Research Article

Circulating Pro-Surfactant Protein B as a Risk Biomarker for Lung Cancer

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Abstract

Background: Our prior studies of lung cancer suggested that a novel biomarker (pro-surfactant protein B or pro-SFTPB) might serve as a predictive marker for this disease. We aimed to determine the potential use of pro-SFTPB for distinguishing lung cancer cases from matched controls as a risk marker.

Methods: Study subjects were drawn from the longitudinal Physicians’ Health Study (PHS). Cases (n = 188) included individuals who were cancer-free at study enrollment but developed lung cancer during follow-up. Controls (n = 337) were subjects who did not develop lung cancer. Cases and controls were matched on date of study enrollment, age at enrollment, and smoking status and amount. Baseline plasma samples drawn at enrollment were analyzed for pro-SFTPB using ELISA to detect differences in protein expression levels for cases and controls.

Results: Pro-SFTPB nondetectable status was significantly associated with lung cancer risk [OR = 5.88; 95% confidence interval (CI) 1.24–27.48]. Among subjects with detectable levels of the protein, increasing plasma concentration of pro-SFTPB was associated with higher lung cancer risk (OR = 1.41 per unit increase in log pro-SFTPB; 95% CI 1.08–1.84).

Conclusion: These results suggest a nonlinear, J-shaped association between plasma pro-SFTPB levels and lung cancer risk, with both nondetectable and higher levels of the marker being associated with lung cancer.

Impact: These results show promise of a risk marker that could contribute to predicting risk for lung cancer development and to narrowing the high-risk population for low-dose computed tomography screening. Cancer Epidemiol Biomarkers Prev; 1–6. ©2013 AACR.

Introduction

Lung cancer is the leading cause of cancer-related death among men, and the second leading cause among women worldwide (1). Despite efforts to improve lung cancer detection and treatment, the prognosis of patients with lung cancer remains poor, with overall 5-year survival rates in the United States of approximately 15%. However, when diagnosed at an early stage, 5-year survival rates for lung cancer approach 50% (2). Recently, the National Lung Screening Trial (NLST) reported that low-dose computed tomography (LDCT) screening reduced lung cancer mortality by 20% in adults who were at high risk of lung cancer (3). Although LDCT screening is a promising approach for early detection, high rates of false positives, cost, and risk from radiation exposure are important limiting factors (4). Determining individual lung cancer risk based on a biomarker profile and known risk factors such as smoking could allow more efficient lung cancer screening. Circulating biomarkers that have been associated with greater risk of developing lung cancer include increased levels of interleukin 8 and surfactant protein D (SP-D; refs. 5, 6). Increased levels of Krebs von Lugeren-6 (KL-6) have also been associated with greater risk of interstitial lung disease and subsequent development of lung cancer (5). We previously showed that levels of circulating mature SFTPB were increased among subjects with lung cancer both at the time of diagnosis and in a prediagnostic setting, compared with matched controls (7). We further examined SFTPB peptide sequences in mouse models of lung adenocarcinoma and in conditioned media from human lung adenocarcinoma cell lines. Mass spectrometry analysis indicated predominant release of an N-terminal pro-peptide containing form of SFTPB in both cell lines and mouse models; we therefore developed a sandwich ELISA assay that detects N-terminal SFTPB pro-peptide. Analysis of samples collected at the time of diagnosis indicated that pro-SFTPB yielded
better discrimination of cases versus controls than mature SFTPB. In this study, we intended to determine whether pro-SFTPB levels were associated with risk of lung cancer in a nested case–control study from the Physicians’ Health Cohort (PHS).

