Pro-Surfactant Protein B As A Biomarker For Lung Cancer Prediction

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Appendix Supplemental Files for

Pro-Surfactant Protein B As A Biomarker For Lung Cancer Prediction

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Appendix Methods

Inclusion Criteria for the Pan-Canadian Study

- Women or men age 50 to 75 years
- Current or former smokers who have smoked cigarettes for 20 years or more. A former smoker is defined as one who has stopped smoking for one or more years.
- An estimated 3-year lung cancer risk of 2% based on the risk prediction model.
- ECOG performance status 0 or 1.
- Capable of providing, informed consent for screening procedures (low dose spiral CT, AFB, spirometry, blood biomarkers).

Exclusion Criteria for the Pan-Canadian Study

- Any medical condition, such as severe heart disease (e.g. unstable angina, chronic congestive heart failure), acute or chronic respiratory failure, bleeding disorder, that in the opinion of the investigator could jeopardize the subject's safety during participation in the study or unlikely to benefit from screening due to shortened life-expectancy from the co-morbidities.
- Diagnosis of cancer except for non-melanomatous skin cancer, localized prostate cancer,carcinoma in situ (CIS) of the cervix, or superficial bladder cancer with the last treatment 5 years or less prior to registration onto this study.
- Ex-smoker for 15 years or more
- On Anti-coagulant treatment such as warfarin or heparin
- Known reaction to Xyocaine, salbutamol, midazolam, and alfentanil
- Pregnancy
- Unwilling to have a spiral chest CT.
- Unwilling to provide written consent.
- Chest CT within 2 years.

Production of recombinant pro-SFTPB protein

Sequence of pulmonary surfactant-associated protein B (SFTPB) was taken from UniProtKB/ Swiss-Prot entry P07988; DNA coding N-terminal pro-peptide (25-200 aa) was synthesized and then optimized using GeneArt (Regensburg, Germany). The synthesized gene was ligated with the pDONR221 vector (Invitrogen, Darmstadt, Germany) and subcloned into pDESTVH8G (modified pTT5V5H8 plasmid from Biotechnology Research Institute, National Research Council Canada, Montreal). After sequence confirmation, plasmid DNA was prepared and transfected into HEK293-EBNA1 cells in suspension with linear PEI for production of recombinant protein according to the method described.[1] The resultant cell culture medium was clarified by

centrifugation (13,000 rpm, 1 hour, 4°C) and filtration (0.45 µ), and bound to Ni2+-NTA resin (25 ml of a 50% slurry, pre-equilibrated in MEB) in batch mode and packed into a chromatographic column connected to an AKTA purifier. The column was washed extensively with MEB to replace 6 M GuHCl with 8 M urea, and eluted using a step imidazole gradient in 8 M urea-MEB. Column fractions containing purified protein, based on SDS-PAGE analysis, were pooled and dialyzed against 20 mM Tris-HCl (pH 8.5) buffer containing 50 mM NaCl. The purified protein preps was analyzed by SDS-PAGE and western blotting, using penta-His mAb, in conjunction with anti-mouse IgG-HRPO conjugate and subsequently confirmed using mass spectrometry.

