Bioinformatics-driven discovery of rational combination for overcoming EGFR-mutant lung cancer resistance to EGFR therapy

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ABSTRACT

Motivation: Non–small-cell lung cancer (NSCLC) is the leading cause of cancer death in the United States. Targeted tyrosine kinase inhibitors (TKIs) directed against the epidermal growth factor receptor (EGFR) have been widely and successfully used in treating NSCLC patients with activating EGFR mutations. Unfortunately, the duration of response is short-lived, and all patients eventually relapse by acquiring resistance mechanisms.

Result: We performed an integrative systems biology approach to determine essential kinases that drive EGFR-TKI resistance in cancer cell lines. We used a series of bioinformatics methods to analyze and integrate the functional genetics screen and RNA-seq data to identify a set of kinases that are critical in survival and proliferation in these TKI-resistant lines. By connecting the essential kinases to compounds using a novel kinase connectivity map (K-Map), we identified and validated bosutinib as an effective compound that could inhibit proliferation and induce apoptosis in TKI-resistant lines. A rational combination of bosutinib and gefitinib showed additive and synergistic effects in cancer cell lines resistant to EGFR TKI alone.

Conclusions: We have demonstrated a bioinformatics-driven discovery roadmap for drug repurposing and development in overcoming resistance in EGFR-mutant NSCLC, which could be generalized to other cancer types in the era of personalized medicine.

Availability and implementation: K-Map can be accessible at: http://tanlab.ucdenver.edu/KMap.

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1 INTRODUCTION

Genomic medicine has dramatically increased our knowledge of the molecular changes that underpin disease states.

Understanding alterations in gene expression can identify proteins and signaling pathways, which might serve as therapeutic targets. Moreover, recent technologic advances in next-generation sequencing facilitate the rapid assessment of gene expression changes in specific patients, allowing for individualized treatment. Although personalized genomics heralds the age of precision medicine, the comprehensive datasets obtained through sequencing require sophisticated bioinformatics tools to identify the critical genes that influence disease. Once these critical genes are identified, the next challenge is to predict what drugs would be useful in reversing the disease states.

The connectivity map represents the first attempt to provide a computational framework to connect genes, drugs and diseases based on gene expression signatures (Lamb et al., 2006). This method assumes gene expression changes could be used as a “universal language” to connect distinct biological states (e.g. diseases), allowing for the successful repurposing of compounds (Hieronymus et al., 2006; Wei et al., 2006). In short, drugs known to be effective in one disease can serve as candidates for use in other diseases marked by similar gene expression changes. The power of this method has inspired other related work (Chung et al., 2014; Li et al., 2009; Zhang and Gant, 2008, 2009) with the goal of improving the utility of the connectivity map in drug repurposing and development.

Non–small-cell lung cancer (NSCLC) serves as an ideal disease for a connectivity map-based approach. NSCLC accounts for ~85% of lung cancers, and is the leading cause of cancer-related death in the United States (Siegel et al., 2013) and worldwide. Comprehensive characterization of cancer genomes have increased our understanding of cancer biology and moved NSCLC beyond standard clinico-pathologic classifications and staging to include molecular characterization based on newly identified oncogenic drivers, such as the mutant epidermal growth factor receptor gene (EGFR) (Pao and Chmielecki, 2010). Although therapies directed against EGFR, such as gefitinib (Hirsch et al., 2003), have revolutionized NSCLC care, the duration of response is short-lived, and all patients eventually relapse through acquired resistance mechanisms.

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(Casás-Selve et al., 2012; Engelman and Jänne, 2008; Ohashi et al., 2013; Ware et al., 2013). Identification of new targeted therapeutics is a priority, yet the ability to translate genomic datasets into new drugs has been limited.

