

Identification and Validation of Differences in Protein Levels in Normal, Premalignant, and Malignant Lung Cells and Tissues Using High-Throughput Western Array and Immunohistochemistry

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Abstract

The identification of proteins, which exhibit different levels in normal, premalignant, and malignant lung cells, could improve early diagnosis and intervention. We compared the levels of proteins in normal human bronchial epithelial (NHBE) and tumorigenic HBE cells (1170-I) by high-throughput immunoblotting (PowerBlot Western Array) using 800 monoclonal antibodies. This analysis revealed that 87 proteins increased by >2-fold, and 45 proteins decreased by >2-fold, in 1170-I compared with NHBE cells. These proteins are involved in DNA synthesis and repair, cell cycle regulation, RNA transcription and degradation, translation, differentiation, angiogenesis, apoptosis, cell adhesion, cytoskeleton and cell motility, and the phosphatidylinositol 3-kinase signaling pathway. Conventional Western blotting using lysates of normal, immortalized, transformed, and tumorigenic HBEs and non-small cell lung cancer cell lines confirmed some of these changes. The expression of several of these proteins has been then analyzed by immunohistochemistry in tissue microarrays containing 323 samples, including normal bronchial epithelium, hyperplasia, squamous metaplasia, dysplasias, squamous cell carcinomas, atypical adenomatous hyperplasia, and adenocarcinomas from 144 patients. The results of the immunohistochemical studies correlated with the Western blotting findings and showed gradual increases (caspase-8, signal transducers and activators of transcription 5, and p70s6K) or decrease (E-cadherin) in levels with tumor progression. These results indicate that the changes in proteins detected in this study may occur early in lung carcinogenesis and persist in lung cancer. In addition, some of the proteins detected by this approach may be novel biomarkers for early detection of lung cancer and novel targets for chemoprevention or therapy. (Cancer Res 2006; 66(23): 11194-206)

Introduction

The leading cause of cancer death among both men and women, lung cancer is predicted to cause 163,510 deaths in the United States in 2005 (1, 2). The poor early detection of lung cancer coupled with

ineffective treatments for advanced disease is responsible for the low (<17%) overall 5-year survival rate (1, 3). Therefore, new approaches to early diagnosis and prognosis of lung cancer are urgently needed (1, 3). The development of genomics has provided opportunities for global analysis of gene expression, and, indeed, several studies have identified differences in gene expression between normal and malignant lung cells using techniques such as serial analysis of gene expression (4) and cDNA microarray (5, 6). However, the levels of mRNA do not always reflect accurately the levels of the corresponding proteins nor do they reveal changes in epigenetic posttranscriptional modulation of proteins (e.g., phosphorylation, acetylation, methylation, ubiquitination, sumoylation, ADP ribosylation, glycosylation, myristoylation, isoprenylation, etc.) or changes in protein degradation rates. Therefore, analysis of differences in protein levels or modifications is important to complement studies on mRNA expression (7–9). Most proteomic studies have used two-dimensional PAGE combined with mass spectrometry (9, 10). More recently, surface-enhanced laser desorption/ionization time-of-flight (TOF) mass spectrometry (MS) using chips with modified surface properties (11) has been used for detection of protein changes between different types of cancer (12–15). However, only limited studies have applied proteomic approaches to lung cancer (9, 16, 17). A recent study has used matrix-assisted laser desorption/ionization MS (MALDI-MS) directly from frozen tissue sections from resected non-small cell lung cancer (NSCLC) for profiling of protein expression to predict histologic groups as well as nodal involvement and survival (18). The specificity and high affinity of antibodies has been applied to proteomic analysis through an antibody-chip, which is a new approach to biomarker discovery for diagnosis and protein target identification (19). Another method known as BD PowerBlot Western Array Screening system (Becton Dickinson Biosciences, San Jose, CA) is a high-throughput Western immunoblotting method, which uses mixtures of subsets of about 800 monoclonal antibodies to evaluate differences in levels of cellular signaling proteins between total cell extracts from different cells or tissues. We used this method to identify protein changes between normal, immortalized, transformed, and tumorigenic lung epithelial cell lines and NSCLC cell lines and examined their differential expression in resected human lung specimens, including normal, premalignant, and malignant tissues, using immunohistochemistry.

Materials and Methods

Cells and culture conditions. Normal bronchial epithelial cells (NHBE) were purchased from Whittaker-Clonetics (San Diego, CA). NSCLC cell lines H460, H1792, SK-MES-1, Calu-1, and A549 were obtained from Dr. Adi Gazdar

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(University of Texas Southwestern, Dallas, TX). Immortalized BEAS-2B cells (20) were obtained from Dr. Curtis Harris (National Cancer Institute, Bethesda, MD). Immortalized 1799, transformed 1198, and tumorigenic 1170-I bronchial epithelial cells derived from BEAS-2B as described by Klein-Szanto et al. (21) were obtained from Dr. Jonathan Kurie (University of Texas M.D. Anderson Cancer Center, Houston, TX) and Dr. Klein Szanto (Fox Chase, Philadelphia, PA). NHBE cells, the BEAS-2B cells, and the 1799 cells were grown in keratinocyte serum-free medium (Life Technologies, Inc., Rockville, MD) supplemented with bovine pituitary extract (50 µg/mL) and epidermal growth factor (5 ng/mL). The 1198 cells and the 1170-I cells were grown in keratinocyte serum-free medium supplemented with bovine pituitary extract and with 3% fetal bovine serum (FBS). NSCLC cell lines were maintained in a 1:1 mixture of DMEM/Ham's F12 medium supplemented with 5% FBS. The cells were maintained in a humidified incubator in a 95% air/5% CO₂ atmosphere at 37°C.

BD PowerBlot Western Array. Total cellular proteins were extracted from NHBE and 1170-I cells using a lysis buffer containing 10 nmol/L Tris (pH 7.4), 1.0 mmol/L sodium orthovanadate, 1.0% SDS. Three milliliters of boiling lysis solution were added per 15-cm plate to cell monolayer that had been washed with PBS. The lysate was heated and sonicated for 10 to 30 seconds. The protein concentration was determined with the Protein Assay kit (Bio-Rad, Hercules, CA). Protein extracts were frozen and sent on dry ice to BD Biosciences for analysis. The samples were analyzed by SDS-PAGE in 16 × 16 × 0.1 cm, 5% to 15% gradient polyacrylamide slab gels. Cell extract containing 400 µg of protein was loaded in one well across the entire width of the gel. The gel was run overnight. The proteins were then transferred electrophoretically to Immobilon-P nylon membrane (Millipore, Bedford, MA) for 1 hour at 1 A. After transfer, the membrane was blocked for 1 hour with 5% milk. Next, the membrane was clamped with a Western blotting manifold that isolates 45 channels across the membrane parallel to the direction of electrophoresis. In each channel, a mixture of four to five antibodies was added and allowed to hybridize for 1 hour. Different sets of antibodies (templates A-D) were used for each membrane. The blot was washed and hybridized for 30 minutes with secondary goat anti-mouse horseradish peroxidase. The membrane was washed and developed with chemiluminescence using SuperSignal West Pico from Pierce Biotechnology (Rockford, IL). Chemiluminescence signals are captured using the Kodak Image Station CCD Camera. Differences in protein level are estimated by densitometric scanning and normalized using internal standards. The method is highly specific and can detect subnanogram levels of protein.

Protein extraction and Western blotting analysis. Cells were washed in PBS and lysed in a buffer containing 50 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 0.1% SDS, 1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 5 µg/mL aprotinin, and 5 µg/mL leupeptin. After incubation on ice for 15 minutes and centrifugation at 12,000 rpm for 10 minutes, the supernatants were collected, and the protein concentration was determined with the Protein Assay kit (Bio-Rad). Protein (50 µg) was electrophoresed through a 10% polyacrylamide gel under nonreducing conditions and transferred to a nitrocellulose membrane (Bio-Rad) by electroblotting. After transfer, the blots were incubated with first antibodies at room temperature for 1 hour. Monoclonal antibodies to Akt, Annexin VI, basic fibroblast growth factor (bFGF), E-cadherin, CaMK kinase (CaMKK), caspase-8, caspase-9, cyclin-dependent kinase 1 (CDK1), cyclin D3, DFF45, Eg5, poly(ADP-ribose) polymerase (PARP), PDK1, p53BP2, p70S6K, phosphatidylinositol 3-kinase (PI3K), and p110α were obtained from BD Transduction Laboratories (Lexington, KY). Monoclonal antibodies to Bad and PTEN were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Monoclonal antibody to p21 was obtained from Exalpha Biologicals (Boston, MA). Monoclonal antibody to Bax and p53 and polyclonal antibody to Bid were obtained from Oncogene Research Products (Boston, MA). Monoclonal antibodies to Bcl-X/L, Bcl-2, caspase-3, caspase-6, and caspase-7 and polyclonal antibodies to cyclin B1 and FGFR-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody to β-actin was obtained from Sigma Chemical Co. (St. Louis, MO). Antibody to galectin-3 was from Vector Laboratories (Burlingame, CA). The membranes were then washed with PBS

and incubated with secondary antibodies for 1 hour at room temperature. The membranes were then washed with PBS, and antibody binding was detected using the enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ) and Hyperfilm MP (Amersham). The films were subjected to scanning densitometry to quantitate band intensity by using the NIH Image software.

