Temporal Molecular and Biological Assessment of an Erlotinib-Resistant Lung Adenocarcinoma Model Reveals Markers of Tumor Progression and Treatment Response

Zoe Weaver1, Simone Difilippantonio1, Julian Carretero3,5,6, Philip L. Martin1, Rajaa El Meskini1, Anthony J. Iacovelli1, Michelle Gumprecht1, Alan Kulaga1, Theresa Guerin1, Jerome Schlomer1, Maureen Baran1, Serguei Kozlov1, Thomas McCann7, Salvador Mena6, Fatima Al-Shahrour4,8, Danny Alexander9, Kwok-Kin Wong3,5, and Terry Van Dyke1,2

Abstract

Patients with lung cancer with activating mutations in the EGF receptor (EGFR) kinase, who are treated long-term with tyrosine kinase inhibitors (TKI), often develop secondary mutations in EGFR associated with resistance. Mice engineered to develop lung adenocarcinomas driven by the human EGFR T790M resistance mutation are similarly resistant to the EGFR TKI erlotinib. By tumor volume endpoint analysis, these mouse tumors respond to BIBW 2992 (an irreversible EGFR/HER2 TKI) and rapamycin combination therapy. To correlate EGFR-driven changes in the lung with response to drug treatment, we conducted an integrative analysis of global transcriptome and metabolite profiling compared with quantitative imaging and histopathology at several time points during tumor progression and treatment. Responses to single-drug treatments were temporary, whereas combination therapy elicited a sustained response. During tumor development, metabolic signatures indicated a shift to high anabolic activity and suppression of antitumor programs with 11 metabolites consistently present in both lung tissue and blood. Combination drug treatment reversed many of the molecular changes found in tumors lung. Data integration linking cancer signaling networks with metabolic activity identified key pathways such as glutamine and glutathione metabolism that signified response to single or dual treatments. Results from combination drug treatment suggest that metabolic transcriptional control through C-MYC and SREBP, as well as ELK1, NRF1, and NRF2, depends on both EGFR and mTORC1 signaling. Our findings establish the importance of kinetic therapeutic studies in preclinical assessment and provide in vivo evidence that TKI-mediated antiproliferative effects also manifest in specific metabolic regulation. Cancer Res; 72(22); 5921–33. ©2012 AACR.

Introduction

Lung cancer is the leading cause of cancer-related deaths (1). Current screening methods for early disease are ineffective at improving the 5-year survival rate, which is less than 4% for advanced non–small cell lung cancer (NSCLC; ref. 2). For most cancers, including NSCLC, traditional cytotoxic chemotherapy has limited efficacy, and patients develop resistance to targeted inhibitors, indicating the need for identification of molecular networks operative in disease and treatment response. In particular, effective preclinical systems are required for guidance on patient stratification, therapeutic directives, and response biomarkers. Such needs are exemplified by the current status of NSCLC clinical management. Activating mutations in EGFR, such as the L858R mutation, are effective predictors of response to EGF receptor (EGFR) tyrosine kinase inhibitor (TKI) therapy (3). While most patients initially respond to EGFR TKIs, the majority relapse within 6 to 12 months, underscoring the importance of identifying therapies that overcome or mitigate such resistance. Strikingly, a single secondary missense mutation in the EGFR TK domain, T790M, is associated with more than 50% of acquired resistance cases (4).

Recent advances in the ability to globally assess molecular networks responsive to biologic and chemical perturbations in cancer have facilitated unbiased discovery in disease...
mechanisms (5). Genetically engineered mouse (GEM) models can facilitate both assessment of disease etiology from initiation through progression as well as treatment responses within the organs/cells of interest (6). Mice engineered to develop erlotinib-resistant lung adenocarcinomas driven by human EGFR L858R;T790M mutations (EGFR-TL; refs. 7, 8) respond to BIBW 2992 (afatinib, an irreversible EGFR/HER2 TK1 currently in clinical trial) and rapamycin combination therapy, based on tumor volume endpoints (9). BIBW 2992 or rapamycin alone minimally inhibit tumor growth. To further assess responses to single and combination therapies and to gain insight into the optimal strategy for preclinical evaluation in robust cancer models, we examined both transcriptional and metabolite profiles in disease induction and therapeutic response with time. These studies underscore the value of temporal well-powered evaluations and offer an opportunity to identify surrogate markers for disease and treatment responses and for tailoring therapeutic regimens.

