Relationship of Deregulated Signaling Converging onto mTOR with Prognosis and Classification of Lung Adenocarcinoma Shown by Two Independent In silico Analyses

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Abstract
There is marked disparity with a slight overlap among prognosis-predictive signatures reported thus far for lung cancers. In this study, we aimed at linking poor prognosis with particular pathways and/or functions of the gene sets involved to better understand the underlying molecular characteristics associated with the prognosis of lung adenocarcinomas. Gene set enrichment analysis identified a gene set down-regulated by rapamycin as the most significant, whereas several others responsive to withdrawal of glucose or amino acids, which are related to signaling converging onto mammalian target of rapamycin (mTOR), were also shown to be significantly associated, in addition to those related to DNA damage response and cell cycle progression. We also used connectivity map (C-MAP) analysis, an independent bioinformatics approach, to search for Food and Drug Administration–approved drugs that potentially transform an unfavorable signature to a favorable one. Those results identified inhibitors of phosphatidylinositol 3-kinase (PI3K) and mTOR, as well as unexpected drugs such as phenothiazine antipsychotics and resveratrol as potential candidates. Experimental validation revealed that the latter unexpected agents also inhibited signaling converging onto mTOR and exhibited antitumor activities. In addition, deregulation of multiple signaling converging onto mTOR was shown to be significantly associated with sensitivity to PI-103, a dual specificity PI3K/mTOR inhibitor that is not contained in the C-MAP database, lending further support for the connection. Our results clearly show the existence of gene set–definable, intrinsic heterogeneities in lung adenocarcinomas, which seem to be related to both clinical behavior and sensitivity to agents affecting the identified pathways. [Cancer Res 2009;69(9):4027–35]

Introduction
Adenocarcinomas exhibit the highest degree of morphologic and clinical diversities among the various types of lung cancers, and it is well recognized that their current pathologic classification and ability to predict postsurgical prognosis are quite inadequate. Although the existence of marked heterogeneity, including their expression profiles, is well appreciated (1), virtually all adenocarcinomas are currently treated similarly under the diagnosis of non–small cell lung cancer. Along this line, better understanding of molecular heterogeneities is considered to be one of the major factors that has significantly improved cure rates of certain types of neoplasia, such as hematologic malignancies and breast cancer. Thus, a better understanding of the underlying molecular mechanisms of the heterogeneities among lung adenocarcinomas is greatly anticipated to contribute to reducing the unbearably large number of deaths by establishing a foundation for rational individualized treatment strategies, as well as development of novel therapeutic methods.

The tumor-node metastasis staging system is currently used as the standard method for predicting prognosis, whereas a number of individualized molecular signature-based predictions that partition patients into distinct prognostic groups have also been developed, including our own, which have shown good results for predicting patients with dismal prognosis (2–6). However, there is a marked discordance with a slight overlap among such predictive signatures thus far reported for lung cancers. This disparity makes it difficult to reconcile them in relation to the underlying molecular characteristics, which has led to criticism stating that such models do not provide biological insight.

In this study, we used two independent bioinformatics approaches, gene set enrichment analysis (GSEA; ref. 7) and connectivity map (C-MAP; ref. 8) analysis, in combination with biological and biochemical experiments for validation, to elucidate the underlying molecular basis for the link of poor prognosis with particular pathways and/or functions of the gene sets involved. Consequently, well-defined and easily interpretable gene sets were identified as associated with fatal outcome, providing insight into the existence of intrinsic heterogeneities in lung adenocarcinomas and the usability of in silico screening for potential antitumor agents with activities to revert a fatal signature to a favorable one.