Case selection and tissue microarray preparation. Formalin-fixed paraffin-embedded specimens from surgically resected primary NSCLC at pathology stages I to IV from patients who had no prior chemotherapy or radiotherapy were obtained from the Lung Cancer Specialized Program of Research Excellence Tissue Bank at the M.D. Anderson Cancer Center. H&E-stained slides of these cases were reviewed by an experienced pathologist (I.W.) and selected to contain normal bronchial epithelium and premalignant lesions besides the cancers. The 323 tissue specimens collected from 144 patients included 29 adenocarcinomas and 30 squamous cell carcinomas (SCC). In addition, we selected 30 normal bronchial epithelia, 74 bronchial hyperplasias, 22 squamous metaplasias, 63 squamous dysplasias and carcinomas *in situ* (16 mild and moderate dysplasias, grouped as low grade and 47 severe dysplasias and carcinomas *in situ*, grouped as high grade), and 75 atypical adenomatous hyperplasias (AAH). Tumors and lesions were classified according the latest WHO 1999 criteria. We considered normal bronchial epithelium, hyperplasia, squamous metaplasia, low-grade dysplasia, high-grade dysplasia, and SCC as representing different steps in the development of SCCs. AAH was considered a premalignant lesion for adenocarcinoma development. Tissue microarrays were prepared with a manual tissue arrayer (Advanced Tissue Arrayer ATA100, Chemicon International, Temecula, CA) using 1-mm-diameter cores in triplicate for tumors and 1.5-mm cores for normal epithelial and premalignant lesions. Some cases were represented in the tissue microarray by several cores. One H&E-stained section for each tissue microarray prepared was examined to determine the presence of the corresponding histology.

Immunohistochemical stainings. Four-micrometer-thick histology sections from tissue microarrays were cut, deparaffinized using standard xylene, and hydrated through graded alcohol into water. Immunohistochemical analysis was done using monoclonal antibodies against E-cadherin (G-10, Santa Cruz Biotechnology) and p70s6k (Thr³⁸⁹, Cell Signaling Technology) and polyclonal antibodies against caspase-8 (E-20, Santa Cruz Biotechnology) and signal transducers and activators of transcription 5 (STAT5; C-17, Santa Cruz Biotechnology). Antigen retrieval was done for all markers using steaming at 95°C for 30 minutes in 10 mmol/L citrate buffer (pH 6; DakoCytomation Target Retrieval, DAKO Corp., Carpinteria, CA). Endogenous peroxidase and nonspecific binding were blocked by using 3% hydrogen peroxide in TBST (DAKO; wash buffer 10×) and with TBST diluted 1:10 in FBS for 30 minutes, respectively. Then, the sections were incubated with primary antibodies (diluted in TBST-FBS solution) for 2 hours at room temperature. Depending on the origin of the primary antibody, DakoCytomation Envision⁺ Dual Link System or LSAB⁺ (DAKO) were used as secondary antibodies, following the corresponding manufacturer's instructions. Diaminobenzidine was used as a chromogen, and commercial hematoxylin was used for counterstaining. Corresponding negative controls were incubated in the absence primary antibody.

A trained pathologist (I.W.) examined immunohistochemical expression of the antigens as follows: E-cadherin in the cell membrane, caspase-8 and STAT5 in the cytoplasm, and p70s6k in the nuclei. Both the extent and intensity of immunopositivity were considered for the membrane and cytoplasmic immunostaining, with intensity ranging from 0 to 3 and the extent of staining from 0% to 100%. The final staining score (range = 0-300) was obtained by multiplying both scores. For nuclear immunostaining, the percentage of positive nuclei in 200 cells examined was calculated. Representative examples of stained tissues were photographed under the microscope.

Statistical analysis. Descriptive statistics were used to quantify the protein fold change in the cell line studies. Summary statistics of each biomarker at each level of histology diagnosis were provided. The distribution of the immunohistochemical expression of the each marker at the various histologies was shown in box plots. Repeated-measures

ANOVA models were fitted to estimate effect of histology on the biomarker scores using SAS (22). All tests are two sided. *P*s < 0.05 were considered statistically significant.

Results

Comparison of protein levels in NHBE and 1170-I cells by PowerBlot analysis. Chemiluminescence images of two PowerBlot Western immunoblots obtained with a subset of the 800 antibodies (Fig. 1A and B) show proteins with increased levels (CaMKK,

procaspase-7, N-cadherin, nexilin, RANBP1, and p70s6k) or decreased levels (γ -catenin) in the tumorigenic 1170-I cells relative to NHBE cells. Additional examples of individual proteins and internal control proteins from NHBE and 1170-I cell lysates, which were found to change after analysis of different blots probed with different subsets of antibodies, are presented in Fig. 1C and D. Table 1 presents the summary of such results obtained for proteins showing mostly >2-fold change (increase or decrease) in tumorigenic versus normal cells. Most of the changes were between 2- and

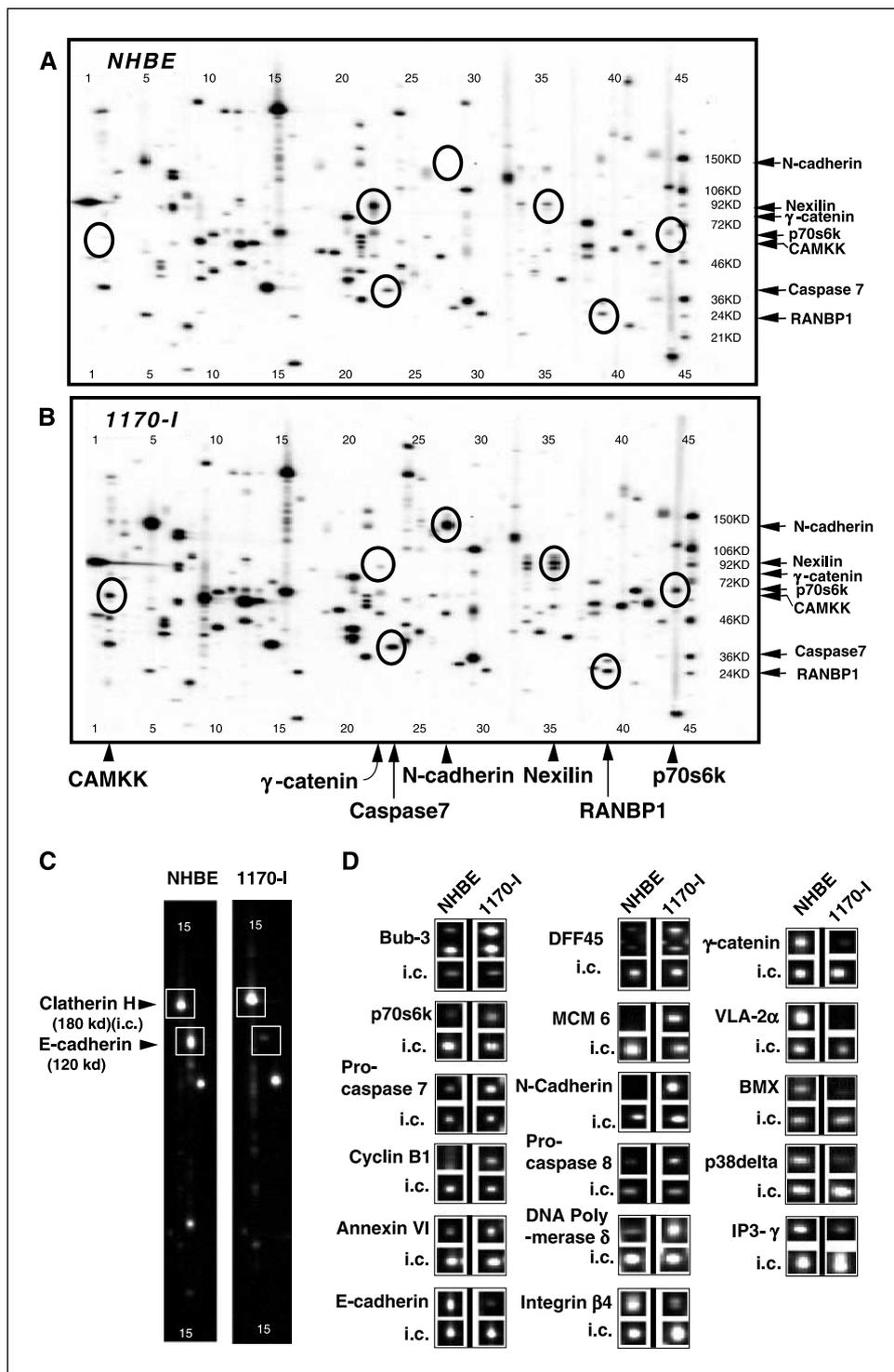


Figure 1. PowerBlot Western array analysis of proteins from normal (NHBE) and tumorigenic (1170-I) lung epithelial cells. Total protein extracts from each of the two cell types were analyzed by SDS-PAGE (extract from one cell type applied across the top of the slab gel) and processed for high-throughput immunoblotting using about 200 antibodies (Becton Dickinson). A and B, mixtures containing four to five antibodies from template B of this collection were added in each of 45 lanes of a manifold placed on top of the nylon membrane onto which the proteins were blotted (see Materials and Methods). The numbers above and below the gel indicate the migration of proteins with defined molecular weights (lane 45). Several proteins with different levels in normal and tumorigenic cells were encircled and identified by name and arrows on the right and below each blot. C and D, examples of PowerBlot Western Array results for individual proteins from different blots probed with different templates. C, segments (lanes 15-17) of two immunoblots probed with the same template of antibodies showing decreased level of E-cadherin in 1170-I cells and no change in another protein (clathrin H) that was selected as an internal control (i.c.). D, examples of changes in levels of different proteins sampled from different blots. Each differentially expressed protein is accompanied by another unrelated and unchanged protein from the same blot as an internal control. The negative of the original data for increased contrast.

