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Prediction of survival in resected non-small cell lung cancer using a protein-expression based risk model: Implications for personalized chemoprevention and therapy

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Abstract

Purpose—Patients with resected non-small cell lung cancer (NSCLC) are at risk for recurrence of disease but we do not have tools to predict which patients are at highest risk. We set out to create a risk model incorporating both clinical data and biomarkers.

Methods—We assembled a comprehensive database with archival tissues and clinical follow-up from patients with NSCLC resected between 2002-2005. Twenty-one proteins identified from our preclinical studies as related to lung carcinogenesis were investigated, including pathways related to metabolism, DNA repair, inflammation and growth factors. Expression of proteins was quantified using immunohistochemistry. Immunohistochemistry was chosen because it is widely available and can be performed on formalin-fixed paraffin-embedded specimens. Cox models were fitted to estimate effects of clinical factors and biomarkers on recurrence free survival (RFS) and overall survival (OS).

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Results—370 patients are included in our analysis. With median follow-up of 5.3 years, median overall survival is 6.4 years. 209 cases with recurrence or death were observed. Multivariate risk models for RFS and OS were developed including relevant biomarkers, age and stage. Increased expression of pAMPK, pmTOR, EpCAM, and CASK were significant ($p<0.05$) predictors for favorable RFS; insulin receptor, CXCR2, and IGF1R predicted for unfavorable RFS. Significant ($p<0.05$) predictors for favorable OS include pAMPK, pmTOR, and EpCAM; CXCR2 and FEN1 predicted unfavorable OS.

Conclusions—We have developed a comprehensive risk model predictive for recurrence in our large retrospective database, which is one of the largest reported series of resected NSCLC.

Keywords

Lung cancer; biomarkers; risk modeling

Introduction

Lung cancer is the leading cause of cancer-related death for both men and women in the United States. Only a minority of patients are diagnosed with disease amenable to surgical resection. The standard of care following surgical resection for patients with stage II or III tumors is adjuvant chemotherapy with a cisplatin-based doublet, but recurrence is common and there are no clinically useful biomarkers to predict the risk of recurrence. As CT-based screening gains wider acceptance, more patients will be diagnosed with early-stage disease, and effective risk stratification models could be very useful.

Numerous risk models for the development of lung cancer have been developed, incorporating clinical factors with or without serum biomarker assays (1-4). Individual biomarkers have been studied, including ERCC1, which predicts for a good prognosis and a lack of benefit to adjuvant chemotherapy (5), and RRM1, which predicts an improved overall survival when expressed at high levels (6). No risk prediction models are widely used in clinical practice, however, and a risk model incorporating tissue biomarkers as well as clinical factors could inform clinical decision making.

Our goal with this project was to develop a risk model for the development of recurrence and metastases in patients following lung cancer resection, and to assess the relationships between biomarkers, clinical patient characteristics, and outcome. Immunohistochemistry (IHC) was chosen because it is a readily available assay in diagnostic pathology labs and can be applied to routine formalin-fixed paraffin embedded tissues. We selected biomarkers belonging to a series of important molecular pathways involved in lung carcinogenesis, including many pathways associated with the hallmarks of cancer (7). These markers have been investigated using *in vitro* and *in vivo* early carcinogenesis models, and were found to be key to the pathogenesis of NSCLC, both adenocarcinoma and squamous cell carcinoma. The markers chosen relate to cell adhesion and extracellular matrix interactions (CASK, CD51 (8), EpCAM (9), SPP1 (10)), inflammation (CXCR2 (11)), growth factors and effector pathways (IGF-1R(12), IGFBP3 (13), insulin receptor (14), pIGF-1R, pEGFR (15, 16)), growth and metabolism (pAkt (17, 18), pSrc (19), pmTor (18), pAMPK (20), pS6 (17), SFN (21), UBE2C), and DNA replication and repair (FEN1, MCM2, MCM6, TPX2 (21,

22)). We then aimed to investigate these biomarkers in early stage lung cancer and to gain a better understanding of the cellular and molecular processes that drive lung carcinogenesis.

Methods

Selection of Biomarkers

Twenty one biomarkers were selected by a team of investigators based on our preclinical work in cell lines as particularly important to lung carcinogenesis. The selected markers were: calcium/calmodulin-dependent serine protein kinase (CASK), CD51 (also known as integrin alpha V), chemokine (C-X-C motif) receptor 2 (CXCR2), epithelial cell adhesion molecule (EpCAM), flap structure specific endonuclease-1 (FEN1), insulin-like growth factor-1 receptor (IGF-1R), insulin-like growth factor binding protein 3 (IGFBP3), insulin receptor, minichromosome maintenance complexes 2 and 6 (MCM2 and MCM6), phospho-Akt, phosphoadenosine monophosphate-activated protein kinase (pAMPK), phospho-epidermal growth factor receptor (pEGFR), pIGF-1R, phospho-mammalian target of rapamycin (pmTOR), pS6, pSrc, stratifen (SFN), secreted phosphoprotein-1 (SPP1), targeting protein for Xk1p2 (TPX2), ubiquitin-conjugating enzyme E2C (UBE2C).