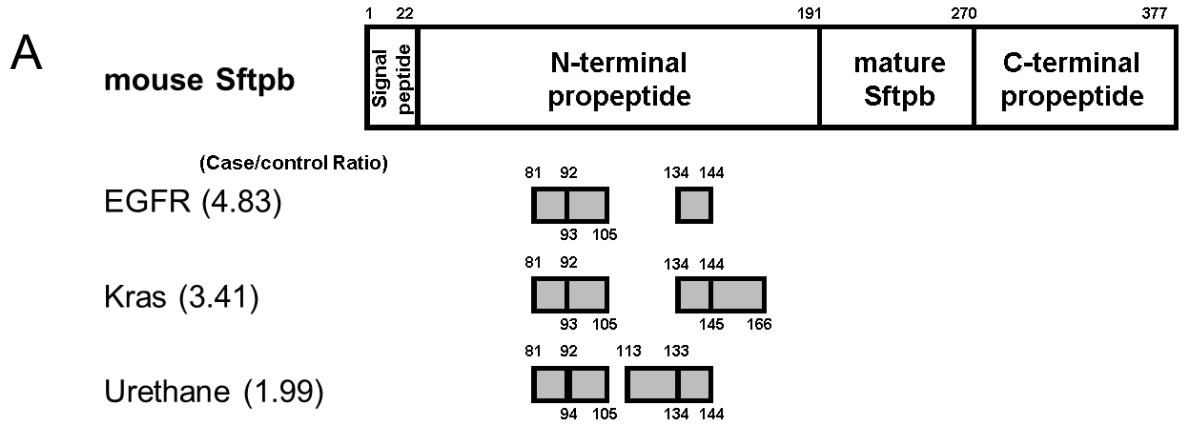
Development of Pro-SFTPB ELISA assay

SFTPB-specific monoclonal antibodies (mAb) and a sandwich ELISA were developed by the Antibody Research Unit of the BC Cancer Agency in Victoria, BC. Costar white high binding 96 well plate (Corning, Corning, NY) were coated with 100µl/well of 1.00 µg/ml purified mAb515 in 0.1M carbonate buffer (33.5 mM Na_2CO_3, 0.1 M $NaHCO_3$, pH 9.6) and incubated overnight at 4°C. Plasma samples with 1:100 dilution and various amounts of N-terminal pro-peptide of SFTPB as standards were added to the wells. Plates were blocked with 200µl/well of Superblock (Pierce, Rockford, IL) and incubated at room temperature (RT) for 2.5hours. Plates were washed with a protocol including six wash steps in TBS/0.1% Tween-20 (TBST) using a Skanwasher plate washer (Molecular Devices, Union City, CA). All samples and controls were assayed in duplicate. Plates were washed and incubated with 100µl per well of 0.5µg/ml biotinylated mAb477 in TBST for 2 hours at RT with shaking. Plates were washed and incubated with 100µl per well streptavidin -alkaline phosphatase conjugae (Applied Biosystems Inc, Foster City, CA) at 1:2500 in TBST for 1 hour on a shaker at RT. After washing, the plates were incubated with 100µl/well of 0.4 mM chemiluminescent CSPD® Substrate with Emerald-II™ Enhancer (Applied Biosystems) at RT for 20min in dark and read on an EnVision multilabel plate reader (PerkinElmer, Waltham, Massachusetts) and analyzed using Envision software 1.12.

Appendix Figures

Figure A1. Schema of mouse and human SFTPB and mass spectrometric identification of SFTPB peptides.

A. Schema of mouse surfactant protein B. Plasma samples from three mouse models of lung adenocarcinoma (EGFR; *TetO-EGFR*^{L858R}*/CCSP-rtTA*, Kras; *TetO-Kras4b*^{G12D}*/CCSP-rtTA*, and Urethane; urethane treated) were analyzed by mass spectrometry in our previous study.[2] Gray bars indicate peptides identified in plasma of each lung adenocarcinoma mouse model. All presented amino acid positions are based on P50405 in UniProtKB.



B. Schema of human SFTPB and identification of peptides by mass spectrometry in the conditioned media of human NSCLC cell lines. Potential SFTPB isoforms were identified in the conditioned media of H3255 and HCC4019. Bars indicate potential SFTPB isoforms and gray regions in the bars indicate identified peptides in the same protein fraction. Numbers indicate the sum of the number of mass spectra counts for each peptide. A black bar indicates the peptide used as an immunogen to develop monoclonal antibodies. All presented amino acid positions are based on P07988 in UniProtKB.