Materials and Methods
Bioinformatics and biostatistics analyses. Clinical characteristics, such as length of the follow-up period, are described in Supplementary Materials and Methods. The Nagoya data set of microarray analysis (GEO GSE 11969), previously reported by our group (1), was used in two independent bioinformatics analyses. Two additional data sets reported by Harvard (9) and Memorial Sloan-Kettering Cancer Center (10) groups were
also used for validation. GSEA was performed using the GSEA version 1.0 program for R (7). C-MAP analysis was performed using the tool described by Lamb and colleagues (8). Detailed methodologic information for the present GSEA and C-MAP analyses is provided in Supplementary Materials and Methods. The CLUSTER program was used for average linkage hierarchical clustering of both genes and cases, whereas the TREEVIEW program was used for display (11). Fisher’s exact test was performed to examine associations of the deregulation score-based clusters with various clinical parameters, whereas Cox regression analysis was used to analyze the relationships among various parameters, including deregulation score-based clusters and postoperative prognosis. All statistical tests were two sided.

Cell lines. ACC-LC-94, ACC-LC-176, and ACC-LC-319 cells were established in our laboratory, whereas the A549, A427, and NCi-H23 cell lines were purchased from the American Type Culture Collection. The SK-LU-1, SK-LC-3, SK-LC-5, and SK-LC-7 cell lines were generously provided by Lloyd J. Old (Memorial Sloan-Kettering Cancer Center). Cells were cultured in RPMI 1640 supplemented with 5% fetal bovine serum (Invitrogen-Life Technologies, Inc.).

Growth inhibition assays. Growth inhibition was assessed by an MTS assay using the TetraColor ONE kit (Seikagaku) according to the manufacturer’s instructions. Each combination of cell line and drug was set up in six replicate wells, and the experiment was repeated at least thrice. Each data point presented in the assay results represents the growth of treated cells compared with untreated cells.

Small interfering RNA knockdown. A synthetic small interfering RNA (siRNA) duplex composed of rictor (Dharmacon) and scramble (QIAGEN) was transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. Twenty-four hours after siRNA transfection, cells were seeded (3 × 10^{3}/100 μL) into 96-well plates, incubated overnight, and treated with or without rapamycin for 72 h. Viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Down-regulation of rictor was confirmed 5 d after transfection by Western blot analysis.

Results

Identification of gene sets associated with relapse in lung adenocarcinomas. We performed GSEA using our previous data set containing 90 lung adenocarcinomas (the Nagoya data set; ref. 1) to identify gene sets with a clear association with relapse. First, data from 42 favorable samples (alive >5 years after surgery without any evidence of relapse) and 33 fatal samples (dead with evidence of relapse after initial surgery) were extracted, and GSEA consequently identified eight gene sets with significant associations with a fatal outcome (Fig. 1A and B; Supplementary Tables S1–S5). A GSEA gene set, which was originally selected because of down-regulation in the presence of rapamycin (Rap_down), exhibited the most significant enrichment of all queried genes associated with relapse-related death. Three other significantly enriched gene sets included those reduced by either glucose deprivation (Glucose_down), leucine deprivation (Leu_down), or glutamine deprivation (Glut_down; Fig. 1B), which seemed to be consistent with the notion that the mammalian target of rapamycin (mTOR) is a converging point of growth factor receptor signaling with nutrient availability and energy status of the cell (12–14). For the sake of convenience, these gene sets are herein termed metabolic pathway gene sets. In addition, gene sets related to cell cycle and DNA damage signaling also showed a significant association with fatal outcome.

Next, we determined whether similar gene sets could be selected by GSEA using the completely independent data set of 62 lung adenocarcinoma cases presented by the Harvard group, for which information regarding both relapse and death are available (9). The Leu_down (P = 0.0238), Cell_Cycle (P = 0.0444), and DNA damage signaling (P = 0.0500) gene sets were shown to be significantly associated with relapse-related death in that data set, whereas Rap_down (P = 0.0833), CR_cell_cycle (P = 0.0851), and Glut_down (P = 0.0976) were also included in the top 10 gene sets, although the results for those did not reach statistical significance. These findings further supported the involvement of these gene sets in signatures associated with clinical behavior (Supplementary Table S6).

