Discovery of New Membrane-Associated Proteins Overexpressed in Small-Cell Lung Cancer

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Introduction: Small-cell lung cancer (SCLC) is the most aggressive subtype of lung cancer, with no early detection strategy or targeted therapy currently available. We hypothesized that difference gel electrophoresis (DIGE) may identify membrane-associated proteins (MAPs) specific to SCLC, advance our understanding of SCLC biology, and discover new biomarkers of SCLC.

Methods: MAP lysates were prepared from three SCLCs, three non–small-cell lung cancers, and three immortalized normal bronchial epithelial cell lines and coanalyzed by DIGE. Subsequent protein identification was performed by mass spectrometry. Proteins were submitted to Ingenuity Pathway Analysis. Candidate biomarkers were validated by Western blotting (WB) and immunohistochemistry (IHC).

Results: Principal component analysis on the global DIGE data set demonstrated that the four replicates derived from each of the nine cell lines clustered closely, as did samples within the same histological group. One hundred thirty-seven proteins were differentially expressed in SCLC compared with non–small-cell lung cancer and immortalized normal bronchial epithelial cells. These proteins were overrepresented in cellular/tissue morphology networks. Dihydropyrimidinase-related protein 2, guanine nucleotide–binding protein alpha-q, laminin receptor 1, pontin, and stathmin 1 were selected as candidate biomarkers among MAPs overexpressed in SCLC. Overexpression of all candidates but RSSA in SCLC was verified by WB and/or IHC on tissue microarrays. These proteins were significantly associated with SCLC histology and survival in univariables analyses.

Conclusion: DIGE analysis of a membrane-associated subproteome discovered overexpression of dihydropyrimidinase-related protein 2, guanine nucleotide–binding protein alpha-q, RUVB1, and stathmin 1 in SCLC. Results were verified by WB and/or IHC in primary tumors, suggesting that investigating their functional relevance in SCLC progression is warranted. Association with survival requires further validation in larger clinical data sets.

Key Words: Biomarker, Membrane-associated, Proteomics, Small-cell lung cancer.

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Small-cell lung cancer (SCLC) represents 10% to 15% of lung cancers and is clinically the most aggressive subtype, with a 5-year overall survival (OS) as low as 5%.[1] Molecular steps leading to SCLC are still poorly understood, and this has translated into the absence of efficient early detection strategies or targeted therapies. In-depth proteomic analysis is therefore needed to improve our understanding of SCLC tumorigenesis and may have implications in the discovery of novel early detection and therapeutic strategies for this aggressive cancer.

Although (epi)genetic alterations are driving carcinogenesis and genomic studies have provided valuable information on cancer biology, the cellular phenotype is determined by proteins and cannot be predicted by genomics alone. Proteomic approaches are therefore powerful tools to study cancer biology.[2,3] Among them, difference gel electrophoresis (DIGE) coupled with protein identification by mass spectrometry (MS) is widely used in comparative proteomics for the detection of protein differences with high reproducibility and reliability.[4–6] However, these studies have not identified key underpinnings of tumor progression or specific molecular targets for SCLC diagnostics or therapeutics.

Membrane-associated proteins (MAPs) are excellent targets for diagnostic and therapeutic interventions. Although membrane proteins constitute only 20% to 30% of human proteome, they represent more than 60% of all drug targets.[7] In this study, we hypothesized that the identification

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of SCLC-specific MAPs by DIGE may advance our understanding of SCLC biology and lead to the discovery of new candidate diagnostic or therapeutic biomarkers. We therefore analyzed MAPs from three SCLC, three non–small-cell lung cancer (NSCLC), and three immortalized normal bronchial epithelial (INBE) cell lines by DIGE.

MATERIALS AND METHODS

Cell Lines and Tissues

Nine cell lines were used for this study: National Cancer Institute (NCI)-H69, NCI-H82, NCI-H209, A549, NCI-H23, NCI-H520, BEAS-2B, HBEC-3KT, and 16-HBE. All were purchased from ATCC (Manassas, VA), except HBEC-3KT (Dr. Minna's gift, University of Texas Southwestern) and 16-HBE (Dr. Gruenert's gift, Children's Hospital, Oakland Research Institute). They were cultured under recommended conditions.

Tissue microarrays (TMAs) made of SCLC and NSCLC specimens were prepared from formalin-fixed, paraffin-embedded tissue blocks[8] retrieved from the Pathology Department's archives at Vanderbilt University Medical Center, Nashville VA Medical Center, and St Thomas Hospital in Nashville, TN. They were obtained between 1996 and 2008 from 136 patients who had surgery or bronchoscopy. Samples were annotated with clinical data elements. The study was approved by the Institutional Review Board at each institution.

