ORIGINAL ARTICLE

Hookah Smoke Mediates Cancer-Associated Epigenomic and Transcriptomic Signatures in Human Respiratory Epithelial Cells

Yin Xiong, PhD,[a] Sichuan Xi, PhD,[a] Sudheer Kumar Gara, PhD,[a] Jigui Shan, PhD,[b] James Gao, PhD,[a] Mary Zhang, MS,[a] Vivek Shukla, PhD,[a] Ruihong Wang, PhD,[a] Chuong D. Hoang, MD,[a] Haobin Chen, MD, PhD,[a] David S. Schrump, MD, MBA[a,*]

[a]*Thoracic Epigenetics Section, Thoracic Surgery Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland*
[b]*The Biomedical Informatics and Data Science, Frederick National Laboratory for Cancer Research, Frederick, Maryland*

Received 27 January 2021; revised 14 April 2021; accepted 23 April 2021
Available online - 8 May 2021

ABSTRACT

Introduction: Although communal smoking of hookah by means of water pipes is perceived to be a safe alternative to cigarette smoking, the effects of hookah smoke in respiratory epithelia have not been well characterized. This study evaluated epigenomic and transcriptomic effects of hookah smoke relative to cigarette smoke in human respiratory epithelial cells.

Methods: Primary normal human small airway epithelial cells from three donors and cdk4 and hTERT-immortalized small airway epithelial cells and human bronchial epithelial cells were cultured for 5 days in normal media with or without cigarette smoke condensates (CSCs) or water pipe condensates (WPCs). Cell count, immunoblot, RNA sequencing, quantitative real-time reverse-transcriptase polymerase chain reaction, methylation-specific polymerase chain reaction, and quantitative chromatin immunoprecipitation techniques were used to compare effects of hookah and cigarette smoke on cell proliferation, global histone marks, gene expression, and promoter-related chromatin structure.

Results: CSC and WPC decreased global H4K16ac and H4K20me3 histone marks and mediated distinct and overlapping cancer-associated transcriptome signatures and pathway modulations that were cell line dependent and stratified across lung cancer cells in a histology-specific manner. Epiregulin encoding a master regulator of EGFR signaling that is overexpressed in lung cancers was up-regulated, whereas FILIP1L and ABI3BP encoding mediators of senescence that are repressed in lung cancers were down-regulated by CSC and WPC. Induction of epiregulin and repression of FILIP1L and ABI3BP by these condensates coincided with unique epigenetic alterations within the respective promoters.

Conclusions: These findings support translational studies to ascertain if hookah-mediated epigenomic and transcriptomic alterations in cultured respiratory epithelia are detectable and clinically relevant in hookah smokers.

Keywords: Hookah smoke; Cigarette smoke; Respiratory epithelia; EREG; FILIP1L; ABI3BP

Introduction

Irrefutable links between cigarette smoking and lung cancer risk[1] have prompted public health measures and

*Corresponding author.

Disclosure: *The authors declare no conflict of interest.*

Address for Correspondence: David S. Schrump, MD, MBA, Thoracic Epigenetics Section, Thoracic Surgery Branch, Center for Cancer Research, National Cancer Institute, Building 10, 4-3942, 10 Center Drive, Bethesda, MD 20892 E-mail: David_Schrump@nih.gov

Cite this article as: Xiong Y, et al. Hookah Smoke Mediates Cancer-Associated Epigenomic and Transcriptomic Signatures in Human Respiratory Epithelial Cells. JTO Clin Res Rep 2021;2:100181

ISSN: 2666-3643

https://doi.org/10.1016/j.jtocrr.2021.100181

legislative efforts in the United States and other industrialized nations to prevent initiation of smoking in minors and reduce cigarette addiction among adults. Decreasing cigarette consumption has been offset by the use of relatively unregulated cigarette alternatives, such as communal smoking of hookah tobacco (shisha) by means of water pipe.[2] Although traditionally observed among various cultures throughout the Middle East, Southwest Asia, and North Africa, water pipe smoking, often known as hookah smoking, is rapidly becoming more prevalent worldwide. In the United States, an estimated 10% to 20% of adolescents and young adults are current hookah smokers.[2–4]

Because shisha burns at a lower temperature than cigarette tobacco (450°C versus 900°C) and is cooled and "filtered" by water, many smokers perceive hookah (particularly flavored blends) to be a safe alternative to cigarettes.[2,5] Nevertheless, during a typical hookah session that lasts approximately 1 hour, individuals inhale considerably more smoke than inhaled by smoking a conventional cigarette (9.8 liter versus 0.55 liter, respectively)[6]; as such, a hookah session results in approximately 1.7-, 6.5-, and 46-fold more nicotine, carbon monoxide, and tar exposures relative to a conventional cigarette.[6–8] Whereas hookah smoke has much lower levels of carcinogenic polyaromatic hydrocarbons (PAHs) per unit volume than cigarette smoke, a typical hookah session delivers approximately 20 times more total PAHs and nearly 50 times more heavy PAHs than a conventional cigarette.[9] Furthermore, relative to conventional cigarette smoke, hookah smoke has higher levels of carcinogenic metals, such as arsenic, lead, and chromium.[10] Thus, depending on which chemicals are used for comparison, carcinogen exposures from a single hookah session have been estimated to be equivalent to smoking 10 to 50 cigarettes.[9,11]

Despite potential health hazards of hookah smoking,[12] associations between this activity and human cancers have not been firmly established. The present study evaluated global epigenomic and transcriptomic effects of hookah smoke relative to conventional cigarette smoke in human respiratory epithelial cells.

Materials and Methods

Informed Consent

Not applicable to this study.

Cell Culture

Normal small airway epithelial cells (SAECs) from a 68-year-old White female smoker (tissue acquisition number: 24517), a 56-year-old black female nonsmoker (tissue acquisition number: 24835), and a 66-year-old Hispanic male nonsmoker (tissue acquisition number: 26789) were obtained from Lonza Inc. (Frederick, MD) and cultured in saline-adenine-glucose-mannitol (SAGM) media (Lonza Inc.). The cdk4 and hTERT-immortalized human bronchial epithelial cell line 3KT, established from a 65-year-old female smoker,[13] was generously provided by John D. Minna (UT Southwestern, Dallas, TX) and cultured in complete keratinocyte serum-free media (Invitrogen, Carlsbad, CA) supplemented with 5 mg/liter epidermal growth factor and 50 mg/liter bovine pituitary extract. The cdk4 and hTERT-immortalized human SAEC (HSAEC) line (H-SAEC-KT; CRL-4050) established from a 22-year-old male smoker was obtained from the American Type Culture Collection and cultured in SAGM media (Lonza Inc.).

Generation of Condensates and Cell Line Exposures

Cigarette smoke condensates (CSCs) were generated from Kentucky 3R4F research cigarettes using a Borgwaldt-LX1 smoking machine (Richmond, VA) and standard Federal Trade Commission smoking conditions (35 mL puff volume, 2.0 s duration, and 1 puff/min, 9 puffs per cigarette). Water pipe condensates (WPCs) were generated from Al-Fakher unflavored shisha tobacco with quick-lighting charcoal disks (Three Kings Brand, Bladel, Holland) on perforated foil above the tobacco using a Borgwaldt-S1000 shisha smoking machine. A total of 170 puffs were used to fully smoke each bowl of hookah; puff time was 20 seconds. At the 100th puff, an additional half charcoal disk was added to the bowl for optimal ignition of the tobacco. The condensates were trapped on preweighed Cambridge glass fiber filters that were then air-died for 1 hour and reweighed, and condensates were extracted from the filters by continuous shaking in corresponding cell culture media without DMSO for 3 hours to yield stock solutions (4 mg tar/mL), which were then further dissolved in SAGM or keratinocyte serum-free media.

For exposure experiments, cells were seeded overnight in six-well plates at 30% to 40% confluency in their respective normal media. On the following day, media was exchanged with normal media with or without CSC or WPC at designated concentrations for 5 days. Media and condensates were changed daily.

RNA Isolation and Quantitative Real-Time Reverse-Transcriptase Polymerase Chain Reaction

Submitted as Supplementary Methods. All primers and antibodies for experiments are listed in Supplementary Table 1.

RNA Sequencing and Data Analysis

Submitted as Supplementary Methods.

Western Blot

Submitted as Supplementary Methods.

Methylation-Specific PCR and Quantitative Chromatin Immunoprecipitation

Submitted as Supplementary Methods.

Statistical Analysis

Data are presented as mean ± SEM. To determine statistical significance, an analysis of variance and *t* test were used with a *p* value of 0.05 considered significant.