Materials and Methods

Tumor induction and sampling

Mice (see Supplementary Materials and Methods) were fed 200 mg/kg doxycycline feed from Research Diets. Blood was taken from the mandibular vein of bitransgenic EGFR-L858R-T790M;CCSP-rtTA mice (E+R+) or mice carrying the CCSP-rtTA driver, but not the EGFR transgene (E−R+) at 2 weeks (n = 6 for both E−R+ and E+R+), and when tumor growth required euthanasia of the mice (terminal at 6–8 weeks; n = 8 for both E+R+ and E−R+), and snap-frozen in an equal volume of 9 mmol/L EDTA in phosphate buffered saline. Lung tissue was harvested and snap-frozen at 2 weeks and terminal stages and age-matched control mice were taken at the same time points postinduction. For treatment studies, blood and lung tissue were harvested after 1 (n = 19), 2 (n = 19), or 4 (n = 40) weeks of daily treatment with drug vehicle, BIBW 2992 (20 mg/kg/d, oral gavage), rapamycin (2 mg/kg/d, intraperitoneally), or the combination of both compounds starting at 4 weeks postinduction.

Histology/pathology

Mice were euthanized at the indicated times and right lungs were inflated with formalin and processed for immunohisto-pathologic analysis. Immunohistochemistry experiments are described in the Supplementary Data. Hematoxylin and eosin slides of lungs from drug- and vehicle-treated tumors were scored blindly by a board certified veterinary pathologist (P.L. Martin) according to a grading scheme that was developed for this study (see Supplementary Data for details). All tumors (4–10) of each experimental group (vehicle- or drug-treated) were analyzed.

Automated image acquisition and quantification

Immunohistochemistry for Ki-67 was conducted on lung sections from each mouse and slides were scanned using the Arioil image scanning and analysis system (Applied Imaging Corp.) at × 20. As the severity of lung disease was heterogeneous between lung lobes, the percentage of Ki-67-positive cells was quantified in the lung lobe that was most representative of the overall histopathologic score. The Kisight image analysis module was used to count the total number of nuclei in the lung section as well as the total number of Ki-67–positive (Brown-DAB) nuclei and Ki-67–negative (blue-hematoxylin) nuclei. For each lung lobe, approximately, 30,000 to 125,000 nuclei were counted.

Gene expression profiling

Preparation of RNA is described in the Supplementary Data. Hybridization to Mouse Gene 1.0 ST chips was conducted following Affymetrix protocols (Affymetrix, Inc.). Hierarchical clustering analysis was conducted using pairwise complete linkage method with the Pearson correlation distance measure using the GenePattern genomic analysis platform. "Single Sample" Gene Set Enrichment Analysis (10, 11) was used to determine the degree of absolute enrichment of gene ontology (GO) biologic processes gene sets (12) in each sample within the gene expression data set. Signature values for each sample were normalized using the entire sample set. Standard gene set enrichment analysis (GSEA) was used to determine the enrichment of transcription factor binding sites between classes. Gene sets sharing a cis-regulatory motif that is conserved across the human, mouse, rat, and dog genomes (13), representing known or predicted regulatory elements in promoters and 3′-UTRs, were obtained from MSigDB (see Supplementary Data for details).

Results

Temporal evaluation of tumor growth and treatment response reveals distinct dynamics of single versus combination therapy

To examine responses elicited during human EGFR L858R-T790M–driven lung adenocarcinoma, as well as experimental treatment-induced regression, we measured changes with time in lung disease severity and tumor burden. Bitransgenic EGFR-L858R-T790M;CCSP-rtTA mice (designated E+R+) are engineered to develop lung adenocarcinoma upon EGFR transgene induction by doxycycline. Mice carrying the CCSP-rtTA driver, but not the EGFR transgene (E−R+), served as controls. E+R+ mice developed lung tumors when continuously exposed to doxycycline, and consistent with previous studies (7), adenomas were detectable by magnetic resonance imaging (MRI) and histology within 2 to 3 weeks postinduction, progressing to adenocarcinomas that often involved entire lobes at terminal stages. Induced E+R+ mice were treated with BIBW 2992, rapamycin, or the combination daily for up to 4 weeks (Fig. 1A). To quantify histopathology response to drug treatment (or lack thereof) in the tumors, severity of disease was scored based on extent and morphology ranging from 0 (no lesions) to 5 (multifocal to coalescing adenocarcinoma; Supplementary Data and Supplementary Fig. S1). The histopathologic severity grade increased over time in the vehicle-treated group, and while each individual drug had some effect in suppressing tumor growth, the combination treatment was most effective (score of 1.9 compared with 4.3 for vehicle-treated after 4 weeks, P = 0.002) (Fig. 1B). Thus, the present expanded study agrees well with previous findings that used smaller cohorts.
We also detected decreased cellular proliferation (by Ki-67 staining) and decrease in overall tumor burden (by hematoxylin and eosin and confirmed by total EGFR staining) in both single and combination treatments. Combination treatment resulted in the greatest decrease in total EGFR, phospho-EGFR, and Ki-67 staining, even after only 1 week of treatment (Supplementary Fig. S2). Automated quantification of Ki-67 staining supports the hypothesis that the strongest effect on proliferation is from BIBW 2992 and rapamycin dual treatment at all time points (Fig. 1C); however, the Ki-67 data indicates an increase in proliferation after 4 weeks even in the combination-treated mice. Immunoblotting confirmed phospho-EGFR or phospho-S6 reduction in lungs of BIBW 2992– or rapamycin-treated mice (respectively) at 4, 8, and 24 hours after single dose, as well as reduction of both in lungs of combination-treated mice (Supplementary Fig. S3). By 24 hours, reduction of phospho-EGFR was more variable, even in the combination-treated mice, indicating the need for continued treatment to achieve target suppression.

