Detecting the Presence and Progression of Premalignant Lung Lesions via Airway Gene Expression

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Abstract

Purpose: Lung cancer is the leading cause of cancer-related death in the United States. The molecular events preceding the onset of disease are poorly understood, and no effective tools exist to identify smokers with premalignant lesions (PMLs) that will progress to invasive cancer. Prior work identified molecular alterations in the smoke-exposed airway field of injury associated with lung cancer. Here, we focus on an earlier stage in the disease process leveraging the airway field of injury to study PMLs and its utility in lung cancer chemoprevention.

Experimental Design: Bronchial epithelial cells from normal appearing bronchial mucosa were profiled by mRNA-Seq from subjects with (n = 50) and without (n = 25) PMLs. Using surrogate variable and gene set enrichment analysis, we identified genes, pathways, and lung cancer-related gene sets differentially expressed between subjects with and without PMLs. A computational pipeline was developed to build and test a chemoprevention-relevant biomarker.

Results: We identified 280 genes in the airway field associated with the presence of PMLs. Among the upregulated genes, oxidative phosphorylation was strongly enriched, and IHC and bioenergetics studies confirmed pathway findings in PMLs. The relationship between PMLs and squamous cell carcinomas (SCC) was also confirmed using published lung cancer datasets. The biomarker performed well predicting the presence of PMLs (AUC = 0.92, n = 17), and changes in the biomarker score associated with progression/stability versus regression of PMLs (AUC = 0.75, n = 51).

Conclusions: Transcriptomic alterations in the airway field of smokers with PMLs reflect metabolic and early lung SCC alterations and may be leveraged to stratify smokers at high risk for PML progression and monitor outcome in chemoprevention trials. Clin Cancer Res; 23(17); 5091–100. ©2017 AACR.

Introduction

Exposure to carcinogens such as cigarette smoke induces smoking-related mRNA and miRNA expression alterations in the cytologically normal epithelium that lines the respiratory tract, creating an airway field of injury (1–6). Gene expression alterations in the airway field of injury were leveraged to build a diagnostic test for early lung cancer detection (7–10). Examination of a gene signature for phosphatidylinositol 3-kinase (PI3K) pathway revealed increased PI3K activation in the airway field of smokers with lung cancer or bronchial premalignant lesions (PMLs; ref. 11). These results suggest the airway field of injury reflects processes associated with a precancerous disease state; however, the molecular changes have not been adequately characterized.

This is an important shortcoming because bronchial PMLs are precursors of squamous cell lung carcinoma (SCC), yet we lack effective tools to identify smokers with PMLs at highest risk of progression to invasive cancer. Several studies report loss of heterozygosity, chromosomal aneusomy, and aberrant methylation and protein expression in bronchial PMLs (12–21). These molecular events are associated with histologic changes that can be reproducibly graded by a pathologist prior to the development of invasive carcinoma. Autofluorescence bronchoscopy can be used to detect and sample PMLs. In high-risk smokers between 40 and 74 years of age who had smoked at least 20 pack-years, the prevalence of PMLs is approximately 13% for moderate dysplasia and 1.6% for carcinoma in situ (CIS; refs. 22, 23). The presence of high-grade PMLs (severe dysplasia or CIS) is a marker of increased lung cancer risk in both the central and peripheral airways, indicating the presence of changes throughout the airway field (24–26).

Molecular characterization of the airway field of injury in smokers with PMLs may provide novel insights into the earliest stages of lung carcinogenesis and identify relatively accessible biomarkers to guide early lung cancer detection and early intervention.
Translational Relevance

Lung cancer prevention could be transformed by novel approaches that enhance the identification of high-risk patients, safe and effective prevention agents, and biomarkers of therapeutic response. We have used mRNA sequencing to identify gene expression alterations in the airway epithelium associated with the presence of bronchial premalignant lesions. Bronchial premalignant lesions are precursors of lung squamous cell carcinoma and are a risk factor for developing lung cancer at the lesion site or elsewhere in the lung. The early molecular changes that we have identified in normal appearing epithelial cells in the premalignant airway may represent possible targets for lung cancer chemoprevention. In addition, gene expression alterations in airway epithelium can be leveraged to develop a biomarker associated with the presence and progression of premalignant lesions that could be used to stratify patients into chemoprevention trials or serve as surrogate markers of efficacy.