Identification of Patients and Gathering of Clinical Data

Patients with early stage (stages I, II, and IIIA) non-small cell lung cancer (NSCLC) who underwent surgical resection at MD Anderson Cancer Center between 2002 and 2005 were eligible for enrollment (Supplementary Figure 1). Patients with stage IIIB or IV disease, surgery less extensive than a lobectomy, or a prior history of malignancy (other than non-melanoma skin cancer) were excluded from this analysis. 370 patients were included in the analysis. Detailed clinical data was obtained from the electronic medical record and follow-up visits and direct contact with patients and/or their families, either by certified letter or telephone. Overall survival (OS) was defined as time from tumor resection to death from any cause; recurrence free survival (RFS) was defined as time from tumor resection to lung cancer recurrence or death.

Lung Tumor Specimens

NSCLC specimens from surgical cases were fixed using standard clinic protocols. Fixation in formalin occurred within 30 minutes of resection and the tissue stayed in formalin for 24 to 48 hours. Archival and de-identified formalin-fixed, paraffin embedded (FFPE) specimens were analyzed. The use of tissues was approved by the Institutional Review Board at MD Anderson Cancer Center. After histological examination of the NSCLC specimens by our dedicated pathologist, the tumor tissue microarrays (TMAs) were constructed by obtaining three 1-mm-diameter cores from each tumor at three different sites (periphery, intermediate, and central). The TMAs were prepared using a manual tissue arrayer (Advanced Tissue Arrayer ATA100; Chemicon International).

Analysis of Biomarkers

Biomarkers examined were: IGF1R, IGFBP3, Insulin receptor, phosphorylated-(p)AKT, phosphorylated-(p) IGF1R, phosphorylated-(p)SRC, phosphorylated-(p)mTOR, phosphorylated-(p)AMPK, phosphorylated-(p)EGFR, pS6, FEN1, MCM2, MCM6, SFN,

TPX2, UBE2C, CASK, CD51, CXCR2, EpCAM, and SPP1. Antibodies were chosen because they were shown to be specific by Western blot analysis using NSCLC cell lines and other cell line models, such as human bronchial epithelial cells. The same NSCLC cell lines tested by Western blot were utilized for IHC optimization using cell line pellets fixed in formalin and embedded in paraffin. Those cell lines were used as controls when the TMAs were assayed by IHC.

IHC (Figure 1) was performed on histology sections of formalin-fixed and paraffin-embedded tissue samples. See supplementary table 1 for details of antibodies used. The sections were deparaffinized, hydrated, subjected to antigen retrieval by heating in a steamer for 20 minutes with 10 mmol/L sodium citrate (pH 6.0), and then incubated in peroxidase blocking reagent (DAKO). Sections were then washed with Tris-containing buffer and incubated overnight at 4°C with the primary antibodies. Subsequently, the sections were washed and incubated with secondary antibodies using the Evison plus labeled polymer kit (DAKO) for 30 minutes followed by incubation with avidin–biotin–peroxidase complex (DAKO) and development with diaminobenzidine chromogen for 5 minutes. Finally, the sections were rinsed in distilled water, counterstained with hematoxylin (DAKO), and mounted on glass slides before evaluation under the microscope. FFPE samples processed similarly, except for the omission of the primary antibody, were used as negative controls.

Experienced lung cancer pathologists blinded to the clinical data examined the immunostainings jointly at the same time using light microscopy to generate one set of readings (P.Y. and I.I.W.). The antigens studied exhibited different patterns of expression, including mainly nuclear (UBE2C, FEN1, MCM2, MCM6, SFN, SPP1, and TPX2), cytoplasmic (p-AMPK, IGF1-R, IGFBP3, insulin receptor, p-Akt, p-S6) and membrane (p-IGF1R, p-Src, p-mTor, EpCAM) expression. The immunostainings were quantified using a 4-value intensity score (0, 1+, 2+, and 3+) and the percentage (0%–100%) of tumor cells with reactivity in each core. The final score was then obtained by multiplying the intensity and reactivity extension values (range, 0–300) as previously reported (23–25). The same two pathologists also scored the samples for necrosis (measured in percentage of cells) and inflammation (graded as mild, moderate, or severe).

Statistical Analysis

Summary statistics, including frequency tabulation, means, standard deviations, median, and range, were given to describe subject characteristics and biomarkers. The continuous markers were dichotomized by either 0 vs positive or median when appropriate after examining the martingale residuals. The Kaplan-Meier method was used to construct overall survival (OS) and recurrence-free survival (RFS) curves and log-rank test was used to test the difference in survival by covariates. Univariate and multivariate Cox model were fitted to estimate the effect of prognostic factors, including age, gender, histology, stage, markers (continuous or dichotomized levels when appropriate) on time to event endpoints, including OS and RFS. All statistical tests were two-sided, and p values of less than 0.05 were considered to be statistically significant.