To attain another quantitative measure of treatment effects, baseline MRI measurements were compared with posttreatment tumor scans (Fig. 1D and E and Supplementary Data). In this model, signals on MRI reflect not only tumor burden, but also associated inflammation. After 1 week of treatment, BIBW 2992 alone resulted in tumor regression.
compared with baseline, whereas in the cohort treated for 2 weeks, tumor growth was inhibited by BIBW 2992 relative to vehicle, but regression was not observed. However, BIBW 2992 treatment together with rapamycin resulted in substantial tumor regression compared with baseline, evidenced by both 2 and 4 week posttreatment times.

Taken together, these results indicate that although BIBW 2992 successfully inhibits its primary target \( \text{EGFR} \) in vivo and elicits a short-term tumor regression response, it does not produce a sustained pathway and growth response unless combined with rapamycin. Importantly, maximal response for either single agent occurs early, with rebound apparent within a few weeks despite continuous treatment. The kinetics of such responses are thus critical in evaluating therapeutic efficacy. During the course of treatment, some tumor regions responded to drug and thus overall tumor burden decreased, which is reflected in the MRI measurements (note that Fig. 1E is an example of the maximum regression response observed). However, as discussed above, at the histopathology level small areas of the affected lungs (below the resolution level of the MRI scan) were observed that were either refractory to treatment or developed resistance as detected by pathology score and Ki-67 data. Endpoint tumor size in this case measures the sum of treatment responses and emerging resistant growth, underscoring the importance of temporal assessment in preclinical models for accurate response measurements.

**Gene expression reflects changes in oncogenic signaling in response to therapy over time**

To further explore the mechanism(s) of synergy in combination treatment, lungs from control \( \text{E}^{-}\text{R}^{+} \), tumor-bearing \( \text{E}^{+}\text{R}^{+} \) induced mice, and drug-treated \( \text{E}^{+}\text{R}^{+} \) mice were analyzed for transcriptome changes. Gene expression data was subjected to supervised hierarchical clustering to compare profiles of untreated tumor-bearing mice to drug-treated for 1 or 4 weeks (Fig. 2A). As expected, vehicle-treated \( \text{E}^{+}\text{R}^{+} \) samples had expression profiles similar to those of untreated \( \text{E}^{+}\text{R}^{+} \) lung samples. The 1-week treated samples showed an almost universal suppression of gene expression compared with untreated or vehicle-treated \( \text{E}^{+}\text{R}^{+} \) animals that did not distinguish between responders and nonresponders by pathology score (Fig. 2A). However, after 4 weeks there were clearly several samples in which the profile more closely matched the normal gene expression profile (\( \text{E}^{-}\text{R}^{+} \) control). These lungs also had lower (less severe) pathology scores than the comparable vehicle-treated or untreated samples. Although most of the combination drug-treated tumors responded at the phenotypic level as seen by MRI scans and histopathology, not all of the samples seemed to be responders at the transcriptome level, indicating some heterogeneity within the tumors as discussed above. These data also point to the variability within treatments due to the mixed tumor cell population.

A single-sample GSEA of GO terms was conducted as a first step to reveal the biologic functions that differentiate normal (\( \text{E}^{-}\text{R}^{-} \)) from tumor-bearing (\( \text{E}^{+}\text{R}^{+} \)) and vehicle- from drug-treated tumors. Among others, GO terms involved in cell metabolism, mitosis, and DNA replication were differentially affected in EGFR tumors (Fig. 2B). We chose 4 genes in the signature (\( \text{BUB1}, \text{TOP2A}, \text{CCNB2}, \text{and } \text{PLK1} \)) for validation by quantitative PCR (qPCR; Supplementary Fig. S4). Of note, in a study of gene expression markers in human NSCLC, \( \text{CCNB2} \) and \( \text{TOP2A} \) were part of an 187-gene signature set distinguishing normal tissues from malignant NSCLCs (14). All 4 genes were downregulated after treatment with BIBW 2992 or rapamycin, and the drug combination caused a further decrease in gene expression levels. Fold-change levels, compared with vehicle-treated controls, were consistently greater after 1 week compared with 2 weeks of drug treatment, again indicating a greater early treatment response on average. The 4-week gene expression data was a more accurate indicator of which individual tumors exhibited a sustained response. We conclude that multiple time points during the course of treatment are crucial to profiling the in vivo activity of cancer drugs.

