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Cancer Res 2007;67:6565-6573.

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Abstract

Squamous cell carcinoma in the lung originates from bronchial epithelial cells that acquire increasingly abnormal phenotypes. Currently, no known biomarkers are clinically efficient for the early detection of premalignant lesions and lung cancer. We sought to identify secreted molecules produced from squamous bronchial epithelial cells cultured with organotypic culture methods. We analyzed protein expression patterns in the apical surface fluid (ASF) from aberrantly differentiated squamous metaplastic normal human tracheobronchial epithelial (NHTBE) and mucous NHTBE cells. Comparative two-dimensional PAGE analysis revealed 174 unique proteins in the ASF of squamous NHTBE cells compared with normal mucociliary differentiated NHTBE cells. Among them, 64 well-separated protein spots were identified by liquid chromatography-tandem mass spectrometry, revealing 22 different proteins in the ASF from squamous NHTBE cells. Expression of six of these proteins [SCC antigen 1 (SCCA1), SCC antigen 2 (SCCA2), S100A8, S100A9, Annexin I, and Annexin II] in the squamous NHTBE cells was further confirmed with immunoblot analysis. Notably, SCCA1 and SCCA2 were verified as being expressed in squamous metaplastic NHTBE cells but not in normal mucous NHTBE or normal bronchial epithelium. Moreover, SCCA1 and SCCA2 expression increased in *in vitro* lung carcinogenesis model cell lines with increasing malignancy. In summary, we identified proteins that are uniquely secreted from squamous metaplastic primary human bronchial epithelial cells cultured by the organotypic air-liquid interface method. These ASF proteins may be used to detect abnormal lesions in the lung without collecting invasive biopsy specimens. [Cancer Res 2007;67(14):6565–73]

Introduction

Lung cancers are largely classified into two major groups based on their histopathologic differences: non–small-cell lung cancer, which is further divided into adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma; and small-cell lung carcinoma. Regardless of its subtype, the 5-year survival rate for lung cancer is only 10% to 15% (1, 2). Disease stage at diagnosis is the most important prognostic variable, primarily because late-stage disease is extremely difficult to cure (3, 4). However, there are currently no known biomarkers that are clinically efficient for the early detection of premalignant lesions and lung cancer. The identification of biomarkers for early abnormal changes in the lung epithelia may help reduce the high mortality rate associated with this deadly disease.

During the development of squamous cell carcinoma in the lung, bronchial epithelial cells exhibit a progressive series of morphologically distinct changes: hyperplasia, squamous metaplasia, dysplasia, carcinoma *in situ*, and finally invasive squamous cell carcinoma. However, there are no biomarkers available for detection of these premalignant changes in the bronchial epithelium, except for histologic examination of biopsied bronchial epithelia. Here, we sought to identify secreted molecules that can be used to monitor these premalignant changes under the assumption that these morphologic changes are accompanied by abnormal secretion of multiple proteins and that identifying the profiles of these proteins will be useful in developing biomarkers for detecting precancerous or cancerous pulmonary lesions, particularly premalignant bronchogenic carcinoma.

However, it is often extremely difficult to identify proteins secreted from epithelial cells because the proteins in bodily fluids, such as bronchial secretions and bronchial lavage fluid, or in cancer tissue specimens include many proteins derived from circulating blood and mixed cell types, including stromal and inflammatory cells.

To identify proteins uniquely secreted from squamous metaplasia of bronchial epithelia, we used an organotypic bronchial epithelial cell culture system. We previously showed that normal human tracheobronchial epithelial (NHTBE) cells cultured by a three-dimensional organotypic culture method (known as the air-liquid interface) recapitulates the *in vivo* mucociliary pseudostratified bronchial epithelium (5). Squamous metaplasia is generated when the same NHTBE cells are grown in retinoic acid (RA)–deficient medium (6–8).

To identify proteins uniquely secreted from squamous metaplastic bronchial epithelia, we compared the secreted protein profiles, or secretomes, of apical surface fluid (ASF) samples from metaplastic squamous NHTBE cell cultures and mucociliary differentiated NHTBE cells. We found that at least 22 proteins in the ASF from the metaplastic squamous NHTBE cells were distinct

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doi:10.1158/0008-5472.CAN-06-2783

from those in the ASF from the mucous NHTBE cells. These proteins, which include SCC antigen 1 (SCCA1), SCC antigen 2 (SCCA2), S100A8, S100A9, Annexin I, and Annexin II, are potential biomarkers for the detection of early metaplastic changes in bronchial epithelial cells.

Materials and Methods

Cell culture. NHTBE cells were cultured by air-liquid interface method as previously described (5–8). Basically, NHTBE cells (Clonetics Corp.) from passage 2 were seeded at a density of 1×10^5 per insert onto 24-mm, uncoated, semipermeable Transwell clear membranes (Costar) in serum-free hormone- and growth factor–supplemented medium (all medium supplements were purchased from Clonetics). NHTBE cell cultures were maintained in RA (5×10^{-8} mol/L)–sufficient media for mucociliary differentiation and in RA-deficient media for squamous metaplasia differentiation. NHTBE cells were grown submerged for the first 7 days, after which time, the air-liquid interface was created. The cells were then cultured in the air-liquid interface condition for 3 weeks, with medium changed every 24 h, as previously described (5, 9). Twenty-eight-day-old cultures with a fully developed mucociliary and squamous metaplasia phenotype were used in all experiments. The ASF (for secretome analysis of the extracellular protein pool) and whole-cell lysates (for proteome analysis of the cellular protein pool) were collected and stored at −80°C until needed.

The non–small-cell lung cancer cell lines NCI-H226, NCI-H292, NCI-H1734, and NCI-H1975 were obtained from the American Type Culture Collection. The cells were grown in a monolayer culture in RPMI 1640 containing 10% fetal bovine serum.

The cell lines for the *in vitro* lung carcinogenesis model, including normal immortalized (BEAS-2B and 1799), transformed (1198), and tumorigenic (1170-I) human bronchial epithelial cell lines, were obtained from Dr. Klein-Szanto (Fox Chase Cancer Center, Philadelphia, PA). All of the human bronchial epithelial cells were grown in keratinocyte serum-free medium (Life Technologies, Gaithersburg, MD) containing epidermal growth factor and bovine pituitary extract, as previously described (10). All cells were cultured at 37°C in a humidified, water-jacketed incubator in 5% CO_2 in air.