Table 1.

A. Proteins increased in 1170-I compared with NHBE cells

Protein	Function	Increased fold
DNA synthesis and DNA repair		
DHFR	A protein that regenerates tetrahydrofolate, which is the coenzyme for thymidilate synthetase	##
DNA pol epsi	Functions in DNA replication and repair	18.9
DNA polyd	Essential for DNA replication with proofreading function	5.0
FEN-1	An exonuclease and endonuclease that is essential for maintaining DNA integrity during replication and after damage by alkylating agents or UV	4.5
Mre 11	Functions in a multiprotein complex that possesses endonuclease and 3' to 5' exonuclease activity during DNA maintenance and repair	##
MSH2	DNA mismatch repair gene	4.2
MSH6	DNA mismatch repair gene	9.0
Mupp 1	Important in protein-protein interactions during G-protein-coupled receptor signaling	##
NMT-2	A myristoyltransferase that catalyzes NH ₂ -terminal myristoylation, a cotranslational lipid modification form at cell-matrix junctions	8.6
PCNA	A nuclear antigen in proliferating cells and a subunit for DNA polymerase δ	6.0
PRK1	Binds to Rho-GTP and may regulate cytoskeleton changes	##
Rad50	Together with Mre11 and Nbs1 forms a complex that is involved in recombination and DNA damage checkpoints in the cellular response to DNA double-strand break	##
TOPO IIa	A ubiquitous ATP-dependent type II topoisomerase that is essential in DNA replication, transcription, chromatin segregation, and cell cycle progression	##
WRN	A RECQ-like helicase, which is deficient in Werner syndrome and plays a critical structural role in DNA double-strand break repair	3.1
XPA	A DNA-binding protein in the nucleotide excision repair pathway, which modulates damage recognition	##
Transcription and RNA degradation		
ARNT-1	Subunit of the hypoxia inducible factor-1, which is a constitutively expressed basic helix-loop-helix transcription factor	##
Ataxin-2	A protein important for RNA splicing or protein complex formation in many normal tissues, whereas polyglutamine containing ataxin-2 leads to neuropathology	##
Dek	A DNA binding protein that functions in transcriptional regulation and signal transduction; DEK-CAN fusion protein is oncogenic	##
LEDGF	A transcription activator that is essential for cell growth and survival	##
RNase H1	Specifically degrades the RNA moiety of RNA-DNA hybrids	5.9
Spot 14	An important transcription factor involved with normal lipogenesis in response to changes in the level of hormones and diet	4.1
STAT5	A member of the STAT family, which are critical mediators of the biological activity of cytokines by promoting transcription of cytokine-inducible genes	##
TEF-1	A member of a family of transcription factors that contain a conserved DNA-binding domain TEA/ATT	11.6
TFIIB	A ubiquitous factor required for transcription initiation by RNA polymerase II	2.3
TIF2	A nuclear receptor coactivator that associates with CREB-binding protein, and MOZ-TIF2 fusion protein is associated with acute myeloid leukemia	##
Translation		
DDX1	A RNA helicase (DEAD box protein) that function in all aspects of RNA metabolism, including translation, ribosome biogenesis, and pre-mRNA splicing	##
IAK1	One of the factors that control polyadenylation and translation in <i>Xenopus</i> oocytes, which also phosphorylates and polyadenylates CPEB	##
p70s6K	A kinase of ribosomal protein S6, which is a regulator of protein synthesis	3.0
Cell cycle regulation		
ALM1	A protein that is essential for cleavage furrowing and onset of cytokinesis in cell cycle	##
Bub3	A protein that associates with Bub1 to sense kinetochore attachment to microtubules during prometaphase to metaphase transition	3.8
CaMKK	An upstream regulator in Ca ²⁺ /CaM-dependent neural processes and a kinase of Akt	##
Cas K II β	A serine/threonine protein kinase, which phosphorylates p34cdc2, c-Jun, and nuclear matrix protein and is required for cell cycle regulation and associated with the nuclear matrix	##
Cdk1	Cdc2, a serine/threonine protein kinase, whose binding with cyclin B initiates the cell's entry into mitosis	##

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Table 1. (Cont'd)

A. Proteins increased in 1170-I compared with NHBE cells

Protein	Function	Increased fold
Chk1	A cell cycle regulatory protein kinase that inactivates Cdc2 and arrest cell cycle	5.0
Cyclin B1	A subunit for the cdc2 (Cdk1) protein kinase	##
Cyclin D3	A D-type cyclin that is associated with Cdk5 and weakly with Cdk2 and downstream of p70S6K in PI3K pathway	1.5*
EGFR	Receptor of epidermal growth factor, is essential for epithelial cell proliferation and differentiation and overexpressed in many epithelial cancers	##
Eg5	A mitotic protein that regulates spindle formation	16.2
HEC	A protein that interacts with Rb and plays a role in chromosome segregation during the M phase of the cell cycle	13.1
MCM6	A nuclear protein that performs essential functions in regulation of chromatin replication	31.6
Metablastin	Regulates the dynamics of microtubule during interphase of the cell cycle by inducing microtubule catastrophes	##
p16ink4	A specific inhibitor of cdk4, and inhibits cdk4-dependent phosphorylation of Rb and Rb-related proteins, p107 and p130	##
PP2C delta	A ubiquitous serine/threonine phosphatase that is important for biological processes, such as cell cycle progression	8.2
Apoptosis		
Akt	A kinase of PI3K pathway that is related to cell survival	2.6*
Bax	A proapoptotic member of Bcl-2 family	2.8*
53BP2	Bind Bcl2, p53, and p65 subunit of nuclear factor-κB to regulate apoptosis	3.9
Bcl-X	A member of the Bcl-2 protein family that is involved in apoptosis	##
Bid	A proapoptotic member of the Bcl2 protein family	##
Caspase-7	A cysteine protease that promote apoptosis	1.7*
Caspase-8	Initiator caspase activated by death receptor and cleaves proapoptotic Bid	
CD40	A member of the TNF receptor family, which involved in B-cell survival, growth, differentiation, and immunoglobulin class switching	2.6
DFF45	A subunit of DFF that is cleaved by caspase-3 and is essential for DNA fragmentation during apoptosis	3.2*
FAF	A protein that binds to the intracellular portion of FAS/Apo-1 and potentiates apoptosis in L-cells	2.4
Metaxin	A protein located on the outer membrane of mitochondria and is required for TNF-induced cell death	##
p53	A tumor suppressor gene	##
PARP	One of the earliest proteins targeted by caspase-3 during apoptosis	4.5*
PK1	A kinase of PI3K pathway that activates downstream Akt	2.0*
Cytoskeleton and cell motility		
cdc42GAP	One of the small GTPases that regulate cytoskeletal reorganization leading to changes in cell morphology and cell motility	3.7
CR1K	A serine/threonine kinase that functions downstream of Rho during cytokinesis and synaptic transmission	##
DMPK	A putative serine/threonine kinase from the Rho-associated enzyme family, which is related to the actin cytoskeleton and membrane proteins	##
PRK1	Binds to Rho-GTP and may regulate cytoskeleton changes	##
tau	A microtubule-associated protein whose dysfunction may lead to some neurodegenerative diseases	7.8
Cell adhesion		
Hic 5	A focal adhesion protein that binds to focal adhesion kinases and PYK2/CAKb	3.4
N-cadherin	A member of the cadherin family of Ca ²⁺ -dependent intercellular adhesion molecules	##
R-cadherin	A member of the cadherin family of Ca ²⁺ -dependent intercellular adhesion molecules	##
MDC9	ADAM9, a member of the ADAM family of metalloproteases, which play important roles in cell-cell fusion, intracellular signaling, and other cellular functions	3.1
Nexilin	A filamentous actin binding protein that is components of the focal contacts	8.2
Symplekin	A component of the submembraneous face of the tight junction in polar epithelial cells	6.6
Angiogenesis		
bFGF	A mitogenic and angiogenic factor	##

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Table 1. (Cont'd)