The predictive accuracy of Cox regression models was quantified by C-index, which provides the area under the receiver operating characteristics curve for censored data (26,

27). A C-index of 0.5 indicates that outcomes are completely random, whereas a C-index of 1 indicates that the model is a perfect predictor. To protect against overfitting during stepwise regression, we used the bootstrap method for internal validation, which allows for computation of an unbiased estimate of predictive accuracy, C-index. We chose bootstrap method because it has been considered as the most efficient among the internal validation methods, data-splitting, cross-validation and Bootstrap (28, 29). Calibration curves, which plot the average Kaplan-Meier estimate against the corresponding 1-, 3- and 5- year predicted probability of OS or RFS rate (by equally dividing patients into 3 groups according to the predicted probability of surviving), were provided to evaluate the performance of the Cox models. We used 200 bootstrap samples in both bootstrap validation and calibration. All computations were carried out in SAS 9.2 and S-plus 8.0 or R 2.12.2.

Results

Patient demographics for the 370 participants with an average age of 65.7 years (standard deviation 10.7, median 66.3, range (32.2, 90)) are shown in Table 1. Our population was evenly split between male and female, and the majority of patients were Caucasian (330 patients, 89%). Over 63% of patients had stage I disease, and most had adenocarcinoma (227, 61%). Most patients were treated only with surgery, though 128 patients (36%) received adjuvant treatment, either with chemotherapy or radiation, and 54 patients (15%) received preoperative therapy. With median follow-up time of 5.3 years, 160 deaths have been observed. A total of 209 cases with recurrence or death have been recorded to date. The median recurrence free survival time is 4.1 years (95% confidence interval (CI): (3.4, 5.3)) and median overall survival is 6.4 years (95% CI: (5.8, not achieved)).

In univariate analysis for patients' clinic-pathological characteristics and treatments, age, gender, stage, and adjuvant/neoadjuvant treatment were significantly associated with OS. Gender, stage, necrosis, inflammation, and adjuvant/neoadjuvant treatment were found to be significantly associated with RFS. Severe inflammation was associated with longer RFS compared to mild inflammation; increased necrosis was associated with shorter RFS. Smoking status was not prognostic in our analysis.

Among the biomarkers examined, we found that the observed five year RFS rate was 50% (95% CI: (44%, 57%)) for patients with positive staining for cytoplasmic pAMPK, and 33% (95% CI: (25%, 44%)) for those with negative results. For patients whose tumors expressed low levels of CXCR2, five year RFS was 49% (95% CI: (42%, 57%)); for those expressing higher levels, 41% (95% CI: (35%, 49%)). Patients with tumors positive for EpCAM had a trend towards improved five year RFS compared to those who did not: 49% (95% CI: (45%, 56%)) vs 39% (95% CI: (31%, 48%)). The five year OS for patients with positive staining for pAMPK was 64% (95% CI: (59%, 71%)) versus 52% (95% CI: (43%, 62%)) for those patients without pAMPK. Five year OS was 66% (95% CI: (60%, 74%)) and 55% (95% CI: (48%, 63%)) for those with low and high levels of staining for CXCR2, respectively; and 66% (95% CI: (60%, 73%)) and 51% (95% CI: (43%, 61%)) for those with positive and negative staining for EpCAM (Table 2). See Figure 2 for Kaplan-Meier curves of RFS and OS by these markers.

Multivariate Cox Model for Recurrence Free Survival

On multivariate analysis, age and stage, but not other clinical variables, remained significant predictors of outcome. Controlling for age and stage, the multivariate Cox model (Table 3) indicates that adjusting for age (hazard ratio (HR) 1.024 per year increase, $p=0.001$) and stage (HR 1.765 (II) and 2.676 (III) compared to stage I, $p=0.002$ and $<.001$, respectively), positive membrane insulin receptor (HR 1.442, $p=0.012$), cytoplasmic CXCR2 above the median (HR 1.360, $p=0.038$), and elevated IGF1R (HR 1.517 per 100 increase, $p=0.040$) were found to be significant predictors for shorter RFS, whereas positive cytoplasmic pAMPK (HR 0.648, $p=0.004$), positive cytoplasmic pmTOR (HR 0.696, $p=0.029$), positive cytoplasmic EpCAM (HR 0.708, $p=0.024$) and higher membrane CASK (0.680 per 100 increase, $p=0.049$) were associated with longer RFS.

Multivariate Cox Model for Overall Survival

The multivariate Cox model for OS (Table 4) includes age, stage, and five biomarkers: age (HR 1.028 per year increase, $p=0.001$), stage (HR 1.425 (II) and 2.620 (III) compared to stage I, $p=0.087$ and $<.001$, respectively), positive cytoplasmic pAMPK (HR 0.669, $p=0.018$), positive cytoplasmic pmTOR (HR 0.662, $p=0.026$), and positive cytoplasmic EpCAM (HR 0.648, $p=0.012$) were significant predictors for longer OS, whereas higher cytoplasmic CXCR2 (HR 1.568, $p=0.007$), and higher nuclear FEN1 (HR 1.424, $p=0.035$) were significant predictors for shorter OS.