We devised an integrative systems biology approach using a functional screen combined with RNA-seq expression data to determine essential kinases other than EGFR that drive proliferation and survival in EGFR-mutant NSCLC. We performed an initial focused, functional genetic screen using short hairpin RNAs (shRNAs) that target the kinome (~600 kinases) in the H1650 lung cancer cell line. This line harbors an EGFR mutation, yet is intrinsically resistant to EGFR TKIs. We used Bioinformatics for Next-Generation Sequencing! (BiNGS!) (Kim and Tan, 2012), a novel bioinformatics pipeline to analyze and interpret functional genomic screening data by next-generation sequencing to identify essential kinases that drive the survival and proliferation signaling pathways of this EGFR-mutant line. Next, we determined the differentially expressed kinases in this cancer cell line by next-generation sequencing (RNA-seq). Integrative analysis was performed to identify kinases that were essential and dysregulated, and the list of essential and functional kinases was connected to explore therapeutic opportunities using the kinase connectivity map (K-Map) (Kim et al., 2013). The efficacy of the K-map prediction of kinase inhibitors was then validated in vitro (Fig. 1).

## 2 MATERIALS AND METHODS

### Cell culture.

H1650 and H1975 cells were obtained from the University of Colorado Cancer Center (UCCC) Tissue Culture Core. All cell lines were routinely cultured in RPMI-1640 growth medium supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA) at 37°C in a humidified 5% CO₂ incubator. Alveolar type II (ATII) cells are primary, normal cells that were isolated and cultured in an air/liquid interface, as previously described (Wang et al., 2007).

### Essential Gene Screen

1. Transduced Cells
2. Day 7
3. Collect shRNAs from cell lines and construct sequencing library
4. Day 9
5. RNAseq Analysis
6. 193 over- and 131 under-expressed kinases in H1650 (FDR ≤ 0.01, FC > 1.25)
7. K-Map Connectivity Results
8. List of Kinases
9. 14 Fast Drop Out Kinases
10. Integrate
11. Experimental Validations

**Fig. 1.** Workflow of the experimental and bioinformatics analyses of this study. Blue boxes represent bioinformatics analyses. The K-Map connectivity results for validation (right).

### Kinome essential screen by next-generation sequencing.

An essential screen identifies genes critical for cell survival by knocking out individual genes in cells (one gene deleted per individual cell). The essential screen is a high-throughput assessment of gene function as opposed to expression. A total of 3 × 10⁶ cells of H1650 were transduced with the short-hairpin loop lentiviral kinome library (~3700 shRNAs targeting ~600 kinases) developed by the RNAi Consortium (TRC 1.0/1.5) and obtained from the UCCC Functional Genomics Shared Resource Core. Cells were transduced with the lentiviral shRNA library using conditions to result in a single shRNA expressed in each infected cell. Cells were cultured and harvested after 2, 7, 14 and 28 days of transduction. shRNAs from surviving cells were extracted, reverse transcribed and barcoded for individual replicates (four replicates per time point). The DNA was purified and amplified, and Illumina adapters were added. The relative abundance of the unique shRNA tags was quantified by the Illumina Genome Analyzer, as previously described (Casás-Selvès et al., 2012; Singleton et al., 2013; Spreafico et al., 2013; Sullivan et al., 2012). Loss of shRNAs represents essential kinases. See Supplementary Methods for details.

**BiNGS! analysis.** We used BiNGS! for analyzing and interpreting the essential screen (Kim and Tan, 2012). In brief, a preprocessing step filtered out erroneous and low-quality reads. Filtered reads were mapped against the shRNA reference library using Bowtie (Langmead et al., 2009). Output from this step is a P x N matrix, where P and N represent shRNA counts and samples, respectively. We also filtered out shRNAs where the median raw count in the time-point group 1 is greater than the maximum raw count in the time-point group 2 if the shRNA is enriched in the time-point group 1, and vice versa. We then used a negative binomial to model the count distribution in the sequencing data using edgeR (Robinson et al., 2010). We computed the q-value of false discovery rate (FDR) for multiple comparisons for these shRNAs, and carried out a meta-analysis by combining adjusted P-values for all shRNAs representing the same gene using weighted Z-transformation (Whitlock, 2005). For each gene, we computed a P-value P(wZ), and we used this P(wZ) to sort the kinase list, as previously described (Casás-Selvès et al., 2012; Singleton et al., 2013). We performed pair-wise comparisons for each of the time points, and we grouped the kinases into three classes using the following rules based on the P(wZ) obtained from each gene (similar
to the classification rules in Marcotte et al., 2012). In this study, we considered kinases that, when deleted by shRNAs, induced cell death in the first time point (day 7) as candidate kinases. These kinases were ‘essential’ for cell survival and they were never recovered in the later time points (day 14 and day 28). See Supplementary Methods for details.