A. Proteins increased in 1170-I compared with NHBE cells

Protein	Function	Increased fold
Other cell signaling		
Adaptin α	A vesicular trafficking-related protein that is associated with the vesicle coats	2.2
AKAP79	One of the PKA type II anchoring proteins, which can form a tertiary complex with PKA and calcineurin	##
Annexin VI	A Ca^{2+} and phospholipid-binding protein that is related to the endocytic pathway	2.4
BAP135	A protein identified as a target for phosphorylation by Bruton's tyrosine kinase in B cells	7.8
iNOS	An enzyme that catalyzes the synthesis of nitric oxide, which is involved in vasorelaxation, neurotransmission, and cytotoxicity	##
IRAK	A serine/threonine kinase that is implicated in IL -1 signaling	##
JAK1	A non-receptor tyrosine kinase, which binds to IFN receptors and act on a non-receptor tyrosine kinase	5.3
80K-H(p90)	A protein substrate phosphorylated in cells stimulated with FGF	1.8*
Lat	An integral membrane protein that couples the activated TCR with downstream signaling molecules during T-cell activation	2.5
Na/K Ch. B2	Na-K-ATPase, an enzyme that has an important role in intestinal sodium and water absorption and is implicated in the metabolism of arachidonate and the release of PG	##
PLC	One type of phospholipase C, which is the enzyme responsible for generating two intracellular second messengers via phosphoinositide hydrolysis	##
p67phox	An adaptor protein, which is a cytosolic component of NADPH oxidase	4.1
p230 trans Golgi	A TGN-specific protein that is important for the biogenesis of special coated vesicles and functions in vesicular transport from the TGN	##
Rag-2	A protein expressed in maturing lymphoid cells which is required for V(D)J DNA recombination at loci for immunoglobulin and T cell receptor genes	3.8
RANBP1	Responsible for the GTP-bound state of Ran and the increased GTP hydrolysis of Ran	##
Sam68	An RNA-binding protein and putative substrate of the insulin receptor in insulin signaling, whose expression is induced by insulin stimulation	##
Sos-1	Son of sevenless, a bifunctional guanine nucleotide exchange factor, which activates Ras and coordinates Ras and Rac activity	##
Tyk-2	A tyrosine kinase belonging to the Janus-activated kinase family, which associates constitutively with a variety of cytokine and hormone receptors	##

B. Proteins decreased in 1170-I compared with NHBE cells

Protein	Function	Decreased fold
Transcription and RNA degradation		
CUL-2	A candidate human tumor suppressor and mediates the function of VHL, which is involved in transcriptional elongation	##
Exportin-t	A ubiquitous tRNA-specific nuclear export receptor related to the RanGTP-binding protein	##
G3BP	A RasGAP-binding protein that may link the Ras pathway to mRNA degradation	2.4
HcKrox	Human cKrox, a Zn finger-containing transcription factor for regulation of several extracellular matrix genes in skin and bone	3.7
p38 delta	A distinct member of mitogen-activated protein kinases, which phosphorylates transcription factors ATF-1 and PHAS-1	7.0
PTRF	Promotes the release of RNA polymerase I and nascent transcript from RNA template terminate transcription	3.1
Sin	Src interacting or signal integrating protein, functions as both an activator and substrate of Src signaling	#
Translation		
Inhibitor 2	A regulatory subunit of protein phosphatase 1, which is involved in protein synthesis, glycogen metabolism, cell division, and neuronal metabolism	9.4
Cell cycle regulation		
14-3-3E	Implicated in cellular processes, such as neurotransmission, phospholipase activity, and calcium signaling and associates with several proteins involved in cell proliferation	2.4
ECA39	A <i>myc</i> target gene that maybe involved in the regulation of cell cycle	2.2
Nek2	A mitotic kinase, which has a role in both maintenance and modulation of centrosome architecture	##

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Table 1. (Cont'd)

B. Proteins decreased in 1170-I compared with NHBE cells

Protein	Function	Decreased fold
Plectin	Is a large cytoskeletal linker protein, which is concentrated at sites of mechanical stress in different cell type	5.6
Rb	A tumor suppressor gene	##
Angiogenesis		
TSP-1	Thrombospondin-1, an antiangiogenic factor binding with high affinity to several angiogenic factors, such as FGF-2, and affecting their location and function	##
csk	COOH-terminal Src kinase, a tyrosine kinase that negatively regulates the activity of Src and related kinases and is important for Src's oncogenic potential and vascular development	##
Apoptosis		
Bruce	One of the ubiquitin activating/conjugating that may be involved in both ubiquitin-dependent proteolysis and antiapoptotic pathways	##
Caspase-3	A cysteine protease that promote apoptosis	2.4*
IGFBP3	A proapoptotic molecule that modulates the biological functions of insulin-like growth factors	##
POSH	A scaffold protein for a multiprotein complex that mediates JNK activation in apoptosis, and contributes to Rac-induced signal transduction pathway	2.2
Cytoskeleton and cell motility		
GRIP	Glutamate receptor interacting protein, an adaptor that links AMPA receptors to cytoskeletal and/or signaling molecules to cluster them at the synapse	##
Moesin P	A protein that localizes to cytoskeleton and modify interactions between cytoskeletal and membrane proteins	4.5
Rho	A member of Ras superfamily of GTPase that regulates cell morphology and motility	##
Cell adhesion		
Brevican	A neural-specific proteoglycan of the brain extracellular matrix, which decorates the surface of large neuronal somata and primary dendrites	##
E-cadherin	A calcium-dependent adhesion molecule	19.7
α -catenin	An E-cadherin-associated protein that is involved in cell adhesion and metastasis	2.2
γ -catenin	Plakoglobin, a component of desmosome	18.0
Integrin β_4	A subunit of integrin, which mediates cell adhesion to extracellular matrix	4.3
VLA-2 α	A member of the integrin superfamily of receptors postulated to have a substantial role in the metastasis	##
Cytoskeleton and cell motility		
GRIP	Glutamate receptor interacting protein, an adaptor that links AMPA receptors to cytoskeletal and/or signaling molecules to cluster them at the synapse	##
Moesin P	A protein that localizes to cytoskeleton and modify interactions between cytoskeletal and membrane proteins	4.5
Rho	A member of Ras superfamily of GTPase that regulates cell morphology and motility	##
Other cell signaling		
AChR β	β -Subunit of acetylcholine receptor, phosphorylation of which is responsible for triggering several effects at the postsynaptic membrane	##
BMX	Bone marrow x kinase, a cytoplasmic tyrosine kinase	##
Cab45	A member of EF-hand Calcium binding proteins	11.6
CASK	A cytosolic protein kinase, which interacts with neuexin	2.4
Caveolin 2	VIP 21, a transmembrane adaptor molecule that can recognize GPI-linked proteins and interact with downstream cytoplasmic signaling molecules	##
Cellugyrin	Represents a ubiquitously expressed four transmembrane domain protein that is closely related to synaptic vesicle protein synaptogyrin	5.5
COX-2	Also known as prostaglandin H synthase, which catalyzes the conversion of arachidonate to prostaglandin H ₂ and induced by IL-1	2.1
EAAT2	The glial glutamate transporter that is primarily responsible for clearance of glutamate from the synaptic cleft	4.1
FKBP 65	FK506-binding protein, immunosuppressive effects of FK506 and FKBP5 results from the inhibition of the calcineurin phosphatase	2.4
4F2hC	Heavy-chain subunit of heterodimeric y+L, which transports L-Arginine	4.0
HO-2	Heme oxygenase-2, a protein that is constitutively expressed in neurons and catalyzes heme breakdown	##

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Table 1. (Cont'd)

B. Proteins decreased in 1170-I compared with NHBE cells

Protein	Function	Decreased fold
HS1	Lck-binding protein 1 (lckBP1), associated with lck and may be an adaptor that regulates cellular tyrosine kinases	##
IP3- γ	A second messenger for many hormones, growth factors, and neurotransmitters	3.2
MCC	The mutated in colorectal cancer gene, a tumor suppressor that is deleted in colorectal cancers	##
NMDAR2B	One of the ionotropic receptors of glutamate, which is a neurotransmitter of synapses	2.9
PKC θ	A serine/threonine protein kinase of the PKC family, which is involved in a number of processes such as growth, differentiation, and cytokine secretion	##
trkB	Receptor of neurotrophins, such as brain-derived neurotrophic factor and NT4 activation triggers multiple signaling pathways	2.7

NOTE: # #, presence versus absence of a protein: fold change is immeasurable. All the increases marked with asterisks were confirmed by conventional Western blotting.

*The increase in protein level is the average of three experiments in which at least one showed <2-fold increase.

5-fold. However, some changes were as high as 31-fold increase and 20-fold decrease. The proteins found to be increased have been grouped in Table 1A according to their functions, including DNA synthesis and DNA repair, transcription and RNA degradation, translation, cell cycle regulation, apoptosis, cytoskeleton structure and cell motility, cell adhesion, angiogenesis, and other functions. Similarly, the proteins found to be decreased were grouped in Table 1B according to function, including transcription and RNA degradation, translation, cell cycle regulation, apoptosis, cytoskeleton structure and cell motility, cell adhesion, angiogenesis, and other functions.