**Tumor induction and drug response result in contrasting metabolomic alterations**

To evaluate additional biologic changes accompanying tumor induction and treatment that may correlate to gene expression responses, we conducted metabolic profiling of blood and lung tissue from induced \( \text{E}^{-}\text{R}^{+} \) and \( \text{E}^{+}\text{R}^{+} \) mice at various time points and from \( \text{E}^{-}\text{R}^{-} \) and \( \text{E}^{+}\text{R}^{-} \) mice treated with the described drug regimen for 4 weeks. Using a high-throughput small-molecule analytic platform (Metabolon, Inc.; see Supplementary Materials and Methods), a total of 457 biochemicals were detected in lung and 324 were detected in blood, including 238 compounds common to both lung and blood, 219 compounds unique to lung, and 86 compounds unique to blood (Supplementary Tables S1 and S2). Statistical comparisons were used to identify biochemicals whose relative levels differed between experimental groups (Supplementary Table S3 and Supplementary Materials and Methods). In lung tissue, there were extensive metabolomic alterations consistent with tumor development within 2 weeks of induction of the EGFR transgene, which were typically stronger at the terminal time point (data summarized in Fig. 3A). These changes were characterized by a shift to high anabolic activity, including diversion of glucose oxidation to the pentose phosphate pathway, elevated amino acid and nucleotide pools, higher levels of polyamines, glycerolipids, and long-chain fatty acids.

Lipid pathway metabolites were upregulated as a result of tumor induction, including fatty acids (used in cell membrane construction, energy metabolism, and as substrates for signaling molecules), and phospholipids (essential for membrane biosynthesis; Fig. 3A). For example, CDP-choline and intermediates to CDP-choline synthesis (choline and choline-phosphate) increased in tumor tissue compared with control lung and decreased upon rapamycin or combination drug treatment. MTORC1 was recently shown to directly induce lipid biosynthesis (15) and therefore, decrease in these metabolites may indicate effective inhibition of mTOR by rapamycin.

Treatment with either BIBW 2992 or rapamycin tended to reverse the metabolic shifts observed during tumor induction in lung tissue; however, rapamycin (or the combination) caused a more dramatic reversal for the vast majority of metabolites than BIBW 2992 alone (Fig. 3A). The majority of
Figure 2. Supervised clustering shows correlation between tumor status, treatment, and pathology score and pathways. A, gene expression profiles of normal lung, lung tumors (2 and 4 weeks postinduction) from untreated EGFR-TL mice, and vehicle, BIBW 2992, rapamycin, or BIBW 2992 plus rapamycin–treated mice at indicated time points. At the bottom of the heat map are the corresponding pathology scores for each lung sample. B, single sample gene set enrichment analysis scores of samples from A. A positive enrichment score (red) indicates a positive correlation between genes in the gene set and the tumor sample expression profile; a negative enrichment score (blue) indicates the reverse (see also Materials and Methods).
metabolomic alterations were in pathways affected downstream of mTOR, for example amino acid synthesis. Individual amino acid pathway metabolites significantly altered in lung tumors as compared with control lung were plotted as Z-scores (Fig. 3B) to examine specific effects of the individual versus the combination treatments. The overall pattern of response is similar in all 3 treatment regimens, not unexpected as both drugs target nodes in the EGFR signaling pathway. Notably, citrate, an intermediate in the tricarboxylic acid (TCA) cycle, is significantly increased in BIBW 2992 and BIBW 2992 + rapamycin combination–treated lung tumors but not by rapamycin alone. As citrate is also an intermediate in fatty acid and phospholipid synthesis, it is possible that the general decrease in lipids upon drug treatment correlates to less diversion of citrate from the TCA cycle to membrane biosynthetic processes.

Thus, this lung cancer model reflects the abnormal metabolism expected in cancer cells, including aberrant nutrient uptake and increased macromolecular synthesis. Moreover, treatment with drugs targeting the EGFR signaling pathway results in reversal of these effects, highlighting the importance of identifying specific metabolites involved in the response.

Key metabolite signature observed in blood and tissue samples during lung tumorigenesis and treatment
In addition to lung tissue, we sampled blood from tumor-bearing animals to determine whether there were metabolic changes that could be used as potential biomarkers indicating the presence of a tumor. Blood was sampled from E→R+ and E+R+ mice at 2 weeks and at terminal stages postinduction for metabolic profiling. The unbiased screen recovered 26 metabolites with statistical significance of P ≤ 0.05. The changes after 2 weeks were more profound, possibly due to wasting at the terminal stages. Relative fold-changes were much lower in blood than tissue, most likely due to the dilution of organ-specific effects by the entire blood volume as well as the homeostatic mechanism, which changes or removes many biochemicals that were identified in blood samples from the first study. Overall, the relative levels of biochemicals that were identified in blood samples collected for the validation study were similar to levels measured in samples collected for the validation study. We identified 11 metabolites from both studies that were significantly changed in blood as well as in the induced lung tissue (Table 1).

