Chronic Cigarette Smoke Mediated Global Changes in Lung Mucoepidermoid Cells: A Phosphoproteomic Analysis

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Abstract

Proteomics analysis of chronic cigarette smoke exposure is a rapidly emerging postgenomics research field. While smoking is a major cause of lung cancer, functional studies using proteomics approaches could enrich our mechanistic understanding of the elusive lung cancer global molecular signaling and cigarette smoke relationship. We report in this study on a stable isotope labeling by amino acids in cell culture-based quantitative phosphoproteomic analysis of a human lung mucoepidermoid carcinoma cell line, H292 cells, chronically exposed to cigarette smoke. Using high resolution Orbitrap Velos mass spectrometer, we identified the hyperphosphorylation of 493 sites, which corresponds to 341 proteins and 195 hypophosphorylated sites, mapping to 142 proteins upon smoke exposure (2.0-fold change). We report differential phosphorylation of multiple kinases, including PAK6, EPHA4, LYN, mitogen-activated protein kinase, and phosphatases, including TMEM55B, PTPN14, TIGAR, among others, in response to chronic cigarette smoke exposure. Bioinformatics analysis revealed that the molecules differentially phosphorylated upon chronic exposure of cigarette smoke are associated with PI3K/AKT/mTOR and CDC42-PAK signaling pathways. These signaling networks are involved in multiple cellular processes, including cell polarity, cytoskeletal remodeling, cellular migration, protein synthesis, autophagy, and apoptosis. The present study contributes to emerging proteomics insights on cigarette smoke mediated global signaling in lung cells, which in turn may aid in development of precision medicine therapeutics and postgenomics biomarkers.

Keywords: phosphoproteomics, cigarette smoke, biomarkers, postgenomics biotechnology, lung cancer

Introduction

SMOKING REMAINS THE LEADING CAUSE of lung cancer mortality worldwide for several decades (Islami et al., 2015). The relative risk of developing lung cancer in male smokers is 23 times more than nonsmokers. Moreover, the 5-year survival rate for lung cancer is only 17% (Siegel et al., 2014). Smoking causes emphysema and chronic bronchitis, the key symptoms of chronic obstructive pulmonary disease

(COPD), which further facilitates the malignant transformation of normal epithelial cells in long-term smokers. Hence, a detailed study on smoke-induced molecular alterations may aid not only in diagnosis and prognosis of lung cancer but also may help in identification of therapeutic targets for non-small cell lung carcinoma (NSCLC) patients specially smokers with better understanding of underlying biological networks associated with smoke-induced lung cancer.

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H292, is a human lung mucoepidermoid carcinoma cell line originally derived from a nonsmoker (Phelps et al., 1996), has been used widely to study the effect of cigarette smoke (Baginski et al., 2006; Luppi et al., 2005; Newland and Richter, 2008; Shao et al., 2004). H292 cells have also been used by researchers for the assessment and reliability of *in vitro* cigarette smoke systems (Adamson et al., 2011) and to study the toxicity of aerosols generated from E-cigarettes or electronic nicotine delivery systems (Leigh et al., 2016). Gene expression studies using H292 cells have reported increased expression of genes associated with oxidative stress, inflammation, DNA damage, and xenobiotic response in response to cigarette smoke (Sekine et al., 2014). Reports indicate that cigarette smoke leads to the activation of mitogen- and stress-activated kinase 1 protein, which in turn is involved in promoting inflammation by increasing IL8 release in lung cells through EGFR/Shp2/mitogen-activated protein kinase (MAPK) signaling (Li et al., 2012).

Cigarette smoke upregulates EGFR mRNA and EGFR tyrosine phosphorylation in H292 cells, which in turn enhances MUC5AC secretion. Mucus secretion entails an important role in COPD progression (Shao et al., 2004; Takeyama et al., 2001). Low concentration of cigarette smoke has been reported to induce proliferation of H292 cells by activation of ERK1/2, while higher concentrations of smoke have an inhibitory effect on cell proliferation through activation of the pro-apoptotic MAP kinases p38 and JNK (Luppi et al., 2005). Cigarette smoke also leads to activation of Rac1, a Rho family GTPase expression, and initiates epithelial-mesenchymal transition (EMT) in lung cells (Shen et al., 2014).

Although these studies have identified certain signaling mechanisms by which cigarette smoke may exert its effects; till date, no effort has been made to study the changes in cellular signaling induced by chronic exposure to cigarette smoke using quantitative phosphoproteomics approach.

To achieve this, we developed a cell line-based model, where H292 cells were chronically treated with cigarette smoke condensate (CSC) for 12 months. We carried out stable isotope labeling by amino acids in cell culture (SILAC)-based quantitative proteomic analysis of H292 cells to investigate the signaling mechanism in response to chronic cigarette smoke exposure. Using titanium dioxide (TiO₂) and P-Tyr-1000 antibody based method for the enrichment of phosphopeptides and phosphotyrosine peptides, respectively, we identified 493 peptides which were hyperphosphorylated and 195 peptides that were hypophosphorylated (2.0-fold) in smoke exposed cells.