Materials and Methods

Subject population used to derive gene signature and biomarker

Bronchial airway brushings were obtained during autofluorescence bronchoscopy procedures between June 2000 and March 2011 from subjects in the British Columbia Lung Health Study (BC-LHS) at the British Columbia Cancer Agency (BCCA; Vancouver, British Columbia, Canada; ref. 27) and between December 2009 and March 2013 from subjects in the High-Risk Lung Cancer Screening Program at Roswell Park Cancer Institute (RPCI; Buffalo, NY; detailed cohort information in the Supplementary Methods). PMLs were sampled (if present) using endobronchial biopsies, graded by a team of pathologists at BCCA or RPCI, and the worst histology observed was recorded. Bronchial brushes of normal appearing epithelium from 84 BCCA subjects (1 brush/subject) with and without PMLs were selected to undergo mRNA sequencing (mRNA-Seq) while ensuring balanced clinical covariates. Fifty-one bronchial brushes of normal appearing epithelium from 23 RPCI subjects were also profiled by mRNA-Seq (18 subjects had 2 procedures, and 5 subjects had 3 procedures) and 32 samples (i.e., “signal”) in at least 15% of the samples. For the RPCI samples, gene counts were computed using RSEM (31) and Bowtie (32) with Ensembl 74 annotation. The data are available from NCBI’s Gene Expression Omnibus using the accession ID GSE79315.

Data analysis for the BCCA samples

Sample and gene filtering yielded 13,870 out of 51,979 genes and 82 samples (n = 2 excluded due to quality or sex annotation mismatches) for analysis. Data from Beane and colleagues (1) were used to predict the smoking status of the 82 samples that was utilized in all subsequent analyses (Supplementary Dataset SD1, Supplementary Fig. S1, and Supplementary Methods). Airway brushings were dichotomized into two groups: samples with no evidence of PMLs (samples with no abnormal fluorescing areas or biopsies having normal or hyperplasia histology, n = 25); and samples with evidence of PMLs (biopsies having mild, moderate, or severe dysplasia, n = 50). Brushes with a worst histology of atypia (n = 7) were excluded from the dichotomized groups. The limma (33), edgeR (34), and sva packages (35) were used to identify differentially expressed genes associated with the presence of PMLs using normalized voom-tranformed (36) data and surrogate variable analysis using the first 7 surrogate variables (Supplementary Table S1). Gene set enrichment analyses were conducted using ROAST (37), GSEA (38), and GSVA (39). The Molecular Signatures Database (MsigDB) v4 Entrez ID Gene Sets were converted to Ensembl IDs using BioMart. Additional gene sets were created from CEL files or RNA-Seq counts from The Cancer Cell Line Compendium (CCLE), SCC tumor, and adjacent normal tissue from TCGA, GSE19188, GSE18842, and GSE4115 (Supplementary Methods).

Cell culture

Human bronchial epithelial biopsy cell cultures (Supplementary Table S2) were obtained from the Colorado Lung SPORE Tissue Bank and cultured in Bronchial Epithelial Growth Media. Human non–small cell lung cancer (NSCLC) cell lines were purchased from ATCC and short tandem repeat profiles were verified at the time of use by the Promega Gene Print 10 system at the Dana Faber Cancer Institute (Boston, MA). H1299, H2085, and SW900 cells were cultured in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin, and H2085 cells were cultured in ALC-4 media. All cells were grown in a 37°C humidified incubator with 5% CO2.

Bioenergetics studies

Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured using the XF96 Extracellular Flux Analyzer instrument (Seahorse Bioscience Inc.). Briefer,
approximately 30,000 cancer cells/well or approximately
40,000 bronchial epithelial biopsy cells/well (higher numbers
due to slow growth rate) were seeded on XF96 cell culture plates
and grown overnight. Prior to running the assay, media were
replaced with Seahorse base media (2 mmol/L l-glutamine)
and placed at 37°C and 0% CO2, for approximately 30 minutes.
The XF Cell Mito Stress Test Kit and protocol were utilized to
test thousands of parameter combinations (6,160
distributions between the complexes I
IV of the ETC and ATP
synthase, were highly activated in the airway field in
the presence of PMLs. We wanted to determine whether the func-
tional activity of these pathways was similarly altered in
PMLs compared with normal tissue. We conducted cellular

Results
Subject population
The study design used 126 bronchial brushings obtained via
autofluorescence bronchoscopy at the BCCA and RPCI for
differential gene expression and pathway analysis, as well as
for biomarker development and validation (Fig. 1). A dataset
consisting of samples (n = 75) collected from BCCA subjects
with PMLs (n = 50) and without PMLs (n = 25) was used to
derive a gene expression signature associated with the presence
of PMLs. Important clinical covariates such as COPD and
reported smoking history as well as alignment statistics from
the mRNA-Seq data were not significantly different between
the two groups (Tables 1 and 2). For biomarker development, the
75 BCCA samples were split by batch and used in biomarker
discovery (n = 58) and validation (n = 17; Supplementary
Tables S4 and S5). The change in biomarker score as a predictor
of progression of PMLs was then tested in the 51 RPCI samples
(Supplementary Tables S5 and S6).