Predictive Accuracy of Models

Predictive accuracy of the models from internal validation demonstrates good accuracy for predicting RFS and OS, with bootstrap-corrected C-index of 0.66 and 0.67 for RFS and OS, respectively. Calibration curves for 1-, 3- and 5-year OS and RFS estimates revealed acceptable model calibration, with good correlation between the OS and RFS estimates from the multivariate Cox model and those derived from Kaplan-Meier estimates (Supplementary Figure 2).

Stage I patients

We further evaluated the prognostic effect of these markers in stage I patients by Kaplan-Meier curves (Supplementary Figure 3) and fitting the same multivariate models for RFS and OS, excluding stage variable (Supplementary Tables 2 and 3). Adjusted for age, cytoplasmic CXCR2, cytoplasmic pAMPK and cytoplasmic pmTOR remained significant factors in RFS, and cytoplasmic pAMPK, cytoplasmic CXCR2 and nuclear FEN1 remained significant factors in OS. Positive cytoplasmic CXCR2 above the median (HR 1.673, $p=0.01$) was a significant predictor for shorter RFS, whereas positive cytoplasmic pAMPK (HR 0.581, $p=0.009$) and positive cytoplasmic pmTOR (HR 0.511, $p=0.003$) were significantly associated with longer RFS. Positive cytoplasmic pAMPK (HR 0.505, $p=0.003$) was a significant factor for longer OS, whereas higher cytoplasmic CXCR2 above median (HR 1.954, $p=0.004$), and higher nuclear FEN1 above median (HR 2.116, $p=0.001$) were significant predictors for shorter OS.

Discussion

We sought to investigate the impact of specific biomarkers and their relationship to outcome in early stage lung cancer patients. Based on our results, we have identified some important biomarker associations and begun early development of a risk model. Risk modeling is an evolving field in cancer biology. Studies incorporating clinical variables have evolved over the years (1, 2), and molecular epidemiology studies have identified germ line markers that predict for risk or benefit with certain interventions, including retinoids (30), statins (31), and celecoxib (32). The Director's Challenge Consortium, which created a large microarray database of resected adenocarcinoma samples, found that models incorporating both clinical data and gene expression data had an improved predictive accuracy compared to models using either alone. Models with only clinical variables were comparable to models with gene expression data alone and no clinical data (33), suggesting the importance of combining the two approaches. Our risk model incorporates both biomarkers and clinical factors and includes all histologic subtypes of non-small cell lung cancer.

We utilized immunohistochemistry because it is a widely available and clinically applicable technique which can be applied to FFPE tissues. We studied a number of phospho-proteins, which are known to be labile, however, we collected these samples using routine clinical standards (fixation in paraffin in about 30 minutes after tissue was sliced and placement in formalin for less than 24 hours). As these markers were prognostic under these conditions, we believe that they could be useful in routine clinical practice as well.

Several of our markers have previously been described as prognostic. In our study, expression of EpCAM predicted for improved OS and RFS. The literature on this topic is mixed, with studies in some malignancies suggesting worse outcomes with higher expression of EpCAM and in other malignancies finding an association with improved prognosis in cancers of the thyroid, kidney, and oral cavity (34, 35). FEN1 was found to be predictive of shorter OS in our study. This protein is involved in the replication and repair of DNA and has been associated with high grade tumors and poor prognosis (36). Another series from our institution confirmed our results by demonstrating that higher expression of FEN1 is a marker of poor prognosis in resected stage I lung cancer (22). IGF-1R predicted for shorter RFS in our group; this has previously been reported (37).

Our findings regarding pAMPK and pmTOR were intriguing; positivity for either marker was associated with improved RFS and OS. The mTOR signaling pathway is a complex pathway involved in energy sensing and control of cell growth (38). It has been implicated in carcinogenesis, and mTOR inhibitors are in clinical use for renal cell carcinoma and neuroendocrine tumors (39, 40). mTOR has been found to be a poor prognostic marker in other malignancies (41, 42), though other groups have reported that it is a marker of good prognosis in resected NSCLC (43, 44). Among its other roles, mTOR negatively regulates autophagy (45), which could explain why we found it to be a marker of good prognosis. Also, activity of mTOR is partially controlled by post-translational mechanisms; therefore, mTOR expression may not correlate with mTOR activity (46). AMPK is a negative regulator of mTOR activity, so it is somewhat surprising that both are found to be markers

of good prognosis; however, mTOR is also regulated by many other mechanisms (38). pAMPK has been reported as a marker of good prognosis elsewhere (20).

Our results suggest that individual protein IHC is unlikely to be clinically useful, as observed differences in outcome between favorable and unfavorable groups are small. Our study is somewhat limited due to a heterogeneous patient population, with multiple different histologies and with some patients who received adjuvant therapy and others who did not. We are not able to create a predictive model for the benefit of adjuvant chemotherapy based on our data. Though there have been numerous efforts by our group and others to create predictive and prognostic risk models, currently these models are not useful to select patients for therapy after surgical resection of NSCLC.

