A genomic approach to identify molecular pathways associated with chemotherapy resistance

Richard F. Riedel,1,2 Alessandro Porrello,1 Emily Pontzer,1 Emily J. Chenette,1 David S. Hsu,1,2 Bala Balakumaran,1 Anil Potti,1,2 Joseph Nevins,1,3 and Phillip G. Febbo1,2,3

1Duke Institute for Genome Sciences and Policy, Duke University and 2Division of Medical Oncology, Department of Medicine, and 3Molecular Genetics and Microbiology, Duke University Medical Center, Durham, North Carolina

Abstract

Resistance to chemotherapy in cancer is common. As gene expression profiling has been shown to anticipate chemotherapeutic resistance, we sought to identify cellular pathways associated with resistance to facilitate effective combination therapy. Gene set enrichment analysis was used to associate pathways with resistance in two data sets: the NCI-60 cancer cell lines deemed sensitive and resistant to specific chemotherapeutic agents (Adriamycin, cyclophosphamide, docetaxel, etoposide, 5-fluorouracil, paclitaxel, and topotecan) and a series of 40 lung cancer cell lines for which sensitivity to cisplatin and docetaxel was determined. Candidate pathways were further screened in silico using the Connectivity Map. The lead candidate pathway was functionally validated in vitro. Gene set enrichment analysis associated the matrix metalloproteinase, p53, methionine metabolism, and free pathways with cytotoxic resistance in the NCI-60 cell lines across multiple agents, but no gene set was common to all drugs. Analysis of the lung cancer cell lines identified the bcl-2 pathway to be associated with cisplatin resistance and the AKT pathway enriched in cisplatin- and docetaxel-resistant cell lines. Results from Connectivity Map supported an association between phosphatidylinositol 3-kinase/AKT and docetaxel resistance but did not support the association with cisplatin. Targeted inhibition of the phosphatidylinositol 3-kinase/AKT pathway with LY294002, in combination with docetaxel, resulted in a synergistic effect in previously docetaxel-resistant cell lines but not with cisplatin. These results support the use of a genomic approach to identify drug-specific targets associated with the development of chemotherapeutic resistance and underscore the importance of disease context in identifying these pathways. [Mol Cancer Ther 2008;7(10):3141–9]

Introduction

The development of chemotherapy resistance poses a significant problem to patients and providers who rely on conventional cytotoxic agents for the treatment of malignant disease. Although the mechanisms underlying the development of resistance are partially understood, the most important mechanisms and associated biological pathways remain unknown. Potential mechanisms include, but are not limited to, the use of multidrug resistance transporters resulting in decreased tumor drug concentrations, reduced drug activation through increased detoxification of drug, alterations in the drug target, and alterations in apoptosis regulator genes (1–3). The identification and subsequent targeting of key molecular pathways associated with resistance may allow for increased response rates and improved clinical outcomes for patients.

Gene expression profiling has proven to be a powerful tool allowing for the characterization of tumors at a molecular level. Microarray analyses allow for quantification of gene expression for thousands of genes within an individual specimen. Individual genomic tumor profiles have been used to identify histologic classes of tumor and develop prediction tools for the development of metastatic disease, disease relapse, prognosis, and response to therapy in a variety of malignancies (4). Recently, we have shown that global gene expression can be used to identify patterns predictive of chemotherapy response and/or resistance (5).

Building on these results, we used integrated genomic methods to identify key biological pathways associated with resistance for a series of commonly used cytotoxic chemotherapeutic agents. We additionally explored disease-specific pathways associated with resistance and sought to determine whether disease context was important. Our work suggests that gene expression patterns associated with sensitivity and/or resistance to chemotherapy may be used to identify signaling pathways that can be interrogated to infer underlying biological mechanisms.
Materials and Methods