RNA-seq of kinome and bioinformatics analysis. Transcriptome libraries were prepared following Applied BioSystems SOLiD Total RNA-Seq protocol. The libraries were sequenced using the Applied BioSystems SOLiD 5500 platform, using 75 base pair by 35 base paired-end reads. Mapping of sequencing reads and quantification of known RefSeq transcripts were performed using LifeScope v2.1 (ABI). Expression values for each transcript were calculated as reads per kilobase of exon model per million mapped reads (RPKM). RPKM is a method of quantifying expression from RNA-seq data by normalizing for total read length and the number of sequencing reads obtained within each sample (Mortazavi et al., 2008). See Supplementary Methods and Supplementary Table S3 for details. To determine the differentially expressed kinases, we computed a FDR on the P-values obtained by t-test. We used FDR ≤5% and fold change >1.25 as thresholds for determining differentially expressed kinases between cancer and normal samples.

K-Map analysis. We have recently developed and implemented a K-Map that systematically connects a kinase profile with a reference kinase inhibitor database and predicts the most effective inhibitor for a queried kinase profile (Kim et al., 2013)(Fig. 2). The K-Map is inspired by the connectivity map concept (Lamb et al., 2006), where the main assumption of this concept is that gene expression profiles could be used as ‘universal language’ to connect between biological states, genes and drugs. The connectivity map has three key components: (i) a reference database that contains a set of predefined gene expression profiles; (ii) a query gene signature; and (iii) a pattern matching algorithm or similarity metric defined between a query gene signature and a reference gene expression profile to quantify the connection (or similarity) between the two biological states. Instead of gene expression signatures, we used the kinase activity profiles as the ‘language’ for connecting kinases with small molecule kinase inhibitors in K-Map to reveal the interactions of kinases and inhibitors (Kim et al., 2013). Figure 2 illustrates the concept of K-Map. Reference Database. We built the reference database based on two recently published comprehensive analyses of kinase inhib
screen, we identified 14 candidate kinases (CALM3, CDK1, CDK6, DDR1, EGFR, EPHA4, GNE, IPMK, MARK3, PBK, PKN1, ROCK1, RPRD1A and TBK1) as essential for survival of this cell line (see Supplementary Fig. S1 and Supplementary Methods for details). Importantly, EGFR was identified from this functional genetic screen as essential in H1650. To determine whether these kinases were mutated in this cell line, we queried the comprehensive curated Catalogue Of Somatic Mutations In Cancer (COSMIC) database (Forbes et al., 2011). According to the COSMIC database, besides EGFR, no other known somatic mutations have been reported in the 13 other essential kinases in H1650.

Differentially expressed kinases identified by the RNA-seq analysis. To determine the differentially expressed kinase genes in H1650, we compared the expressed kinome of H1650 defined by targeted RNA-seq with four normal, human ATII cells, the putative cell of origin for lung adenocarcinomas, a histologic subtype of NSCLC (Xu et al., 2012). From the RNA-seq data, we focused on the 611 kinases that were the same kinases targeted in the functional genetic screens. We identified 193 kinases overexpressed and 131 underexpressed comparing the H1650 data with the normal ATII RNA-seq data (FDR <0.05, fold change >1.25). Again, EGFR was the top overexpressed gene in the list, supporting H1650’s dependence on this oncogene. The heat map of this analysis is illustrated in Supplementary Figure S2 and Supplementary Table S1.