Bioinformatics analysis revealed perturbations in pathways regulating cytoskeletal remodeling, cell survival, and proliferation. In particular, we identified that molecules differentially phosphorylated upon chronic exposure of cigarette smoke are associated with PI3K/AKT/mTOR and CDC42-PAK signaling networks, which are known to be involved in several cellular processes such as cell polarity, cytoskeletal remodeling, cell migration, protein synthesis, autophagy, and apoptosis.

Materials and Methods

Cell culture, SILAC labeling, and trypsin digestion

Human lung mucoepidermoid carcinoma cell line NCI-H292 was purchased from American Type Culture Collection (ATCC, Manassas, VA). H292 cells were cultured in Dulbecco's mod-

ified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Clontech, Mountain View, CA) and 1% penicillin/streptomycin solution and maintained in humidified incubator at 37°C with 5.0% CO₂.

To study the effect of the CSC on H292 cells, the cells were chronically treated with 0.1% CSC (Murty Pharmaceuticals, Inc., KY) for 12 months. Cells cultured in normal condition and in the presence of smoke were labeled as H292-P (parental) and H292-S (smoke), respectively. The H292-parental cells were then adapted to SILAC media by growing in DMEM lacking normal lysine and arginine amino acids (Carlsbad, CA), but supplemented with ¹³C₆-Lysine and ¹³C₆-Arginine (Cambridge Isotope Laboratories, Andover, MA).

SILAC labeled H292-parental cells and H292-smoke cells were washed with ice-cold phosphate buffered saline following serum starvation and lysed in urea lysis buffer (20 mM HEPES pH 8.0, 9.0 M urea, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM phosphoglycerophosphate). Bicinchoninic acid assay was used to estimate protein amounts. Equal amount of protein from both conditions was mixed followed by reduction and alkylation using dithiothreitol (5 mM) and iodoacetamide (10 mM), respectively. Urea concentration in the sample was brought to <2 M by diluting it with HEPES buffer (20 mM) and digested overnight at 37°C using trypsin (Worthington Biochemical Corp.). The digested peptides were cleaned using Sep-Pak C₁₈ column, and the eluted peptides were lyophilized and stored at -80°C till further analysis.

Immunoaffinity enrichment of tyrosine phosphopeptides

Immunoaffinity enrichment of phosphopeptides was carried out according to previously described protocol (Rush et al., 2005). Briefly, 30 mg of lyophilized peptides were resuspended in 1.4 mL of Immunoaffinity purification (IAP) buffer (50 mM MOPS pH 7.2, 10 mM sodium phosphate, 50 mM NaCl) and adjusted to pH 7.2 using 1 M Tris Base. After washing P-Tyr-1000 beads (Cell Signaling Technology, Beverly, MA) twice with IAP buffer at 4°C, the peptides were incubated with P-Tyr-1000 beads for 20 min. Beads with bound peptides were washed thrice with ice-cold IAP buffer and then twice with ice-cold water. Peptides were eluted twice using 0.15% trifluoroacetic acid (TFA) concentrated by vacuum centrifugation followed by desalting using C₁₈ StageTips before mass spectrometry analysis.

Basic pH reversed-phase liquid chromatography and enrichment of TiO₂-based phosphopeptides

Lyophilized peptides were resuspended in 7 mM triethylammonium bicarbonate (TEABC) (pH 9) and fractionated using basic pH reversed-phase liquid chromatography (BRPLC). The dissolved peptides were loaded onto XBridge BEH C₁₈ Column (Waters, United Kingdom) and resolved with an increasing gradient of 7 mM TEABC and 90% acetonitrile (pH 9) over 30 min duration at a flow rate of 1 mL/min using Agilent 1100LC system. A total of 96 fractions collected during fractionation were further pooled to 12 fractions and dried in SpeedVac. Each of the fractions was subjected to phosphopeptide enrichment using TiO₂ based method.

The TiO₂ beads (Titansphere; GL Sciences, Inc.) were suspended in DHB solution (80% ACN, 1% TFA, and 5%

2,5-Dihydroxybenzoic acid) at room temperature for 1 h. Each of the fractions was then resuspended in 5% DHB solution and incubated with TiO₂ beads for 30 min at room temperature with gentle rotation. TiO₂ beads enriched with phosphopeptides were washed thrice with DHB solution and then with 40% ACN twice. The enriched phosphopeptides were eluted thrice into tubes containing 20% TFA on ice using 2% ammonia solution. The peptides were concentrated by vacuum centrifugation and subjected to C₁₈ StageTips cleanup before mass spectrometry analysis.

Liquid chromatography-tandem mass spectrometry and data analysis

brPLC fractionated phosphopeptides were analyzed using LTQ-Orbitrap Velos mass spectrometer (Thermo Electron, Bremen, Germany) interfaced with EASY-nLC II nanoflow liquid chromatography system (Thermo Scientific, Odense, Southern Denmark). Each fraction was reconstituted in Solvent A (0.1% Formic acid) and loaded on trap column (75 μ m $\times$ 2 cm) packed with Magic C₁₈ AQ (Michrom Biosciences, Inc., Auburn, CA) (5 μ m particle size, pore size 100 Å). Peptides were resolved on an analytical column (75 μ m $\times$ 15 cm) at a flow rate of 350 nL/min using a linear gradient from 5% to 60% ACN in a 100 min run. MS data were acquired using scan range of 350–1800 m/z at mass resolution of 60,000, and MS/MS data were acquired using resolution of 15,000 at m/z of 400. HCD fragmentation for MS/MS analysis of the 15 most abundant ions was carried out with isolation width of 1.90 m/z and normalized collision energy of 39%.