NCI-60 Cancer Cell Line Drug Sensitivity

Using publicly available chemotherapy sensitivity data for the NCI-60 series of cancer cell lines, cell lines were selected representing the extremes of sensitivity to specific chemotherapeutic agents as described previously (5). Cell lines with mean GI50 and confidence intervals greater than the overall mean GI50 - 1 SD from all samples were deemed sensitive (S), whereas those with mean GI50 and confidence intervals greater than the overall mean GI50 + 1 SD from all samples were deemed resistant (R). Publicly available total growth-inhibitory and LC50 doses of the sensitive and resistant subsets were correlated with the respective GI50 data to ascertain consistency between the total growth inhibition, LC50, and GI50 data. Cell lines with low GI50/LC50 total growth inhibition needed to have a low LC50 to be considered sensitive. Likewise, those with the highest total growth inhibition and LC50 for a given drug were considered resistant. The chemotherapy agents and number of cell lines designated for a particular phenotype are as follows: Adriamycin (10R:12S), cyclophosphamide (8R:8S), docetaxel (7R:7S), etoposide (9R:8S), 5-fluorouracil (8R:7S), paclitaxel (9R:8S), and topotecan (13R:10S). MAS 5.0 normalized U133A gene expression data for each cell line were used for all subsequent computational analyses.

Gene Set Enrichment Analysis

Gene set enrichment analysis (GSEA) version 2.05 was done for each chemotherapy agent based on the predefined phenotype according to previously published methods (6). Gene sets (Supplementary Table S1) were preprocessed to exclude gene sets with <10 and >500 genes. As recommended, 1,000 iterations were done per analysis using the default weighted enrichment statistic and a signal-to-noise metric to rank genes based on their differential expression across sensitive and resistant cell lines. Gene sets with a nominal P ≤ 0.05 were taken further in the discovery mode (7, 8).

Lung Cancer Cell Line Sensitivity

To assess disease context as a function of chemotherapy resistance, a spectrum of chemotherapy sensitivity was defined for a series of publicly available lung cancer cell lines to two commercially available agents: cisplatin (Platinol) and docetaxel (Taxotere). A National Cancer Institute cytotoxicity assay using the 1 + 2 screening method was done (9). Cells were grown according to media recommendations by the commercial vendor (Amer-}

Connectivity Map

To complement findings from GSEA, the top genes (50, 100, and 200) up-regulated and down-regulated for docetaxel resistance, based on a rank-ordered gene list provided by GSEA, were analyzed using the Connectivity Map (cmap) in an attempt to link genes associated with a phenotype with potential therapeutic agents (11). Using cmap, an imported query was compared with predefined signatures of therapeutic compounds and ranked according to a connectivity score (+1 to -1), representing relative similarity to the imported gene lists. Compounds with negative connectivity scores, representing genes expressed in a dissimilar fashion to the imported query, were taken forward for functional validation in an attempt to confer sensitivity in previously resistant cell lines when combined with conventional cytotoxic agents. Results were used in the discovery mode to identify potential therapeutic agents targeting biological pathways identified by GSEA results.

Synergy Experiments: Targeted Inhibition in Combination with Conventional Cytotoxic Agents

Synergy for the combination of targeted pathway inhibition with conventional cytotoxic therapy was assessed.

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4 http://dtp.nci.nih.gov/docs/cancer/cancer_data.html
5 http://www.broad.mit.edu/gsea
6 http://www.broad.mit.edu/gsea/msigdb
7 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
8 http://www.affymetrix.com/support/technical/byproduct.affx?product=biotencyclargetargetlabel
9 http://www.broad.mit.edu/cmap
using Calcsyn (Biosoft). Briefly, serial 2-fold dilutions of docetaxel (0.25-128 nmol/L) and either LY294002 (50 nmol/L-25.6 µmol/L) or SU6656 (50 nmol/L-25.6 µmol/L) were applied to docetaxel-resistant, docetaxel-sensitive, and cisplatin-resistant cell lines. The combination of agents was additionally applied to cell lines as a fixed-ratio serial dilution of 1:200 for docetaxel/LY294002 and docetaxel/SU6656. LY294002 and SU6656 were obtained from a commercial source (Sigma-Aldrich). Cytotoxicity was assessed at days 0 and 5 with propidium iodide as described above. A combination index (CI) was calculated based on the Chou and Talalay method with a CI < 1 implying synergy, CI > 1 antagonism, and CI = 1 additive (12). Experiments were done in triplicate to obtain mean CI and SD.