Preparation and analysis of protein extracts. To analyze protein expression patterns in the ASF, the apical surfaces of the 4-week cultures of NHTBE cells were vigorously washed with PBS containing EDTA-free complete protease inhibitor cocktail (Roche Applied Science) and then the ASF of mucous and squamous metaplastic NHTBE cells was collected. Next, to extract the proteins from the ASF, trichloroacetic acid, at a final concentration of 20%, was added to the thawed ASF, and the mixture was incubated for 15 min on ice and then centrifuged at 10,000 × *g* at 4°C for 15 min. The pellet was then washed with cold acetone, allowed to air-dry, and resuspended in rehydration buffer containing 7 mol/L urea, 2 mol/L thiourea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonic acid, 100 mmol/L DTT, 0.1% (w/v) BioLytes (Bio-Rad Laboratories), and a trace amount of bromophenol blue. Insoluble materials were removed by centrifugation.

To analyze protein expression patterns in whole-cell lysates, the cells were removed immediately and stored at −80°C after being frozen in liquid nitrogen before their preparation for two-dimensional PAGE analysis. The cells were collected and homogenized in 500 μL of lysis buffer [7 mol/L urea, 2 mol/L thiourea, 2% CHAPS, 2% Pharmalyte (pH 3–10), 100 mmol/L DTT] containing complete EDTA-free protease inhibitors (Roche Diagnostics). The solubilized proteins were separated from nonsolubilized cellular components by centrifugation (12,000 × *g* at 4°C for 1 h). The supernatant was transferred into a new microcentrifuge tube.

The protein concentrations of ASF and whole-cell lysates were determined using a detergent-compatible protein assay kit (Bio-Rad) according to the manufacturer's instructions.

Two-dimensional PAGE and image analyses. To analyze differential protein expression, 200 μg of protein extracted from the ASF and whole-cell lysates were subjected to two-dimensional PAGE analysis as previously described (11). The samples were loaded onto 11-cm immobilized pH gradient strips (pH 3–10, nonlinear), which were rehydrated for 12 h at 50 V. Isoelectric focusing was conducted using a Protean isoelectric focusing cell system for 40,000 V/h according to the manufacturer's instructions. Focused strips were equilibrated with 6.0 mol/L urea, 20% (v/v) glycerol, 2% SDS, 2.5% acrylamide, and 0.2 mmol/L tributyl phosphine in 150 mmol/L Tris-HCl (pH 8.8) for 1 h. The equilibrated strips were applied directly to 4% to 20% gradient SDS-PAGE gel (Criterion, Bio-Rad) and separated overnight at a constant voltage of 40 V. For analytic purposes, the gels were silver stained using a Silver Stain Plus kit (Bio-Rad) according to the manufacturer's instructions. The stained gels were scanned using a Fluor-S MultiImager (Bio-Rad) and analyzed with PDQuest software V9.0 (Bio-Rad). The images were exported in the form of 16-bit tagged image file format files for analysis. The digitalized images of differentially expressed protein spots were compared using a color channel matching method with the PDQuest software.

Identification of differentially expressed proteins. After being excised, the silver-stained spots were destained by the method of Switzer et al. (12) and digested in gel with 200 ng of modified trypsin (Promega). The resulting peptides were analyzed by nano-liquid chromatography-tandem mass spectrometry with on-line desalting with a Famos autosampler, an Ultimate nano-LC module, and a Switchos precolumn switching device (LC Packings/Dionex). Electrospray ion-trap mass spectrometry was done using an LTQ linear ion-trap mass spectrometer (Thermo). The fragment spectra were analyzed using the National Center for Biotechnology Information nonredundant protein database and the Mascot search engine (Matrix Science).

Western blot and dot-blot analyses. To determine endogenous protein expression levels, we prepared whole-cell lysates using 2× SDS Laemmli lysis buffer [250 mmol/L Tris-HCl (pH 6.5), 2% SDS, 4% β-mercaptoethanol, 0.02% bromophenol blue, and 10% glycerol]. Equal amounts of each lysate (20 μg) were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% skim milk in PBST (PBS containing 0.1% Tween 20) for 1 h at room temperature and then incubated overnight with primary antibody in PBST containing 2.5% bovine serum albumin. The membranes were then washed with PBST and incubated for 1 h at room temperature with peroxidase-conjugated secondary antibody (Pierce) and visualized using an enhanced chemiluminescence system (Amersham Biosciences). Equal protein loading was confirmed by stripping the blots and reprobing them with antibody against β-actin (Sigma-Aldrich).

Mouse anti-human monoclonal antibodies against SCCA1 and SCCA2 were purchased from Santa Cruz Biotechnology. Mouse anti-human monoclonal antibodies against S100A8, S100A9, Annexin I, and Annexin II were purchased from Transduction Laboratories. To determine the proportion of each secreted protein in the ASF, equal volumes of ASF or the medium from the cultured NHTBE cells were applied to nitrocellulose membranes using a Minifold I dot-blot system (Schleicher & Schuell), and then dot-blotting was done.

Tissue microarrays construction and immunohistochemical staining. The Tissue Bank at M.D. Anderson Cancer Center from 1997 to 2001 was searched for formalin-fixed, paraffin-embedded material from surgically resected lung cancer tissue specimens. The specimens obtained from 303 non–small-cell lung cancer cell lines were histologically examined, classified using the 2004 WHO classification, and selected for tissue microarray construction. They consisted of 114 squamous cell carcinomas and 189 adenocarcinomas. Use of the specimens for research was approved by our institutional review board. After histologic examination, tumor tissue microarrays were prepared using triplicate 1-mm-diameter cores per tumor, obtaining tissues from central, intermediate, and peripheral tumor areas. In five cases from each tumor histology, adjacent normal bronchial epithelia were also selected for immunohistochemical expression analysis using whole-section specimens. Five-micron histologic sections were deparaffinized, hydrated, and pretreated for 5 min with proteinase K for antigen retrieval. Peroxide blocking was done with 3% H_2O_2 at room temperature for 15 min, followed by 10% bovine serum albumin in PBST for 30 min at room temperature. The slides were incubated with mouse anti-human