Confirmation of protein changes by conventional Western blotting. A subset of 25 proteins was selected from Table 2 for validation using conventional Western blotting (one antibody per blot) under conditions that were optimal for each antibody (Fig. 2A-D). This method confirmed the PowerBlot results for 20 of 25 (80%) of these proteins. They included Cdk1, cyclin B1, cyclin D3, bFGF, PDK1, CaMKK, Akt, p70s6K, Bax, procaspase-7, procaspase-8, DFF45, PARP, Annexin VI, p53, 53BP3, C-cbl, Eg5, TFIIB, and E-cadherin. The PowerBlot data on changes in the levels of procaspase-3, procaspase-6, 80K-H, p38 δ , and Bid between NHBE and 1170-I cells could not be confirmed. During the validation experiments, we also compared the levels of different proteins in normal HBE cells and the isogenic family of cell lines that includes immortalized (BEAS-2B and 1799), transformed (1198), and tumorigenic (1170-I) HBE cells as well as several cell lines established from human NSCLC tumors (e.g., H460, H1792, SK-MES-1, A549, and Calu-1). Examples of the Western blotting results of proteins involved in cell cycle regulation, apoptosis, and PI3K/Akt signaling pathway are presented in Fig. 2 (note the lanes marked NHBE and 1170-I enclosed within dashed boxes). The levels of most of the proteins analyzed by Western blotting (e.g., E-cadherin, cyclin B1, procaspase-3, procaspase-7, procaspase-8, PARP, DFF45, 53BP2, PDK1, PI3K, CaMKK, p70s6k, and cyclin D3) were similar in 1170-I and the NSCLC cell lines. Other proteins (e.g., Annexin VI, Eg5, CDK1, p53, and Bax) showed different levels in 1170-I cells and NSCLC cell lines. The levels of PDK1 and Akt were similar in 1170-I cells and in two of four NSCLC cell lines.

A graphic comparison of the levels of proteins in the *in vitro* lung carcinogenesis model obtained after scanning the Western blots and quantitative analysis (Fig. 3) has shown that many of the changes observed with 1170-I cells are also observed in immortalized cells except for E-cadherin, which decreased only in transformed and tumorigenic HBE cells and Annexin VI, which increased in one of the immortalized cells (1799) but not in BEAS-2B (Fig. 3). For most proteins, changes seem to occur gradually as cells progress through stages of increased malignancy (Fig. 3).

Confirmation of protein changes by immunohistochemical analysis of normal, premalignant, and malignant lung tissues.

To begin to assess the relevance of the data obtained using the *in vitro* cell carcinogenesis model for changes in proteins during lung carcinogenesis *in vivo*, we did immunohistochemical analysis of the expression of E-cadherin, caspase-8, p70s6K, and STAT5 selected as representatives of proteins involved in cell adhesion, apoptosis, and growth factor signaling, respectively, using tissue microarrays containing 323 samples, including normal, premalignant, and malignant (NSCLC) tissue samples from lungs resected from 144 lung cancer patients (Table 2). Examples of the immunohistochemistry results are presented in Fig. 4. The level of E-cadherin, which is high in normal bronchial epithelium, decreased progressively in premalignant tissue and carcinoma. In contrast, the levels of caspase-8, STAT5, and nuclear p70s6K increased in the premalignant and malignant tissue samples. The semiquantitative immunohistochemistry results are presented in Fig. 5. The repeated-measures ANOVA showed that the level of E-cadherin decreased significantly in high-grade dysplasia and SCC as well as in AAH and adenocarcinoma relative to normal epithelium (Fig. 5A). The level of caspase-8 showed a trend for increase in high-grade dysplasia and a significant increase in SCC and adenocarcinoma; however, it decreased significantly in AAH (Fig. 5B). The level of p70s6K increased significantly in hyperplasia, high-grade dysplasia, and adenocarcinoma but did not show statistically significant changes in the other histologies (Fig. 5C). Cytoplasmic STAT5 level increased significantly in high-grade dysplasia, SCC, AAH, and adenocarcinoma, whereas the nuclear STAT5 showed increases in high-grade dysplasia, AAH, and adenocarcinoma but not in SCC and the other histologies (Fig. 5D and E).

Because normal bronchial epithelial cells may not be the precursor of AAH and adenocarcinoma, we also compared the levels of expression of the different proteins in adenocarcinoma and AAH and found that the difference in the levels of caspase-8 and nuclear p70s6K were statistically significant, whereas the differences in the level of E-cadherin and cytoplasmic and nuclear STAT5 were not (Fig. 5). There were no correlations between the changes in protein levels detected by immunohistochemistry and the gender, smoking status, or tumor-node-metastasis stage of the cancer patients from whom the tissues were obtained.

Discussion

The identification of biomarkers of early lung cancer is expected to improve the long-term survival of lung cancer patients. The application of high-throughput technologies to identify genes and proteins that are expressed differentially in tumor cells and normal tissues seems to be promising (23). Gene expression profiling is considered a standard procedure for high-throughput analysis of normal and malignant tissues. However, the reported lack of correlation between mRNA and protein expression has highlighted the importance of conducting parallel proteomics studies to complement cDNA or oligonucleotide array data. In this study, we compared and contrasted the levels of nearly 800 proteins

related to cell growth, differentiation, and apoptosis in normal and tumorigenic human lung epithelial cells by using the PowerBlot technique, which has been used previously to identify protein changes in head and neck squamous carcinoma cells (HNSCC) after docetaxel (24) or retinoid (25) treatments. We detected changes in 135 proteins (87 increased and 48 decreased) in tumorigenic 1170-I cells relative to normal cells. These proteins execute diverse functions associated with DNA synthesis and repair, RNA transcription and degradation, translation, cell cycle regulation, and apoptosis, cytoskeleton structure, cell adhesion and motility, angiogenesis, and other functions. Understanding the mechanisms underlying the changes in the levels of these proteins and how they are related to lung carcinogenesis will obviously require additional experiments, such as functional analyses using gene silencing and gene transfection experiments.

Our analysis does not indicate whether a change in protein level is the result of increased gene expression or stabilization of mRNA or protein. Recently, Yoo et al. (24) found a 35% discrepancy between PowerBlot analysis data (protein levels) and cDNA array data (mRNA expression) in untreated and docetaxel-treated HNSCC cells and proposed that this discrepancy is due to posttranslational changes in proteins. In addition, changes in mRNA and protein stability may also account for differences between mRNA and protein analyses.

Table 2. Demographic and clinical-pathologic data regarding the lung cancer and respiratory epithelial tissue specimens studied for immunohistochemical analyses

	ADCA	SCC	Normal bronchial epithelium	Hyperplasia	Squamous metaplasia	Low-grade dysplasia	High-grade dysplasia	AAH	Total
No. patients*	29	30	13	40	13	10	22	22	144
No. tissue specimens	—	—	30	74	22	16	47	75	323
Age	69 (40-84)	66 (47-90)	68 (55-73)	67 (47-80)	64 (47-79)	67 (60-80)	68 (59-80)	67 (47-80)	66.4 (40-90)
Gender									
Male	11	21	7	27	7	5	19	5	72
Female	18	9	6	13	6	5	3	17	72
TNM									
I (A-B)	25	15	5	10	3	4	9	15	75
II (A-B)	3	14	3	8	4	0	4	0	28
III (A-B)	1	1	0	7	2	0	4	2	13
IV	0	0	0	0	0	0	0	2	2
Unknown		0	5	15	4	6	5	3	28
Smoking status									
Current	14	15	4	11	2	3	5	9	54
Former	10	12	1	10	4	1	7	6	43
Never	4	1	2	4	3	1	3	4	17
Unknown	—	2	6	15	4	5	5	3	30
Ethnicity									
White	28	25	12	35	10	9	21	20	133
Other [†]	1	5	1	5	3	1	1	2	11
NSCLC [‡]									
ADCA	—	—	6	17	7	2	3	19	44
SCC			6	17	3	4	18	1	31
Other [§]			1	6	3	4	1	2	10

Abbreviations: ADCA, adenocarcinoma; TNM, tumor-nodes-metastasis.

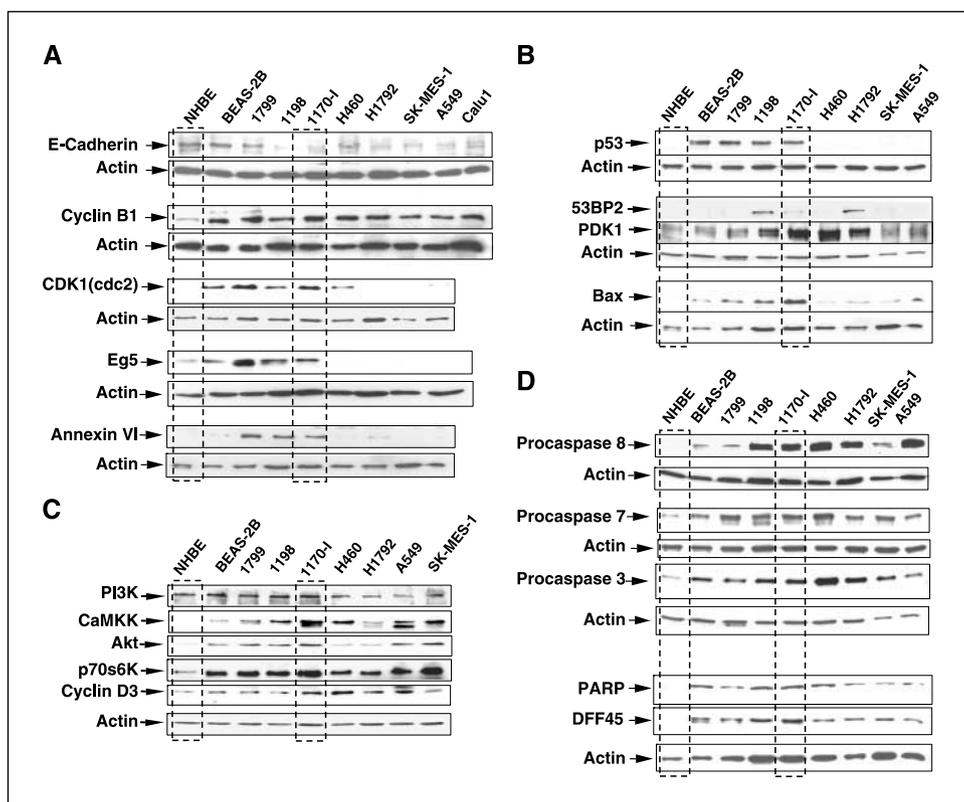
*Multiple samples originated from a subset of patients.