![Z-score plot of amino acid pathway only from gene expression and metabolomics in a lung cancer model](image-url)
Evidence of elevated amino acid pools observed in lung were reflected in blood. The branched chain amino acid conjugates propionyl-carnitine and deoxycarnitine were significantly increased in E\(\times\)R\(+\) blood and lung tissue compared with control E\(-\)R\(+\). These intermediates of valine, leucine, and isoleucine catabolism are indicative of oxidative stress or a shortage of coenzyme A and can build up when the pathway proceeds at rates in excess of downstream capacity to use metabolites in the TCA cycle. The amino acids glutamine, threonine, serine, arginine, and tryptophan were also increased (Table 1), suggestive of increased amino acid synthesis or enhanced protein turnover due to muscle wasting during cancer development. Unlike the other amino acids, aspartate was upregulated in lung and downregulated in blood. Using liquid chromatography-tandem mass spectrometry (LC-MS/MS) quantification, we verified that glutamine, tryptophan, and arginine were elevated in E\(\times\)R\(+\) blood and tumors compared with E\(-\)R\(+\) control samples induced for 2 weeks, whereas aspartate was upregulated in lung only (Table 1; MS-targeted assay). In addition, we detected the levels of these amino acids in induced, tumor-bearing lungs after 2 weeks of BBW 2992, rapamycin, or combination drug treatment (Supplementary Fig. S5). As expected, drug treatment reversed the higher levels of the metabolites observed in the vehicle-treated tumors, though in some cases (aspartate, glutamate) the combination treatment did not decrease the levels beyond the individual treatments. This could be due to the natural variation in a smaller number of samples, or a difference in the 2 week treatment compared with the original analysis after 4 weeks of treatment. The amino acids identified are members of key pathways affected in the treatment response (see below) underscoring the potential significance of small changes in their relative quantities.

**Integration of metabolite and gene expression data highlights pathways essential to drug response**

While the examination of gene expression or metabolic signatures was informative, we speculated that combining the datasets could reveal the most significant changes. To test this, we conducted pathway enrichment analysis on metabolic and transcriptomic signatures. Gene expression and metabolite data from control E\(-\)R\(+\) mice, induced E\(\times\)R\(+\) mice and mice treated with the drug combination were mapped together onto Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Supplementary Fig. S6). Each dataset alone did not show significant enrichment in most pathways, but merging the 2 datasets points to key pathways in cancer cell energy production, such as the pentose phosphate pathway, glutathione metabolism, and glycolysis and gluconeogenesis that are differentially regulated in untreated versus treated tumors. There were 46 significantly enriched pathways that are upregulated in E\(\times\)R\(+\) tumors compared with E\(-\)R\(+\) and of these, 29 were reversed in direction by drug treatment. Two of the pathways, "alanine, aspartate, and glutamate metabolism" and "glycolysis and gluconeogenesis" are also of note as, in a study of gene expression profiles in tumor samples from patients with lung adenocarcinoma, gene sets representing those pathways were enriched in patients with poor outcome (11).

Individual pathway maps are informative to highlight the concordance between the metabolites altered in key energy pathways and changes in expression of the enzymes that control production of individual metabolites. For example, both oxidized (GSSG) and reduced glutathione (GSH), as well as amino acids used to synthesize them, were elevated in tumor development (Fig. 4A). Antioxidant enzymes related to glutathione synthesis and coordinately regulated through antioxidant response elements (AREs) and NRF2 binding, such as gamma-glutamylcysteine synthetase (\(\gamma\)-GCS), glutathione reductase, and glutaredoxin were correspondingly upregulated in tumors (Fig. 4A). The glutathione pathway is involved in protection from oxidative stress, a characteristic of rapidly growing cells, by providing a substrate for neutralization of reactive oxygen species (ROS; ref. 16). ROS production and subsequent oxidative stress have been implicated specifically in carcinogenesis driven by oncogenes such as KRAS (17) and EGFR (18). Alternatively, ROS production can occur due to intratumoral hypoxic conditions, leading to oxidative stress, which causes the cells to oxidize GSH to GSSG and results in the activation of glutathione synthesis (19). A hypoxia-related gene signature was overexpressed in E\(\times\)B\(+\) versus E\(-\)B\(+\) lung (Supplementary Fig. S7). For example, GLUT1, a target of hypoxia-inducible factor-1 and mediator of glucose uptake (20), was upregulated in tumors reflecting hypoxic conditions and correlated to an increase in glycolytic metabolites and other glycolytic enzymes (Fig. 4B). After treatment with the drug combination, oxidized, and reduced glutathione as well as the hypoxia-inducible factor-1 target gene signature were downregulated (Fig. 4B, Supplementary Fig. S7), indicating a decrease in energy needs in the tissue.