Mass spectrometry data (.raw) were searched against human RefSeq database (RefSeq59) appended with frequently observed contaminants using Mascot (v2.2) and SEQUEST search algorithms through the Proteome Discoverer platform (v1.4, Thermo Scientific, Bremen, Germany). The search parameters for both algorithms included maximum of two missed cleavage, oxidation of methionine, carbamidomethylation at cysteine as a fixed modification, phosphorylation at serine, threonine, and tyrosine and SILAC labels ¹³C₆-Lysine; ¹³C₆-Arginine as dynamic modifications.

The precursor mass error tolerance of 20 ppm and fragment mass error tolerance of 0.1 Da were considered during analysis. The data were searched against a decoy database, and peptides identified at <1% false discovery rate (FDR) were considered further for protein identification. The phosphorylation probability at each S/T/Y site was calculated using the PhosphoRS node (Version 3.0) in the Proteome Discoverer, and peptides with more than 75% site localization probability were considered for further analysis. Enriched motifs were identified using motif-X algorithm (Schwartz and Gygi, 2005) for which phospho window of 15aa long was used to extract consensus motif.

Data availability

Mass spectrometry raw data have been submitted to ProteomeXchange Consortium (www.proteomecentral.proteomexchange.org) through the PRIDE public data repository which can be accessed using the data identifier—PXD006672.

Invasion assays. The invasion assay was performed using a transwell system (BD Biosciences, San Jose, CA) with Matrigel-coated polyethylene terephthalate (PET) filters similarly to previously described protocol (Subbannayya et al.,

2015). Briefly, 20,000 cells in 500 μ L of serum-free media were seeded on the Matrigel-coated PET membrane in the upper compartment of transwell system. The lower compartment was filled with 500 μ L of complete media. After an incubation period of 48 h at 37°C, migrated cells on the lower surface of the membrane were fixed and stained with methylene blue. Each experiment was performed in triplicate, and the experiments were repeated thrice.

Western blotting. Whole cell extracts of H292 parental and H292 smoke cells were prepared in modified RIPA lysis buffer (Merck Millipore, Billerica, MA) containing protease inhibitors (Roche, Indianapolis, IN) and phosphatase inhibitors (Thermo Scientific). Western blot analysis was performed as previously described using 40 μ g protein lysates. Nitrocellulose membranes were hybridized with primary antibodies and developed using Luminol reagent (Santa Cruz Biotechnology, Dallas, TX) as per the manufacturer's instructions. The antibodies against following molecules were purchased from Cell Signaling Technology—Bcl-xL, Bcl-2, Bax, E-Cad, N-Cad, Slug, Snail, Twist, STAT3, phospho-STAT3 (S727), NF- κ B, phospho-NF- κ B (S276 and S468), AKT, and phospho-AKT (S473 and T308). Beta-actin antibody was obtained from Sigma (St. Louis, MO).

Results and Discussion

Cigarette smoke enhances survival proteins in lung cells

To understand the effects of chronic cigarette smoke exposure in lung cancer cells, we developed a cell line model using spontaneously immortalized mucoepidermoid cell line H292. These cells were exposed to CSC (0.1%) for 12 months and were designated as H292-S, and the cells not exposed to smoke (parental) were referred as H292-P. We observed alterations in cellular morphology (Fig. 1A) and invasion property of cells chronically exposed to CSC. Matrigel based invasion assays showed increase in invasive property of H292-S, compared with H292-P cells (Fig. 1B). We studied the expression of BCL-2 family proteins in response to cigarette smoke. We observed an increased ratio of both Bcl-xL and Bcl-2 to Bax in H292-smoke cells compared to the parental cells (Fig. 1C). The increased ratio of Bcl-xL and Bcl-2 to Bax indicates that the cells gain an advantage toward survival evading apoptosis (Cheng et al., 2001) and that chronic exposure of cigarette smoke results in oncogenic transformation in lung cells.

Cigarette smoke induces early EMT in lung cells

Several studies have reported that cigarette smoke exposure induces EMT in various cell types, including lung cells (Jeon et al., 2016; Kim et al., 2017; Vu et al., 2016). The smoke induced EMT is known to enhance the migratory and invasive potential of cells through several pathways, including MAPK pathways (Park and Kim, 2017; Sun et al., 2017; Yu et al., 2017). Hence, we sought to check the expression of EMT markers in H292-P and H292-S cells. We observed a decrease in processed E-cadherin and an increase in the expression of N-cadherin and Twist in the H292-S cells compared to the parental cells, indicating early signs of EMT in the smoke treated cells (Fig. 1D). Expression of Vimentin was not detectable in either the parental or the smoke treated cells (data not shown).

FIG. 1. Chronic exposure to cigarette smoke increases invasive potential of cells and enhances cell survival proteins. **(A)** Cellular morphology of parental (H292-P) and smoke treated cells (H292-S). **(B)** Invasive ability of parental (H292-P) and smoke treated cells (H292-S) (* $p < 0.05$). **(C)** Western blot analysis of cell survival proteins in H292-P and H292-S cells **(D)** Western blot analysis of epithelial-mesenchymal transition marker proteins in H292-P and H292-S. β -actin serves as a loading control. **(E)** Western blot analysis of STAT3 (S727) and NF- κ B (S276 and S468) activation sites in H292-P and H292-S. NF- κ B, NF-kappa-B; STAT, signal transducer and activator of transcription.