[†]Included three Hispanics, five African Americans, and three Asians.

[‡]Original tumors from where preneoplasia specimens were obtained.

[§]Other NSCLC (two large cell carcinomas, two nonclassified NSCLCs, one pleomorphic NSCLC, and five carcinoids).

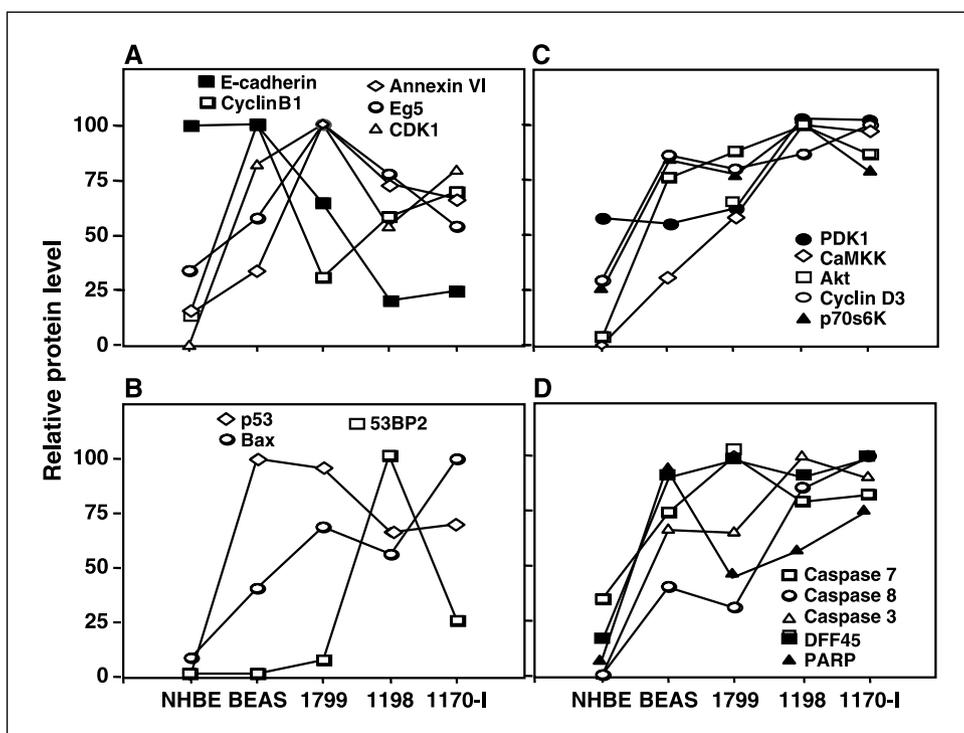
Figure 2. Conventional Western blotting analysis of proteins from normal, immortalized, transformed, tumorigenic, and malignant lung epithelial cell lines. Total cell proteins were extracted from normal NHBE cells and the indicated cell lines and separated in different lanes of the same slab polyacrylamide gel, and then processed for conventional Western blotting using one primary antibody at a time. Five different slab gels were analyzed with one antibody at a time. The primary antibodies were as follows: (A) E-cadherin, cyclin B1, CDK1 (cdc2), Eg5, and Annexin VI; (B) p53, 53BP2, PDK1, and Bax; (C) PI3K, CaMKK, Akt, p70s6K, and cyclin D3; (D) procaspase-8, procaspase-7, procaspase-3, PARP, and DFF45. Each blot was stripped and reprobbed with antibodies against β -actin as an internal control for loading.



Others have used proteomic-based approaches to study lung physiology and cancer. Two-dimensional PAGE was used to separate proteins expressed differentially between human lung squamous carcinoma and surrounding normal bronchial epithelial tissue and MALDI-TOF MS and database searching were used to

identify some of these proteins (16). The MALDI-TOF MS method has also been applied directly to frozen sections for profiling of protein expression in surgically resected normal and malignant lung tissues (18). The PowerBlot Western array complements these techniques because it is sensitive down to the subnanogram level,

Figure 3. Quantitative depiction of differences in protein levels among normal, immortalized, transformed, and tumorigenic cells based on scans of the Western blots in Fig. 2. A, proteins involved in cell cycle and cell adhesion. B, p53-related proteins. C, proteins related to the PI3K/Akt pathway. D, caspases and caspase-3 substrates.



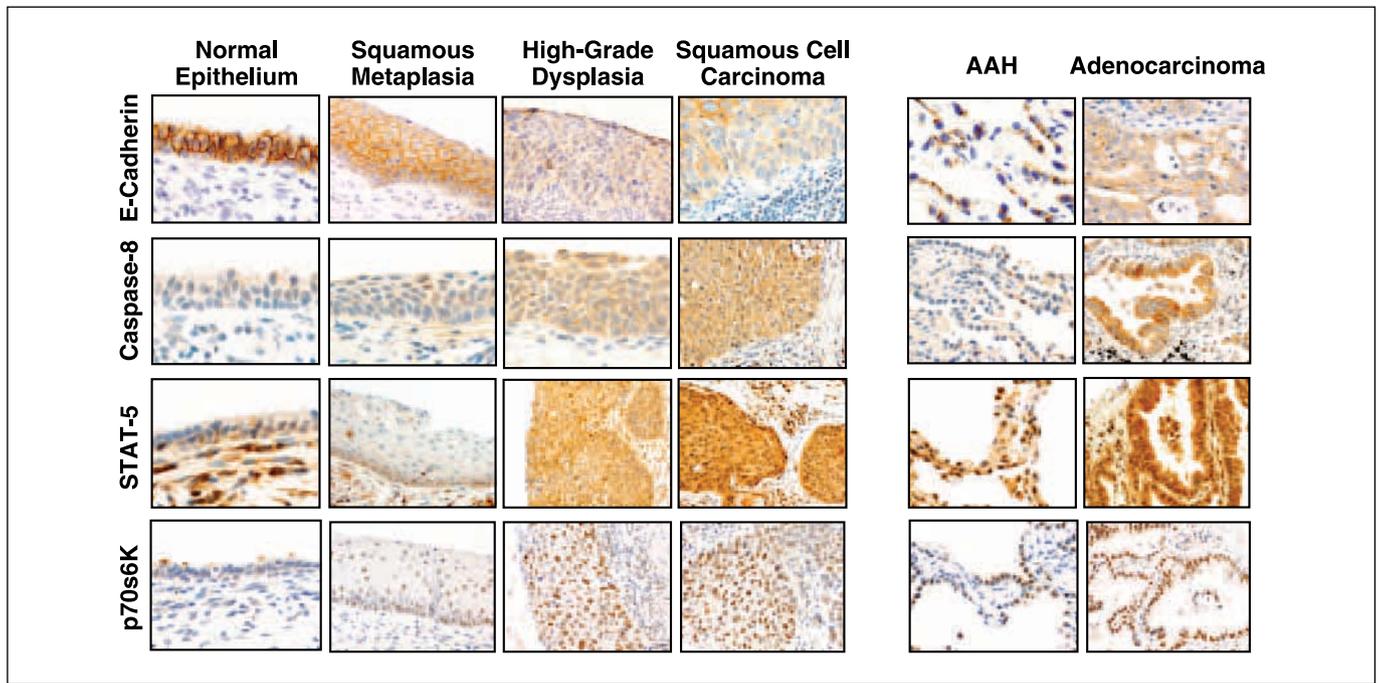


Figure 4. Representative examples of photomicrographs showing the immunohistochemical expression of E-cadherin, caspase-8, STAT5, and p70s6K in the sequential pathogenesis of human NSCLCs of the squamous cell carcinoma and adenocarcinoma histologies.

which cannot be accomplished for low-abundance proteins using two-dimensional PAGE/MS, and because the proteins' identities are well established by the corresponding antibodies.

