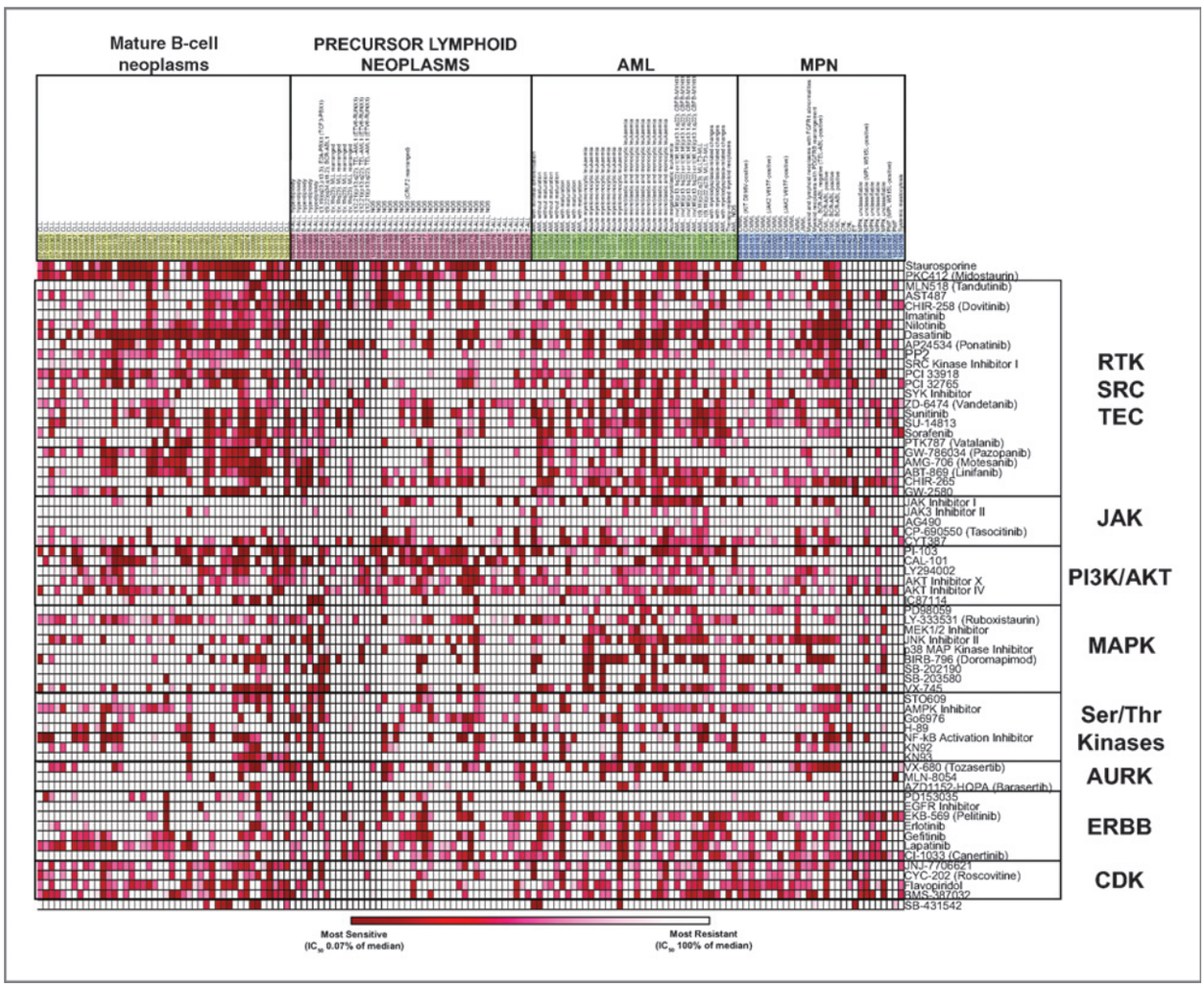

Figure 1. Response of 151 leukemia patient samples to 66 small-molecule kinase inhibitors. Mononuclear cells were obtained from 151 primary leukemia patient samples and IC_{50} values for 66 small-molecule kinase inhibitors were tabulated as described in Supplementary Fig. S1. The percentage of median IC_{50} for each patient for every drug is expressed on this heatmap where darkest red indicates most sensitive (most sensitive patient sample to any drug was 0.07% of median) and white indicates completely insensitive (IC_{50} for patient is at or above 100% of median). Patient samples are arranged by diagnosis (ALL, red; AML, green; CLL, yellow; MPN, blue). Kinase inhibitors are arranged on the basis of family of primary kinase targets for each drug. Numerical input for this heatmap is found in Supplementary Table S3.

by elimination of targets of drugs to which a sample is not sensitive.