Associations of deregulation of GSEA-identified gene sets with various clinicopathologic characteristics. To investigate associations between the levels of perturbation in each of the GSEA-identified gene sets and various clinicopathologic characteristics, we defined a “deregulation index” for each gene set, which was calculated by averaging the normalized gene expression values. The deregulation index values clearly separated between fatal and favorable prognosis (Fig. 1C; Supplementary Fig. S1), as well as between terminal respiratory unit (TRU) and non-TRU types, our previously proposed expression profile-substantiated classification of adenocarcinomas (Supplementary Fig. S2; ref. 1). Interestingly, even tumors from patients with a favorable prognosis exhibited a more prominent perturbation state than normal lung tissue samples. Hierarchical clustering analysis using the deregulation indices of the four metabolic pathway gene sets was also performed, which resulted in clear separation of two major branches (Fig. 2A). The right branch preferentially consisted of tumors with pronounced deregulation and characteristically harbored male gender and positive smoking history (Supplementary Table S7; P = 0.019 and P = 0.002, respectively). We also noted a significant association of deregulation state with the presence of bronchioloalveolar carcinoma features (P = 0.002), as well as with TRU type (P < 0.001; Supplementary Table S7). Mutations in the epidermal growth factor receptor (EGFR) and K-ras genes did not show any associations (P = 0.659 for EGFR and P = 0.735 for K-ras), although there was a tendency for a more prevalent presence of p53 mutations in the right branch with greater deregulation (P = 0.071 for p53). Kaplan-Meier survival curves showed a significant difference in relapse-free survival between the high-deregulation and low-deregulation clusters, whereas multivariate Cox regression analysis revealed that deregulation cluster discrimination was a significant independent prognostic factor (P = 0.009) in addition to disease stage (P = 0.009; Fig. 2B).

The associations of deregulation state with prognosis and other variables were further validated using another independent data set from the Memorial Sloan-Kettering Cancer Center (10). Hierarchical clustering analysis based on the deregulation indices of the four metabolic pathway gene sets resulted in clear separation of two major branches, which showed that cases in the cluster with pronounced deregulation characteristically harbored positive smoking history (P = 0.010; Supplementary Table S8; Fig. 2C). Notably, we confirmed that cases with pronounced deregulation had significantly worse prognosis than those in the other cluster (Fig. 2D), whereas multivariate Cox regression analysis revealed that deregulation cluster discrimination was significantly associated with postoperative prognosis independent of disease stage (P = 0.012), further supporting the notion that deregulation of the metabolic pathways was a generic signature observed in patients with a high risk for relapse and fatal prognosis.