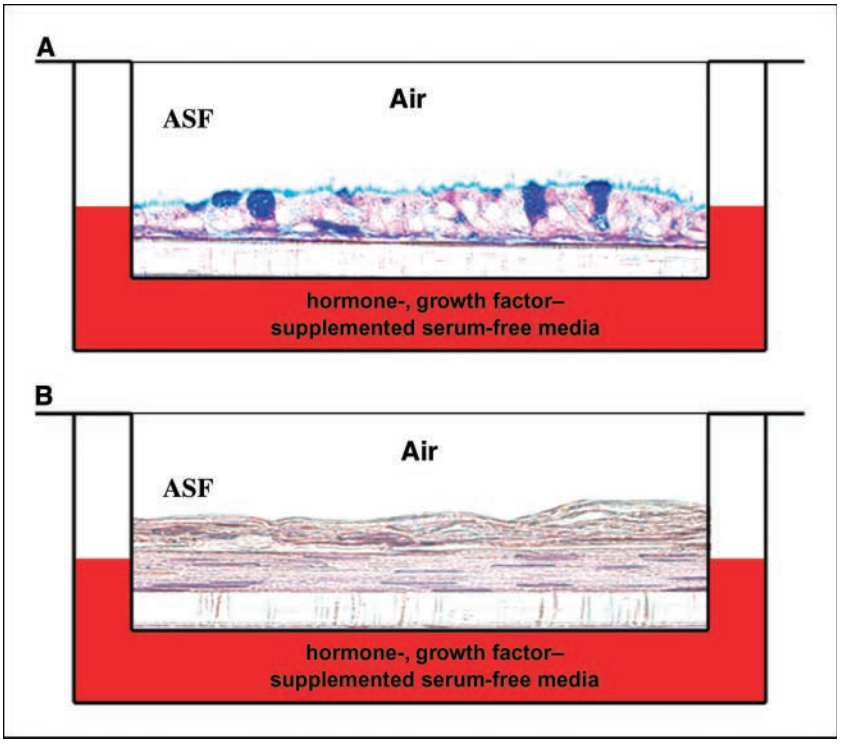

Figure 1. Histologic presentation of mucociliary and squamous metaplastic NHTBE cell cultures maintained by a three-dimensional organotypic air-liquid interface culture method. *A,* histomorphologic appearance of NHTBE cell cultures grown with (*A*) or without (*B*) RA for 4 wks. After 4 wks in culture, ASF and whole-cell lysates of the mucous and squamous differentiated NHTBE cells were prepared and analyzed as described in Materials and Methods.

SCCA1 and mouse anti-human SCCA2 (Santa Cruz Biotechnology) at 1:50 dilution for 90 min at room temperature. After washing with PBS, incubation with biotin-labeled secondary antibody was done for 15 min, followed by incubation with a 1:40 solution of streptavidin-peroxidase for 15 min. The staining was then developed with 0.05% 3′,3-diaminobenzidine tetrahydrochloride that had been freshly prepared in 0.05 mol/L Tris buffer (pH 7.6) containing 0.024% H_2O_2, and then the sections were counterstained with hematoxylin, dehydrated, and mounted. As negative control, we used the same specimens used as positive controls, replacing the primary antibody with PBS. The specimens were examined for SCCA1 and SCCA2 immunohistochemical expression in tumor cells and normal bronchial epithelial cells using light microscopy.

Immunofluorescence assays. SCCA1 and SCCA2 expression was analyzed by immunofluorescence in non–small-cell lung cancer cells and cell lines used in the *in vitro* lung carcinogenesis model (BEAS-2B, 1799, 1198, and 1170-I). The cells were grown in chamber slides until 60% confluent and then fixed with 4% (v/v) paraformaldehyde in PBS for 20 min at room temperature. The cells were then permeabilized with PBS containing 0.5% Triton X-100, blocked in 5% normal goat serum for 1 h, and incubated with antihuman SCCA1 and antihuman SCCA2 antibodies (each diluted 1:500) for 2 h. After being washed with PBS, the cells were further incubated with Alexa Fluor 488–conjugated goat anti-mouse secondary antibody (Molecular Probes) for 1 h, and nuclei were counterstained with 4′6′-diamidino-2-phenylindole for 30 min. After being washed again with PBS, the cells were mounted using a SlowFade antifading kit (Molecular Probes). Finally, the stained cells were visualized under an Axioskop 40 microscope (Carl Zeiss) and images were captured at ×400 magnification and stored using Axiovision software (Carl Zeiss) according to the manufacturer's instructions.

Results

Collection of ASF from normal mucociliary differentiated and squamous metaplastic primary NHTBE cell cultures. To investigate the differentially expressed proteins in ASF, we cultured NHTBE cells with or without RA using a three-dimensional air-liquid interface culture method. NHTBE cells cultured with RA differentiated into mucous and ciliated differentiated cells (Fig. 1*A*). In contrast, NHTBE cells cultured without RA differentiated into squamous metaplastic cells that showed typical squamous cell layers (Fig. 1*B*). In this system, the basal and apical compartments of confluent cell cultures are completely separated, leaving the ASF free from the protein components in the culture medium in the basal compartment. The NHTBE cell cultures develop fully differentiated mucociliary epithelia when maintained in serum-free medium supplemented with RA and growth factors in the basal compartment and exposed to air on the apical side. Proteins in the basal compartment medium are prevented from leaking into the apical side by the tight junctions in the epithelial cell layer. Thus, unlike the secreted proteins from the bronchial surface fluid *in vivo*, ASF from cultured bronchial epithelial cells contains proteins secreted only by bronchial epithelial cells.

Identification of differentially expressed proteins in squamous NHTBE cells. As a first step toward identifying proteins that are differentially represented in the ASF from squamous metaplastic NHTBE cells, we determined protein expression profiles with independent secretomes and proteomes of mucous and squamous differentiated NHTBE cells using two-dimensional PAGE analysis.