Results

Growth Inhibitory Effects of WPC and CSC in Cultured Cells

Preliminary experiments were performed to define exposure conditions for subsequent comparative studies. Quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis revealed that CSC and WPC mediated dose-dependent up-regulation of xenobiotic response genes in cultured respiratory epithelial cells (representative results pertaining to CYP1A1 are depicted in Supplementary Fig. 1*A*). CYP1A1 induction by 1 mg/mL and 2 mg/mL WPC approximated that observed for 0.05 and 0.1 mg/mL CSC, which we have used previously in our smoke exposure studies.[14] Additional experiments revealed that CSC and WPC mediated dose-dependent growth inhibition in SAEC, human bronchial epithelial cell (HBEC), and HSAEC (Supplementary Fig. 1*B*). The growth inhibitory effects of 1 mg/mL and 2 mg/mL WPC approximated those observed for 0.05 and 0.1 mg/mL CSC. Because we have previously estimated these CSC concentrations to correspond to approximately 0.5 to 1 pack-per-day exposures in humans,[14] and because a hookah session yields approximately 46-fold more tar than a conventional cigarette,[6] we chose to use 1 mg/mL and 2 mg/mL WPC for definitive experiments. These WPC concentrations were twofold to sixfold lower than those used by Rammah et al.[15] and Shihadeh et al.[16] in their studies pertaining to the effects of WPC on growth and signal transduction in lung cancer cells.

"Cancer-Associated" Histone Alterations Mediated by WPC and CSC

We have previously revealed that CSC mediates time- and dose-dependent decreases in global H4K16ac and H4K20me3 levels in SAEC and HBEC.[14] As such, immunoblot experiments were performed to evaluate if WPC could induce similar histone alterations in respiratory epithelia. As revealed in Supplementary Figure 2, WPC mediated dose-dependent decreases in both histone marks in SAEC, HBEC, and HSAEC.

Transcriptome Profiles Mediated by CSC and WPC in SAEC

RNA sequencing (RNA-seq) experiments were performed to compare transcriptome signatures mediated by CSC and WPC in respiratory epithelial cells. Using criteria of twofold or greater change relative to controls, 4142 plus or minus 160 transcripts were differentially expressed in each of the three SAEC lines after 5-day exposure to 0.05 mg/mL CSC, of which 916 transcripts (~22%) were modulated in all three lines, possibly reflecting differences in genders, ethnicities, and smoking histories of the donors (Fig. 1*A*; left panel). More transcripts (6222 plus or minus 98; $p = 0.0001$) were modulated in SAEC after exposure to WPC 1 mg/mL; once again, a relatively limited number of transcripts (1320; 21%) were differentially expressed in all three lines (Fig. 1*A*; middle panel). A total of 249 transcripts (22%) were modulated by both condensates, of which 75 (30%) are cancer associated (Fig. 1*A*; right panel; Supplementary Table 2). Top canonical pathways associated with these common transcripts included xenobiotic metabolism, aryl hydrocarbon synthesis, and retinoate/retinol biosynthesis (Supplementary Fig. 3*A*; left panel). Top diseases and biofunctions associated with these transcripts are depicted in Supplementary Figure 3*A* (right panel); notably, cancer was ranked 24th. The top 100 transcripts up-regulated and down-regulated in all three SAEC lines by "low dose" CSC or WPC alone are listed in Supplementary Table 3.

A total of 4956 plus or minus 204 transcripts were modulated by CSC 0.1 mg/mL in each of the three SAEC lines, of which 1289 (~26%) were differentially expressed in all three lines (Fig. 1*B*; left panel). In comparison, 6999 plus or minus 375 transcripts ($p = 0.026$) were differentially expressed in each of the three SAEC lines after exposure to WPC 2 mg/mL, of which 1686 (~24%) were modulated across all three lines (Fig. 1*B*; middle panel). A total of 388 transcripts (~26%) were common to 0.1 mg/mL CSC and 2 mg/mL WPC exposures across the three lines (Fig. 1*B*; right panel; Supplementary Table 4); 191 (49%) are cancer-associated. Four of the top 10 canonical pathways and five of the top 16 diseases (31%) and biofunctions often associated with 0.1 mg/mL CSC and 2 mg/mL WPC exposures were also observed for the lower-dose condensate exposures (Fig. 1*C*; left panel and Supplementary Fig. 3*A*; left panel); notably, cancer emerged as the second highest ranked diseases and biofunctions associated with transcripts often regulated by "high dose" CSC and WPC, reflecting dose-dependent activation of pathways, such as NRF2-mediated oxidative stress response (Fig. 1*C*; right panel and Supplementary Fig. 3*A*; right panel). The top 100 transcripts uniquely up- and down-regulated in SAEC by "high dose" CSC or WPC are listed in Supplementary Table 5.

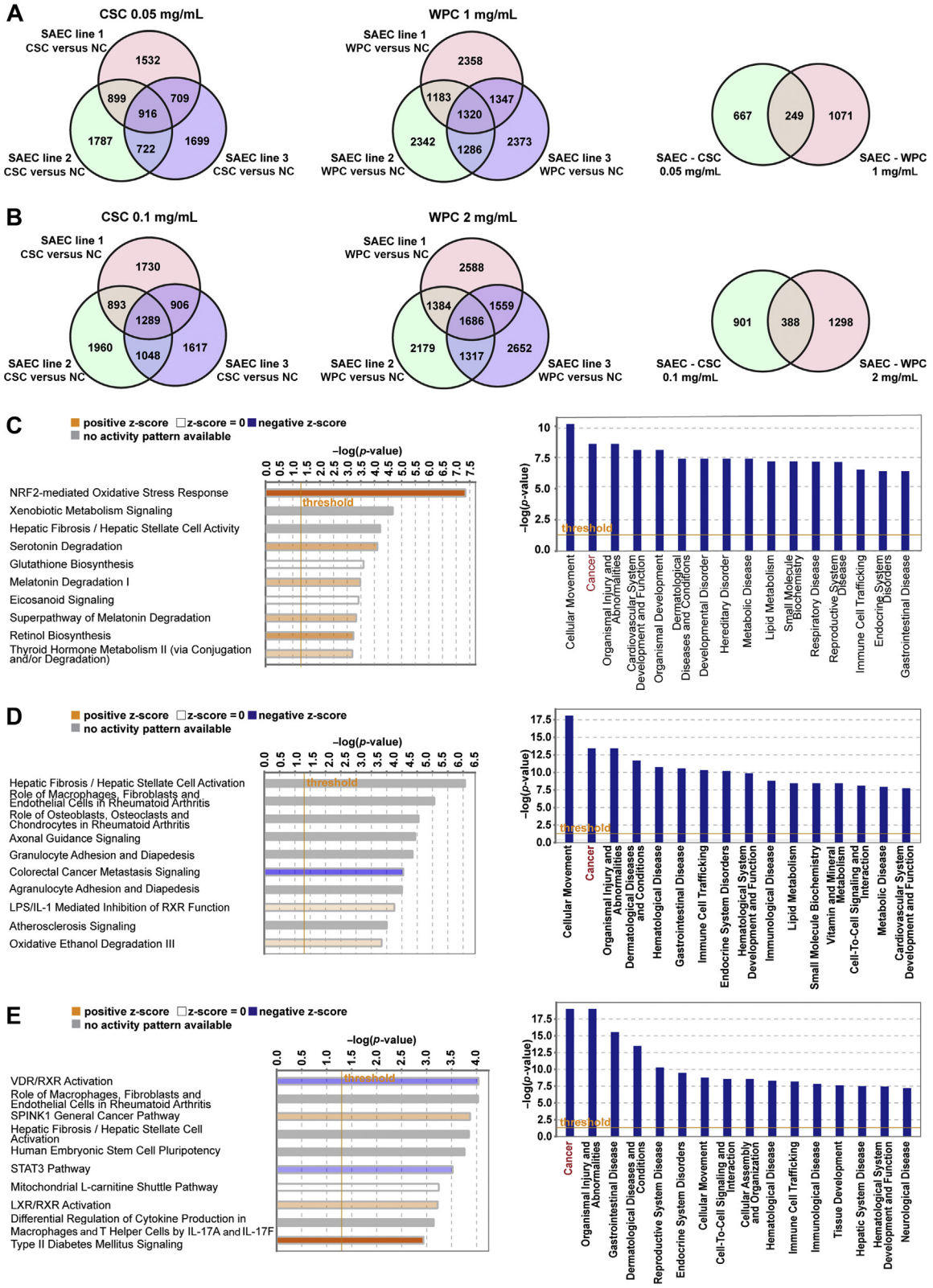

Figure 1. (*A*) Venn diagrams depicting genes modulated by CSC 0.05 mg/mL (left panel), WPC 1 mg/mL (middle panel), and CSC 0.05 mg/mL and WPC 1 mg/mL (right panel) in three SAEC lines. (*B*) Venn diagrams depicting genes modulated by CSC 0.1 mg/mL (left panel), WPC 2 mg/mL (middle panel), and CSC 0.1 mg/mL and WPC 2 mg/mL (right panel) in three SAEC lines. (*C*) Top canonical pathways (left panel) and top diseases and biofunctions (right panel) associated with genes often regulated by CSC 0.1 mg/mL and WPC 2 mg/mL in SAEC. (*D*) Top canonical pathways (left panel) and top diseases and biofunctions (right panel) associated with genes uniquely regulated by CSC 0.1 mg/mL in SAEC. (*E*) Top canonical pathways (left panel) and top diseases and biofunctions (right panel) associated with genes uniquely regulated by WPC 2 mg/mL in SAEC. CSC, cigarette smoke condensate; NC, normal control; SAEC, small airway epithelial cell; WPC, water pipe condensate.