Integrating a functional kinome genetic screen and RNA-seq analysis. To refine the panel of identified key driving survival and proliferation of H1650 cells, we sought to integrate the candidate kinase genes identified from the essential kinome screen with differential kinase transcriptional profiling, two different yet complementary approaches. Transcriptional profiling analysis identifies kinases that are differentially expressed compared with normal lung ATII cells, yet provides no insight on kinase function. Conversely, the functional genetic screen allows discovery of kinases required for cell survival, yet provides no information specific to transformation. Therefore, integrating these two analyses will provide a list of functionally essential, potentially transformative kinases in H1650. By comparing the candidate gene lists obtained from the two approaches (Fig. 1), seven kinases (CDK6, EGFR, MARK3, PBK, TBK1, DDR1 and EPHA4) were found to be common to both lists.

Connecting the essential, potentially transformative kinases to therapeutics using the K-Map. We next asked what compounds could inhibit these essential and possibly transformative kinases and serve as a potential therapy to inhibit proliferation of this cell line. We queried the K-Map using the seven essential and potentially transformative kinases to connect them to drugs based on two different kinase activity assays (IC50 and Kd). The top connection in both assays was staurosporine, a natural product isolated from the bacterium Streptomyces staurosporues. Staurosporine is a potent, general ATP-binding site inhibitor across kinases, lacking any specificity (Anastassiadis et al., 2011; Davis et al., 2011). Interestingly, bosutinib, a Src and Abl dual inhibitor, was also positively connected and ranked #3 and #29 in the Kd and IC50 assays, respectively (Fig. 1 and Supplementary Table S2). As expected, the two FDA-approved EGFR-specific TKIs for treating NSCLC patients, gefitinib and erlotinib, were not connected by the K-Map as effective therapy for the queried essential kinases (Fig. 1).

Experimental validation of the compounds identified by K-map analysis. To test whether compounds identified by the K-Map could provide better growth inhibition of H1650 cells compared with the current standard of care with EGFR-specific TKIs, we selected bosutinib (Fig. 1) as the candidate compound prediction of the K-map and validated its effect on cell survival. In comparison, we selected gefitinib, a drug approved for use in EGFR-mutant NSCLC, which was not identified using our strategy and therefore, we predicted, would be less efficacious in H1650 cells (Fig. 1). To further test our algorithm and K-Map, we identified sorafenib (connectivity scores of 0.234 and 0.226 in both kinase assays, Fig. 1) as a compound which was predicted to be ineffective against the H1650 cell line.

To evaluate the sensitivity of bosutinib, gefitinib and sorafenib, H1650 NSCLC cells were exposed to increasing concentrations of these individual drugs and assessed for proliferation. As depicted in Figure 3A, bosutinib had the lowest IC50 among the three tested compounds. Conversely, gefitinib and sorafenib had higher IC50 values (IC50 >10 μM), indicating both drugs were less effective in inhibiting the growth of H1650 cells. As an additional negative control, we validated CI-1040, a compound that has a connectivity score of 0 (minimal effect and ranked #238 of 250 drugs) and was the least effective of the four drugs tested (IC50 ~34 μM) in H1650 (Supplementary Fig. S3).

To estimate the statistical significance of the connection, we used the connection testing proposed by Zhang and Gant (2008, 2009), where the two-tailed P-value associated with the observed connection score is the number of times the connection score obtained by a random gene signature with the same number of
genes when queried to the database. Given a reference database and a query signature $S$ with $m$ kinases (here, $m = 7$), the K-Map connectivity score is $KS_m$ and the two-tailed $P$-value is estimated as: $p = \text{Prob}\{|KS_m\geq |KS_0|\}$, where $KS_0$ is the connection score obtained from the random query signature with $m$ kinases. For this particular study, we performed 500 permutations, and computed the $P$-value of the bosutinib connection as $p = 0.052$ (see Supplementary Methods for details). As demonstrated by the experimental validation, this indicates that the connection is not a false positive identified by the method.