Phosphoproteomic analysis in cigarette smoke treated lung mucoepidermoid cancer cells

We used SILAC based phosphoproteomics approach to study signaling perturbations in smoke treated cells. The

pSer/pThr/pTyr containing peptides were enriched using titanium dioxide (TiO_2) beads, and immunoaffinity based approach was used to enrich phosphotyrosine containing peptides. The bRPLC coupled with LTQ-Orbitrap Velos mass spectrometer was used to analyze enriched phosphopeptides.

The schematic workflow used to carry out study SILAC-based phosphoproteomics is shown in Supplementary Figure S1. Using high resolution mass spectrometry, we identified a total of 2615 unique phosphopeptides (2472 unique phosphorylation sites) corresponding to 1436 proteins. The unique phosphorylation sites comprise 1895 serine, 331 threonine, and 246 tyrosine sites.

Using phosphoRS probability cutoff of 75%, we identified 493 hyperphosphorylated sites corresponding to 341 proteins and 195 hypophosphorylated sites mapped to 142 proteins, respectively. The list of identified phosphopeptides is provided in Supplementary Table S1A, and list of unmodified peptides is provided in Supplementary Table S1B.

Functional annotation of phosphoproteome in response to cigarette smoke

Since we observed widespread signaling alterations in cigarette smoke exposed cells, we performed bioinformatics analysis of the differentially phosphorylated proteins to categorize them based on their cellular localization and biological function. The classification of proteins was done using human protein reference database (HPRD) which is a gene ontology compliant database (Keshava Prasad et al., 2009; Prasad et al., 2009). Functional enrichment of differentially phosphorylated proteins using HPRD as backend database was done using FunRich tool (Pathan et al., 2015).

Our analysis revealed diverse molecular functions for the proteins which were differentially phosphorylated upon chronic cigarette smoke exposure. A large fraction of differentially phosphorylated proteins were from transcription regulatory activity (7.1%), which includes nuclear factor- κ B (NFKB2), signal transducer and activator of transcription (STAT1 and STAT3), and transcription factor AP-1 (JUN). The transcription factors, STAT3 and NFKB2, create an inflammatory microenvironment in bronchial epithelium upon chronic exposure of cigarette smoke. These inflammation mediators play significant role in signal transduction leading to progression and transformation of COPD into pro-cancerous lesions followed by lung cancer (Di Stefano et al., 2002; Sekine et al., 2014; Tang et al., 2006; Tichelaar et al., 2005).

In our study, we identified hyperphosphorylation of STAT3 at S727 (2.1-fold). This was further validated by Western blot (Fig. 1E). We observed hyperphosphorylation of NFKB2 (S222; 27.5-fold) in response to cigarette smoke. In addition, we evaluated the expression of NF- κ B activation sites (S276 and S468) using western blot. We found hyperphosphorylation of both the activation sites in H292-S cells (Fig. 1E). Long-term activation of NF- κ B in mice resulted in macrophage and lymphocyte infiltration in airway epithelial cells, which substantiated the critical role of NF- κ B in chronic airway inflammation leading to progressive lung carcinogenesis (Zaynagetdinov et al., 2012). In addition to STAT3, we also observed hyperphosphorylation of STAT1 at S727 (7.7-fold) and Y701 (14.8-fold).

Activation of STAT1 in respiratory epithelial cells has been shown to potentiate CSE-induced cytotoxicity (Wang et al., 2014). Overexpression and hyperphosphorylation of JUN are reported in NSCLC (Heasley and Winn, 2008; Szabo et al., 1996). We observed hyperphosphorylation of JUN (6.6-fold) at S63 in smoke exposed cells. Other differentially phosphorylated proteins were involved in cytoskeletal re-

modeling (4.0%), structural molecular activity (3.2%), and cell adhesion (3.6%) (Supplementary Fig. S2A).

Further analysis revealed that majority of differentially phosphorylated proteins were localized to cytoplasm (66.4%) followed by nucleus (63.3%), plasma membrane (25.3%), and cytoskeleton (4.4%) (Supplementary Fig. S2B). We carried out ingenuity pathway analysis based grouping of differentially phosphorylated proteins into networks and canonical pathways. The top enriched pathways identified were those associated with cytoskeletal arrangement, cell motility, cellular assembly and organization, cell cycle, cell death and survival, cell-to-cell signaling, and cancer progression (Table 1). The proteins in significantly enriched networks were found to be associated with Rho GTPase-PAK signaling and PI3K/AKT/mTOR signaling.

Alteration of Protein kinases and phosphatases in cigarette smoke induced signaling

Protein kinases and phosphatases play key regulatory role in signaling cascade. The 518 differentially phosphorylated proteins identified in our dataset include 48 kinases and 10 phosphatases. The differentially regulated kinases in smoke treated cells were mapped in kinome tree (Fig. 2) using Kin-Map (Eid et al., 2017). Kinome tree classified these proteins into six out of eight typical groups [TK (tyrosine kinase); TKL (tyrosine kinase like); AGC (containing protein kinases A, G, and C); CAMK (calcium/calmodulin-dependent protein kinase); CK1 (casein kinase 1); CMGC (containing cyclin-dependent kinase, MAPK, glycogen synthase kinase 3, and CDC2 like); and STE (homologs of yeast sterile 7, sterile 11, and sterile 20)] and two atypical families (pyruvate dehydrogenase kinase and TIF family of protein kinases). A total of 43 kinases out of 48 kinases were mapped in Kinome tree.