The results from AML patient 08024 illustrate our target identification strategy. Malignant cells from this patient were hypersensitive to 3 drugs: AST-487, sunitinib, and SU-14813. As noted above, drug hypersensitivity is defined by comparison of the IC_{50} values for this individual sample with the median IC_{50} values that these drugs achieved across the entire cohort. Cells from patient 08024 exhibited IC_{50} values that were at least 5-fold lower than the cohort median IC_{50} values for each of these 3 drugs (Fig. 2A). Analysis of the known targets of these 3 compounds revealed 4 kinases, KIT, PDGFR, CSF1R, and FLT3, that are common targets between all 3 drugs (Fig. 2B). Because this sample was also unaffected by the 2 drugs, dasatinib and nilotinib (no difference between patient 08024 IC_{50} and median IC_{50}; Fig. 2A), the kinase targets of these 2 drugs can be eliminated from consideration as kinases that could mechanistically explain cell viability in this patient sample. The kinase targets that are effectively inhibited by dasatinib and/or nilotinib are shaded blue, whereas the kinases not targeted by either dasatinib or nilotinib are shaded red (Fig. 2B). This reveals that the only kinase target in common between AST-487, sunitinib, and SU-14813 that is also not a target of dasatinib or nilotinib is FLT3. Further analysis of AML patient 08024 revealed presence of the *FLT3-ITD* and no wild-type *FLT3* alleles (Fig. 2C).

Development of a customized algorithm to automate oncogenic pathway prediction

Application of the logic illustrated in Fig. 2B can be conducted manually for a small number of kinase inhibitors; however, expansion of this process to evaluate data from the

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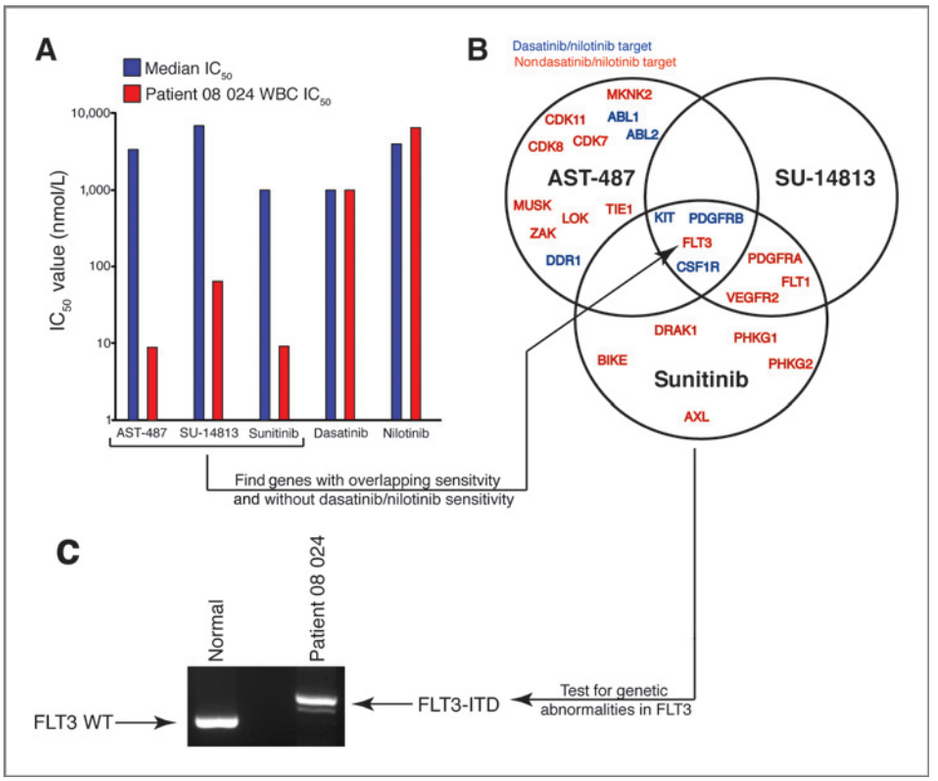

Figure 2. Logical prediction of kinase targets based on drug sensitivity/resistance. To predict operational kinase targets in Patient 08024, response to 5 kinase inhibitors was considered. A, patient 08024 exhibited at least 5-fold lower IC_{50} than median to AST-487, SU-14813, and sunitinib (effective drugs), whereas no difference is seen between median and patient 08024 IC_{50}s for dasatinib and nilotinib (ineffective drugs). To predict targets that underlie this sensitivity pattern, the overlap of kinase targets between the 3 effective drugs (AST-487, SU-14813, and sunitinib) is shown in B revealing that only KIT, PDGFR, CSF1R, and FLT3 are common kinase targets between these drugs. To distinguish between these 4 targets, their responsiveness to the ineffective drugs (dasatinib and nilotinib) was considered. The kinase targets in blue (*KIT*, *PDGFR*, *CSF1R*) are good targets of dasatinib and/or nilotinib, whereas the kinase targets in red (*FLT3*) were not effectively inhibited by dasatinib or nilotinib. Elimination of these dasatinib/nilotinib targets (in blue) leaves only FLT3 as a potential kinase target for explanation of the functional response to these 5 drugs. Follow-up PCR analysis revealed this AML patient sample to exhibit *FLT3-ITD* with loss of the wild-type *FLT3* allele (C).