We found that a small proportion (~40%) of the secreted proteins appeared to be intracellular proteins (data not shown). However, a substantial proportion of secreted proteins were uniquely represented in the ASF from squamous and mucous cultures compared with their respective intracellular proteomes (Fig. 2*A*). For example, for the squamous differentiated NHTBE cells, image analysis showed that only 39% of the spots from the extracellular ASF secretome (Fig. 2*A-a, red*) and intracellular proteome (*d*; *green*) overlapped (*g*). Similarly, for the mucous

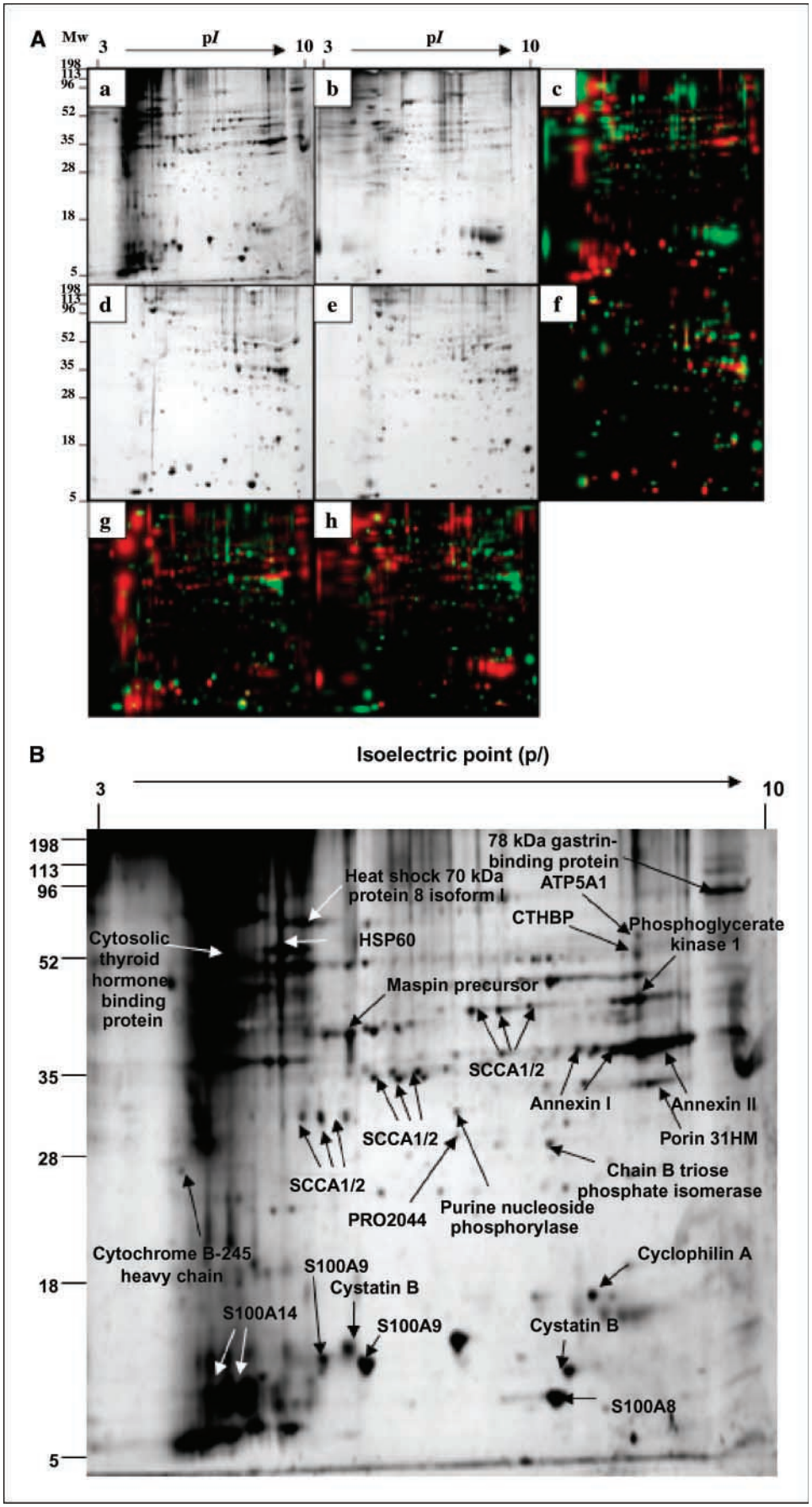

Figure 2. Comparison of protein expression in squamous and mucous differentiated NHTBE cells determined by two-dimensional PAGE analysis. *A,* representative images from two-dimensional PAGE analysis showing ASF from squamous differentiated NHTBE cells (*a*; *red channel*); ASF from mucous differentiated NHTBE cells (*b*; *green channel*); merged images of *a* and *b* (*c*); whole-cell lysate from squamous differentiated NHTBE cells (*d*; *green channel*); whole-cell lysate from mucous differentiated NHTBE cells (*e*; *red channel*); merged image of *d* and *e* (*f*); merged image of *a* and *d* (*g*); and merged image of *b* and *e* (*h*). Representative of three independent experiments. *B,* representative two-dimensional PAGE images of ASF from squamous differentiated NHTBE cells. *Arrows with annotations,* protein spots identified by liquid chromatography-tandem mass spectrometry. The identified proteins are listed in Table 1.

differentiated NHTBE cells, only 40% of the spots matched when the secretome (*b*; *green*) and proteome (*e*; *red*) matched (*h*). These results clearly indicated that a unique panel of proteins could be detected in the ASF that were not detected intracellularly by two-dimensional PAGE.

Comparative image analysis of the secretomes and proteomes of squamous and mucous NHTBE cells revealed considerable discrepancy. Only 35% of the spots matched in the secretomes of the two cultures (Fig. 2*A-c*). Similarly, only 41% of the spots matched in the proteomes of the two cultures (Fig. 2*A-f*). The analysis yielded more than 174 protein spots that seemed to be expressed differently in the ASF from the squamous differentiated NHTBE cells but not in the ASF from the mucous differentiated NHTBE cells. Of those 174 proteins, 64 well-separated protein spots were isolated, processed, and identified by liquid chromatography-tandem mass spectrometry. A summary of all matches identified for each spot is presented in Table 1, and the corresponding spots are labeled in Fig. 2*B*. As shown in Fig. 2*B*, we found more than 11 spots [isoelectric point (p*I*), 5.5–7.55] of SCCA1 and SCCA2 proteins, which were expressed in squamous cell but not mucous cell ASF. In addition, S100A8, S100A9, Annexin I, and Annexin II were identified in the squamous cell ASF but not in the mucous cell ASF.