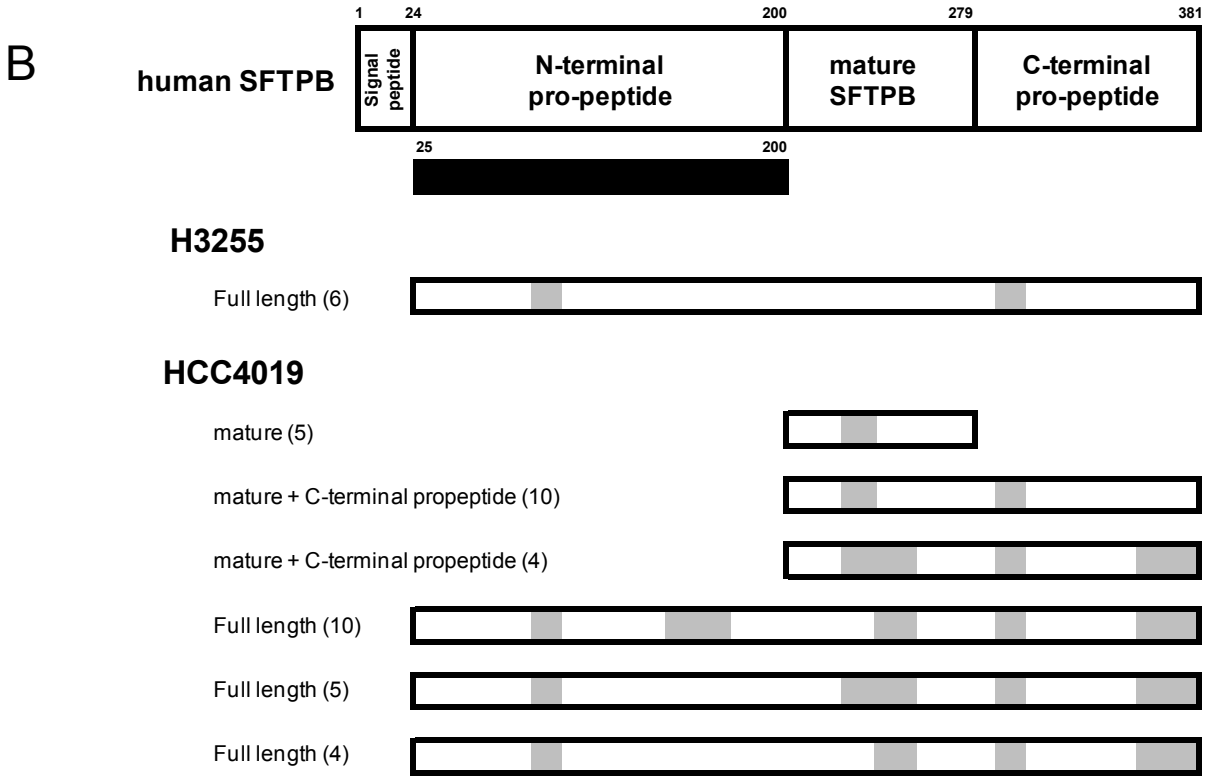
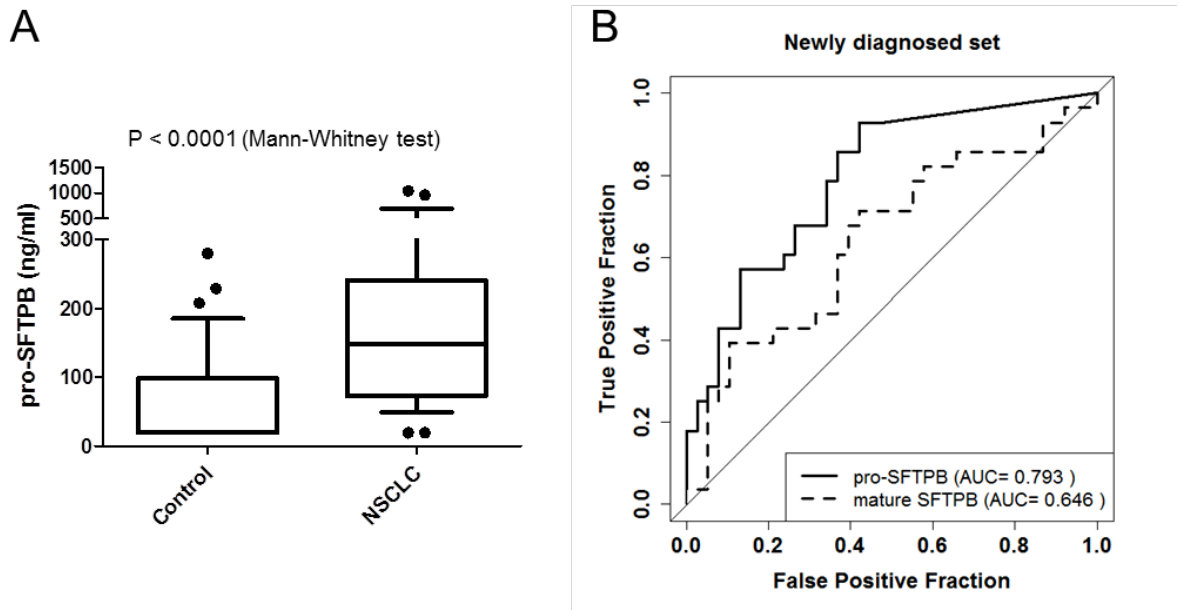