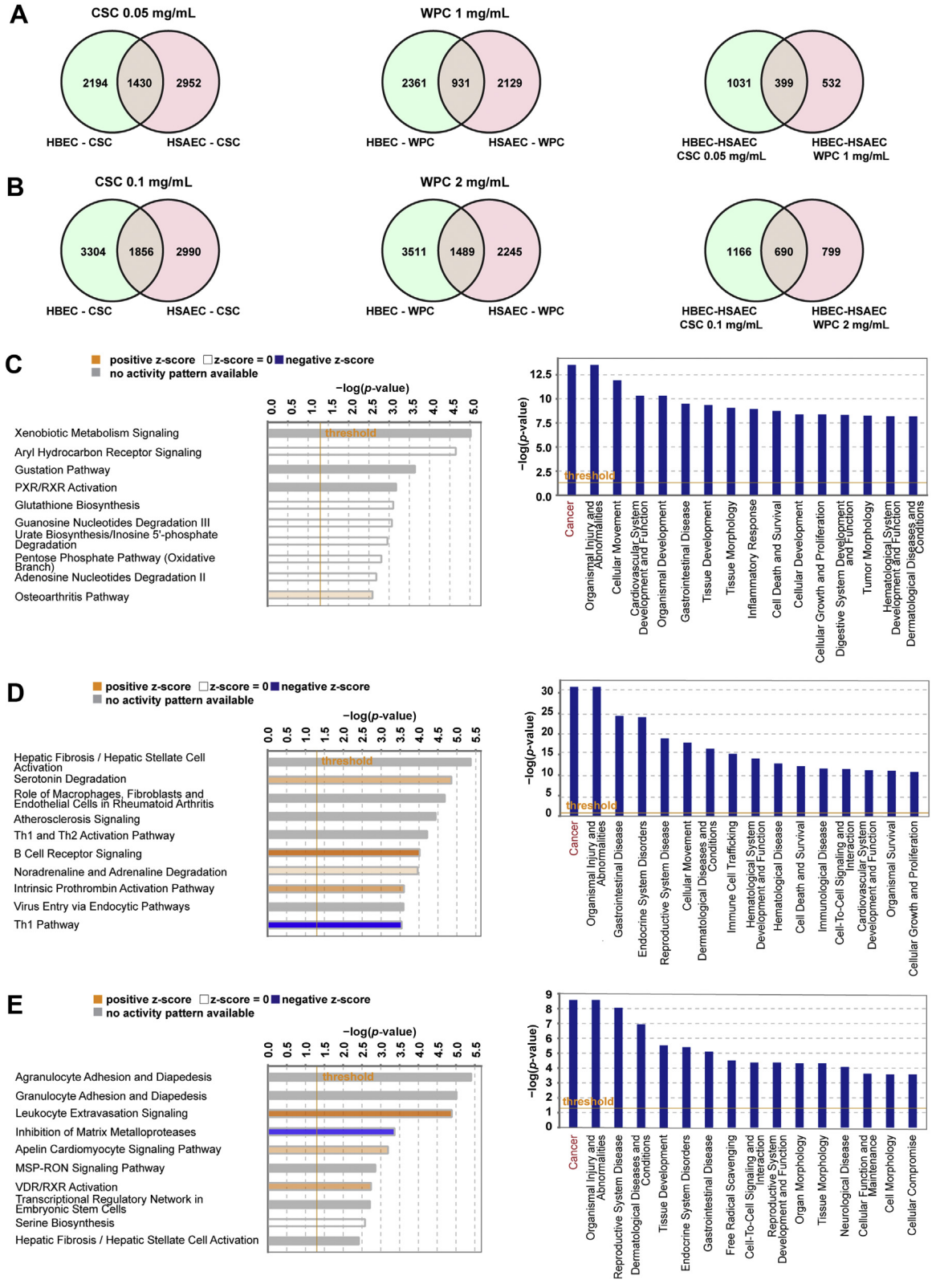

Figure 2. (*A*) Venn diagrams depicting genes modulated by CSC 0.05 mg/mL (left panel), WPC 1 mg/mL (middle panel), and CSC 0.05 mg/mL and WPC 1 mg/mL (right panel) in HBEC and HSAEC. (*B*) Venn diagrams depicting genes modulated by CSC 0.1 mg/mL (left panel), WPC 2 mg/mL (middle panel), and CSC 0.1 mg/mL and WPC 2 mg/mL (right panel) in HBEC and HSAEC. (*C*) Top canonical pathways (left panel) and top diseases and biofunctions (right panel) associated with genes often regulated by CSC 0.1 mg/mL and WPC 2 mg/mL in HBEC and HSAEC. (*D*) Top canonical pathways (left panel) and top diseases and

IPA was performed to evaluate the extent to which common pathways rather than individual transcripts were modulated by the different condensates. Of 667 transcripts uniquely regulated by CSC 0.05 mg/mL (Fig. 1*A*; right panel), 346 (52%) are associated with cancer. In contrast, of 1071 transcripts uniquely modulated by WPC 1 mg/mL (Fig. 1*A*; right panel), 295 (28%) are cancer associated. There were no canonical pathways common to these unique signatures (Supplementary Fig. 3*B* and *C*; left panels). Nevertheless, 6 of the top 16 (38%) diseases and biofunctions associated with transcriptome signatures uniquely associated with either CSC 0.05 mg/mL or WPC 1 mg/mL in SAEC were modulated by both condensates; cancer was the top ranked diseases and biofunctions associated with both unique signatures (Supplementary Fig. 3*B* and *C*; right panels).

Of 901 transcripts uniquely modulated in SAEC by 0.1 mg/mL CSC (Fig. 1*B*; right panel), 525 (58%) are cancer associated. In contrast, only 495 (38%) of 1298 transcripts unique to WPC 2 mg/mL exposures (Fig. 1*B*; right panel) are associated with cancer. Only two of the top 10 canonical pathways (hepatic fibrosis/hepatic stellate cell activation; role of macrophages, fibroblasts, and endothelial cells in rheumatoid arthritis) were associated with both exposures (Fig. 1*D* and *E* left panels). In contrast, 11 of the top 16 diseases (69%) and biofunctions were common to both unique signatures, with cancer ranking number 2 and number 1 for CSC and WPC exposures, respectively (Fig. 1*D* and *E*; right panels).

Transcriptome Effects of CSC and WPC in Immortalized Respiratory Epithelial Cells

Because immortalized respiratory epithelial cells are often used to evaluate effects of tobacco carcinogens, additional RNA-seq experiments were performed to evaluate transcriptome signatures mediated by CSC and WPC in HBEC and HSAEC-KT (hereafter referred to as HSAEC). Using criteria of twofold or greater change relative to controls, 3624 transcripts were differentially expressed in HBEC whereas 4382 transcripts were modulated in HSAEC after 5-day exposure to CSC 0.05 mg/mL; 1430 transcripts (∼39% and 32% of differentially expressed transcripts in HBEC and HSAEC, respectively) were modulated in both cell lines (Fig. 2*A*; left panel). WPC 1 mg/mL modulated expression of 3292 and 3060 transcripts in HBEC and HSAEC, respectively, of which 931 (∼28 and 30%) were differentially expressed in both cell lines (Fig. 2*A*; middle panel). A total of 399 transcripts (28% and 43%, respectively) were modulated by both condensates (Fig. 2*A*; right panel; Supplementary Table 6), of which 115 (29%) are cancer associated. Consistent with what was observed in SAEC, top canonical pathways linked with these 399 transcripts in HBEC and HSAEC (Supplementary Fig. 4*A*; left panel) included xenobiotic metabolism signaling, aryl hydrocarbon receptor signaling, and nicotine degradation. Top diseases and biofunctions included cancer and various organ development and metabolic functions (Supplementary Fig. 4*A*; right panel). The top 100 transcripts uniquely modulated by CSC 0.05 mg/mL and WPC 1 mg/mL in both HBEC and HSAEC are listed in Supplementary Table 7.

A total of 5160 and 4846 transcripts were differentially expressed in HBEC and HSAEC, respectively, after exposure to 0.1 mg/mL CSC, of which 1856 transcripts (∼37%) were modulated in both cell lines (Fig. 2*B*; left panel). A total of 5000 and 3914 transcripts were modulated in HBEC and HSAEC, respectively, after exposure to 2 mg/mL WPC. A total of 1489 transcripts (30% and 38% of differentially expressed transcripts in HBEC and HSAEC, respectively) were modulated in both cell lines (Fig. 2*B*; middle panel). A total of 690 transcripts (37% and 46% of differentially expressed genes modulated by CSC and WPC, respectively) were modulated by both condensates in both cell lines (Fig. 3*B*; right panel; Supplementary Table 8), of which 339 (42%) are cancer associated. Top canonical pathways associated with these common transcripts included xenobiotic metabolism signaling, aryl hydrocarbon receptor signaling, and retinoic acid receptor activation (Fig. 2*C*; left panel). Top diseases and biofunctions included cancer (highest ranked) and functions related to tissue/organ development/morphology (Fig. 3*C*; right panels). The top 100 transcripts uniquely modulated in HBEC and HSAEC by CSC 0.1 mg/mL or WPC 2 mg/mL are listed in Supplementary Table 9.