entire panel of 66 drugs requires computational support. We have developed an algorithm to carry out the same logical steps outlined in Fig. 2, for all 66 kinase inhibitors in 4 steps (Sweave document with instructions for generating application to perform this algorithm can be found in Supplementary Material). First, the kinase target information for each drug was curated from published sources (35, 37–43) into a database (Fig. 3A and Supplementary Table 1). Second, the K_d or IC_{50} values (depending on the format of assay used to define drug targets) for each drug were subdivided into 5 tiers on a Log_{10} scale. The first tier is defined as the kinase with the lowest K_d or IC_{50} value as well as all other kinases with K_d or IC_{50} values less than or equal to 10-fold than lowest value. Kinases with K_d or IC_{50} values ranging from 10-fold to 100-fold, the lowest values are considered tier 2 targets. Each subsequent Log_{10} increase in K_d or IC_{50} comprises a new tier of kinase targets (Fig. 3A; bottom). Next, the IC_{50} results from an individual patient sample are subdivided into drugs to which the sample was hypersensitive (defined by a patient-specific IC_{50} that is 5-fold less than the cohort median IC_{50}) as well as the drugs to which the sample is not sensitive (Fig. 3B). We next devised a scoring system that assigns points to each kinase based on whether inhibitors defined to block that kinase target were effective (positive points awarded to the target's score) or ineffective (negative points deducted from the target's score). The addition or subtraction of points is conducted in a graded manner with tier 1 kinases having the most points added or subtracted from their score and the score for kinases from each subsequent tier being modified to lesser degrees (Fig. 3C). Finally, the cumulative scores for each kinase are tabulated and ranked such that the highest scoring kinases for any given patient are those kinases predicted to be most probable in explaining the observed drug response and, therefore, the most probable in playing a pathogenic role for that patient's malignancy (Fig. 3D).

Application of oncoprotein prediction algorithm to four proof-of-principle examples

To test this algorithm, we chose 3 specimens from patients with leukemia with known, dysregulated tyrosine kinase pathways and one specimen from a patient without a known kinase mutation. The first example, AML patient 08024, was described in Fig. 3 whereby the *FLT3-ITD* gene target was predicted on the basis of analysis of the response pattern of the cells to 5

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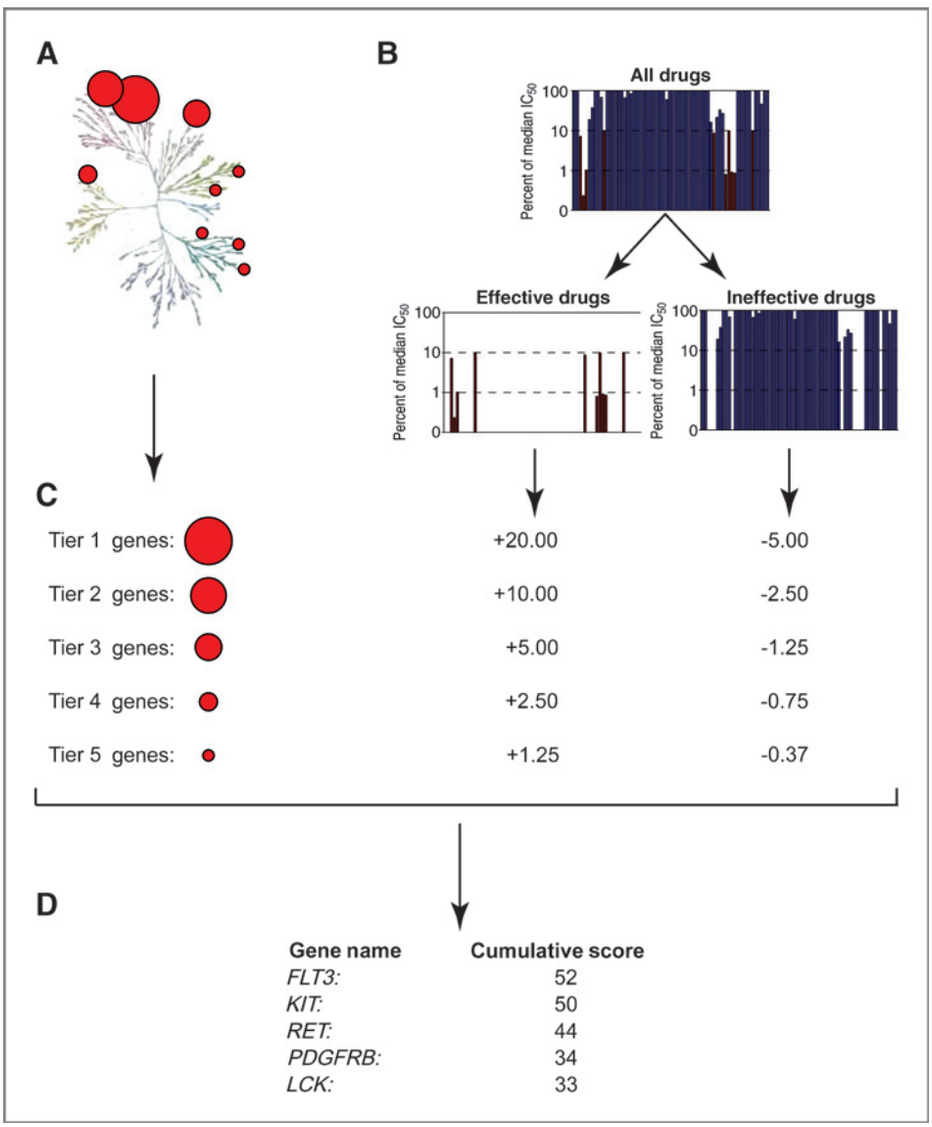