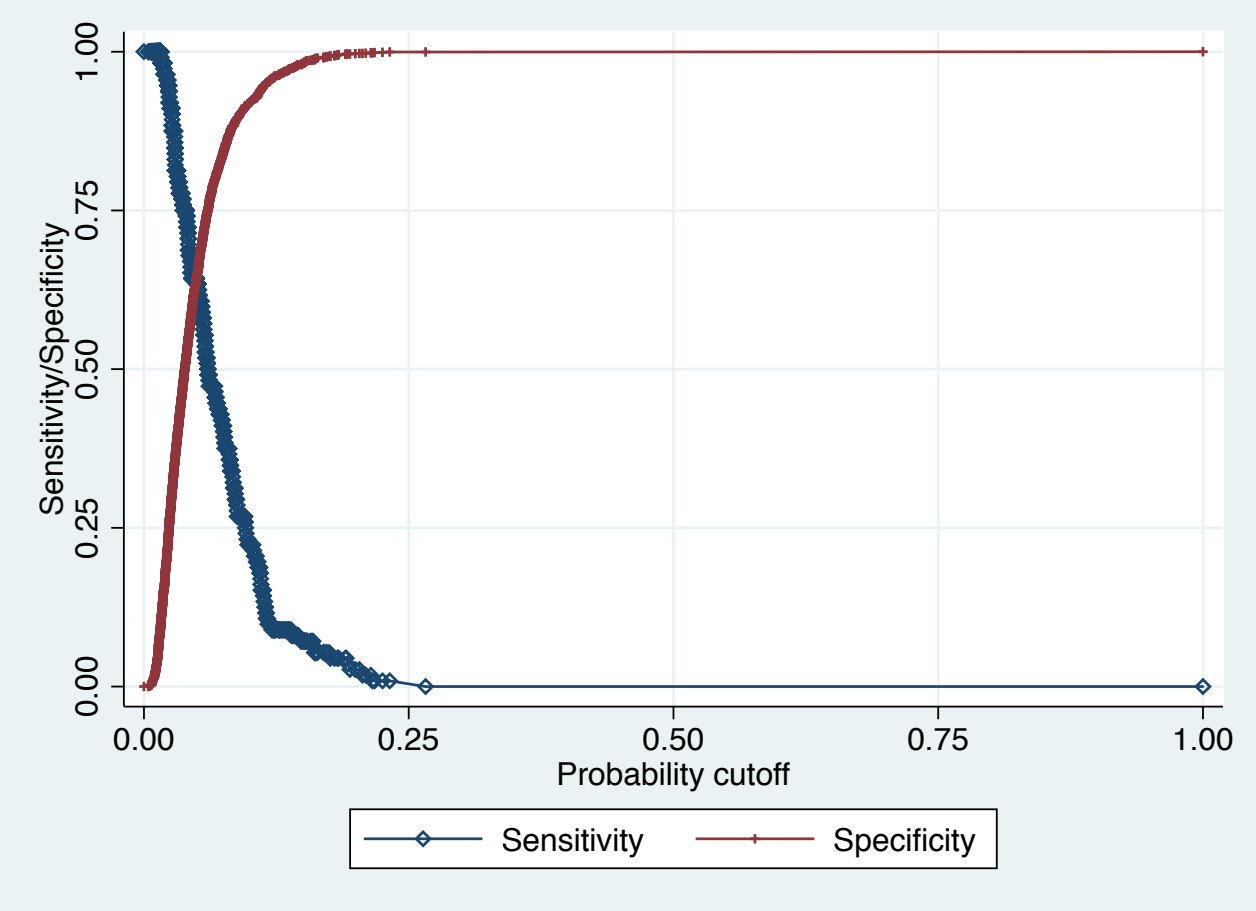
Figure A2. Plasma pro-SFTPB levels in newly diagnosed NSCLC set.