**Materials and Methods**

**Study populations**

The PHS cohort comprises two groups: PHS I and II. PHS I began in 1982 as a randomized trial of aspirin and beta-carotene for the primary prevention of heart disease and cancer among 22,071 male, Caucasian physicians initially aged 40 to 84 years. Men were excluded from the study if they had a history of cardiovascular disease (CVD), cancer (except non–melanoma skin cancer), and contraindications to aspirin use or were users of aspirin, or took platelet-active medications or vitamin A supplements. The aspirin and beta-carotene components of the PHS I trial have previously been reported (8, 9). The PHS II was a randomized trial that began in 1997 to evaluate the impact of beta-carotene, vitamin C, vitamin E, and a daily multivitamin on the prevention of cancer, CVD, age-related eye disease, and decline in cognitive function. The PHS II included 14,641 men, with 7,641 participants from the PHS I plus 7,000 new physicians, bringing the total number of PHS participants to 29,071. In PHS II, neither vitamin C nor vitamin E had an effect on CVD (10) or cancer (11). Follow-up of all PHS participants for major morbidity and mortality continues through annual questionnaires and endpoint follow-up. Written informed consent was obtained from each participant and the study was approved by the Human Research Committee at Brigham and Women’s Hospital (Boston, MA). At baseline for PHS I and II, participants were sent blood kits and asked to have their blood drawn, fractionated by centrifugation, and packed on dry ice for return within 24 hours by overnight courier. Prerandomization blood specimens were obtained from 14,916 (67.6%) of 22,071 PHS I participants and 11,133 (76.0%) of 14,641 PHS II participants. Upon receipt in the central laboratory, blood components were immediately aliquoted, labeled, frozen, and stored at −82°C for PHS I samples and in liquid nitrogen at −170°C for PHS II samples. Eligible cases for the pro-SFTPB assays were subjects free of baseline cancer, who developed lung cancer during follow-up and had plasma samples collected at baseline and available for laboratory analyses. Up to two controls who remained free of cancer were randomly selected and matched to cases based on date of recruitment into the cohort (±24 months), age at recruitment (±36 months), PHS I or II group, smoking status (never, former, current), and among current smokers, categories of cigarettes smoked per day (1–19, 20–39, 40 or more).

**Pro-SFTPB assay**

Samples were blinded and analyzed using anti-pro-SFTPB mouse monoclonal antibodies (#515 and #464) developed against the N-terminal pro-peptide of human SFTPB. Ninety-six–well polystyrene plates (Corning) were coated with 1 μg/mL of anti-pro-SFTPB mouse monoclonal antibody (#515) and blocked with 3% bovine serum albumin blocking buffer. Plasma samples with 1:100 dilution and various amounts of N-terminal pro-peptide of SFTPB as standards were added to the wells. Anti-pro-SFTPB mouse monoclonal antibody (#464) was biotinylated with EZ-Link Sulfo-NHS- LC-Biotin (Thermo Scientific) and used for incubation at 0.5 μg/mL. After washing, each well was incubated with Streptavidin-HRP followed by incubation of color reagents and adding stop solution (R&D Systems). The absorbance was measured at 450 nm with a SpectraMax M5 microplate reader (Molecular Devices).

**Statistical analyses**

Descriptive statistics on age, duration of follow-up, pro-SFTPB detection status (detectable or nondetectable), and smoking status as well as the number of cigarettes smoked per day were compared for cases and controls. Cancer histology (adenocarcinoma or nonadenocarcinoma) and metastatic status (metastatic or non-metastatic) was also assessed for lung cancer cases. Blood samples from 53 of the PHS subjects (10.1%) were found to have levels of pro-SFTPB below the detection limit of the ELISA. These samples were assigned a value of 1.56 ng/mL, which corresponds to one-half of the detection limit (12). Pro-SFTPB levels were natural log-transformed to produce a more normal distribution of values. Using data from the controls with detectable pro-SFTPB levels, multivariable generalized estimating equations (GEE; ref. 13) assessed associations between smoking status and age at enrollment and pro-SFTPB levels. GEE analyses account for non-independence of measures between pairs of controls matched to the same case. Conditional logistic regression analysis estimated the ORs (equivalent here to relative risk or RR) of lung cancer incidence in relation to baseline biomarker levels. The regression model included a variable for the natural log-transformed pro-SFTPB concentration levels and a dichotomous variable indicating samples that were above versus below the detection limit. Thus the regression model estimated an OR for the risk of lung cancer associated with having nondetectable pro-SFTPB levels and an OR associated with the per unit increase in log-transformed pro-SFTPB concentration. Analyses were conducted among all subjects, then by strata of baseline smoking status (never, former, current), age (at median age of 65 years), and median follow-up (94.6 months) among cases. Polychotomous analyses were also conducted comparing adenocarcinoma cases to their matched controls and nonadenocarcinoma cases to their matched controls. Similarly, polychotomous analyses were completed comparing cases with metastatic disease to their matched controls and cases with nonmetastatic disease to their matched controls.
Results

To assess the potential of pro-SFTPB as a risk marker for lung cancer, we examined pro-SFTPB levels in plasma samples drawn from the prospective PHS set. Table 1 presents the characteristics of the PHS cases and controls. The average follow-up period between the baseline collection of blood samples and diagnosis/matching was 117.6 months (range 4.8–304.8 months). A total of 188 cases and 337 matched controls were included from the PHS cohort, as 39 cases had one matched control and 149 cases had two matched controls (Table 1). Among controls, in multivariable GEE analyses, plasma levels of pro-SFTPB were higher among current smokers (+142%; \( P < 0.001 \)) and former smokers (+21%; \( P = 0.09 \)) than never smokers and increased with age (+18.1% per 10 year difference in age; \( P = 0.02 \); see Fig. 1).