http://www.broad.mit.edu/cmap/
Search for Food and Drug Administration–approved drugs with potential to revert fatal signatures of the gene sets. To identify agents with antitumor effects by searching for those that potentially alter a fatal prognosis–associated signature to a favorable prognosis–associated one, we performed C-MAP analysis, another completely distinct bioinformatics method. C-MAP is a library of signatures of drug response in a database used for queries regarding expression information to clarify potential association with other biological contexts, thereby linking otherwise unrelated physiologic events (8, 15). For this analysis, we selected 477 probes derived from 332 up-regulated and 145 down-regulated genes that were significantly differentially expressed ($P < 0.05$ by $t$ test) between those who survived without relapse for $>5$ years and those who died with relapse in 75 cases of lung adenocarcinomas in the Nagoya data set and used them as query items to compare to rank-ordered lists for each drug treatment in the C-MAP database. We identified multiple Food and Drug Administration (FDA)–approved drugs with a significantly biased appearance of the query genes closer to the top of the rank-ordered list, indicating the induction of significant down-regulation in 332 genes with relapse-related death-associated up-regulation by a given drug treatment, as well as significant up-regulation of the 145 genes down-regulated by the treatment (Table 1). The results of Kolmogorov-Smirnov scanning of the sixth-ranked sirolimus in the C-MAP analysis are shown in Fig. 3A, which showed enrichment of the favorable (down-regulated) and fatal (up-regulated) signatures, whereas summaries of all instances of sirolimus and LY-294002 are shown in Fig. 3B. Wortmannin and LY-294002, inhibitors of phosphoinositide...
3-kinase (PI3K), as well as sirolimus (rapamycin), which inhibits mTOR, were ranked near the top of the C-MAP list of FDA-approved drugs (Supplementary Table S9 and Supplementary Fig. S3 showing results of the permutation test). In addition to the cancer-related kinase inhibitors, C-MAP analysis also identified several other unexpected agents with distant connections to cancer treatment, which included phenothiazine antipsychotics (prochlorperazine, trifluoperazine, fluphenazine, and thioridazine) and resveratrol, raising the possibility that these agents may also inhibit the same signaling pathways, exhibiting antitumor effects.
Finally, we analyzed whether changes in gene expression before and after rapamycin treatment in the ACC-LC-94 and A549 lung cancer cell lines corresponded to those in MCF-7, because the C-MAP database is mainly based on the expression profiling results in the MCF-7 breast cancer cell line. As a result, C-MAP analysis using the extracted probe sets exhibiting a significant response to rapamycin faithfully selected sirolimus (rapamycin) as significant (Supplementary Tables S10 and S11; \( P = 0.0079 \) in ACC-LC-94 and \( P = 0.0105 \) in A549 by permutation test).

**Experimental validation of results obtained by the C-MAP analysis.** To experimentally validate our results obtained by C-MAP analysis, we examined the growth inhibitory activities of the C-MAP–identified agents using a panel of lung cancer cell lines. Rapamycin and LY-294002 exhibited growth inhibitory activities in MTT assays, as expected, whereas prochlorperazine and resveratrol treatments also resulted in growth inhibition, although rapamycin did not reach 50% growth inhibition with most of the lung cancer cell lines tested in this study (Supplementary Fig. S4). Notably, there was a significant inhibition of phosphorylation of p70 S6K, a downstream effector of mTOR, in the A549 adenocarcinoma cell line by treatment with not only rapamycin and LY-294002 but also prochlorperazine and resveratrol. Inhibition of AKT phosphorylation was also detected in A549 cells treated with resveratrol or LY-294002, whereas prochlorperazine elicited a delayed induction of AKT similar to that observed with rapamycin (Fig. 3C). In addition, the expression of EGFR was down-regulated by treatment with prochlorperazine via a currently unknown mechanism (Supplementary Fig. S5). In addition to down-regulation of the prosurvival effectors, resveratrol induced marked phosphorylation of p38 MAP kinase, a proapoptotic effector, as well as modest phosphorylation of AMP kinase, which is activated by deprivation of cellular energy (13, 14), leading to inhibition of mTOR activity (Fig. 3D). These results showed that these compounds, which were identified *in silico*, as drugs with potential activities to alter a relapse-related death signature have effects on pathways converging onto mTOR with antitumor activities *in vitro*.

**Requirement of inhibition of both mTORC1 and TORC2 for growth suppression of lung cancer cells.** The modest growth inhibitory activity seen for the mTOR-inhibiting rapamycin led us to investigate the contribution of a delayed reactivation of AKT induced by rapamycin. Rapamycin treatment diminished the binding of mTOR with raptor, although a complex of mTOR and rictor (mTORC2) was still formed in the presence of rapamycin (Fig. 4A). siRNA-mediated knockdown of rictor caused a significant reduction in cell growth, which was enhanced by combined use with rapamycin (Fig. 4B). A reduction in the expression of rictor decreased the levels of phosphorylation of both AKT and FoxO3a, well-established direct substrates of AKT, whereas the phosphorylation of S6 kinase was marginally inhibited by knockdown of rictor (Fig. 4C), showing that simultaneous inhibition of mTORC1 and mTORC2 effectively inhibits cell growth in lung adenocarcinomas. In line with this observation, combined treatment of rapamycin with LY-294002 exhibited inhibition of rapamycin-mediated reactivation of AKT, in association with a more pronounced cell growth inhibition in lung cancer cell lines (Supplementary Fig. S6). We also found that concomitant treatment with resveratrol similarly inhibited reactivation of AKT in lung cancer cell lines treated with rapamycin (Fig. 4D).