MAP Extraction

MAP extraction was performed four independent times for each cell line with the ProteoExtract Native Membrane Protein Extraction Kit (EMD Chemicals, San Diego, CA) per manufacturer's protocol.

DIGE and MS

Minimal labeling was performed with N-hydroxysuccinimide–ester dyes Cy2/3/5 using the mixed internal standard methodology as described previously,[9] with 150 μg of protein of each of the 36 experimental samples. DIGE-associated instrumentation was manufactured by GE Healthcare (Piscataway, NJ). Isoelectric focusing (pH 4–7, 24 cm) was performed after passive rehydration with sample and then focused using a manifold-equipped IPGphor per manufacturer's protocol. Second-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed using handcast gels for which one plate was pre-silanized using an Ettan DALT 12U per manufacturer's protocol. Cy2/3/5-specific 16-bit data files were acquired at 100-μm resolution separately by dye-specific excitation and emission wavelengths using a Typhoon 9400 Variable Mode Imager and analyzed using DeCyder_6.5 software. Gels were counterstained for total protein content with SyproRuby (Molecular Probes/Invitrogen, Grand Island, NY), followed by robot excision of proteins of interest and subsequent processing for trypsin digestion and MS using an automated Spot Handling Workstation. Resulting peptides were subjected to C18 reverse-phase liquid chromatography coupled in-line with tandem MS using an LTQ Orbitrap tandem MS equipped with MicroAS autosampler and Surveyor HPLC pump, nanospray source, and Xcalibur_2.0 instrument control (Thermo Scientific, San Jose, CA). Tandem MS/MS data were searched against proteins extracted from UniProtKB database (www.uniprot.org), with taxonomy tag "Homo sapiens (human)" acquired on July 2009 (173,736 entries). This database was concatenated with protein sequences in reverse to enable false discovery rate calculations and also contained common laboratory contaminants. Searches were performed using Sequest and X! Tandem algorithms, allowing for cysteine carbamidomethylation and partial methionine oxidation. Results were validated and assembled into protein identifications using Scaffold_3.1.2 (Proteome Software Inc., Portland, OR). Peptide identifications were accepted if they could be established at more than 95.0% probability as specified by Peptide Prophet algorithm.[10] Protein identifications were accepted if they could be established at more than 99.0% probability and contained two or more identified peptides. Protein probabilities were assigned by Protein Prophet algorithm.[11] Proteins containing similar peptides and not differentiated based on MS/MS alone were grouped to satisfy parsimony principles.

Ingenuity Pathway Analysis

Biological processes, molecular functions, and genetic/protein networks of identified proteins were analyzed using Ingenuity Pathway Analysis (IPA) tools (Ingenuity Systems, Mountain View, CA). The score associated with a particular network was the likehood of differentially expressed proteins being found together due to chance.

Western Blotting

Blots were probed with antibodies against human v-Akt murine thymoma viral oncogene homolog 1 (AKT) (1:1000; Cell Signaling, Danvers, MA), dihydropyrimidinase-related protein 2 (DPYL2) (1:1000; LSBio, Seattle, WA), phospho-DPYL2 (Thr^{514}/Ser^{518}) (1:1000; LSBio), E-cadherin (1:1000; BD Transduction Laboratories, San Jose, CA), endothelial growth factor receptor (EGFR) (1:750; Santa Cruz Biotechnology, Santa Cruz, CA), guanine nucleotide–binding protein alpha-q (GNAQ) (1:900; Abcam, Cambridge, MA), neural cell adhesion molecule (NCAM) (1:500; Sigma, St. Louis, MO), laminin receptor 1 (RSSA) (1:1000; Sigma), pontin (RUVB1) (1:1000; Atlas Antibodies, Stockholm, Sweden), stathmin 1 (STMN1) (1:1000; Cell Signaling), and phospho-STMN1 (Ser^{16}) (1:1000; Cell Signaling). Protein expression was normalized to Actin (1:5000; Sigma).

Immunohistochemistry

Tissue sections were stained following previously reported protocol,[12] using antibodies against human DPYL2 (1:500; LSBio), GNAQ (1:100; Abcam), RSSA (1:150; LSBio), RUVB1 (1:100; Atlas Antibodies), and STMN1 (1:50; Cell Signaling). Staining intensity of cases represented in triplicate was evaluated by two independent observers (B.W., S.O.) as follows: 0, no staining; 1, weak; 2, moderate; and 3, strong. Staining intensity was then multiplied by stained tumor cell percentage to obtain the final staining score (range, 0–300).