Figure 3. Development of an algorithm for kinase target prediction based on kinase inhibitor efficacy. Automation of kinase target prediction based on kinase inhibitor sensitivity patterns proceeds in several steps: A, the kinase targets of each kinase inhibitor are curated into a single database from published sources. The kinase target with lowest K_d/IC_{50} for a given inhibitor as well as all kinase targets with K_d/IC_{50} less than or equal to 10-fold than lowest value are considered tier 1 targets. Targets with K_d/IC_{50} within each subsequent 10-fold increase in K_d/IC_{50} are considered to be in tiers 2 to 5, respectively. Illustration reproduced courtesy of Cell Signal Technology, Inc. (www.cellsignal.com). B, drug IC_{50}s for an individual patient sample are compared with median drug IC_{50}s to determine effective and ineffective drugs, as described in Fig. 1. C, kinase targets of effective drugs are given positive points and kinase targets of ineffective drugs receive negative points, both in a tiered fashion based on the target tiers defined in A. D, total scores are tabulated for every kinase target and ranked such that targets with the highest points are predicted to be most probable for operational involvement in preservation of cell viability and, therefore, most likely to mechanistically underlie the observed drug sensitivity pattern.

small-molecule kinase inhibitors. To determine whether our algorithm would also successfully identify FLT3 as a high probability target when applying data from all 66 drugs, we conducted the algorithm on kinase inhibitor panel screening outcomes from this patient sample. This exercise revealed that FLT3 was the second highest scoring kinase on the target list with a score of 89 points (Fig. 4A). The highest scoring kinase (EGFR) scored 90 points due to near complete hypersensitivity of this specimen to ERBB family inhibitors. Analysis of target profiles of these ERBB family inhibitors reveals that they do not exhibit off-target effects against FLT3 (37), indicating that there may be cross-talk between the *FLT3-ITD* oncogene and ERBB family members. We also applied this screen to cells from a patient with CML in blast crisis. In this case, the algorithm correctly identified ABL1 as the top-scoring kinase (Fig. 4B). Finally, we applied this technique to cells from a patient with MPN positive for the oncogene, *MPL-W515L*. In this case, 3 of the top 5 targets are JAK family kinases, which represent therapeutic targets downstream of MPL (MPL is not a kinase, but signals through JAK kinases; refs. 44, 45; Fig. 4C). Thus, application of this algorithm to 3 proof-of-principle examples shows that the approach correctly identifies known oncogenic signaling pathways in each case. In addition, a patient with AML (08102) with no known mutations in tyrosine kinases exhibited hypersensitivity to 12 of the kinase inhibitors on our panel (Fig. 4D). Among the kinases predicted by our algorithm to be likely involved in pathogenesis of this specimen was FLT3, which is commonly mutated in AML (Fig. 4E). However, as noted above, this patient did not exhibit any of the common FLT3 abnormalities. This prompted us to conduct more extensive sequencing of *FLT3*, and we identified a point mutation in the extracellular region of FLT3 (S451F; Fig. 4F), this rare mutation has been previously shown to exhibit transformative capacity (46).