Glutamine levels were significantly lowered by BBW 2992 treatment or BBW 2992 + rapamycin, but not by rapamycin alone (Fig. 4A). Glutamine is the major nutrient consumed by cancer cells besides glucose and is a precursor of glutathione as well as a source of TCA cycle intermediates. Glutamine metabolism is increased by C-MYC, and downstream gene targets of C-MYC like the glycolytic enzyme enolase, are upregulated in
Figure 5. Oncogenic transcription factor activity is linked to downstream alteration of key metabolic pathways. A, gene set enrichment analysis of TFBS in expression profiles from normal lung, lung tumors from untreated EGFR-TL mice, and vehicle, BIBW 2992, rapamycin or BIBW 2992 plus rapamycin-treated EGFR-TL mice. Heat map shows significant enrichments of TFBS across indicated comparisons. Color scale depicts the logarithm of P value (red, overrepresentation; blue, underrepresentation). B, leading edge analysis of metabolic genes that have TFBS in their promoters, in normal lung, lung tumors from untreated, and vehicle, BIBW 2992, rapamycin or BIBW 2992 plus rapamycin-treated EGFR-TL mice. Heat map shows relative expression levels of genes annotated to KEGG metabolic pathways across sample set. Black boxes (middle) represent the presence of an indicated TFBS in the promoters.
higher levels in E
 ty-glucosamine, which is an important precursor for protein
glycosylation and polysaccharide synthesis, were present in
higher levels in E+R+ tumor tissue versus E−R+ control
lungs (Fig. 4B). While glucose itself is lower in tumor tissue,
it is not a good indicator of tumor glucose levels as it may
simply indicate binding of glucose to dead cells. The phos-
phorylated products fructose-6-phosphate (F-6-P) and fruc-
tose-1,6-bisphosphate (F-1,6-BP) are higher in tumors and
phosphofructokinase (PFK), which catalyzes the conversion
of F-6-P to F-1,6-BP, is correspondingly upregulated. There
was also strong evidence for activation of the pentose
phosphate pathway, which provides precursors for growth
as well as the NADPH needed for various anabolic reactions
(Fig. 4B). For example, expression of transketolase (TKT),
which catalyzes the formation of sedoheptulose as well as
the conversion of xylulose-5-phosphate back to F-6-P in the
pentose phosphate pathway, was oppositely regulated in
tumors versus combination-treated lungs.

Of note, pyruvate and lactate, the oxidation product of
pyruvate, were higher in tumor tissue, as was lactate dehy-
drogenase (LDH) gene expression. This is consistent with
previous observations made in tumor tissue, in which pyruvate is converted to lactate (Warburg effect) avoiding
energy-producing oxidative phosphorylation. Pyruvate was
lowered only by the combination drug treatment, indicating
that full suppression of the pathway may be necessary to
inhibit glycolysis.

**Oncogenic transcription factor activity may be
responsible for downstream alteration of key metabolic
pathways**

Recent studies have suggested that oncoproteins can direct-
ly reprogram tumor cell metabolism, and several oncoproteins
and tumor suppressors have been linked to the regulation of
downstream metabolic processes through transcription factor
activity (21). To identify candidate transcription factors down-
stream of oncopgenic EGFR activation in our model, we used
GSEA and a library of gene sets containing genes with a cis-
regulatory element and conserved DNA motifs, representing
known or predicted regulatory elements in promoters and 3’-
UTRs (13). This bioinformatic methodology was used to iden-
tify overrepresented transcription factor binding sites (TFBS)
that were present in the promoters of the genes included in
gene expression profiles of tumors from untreated E+R+ mice,
and vehicle, BIBW 2992, rapamycin or BIBW 2992 plus rapa-
mycin-treated E+R+ mice. Our analysis revealed a cluster of 12
transcription factors that were significantly enriched (FDR
value < 0.05) in EGFR tumors compared with normal lung,
for example MYC and SREBP1 (Fig. 5A). In contrast, after
the first week of drug treatment, the targets of some of these
transcription factors were underrepresented, for example
NRF1 and NRF2.