Prominent among the proteins that are increased in the tumorigenic cells is the group linked to DNA synthesis and repair,

which is expected from highly proliferative malignant cells (21). For example, proliferating cell nuclear antigen, a subunit of DNA polymerase δ , which increased in the 1170-I cells, is associated with DNA replication and with poor prognosis of NSCLC patients (26) and with resistance of lung cancer cells to chemotherapy and

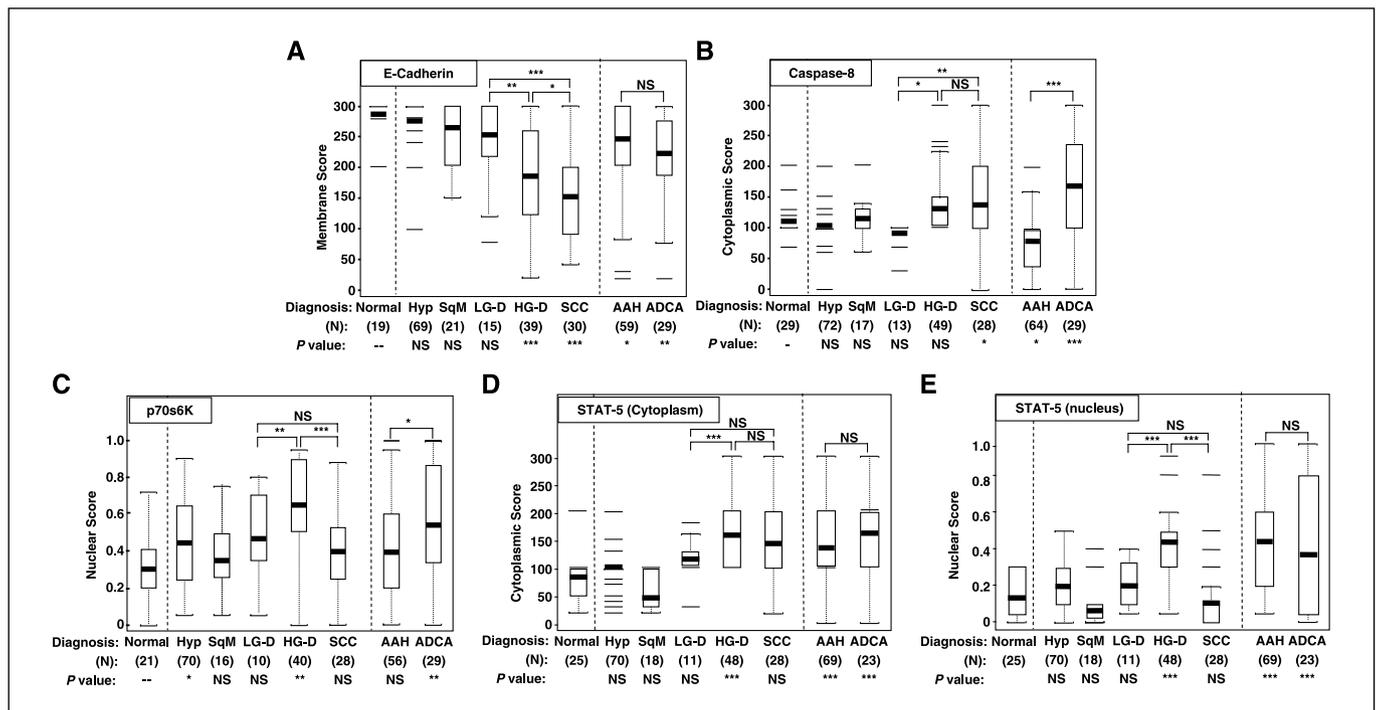


Figure 5. Box plots of immunohistochemical expression scores for E-cadherin (A), caspase-8 (B), p70s6K (C), cytoplasmic STAT5 (D), and nuclear STAT5 (E) in the sequential pathogenesis of lung cancer. Columns, mean; bars, SE. Numbers under histology represent number of specimens (N) examined in each category. Hyp, hyperplasia; SqM, squamous metaplasia; LG-D, low-grade dysplasia; HG-D, high-grade dysplasia; ADCA, lung adenocarcinoma. NS, nonsignificant ($P > 0.05$). *, $P = 0.01$ to 0.05 ; **, $P = 0.001$ to 0.01 ; ***, $P < 0.001$.

radiation treatment (27). The expression of MSH2 has been correlated with p53 overexpression in NSCLC samples (28). However, in the 1170-I cells p53 is inactivated by SV40 large T antigen; therefore, the increase in MSH2 protein is independent of p53 function.

Several of the proteins that show increased levels in the tumorigenic 1170-I cells had been reported to function as oncogenes. For example, STAT5 (29) has been found to activate cyclin D1, c-Myc, and Bcl-XL expression; to promote cell cycle progression and cellular transformation; and to prevent apoptosis (30). TEF-1 can promote immortalization and transformation (31). Some of the proteins found to decrease in the tumorigenic cells, such as CUL-2, Rb, E-cadherin, and γ -catenin, are known to play a role as tumor suppressors, and their decreased expression may be related to the tumorigenic phenotype. The concurrent decrease in E-cadherin and γ -catenin may decrease cell adhesion and contribute to increased malignant behavior (32).

The increases in components of the PI3K/Akt cell survival pathway (PDK1, Akt, CaMKK, p70S6K, and cyclin D3) as well as the decrease in the proapoptotic IGFBP3 are expected to have contributed to the tumorigenic phenotype in 1170-I cells. In contrast, the increase in the proapoptotic proteins Bax, Bid, and procaspase-7 and procaspase-8, which were detected by the PowerBlot and confirmed by Western blotting and immunohistochemistry (caspase-8), and in procaspase-3 observed only by Western blotting, are not easy to reconcile because they are not related to rates of spontaneous apoptosis measured by the terminal deoxynucleotidyl transferase-mediated nick-end labeling assay in the various normal, immortalized, transformed, and tumorigenic lung epithelial cells (data not shown). Malignant lung epithelial cells may be resistant to the proapoptotic caspases that they contain possibly as a result of high levels of antiapoptotic proteins (33).

The immortalized (BEAS-2B and 1799), transformed (1198), and tumorigenic (1170-I) cells (21) have been chosen because we surmised that a comparison of protein changes among these cells could provide a good starting point to identify markers for diagnosis and targets for intervention. The changes in protein levels among these cell lines indicate that the levels of some proteins changed "early" in this carcinogenesis model (i.e., between normal and immortalized cells), and this change was sustained through the tumorigenic state. The level of other proteins was changed progressively. We also analyzed protein levels in five cell lines derived from human NSCLC tumors. Only four cell lines (BEAS-2B, 1799, 1198, and 1170-I) showed the p53 protein band in Western blotting. This result could be expected because these cell lines were immortalized by SV40 large T antigen, which forms a complex with p53 to suppress its transcriptional activity (34) but, within the complex, p53 may be protected from degradation. The increase in cyclin B1 and CDK1 in immortalized and transformed cells (Fig. 3A) may be explained by the increased proliferation of these cells relative to NHBE, which results in a higher proportion of cells in the S and G₂-M phases of the cell cycle. Others have reported that cyclin B1 expression in NSCLC tumors *in vivo* is associated with higher Ki-67 proliferative index and a poorer prognosis (35). Likewise, Eg5, a kinesin implicated in the formation of the bipolar spindle and its movement before and during anaphase, is essential for mitosis (36). Its levels increased in the immortalized, transformed, and tumorigenic lung epithelial cells (Fig. 2A). Interestingly, Eg5 has been implicated as an oncogene in the development of B-cell leukemias (37). Because Eg5 is

phosphorylated by Cdc2 (CDK1)/cyclin B (38), the increase in its level may be related to the increase in both CDK1 and cyclin B1 observed in the same cells (Fig. 2). The increase in cyclin B1 was observed in the *in vitro* carcinogenesis model as well as in cell lines derived from human cancers (e.g., H460, H1792, SK-MES-1, and A549), in contrast with the increases in CDK1, Eg5, Annexin VI, and Bax, which were not observed in the NSCLC cell lines.

Studies with tissue specimens from human NSCLC tumors have found strong procaspase-3 protein by immunostaining had a poor 5-year survival (39). Caspase-8 has been detected in all 22 cases of NSCLC tissue samples analyzed in another study (40).

Our finding of an increase in the levels of Akt and the increases in the levels of the Akt downstream targets p70S6K and cyclin D3 (Fig. 2C) supports the contention that the PI3K/Akt pathway may be activated during the progression of cells to malignancy (41, 42). PI3K protein levels did not increase much; however, CaMKK, which can phosphorylate Akt on Thr³⁰⁸ (43), did show a gradual increase in the carcinogenesis model.

To begin to determine whether some of these protein changes occur *in vivo*, we used tissue microarrays prepared from resected human lung tissue specimens, which included normal, hyperplasia, squamous metaplasias, dysplasias, SCCs, AAHs, and adenocarcinomas for immunohistochemical analyses. To the best of our knowledge, this is the first time that a tissue microarray with so many premalignant lung tissue samples has been created and analyzed. In general, there was a good relationship between the changes observed in the levels of the four representative proteins (E-cadherin, caspase-8, p70s6K, and STAT5) detected by the PowerBlot, the conventional Western blotting, and immunohistochemical analysis. Many previous studies have compared levels of proteins in normal and malignant lung tissue samples by immunohistochemistry. However, our tissue microarray allowed us to compare for the first time the levels of proteins in different stages of SCC development, including low-grade dysplasia, high-grade dysplasias (including carcinoma *in situ*), and SCC. Interestingly, for E-cadherin, there was a progressive change (decrease) at different stages between normal and SCC, whereas the levels of nuclear STAT5 and p70s6K in SCCs were lower than in the high-grade dysplasias. In addition, our findings highlight the importance of validating immunoblotting blotting data obtained with cell or tissue lysates by immunohistochemistry using histologic sections because some proteins may be located in different cell compartments. For example, we found that STAT5 is located in both the nucleus and the cytoplasm, and its level in these two cellular compartments changes in a distinct fashion at different stages of SCC development. Specifically, the cytoplasmic STAT5 level is similar in SCC and high-grade dysplasia, and both are higher than levels in normal bronchial epithelial cells; in contrast, the level of nuclear STAT5 is higher in high-grade dysplasia than in normal cells but drops to normal levels in SCCs.