Detection of pAKT
Select docetaxel-resistant cell lines (H322, H460, and H2030) were grown to 70% confluence in recommended growth medium per the commercial vendor (American Type Culture Collection) with minor modification. EC_{50} concentrations for docetaxel, LY294002, and the combination, based on single-agent cytotoxicity assays as determined by Calcsyn (Biosoft), were added to each cell line with protein lysates isolated at 0, 3, 6, 9, and 12 h post-drug exposure. Western blot analysis was done to confirm phosphatidylinositol 3-kinase (PI3K)/AKT pathway inhibition using commercially available antibodies to pAKT (Ser473), total AKT, and β-actin. Briefly, 30 to 50 mg protein lysate was electrophoresed on a 10% Tris-HCl gel (Bio-Rad Ready Gel, Bio-Rad Laboratories) and subsequently transferred to a biomembrane blot (Immobilon-P, Millipore) using a semidry Electrophoretic Transfer Cell, Bio-Rad Laboratories). Blots were blocked in 5% milk for 60 min before being hybridized overnight at 4°C with 1:1,000 dilution of primary antibody (Ser473 pAKT, total AKT, or β-actin control; Cell Signaling Technology). After primary hybridization, blots were washed for 15 min (5 min × 3), in 0.5% TBS-Tween 20 and then hybridized to a 1:1,000 dilution of secondary antibody for 60 min. Blots were then washed with 0.5% TBS-Tween 20 for 25 min (5 min × 5) and exposed to a chemiluminescence reagent (Western Lightning Western Blot Chemiluminescence Reagent; PerkinElmer Life and Analytical Sciences) for radiographic detection of protein.

Results

GSEA Analysis for NCI-60 Cancer Cell Lines
Previous work has shown that differential gene expression between NCI-60 cell lines sensitive or resistant to chemotherapy can be used to create expression-based signatures predicting response or resistance in human tumors (5, 13, 14). Here, we investigated the biology underlying the differential gene expression across cell lines to discover mechanisms of resistance and targets for combination therapy. By applying GSEA (6), a computational method that identifies shared differential gene expression of predefined, functionally related gene sets representing biological pathways,10 we identified biological pathways associated with resistance for each chemotherapy agent tested. Previously described associations between biological mechanisms and chemotherapy resistance were identified including p53 with Adriamycin, K-ras with etoposide, mammalian target of rapamycin with paclitaxel, and mitogen-activated protein kinase with topotecan (15–18). In addition to known associations, potentially novel pathways associated with resistance were identified for each agent (Supplementary Table 2). No pathways were common to all agents and no pathway was found to be significantly enriched in resistant versus sensitive cell lines across all agents investigated. Four pathways, however, were associated with resistance to more than one agent including Free Pathway (free radical-induced apoptosis), matrix metalloproteinase, p53, and methionine metabolism (Table 1).

Cytotoxicity Assays for Lung Cancer Cell Lines
Given the broad but sparse representation of specific cancer types in the NCI-60 and the potential importance of cellular context in the biological mechanisms resulting in resistance, we sought to determine molecular pathways associated with chemotherapy resistance in a large collection of non-small cell lung cancer cell lines. The in vitro sensitivity for 40 publicly available lung cancer cell lines was determined to both cisplatin (Fig. 1A) and docetaxel (Fig. 1B), two chemotherapy agents frequently used in combination for patients with advanced lung cancer. As with the NCI-60 cell lines, this set of lung cancer cell lines had a broad range of sensitivity to both agents with a 21-fold difference between the least (mean EC_{50}...
4,662 nmol/L) and most sensitive (mean EC₅₀, 221 nmol/L) cell lines to cisplatin (mean EC₅₀ across all samples, 1,662 ± 398 nmol/L) and a 17-fold difference between the least sensitive (mean EC₅₀, 4.8 nmol/L) and the most sensitive (mean EC₅₀, 0.28 nmol/L) cell lines to docetaxel was noted with mean EC₅₀ across all samples of 0.92 ± 0.3 nmol/L.

**GSEA Analysis for Lung Cancer Cell Lines**

Using previously established thresholds to identify lung cancer cell lines as either sensitive or resistant (EC₅₀ > 1 SD away from mean EC₅₀, ref. 5), we identified pathways associated with resistance to cisplatin and docetaxel using GSEA (Table 2). Twenty pathways were associated with docetaxel resistance and 23 pathways with cisplatin resistance (at nominal P ≤ 0.05). Of these, the bcl-2 pathway was associated with cisplatin resistance (at nominal P = 0.002), consistent with previously known associations (19–21). Two pathways were associated with resistance to both agents (AKT and SA Programmed Cell Death). Importantly, AKT gene sets along with PI3K-specific gene sets were consistently associated with docetaxel resistance.