Overall, conditional logistic regression analyses that accounted for matching factors found that pro-SFTPB nondetectable status was significantly associated with lung cancer risk [OR = 5.88; 95% confidence interval (CI), 1.24–27.84]. Increasing concentration of plasma pro-SFTPB was also associated with higher lung cancer risk.

### Table 1. Descriptive statistics for cases of lung cancer and controls in the PHS

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Cases (( N = 188 )) Mean (SD)</th>
<th>Controls (( N = 337 )) Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>64.5 (10.0)</td>
<td>64.7 (10.1)</td>
</tr>
<tr>
<td>Follow-up in months [median (25th, 75th percentile)]</td>
<td>94.6 (62.7, 148.9)</td>
<td>111.2 (74.0, 163.3)</td>
</tr>
<tr>
<td>Blood pro-SFTPB (ng/mL)</td>
<td>325.6 (313.9)</td>
<td>260.8 (289.5)</td>
</tr>
<tr>
<td>Detectable pro-SFTPB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>170 (90)</td>
<td>304 (90)</td>
</tr>
<tr>
<td>No</td>
<td>18 (10)</td>
<td>35 (10)</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smoker</td>
<td>41 (22)</td>
<td>80 (24)</td>
</tr>
<tr>
<td>Former smoker</td>
<td>96 (51)</td>
<td>185 (55)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>51 (27)</td>
<td>72 (21)</td>
</tr>
<tr>
<td>Cigarettes per day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–19</td>
<td>12 (23)</td>
<td>13 (18)</td>
</tr>
<tr>
<td>20–39</td>
<td>28 (55)</td>
<td>42 (58)</td>
</tr>
<tr>
<td>40 or more</td>
<td>11 (22)</td>
<td>17 (24)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>78 (42)</td>
<td>NA</td>
</tr>
<tr>
<td>Nonadenocarcinoma</td>
<td>110 (58)</td>
<td>NA</td>
</tr>
<tr>
<td>Metastatic disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>84 (45)</td>
<td>NA</td>
</tr>
<tr>
<td>No</td>
<td>104 (55)</td>
<td>NA</td>
</tr>
</tbody>
</table>

NOTE: Means and percentages for matching variables are not equal between the case and control series because some matched sets have two controls for each case and some have one control per case.

![Figure 1. Associations between pro-SFTPB and smoking status and age.](image-url)
We have validated pro-SFTPB as a promising new biomarker of lung cancer risk. In this study, initial plasma concentrations of pro-SFTPB were higher in never smokers (OR = 1.41 per unit difference in log pro-SFTPB; 95% CI, 1.08–1.84; Table 2). These results suggest a non-linear, J-shaped association between plasma pro-SFTPB levels and lung cancer risk, with both nondetectable and higher levels of the marker being associated with lung cancer.

Table 3 portrays the results stratified by smoking status, age at enrollment, and duration of follow-up. In terms of smoking status, OR for pro-SFTPB nondetectable status was higher in never smokers (OR = 9.25; 95% CI, 0.56–154.09), whereas OR for log-transformed pro-SFTPB concentration was higher in current smokers (OR = 2.08 per unit difference in log pro-SFTPB; 95% CI, 0.97–4.49). ORs for both pro-SFTPB nondetectable status and log-transformed pro-SFTPB concentration were numerically larger among those with a longer follow-up and who were of younger age at enrollment. However, tests for interaction in these analyses did not reach statistical significance.

Table 4 presents the results of the polychotomous analyses in which cases were subgrouped by histology and metastatic status and compared with their respective controls. While the association between pro-SFTPB nondetectable status and lung cancer was numerically larger for nonadenocarcinomas and for cases with metastatic disease, the CIs were wide and overlapped, providing insufficient evidence for etiologic heterogeneity (Table 3) due to the constraint of sample size limitations. Therefore, one cannot conclude that risk associated with pro-SFTPB varies by lung cancer risk factors or tumor characteristics.