Transcriptomic alterations in the airway field of injury
associated with the presence of PMLs
We identified 280 genes significantly differentially expressed
between subjects with and without PMLs (FDR < 0.002; Fig. 2).
Utilizing the MSigDB v4 canonical pathways, we identified 170
pathways significantly enriched in genes up- or downregulated
in the presence of PMLs using ROAST (FDR < 0.05; Supplementary
Dataset SD2; ref. 37). Pathways involved in oxidative phosphor-
ylation (OXPHOS), the electron transport chain (ETC), and
mitochondrial protein transport were strongly enriched among
genes upregulated in the airways of subjects with PMLs. Other
upregulated pathways included DNA repair and the HIF1A path-
way. Downregulated pathways included the STAT3 pathway, the
JAK/STAT pathway, IL4 signaling, RAC1-regulatory pathway,
NCAM1 interactions, collagen formation, and extracellular matrix
organization.

OXPHOS is increased in PML cell cultures and biopsies of
increasing severity
The ETC and OXPHOS pathways, which involve genes
distributed between the complexes I–IV of the ETC and ATP
synthase, were highly activated in the airway field in
the presence of PMLs. We wanted to determine whether the func-
tional activity of these pathways was similarly altered in
PMLs compared with normal tissue. We conducted cellular
bioenergetics by measuring OCR as a measure of ETC/OXPHOS (aerobic respiration), ECAR as a measure of glycolysis (anaerobic respiration), and MitoTraker Green FM as a measure of mitochondrial content in primary cell cultures derived from bronchial biopsies. In addition, we performed IHC of select OXPHOS-related genes in mouse and human dysplastic lesions and normal tissue to measure protein levels.

We established a significant concordance between ETC/OXPHOS gene expression and cellular bioenergetics in NSCLC cell lines (Supplementary Fig. S3A–S3F). Next, using primary cell cultures derived from normal to severe dysplastic tissue (Supplementary Table S2), we observed that the mean baseline OCR/ECAR values were 2.5/1.5-fold higher in the cultures from PMLs compared with controls \( (P = 0.035; \text{Fig. 3A}) \), reflecting predictions based on mRNA-Seq field data (Supplementary Fig. S3G and S3H). There was a greater reduction in OCR in PMLs immediately following oligomycin treatment \( (P = 0.022) \), suggesting an increased dependence on OXPHOS for ATP production to meet energetic demands. In addition, the mean spare respiratory capacity following the release of the proton gradient was elevated by approximately 1.5-fold in the PML cultures compared with controls, indicating increased ability to respond to energy demands \( (42) \). Finally, treatment with antimycin A resulted in a greater reduction of OCR in PML cultures \( (P < 0.001, \text{Fig. 3B}) \), suggesting that oxygen consumption in the lesions is dependent on increased ETC components in complex III. No significant changes to ECAR were detected in response to mitochondrial perturbations. Furthermore, to examine whether the increased OXPHOS was a result of increased mitochondrial biogenesis in PML cultures, cells were incubated with MitoTraker FM to stain for mitochondrial content, and fluorescence enumerated using flow cytometry revealed no significant difference between PML and controls \( (P = 0.15; \text{Fig. 3C and D}) \).