Of 1031 transcripts uniquely modulated in HBEC and HSAEC by 0.05 mg/mL CSC (Fig. 2*A*; right panel), 536 (52%) are associated with cancer. In contrast, only 149 of 532 (28%) transcripts unique to the 1 mg/mL WPC signatures (Fig. 2*A*; right panel) are cancer associated. Despite no overlap among the top 10 canonical pathways (Supplementary Fig. 4*B* and *C*; left panels), cancer was the highest ranked diseases and biofunctions associated with both unique

biofunctions (right panel) associated with genes uniquely regulated by CSC 0.1 mg/mL in HBEC and HSAEC. (*E*) Top canonical pathways (left panel) and top diseases and biofunctions (right panel) associated with genes uniquely regulated by WPC 2 mg/mL in HBEC and HSAEC. CSC, cigarette smoke condensate; HBEC, human bronchial epithelial cell; HSAEC, human small airway epithelial cell; Th, T helper; WPC, water pipe condensate.

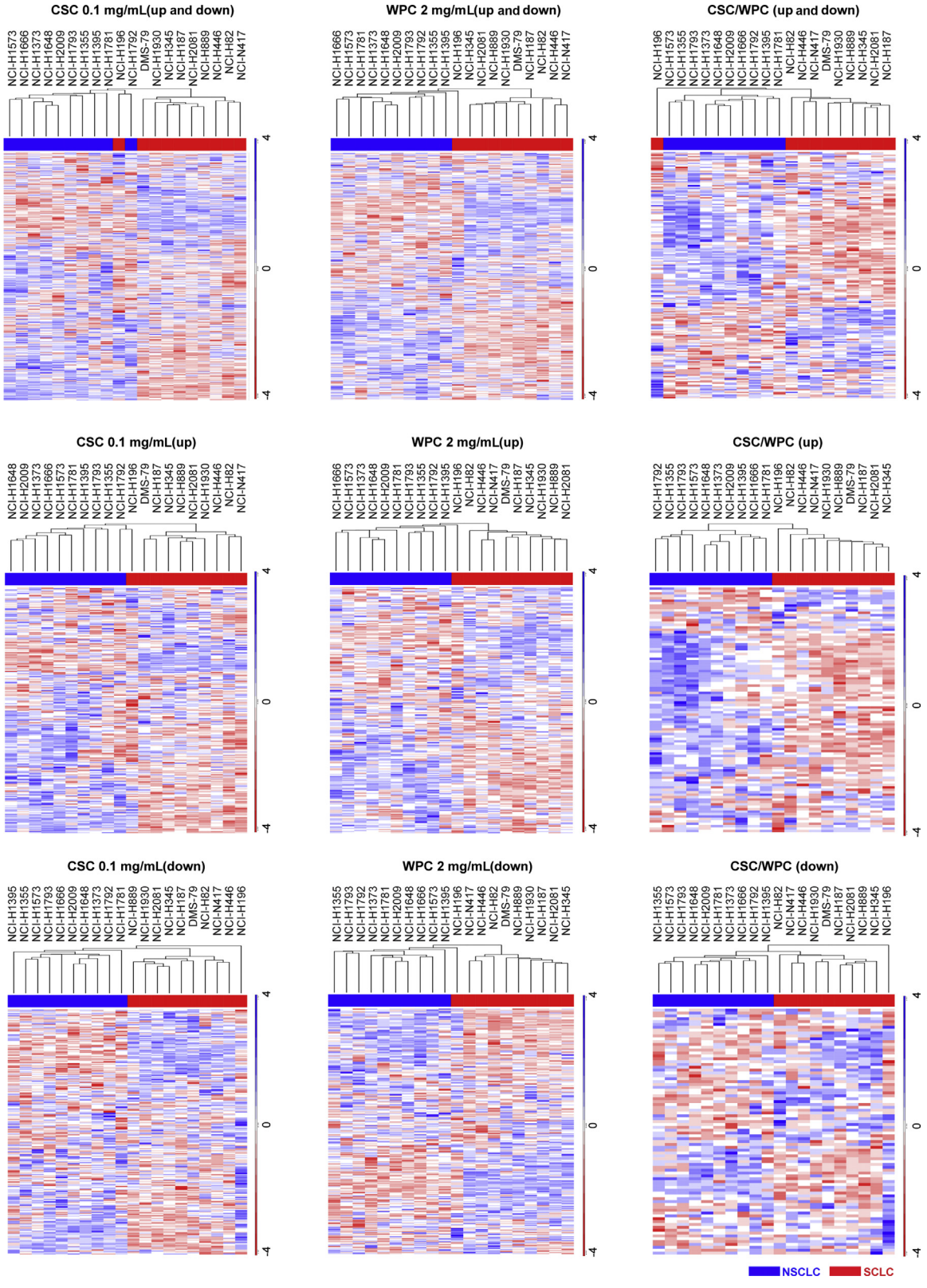

Figure 3. Unsupervised hierarchical clustering of transcripts uniquely modulated across three SAEC lines by CSC 0.1 mg/mL or WPC 2 mg/mL or often regulated by CSC 0.1 mg/mL and WPC 2 mg/mL in NSCLC versus SCLC lines. The signatures segregated lung cancer lines on the basis of histology. CSC, cigarette smoke condensate; SAEC, small airway epithelial cell; WPC, water pipe condensate.

signatures (Supplementary Fig. 4*B* and *C*; right panels). Five additional diseases and biofunctions, including organismal injury and abnormalities, tissue development, and cell-to-cell signaling and interaction, were common to both unique signatures.

A similar phenomenon was observed for "high dose" condensate exposures. Of 1166 and 799 transcripts uniquely regulated in HBEC and HSAEC by CSC 0.1 mg/mL or WPC 2 mg/mL, respectively, (Fig. 2*B*; right panel), 651 (56%) and 339 (42%) are cancer associated. Hepatic fibrosis/hepatic stellate cell activation was the only top canonical pathway associated with both unique signatures (Fig. 2*D* and *E*; left panels). Despite this observation, 7 of the top 16 (44%) diseases and biofunctions were common to the two signatures; once again, cancer was the highest ranked diseases and biofunctions associated with both signatures (Fig. 2*D* and *E*; right panels).

Cancer Histology-Related Transcriptome Signatures Mediated by CSC and WPC

Additional experiments were performed to compare transcriptome signatures mediated by short-term CSC and WPC in human respiratory epithelial cells with those observed in untreated lung cancer cells. Briefly, cancer-associated transcripts uniquely modulated by CSC 0.1 mg/mL or WPC 2 mg/mL or often regulated by these exposures in SAEC or HBEC and HSAEC were compared with expression levels of these transcripts in 10 NSCLC and 10 SCLC lines relative to untreated SAEC using publicly accessible databases.[17,18] Unsupervised, hierarchical cluster analysis revealed transcriptome signatures uniquely associated with CSC or WPC exposures or common to both segregated cancer cell lines on the basis of NSCLC versus SCLC histology in SAEC (Fig. 3). A similar phenomenon was observed after analysis of HBEC and HSAEC (Supplementary Fig. 5).

Overlap of CSC- and WPC-Mediated Transcriptome Signatures in SAEC, HBEC, and HSAEC

A total of 211 transcripts were often modulated by CSC 0.05 mg/mL in the three SAEC cultures, HBEC, and HSAEC (Fig. 4*A*; top left panel; Supplementary Table 10); 85 (40%) are cancer associated. In contrast, 329 transcripts were often regulated by CSC 0.1 mg/mL across the five lines, of which 172 (52%) are cancer associated (Fig. 4*A*; lower left panel, Supplementary Table 11). A total of 90 transcripts were common to both doses of CSC across the five cell lines (Supplementary Table 12); 40 (44%) are cancer associated. Although xenobiotic metabolism signaling was associated with both CSC exposures, NRF2-mediated oxidative stress response emerged as a top canonical pathway after high-dose CSC treatments (Fig. 4*A*; upper and lower middle panels). Cancer and organismal injury and abnormalities, which were the fourth and fifth highest ranked diseases and biofunctions associated with low-dose CSC exposure across the five lines, became the first and second highest diseases and biofunctions associated with high-dose CSC exposures, suggestive of a biological threshold of carcinogen exposure in these cells (Fig. 4*A*; upper and lower right panels). No overlap was observed among the top six common diseases and biofunctions associated with the two treatment groups.