Validation of differentially expressed proteins in ASF. To verify the differential expression of proteins in the squamous cell ASF, we used dot-blot analysis to examine the expression of a selected panel of proteins, including SCCA1, SCCA2, S100A8, S100A9, Annexin I, and Annexin II, in the ASF from squamous and mucous NHTBE cells. The selected proteins were detected only in the ASF from squamous differentiated NHTBE cells (Fig. 3*A*). The presence of the MUC5AC protein in the ASF was used as an indicator of mucous differentiation of NHTBE cells because MUC5AC is exclusively produced by the mucous goblet cells in the bronchial epithelia.

We next examined the cellular expression of the selected proteins by Western blot analysis. SCCA1 and SCCA2 were expressed only in squamous differentiated cells (Fig. 3*B*). S100A8 and S100A9 were much more highly expressed in squamous differentiated cells than in mucous differentiated cells. In contrast, Annexin I and Annexin II were expressed at the same intracellular levels in both cell types, despite being detected only in the squamous cell ASF.

Together, these results clearly showed that SCCA1 and SCCA2 in the ASF are secreted markers of metaplastic squamous differentiation in bronchial epithelial cells.

SCCA1 and SCCA2 expression levels in transformed and tumorigenic human bronchial epithelial cells and non–small-cell lung cancer cells. To further investigate whether SCCA1 and SCCA2 expression is abnormally regulated during lung carcinogenesis, we measured the expression of these proteins in an *in vitro* lung carcinogenesis model. The system was composed of syngeneic cell lines: the immortalized human bronchial epithelial cell lines BEAS-2B and 1799 and the tumorigenic cell lines 1198 (transformed) and 1170-I (tumorigenic). Western blot (Fig. 4*A*) and immunofluorescence (Fig. 4*B*) analyses showed that SCCA1 expression and SCCA2 expression were much higher in tumorigenic cell lines (1198 and 1170-I) than in the BEAS-2B and 1799 cell lines. These results showed that expression of these proteins increased during progression to the tumorigenic phenotype.

To confirm the elevated expression of SCCA1 and SCCA2 in tumorigenic cell lines, we next examined the expression of SCCA1 and SCCA2 in four established non–small-cell lung cancer cell lines (two squamous cell carcinomas and two adenocarcinomas). Western blotting (Fig. 4*C*) and immunofluorescence (Fig. 4*D*) analyses showed that SCCA1 and SCCA2 were expressed only in the squamous cell carcinoma cell lines (H226 and H292); their expression was undetectable in the

Table 1. Uniquely expressed proteins in ASF from squamous metaplastic NHTBE cells

Spot	Protein	Accession no.	M_r (kDa)	p*I*	Mascot scores	Identified peptide no.
1	S100A8	NP_002955	10.9	6.7	692	10
2	Cystatin B	AAF44059	15.2	5.9	566	5
3	S100A9	NP_002956	13.2	5.6	547	14
4	S100A14	NP_065723	11.6	5.4	102	11
5	Chain A, cyclophilin A complexed with cyclosporin A	3CYSA	17.9	8.1	227	9
6	Annexin I	NP_000691	37.3	8.1	157	5
7	Annexin II	NP_004030	36.6	8.5	149	9
8	SCCA1	P29508	42.1	5.3	809	19
9	SCCA2	P48594	42.1	7.0	843	18
10	Maspin precursor (protease inhibitor 5)	P36952	42.0	5.2	377	5
11	Phosphoglycerate kinase 1	P00558	45.0	8.5	939	17
12	Trifunctional enzyme α subunit	P40939	78.0	9.0	144	13
13	60-kDa heat shock protein	P10809	60.0	4.5	567	8
14	70-kDa heat-shock protein 8 isoform 1	NP_006588	69.7	8.0	663	9
15	Cytosolic thyroid hormone-binding protein	P14618	57.3	8.5	186	3
16	Protein disulfide-isomerase precursor	P02737	55.0	4.0	486	14
17	ATP5A1 protein	AAH39135	60.9	8.5	826	14
18	Purine nucleoside phosphorylase	P00491	31.2	5.6	188	15
19	Porin 31HM	AAB20246	32.8	8.8	289	4
20	PRO2044	AAF22034	29.0	6.0	102	2
21	Chain B, triose phosphate isomerase	1HTIB	26.7	6.5	610	9
22	Cytochrome B-245 heavy chain	P04839	20.5	4.0	301	5

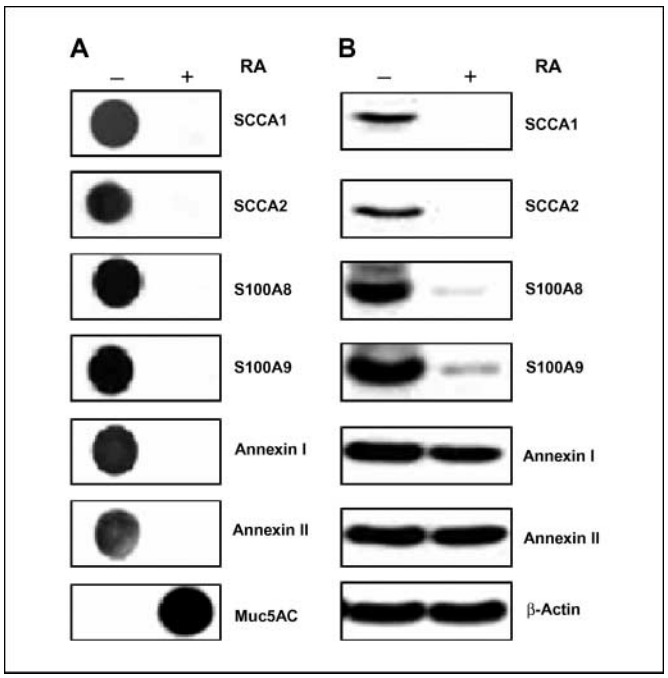

Figure 3. Dot-blot and Western blot analyses of differentially expressed proteins. ASF and whole-cell lysates from squamous differentiated (*−RA*) and mucous differentiated (*+RA*) NHTBE cells were analyzed by dot-blot (*A*) and Western blot (*B*) analyses as described in Materials and Methods. MUC5AC was used as an indicator of mucous cell differentiation. β-Actin was used as a loading control for Western blot analysis. Representative of three independent experiments.

adenocarcinoma cell lines (H1734 and H1975). These results showed that SCCA1 and SCCA2 are expressed only in squamous cell carcinoma cell lines.