Figure 1.
Study design. Flow diagram depicting use of bronchial brushings collected from subjects with (red, \( n = 50 \)) and without (gray, \( n = 25 \)) PMLs from the BCCA as part of the BC-LHS (top panel) for differential gene expression/pathway analysis and for biomarker development. Independent human and mouse bronchial biopsies and biopsy cell cultures were used to validate these findings via mitochondrial enumeration, bioenergetics, and IHC (left panel). Biomarker development was conducted by splitting samples from the BC-LHS into a discovery \( (n = 58) \) and a validation set \( (validation 1, n = 17; \text{right panel}) \). The discovery set was used to create the gene expression–based biomarker to detect the presence of PMLs in the airway field of injury. The biomarker was tested on the BC-LHS validation set and an external validation set (bottom panel) from RPCI \( (validation 2, n = 28 \text{ matched time point pairs, stable/progressing pairs in yellow and regressing pairs in blue}) \).
that appear normal. Can be observed throughout the respiratory tract, even in cells supporting the concept that early events in lung carcinogenesis (GSE4115, Fig. 4C; Supplementary Dataset SD3). These results are dysregulated in the airway and PMLs, we examined the relationship between PML-associated gene expression alterations in the airway and their outcome over time. Our results suggest that PMLs are more ETC- and OXPHOS-dependent and express OXPHOS-related proteins at higher levels compared with normal tissue.

PML-associated gene expression alterations in the airway field are involved in lung squamous cell carcinogenesis

To further extend the connection between the airway field and PMLs, we examined the relationship between PML-associated genes in the airway field and other lung cancer-related datasets. We identified genes differentially expressed between lung tumor tissue (primarily squamous) and normal lung tissue in three different datasets (TCGA, GSE19188, and GSE18842). Genes associated with lung cancer in all datasets were significantly (FDR < 0.05) enriched by GSEA, concordantly with gene expression changes associated with the presence of PMLs in the field (Fig. 4A; Supplementary Dataset SD3). Extending beyond the lung tumor, similar enrichment (FDR < 0.05) was found using early, stepwise, and late gene expression changes in SCC identified by Ooi and colleagues (Fig. 4B; Supplementary Dataset SD3; ref. 43) and among genes associated with lung cancer in the airway field of injury (GSE4115, Fig. 4C; Supplementary Dataset SD3). These results support the concept that early events in lung carcinogenesis can be observed throughout the respiratory tract, even in cells that appear normal.

Development and validation of a biomarker for PML detection and monitoring

The airway brushings from BCCA subjects with and without PMLs were leveraged to build a biomarker predictive of the presence of PMLs. The biomarker consisted of 200 genes (of which 91 overlapped with the gene signature in Fig. 2) and achieved a ROC curve AUC of 0.92, sensitivity of 0.75 (9/12 samples with PMLs predicted correctly), and specificity of 1.00 (5/5 samples without PMLs predicted correctly) in independent validation samples (n = 17; Fig. 5A). In addition, the biomarker was used to score an independent set of longitudinally collected bronchial brushings from RPCI subjects (Fig. 1). Biomarker scores were calculated for each sample, and the difference in biomarker scores between sequential procedures (n = 28 time point pairs, Supplemental Methods) was predictive of whether the worst PML histology observed during the baseline procedure progressed or whether it was stable or progressed with an AUC of 0.75 (Fig. 5B).

Discussion

In this study, we identified a PML-associated gene expression signature in bronchial brushings obtained from normal appearing mucosa and characterized the biological pathways that are dysregulated in the airway field of injury. We established that the PML-associated airway field harbors alterations observed in PMLs and in SCC. This evidence motivated the development of a biomarker that reflects the presence of PMLs and their outcome over time. Our findings provide novel insights into the earliest molecular events associated with lung cancer development.

Table 1. Demographic and clinical characteristics stratified by premalignant lesion status

| Factors                    | Overall (n = 82) | No lesions (n = 25) | Lesions (n = 50) | P
|---------------------------|------------------|---------------------|------------------|---
| Age                       | 62.9 (7.2)       | 64.5 (5.8)          | 62.2 (8.0)       | 0.16
| Male                      | 54/82 (65.9)     | 16/25 (64)          | 35/50 (70)       | 0.61
| Current smoker            | 40/82 (48.8)     | 11/25 (44)          | 25/50 (50)       | 0.81
| Pack-years                | 47.3 (15.7)      | 47.6 (17.9)         | 47.2 (15.2)      | 0.93
| FEV1% predicted           | 82.5 (18.6)      | 84.5 (17.9)         | 81.7 (19.2)      | 0.54
| FEV1/FVC ratio            | 71.2 (7.9)       | 75.4 (7.4)          | 69.6 (8.1)       | 0.05
| COPD (FEV1% < 80 & FEV1/FVC < 70) | 24/82 (29.3) | 5/25 (20)          | 17/50 (34)       | 0.28