A total of 161 and 261 transcripts were often regulated by WPC 1 mg/mL and WPC 2 mg/mL, across the five lines (Fig. 4*B*; upper and lower left panels; Supplementary Tables 13 and 14), of which 38 (24%) and 111 (42%), respectively, are cancer associated. In addition, 66 transcripts (including 10 cancer-associated transcripts; 15%) were associated with both WPC exposures (Supplementary Table 15). Of the top six canonical pathways, xenobiotic metabolism signaling was the only one associated with both WPC signatures; NRF2-mediated oxidative stress response emerged as a top canonical pathway after high-dose WPC exposures (Fig. 4*B*; upper and lower middle panels). Four of the top diseases and biofunctions associated with low-dose WPC exposure involved metabolism or energy production. Although not among the top diseases and biofunctions associated with low-dose WPC treatment, cancer and organismal injury and abnormalities emerged as top diseases and biofunctions after "high dose" WPC exposures (Fig. 4*B*; upper and lower right panels).

A total of 61 transcripts were often regulated by CSC 0.05 mg/mL and WPC 1 mg/mL across the five cell lines, of which 13 (21%) are cancer associated; in contrast, 96 transcripts, including 27 (28%) cancer-associated transcripts, were often regulated by CSC 0.1 mg/mL and WPC 2 mg/mL across these lines (Fig. 4*C*; upper and lower left panels; Supplementary Tables 16 and 17). Among the top canonical pathways, only xenobiotic metabolism signaling was common to all four condensate exposures across the cell lines; consistent with aforementioned results. NRF2-mediated oxidative stress response emerged as the top canonical pathway after high-dose CSC and WPC exposures (Fig. 4*C*; upper and lower middle panels). Although metabolism and energy production primarily accounted for the six top diseases and biofunctions associated with low-dose CSC and WPC exposures across the five cell lines, cancer and organismal injury and abnormalities emerged as number 1 and number 3 among the top diseases and biofunctions associated with high-dose CSC and WPC treatments, respectively (Fig. 4*C*; upper and lower right panels). These findings are consistent with dose-dependent toxicities of cigarette and water pipe smoke in respiratory epithelial cells.

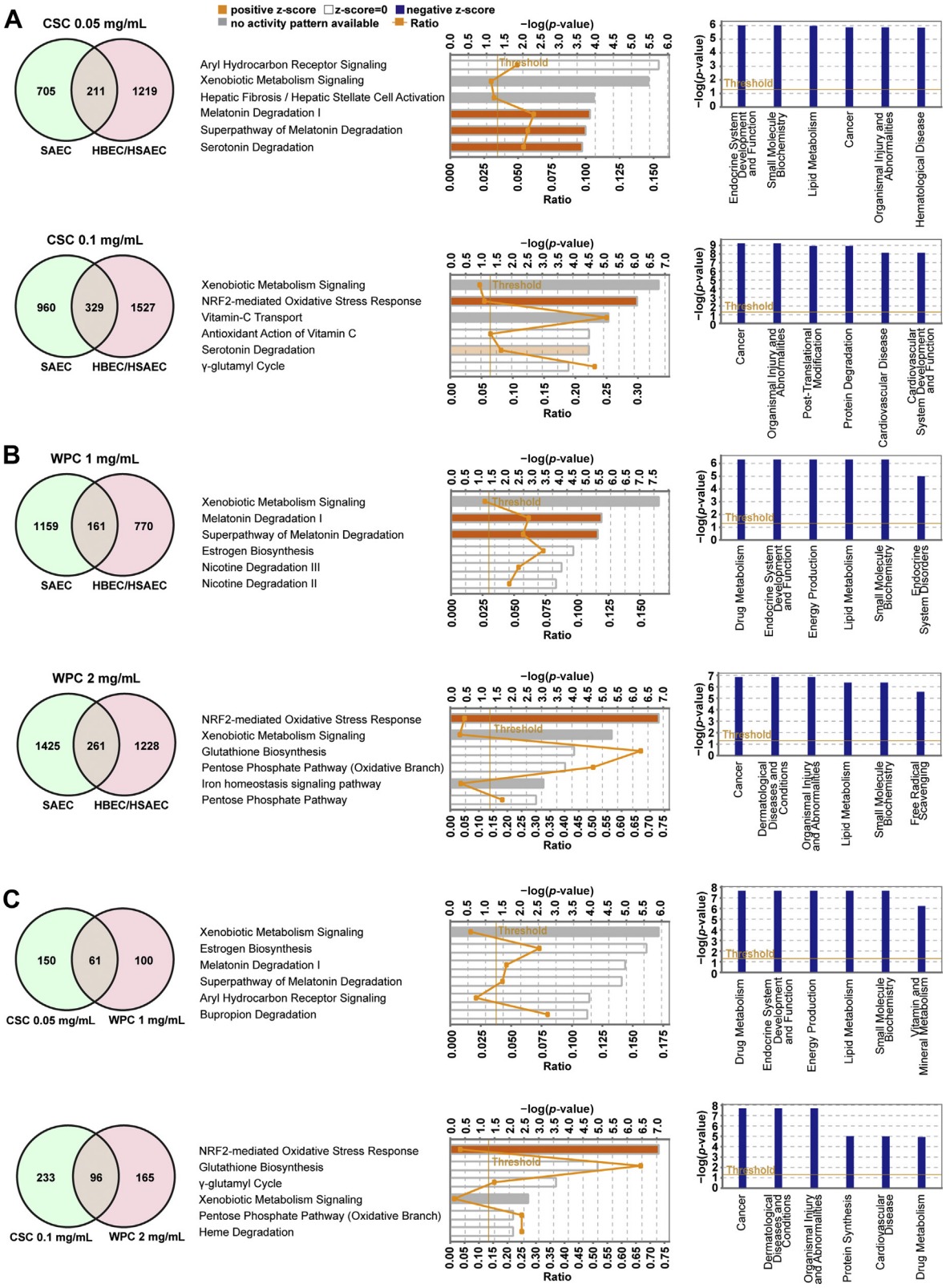

Figure 4. Transcripts often regulated by (*A*) CSC 0.05 mg/mL or CSC 0.1 mg/mL, (*B*) WPC 1 mg/mL or WPC 2 mg/mL, and (*C*) CSC 0.05 mg/mL and WPC 1 mg/mL or CSC 0.1 mg/mL and WPC 2 mg/mL in three short-term SAEC lines and immortalized HBEC and HSAEC (left panels) with corresponding top canonical pathways and diseases and biofunctions (middle and right panels, respectively). CSC, cigarette smoke condensate; HBEC, human bronchial epithelial cell; HSAEC, human small airway epithelial cell; SAEC, small airway epithelial cell; WPC, water pipe condensate.

Mechanism of CSC- and WPC-Mediated Up-Regulation of Epiregulin in Respiratory Epithelial Cells

Additional experiments were performed to validate results of RNA-seq experiments focusing on several putative cancer-associated transcripts. Epiregulin (EREG), which is up-regulated by means of epigenetic mechanisms during colon cancer progression,[19] was induced by high- and low-dose CSC and WPC exposures in all five lines. qRT-PCR, immunoblot, and methylation-specific PCR experiments revealed dose-dependent up-regulation of EREG in SAEC and HBEC after CSC or WPC exposures (Fig. 5*A*) that coincided with DNA demethylation within the EREG promoter (Fig. 5*B*); this region contains recognition elements for several transcription factors, including SP1, which has been found to activate stem cell genes in response to cigarette smoke.[20] Quantitative chromatin immunoprecipitation experiments revealed no appreciable changes in occupancy of either the maintenance DNMT1 or the de-novo transferase DNMT3b within the EREG promoter; however; CSC and WPC induced recruitment of the TET2 to the EREG promoter (Fig. 5*C*), which coincided with an increase in H3K27Ac (histone activation mark) and increased occupancy of SP1 (Fig. 5*D*; left panel). Consistent with these findings, mithramycin, a chemotherapeutic agent that inhibits binding of SP1 to GC-rich DNA, markedly attenuated induction of EREG by CSC and WPC in respiratory epithelial cells (Fig. 5*D*; right panel).

Epigenetic Alterations Coinciding With CSC- and WPC-Mediated Repression of FILIP1L and ABI3BP in Respiratory Epithelial Cells

Several malignancies, including lung cancers, exhibit down-regulation of FILIP1L, which coincides with DNA hypermethylation within the promoter of variant 2.[21,22] ABI3BP has been reported to be differentially spliced and down-regulated by unknown mechanisms in lung cancers.[23,24] As such, experiments were performed to evaluate mechanisms by which FILIP1L and ABI3BP are repressed by CSC and WPC in human respiratory epithelial cells. Review of TCGA and Oncomine data sets confirmed repression of FILIP1L and ABI3BP in lung cancers relative to normal lung (Fig. 6*A* and *D*). qRT-PCR experiments using primers recognizing all FILIP1L transcripts and primers specific for variant 2 revealed dose-dependent repression of FILIP1L in SAEC and to a somewhat lesser extent, HBEC after CSC or WPC exposures (Fig. 6*B*; left and right panels, respectively). Consistent with previous observations,[21] FILIP1L protein levels were not detected in proliferating SAEC or HBEC (data not found). In addition, consistent with bisulfite sequencing analysis of the FILIP1L promoter in cultured lung cancer cells,[21] methylation-specific PCR experiments revealed that CSC and WPC increased DNA methylation within a CpG island in the promoter region of variant 2 in SAEC and HBEC (Fig. 6*C*). Additional qRT-PCR and immunoblot experiments confirmed that CSC and WPC mediated dose-dependent decreases in ABI3BP in SAEC and HBEC (Fig. 6*E*). No CpG island was identified within the ABI3BP promoter, suggesting DNA methylation is not a primary mechanism of CSC- and WPC-mediated repression of this putative tumor-suppressor gene in respiratory epithelial cells. Quantitative chromatin immunoprecipitation experiments revealed that down-regulation of ABI3BP coincided with increased occupancy of EZH2 (a core component of PRC-2) with a concomitant increase in the PRC-2–mediated repressive histone mark, H3K27me3, and a decrease in the histone activation mark H3K4me3 within the ABI3BP regulatory region (Fig. 6*F*). Collectively, these findings reveal that CSC and WPC silence mediators of senescence in human respiratory epithelial cells by DNA methylation and polycomb mechanisms.