![Sensitivity spectrum to cisplatin (A) and docetaxel (B) in a series of lung cancer cell lines (n = 40). Cell lines included in sensitive or resistant phenotypes are noted with exceptions (*, no expression data available; **, error bars overlapped within 1 SD of overall mean).](image-url)
regardless of the method used to derive microarray data (MAS5 versus RMA) or empirical thresholds used to identify sensitive and resistant cell lines (mean EC50 >0.5 or >1 SD with or without overlapping error bars). Thus, the association of AKT with docetaxel resistance was sufficiently robust to not be significantly affected by changes in data processing and that the association of AKT with docetaxel resistance may be reflective of increased PI3K activity.

When GSEA results for docetaxel from the NCI-60 cancer cell line analysis are compared with the lung cancer cell line analysis, only one pathway without prior association.

### Table 2. Top GSEA pathways associated with cisplatin and docetaxel resistance in lung cancer cell lines with a nominal $P < 0.05$ (common pathways shaded)

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<th>Pathway</th>
<th>No. genes/set</th>
<th>Nominal $P$</th>
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<th>No. genes/set</th>
<th>Nominal $P$</th>
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<td>MAP00020 Citrate Cycle TCA Cycle</td>
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<td>MAP00632 Benzoate Degradation</td>
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<tr>
<td>Fatty Acid Degradation</td>
<td>51</td>
<td>0.05</td>
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**Figure 2.** Results of cmap screen for compounds with expression signatures opposite those of imported query for docetaxel resistance (screen shot from Web browser).
with docetaxel resistance was common to both (Benzoate Degradation). This observation, combined with those from the NCI-60 across different agents, shows the effect of disease context and drug specificity on pathway-associated resistance mechanisms.

**cmap Implicates PI3K Pathway and Docetaxel Resistance but Not Cisplatin Resistance**

Although GSEA is one approach with which to associate biological processes with gene expression differences across specific phenotypes (chemotherapy resistance), alternative methods are available. The cmap is one such method that compares lists of differential expressed genes to a library of experiments assessing the effect of small molecules and genetic events on gene expression. Using the cmap, LY294002, a direct PI3K inhibitor, was found to be highly ranked among compounds with antagonistic effects on genes associated with docetaxel resistance (connectivity score -1; Fig. 2). A query for compounds based on the imported genes for cisplatin resistance revealed numerous compounds of potential interest, including thalidomide but did not include LY294002 (Fig. 3). To determine how sensitive the association between LY294002 and docetaxel was to the number of differentially expressed genes included in the query, the top 100 and 200 genes up-regulated and down-regulated for both cisplatin and docetaxel resistance were also imported into the cmap. LY294002 was consistently antagonistic to the genes associated with docetaxel resistance (data not shown). In contrast, across the cisplatin experiments, LY294002 was never ranked in the top 25 and had a consistently weaker antagonism of cisplatin resistance.

**PI3K/AKT Pathway Inhibition and Synergy Assessment**

The results from GSEA and the cmap associated the PI3K/AKT activity with resistance to docetaxel and served as the rationale to test for synergy between docetaxel and PI3K inhibition (with LY294002) in docetaxel-resistant lung cancer cell lines. When fixed dilutions of docetaxel and LY294002, separately and in combination, were applied to the lung cancer cell lines most resistant to docetaxel, the CIs were all <1, suggesting synergy [H322 (mean CI, 0.56 ± 0.16), H460 (mean CI, 0.74 ± 0.16), and H2030 (mean CI, 0.64 ± 0.28; Fig. 4)]. PI3K inhibition, as shown by Western blot analysis for phosphorylated AKT, was seen consistently with LY294002 treatment and was generally not affected by treatment with docetaxel (Fig. 5). Interestingly, in a single cell line, phosphorylated AKT increased at 12 h of docetaxel treatment. The association of docetaxel

![Figure 3. Results of cmap screen for compounds with expression signatures opposite those of imported query for cisplatin resistance (screen shot from Web browser).](image)