Histology

|                | Overall (n = 82) | No lesions (n = 25) | Lesions (n = 50) | P
|----------------|------------------|---------------------|------------------|---
| Normal         | 15/82 (18.6)     | 2/25 (8)            | 13/50 (26)       | 0.28
| Hyperplasia    | 15/82 (18.6)     | 2/25 (8)            | 13/50 (26)       | 0.28
| Metaplasia     | 7/82 (8.5)       | 2/25 (8)            | 5/50 (10)        | <0.001
| Mild dysplasia | 35/82 (42.7)     | 35/50 (70)          |                  | 0.76
| Moderate dysplasia | 12/82 (14.6) | 12/50 (24)          |                  | 0.76
| Severe dysplasia | 5/82 (6.2)      | 5/50 (10)           |                  | 0.76

NOTE: Data are means (SD) for continuous variables and proportions with percentages for dichotomous variables. Two sample t tests were used for continuous variables; Fisher exact test was used for categorical variables. Brushes with a worst histology of metaplasia (n = 7) were excluded from the dichotomized groups.

*P values are for the comparison of subjects with and without premalignant lesions.

Table 2. Alignment statistics stratified by premalignant lesion status

| Factor                  | Overall (n = 82) | No lesions (n = 25) | Lesions (n = 50) | P
|-------------------------|------------------|---------------------|------------------|---
| Total alignments        | 90 M (17 M)      | 90 M (15 M)         | 91 M (19 M)      | 0.78
| Unique alignments       | 83 M (16 M)      | 83 M (14 M)         | 84 M (17 M)      | 0.76
| Properly paired alignments | 66 M (12 M)   | 66 M (11 M)         | 67 M (14 M)      | 0.75
| Genebody 80/20 ratio    | 1.3 (0.2)        | 1.3 (0.2)           | 1.3 (0.2)        | 0.84
| Mean GC content         | 47.8 (3.4)       | 47.4 (2.9)          | 48.2 (3.7)       | 0.54

NOTE: Data are means (SD) for continuous variables and proportions with percentages for dichotomous variables. Reads are expressed in millions denoted by M. Two sample t tests were used for continuous variables; Fisher exact test was used for factors. Brushes with a worst histology of metaplasia (n = 7) were excluded from the dichotomized groups.

*P values are for the comparison of subjects with and without premalignant lesions.
carcinogenesis and have the potential to impact lung cancer prevention by providing novel targets (e.g., OXPHOS) and potential biomarkers for risk stratification and monitoring the efficacy of chemoprevention agents.

The first major finding of our study was the identification of a PML-associated field of injury. The most significantly enriched pathways among upregulated genes in subjects with PMLs were OXPHOS, ETC, and mitochondrial protein transport. These pathways efficiently generate energy in the form of ATP by utilizing the ETC in the mitochondria. During cancer development, energy metabolism alterations are described as an increase in glycolysis and suppression of OXPHOS, known as the Warburg effect (44); however, recent studies demonstrate that OXPHOS is maintained in many tumors and can be important for progression (45). We wanted to assay for OXPHOS activation in PMLs, as it may support PML progression by generating reactive oxygen species (ROS) that can induce oxidative stress, increase DNA damage, and HIF1A pathway activation (pathways observed in our analysis).

We observed increases in both the basal OCR and the spare respiratory capacity in the PML biopsies, suggesting that PML-derived cell cultures are more ETC and OXPHOS dependent than the non-PML cultures. We also further demonstrated the increase in ETC activity marked by positive COX IV staining associated with increasing PML histologic grade. Several members of the mitochondrial protein import machinery (46) were significantly upregulated (FDR < 0.05) in airways with PMLs, including members of the TOM complex (TOMM22, TOMM7, and TOMM20) and TIM23 complex (TIMM23, TIMM21, and TIMM17A). We observed positive staining of TOMM22 with increasing PML grade, suggesting that increased import of precursor proteins from the endoplasmic reticulum may be required to meet the energy demands of PMLs. Measurements of mitochondrial content indicated no significant differences between the normal and PML-derived cultures, and transcriptional levels of PPARGC1A, associated with mitochondrial biogenesis, were not different between subjects with and without PML, indicating that increases in OXPHOS are likely independent of mitochondrial number (47–49). Increases in OXPHOS have also been demonstrated to be associated with PML progression in Barrett's esophagus and esophageal dysplasia (50), cervical dysplasia (51), and the dysplastic lesions that precede oral SCC (52). Collectively, these data suggest that the OXPHOS pathway may be a target for early intervention. Preclinical studies in the NTCU mouse model of lung SCC demonstrate the potential for targeting mitochondrial respiration by using the natural product honokiol to inhibit tumor development (53). Further investigations into the role of cellular energy metabolism in the development and progression of PMLs are needed to fully understand how to best target it for intervention in lung cancer.