Lung cancer is a deadly malignancy, and cancer prevention and early detection are important goals. By gaining a better understanding of the biology of lung carcinogenesis, we hope to use this knowledge to accurately assess risk and personalize chemoprevention strategies following lung cancer resection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Spitz MR, Hong WK, Amos CI, Wu X, Schabath MB, Dong Q, et al. A risk model for prediction of lung cancer. *J Natl Cancer Inst.* 2007; 99:715–26. [PubMed: 17470739]
2. Spitz MR, Etzel CJ, Dong Q, Amos CI, Wei Q, Wu X, et al. An expanded risk prediction model for lung cancer. *Cancer Prev Res (Phila Pa).* 2008; 1:250–4.
3. Cassidy A, Myles JP, van Tongeren M, Page RD, Liloglou T, Duffy SW, et al. The LLP risk model: an individual risk prediction model for lung cancer. *Br J Cancer.* 2008; 98:270–6. [PubMed: 18087271]
4. De Torres JP, Bastarrika G, Wisnivesky JP, Alcaide AB, Campo A, Seijo LM, et al. Assessing the relationship between lung cancer risk and emphysema detected on low-dose CT of the chest. *Chest.* 2007; 132:1932–8. [PubMed: 18079226]
5. Olaussen KA, Dunant A, Fouret P, Brambilla E, Andre F, Haddad V, et al. DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. *N Engl J Med.* 2006; 355:983–91. [PubMed: 16957145]
6. Zheng Z, Chen T, Li X, Haura E, Sharma A, Bepler G. DNA synthesis and repair genes RRM1 and ERCC1 in lung cancer. *N Engl J Med.* 2007; 356:800–8. [PubMed: 17314339]
7. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011; 144:646–74. [PubMed: 21376230]

8. Jin Y, Tong DY, Chen JN, Feng ZY, Yang JY, Shao CK, et al. Overexpression of osteopontin, alphavbeta3 and Pim-1 associated with prognostically important clinicopathologic variables in non-small cell lung cancer. *PLoS One*. 2012; 7:e48575. [PubMed: 23119061]
9. Chaves-Perez A, Mack B, Maetzel D, Kremling H, Eggert C, Harreus U, et al. EpCAM regulates cell cycle progression via control of cyclin D1 expression. *Oncogene*. 2012
10. Byers LA, Holsinger FC, Kies MS, William WN, El-Naggar AK, Lee JJ, et al. Serum signature of hypoxia-regulated factors is associated with progression after induction therapy in head and neck squamous cell cancer. *Mol Cancer Ther*. 2010; 9:1755–63. [PubMed: 20530716]
11. Sun H, Chung WC, Ryu SH, Ju Z, Tran HT, Kim E, et al. Cyclic AMP-responsive element binding protein- and nuclear factor-kappaB-regulated CXC chemokine gene expression in lung carcinogenesis. *Cancer Prev Res (Phila)*. 2008; 1:316–28. [PubMed: 19138976]
12. Kim WY, Jin Q, Oh SH, Kim ES, Yang YJ, Lee DH, et al. Elevated epithelial insulin-like growth factor expression is a risk factor for lung cancer development. *Cancer Res*. 2009; 69:7439–48. [PubMed: 19738076]
13. Kim WY, Kim MJ, Moon H, Yuan P, Kim JS, Woo JK, et al. Differential impacts of insulin-like growth factor-binding protein-3 (IGFBP-3) in epithelial IGF-induced lung cancer development. *Endocrinology*. 2011; 152:2164–73. [PubMed: 21447628]
14. Kim JS, Kim ES, Liu D, Lee JJ, Solis L, Behrens C, et al. Prognostic impact of insulin receptor expression on survival of patients with nonsmall cell lung cancer. *Cancer*. 2012; 118:2454–65. [PubMed: 21952750]
15. Morgillo F, Woo JK, Kim ES, Hong WK, Lee HY. Heterodimerization of insulin-like growth factor receptor/epidermal growth factor receptor and induction of survivin expression counteract the antitumor action of erlotinib. *Cancer Res*. 2006; 66:10100–11. [PubMed: 17047074]
16. Tang XM, Varella-Garcia M, Xavier AC, Massarelli E, Ozburn N, Moran C, et al. Epidermal growth factor receptor abnormalities in the pathogenesis and progression of lung adenocarcinomas. *Cancer Prev Res*. 2008; 1:192–200.
17. Shen J, Behrens C, Wistuba II, Feng L, Lee JJ, Hong WK, et al. Identification and validation of differences in protein levels in normal, premalignant, and malignant lung cells and tissues using high-throughput Western Array and immunohistochemistry. *Cancer Res*. 2006; 66:11194–206. [PubMed: 17145864]
18. Jin Q, Feng L, Behrens C, Bekele BN, Wistuba II, Hong WK, et al. Implication of AMP-activated protein kinase and Akt-regulated survivin in lung cancer chemopreventive activities of deguelin. *Cancer Res*. 2007; 67:11630–9. [PubMed: 18089792]
19. Kim WY, Chang DJ, Hennessy B, Kang HJ, Yoo J, Han SH, et al. A novel derivative of the natural agent deguelin for cancer chemoprevention and therapy. *Cancer Prev Res (Phila)*. 2008; 1:577–87. [PubMed: 19139008]
20. William WN, Kim JS, Liu DD, Solis L, Behrens C, Lee JJ, et al. The impact of phosphorylated AMP-activated protein kinase expression on lung cancer survival. *Ann Oncol*. 2012; 23:78–85. [PubMed: 21430184]
21. Kadara H, Lacroix L, Behrens C, Solis L, Gu X, Lee JJ, et al. Identification of gene signatures and molecular markers for human lung cancer prognosis using an in vitro lung carcinogenesis system. *Cancer Prev Res (Phila)*. 2009; 2:702–11. [PubMed: 19638491]
22. Kadara H, Behrens C, Yuan P, Solis L, Liu D, Gu X, et al. A five-gene and corresponding protein signature for stage-I lung adenocarcinoma prognosis. *Clin Cancer Res*. 2011; 17:1490–501. [PubMed: 21163870]
23. Prudkin L, Behrens C, Liu DD, Zhou X, Ozburn NC, Bekele BN, et al. Loss and reduction of FUS1 protein expression is a frequent phenomenon in the pathogenesis of lung cancer. *Clin Cancer Res*. 2008; 14:41–7. [PubMed: 18172250]
24. Behrens C, Lin HY, Lee JJ, Raso MG, Hong WK, Wistuba II, et al. Immunohistochemical expression of basic fibroblast growth factor and fibroblast growth factor receptors 1 and 2 in the pathogenesis of lung cancer. *Clin Cancer Res*. 2008; 14:6014–22. [PubMed: 18829480]
25. Behrens C, Feng L, Kadara H, Kim HJ, Lee JJ, Mehran R, et al. Expression of interleukin-1 receptor-associated kinase-1 in non-small cell lung carcinoma and preneoplastic lesions. *Clin Cancer Res*. 2010; 16:34–44. [PubMed: 20028769]