![Figure 4. CI for the combination of docetaxel and LY294002 in three docetaxel-resistant cell lines (H322, H460, and H2030) and one docetaxel-sensitive cell line (H1373). CI suggests synergy (CI < 1), additivity (CI = 1), or antagonism (CI > 1).](image)
resistance and PI3K activity appears specific as a docetaxel-sensitive cell line (H1373) did not reveal synergy for the combination (CI, 1.01 ± 0.60; Fig. 4) nor did the combination of cisplatin and LY294002 in docetaxel-resistant cell lines H322 (CI, 2.66 ± 0.19), H460 (CI, 1.30 ± 0.29), H2030 (CI, 2.09 ± 0.34), or a cisplatin-resistant cell line H1703 (CI, 1.15 ± 0.22; Fig. 6). Finally, the combination of docetaxel with a Src inhibitor (SU6656) in docetaxel-resistant cell lines additionally failed to reveal synergy (Fig. 7), thus strengthening the observed association of docetaxel resistance with the PI3K/AKT pathway.

**Discussion**

Resistance to chemotherapy is a universal concern for cancer patients and their providers. Building on the successes of multiple groups using *in vitro* derived cell line sensitivity and gene expression data to build predictive models that anticipate patient response or resistance to cytotoxic therapy, we used integrated computational approaches to identify biological pathways implicated by differential gene expression between sensitive and resistant cell lines to identify rational targets for combination therapy.

Although common mechanisms of resistance across cytotoxic agents and types of cancer would facilitate standard approaches, our analysis suggests that the biology associated with resistance is relatively specific to agents and significantly affected by the context of the cell lines tested. No single biological pathway was associated with all cytotoxic agents when the NCI-60 cell line data were analyzed for Adriamycin, cyclophosphamide, docetaxel, etoposide, 5-fluorouracil, paclitaxel, and topotecan. In fact, using a previously established level of statistical significance (at nominal $P \leq 0.05$), only four pathways were associated with resistance to two agents and no pathway was found to be common to more than two agents. Furthermore, the pathways associated with docetaxel resistance by the NCI-60 data were significantly different from those identified in the lung cancer cell lines. Thus, future approaches at identifying molecular pathways associated with resistance are likely to be most successful when focused on specific agents in specific disease states.

The implication of commonly known mechanisms of resistance with specific agents suggests that our approach identifies important associations and the lack of common mechanisms across all agents is not a false negative. As an example, our results identified a gene set involved with Bcl-2 regulation to be significantly enriched for cisplatin resistance. Members of the Bcl-2 family are known to be overexpressed in non-small cell lung cancer cells and associated with altered apoptosis. As a result, Bcl-2 has been the target of antisense strategies in an attempt to increase chemotherapeutic effectiveness (22) and a recent study reported the effects between GX15-070, a pan-Bcl-2 inhibitor, and cisplatin in the treatment of non-small cell lung cancer cell lines (23). In addition, our analysis found cisplatin resistance to be associated with three gene sets (ATP Synthesis, Photosynthesis, and Type III Secretion System) involved in the production of the H+-ATPase. Cisplatin-resistant cell lines have been shown previously to have significantly increased intracellular pH compared with sensitive cell lines with an ability to confer sensitivity through the concomitant use of H+-ATPase inhibitors, such as bafilomycin (24).

Interestingly, little overlap was observed in the pathways associated with resistance for docetaxel and paclitaxel. Although initially surprising, it is important to note that docetaxel is currently Food and Drug Administration approved for the second-line treatment of non-small cell lung cancer cell lines.

Figure 5. Western blot assessment of AKT pathway inhibition in docetaxel-resistant H322 (A), H460 (B), and H2030 (C) cell lines after timed exposure to docetaxel, LY294002, and the combination.

Figure 6. CI for the combination of cisplatin and LY294002 in three docetaxel-resistant cell lines (H322, H460, and H2030) and one cisplatin-resistant cell line (H703). CI suggests synergy (CI < 1), additivity (CI = 1), or antagonism (CI > 1).
Figure 7. CI for the combination of docetaxel and SU6656 in three docetaxel-resistant cell lines (H322, H460, and H2030). CI suggests synergy (CI < 1), additivity (CI = 1), or antagonism (CI > 1).