Discussion

We have validated pro-SFTPB as a promising new biomarker of lung cancer risk.
Table 4. Polychotomous analyses of lung cancer risk

<table>
<thead>
<tr>
<th>Segregating cases by histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases with adeno histology</td>
</tr>
<tr>
<td>versus their controls</td>
</tr>
<tr>
<td>((N=78\text{ case-control sets}))</td>
</tr>
<tr>
<td>(\text{versus their controls})</td>
</tr>
<tr>
<td>Detectable</td>
</tr>
<tr>
<td>Nondetectable</td>
</tr>
<tr>
<td>Ln concentration</td>
</tr>
<tr>
<td>(3.30\ (0.29, 37.41) P = 0.34)</td>
</tr>
<tr>
<td>(1.40\ (0.89, 2.23) P = 0.15)</td>
</tr>
</tbody>
</table>

NOTE: ORs and 95% CI reported as OR (95% CI), OR for nondetectable status and Ln of concentration are mutually adjusted and further adjusted for matching variables. Ln concentration, log-transformed concentration.

Pro-SFTPB levels were associated with smoking status, age, and higher risk of lung cancer in men with up to 23.7 years follow-up. This work was preceded by studies in the samples collected at the time of diagnosis and two independent pre-diagnostic lung cancer cohorts using a newly developed ELISA against the N-terminal pro-peptide of SFTP B (submitted), based on mass spectrometric findings in lung adenocarcinoma mouse models and human lung adenocarcinoma cell lines (7).

Increased pro-SFTPB levels were associated with smoking status and age in control subjects, and the risk of lung cancer, concordant with the previous studies in the general population (14,15), as well as our previous studies in prediagnostic cohorts (submitted). It was surprising that pro-SFTPB nondetectable status was also significantly associated with lung cancer risk, predominantly in never smokers. The mechanism behind increased risk of lung cancer and decreased circulating pro-SFTPB levels needs to be elucidated. However, decreased concentrations of surfactant protein B in bronchoalveolar lavage fluid are associated with acute respiratory distress syndrome (16), lung injury induced by endotoxin (17), and late asthmatic response (18). Interestingly, lung sftp b gene expression levels were decreased in mice by exposure to nickel (19), one of the occupational carcinogens for lung cancer (20). These findings suggest that decreased plasma pro-SFTPB levels may reflect some pathologic conditions that are associated with an increased risk of lung cancer, especially in never smokers. Currently, no biomarker or prediction model has enough potential to identify a high risk group for lung cancer among never smokers. Thus, it is critical to develop a risk prediction model for never smoker lung cancer in larger cohorts, integrating circulating pro-SFTPB levels and known risk factors of never smoker lung cancer (21).

Interestingly, both pro-SFTPB nondetectable status and log-transformed pro-SFTPB concentration were more strongly associated with the risk of nonadenocarcinoma than adenocarcinoma, although CIs overlap with each other. Increased pro-SFTPB levels may also reflect pathologic conditions of the lung, as well as decreased pro-SFTPB levels as we mentioned above. While a transcription factor thyroid transcription factor 1 (TITF1)/NK2 homeobox 1 (NKX2-1), which regulates surfactant gene expression, decreases in sites of acute epithelial injury, TITF1 is markedly increased in regions of lung parenchyma undergoing regeneration and repair (22). Thus, circulating proSFTPB levels might be altered under different pathologic lung conditions caused by smoking, genetic, hormonal, and viral factors, which would result in the occurrence of different histologic subtypes of lung cancer (21).

On the basis of the results in the NLST study (3), the American Cancer Society (ACS) recently published lung cancer screening guidelines (23). In the guidelines, the ACS recommends that clinicians should initiate a discussion about lung cancer screening with subjects who meet the NLST criteria (e.g., aged 55–74 years, ≥30 pack-year smoking history, and <15 years since quitting). In this study, plasma pro-SFTPB levels are associated with an increased risk of lung cancer in middle-aged and older men. Thus pro-SFTPB might improve selection criteria for lung cancer screening or even might provide an opportunity to propose a personalized screening program, such as intensity of follow-up. Further investigations are needed to clarify the relationship of circulating pro-SFTPB levels and lung function and other lung disease, such as chronic obstructive pulmonary disease. In addition, further studies of pro-SFTPB in presymptomatic subjects or in screening subjects combined with
LDCT are of particular interest in improving lung cancer survival.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed by the authors.

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Taguchi, J.M. Gaziano, H.D. Sesso, F. Perera
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Taguchi, S.M. Hanash, A.G. Rundle, I.W. McKeage
Writing, review, and/or revision of the manuscript: A. Taguchi, S.M. Hanash, A.G. Rundle, I.W. McKeage, D.L. Tang, S. Darakjy, J.M. Gaziano, H.D. Sesso, F. Perera

References
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27. Study supervision: S.M. Hanash, S. Darakjy, H.D. Sesso

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