**Association of deregulation of GSEA-identified gene sets with sensitivity to the multikinase inhibitor PI-103.** Our findings of effective growth inhibition of lung adenocarcinoma cells by combined inhibition of signaling involving PI3K, mTORC1, and mTORC2 led us to examine the inhibitory effects of PI-103, a small molecule with the capability to inhibit these three (Supplementary Fig. S7), in relation to the deregulation state of signaling converging onto mTOR (16, 17). Treatment with PI-103 clearly inhibited phosphorylation of AKT and S6k, as well as PDK1 downstream of PI3K in a dose-dependent manner in

<table>
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<tr>
<th>Rank</th>
<th>C-MAP name</th>
<th>Dose</th>
<th>Cell</th>
<th>Remarks</th>
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<td>1 μmol/L</td>
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<td>PI3K inhibitor</td>
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<tr>
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<td>mTOR inhibitor</td>
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<tr>
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<td>11 μmol/L</td>
<td>MCF7</td>
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A549 and ACC-LC-319 cells (Fig. 5A). The level required for 50% growth inhibition in the panel of 11 lung adenocarcinoma cell lines ranged from 0.2 to 2.7 μmol/L (Supplementary Table S12). Intriguingly, the cumulative deregulation score, which we defined on the basis of the cumulative deregulation of each gene set, including Rap_down, Glucose_down, Leu_down, and Glut_down, was found to be associated with sensitivities to PI-103 in each lung adenocarcinoma cell line (Fig. 5B; \( r = 0.667, P = 0.025 \) by Pearson’s correlation coefficient), suggesting the potential usefulness of such a cumulative deregulation score for predicting the sensitivity to PI-103 and possibly of other agents simultaneously affecting multiple molecules in pathways converging onto mTOR.

**Discussion**

Rapid proliferation of cancer cells causes an insufficient blood supply, which elicits a variety of environmental stresses, such as deprivation of growth factors, nutrients, and/or oxygen. However, cancer cells are thought to be capable of surviving in spite of such environmental stresses.
constraints, which conceivably leads to disease progression and ultimately confers poor prognosis in affected patients. A number of studies have aimed at identifying a list of genes related to lung cancer prognosis (2, 3, 10, 18–21); however, criticism is frequently raised noting that the gene lists used in such prognosis prediction classifiers have minimal overlaps and provide little biological insight into the underlying mechanisms. In a related study, Bild and colleagues analyzed artificially generated cells with overexpression of various oncogenes in vitro and identified oncogenic pathway signatures with a potential to separate lung cancer patients into subgroups in relation to outcome, including drug sensitivity (22). In contrast, the present study, which used two independent bioinformatics approaches, namely GSEA and C-MAP analysis, directly used expression profiles of patient tumors. We specifically designed our study to focus on the underlying molecular basis linking poor prognosis with particular pathways and/or functions of the gene sets involved, which showed the association of deregulation of multiple signaling converging onto mTOR with relapse and death in lung adenocarcinoma patients.