26. Harrel, F. Regression modeling strategies. New York: Springer-Verlag; 2001.
27. Hanley JA, McNeil BJ. The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology*. 1982; 143:29–36. [PubMed: 7063747]
28. Ephron B, Tibishirani R. Improvements on cross-validation: the .632+ bootstrap method. *JASA*. 1997; 92:548–60.
29. Harrell, FE. Regression Modeling Strategies, with Applications to Linear Models, Logistic Regression, and Survival Analysis. Springer; 2011.
30. Lee JJ, Wu X, Hildebrandt MA, Yang H, Khuri FR, Kim E, et al. Global assessment of genetic variation influencing response to retinoid chemoprevention in head and neck cancer patients. *Cancer Prev Res (Phila)*. 2011; 4:185–93. [PubMed: 21292633]
31. Lipkin SM, Chao EC, Moreno V, Rozek LS, Rennert H, Pinchev M, et al. Genetic variation in 3-hydroxy-3-methylglutaryl CoA reductase modifies the chemopreventive activity of statins for colorectal cancer. *Cancer Prev Res (Phila Pa)*. 2010; 3:597–603.
32. Chan AT, Zauber AG, Hsu M, Breazna A, Hunter DJ, Rosenstein RB, et al. Cytochrome P450 2C9 variants influence response to celecoxib for prevention of colorectal adenoma. *Gastroenterology*. 2009; 136:2127–36 e1. [PubMed: 19233181]
33. Shedden K, Taylor JMG, Enkemann SA, Tsao MS, Yeatman TJ, Gerald WL, et al. Gene expression-based survival prediction in lung adenocarcinoma: a multi-site, blinded validation study. *Nature Medicine*. 2008; 14:822–7.
34. Fong D, Steurer M, Obrist P, Barbieri V, Margreiter R, Amberger A, et al. Ep-CAM expression in pancreatic and ampullary carcinomas: frequency and prognostic relevance. *J Clin Pathol*. 2008; 61:31–5. [PubMed: 16775119]
35. Seligson DB, Pantuck AJ, Liu X, Huang Y, Horvath S, Bui MH, et al. Epithelial cell adhesion molecule (KSA) expression: pathobiology and its role as an independent predictor of survival in renal cell carcinoma. *Clin Cancer Res*. 2004; 10:2659–69. [PubMed: 15102668]
36. Lam JS, Seligson DB, Yu H, Li A, Eeva M, Pantuck AJ, et al. Flap endonuclease 1 is overexpressed in prostate cancer and is associated with a high Gleason score. *BJU Int*. 2006; 98:445–51. [PubMed: 16879693]
37. Nakagawa M, Uramoto H, Oka S, Chikaishi Y, Iwanami T, Shimokawa H, et al. Clinical significance of IGF1R expression in non-small-cell lung cancer. *Clin Lung Cancer*. 2012; 13:136–42. [PubMed: 22133293]
38. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol*. 2011; 12:21–35. [PubMed: 21157483]
39. Motzer RJ, Escudier B, Oudard S, Hutson TE, Porta C, Bracarda S, et al. Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial. *Lancet*. 2008; 372:449–56. [PubMed: 18653228]
40. Yao JC, Phan AT, Chang DZ, Wolff RA, Hess K, Gupta S, et al. Efficacy of RAD001 (everolimus) and octreotide LAR in advanced low- to intermediate-grade neuroendocrine tumors: results of a phase II study. *J Clin Oncol*. 2008; 26:4311–8. [PubMed: 18779618]
41. Pantuck AJ, Seligson DB, Klatte T, Yu H, Leppert JT, Moore L, et al. Prognostic relevance of the mTOR pathway in renal cell carcinoma: implications for molecular patient selection for targeted therapy. *Cancer*. 2007; 109:2257–67. [PubMed: 17440983]
42. Prins MJ, Verhage RJ, Ruurda JP, ten Kate FJ, van Hillegersberg R. Over-expression of phosphorylated mammalian target of rapamycin is associated with poor survival in oesophageal adenocarcinoma: a tissue microarray study. *J Clin Pathol*. 2013; 66:224–8. [PubMed: 23162107]
43. Anagnostou VK, Bepler G, Syrigos KN, Tanoue L, Gettinger S, Homer RJ, et al. High expression of mammalian target of rapamycin is associated with better outcome for patients with early stage lung adenocarcinoma. *Clin Cancer Res*. 2009; 15:4157–64. [PubMed: 19509151]
44. Oh MH, Lee HJ, Yoo SB, Xu X, Choi JS, Kim YH, et al. Clinicopathological correlations of mTOR and pAkt expression in non-small cell lung cancer. *Virchows Arch*. 2012; 460:601–9. [PubMed: 22562131]
45. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol*. 2011; 13:132–41. [PubMed: 21258367]