SCCA1 and SCCA2 expression levels in normal and malignant lung tissue specimens. To determine whether SCCA1 and SCCA2 expression is also elevated in non–small-cell lung cancer tissue specimens and the specificity of these proteins expression in adenocarcinoma and squamous cell carcinoma histologies, we did immunohistochemical analysis using tumor tissue microarray samples (Fig. 5). As shown in Fig. 5, SCCA1 and SCCA2 immunostainings were detected in the cytoplasm of tumor cells in both tumor histology specimens. However, the protein expression frequency was obviously higher in squamous cell carcinoma [positive, SCCA1 42.4% (n = 45) and SCCA2 65.7% (n = 69); negative, SCCA1 57.6% (n = 61) and SCCA2 34.3% (n = 36)] compared with adenocarcinoma [positive, SCCA1 33.3% (n = 58) and SCCA2 39.8% (n = 70); negative, SCCA1 66.7% (n = 116) and SCCA2 60.2% (n = 106)] histology, especially for SCCA2. In contrast, the expression of the two proteins was undetectable in normal bronchial epithelia adjacent to tumors from each histology (Fig. 5, *top*). These results indicate that SCCA1 and SCCA2 are not expressed in normal bronchial epithelium and they are highly expressed in squamous cell carcinoma of the lung. Somewhat surprising, approximately one third of adenocarcinomas showed SSCA1 and SCCA2 immunohistochemical expression.

Discussion

The discovery of new biomarkers holds promise for the early detection of precancerous lesions and the potential to reduce cancer mortality. However, no systematic approach has been applied to study the composition of the secretory proteome of bronchial epithelia cells. To accomplish this goal, we have applied a novel *in vitro* approach to identify new biomarkers using primary bronchial epithelial cells cultured by the organotypic air-liquid interface method, which allows the development of normal mucociliary cells or squamous metaplasia depending on the presence or absence of RA (5). Similarly, a previous study showed that the secretory protein profiles of adipose cells from the epididymal, inguinal, and omental fat pads of male rats differ from the protein profiles of whole-cell lysates (13).

Comparative analysis of the secretomes in the ASF from squamous and mucous NHTBE cells revealed at least 22 proteins that were unique in the ASF from squamous metaplastic NHTBE cells. Six proteins (SCCA1, SCCA2, S100A8, S100A9, Annexin I, and Annexin II) were initially selected for verification and further characterization because these proteins were implicated in cancer as described below.

Particularly, SCCA1 and SCCA2 were of interest because of increasing number of reports suggesting that SCCA may be a marker for squamous cell carcinoma. For example, SCCA has been implicated as a circulating tumor marker for squamous cell carcinoma of the cervix, head and neck, lung, and esophagus (14–17). Body et al. (18) reported elevated SCCA levels in patients with advanced lung cancer. Several studies show that increased serum SCCA levels correlated with advance lung cancer (19–23). In a previous study, it was reported that SCCA levels are increased in squamous cell carcinoma patients with correlation of disease extension (24). Consistent with our results, it was also reported that overexpression of SCCA1 and SCCA2 was found in 43% of lung tumors compared with corresponding normal tissue (25). In addition, overexpression of SCCA2 to SCCA1 was proposed as a marker of squamous cell carcinoma of the lung (26). However, our finding of approximately one third of adenocarcinomas showing SCCA1 and SCCA2 immunohistochemical expression suggests that these markers are not specific for lung squamous cell carcinoma. Nevertheless, it is noteworthy that the expression of SCCA1 and SCCA2 was low or undetectable in normal bronchial epithelia adjacent to tumors, and expression of the two proteins was obviously higher in squamous cell carcinoma (SCCA1, 42.4%; SCCA2, 65.7%) compared with adenocarcinoma (SCCA1, 33.3%; SCCA2, 39.8%) histology, especially for SCCA2.

Interestingly, the expression of the two proteins was also gradually augmented with increasing malignancy in lung carcinogenesis model cell lines, and the two proteins were overexpressed in non–small-cell lung cancer cell lines. This result is somewhat consistent with previous reports that SCCA is not present in respiratory secretions from the normal epithelium of the lung (27) but is present in secretions from areas of squamous metaplasia in the lung (28). Although SCCA activity was previously detected in inflammatory lung disease (27), SCCA may be secreted from nonsquamous epithelial cells undergoing squamous metaplastic changes in response to severe inflammation. Our finding that the expression of SCCA1 and SCCA2 increased with increasing malignancy suggests that the two proteins are associated with tumor progression. Thus, the presence of SCCA in the ASF of epithelial cells undergoing early abnormal changes suggests that SCCA1 and SCCA2 can be used as biomarkers for early detection of premalignant bronchial lesions and lung squamous cell carcinoma. However, further analysis of the expression of these two makers in

preneoplastic lesions for both squamous cell carcinoma and adenocarcinoma of the lung is needed.

The second pair of proteins we examined, S100A8 and S100A9, belong to the S100 family, one of the largest subfamilies of the elongation factor-hand calcium-binding proteins (29, 30). Although the role of these proteins in squamous metaplasia is not known, S100 family proteins have been shown to be involved in metastasis of cancer cells. For example, S100A6, S100A8, S100A9, and S100A11 are overexpressed in lung cancer and are involved in migration of cancer cells during metastasis (31, 32).