Discussion

Water pipe smoking has replaced cigarettes as the most popular method of tobacco use among youth in the Middle East, and it is second only to cigarettes in several other parts of the world.[25] Although hookah smoking is perceived to be a safe alternative to cigarettes, two recent meta-analyses have revealed positive associations between water pipe smoking and lung cancer with ORs equaling 2.12 and 4.58, with 95% confidence intervals of 1.32 to 3.42, and 2.61 to 8.03, respectively.[26,27] Nevertheless, associations between hookah smoking and lung cancer risk have not been firmly established in part because most studies have not controlled for concomitant cigarette smoking in hookah smokers, making it difficult to ascribe risks specifically to hookah smoke, cigarette smoke, or both. Of particular concern regarding the globalization of hookah smoking are observations that in addition to inherent health risks, this activity increases susceptibility to initiation of cigarette smoking; as such, hookah smoking has been referred to as a "gateway" to cigarette addiction.[28]

In this study, we observed that similar to CSC, WPC mediates dose-dependent reductions in global levels of H4K20me3 and H4K16ac in respiratory epithelial cells. These histone alterations, which have been designated "hallmarks of cancer" have been linked to epigenetic derepression of DNA repeats and retrotransposons resulting in genomic instability.[29,30] Loss of H4K20me3 and decreased H4K16ac levels have been observed in large percentages of human lung cancers and their

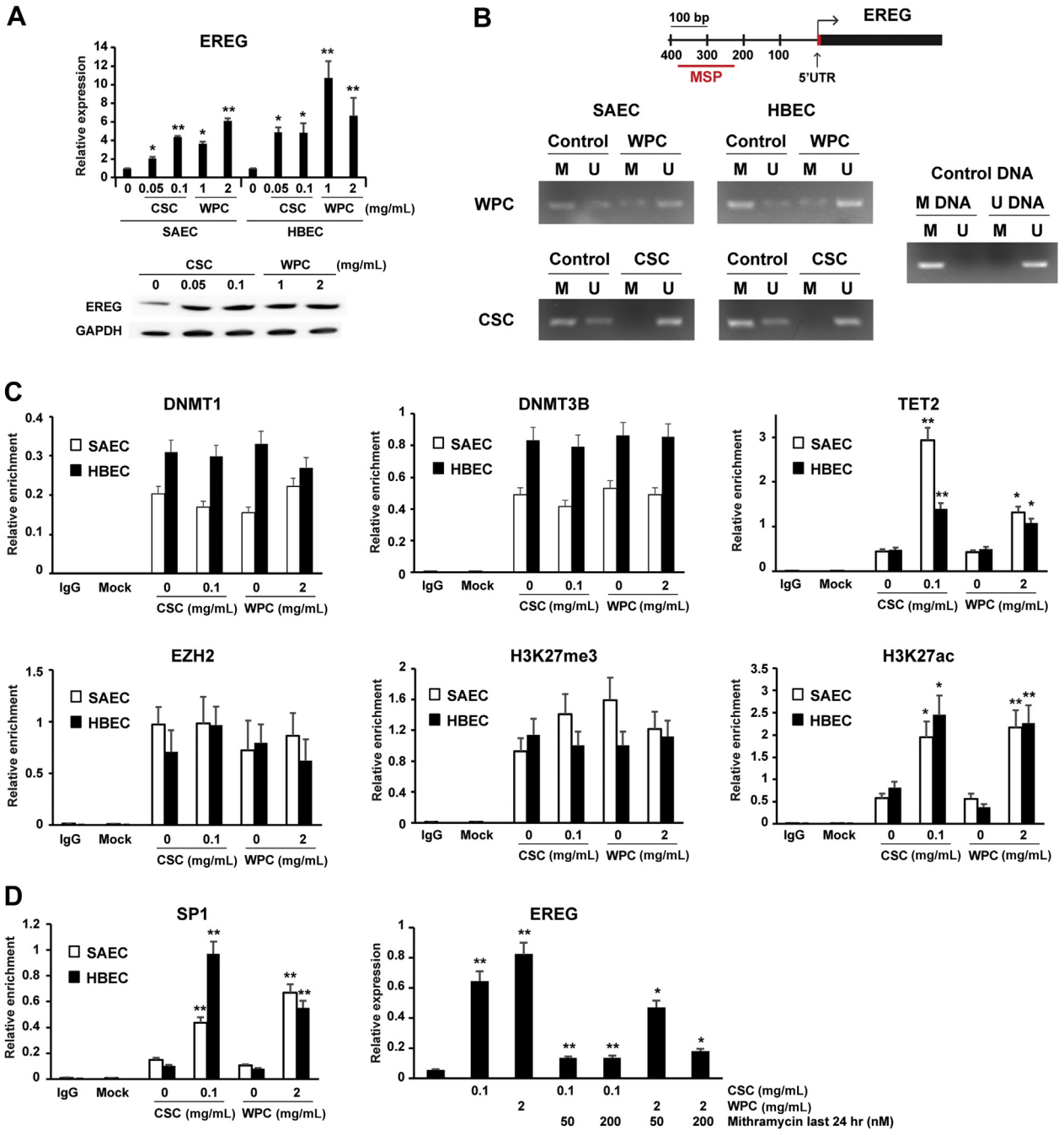

Figure 5. (*A*) Upper and lower panels: qRT-PCR and corresponding immunoblot analysis of EREG expression in SAEC and HBEC after 5-day exposure to CSC or WPC. (*B*) MSP analysis revealing that CSC and WPC mediate demethylation within a CpG island in the EREG regulatory region. (*C*) qChIP analysis revealing that up-regulation of EREG coincides with recruitment of TET2 and SP1 to the EREG regulatory region. (*D*) qRT-PCR analysis revealing that mithramycin exposure during the past 24 hours of 5-day CSC or WPC treatment markedly attenuates up-regulation of EREG in HBEC. *$p < 0.05$; **$p < 0.01$. 5'UTR, 5′ untranslated region; bp, base pair; CSC, cigarette smoke condensate; EREG, epiregulin; HBEC, human bronchial epithelial cell; IgG, immunoglobulin G; M, methylated; MSP, methylation-specific PCR; qChIP, quantitative chromatin immunoprecipitation; qRT-PCR, quantitative real-time reverse-transcriptase polymerase chain reaction; SAEC, small airway epithelial cell; U, unmethylated; WPC, water pipe condensate.

precursor lesions.[31] The mechanisms by which CSC and WPC mediate these rapid and dynamic histone alterations are a focus of ongoing investigation in our laboratory.

Our analysis revealed marked variations in transcriptome signatures mediated by CSC and WPC across the three SAEC cultures, HBEC, and HSAEC possibly reflecting differences pertaining to smoking status of