46. Gingras AC, Raught B, Sonenberg N. Regulation of translation initiation by FRAP/mTOR. *Genes Dev.* 2001; 15:807–26. [PubMed: 11297505]

Translational Relevance

Many patients with surgically resected non-small lung cancer will eventually develop tumor recurrence or metastasis. We are not yet able to predict who is at highest risk for recurrent disease. In this study, we evaluated a number of biomarkers identified in our laboratories as particularly important in carcinogenesis and correlated their expression to prognosis of patients with surgically resected tumors to create a risk model for tumor recurrence and survival. With an improved understanding of the mechanisms of early stage disease, our goals are to create a program of personalized chemoprevention and therapy for all patients at risk for lung cancer. For example, studies like this could help us to improve the adjuvant treatment of patients with cancer, by sparing toxicities of chemotherapy for those with a good prognosis and identifying patients with poor prognosis who may benefit from clinical trials utilizing novel therapeutics.

Figure 1.

Photomicrographs showing immunohistochemical expression in malignant cells of NSCLC tissue specimens of the five prognostic protein markers by histologic type. Cases with high and low expression are represented. p-AMPK, p-mTOR, EpCAM and CXCR2 show mostly cytoplasmic expression in malignant cells. p-mTOR and EpCAM show also distinct membrane staining. FEN1 show nuclear expression. Original magnification, $\times 200$.

Figure 2

Figure 2.

Kaplan-Meier curves for RFS and OS by various marker groups. (A) RFS and (B) OS by cytoplasmic expression of pAMPK, positive (blue line) vs negative (black line). (C) RFS and (D) OS by cytoplasmic expression of CXCR2, at or above the median (blue line) vs below the median (black line). (E) RFS and (F) OS by cytoplasmic expression of EPCAM, positive (blue line) vs negative (black line)

Table 1
Demographic Data

		N (%)	RFS HR (95% CI)	OS HR (95% CI)
Age		Median 65.7 (range: 32-90)		
Gender	Female	184 (49.7%)	1	1
	Male	186 (50.3%)	1.37 (1.04, 1.80)*	1.48 (1.08, 2.03)*
Race	African American	21 (5.7%)	1 [#]	1
	Other	19 (5.1%)		
	Caucasian	330 (89.2%)	0.82 (0.53, 1.27)	0.77 (0.48, 1.24)
Smoking status	Never	38 (10.3%)	1	1
	Former	170 (45.9%)	1.05 (0.65, 1.71)	1.02 (0.58, 1.78)
	Current	162 (43.8%)	1.10 (0.68, 1.79)	1.22 (0.70, 2.13)
Stage	I	234 (63.2%)	1	1
	II	75 (20.3%)	1.68 (1.20, 2.36)*	1.49 (1.02, 2.20)*
	IIIA	61 (16.5%)	2.59 (1.84, 3.64)*	2.29 (1.56, 3.38)*
Histologic subtype	Squamous	126 (34.1%)	1	1
	Adenocarcinoma	227 (61.4%)	0.81 (0.61, 1.07)	0.83 (0.60, 1.15)
	Other**	17 (4.6%)	0.64 (0.31, 1.32)	0.69 (0.30, 1.60)
Neoadjuvant treatment	No	313 (85.3%)	1	1
	Yes	54 (14.7%)	1.69 (1.19, 2.41)*	1.73 (1.17, 2.56)*
	Unknown ^{##}	3		
Adjuvant treatment	No	224 (63.6%)	1	1
	Yes	128 (36.4%)	1.23 (0.93, 1.65)	1.00 (0.71, 1.40)
	Chemotherapy	103 (29.2%)		
	Radiation	46 (13.1%)		
	Both	21 (5.9%)		
	Unknown ^{##}	18		

* P<0.05.