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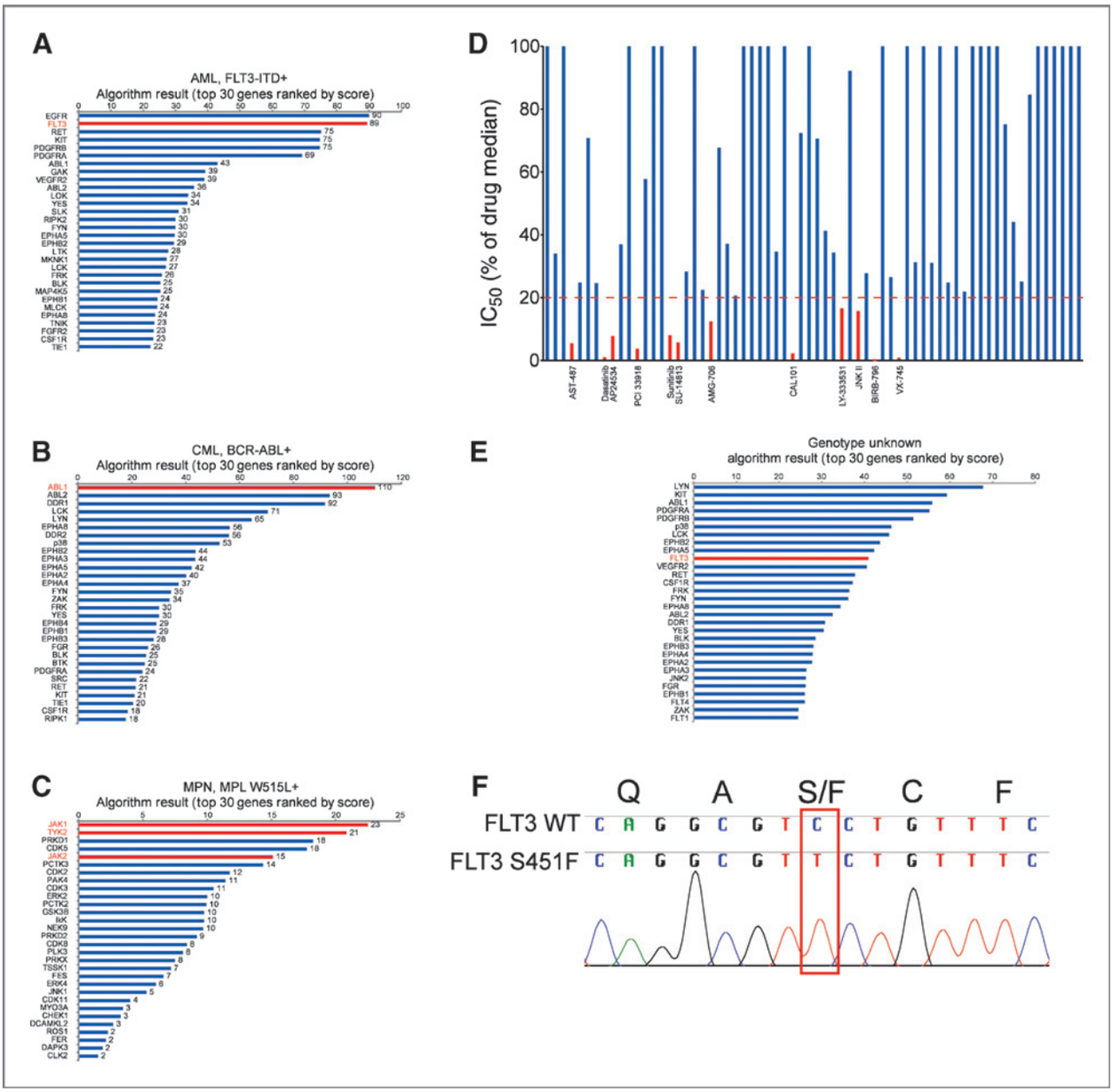

Figure 4. Algorithm kinase target scores for 4 leukemia patient samples with known oncogenic lesions. Proof-of-principle of performance of algorithm described in Fig. 3. The algorithm kinase target scores from 3 patient samples with known genetic etiology are considered. The first sample (A) is an AML patient with *FLT3-ITD*, and FLT3 ranks as the second highest kinase target. EGFR is the highest scoring kinase in this example due to complete hypersensitivity of the specimen to ERBB family inhibitors. This may reveal a mechanistic link between FLT3 and ERBB family signaling, as the ERBB family inhibitors do not exhibit affinity for FLT3. The second sample (B) is derived from a patient with CML in blast crisis with no kinase domain mutations in BCR-ABL. ABL1 is the top scoring kinase for this sample. The third example (C) is an MPN patient with an MPL-W515L mutation. Although MPL is not a kinase (and is not scored by the algorithm), it signals primarily through JAK kinases. Notably, JAK1, JAK2, and TYK2 are 3 of the top 5 scoring kinases for this example. A fourth patient with no known kinase mutations exhibited sensitivity to a variety of kinase inhibitors on our panel (D). The algorithm described in Fig. 3 predicted this pattern to be driven by dependence on a variety of type III receptor tyrosine kinases, including FLT3, which is commonly mutated in AML (E). Because this patient did not exhibit any of the FLT3 mutations commonly observed in AML, we sequenced full-length FLT3 and identified a rare point mutation in the FLT3 extracellular domain (S451F), previously shown to exhibit transformative capacity. The sequence trace of this mutant FLT3 allele is shown in F.