We identified differential phosphorylation of receptor tyrosine kinases which includes ephrin type-A receptor 4 (EPHA4), epithelial discoidin domain-containing receptor 1, and AXL receptor tyrosine kinase (AXL). Overexpression of EPHA4 is reported in various carcinomas such as gastric cancer (Miyazaki et al., 2013) and pancreatic cancer (Liu et al., 2014). EPHA4 is a transmembrane receptor tyrosine kinase, which interacts with cyclin-dependent kinase 5 and enhances the expression of p-AKT (Ding et al., 2017). We identified hyperphosphorylation of EPHA4 at Y602 (3.6-fold) in our data. We report hyperphosphorylation of this site in response to cigarette smoke, indicating that these sites may contribute to smoke-induced cellular transformation.

We found hyperphosphorylation of Src family tyrosine-protein kinase Lyn at Y32 (5.7-fold), Y194 (2.0-fold), and Y487 (3.3-fold). Lyn has been reported to be expressed in lung alveoli epithelial lining, and knockout studies in mouse have established role of Lyn in lung physiology and smoke associated cytotoxicity (Wang et al., 2014). In presence of RACK1 and PKC β II, Lyn binds to EGFR and leads to its activation through PI3K and c-Met mediated signaling cascade in NSCLC (Sutton et al., 2013).

In addition, Src family kinases are known to interact with neural precursor cell expressed, developmentally down-regulated 9 (NEDD9) (Cabodi et al., 2010; Manie et al., 1997). NEDD9 is hyperphosphorylated at multiple tyrosine residues in our data—Y241 (4.2-fold), Y214 (3.7-fold), Y345 (3.5-fold), Y177 (10.0-fold), Y92 (3.3-fold), Y166 (3.0-fold),

TABLE 1. TOP SIX BIOLOGICAL NETWORKS IDENTIFIED BY INGENUITY PATHWAY ANALYSIS

<i>Top networks</i>	<i>Associated molecules</i>	<i>IPA score</i>	<i>Focus molecules</i>
Cellular Assembly and Organization, Infectious Diseases, Dermatological Diseases and Conditions	Akt, <i>AP3D1</i> , <i>CLASRP</i> , Collagen type I, <i>CRTC2</i> , DAP, <i>EIF4ENIF1</i> , <i>EPHA4</i> , <i>HAUS6</i> , <i>HNRNPA1</i> , <i>HNRNPK</i> , Hsp90, <i>KHDRBS1</i> , <i>LDLR</i> , <i>LIMA1</i> , <i>LMNA</i> , <i>MATR3</i> , <i>MEPCE</i> , <i>NOB1</i> , <i>OSBPL8</i> , P-TEFb, <i>PALLD</i> , <i>PDLIM5</i> , <i>PI4K2A</i> , <i>PPP1R18</i> , <i>PRRC2A</i> , <i>RAVER1</i> , <i>RBMX</i> , <i>SEC16A</i> , <i>SRRM2</i> , <i>SYNPO</i> , <i>TNKS1BP1</i> , WASHC2A/WASHC2C, <i>XRN2</i> , <i>ZNF318</i>	42	31
Cell Cycle, Gene Expression, Cell Death and Survival	Ap1, <i>CELSR1</i> , estrogen receptor, <i>FAT1</i> , <i>FOSL2</i> , <i>GAPDH</i> , <i>GORASP2</i> , <i>HSF1</i> , <i>IFI16</i> , IFN Beta, <i>IL1A</i> , <i>Interferon</i>	36	28
Cell Cycle, Cancer, Organismal Injury and Abnormalities	14-3-3 proteins, <i>CDC27</i> , <i>CDC42BPG</i> , cytochrome-c oxidase, <i>DDX17</i> , <i>ELF4</i> , <i>EPHB2</i> , <i>ERBB2</i> , <i>G6PD</i> , <i>GDF15</i> , Gsk3, <i>Hdac</i> , <i>HDAC1</i> , <i>HDAC7</i> , <i>Histone h4</i> , Hsp27, Hsp70, <i>KANK2</i> , <i>MAPK13</i> , <i>MARK2</i> , <i>MDM2</i> , Mek, <i>MPRIP</i> , <i>NCAPD3</i> , <i>NELFB</i> , <i>PAK6</i> , <i>PCMI</i> , <i>PGRMC1</i> , <i>PML</i> , PP1-C, <i>RB1</i> , <i>RPL11</i> , <i>SMC4</i> , <i>SP100</i> , <i>UBR5</i>	32	26
Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization, Cellular Function and Maintenance	<i>AKAP13</i> , <i>AKT1S1</i> , <i>ARFGEF2</i> , <i>BAG3</i> , calpain, <i>CFL1</i> , Creb, <i>CTTN</i> , <i>EPS8</i> , FActin, <i>Filamin</i> , <i>Focal adhesion kinase</i> , <i>GIT1</i> , <i>HMGA1</i> , <i>HSPB8</i> , <i>HTT</i> , <i>INPPL1</i> , <i>IQGAP1</i> , <i>ITGB1</i> , <i>LRCH3</i> , <i>NFκB (complex)</i> , <i>PAK4</i> , Pka catalytic subunit, <i>PKN2</i> , <i>PRKD2</i> , <i>PXN</i> , <i>RPS6KA1</i> , Shc, <i>SOS1</i> , SRC (family), <i>SRCAP</i> , <i>Talin</i> , <i>TJP2</i> , <i>TNIP1</i> , <i>UBXN1</i>	30	25
Cell Death and Survival, Cancer, Organismal Injury and Abnormalities	20s proteasome, 26sProteasome, <i>Alpha tubulin</i> , <i>ANXA2</i> , <i>ATRX</i> , <i>BCL2L12</i> , <i>BICD2</i> , <i>CLCN7</i> , <i>CLIP1</i> , <i>COPB2</i> , Cyclin D, <i>Cyclin</i>	30	25
Cellular Assembly and Organization, Cellular Function and Maintenance, Cellular Movement	Actin, <i>ARHGEF17</i> , <i>ARPC1B</i> , <i>ATF2</i> , <i>BAHD1</i> , <i>CANX</i> , <i>CD3</i> , <i>CDC42EP3</i> , <i>EPS8L1</i> , <i>EPS8L2</i> , <i>FLNA</i> , <i>IRF6</i> , <i>LMO7</i> , <i>MAPK14</i> , <i>MBD1</i> , <i>MKL1</i> , <i>MTORC1</i> , <i>NCK1</i> , <i>P38 MAPK</i> , <i>PI3K</i>	28	24