The other proteins we examined were Annexins I and II. Annexin I has been shown to be involved in membrane fusion, intracellular signal transduction, and cell differentiation. This protein is not found in normal tissue but is present in various tumor vessels (33) and diffusely in neoplastic lung tissue (34). Although Annexin I is a major component of the cilia of human bronchial cells and a fetal lung marker that is a substrate of the epidermal growth factor receptor (33, 35, 36), we found it only in the ASF of squamous NHTBE cells, not in the ASF of mucous NHTBE cells. It is not known how and why squamous NHTBE cells secrete Annexin I.

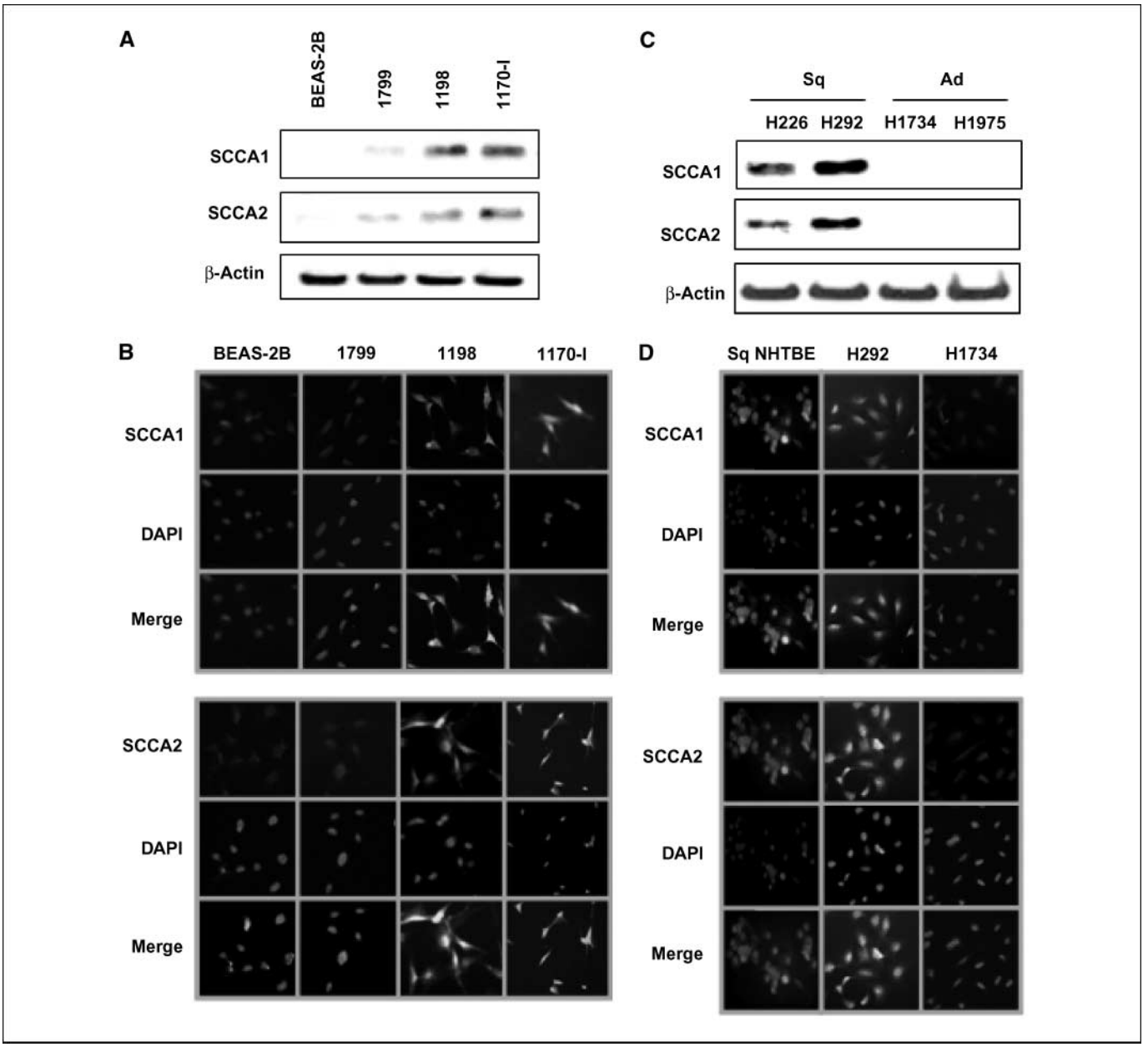

Figure 4. Expression of SCCA1 and SCCA2 in syngeneic *in vitro* lung carcinogenesis model cell lines and non–small-cell lung cancer cell lines. *A,* equal amounts of whole-cell lysates isolated from immortalized human bronchial epithelial cells (BEAS-2B and 1799), transformed cells (1198), and malignant cells (1170-I) were subjected to Western blot analysis with anti-SCCA1 and anti-SCCA2 antibodies. *B,* cells grown on chamber slides were subjected to immunofluorescence analysis with the same antibodies. Nuclei were stained with 4′6′-diamidino-2-phenylindole (DAPI) and the images were merged. *C,* equal amounts of whole-cell lysates from non–small-cell lung cancer cell lines (squamous cell carcinoma cell lines H226 and H292 and adenocarcinoma cell lines H1734 and H1975) were subjected to Western blot analysis with anti-SCCA1 and anti-SCCA2 antibodies. *D,* cells grown on chamber slides were subjected to immunofluorescence analysis with the same antibodies. Nuclei were stained with DAPI and the images were merged. Representative of three independent experiments.