Algorithmic prediction of oncogenic signaling pathways in 151 patient samples

To better understand the kinase targets that might underlie drug sensitivity patterns in our entire cohort of leukemia patient samples, we applied our target prediction algorithm to all 151 specimens that were interrogated by the kinase inhibitor panel. The data reveal a heterogeneous list of probable kinase targets that arise in a highly patient-specific manner (Supplementary Table S5). One clear conclusion from these results is the need for a personalized approach to application of kinase-targeted

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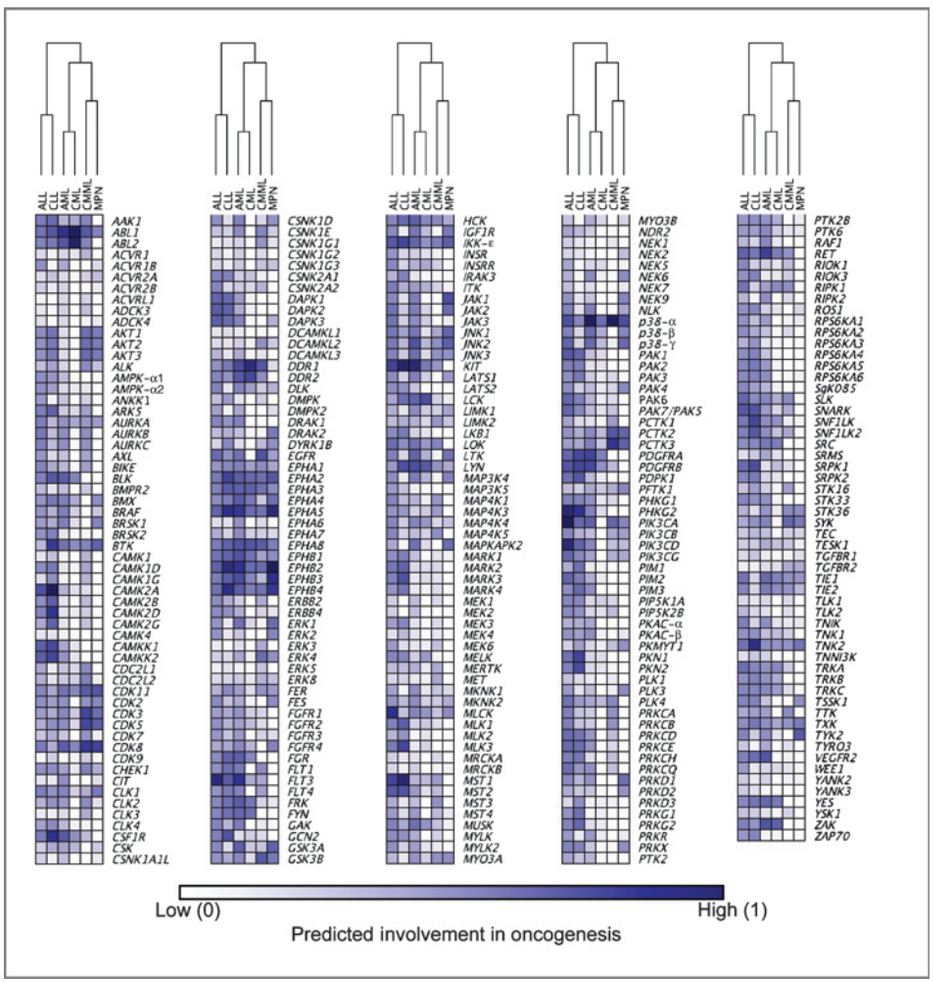

Figure 5. Cumulative algorithm kinase target scores by diagnosis. To gain insight into targets that are frequently and infrequently predicted in various diagnostic subsets of leukemia, we tabulated cumulative kinase target scores for ALL, AML, CLL, CML, CMML, and other MPN. Target scores for all 151 patients were tabulated by the algorithm described in Fig. 4 (individual patient target scores can be found in Supplementary Table S5). To compute cumulative scores, all negative values were eliminated and remaining positive values were normalized for each patient to that patient's respective highest scoring kinase, such that all scores were on the same scale from 0 to 1 for every patient. The mean of these normalized scores for each kinase was then calculated for every patient within the above diagnostic leukemia subsets. These average target scores for each leukemia subset were once again normalized to the highest scoring respective gene from within each leukemia subset such that each subset was represented on the same 0 to 1 scale. These values are illustrated on a heatmap where darkest blue indicates targets most frequently predicted to be operationally important within each respective leukemia subset and white indicating no evidence for functional importance. Notably, as a proof-of-principle, ABL1 is the highest cumulative scoring kinase in CML. The numerical input for this heatmap is found in Supplementary Table S6.

therapies. However, the data can also be used to identify kinases that may be more frequently implicated in any one diagnostic subset of malignancy. As such, we tabulated the average kinase scores for each patient within the broad diagnostic categories of ALL, CLL, AML, CML, CMML, and other MPN. Kinase scores were normalized for comparison between patients by dividing all scores of a given profile by the maximum score for that profile. Negative scores were removed resulting in a normalized profile score between 0 and 1. Next, we computed average normalized scores for each kinase across diagnostic groups. Finally, the kinase scores for each diagnostic group were again normalized to the highest scoring kinase within each group so that each diagnostic category would be represented on the same scale. These values were then expressed on a heatmap to visually represent the kinases that are predicted to be more frequently involved in the pathogenesis of each of these broad diagnostic subsets of hematologic malignancies (Fig. 5; input data found in Supplementary Table S6). As anticipated, ABL1 is the highest cumulative scoring kinase among CML patients tested, serving as a proof-of-principle for this strategy. Interestingly, CaM kinases seem to score highly in lymphoid but not myeloid malignancies. One possible link between that CaM kinases and lymphoid malignancies may be the activation of CBP. Recent studies have identified mutations in CBP (and loss of activity) as a strong correlation with relapse in ALL (47). It has been previously established that phosphorylation of CBP by CaM kinase IV is important for its activity (48). Therefore, inhibition of CaM kinases may provide further insight to the functional