Molecules identified in our study are in italics.
IPA, ingenuity pathway analysis.

and Y629/Y631 (2.2-fold). NEDD9 is highly expressed and abnormally phosphorylated in lung and breast cancer cells (Law et al., 1998). NEDD9 is known to alter various cellular processes through dynamic regulation of cytoskeleton, cellular transformation, and neoplastic growth leading to tumor progression, metastasis, and resistance to anticancer drugs in multiple tumor types, including breast cancer, lung cancer, glioblastoma, and melanoma (Guerrero et al., 2012; Tikhmyanova et al., 2010).

We have identified several members of MAPK signaling pathways which were differentially phosphorylated in response to cigarette smoke exposure. MAPK signaling is associated with cell proliferation, malignant transformation, stress response, and drug resistance (Coulthard et al., 2009; McCubrey et al., 2007). We observed increased phosphorylation of Y185 and T180/Y182 residues in MAPK12 (3.1-fold) and MAPK14 (2.3-fold), respectively. Apart from these known phosphorylation sites, we have also identified hyperphosphorylation at T76/Y78 (8.0-fold) residue in MAPK10.

In addition to kinases, we also identified altered phosphorylation of multiple phosphatases upon cigarette smoke exposure. We observed hyperphosphorylation of several

phosphatases, which includes phosphatidylinositol 4,5-bisphosphate 4-phosphatase isoform 2 (TMEM55B), TP53-induced glycolysis and apoptosis regulator (TIGAR), and phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2 (INPPL1). TMEM55B is an important regulator of cellular cholesterol metabolism and its knockdown in hepatoma cells is reported to reduce low-density lipoprotein receptors (LDLRs) (Medina et al., 2014). Increased expression of LDLRs is reported in pancreatic tumor cells and blocking LDLR expression reduces proliferative potential and also sensitizes cells toward cytotoxic drugs (Guillaumond et al., 2015).

However, the involvement of TMEM55B in cancer is not reported. We report hyperphosphorylation of TMEM55 Bat S162 (9.9-fold) in response to cigarette smoke, indicating possible role of smoke-induced metabolic transformation in rapidly dividing cells. TIGAR shares bisphosphatase domain of glycolytic fructose-2,6-bisphosphatase enzyme (Bensaad et al., 2006), and higher activity of TIGAR depletes intracellular fructose-2,6-bisphosphate thereby reducing the rate of glycolysis (Al-Khayal et al., 2016). Higher expression of TIGAR is reported in various cancers like breast (Ko et al., 2016), colorectal (Ahmad et al., 2017), and lung (Chen et al.,

TABLE 2. THE PARTIAL LIST OF KINASES/PHOSPHATASES REGULATED BY CIGARETTE SMOKE EXPOSURE

Gene symbol	Protein	Site	Phosphopeptide sequence	H292-S/H292-P
MAPK10	Mitogen-activated protein kinase 10	T81	TAGTSFMMtPyVVTR	8.0
EPHA4	Ephrin type-A receptor 4	Y602	TYVDPFTyEDPNQAVR	3.6
LYN	Tyrosine-protein kinase Lyn isoform	Y32	TIyVRDPTSNIK	5.7
LATS1	Serine/threonine-protein kinase LATS1	S613	QITTsPITVR	0.5
AXL	Tyrosine-protein kinase receptor UFO precursor	Y702	IYNGDyYR	0.3
TMEM55B	Phosphatidylinositol 4,5-bisphosphate 4-phosphatase isoform 2	S162	IINLGpVHPGPLsP	9.9
TIGAR	TP53-induced glycolysis and apoptosis regulator	S154	EADQKEQFsQGSPS	2.8
PTPN14	Tyrosine-protein phosphatase nonreceptor type 14	S456	NCLETSLAEIFPLGK	0.3
			GILHTDSQSQsLR	