**Generalizing the K-map prediction to acquired EGFR-TKI resistance.** Acquired resistance to EGFR TKIs after therapy is universal in lung cancer patients limiting their usefulness as a treatment of NSCLC. Drugs that can overcome this resistance would dramatically impact lung cancer care. To explore whether bosutinib can be extrapolated as an effective therapy for an acquired mutation conferring resistance to EGFR TKIs, we tested H1975, a NSCLC line that harbors EGFR T790M, a gatekeeper mutation that is commonly acquired during treatment with EGFR TKIs and renders standard EGFR TKIs ineffective. As illustrated in Figure 3B, the IC$_{50}$ value of bosutinib on H1975 was $\sim$3.7 $\mu$M. As a negative control, we also determined the IC$_{50}$ value of gefitinib, sorafenib and CI-1040 for this cell line as $\sim$12, $\sim$17 and $\sim$31 $\mu$M, respectively (Fig. 3B and Supplementary Fig. S3). Thus, bosutinib is effective in inhibiting the proliferation of a NSCLC line with an acquired EGFR resistance mutation.

**Rational combination of bosutinib and gefitinib shows synergistic effects in EGFR-mutant NSCLC cells.** We further hypothesized that the addition of bosutinib to gefitinib would be synergistic in EGFR-mutant NSCLC cells resistant to single agent EGFR-TKI treatment. Indeed, the combination of bosutinib and gefitinib demonstrated additive or synergistic effects in both EGFR-mutant NSCLC lines (Fig. 3C and D). Combination therapy was significantly improved in H1650 and H1975 ($P<0.05$, Welch two sample $t$-test) in inhibiting cell proliferation when compared with individual drug alone (Fig. 3C and D). Based on the additivism models, in H1650 combination therapy was synergistic (HSA = 0.34 and Bliss = 0.10), whereas in H1975 was additive (HSA = 0.19 and Bliss = -0.04) (see Supplementary Methods for details). Immunoblotting of H1650 and H1975 revealed an increase of cleaved caspase-3 (Casp3), an apoptotic marker, in the bosutinib and combination treated cells as compared with gefitinib alone and vehicle as early as 48 h after treatment (Fig. 3E and F).

**4 DISCUSSION**

Functional genetic screens have the potential to identify genes essential for cancer cell survival and proliferation, providing a ‘functional’ map of human cancer. Complementing the functional genetic screen with comprehensive genomics studies such as transcriptional profiling could reveal the ‘vulnerable targets’ for targeted treatment of cancer cells. Here, we performed a systematic and unbiased approach to determine essential kinases driving survival mechanisms of EGFR-mutant NSCLC lines resistant to EGFR TKIs. Using a series of novel bioinformatics analyses, specifically connecting the essential kinases with small molecules based on inhibition activities, we have identified that bosutinib effectively inhibits the essential kinases in H1650 resulting in cell death. We validated bosutinib in H1650 (intrinsic EGFR-TKI resistance) and H1975 (acquired gatekeeper T790M mutation) and demonstrated that this compound inhibited cell proliferation and induced apoptosis in these cancer cell lines that are resistant to EGFR-specific TKIs better than standard care.

Our strategy combines high-throughput genetic screens with a new computational technique (BiNGS!) to rapidly identify kinases that are essential for cancer cell survival. As described earlier, the H1650 cell line is driven by an activating EGFR mutation; therefore, it was expected that EGFR would be one of the seven essential kinases found in the overlapping gene lists. Other differentially expressed and essential kinases identified in this study have been previously reported to play an important role in cancer cells. For example, MARK3 has been shown to play a role in regulating cell cycle progression in cancer cells (Sha et al., 2007). 

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