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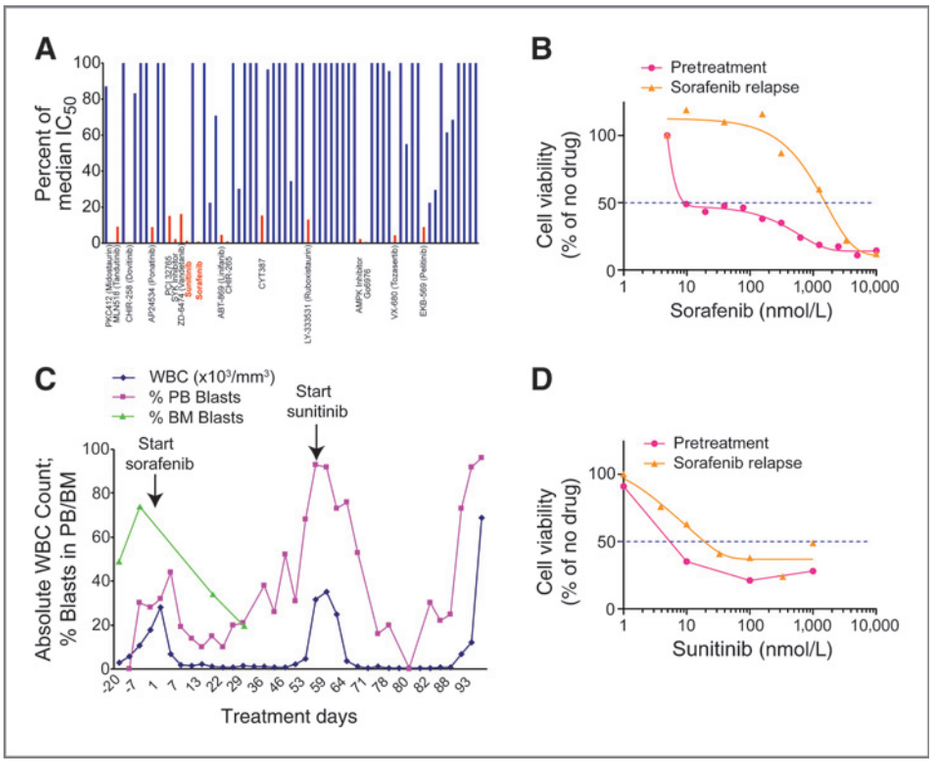

Figure 6. Correlation of *in vitro* sensitivity with clinical patient response. A, a specimen from a multiple-relapsed AML patient was evaluated on the kinase inhibitor panel and exhibited hypersensitivity to a variety of kinase inhibitors, including 2 that are FDA-approved (sorafenib and sunitinib). B, this patient was treated on sorafenib and subsequently treated on sunitinib and responded well to both treatments with rapid reductions in WBC counts, peripheral blast percentage, and bone marrow blast percentage. C, *in vitro* response of this patient's cells to sorafenib mimicked the clinical response whereby pretreatment cells were highly sensitive and cells taken after sorafenib relapse exhibited an IC_{50} greater than logs higher than the pretreatment cells. D *in vitro* response of this patient's cells to sunitinib was consistent with the clinical response whereby cells were highly sensitive to sunitinib before sorafenib treatment and at sorafenib relapse, at which time the patient responded beneficially to treatment with sunitinib.

importance of CBP in lymphoid viability. PI3K and AKT isoforms score highly in lymphoid malignancies as well as MPN, but are infrequently represented in patients with AML. BTK scores highly in patients with CLL, consistent with the notion that B-cell receptor signaling, which depends partly on BTK, is critical for viability of cells from many patients with CLL (28, 30). Cyclin-dependent kinases are predicted to be involved more frequently in MPN than in AML or lymphoid malignancies. Ephrin receptors score strongly across all diagnostic groups. In addition, p38 appears frequently involved in all malignancy subsets, and especially so in AML and CMML. There are a wide diversity of genes and signaling pathways that are predicted to play a role in the pathogenesis of each type of hematologic malignancy. However, our drug target scoring algorithm indicates particular pathways are more frequently represented in some diagnostic subsets than in others. Follow-up investigation will be required to validate the complete genetic etiology of these observations.