To identify metabolic genes that drive the enrichment for
each transcription factor gene set, we ran a leading edge
analysis of GSEA results described above. By grouping those
leading edge genes (i.e., genes with the highest enrichment
scores in EGFR tumors) included in TFBS gene sets that were
significantly underrepresented after drug treatments (GABP,
NRF1, NRF2, SREBP, ELK1 and C-MYC), we were able to
categorize them into metabolic processes. As shown in Fig.
5B, the strongest downregulation of metabolic genes activated
in EGFR-TL tumors seemed to be after only 1 week of treat-
ment, particularly in response to the combination of BIBW and
rapamycin. Key genes, such as hexokinase 2 (HK2) and lactate
dehydrogenase A (LDHA: glycolysis), and GPX1 and GSS (glu-
tathione metabolism) were affected. After 4 weeks of treat-
ment, the single-drug regimens were unable to repress most of
the metabolic genes included in the analysis. Only the drug
combination reduced gene expression levels more consistent-
ly, suggesting that transcriptional control of metabolic path-
ways through C-MYC and SREBP, as well as ELK1, NRF1 and
NRF2, depends on both EGFR and mTORC1 signaling.

**Discussion**

Presently, human clinical trials using BIBW 2992 (afatinib)
and rapamycin combination are ongoing (22, 23). The unbiased
profiling approach described here highlights the utility of
GE Ms in rapidly identifying clinically testable hypotheses that
might immediately impact the analysis of these ongoing clin-
tical trials, and the design of subsequent human studies. To
that point, we observed that several of the prominent pathways
modulated during EGFR-driven tumorigenesis were reversed
upon combination drug treatment, revealing possible reasons
for increased efficacy.

Because of their controlled genetics and environment,
mouse models represent ideal tools for the identification of
candidate biomarkers. Recent work comparing the plasma
proteome of mouse ovarian cancer models with human ovar-
ian cancer cell lines and tissue samples from patients resulted
in promising biomarker candidates (24); another study in a
mouse pancreatic cancer model revealed proteins that could
discriminate human pancreatic cancer sera from controls (25);
and protein signatures have been found that distinguish
between different types of lung cancer in the mouse (26). We
previously identified metabolomic changes in a mouse model
of serous epithelial ovarian cancer that overlapped with find-
ings obtained from tissue of human patients with metastatic
ovarian cancer (27, 28). As energy metabolism has long been
known to be aberrant in cancer, in this study we conducted
metabolic profiling in the EGFR-TL mouse model to iden-
tify biochemical processes that might be perturbed both in
tumor development and drug treatment. By comparing
tissue and blood metabolomic changes during tumor progres-
sion we identified disease-associated changes. Several of the
same metabolites have recently been shown to be altered in
human lung cancer tissue and sera from patients with squa-
rous cell carcinoma and small cell lung cancer as well as
adenocarcinoma (29), indicating fidelity of the mouse models
in mimicking the human condition. Furthermore, they show
that the molecular interactions between the tumor and host
may be similar in these 2 species. Because the dilution factor
represented by the movement of a compound from the tumor
to blood is large, and at the same time other organs may be acting to clear or modify the compound, the ability to validate the blood markers by correlation to lung tumor tissue metabolites in the mouse model is crucial.

As seen in patients (29), murine lung cancer tissue contained higher levels of most amino acids compared with normal lung tissue, including higher levels of glutamine and tryptophan. It is remarkable that high levels of glutamine are maintained although large amounts of glutamine are consumed in cancer cells through glutamine metabolism. Notably, treatment with BIBW 2992 and the combination of BIBW 2992 and rapamycin downregulated the levels of glutamine in the lung tissue, indicating the potential importance of this key source of energy in maintaining cancer cell growth. This could also offer a clue to the synergistic effects of the drug treatment with rapamycin, as glutamine depletion can cause apoptosis in lung cells by affecting pathways that do not require mTOR activity; instead they are MYC-regulated (30). Glutamine can also maintain the TCA cycle when glucose levels are limiting. Growth factor signaling through the PI3K-Akt-mTOR pathway in EGFR-overexpressing mice may be further activated by higher glutamine levels, resulting in increased protein translation (31).

MTORC1, the target of rapamycin and a key regulator of cell growth and proliferation downstream of oncogenic signaling pathways, including EGFR, has been linked previously to cellular metabolism regulation through the transcriptional control of metabolic genes (32, 33), and other recent studies have also linked metabolism to specific oncogenic signaling pathways (34, 35). In our analysis, relating transcriptional control elements from the gene expression data to metabolic processes revealed functional connections between known oncogenes and metabolite changes in the lung tumors. After the first week of drug treatment, binding sites for several transcription factors enriched in tumor samples were underrepresented, for example NRF1 and NRF2. Both BIBW 2992 and rapamycin treatments result in a decrease in the target genes for NRF2 after 1 week, but after 4 weeks only, the dual treatment suppresses expression of those genes. NRF2 is a transcriptional activator of cytoprotective enzymes including those involved in glutathione synthesis, and has increased activation in lung cancer tissue and cell lines (36). Therefore, NRF2 activation of glutathione metabolism may be a previously unappreciated mediator of oncogenesis which is inhibited by the combination drug regimen used here.