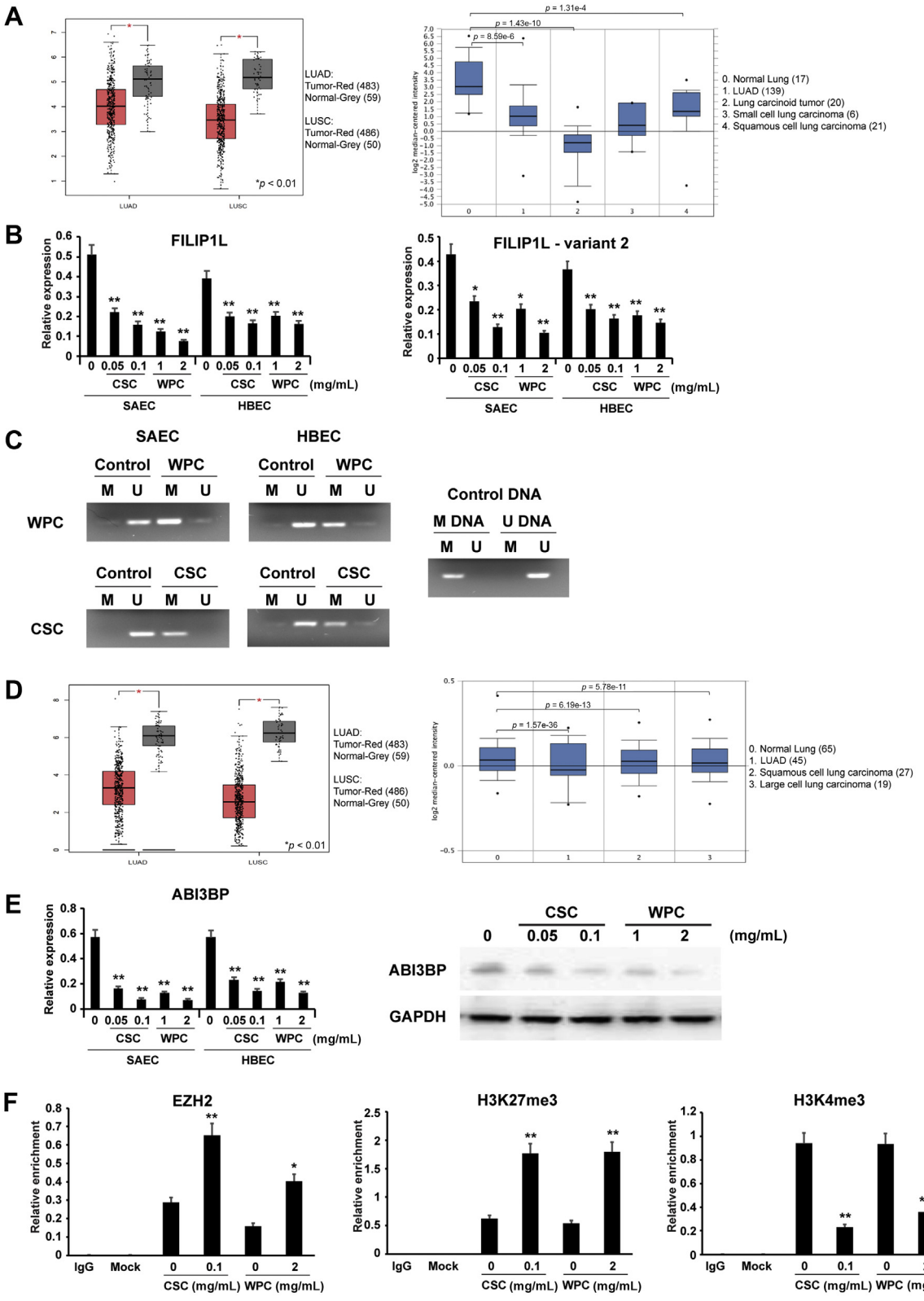

Figure 6. (*A*) TCGA (left panel) and Oncomine (right panel) quantifications of *FILIP1L* expression in lung cancers relative to normal lung (LUAD, LUSC). (*B*) qRT-PCR analysis revealing dose-dependent repression of *FILIP1L* in SAEC and HBEC after CSC or WPC exposure. (*C*) MSP analysis revealing that CSC and WPC exposures increase DNA methylation within a CpG island in the regulatory region of variant 2. (*D*) TCGA (left panel) and Oncomine (right panel) quantifications of *ABI3BP* expression in lung cancers relative to normal lung (LUAD, LUSC). (*E*) Left panel: qRT-PCR analysis revealing dose-dependent repression of *ABI3BP* in SAEC and HBEC after CSC or WPC exposure; right panel: immunoblot revealing that CSC and WPC decrease ABI3BP protein

the donors and metabolism of tobacco components and immortalization status of the lines. Overall, there was a tendency for the immortalized lines (HBEC; HSAEC) to be more responsive to CSC and less responsive to WPC compared with SAEC. In addition, more cancer-associated transcripts were modulated by CSC than WPC across all five lines (Supplementary Table 18). Relatively limited overlap of transcriptome signatures was observed between CSC and WPC in either SAEC cultures or HBEC and HSAEC. Presently, we are unable to ascribe these differences to variations in either unique components or relative levels of specific carcinogens because the complexities of the condensates have precluded simple comparison by mass spectroscopy or high-performance liquid chromatography techniques, and there are no published data pertaining to this issue. In addition, limited overlap of CSC- or WPC-mediated transcriptome signatures was observed between short-term SAEC and immortalized HBEC and HSAEC, suggesting immortalization affects plasticity of transcriptional responses to the condensates. Such heterogeneous responses to CSC and WPC exposures highlight potential limitations of using only one or two cell lines to identify potential biomarkers of malignant transformation in respiratory epithelia of smokers.

Our analysis identified 90 transcripts that were often regulated by both doses of CSC across all five respiratory epithelial cell lines and 66 transcripts that were differentially expressed in all five lines after low- and high-dose WPC exposure that conceivably could prove useful biomarkers of carcinogen exposure in vivo. Notably, short-term WPC and CSC exposures mediated epigenetic dysregulation of EREG, FILIP1L, and ABI3BP in human respiratory cells. EREG encodes one of seven ligands that interact with erbB receptors; up-regulation of EREG promotes lung cancers in mice and enhances proliferation, metastatic potential, and stemness of cancer cells.[32–34] Nearly 70% of NSCLC exhibit overexpression of EREG, with levels tending to be higher in smokers and adenocarcinomas.[32] FILIP1L (also known as DOC1) is up-regulated in prostate epithelial cells as they senesce[35] and inhibits angiogenesis, adhesion, migration, invasion, epithelial-to-mesenchymal transition, and metastatic potential of cancer cells by facilitating degradation of β-catenin and subsequent attenuation of Wnt signaling.[22] ABI3BP has been implicated in replicative senescence, maintenance of chromosomal stability, and differentiation of normal stem cells by means of mechanisms that remain elusive.[23,36–38] Although our findings suggest a strong selection pressure to up-regulate EREG and silence FLILIP1L and ABI3BP during human pulmonary carcinogenesis, additional studies are necessary to determine if these epigenetic perturbations (which conceivably promote the acquisition of stem-like phenotypes) are relevant biomarkers of carcinogen exposure and lung cancer risk in hookah and cigarette smokers.

One unexpected and potentially intriguing aspect of our analysis pertains to our observations that transcripts uniquely associated with either CSC or WPC exposures in normal respiratory epithelial cells seemed to stratify lung cancer lines on the basis of nonsmall cell versus small cell histology. Studies are in progress to determine the biological significance of these findings and to ascertain if these signatures might be useful for risk assessment and chemoprevention in smokers with no clinical evidence of malignancy.

A potential limitation of our study pertains to the extent to which our in vitro model reflects "real life" hookah smoke exposures.[39] Given the wide variability in levels of individual components in hookah versus cigarette smoke, we chose to use tar contents to determine the condensate concentrations, attempting to model exposures in daily hookah smokers relative to $^1/_2$ to 1 pack-per-day cigarette smokers. Because of the coronavirus disease pandemic, we have been unable to obtain bronchoscopic biopsy samples of respiratory epithelia from asymptomatic hookah smokers to validate our in vitro findings. The fact that some of the transcripts often regulated by WPC across the five cell lines have also been detected in respiratory epithelial cells from "light" (intermittent) hookah smokers[40] suggests that our model does indeed reflect in vivo exposures. Collectively, our findings support additional studies to ascertain if hookah-mediated epigenomic and transcriptomic alterations in cultured respiratory epithelial cells are detectable and clinically relevant in hookah smokers.

levels in HBEC. (*F*) qChIP analysis revealing that CSC (0.1 mg/mL) or WPC (2 mg/mL) exposures decrease H3K4me3 levels while increasing H3K27me3 levels within the ABI3BP regulatory region. Increased H3K27me3 levels coincide with recruitment of EZH2, consistent with PRC-2 mediated repression. *$p < 0.05$; **$p < 0.01$. CSC, cigarette smoke condensate; HBEC, human bronchial epithelial cell; IgG, immunoglobulin G; LUAD, lung adenocarcinoma; LUSC, lung squamous cell lung cancer; M, methylated; MSP, methylation-specific PCR; qChIP, quantitative chromatin immunoprecipitation; qRT-PCR, quantitative real-time reverse-transcriptase polymerase chain reaction; SAEC, small airway epithelial cell; TCGA, The Cancer Genome Atlas; U, unmethylated; WPC, water pipe condensate.

CRediT Authorship Contribution Statement

Yin Xiong, Sichuan Xi, Mary Zhang, Haobin Chen, David S. Schrump: Design, Conduction of experiments.

Yin Xiong, Sudheer Kumar Gara, Jigui Shan, James Gao, Vivek Shukla, Ruihong Wang, Chuong D. Hoang, Haobin Chen, David S. Schrump: Data analysis.

Yin Xiong, Sudheer Kumar Gara, Mary Zhang, Chuong D. Hoang, Haobin Chen, David S. Schrump: Manuscript preparation.

Yin Xiong, Sichuan Xi, Sudheer Kumar Gara, Jigui Shan, James Gao, Mary Zhang, Vivek Shukla, Ruihong Wang, Chuong D. Hoang, Haobin Chen, David S. Schrump: Manuscript approval.