Clinical relevance of *in vitro* drug sensitivity/resistance

The clinical use of this type of test is predicated on a meaningful correlation between *in vitro* and an *in vivo* response to kinase inhibitors. As a proof-of-concept, we tested this correlation in a patient with refractory AML. A 36 year-old patient with a white blood cell (WBC) count of 133,000 was diagnosed with AML with inversion of chromosome 2 and trisomy 8. The FLT3-ITD was noted to be weakly positive with an allelic ratio of 0.02. Following leukapheresis and a standard 7+3 induction therapy, he was found to have refractory AML and was reinduced with HAM chemotherapy. Although he achieved a remission and soon after underwent an unrelated donor transplant, he relapsed 60 days later. The donor was not available for donor leukocyte infusions and the patient was refractory to FLAG-IDA salvage therapy. Our inhibitor panel showed dramatic sensitivity to numerous kinase inhibitors, including several drugs that are already FDA-approved and have been used for treatment of AML (Fig. 6A). Because this patient had no other standard therapeutic options, he elected treatment with one of the inhibitors predicted to be effective by the inhibitor panel assay. Daily treatment with this drug, sorafenib, induced a rapid normalization of WBC counts with decreased blasts in both the peripheral blood and bone marrow that was maintained for more than 2 months (Fig. 6B). At the time of relapse, a repeat kinase inhibitor sensitivity panel showed that the *in vitro* response to sorafenib was approximately 3 logs less than the pretreatment cells (Fig. 6C). Interestingly, the analysis also showed that this relapsed AML remained highly sensitive to another FDA-approved kinase inhibitor, sunitinib. (Fig. 6D).

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Treatment with daily sunitinib resulted in a significant initial response with rapid reduction in WBC counts and peripheral leukemic blasts for a period of 4 weeks.

Discussion

Here, we show that functional screening of primary cells from leukemia patients with a panel of kinase inhibitors can identify effective kinase inhibitors in 70% of patients in just 3 days, including 40% of patient samples that were hypersensitive to drugs already approved for clinical use. We also show an algorithm that uses the partially overlapping kinase target spectra for each drug for prediction of critical kinase targets that underlie inhibitor sensitivity patterns. Finally, using this algorithm, we identify and rank probable kinase targets in 151 patients with a variety of hematologic malignancies.

The predicted kinase targets for these patient samples show a great deal of heterogeneity, even within diagnostic subsets. This finding highlights one of the strengths of this kinase inhibitor screen—kinase targets and patient-specific therapeutic options are detected regardless of the frequency with which these targeted therapies would be applicable within the given disease subset. In addition, although our target prediction algorithm can suggest candidate pathways for follow-up validation, this technique is empirical in that it does not require specific knowledge of any genetic lesion or biomarker to uncover potential therapeutic options for patients.

It is likely that the algorithm shown here exhibits both false-positive and false-negative kinase targets. Because the algorithm is driven by our knowledge of potential target spectra of kinase inhibitors that are present on the panel, there is certainly an opportunity for target bias due to pathways that are over- or under-represented on the panel. As drug development continues and the panel expands to include other drugs that offer more complete and even coverage of the kinome, this pitfall will be diminished. In addition, further profiling of these inhibitors to fully delineate target spectra will also improve the algorithm output as this output is largely driven, and limited, by our knowledge of these target spectra.

It is also possible that the setup of the assay could lead to false-negative results, as the technique relies on a short 3-day window for assessment of drug sensitivity. For this reason, interpretation of negative results from the drug assay (i.e., lack of sensitivity to a particular drug) must be taken with caution. Correlation of results from this screen with clinical cases in which patients are treated with drugs from the panel will help inform the full clinical relevance of both positive and negative assay results.

It will be critical to integrate this kinase inhibitor screen with other cutting edge techniques. We have already begun to apply this assay in conjunction with a related siRNA screen (49, 50), and initial results obtained within 4 days of receiving fresh primary samples indicate a high concordance of siRNA and kinase inhibitor sensitivities. In addition, application of genomics techniques such as gene expression microarray and deep sequencing in parallel with these functional screening tools will undoubtedly accelerate our understanding of the precise molecular events that underlie the observed kinase sensitivity patterns. Matching of these patient genotypes with drug sensitivity patterns will ultimately enable patients to be treated on the basis of tumor genotypes, and this drug assay and kinase target algorithm offer useful modalities for translation of tumor genotypes into therapeutically relevant clinical strategies. Hence, the kinase inhibitor screen illustrated here represents advancement toward individual patient-tailored cancer therapy. Malignant cells from patients with cancer can be economically screened to identify effective targeted therapies within 3 days, thus matching the appropriate drugs with the appropriate patients on a timescale that affords opportunity for informed, mechanism-based intervention.

Disclosure of Potential Conflicts of Interest

S.E. Spurgeon has a honoraria from Speakers Bureau from GSK and Millenium. B.J. Druker has ownership interest (including patents) and is a consultant/advisory board member in Blueprint Medicines. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.W. Tyner, W.F. Yang, L.B. Fletcher, J. Bryant, J.M. Glover, B.H. Chang, S.E. Spurgeon, W.H. Fleming, T. Kovacsovics, J.R. Gotlib, S.T. Oh, M.W. Deininger, C.M. Zwaan, M.L. den Boer, M.M. van den Heuvel-Eibrink, M.M. Loriaux

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Kinase Pathway Dependence in Primary Human Leukemias Determined by Rapid Inhibitor Screening

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