The definitive assessment of the synergy between PI3K/AKT inhibition and docetaxel resistance in human lung cancer tumors will require a clinical trial. Given the importance of the PI3K/AKT pathway, there are a growing number of inhibitors under commercial development. Our findings suggest that the most efficient means of developing inhibitors is to perform trials where patients are chosen based on their anticipated resistance to docetaxel. Specifically, microarray analysis can be done on patients with advanced lung cancer and a docetaxel sensitivity predictor applied. For patients predicted to be sensitive, docetaxel alone or as part of an established doublet (with cisplatin) would be administered, whereas patients predicted to be resistant would receive docetaxel combined with a PI3K/AKT inhibitor or other cytotoxic agents. Clearly, such a trial design is dependent on phase I trials showing safety and establishing combination doses. Our institution currently has a protocol using a microarray-based cisplatin predictor
to stratify patients between cisplatin-based treatment (if predicted to be sensitive) or pemetrexed-based therapy (if predicted to be cisplatin resistant). This trial uses information from the individual’s tumor to determine therapy and represents a model for how medical oncology can move beyond empiricism to personalized, molecular-based care.

In conclusion, the mechanisms underlying the development of chemotherapy resistance are only partially understood with the most important mechanisms and key biological pathways remaining unknown. Gene expression profiling identifies involved genes and pathways associated with resistance and allows for the prediction of therapeutic response. Novel computational methods allow for the determination of appropriate biological relevance and identification of pathways that may serve as targets for novel therapeutics. By combining standard chemotherapy with pathway-specific therapies, one takes a crucial step in developing individualized, patient-specific therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. John N. Weinstein (Genomics and Bioinformatics Group, National Cancer Institute) for providing U133A expression data for the NCI-60 series of cancer cell lines.

References


Mol Cancer Ther 2008;7(10), October 2008

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Retraction in Part: A Genomic Approach to Identify Molecular Pathways Associated with Chemotherapy Resistance

We wish to retract Table 1 and Supplemental Table 1 from our article entitled "A genomic approach to identify molecular pathways associated with chemotherapy resistance," which was published in the October 2008 issue of Molecular Cancer Therapeutics (1).

Using previously published annotations for chemotherapy sensitivity in the NCI-60 series of cancer cell lines (2), we performed gene set enrichment analysis on predefined groups of sensitive and resistant NCI-60 cell lines for a range of chemotherapies to identify biological pathways associated with resistance. We purposefully used the annotations for sensitivity and resistance published in the Nature Medicine article and applied a complementary computational approach in order to glean biological insight from the differential gene expression. The article upon which our annotations were based has now been retracted (3). After re-examination, the annotations for the cell lines with respect to chemotherapy sensitivity were erroneous. Thus, our manuscript propagates this error and the results in Table 1 and Supplemental Table 1 from our manuscript are invalid.

The majority of the paper reports our work including in vitro sensitivity testing for 40 lung cancer cell lines, identification of pathways associated with resistance to tested agents, and functional validation of a lead candidate pathway in vitro. These data appear in the remaining Figures 1–7 and Table 2 of the paper and we remain confident in our analysis and findings.

Richard F. Riedel
1Duke Institute for Genome Sciences and Policy, Duke University and 2Division of Medical Oncology, Department of Medicine

Alessandro Porrello
1Duke Institute for Genome Sciences and Policy, Duke University

Emily Pontzer
1Duke Institute for Genome Sciences and Policy, Duke University

Emily J. Chenette
1Duke Institute for Genome Sciences and Policy, Duke University

David S. Hsu
1Duke Institute for Genome Sciences and Policy, Duke University and 2Division of Medical Oncology, Department of Medicine, Duke University

Bala Balakumaran
1Duke Institute for Genome Sciences and Policy, Duke University

Anil Potti
1Duke Institute for Genome Sciences and Policy, Duke University and 2Division of Medical Oncology, Department of Medicine, Duke University

*Former Institute
Joseph Nevins
1Duke Institute for Genome Sciences and Policy, Duke University and
3Molecular Genetics and Microbiology, Duke University Medical Center,
Durham, North Carolina

Phillip G. Febbo**
1Duke Institute for Genome Sciences and Policy, Duke University and
2Division of Medical Oncology, Department of Medicine, and
3Molecular Genetics and Microbiology, Duke University Medical Center, Durham, North Carolina

**Current Institution
Departments of Medicine and Urology, University of California, San Francisco Medical School, San Francisco, CA

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Published OnlineFirst March 29, 2012.
doi: 10.1158/1535-7163.MCT-12-0210
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Molecular Cancer Therapeutics

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