of all phosphopeptides that possess the enriched motifs is provided in Supplementary Table S2. Consensus AKT motif (RxxS) was enriched in smoke treated cells, which suggest activation of PI3K/AKT pathway. This is supported by our western blot data where we observe activation of AKT sites (S473 and T308) in H292-S cells (Supplementary Fig. S3B). Hyperphosphorylation of AKT1S1 (AKT1 substrate 1) by AKT leads to its inactivation. We identified hyperphosphorylation of AKT1S1 (S88 and S92) (2.4-fold) upon chronic cigarette smoke exposure. AKT1S1 is negative regulator of the mammalian target of rapamycin complex 1 (mTORC1).

In addition, we also identified PKM which is known to activate mTOR pathway by phosphorylating AKT1S1 (He et al., 2016). The key downstream signaling molecules of mTORC1 which were significantly hyperphosphorylated in our data are unc-51 like autophagy activating kinase 1 (ULK1) [S450 (3.2-fold) and S638 (2.4-fold)], ribosomal protein S6 kinases [RPS6KA4 (S347) (3.9-fold) and RPS6KA1 (T573) (2.1-fold)], eukaryotic translation initiation factor 4E transporter (EIF4G1) (S1067) (2.4-fold), and 40S ribosomal protein S6 (RPS6) (S236) (2.0-fold). Hyperphosphorylation of ULK1 has been associated with increased disease progression and survival in cancer, including NSCLC (Caino et al., 2013; Xu et al., 2013).

FIG. 3. The signaling network of PI3K/AKT/mTOR pathway based on differentially phosphorylated molecules upon chronic exposure to cigarette smoke.

Recently, high expression of ULK1 also has been related with therapeutic resistance in human nasopharyngeal carcinoma (Yun et al., 2015). The MS and MS/MS spectrum for IGF1R (Y1164) and ULK1 (S450) are depicted in Supplementary Figure. S4A and B. The signaling network of PI3K/AKT/mTOR pathway based on differentially phosphorylated molecule identified in our data upon cigarette smoke exposure is represented in Figure 3.

Cigarette smoke induces cytoskeletal remodeling in lung cells through CDC42-PAK signaling pathway

CDC42 has been associated with epithelial cell morphogenesis by regulating cellular polarity, controlling spindle fiber orientation during mitotic cell division, and formation of cell-cell junctions. We identified hyperphosphorylation of cdc42 effector protein 3 (CDC42EP3) at S89 (3.7-fold).

FIG. 4. Representative MS/MS spectra depicting the hyperphosphorylation of (A) IQ motif containing GTPase activating protein 1 (IQGAP1) (2.1-fold), (B) p21 (RAC1) activated kinase 4 (PAK4) (2.0-fold).

CDC42 is active when bound to GTP, which is regulated by guanine nucleotide exchange factors (GEFs). We identified hyperphosphorylation of many GEFs (ARHGEF5, ARHGEF7, ARHGEF17, and ARHGEF18) in our data. TNK2 is a downstream target of CDC42; phosphorylation of TNK2 enhances the activity of GEFs toward CDC42 and maintains CDC42 in its active state bound to GTP (Kato-Stankiewicz et al., 2001). We identified hyperphosphorylation of TNK2 (activated CDC42 kinase 1) at Y827 (2.0-fold).

In addition, we identified hyperphosphorylation of PAK family members [PAK6 at S560 (3.0-fold) and PAK4 at S181

(2.0-fold)] upon cigarette smoke exposure. PAKs are immediate downstream effector molecules of Rho GTPase (Rac and CDC42) and important regulators of cytoskeletal dynamics leading to motile phenotype. PAK4 is an important target of CDC42, which is required for apical and tight junction formation in human bronchial epithelial cells (DeLong, 2010; Zhao and Manser, 2012). Knockdown of PAK4 also has been shown to suppress cell migration and invasion in A549 human lung cancer cells through inhibition of CREB, NF- κ B, and β -catenin pathway (Ryu et al., 2014). Studies from our group have reported hyperphosphorylation of PAK6 in

FIG. 5. A curated network of CDC42-PAK signaling upon chronic exposure of cigarette smoke.

response to cigarette smoke and that PAK6 can act as a potential therapeutic target in NSCLC, specifically in smokers (Raja et al., 2016). Apart from hyperphosphorylation of PAK, we also identified hyperphosphorylation of multiple known CDC42 and PAK substrates.

We have identified hyperphosphorylation of multiple signaling molecules associated with the CDC42-PAK signaling, which includes stathmin [(STMN1) (S38) (2.0-fold) and (S25) (2.6-fold)], cofilin-1 (CFL1) (Y89) (3.4-fold), MAPK [MAPK10 (Y78) (8.0-fold), MAPK12 (Y185) (3.1-fold), MAP2K2 (S23) (7.9-fold)], BCL-2 associated athanogene 3 (BAG3) (S377) (4.1-fold), Microtubule-associated protein 1B (MAP1B) (S1016) (24.5-fold). Substrates of CDC42 which were hyperphosphorylated include IQ motif containing GTPase activating protein 1 (IQGAP1) (S1443) (2.1-fold), Wiskott–Aldrich syndrome protein (WASL) (Y256) (2.2-fold), brain-specific angiogenesis inhibitor 1-associated protein 2 [(BAIAP2) (Y505) (3.4-fold) and (S33b) (3.2-fold)].