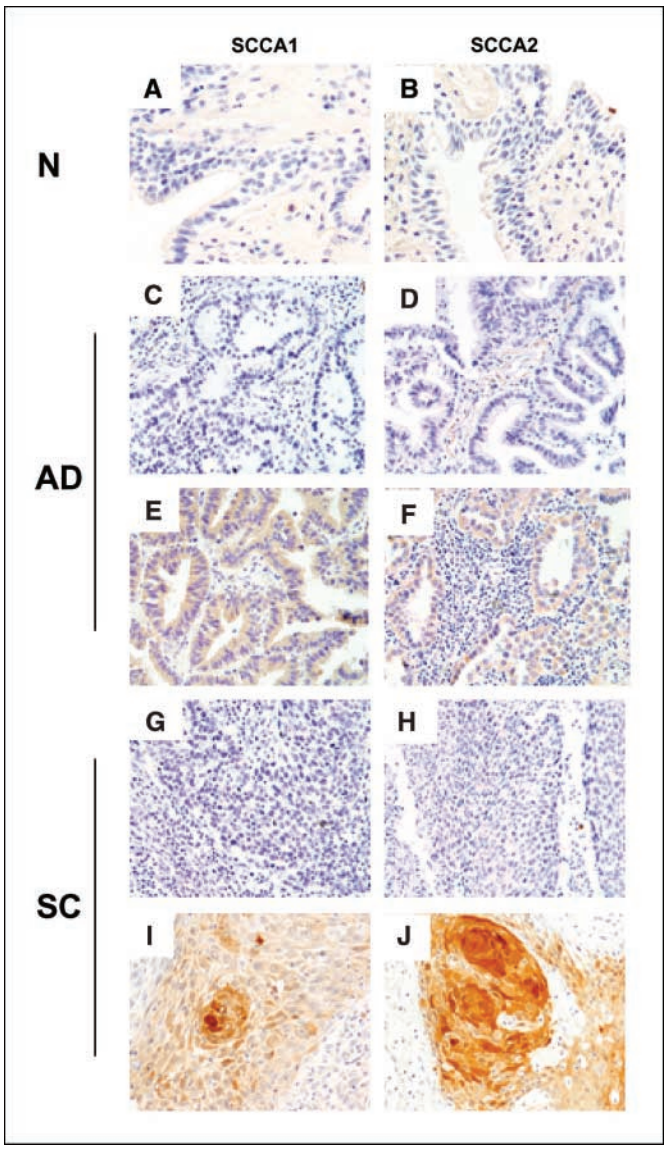

Figure 5. Microphotographs showing immunohistochemical expression of SCCA1 and SCCA2 in normal bronchial epithelium and non–small-cell lung cancer tumor tissue microarray tissue specimens. Normal bronchial epithelium (*A* and *B*), adenocarcinoma (*C–F*), and squamous cell carcinoma (*G–J*) tissue sections were subjected to immunohistochemical analysis with antihuman SCCA1 (*left*) and antihuman SCCA2 (*right*) antibodies. As negative control, we used the same specimens used as positive controls, replacing the primary antibody with PBS (*C, D, G*, and *H*). *N,* normal bronchial epithelium; *AD,* adenocarcinoma; *SC,* squamous cell carcinoma.

Whereas the role of Annexin II in lung cancer has not been clearly elucidated, autoantibodies against it have been detected in sera from lung cancer patients (37).

Only some of the proteins that we identified in this study as being secreted from squamous metaplastic bronchial epithelial cells were reported in previous studies of biomarkers for early lung cancer (38, 39). This discrepancy may be related to the sources of the samples and the model system used. Our organotypic air-liquid interface NHTBE cell culture system, which recapitulates *in vivo* mucociliary differentiated bronchial epithelium, has several advantages over the approaches used in other studies that have relied on cancer tissues or body fluids. NHTBE cells cultured by this method develop a squamous metaplastic phenotype when the cells are maintained in RA-deficient medium, whereas the same NHTBE cells develop a mucociliary phenotype in RA-sufficient medium. Thus, heterogeneity due to different cell types is excluded. In addition, the basal medium and ASF of confluent cell cultures are completely separated, meaning that the ASF is not contaminated with or diluted by the protein components in the culture medium in the basal compartment. Finally, the proteins in the ASF are secreted only from homogeneous bronchial epithelial cells and also enriched without dilution by culture medium or other types of cells, whereas the proteins in bronchial lavage fluid or cancer tissue specimens include many proteins derived from various cell types, such as stromal cells, inflammatory cells infiltrating cancer tissues, and circulating blood cells.

However, there is a potential limitation to our study. We used cultured squamous metaplastic NHTBE cells as a model for early abnormal changes indicative of carcinogenesis, but the classification of metaplastic lesions as precancerous has been controversial (40, 41). For example, metaplastic lesions are frequently associated with chronic inflammation, and they can be induced by mechanical trauma. In addition, the molecular changes in these lesions are similar to those in histologically normal epithelial cells in chronic smokers. It is also not clear whether the presence of such lesions indicates an increased risk for developing lung cancer. Nonetheless, the development of squamous cell lung cancer involves a series of morphologically distinct changes, often progressing from hyperplasia to metaplasia, dysplasia, and finally carcinoma *in situ* (42).

Although we verified only six of 22 proteins, the other proteins we identified in the ASF (e.g., cyclophilin, cystatin, heat shock proteins, and maspin) are also reportedly associated with carcinogenesis or lung cancer progression (25, 43–46). Additional studies are needed to elucidate the potential of this larger panel of proteins to be biomarkers for the early detection of premalignant squamous cell carcinoma lesions.

In conclusion, we identified 22 proteins that were overexpressed in the ASF from cultured squamous NHTBE cells but not in the ASF from mucous NHTBE cells. We further characterized the overexpression of six of these proteins—SCCA1, SCCA2, S100A8, S100A9, Annexin I, and Annexin II—in the ASF from squamous and mucous NHTBE cells. Our findings suggest that proteins secreted from bronchial epithelial cells are potential markers of early squamous metaplastic or precancerous changes. However, further retrospective and prospective studies are required to determine whether these proteins are aberrantly overrepresented in bronchial secretions from patients who are at high risk of developing lung cancer. Our approach involving analysis of ASF from primary bronchial epithelial cells using organotypic air-liquid interface culturing can be extended to other types of cells, such as adenocarcinoma or squamous cell carcinoma, to identify proteins secreted directly from cancer cells without interference from the culture medium.

Acknowledgments

Received 7/27/2006; revised 2/8/2007; accepted 4/19/2007.

Grant support: U.S. Department of Defense grant DAMD 17-02-1-0706 01 (J.S. Koo and W.K. Hong); National Heart, Lung, and Blood Institute grant R01-HL-077556 (J.S. Koo); and National Cancer Institute Core Grant CA-16672 (to The University of Texas M. D. Anderson Cancer Center).

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We thank Dr. Won Sup Lee for helpful discussion.

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