In conclusion, we have shown for the first time the usefulness of a high-throughput immunoblotting approach combined with an *in vitro* carcinogenesis cell model and tissue array to identify a variety of changes that occur in the level of different proteins involved in fundamental cellular processes potentially associated with lung carcinogenesis. Some of these proteins change early (between normal and immortalized cells), and others change late (between transformed and tumorigenic cells). They may serve as markers for diagnosis or prognosis and are potential targets for intervention in the carcinogenesis process by chemoprevention or therapy.

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References

1. Forgacs E, Zochbauer-Muller S, Olah E, Minna JD. Molecular genetic abnormalities in the pathogenesis of human lung cancer. *Pathol Oncol Res* 2001;7:6-13.
2. Jemal A, Murray T, Ward E, et al. Cancer statistics, 2005. *CA Cancer J Clin* 2005;55:10-30.
3. Montuenga LM, Mulshine JL. New molecular strategies for early lung cancer detection. *Cancer Invest* 2000;18:555-63.
4. Nacht M, Dracheva T, Gao Y, et al. Molecular characteristics of non-small cell lung cancer. *Proc Natl Acad Sci U S A* 2001;98:15203-8.
5. Wang T, Hopkins D, Schmidt C, et al. Identification of genes differentially over-expressed in lung squamous cell carcinoma using combination of cDNA subtraction and microarray analysis. *Oncogene* 2000;19:1519-28.
6. Goodwin LO, Mason JM, Hajdu SI. Gene expression patterns of paired bronchioloalveolar carcinoma and benign lung tissue. *Ann Clin Lab Sci* 2001;31:369-75.
7. Hermeking H. Serial analysis of gene expression and cancer. *Curr Opin Oncol* 2003;15:44-9.
8. Anderson L, Seilhamer J. A comparison of selected mRNA and protein abundances in human liver. *Electrophoresis* 1997;18:533-7.
9. Celis JE, Gromov P. Proteomics in translational cancer research: toward an integrated approach. *Cancer Cell* 2003;3:9-15.
10. Hanash S. Disease proteomics. *Nature* 2003;422:226-32.
11. Chapman K. The ProteinChip Biomarker System from Ciphergen Biosystems: a novel proteomics platform for rapid biomarker discovery and validation. *Biochem Soc Trans* 2002;30:82-7.
12. Petricoin EF, Liotta LA. Mass spectrometry-based diagnostics: the upcoming Revolution in disease detection. *Clin Chem* 2003;49:533-4.
13. Jain KK. Role of proteomics in diagnosis of cancer. *Technol Cancer Res Treat* 2002;1:281-6.
14. Yip TT, Lomas L. SELDI ProteinChip array in oncoproteomic research. *Technol Cancer Res Treat* 2002;1:273-80.
15. Walter G, Bussow K, Lueking A, Glokler J. High-throughput protein arrays: prospects for molecular diagnostics. *Trends Mol Med* 2002;8:250-3.
16. Li C, Chen Z, Xiao Z, et al. Comparative proteomics analysis of human lung squamous carcinoma. *Biochem Biophys Res Commun* 2003;309:253-60.
17. Oh JM, Brichory F, Puravs E, et al. Molecular biology and early diagnosis in lung cancer. *Lung Cancer* 2002;38: S5-8.
18. Yanagisawa K, Shyr Y, Xu BJ, et al. Proteomic patterns of tumour subsets in non-small-cell lung cancer. *Lancet* 2003;362:433-9.
19. Valle RP, Chavany C, Zhukov TA, Jendoubi M. New approaches for biomarker discovery in lung cancer. *Expert Rev Mol Diagn* 2003;3:55-67.
20. Reddel RR, Ke Y, Gerwin BI, et al. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 early region genes. *Cancer Res* 1988;48:1904-9.
21. Klein-Szanto AJ, Iizasa T, Momiki S, et al. A tobacco-specific N-nitrosamine or cigarette smoke condensate causes neoplastic transformation of xenotransplanted human bronchial epithelial cells. *Proc Natl Acad Sci U S A* 1992;89:6693-7.
22. SAS user guide version 8. Cary (NC): SAS Institute, Inc.; 1999.
23. Franklin WA. Premalignant evolution of lung cancer: Gilles F. Filley lecture. *Chest* 2004;125:90-4.
24. Yoo GH, Piechocki MP, Ensley JF, et al. Docetaxel induced gene expression patterns in head and neck squamous cell carcinoma using cDNA microarray and PowerBlot. *Clin Cancer Res* 2002;8:3910-21.
25. Kim HJ, Lotan R. Identification of retinoid-modulated proteins in squamous carcinoma cells using high-throughput immunoblotting. *Cancer Res* 2004;64:2439-48.
26. Caputi M, Esposito V, Groger AM, et al. Prognostic role of proliferating cell nuclear antigen in lung cancer: an immunohistochemical analysis. *In vivo* 1998;12:85-8.
27. Joseph B, Lewensohn R, Zhivotovsky B. Role of apoptosis in the response of lung carcinomas to anti-cancer treatment. *Ann N Y Acad Sci* 2000;926:204-16.
28. Xinarianos G, Liloglou T, Prime W, et al. p53 status correlates with the differential expression of the DNA mismatch repair protein MSH2 in non-small cell lung carcinoma. *Int J Cancer* 2002;101:248-52.
29. Xi S, Zhang Q, Gooding WE, Smithgall TE, Grandis JR. Constitutive activation of Stat5b contributes to carcinogenesis *in vivo*. *Cancer Res* 2003;63:6763-71.
30. Calo V, Migliavacca M, Bazan V, et al. STAT proteins: from normal control of cellular events to tumorigenesis. *J Cell Physiol* 2003;197:157-68.
31. Ishiji T, Lacey MJ, Parkkinen S, et al. Transcriptional enhancer factor (TEF)-1 and its cell-specific co-activator activate human papillomavirus-16 E6 and E7 oncogene transcription in keratinocytes and cervical carcinoma cells. *EMBO J* 1992;11:2271-81.
32. Pirinen RT, Hirvikoski P, Johansson RT, Hollmen S, Kosma V-M. Reduced expression of α -catenin, β -catenin, and γ -catenin is associated with high cell proliferative activity and poor differentiation in non-small cell lung cancer. *J Clin Pathol* 2001;54:391-5.
33. Hofmann HS, Simm A, Hammer A, Silber RE, Bartling B. Expression of inhibitors of apoptosis (IAP) proteins in non-small cell human lung cancer. *J Cancer Res Clin Oncol* 2002;128:554-60.
34. Pipas JM, Levine AJ. Role of T antigen interactions with p53 in tumorigenesis. *Semin Cancer Biol* 2001;11:23-30.
35. Yoshida T, Tanaka S, Mogi A, Shitara Y, Kuwano H. The clinical significance of cyclin B1 and Wee1 expression in non-small-cell lung cancer. *Ann Oncol* 2004;15:252-6.
36. Heald R. Motor function in the mitotic spindle. *Cell* 2000;102:399-402.
37. Hansen GM, Justice MJ. Activation of Hex and mEg5 by retroviral insertion may contribute to mouse B-cell leukemia. *Oncogene* 1999;18:6531-9.
38. Nigg EA, Blangy A, Lane HA. Dynamic changes in nuclear architecture during mitosis: on the role of protein phosphorylation in spindle assembly and chromosome segregation. *Exp Cell Res* 1996;229:174-80.
39. Takata T, Tanaka F, Yamada T, et al. Clinical significance of caspase-3 expression in pathologic-stage I, nonsmall-cell lung cancer. *Int J Cancer* 2001;96 Suppl: 54-60.
40. Shivapurkar N, Toyooka S, Eby MT, et al. Differential inactivation of caspase-8 in lung cancers. *Cancer Biol Ther* 2002;1:65-9.
41. West KA, Linnoila IR, Belinsky SA, Harris CC, Dennis PA. Tobacco carcinogen-induced cellular transformation increases activation of the phosphatidylinositol 3'-kinase/Akt pathway *in vitro* and *in vivo*. *Cancer Res* 2004;64:446-51.
42. Chun KH, Kosmeder JW II, Sun S, et al. Effects of deguelin on the phosphatidylinositol 3-kinase/Akt pathway and apoptosis in premalignant human bronchial epithelial cells. *J Natl Cancer Inst* 2003;95:291-302.
43. Yano S, Tokumitsu H, Soderling TR. Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. *Nature* 1998;396: 584-7.