[#] African American and other races together as the reference group.

** Other histologies include adenosquamous carcinoma (11), large cell carcinoma (1), sarcomatoid carcinoma (2), and non-small cell lung carcinoma not otherwise specified (3)

^{##} These patients were not included in final multivariate model

Table 2
2- and 5- Year Recurrence Free Survival Rates and Overall Survival Rates by Stage and Biomarker Groups

Variable	RFS(95% CI)			OS (95% CI)		
	2-year	5-year	5-year	2-year	5-year	5-year
Stage						
I	74% (69%, 80%)	54% (48%, 61%)	87% (82%, 91%)	68% (62%, 75%)		
II	57% (47%, 70%)	35% (25%, 48%)	83% (74%, 92%)	51% (41%, 64%)		
III	41% (30%, 55%)	24% (15%, 38%)	57% (46%, 71%)	44% (33%, 59%)		
m-Insulin receptor						
0	70% (64%, 76%)	48% (42%, 55%)	84% (79%, 89%)	62% (56%, 70%)		
Positive	58% (50%, 66%)	40% (33%, 49%)	76% (69%, 83%)	58% (50%, 66%)		
c-pAMPK						
0	55% (47%, 65%)	33% (25%, 44%)	77% (70%, 86%)	52% (43%, 62%)		
Positive	69% (64%, 75%)	50% (44%, 57%)	82% (78%, 87%)	64% (59%, 71%)		
c-pmTOR						
0	53% (44%, 65%)	34% (25%, 45%)	69% (60%, 80%)	48% (38%, 60%)		
Positive	69% (64%, 74%)	49% (43%, 55%)	84% (80%, 89%)	65% (59%, 71%)		
c-CXCR2						
<Median	70% (63%, 77%)	49% (42%, 57%)	83% (78%, 89%)	66% (60%, 74%)		
>=Median	61% (54%, 68%)	41% (35%, 49%)	78% (73%, 85%)	55% (48%, 63%)		
c-EPCAM						
0	58% (50%, 67%)	39% (31%, 48%)	76% (69%, 84%)	51% (43%, 61%)		
Positive	69% (63%, 75%)	49% (43%, 56%)	84% (79%, 89%)	66% (60%, 73%)		
n-FENI						
<Median	69% (63%, 77%)	50% (43%, 58%)	85% (80%, 91%)	68% (62%, 76%)		
>=Median	61% (54%, 68%)	41% (34%, 49%)	76% (70%, 83%)	54% (47%, 62%)		

Table 3
Multicovariate Cox Model for Recurrence Free Survival

Variable	Hazard Ratio (95% CI)	P-value
<i>Age</i>	1.024 (1.009, 1.039)	0.001
<i>Stage (II vs I)</i>	1.765 (1.239, 2.516)	0.002
<i>(III vs I)</i>	2.676 (1.873, 3.824)	<0.001
<i>c-IGF-1R (per 100 increase)</i>	1.517 (1.018, 2.259)	0.040
<i>m-Insulin receptor (Pos vs 0)</i>	1.442 (1.085, 1.915)	0.012
<i>c-pAMPK (Pos vs 0)</i>	0.648 (0.483, 0.870)	0.004
<i>c-pmTOR (Pos vs 0)</i>	0.696 (0.502, 0.963)	0.029
<i>c-CXCR2 (above vs below median)</i>	1.360 (1.017, 1.820)	0.038
<i>c-EPCAM (Pos vs 0)</i>	0.708 (0.524, 0.956)	0.024
<i>m-CASK (per 100 increase)</i>	0.680 (0.470, 0.998)	0.049

c: cytoplasmic, m: membrane

Table 4
Multicovariate Cox Model for Overall Survival

Variable	Hazard Ratio (95% CI)	P-value
<i>Age</i>	1.028 (1.011, 1.046)	0.001
<i>Stage (II vs I)</i>	1.425 (0.950, 2.140)	0.087
<i>(III vs I)</i>	2.620 (1.737, 3.951)	<0.001
<i>c-pAMPK (Pos vs 0)</i>	0.669 (0.480, 0.932)	0.018
<i>c-pmTOR (Pos vs 0)</i>	0.662 (0.460, 0.952)	0.026
<i>c-CXCR2 (above vs below median)</i>	1.568 (1.132, 2.173)	0.007
<i>c-EPCAM (Pos vs 0)</i>	0.648 (0.461, 0.910)	0.012
<i>n-FEN1 (above vs below median)</i>	1.424 (1.024, 1.980)	0.035

c: cytoplasmic, n: nuclear