Activation of the PI3K-AKT-mTOR axis plays a key role in growth factor receptor–mediated signaling for cancer cell growth and survival, whereas LKB1, which is inactivated in a fraction of lung adenocarcinomas (23, 24), activates AMP kinase in microenvironmental conditions, including insufficient nutrients, and leads to inhibition of mTOR activity in an AKT-independent manner (13, 14). In addition, amino acids, such as leucine, regulate mTOR signaling, which involves raptor, a ras homologue enriched in the brain, and a GTPase that activates mTOR, which are directly associated with mTOR (25, 26). This study is the first to report that the expression state of genes affected by withdrawal of amino acids is significantly altered in association with poor prognosis, suggesting that adaptation to an insufficient availability of amino acids may be a prerequisite for the progression of lung adenocarcinomas. In addition, the expression state of genes responsive to glucose withdrawal was found to be altered in tumors associated with a fatal outcome at a more pronounced level, a finding consistent with a previous report by Chen and colleagues that found increased expression of glycolytic enzymes in association with poor prognosis (27). In our multivariate Cox regression analysis, deregulation of mTOR-converging signaling seemed to be an independent prognostic factor in addition to disease stage, whereas such statistical significance was not observed with various other parameters.
including mutations of \textit{EGFR}, \textit{K-ras}, and \textit{p53}, as well as bronchioloalveolar carcinoma features and TRU/non-TRU classification. Multivariate Cox regression analysis of the Memorial Sloan-Kettering Cancer Center data sets also showed similar associations, supporting the notion that deregulation of mTOR-converging signaling may be a generic signature in patients with a high risk for relapse and fatal outcome. It is interesting that two independent groups recently reported a lack of prognostic value of \textit{LKB1} inactivation in lung cancers (28, 29), whereas conflicting results were presented in terms of the association between poor prognosis and AKT phosphorylation (30–32). Therefore, it is possible that an altered state of multiple signaling converging onto mTOR may be more reflected in clinical behavior rather than a given molecule in the pathways.

Also interesting is that our \textit{in silico} search using C-MAP analysis for drugs with activities that potentially revert a fatal prognosis-associated signature to a favorable prognosis-associated one resulted in identification of not only well-known inhibitors of PI3K or mTOR, but also several unexpected ones, such as those with antitumor activities \textit{in vitro}. It has been reported that whereas rapamycin and its derivatives exhibit considerable antitumor activity in certain tumor types, including renal cell carcinoma, mantle cell lymphoma, and endometrial cancers, lung cancers are rather resistant with response rates of <10\% (33). In this connection, the present results obtained with siRNA-mediated silencing of rictor in the presence of rapamycin suggest that enhanced effectiveness may be attained by concomitant inhibition of mTORC1 and mTORC2. We also unexpectedly identified drugs, including phenothiazine antipsychotics (prochlorperazine, trifluoperazine, fluphenazine, and thioridazine), an antihelminthic (pyrimidin), and resveratrol, a chemopreventive agent (34). In addition, prochlorperazine was shown here for the first time to inhibit mTOR-mediated signaling and induce a delayed phosphorylation of AKT in a manner similar to rapamycin. It is also important to note that both rapamycin and prochlorperazine have abilities to promote autophagy (35), a cellular response to a growth factor/nutrient-deprived condition. Pyrimidin was previously shown to inhibit cancer cell survival by blocking AKT signaling specifically in a nutrient-starved condition, which is also consistent with the present findings (36). Interestingly, whereas this study showed that PI-103, a dual specificity kinase inhibitor, inhibited growth of lung adenocarcinoma cell lines through concurrent inhibition of PI3K and mTOR without AKT feedback activation, we observed an inverse relationship between cumulative deregulation score, i.e., extent of augmentation in the expression signatures of gene sets related to mTOR-converging signaling, and sensitivity to PI-103, providing further support to a functionally relevant connection.

In conclusion, using two independent bioinformatics approaches, we identified a significant association between deregulation of signaling converging onto mTOR and poor prognosis in lung adenocarcinomas, suggesting the existence of gene set-definable, intrinsic heterogeneities in this devastating tumor type. In addition, our results lend support to the notion that compounds identified by \textit{in silico} screening as having potential activities to alter signatures associated with relapse and death to favorable ones can be anticipated to show antitumor activities and inhibitory effects on pathways converging onto mTOR.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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