In addition, we extended the connection between the PML-associated airway field and PMLs beyond the OXPHOS pathway to processes associated with squamous cell lung carcinogenesis. By examining gene sets from multiple external studies representative of lung cancer–related processes occurring in the tumor, adjacent to the tumor, and in the upper airway, we found significant concordant relationships between the PML-associated field and processes associated with SCC tumors. Genes are similarly altered in these varied cancer-associated contexts, and thus, tissues in the field both adjacent to and far away from the tumor may reflect basic processes and mechanisms of lung carcinogenesis, such as DNA damage, as hypothesized earlier.

These observations motivated us to pursue the most translational aspect of this study, a biomarker that can detect PMLs and monitor their progression over time. The 200-gene biomarker, measured in normal appearing bronchial mucosa, achieved high performance detecting the presence of PMLs in a small test set (AUC = 0.92). This biomarker may increase the sensitivity of bronchoscopy in detecting the presence of PMLs.
(which can be difficult to observe under white light) and thus improve identification of high-risk smokers that should be targeted for aggressive lung cancer screening programs. In addition, the biomarker may offer wider clinical utility in early intervention trials by serving as an intermediate endpoint of efficacy (beyond Ki-67 staining for proliferation, and changes in biopsy histology). Toward this goal, we demonstrated that the change in biomarker scores over time reflects contemporaneous regressive or progressive/stable disease (AUC = 0.75). This result suggests that the airway field of injury in the presence of PMLs is dynamic and that capturing the gene expression longitudinally may allow for further stratification of high-risk subjects. The potential clinical utility of the biomarker is further supported by recent work demonstrating a significant association between the development of incident lung SCC and the frequency of sites that persist or progress to high-grade dysplasia (24).

Further development and testing in a larger cohort is needed to confirm the biomarker’s performance, utility, and ability to predict future PML progression or regression. In addition, longitudinal and spatial sampling would provide a greater understanding of the dynamic relationship between the normal epithelium and the PMLs as they regress or progress to SCC. Longitudinal studies would allow for more accurate monitoring of premalignant lesions via airway gene expression.

Figure 3.

OXPHOS upregulation in premalignant lesion biopsies. A, The mean baseline OCR/ECAR ratio measured in human bronchial biopsy cultures from PMLs (pink, n = 6) was 2.5-fold higher than the biopsies of normal airway epithelium (gray, n = 6; P = 0.035). B, Bioenergetic studies testing mitochondrial function demonstrate PMLs (red) have a significantly (~1.5-fold) higher maximal respiration (P = 0.022). Error bars (A and B), SEM. C and D, Mitochondrial enumeration by FACS analysis of MitoTracker GFP suggests increased OCR is not reliant on increased mitochondria as the difference in GFP per cell was not significant (P = 0.150). E, Representative images of TOMM22 and COX IV staining in which expression of both proteins is increased in low and moderate dysplastic lesions in both human and NTCU-mouse PMLs (magnification, >400).
characterization of the time intervals needed to observe gene expression dynamics both in the PMLs and in the airway field of injury. Spatial sampling throughout the respiratory tract, including the more accessible nasal airway that shares the tobacco-related injury with the bronchial airways (54), would allow for evaluation of the impact of distance between the PMLs and the brushing site, the range of PML histologies, and the multiplicity of PMLs that can be present simultaneously in a patient and influence the PML-associated airway field.

In light of these challenges and opportunities for future work, we have comprehensively profiled gene expression changes in airway epithelial cells in the presence of PMLs that suggest great clinical utility. Moving therapeutics and detection strategies toward an earlier stage in the disease process via molecular characterization of premalignant disease holds great promise (55, 56), and this study represents an important step toward a precision medicine approach to lung cancer prevention.
Disclosure of Potential Conflicts of Interest

J. Beane reports receiving commercial research grants from Janssen Pharmaceuticals. A.M. Tassinari reports receiving a commercial research grant from Janssen Pharmaceuticals. M.E. Lenburg reports receiving commercial research grants from Janssen Research and Development, Inc. and is a consultant/advisory board member for Varacayte. A.E. Spira reports receiving commercial research grants from Janssen Pharmaceuticals and is a consultant/advisory board member for Janssen Pharmaceuticals and Varacayte. No potential conflicts of interest were disclosed by the other authors.

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