Acknowledgments

This work was supported by the National Cancer Institute intramural fund ZIA BC 011115 (Dr. Schrump), the Food and Drug Administration IAA NRC 13023-8015513 (Dr. Schrump), and the Stephen J. Solarz Memorial Fund at the Foundation of The National Institutes of Health. The authors express their appreciation to Shih-Hsin Hsiao, Yi Liu, and Christopher Tao for technical assistance and Ms. Julie Hong and Ms. Jan Pappas for administrative support.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *JTO Clinical and Research Reports* at www.jtocrr.org and at https://doi.org/10.1016/j.jtocrr.2021.100181.

References

1. Proctor RN. The history of the discovery of the cigarette-lung cancer link: evidentiary traditions, corporate denial, global toll. *Tob Control*. 2012;21:87–91.
2. Maziak W, Sharma E. Building the evidence base for waterpipe regulation and policy. *Tob Control*. 2020;29(suppl 2):s59–s61.
3. Anic GM, Sawdey MD, Jamal A, Trivers KF. Frequency of use among middle and high school student tobacco product users - United States, 2015–2017. *MMWR Morb Mortal Wkly Rep*. 2018;67:1353–1357.
4. Azagba S, Latham K, Shan L. Waterpipe tobacco smoking trends among middle and high school students in the United States from 2011 to 2017. *Drug Alcohol Depend*. 2019;200:19–25.
5. Bernd K, DeGrood D, Stadtler H, et al. Contributions of charcoal, tobacco, and syrup to the toxicity and particle distribution of waterpipe tobacco smoke. *Toxicol Lett*. 2019;313:60–65.
6. Cobb C, Ward KD, Maziak W, Shihadeh AL, Eissenberg T. Waterpipe tobacco smoking: an emerging health crisis in the United States. *Am J Health Behav*. 2010;34:275–285.
7. Shihadeh A, Saleh R. Polycyclic aromatic hydrocarbons, carbon monoxide, "tar", and nicotine in the mainstream smoke aerosol of the narghile water pipe. *Food Chem Toxicol*. 2005;43:655–661.
8. Djordjevic MV, Stellman SD, Zang E. Doses of nicotine and lung carcinogens delivered to cigarette smokers. *J Natl Cancer Inst*. 2000;92:106–111.
9. Sepetdjian E, Shihadeh A, Saliba NA. Measurement of 16 polycyclic aromatic hydrocarbons in narghile waterpipe tobacco smoke. *Food Chem Toxicol*. 2008;46:1582–1590.
10. Shihadeh A. Investigation of mainstream smoke aerosol of the argileh water pipe. *Food Chem Toxicol*. 2003;41:143–152.
11. Neergaard J, Singh P, Job J, Montgomery S. Waterpipe smoking and nicotine exposure: a review of the current evidence. *Nicotine Tob Res*. 2007;9:987–994.
12. Patel MP, Khangoora VS, Marik PE. A review of the pulmonary and health impact of hookah use. *Ann Am Thorac Soc*. 2019;16:1215–1219.
13. Ramirez RD, Sheridan S, Girard L, et al. Immortalization of human bronchial epithelial cells in the absence of viral oncoproteins. *Cancer Res*. 2004;64:9027–9034.
14. Liu F, Killian JK, Yang M, et al. Epigenomic alterations and gene expression profiles in respiratory epithelia exposed to cigarette smoke condensate. *Oncogene*. 2010;29:3650–3664.
15. Rammah M, Dandachi F, Salman R, Shihadeh A, El-Sabban M. In vitro cytotoxicity and mutagenicity of mainstream waterpipe smoke and its functional consequences on alveolar type II derived cells. *Toxicol Lett*. 2012;211:220–231.
16. Shihadeh A, Eissenberg T, Rammah M, Salman R, Jaroudi E, El-Sabban M. Comparison of tobacco-containing and tobacco-free waterpipe products: effects on human alveolar cells. *Nicotine Tob Res*. 2014;16:496–499.
17. Iorio F, Knijnenburg TA, Vis DJ, et al. A landscape of pharmacogenomic interactions in cancer. *Cell*. 2016;166:740–754.
18. Dogan I, Kawabata S, Bergbower E, et al. SOX2 expression is an early event in a murine model of EGFR mutant lung cancer and promotes proliferation of a subset of EGFR mutant lung adenocarcinoma cell lines. *Lung Cancer*. 2014;85:1–6.
19. Qu X, Sandmann T, Frierson H Jr, et al. Integrated genomic analysis of colorectal cancer progression reveals activation of EGFR through demethylation of the EREG promoter. *Oncogene*. 2016;35:6403–6415.
20. Zhang M, Mathur A, Zhang Y, et al. Mithramycin represses basal and cigarette smoke-induced expression of ABCG2 and inhibits stem cell signaling in lung and esophageal cancer cells. *Cancer Res*. 2012;72:4178–4192.
21. Kwon M, Lee SJ, Reddy S, Rybak Y, Adem A, Libutti SK. Down-regulation of filamin A interacting protein 1-like is associated with promoter methylation and an invasive phenotype in breast, colon, lung and pancreatic cancers [corrected]. *PLoS One*. 2013;8:e82620.
22. Kwon M, Kim JH, Rybak Y, et al. Reduced expression of FILIP1L, a novel WNT pathway inhibitor, is associated with poor survival, progression and chemoresistance in ovarian cancer. *Oncotarget*. 2016;7:77052–77070.

23. Hodgkinson CP, Naidoo V, Patti KG, et al. Abi3bp is a multifunctional autocrine/paracrine factor that regulates mesenchymal stem cell biology. *Stem Cells*. 2013;31:1669–1682.
24. Terauchi K, Shimada J, Uekawa N, Yaoi T, Maruyama M, Fushiki S. Cancer-associated loss of TARSH gene expression in human primary lung cancer. *J Cancer Res Clin Oncol*. 2006;132:28–34.
25. Maziak W, Taleb ZB, Bahelah R, et al. The global epidemiology of waterpipe smoking. *Tob Control*. 2015;24(suppl 1):i3–i12.
26. Waziry R, Jawad M, Ballout RA, Al Akel M, Akl EA. The effects of waterpipe tobacco smoking on health outcomes: an updated systematic review and meta-analysis. *Int J Epidemiol*. 2017;46:32–43.
27. Montazeri Z, Nyiraneza C, El-Katerji H, Little J. Waterpipe smoking and cancer: systematic review and meta-analysis. *Tob Control*. 2017;26:92–97.
28. Salloum RG, Haider MR, Barnett TE, et al. Waterpipe tobacco smoking and susceptibility to cigarette smoking among young adults in the United States, 2012–2013. *Prev Chronic Dis*. 2016;13:E24.
29. Fraga MF, Ballestar E, Villar-Garea A, et al. Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. *Nat Genet*. 2005;37:391–400.
30. Jørgensen S, Schotta G, Sørensen CS. Histone H4 lysine 20 methylation: key player in epigenetic regulation of genomic integrity. *Nucleic Acids Res*. 2013;41:2797–2806.
31. Van Den Broeck A, Brambilla E, Moro-Sibilot D, et al. Loss of histone H4K20 trimethylation occurs in preneoplasia and influences prognosis of non-small cell lung cancer. *Clin Cancer Res*. 2008;14:7237–7245.
32. Sunaga N, Kaira K. Epiregulin as a therapeutic target in non-small-cell lung cancer. *Lung Cancer (Auckl)*. 2015;6:91–98.
33. Sunaga N, Kaira K, Imai H, et al. Oncogenic KRAS-induced epiregulin overexpression contributes to aggressive phenotype and is a promising therapeutic target in non-small-cell lung cancer. *Oncogene*. 2013;32:4034–4042.
34. Sun L, Pan J, Yu L, et al. Tumor endothelial cells promote metastasis and cancer stem cell-like phenotype through elevated epiregulin in esophageal cancer. *Am J Cancer Res*. 2016;6:2277–2288.
35. Desotelle J, Truong M, Ewald J, et al. CpG island hypermethylation frequently silences FILIP1L isoform 2 expression in prostate cancer. *J Urol*. 2013;189:329–335.
36. Uekawa N, Terauchi K, Nishikimi A, Shimada J, Maruyama M. Expression of TARSH gene in MEFs senescence and its potential implication in human lung cancer. *Biochem Biophys Res Commun*. 2005;329:1031–1038.
37. Latini FR, Hemerly JP, Freitas BC, Oler G, Riggins GJ, Cerutti JM. ABI3 ectopic expression reduces in vitro and in vivo cell growth properties while inducing senescence. *BMC Cancer*. 2011;11:11.
38. Latini FR, Hemerly JP, Oler G, Riggins GJ, Cerutti JM. Re-expression of ABI3-binding protein suppresses thyroid tumor growth by promoting senescence and inhibiting invasion. *Endocr Relat Cancer*. 2008;15:787–799.
39. Jawad M, Eissenberg T, Salman R, et al. Toxicant inhalation among singleton waterpipe tobacco users in natural settings. *Tob Control*. 2019;28:181–188.
40. Walters MS, Salit J, Ju JH, et al. Waterpipe smoking induces epigenetic changes in the small airway epithelium. *PLoS One*. 2017;12:e0171112.