It was recently observed that mTORC1 signaling increases flux through the pentose phosphate pathway in some measure through activation of SREBP1 (15). SREBP1 is a transcriptional regulator of genes involved in de novo lipid and sterol biosynthesis and is itself regulated by mTOR (32). Interestingly, although rapamycin alone can inhibit mTOR, the SREBP1 gene target set is underrepresented in rapamycin-treated lungs only after 4 weeks of treatment, and even more significantly in the combination-treated samples. Targets of C-MYC, a key transcription factor driver in metabolic processes in cancer, are enriched in E-I R+ tumors and underrepresented in rapamycin and combination-treated lungs after 1 or 4 weeks of drug treatment.

In summary, this study combines transcriptional and metabolic analyses of a complex in vivo model system to examine temporal responses after drug treatment. Examination of lung tumor progression in EGFR-overexpressing TKI-resistant mice as well as a drug regimen known to result in tumor regression revealed marked changes in gene expression and biochemical pathways during tumor growth that were in many cases reversed when treatment was effective, and were different depending on the specific treatment and duration of treatment. These studies can be used to guide future analyses of drug combinations for human disease with targeted agents aimed at resistance by simultaneous blockade of relevant pathways.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Disclaimer
The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does the mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

Authors' Contributions
Conception and design: Z. Weaver, S. DiFliппantionato, T.V. Dyke
Development of methodology: Z. Weaver, J. Carretero, S. Mena, K.K. Wong, T.V. Dyke
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Weaver, J. Carretero, P.L. Martin, R. El Meskini, A.J. lacevelli, M. Gumprecht, A. Kulała, T. Guerin, J. Schlomer, M. Baran, T. McCann, D. Alexander, K.K. Wong
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Weaver, S. DiFliппantionato, P.L. Martin, T. McCann, F. Al-Shahrour, D. Alexander, K.K. Wong, T.V. Dyke
Writing, review, and/or revision of the manuscript: Z. Weaver, S. DiFliппantionato, J. Carretero, S. Kozlov, D. Alexander, K.K. Wong, T.V. Dyke
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Kozlov, S. Mena, K.K. Wong, T.V. Dyke
Study supervision: Z. Weaver, S. DiFliппantionato, K.K. Wong, T.V. Dyke

Acknowledgments
The authors thank Catherine Drennan, Melanie Gordon, SAIC's Laboratory Animal Sciences Program, Small Animal Imaging Program, Pathology/Histotechnology Laboratory, and the Laboratory of Molecular Technology for technical assistance; Dres. Marcelino Bernardo and Peter Choyke for MRI analysis; Maria L. Rodriguez for assistance with HPLC-MS, Patti Lamb for administrative technology laboratory, and the Laboratory of Molecular Technology for technical assistance; and Lionel Feigenbaum for program support.

Grant Support
This research was supported with federal funds from the National Cancer Institute, Intramural Research Program, NIH.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 6, 2012; revised August 8, 2012; accepted August 23, 2012; published OnlineFirst September 11, 2012.

References
Gene Expression and Metabolomics in a Lung Cancer Model


Temporal Molecular and Biological Assessment of an Erlotinib-Resistant Lung Adenocarcinoma Model Reveals Markers of Tumor Progression and Treatment Response

Zoë Weaver, Simone Difilippantonio, Julian Carretero, et al.


<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-12-0736</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://cancerres.aacrjournals.org/content/suppl/2012/09/11/0008-5472.CAN-12-0736.DC1">http://cancerres.aacrjournals.org/content/suppl/2012/09/11/0008-5472.CAN-12-0736.DC1</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cited articles</th>
<th>This article cites 31 articles, 10 of which you can access for free at: <a href="http://cancerres.aacrjournals.org/content/72/22/5921.full.html#ref-list-1">http://cancerres.aacrjournals.org/content/72/22/5921.full.html#ref-list-1</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Citing articles</td>
<td>This article has been cited by 4 HighWire-hosted articles. Access the articles at: <a href="http://cancerres.aacrjournals.org/content/72/22/5921.full.html#related-urls">http://cancerres.aacrjournals.org/content/72/22/5921.full.html#related-urls</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>E-mail alerts</th>
<th>Sign up to receive free email-alerts related to this article or journal.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reprints and Subscriptions</td>
<td>To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at <a href="mailto:pubs@aacr.org">pubs@aacr.org</a>.</td>
</tr>
<tr>
<td>Permissions</td>
<td>To request permission to re-use all or part of this article, contact the AACR Publications Department at <a href="mailto:permissions@aacr.org">permissions@aacr.org</a>.</td>
</tr>
</tbody>
</table>