The MS and MS/MS spectra for IQGAP1 (S1443) and PAK4 (S181) are depicted in Figure 4A and B. CDC42 forms a tripartite complex with IQGAP1 and CAP-Gly domain-containing linker protein 1 (CLIP1) which helps in microtubule orientation and cellular polarization (Fukata et al., 2002). The actin-related protein 2/3 complex subunit 1B (ARPC1B) belongs to Arp2/3 complex. Arp2/3 complex is known to be activated by Wiskott–Aldrich syndrome protein family and is an important regulator of Actin polymerization. We identified hyperphosphorylation of ARPC1B at S310 (4.2-fold) in our data.

Interestingly, cytoskeletal remodeling associated with actin polymerization is known to inhibit LATS1 (Codellia et al., 2014). LATS1 mediates the phosphorylation and inhibition of the transcription coactivators (yes associated protein) YAP and (transcriptional coactivator with PDZ-binding motif) TAZ, major downstream effectors of the Hippo pathway. We identified hypophosphorylation of LATS1 at S613 in our data. In addition, the expression of LATS1 was found to be significantly lower in NSCLC patients (Lin et al., 2014).

Taken together, our data suggest that chronic exposure to cigarette smoke activates the signaling cascade of CDC42-PAK in an orchestrated manner. A schematic representation of enrichment of CDC42-PAK pathway upon chronic cigarette smoke exposure is provided in Figure 5. Pathway was drawn using “PathVisio” (www.PathVisio.org) (van Iersel et al., 2008).

Conclusions

Proteomics is a rapidly emerging specialty of post-genomics medicine and one of the key focus areas of environmental health research agenda (Hayes et al., 2016; Murthy et al., 2016; Ramos et al., 2016). Relatively little research exists on the long-term effects of cigarette smoke on lung cells using proteomics biotechnology. Several studies have demonstrated the ill effects of cigarette smoking, including DNA damage, oxidative stress, inflammation, and xenobiotic response. Earlier studies from our group have demonstrated the effect of cigarette smoke exposure on primary skin keratinocytes and identified dysregulation of proteins related to skin barrier maintenance, integrity, and oxidative stress in response to cigarette smoke. In this study, we investigated the alterations in global phosphoproteome (serine/threonine and tyrosine) upon chronic exposure to cigarette smoke in lung cells.

We report dysregulation of multiple kinases and phosphatases in response to cigarette smoke exposure. These were associated with regulation of many cellular processes such as cytoskeletal remodeling and cell proliferation, migration, and invasion. Taken together, we observe the activation of PI3K/AKT/mTOR and CDC42-PAK pathway in the lung cells chronically exposed to cigarette smoke. Taken together our studies indicate that the effect of cigarette smoke may vary depending on the cell or tissue. It is known that smokers are more prone to infections like pneumonia (Bagaitkar et al., 2008; Bok et al., 2008; Kono et al., 2012). Reports indicate that the internalization of *Pneumococci* by respiratory epithelial cells is dependent on coactivation of CDC42, PI3K, and AKT, a phenomenon observed in our model cell line (Agarwal and Hammerschmidt, 2009).

Our data collectively indicate that the cell model presented in this study might serve as a rich resource to understand not only the molecular mechanism of cigarette smoke induced transformation but also aid in understanding and evaluating the cause of susceptibility of smokers to diseases impacted by long-term smoke. The present study will, therefore, not only aid in better understanding of smoke mediated signaling in lung cancer cells but also permit the multiple kinases identified in our study to serve as potential molecular biomarker targets in lung cancer among smokers where the first line of therapy has shown limited success.

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Author Disclosure Statement

The authors' declare that no conflicting financial interests exist.

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Abbreviations Used

ARPC1B = actin-related protein 2/3 complex subunit 1B

bRPLC = basic pH reversed-phase liquid chromatography

COPD = chronic obstructive pulmonary disease

CSC = cigarette smoke condensate

DMEM = Dulbecco's modified Eagle's medium

EMT = epithelial-mesenchymal transition

EPHA4 = ephrin type-A receptor 4

FDR = false discovery rate

GEFs = guanine nucleotide exchange factors

PET = polyethylene terephthalate

IAP = immunoaffinity purification

IGF1R = insulin-like growth factor 1 receptor

IRS1 = insulin receptor substrate 1

LDLR = low-density lipoprotein receptor

MAPK = mitogen-activated protein kinase

mTORC1 = mammalian target of rapamycin complex 1

NEDD9 = neural precursor cell expressed, developmentally downregulated 9

NSCLC = non-small cell lung carcinoma

HPRD = human protein reference database

SILAC = stable isotope labeling by amino acids in cell culture

STAT = signal transducer and activator of transcription

TEABC = triethylammonium bicarbonate

TIGAR = TP53-induced glycolysis and apoptosis regulator

TiO₂ = titanium dioxide

ULK1 = unc-51 like autophagy activating kinase 1