A. Levels of pro-SFTPB in plasmas from newly diagnosed NSCLC subjects and from non-cancer controls. Columns indicate 25th and 75th percentiles, horizontal lines in columns indicate median, bars indicate 10th and 90th percentiles, and black dots indicate data outside the 10th and 90th percentiles.

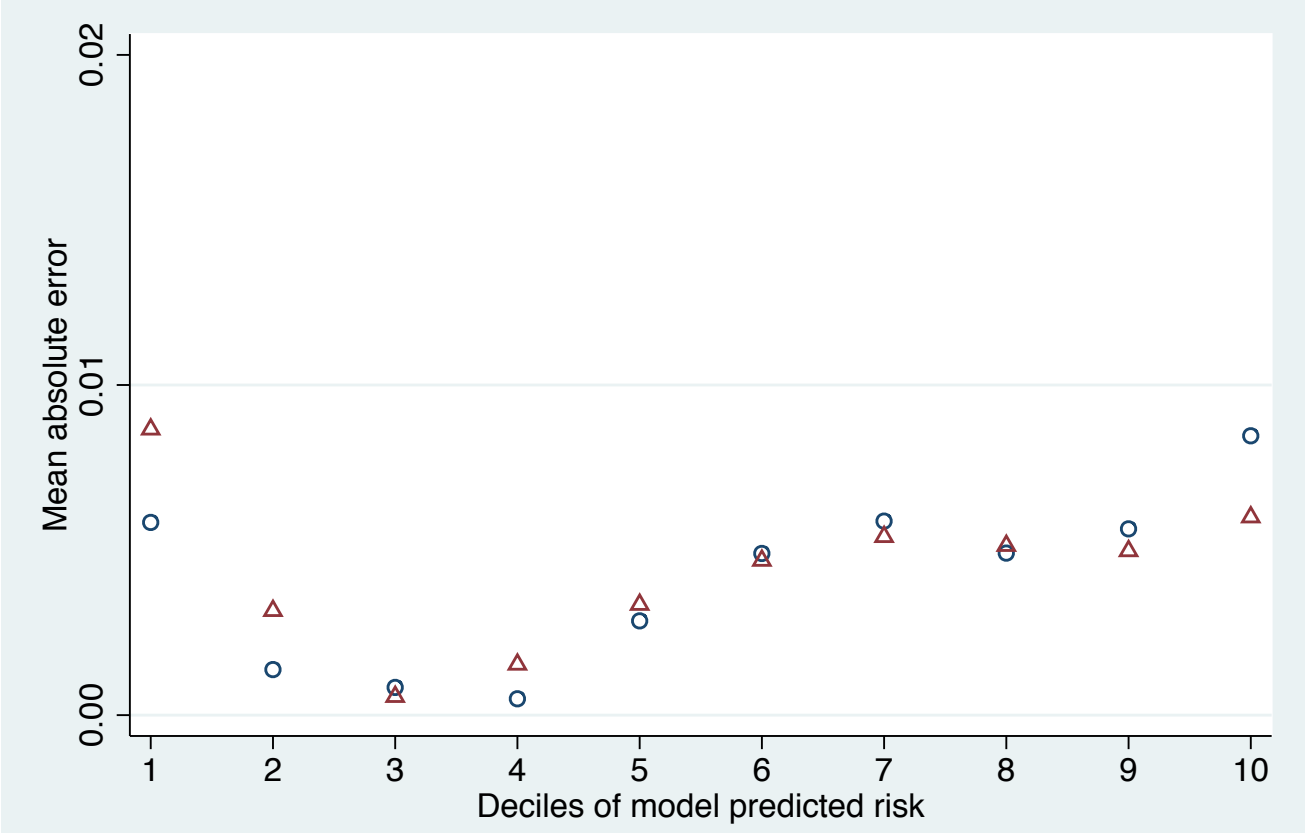
B. ROC analysis of pro-SFTPB and mature SFTPB. Mature SFTPB was assayed in the previous study.[2]. AUC, area under the curve.

Figure A3. Sensitivity and specificity for the unadjusted logistic regression model of log-transformed pro-SFTPB predicting lung cancer in the Pan-Canadian Early Detection of Lung Cancer Study.



Abbreviations: pro-SFTPB, pro-surfactant protein B

Figure A4. Mean absolute error (observed – predicted probabilities) for prediction models with (Table 3) and without log-transformed pro-SFTPB.

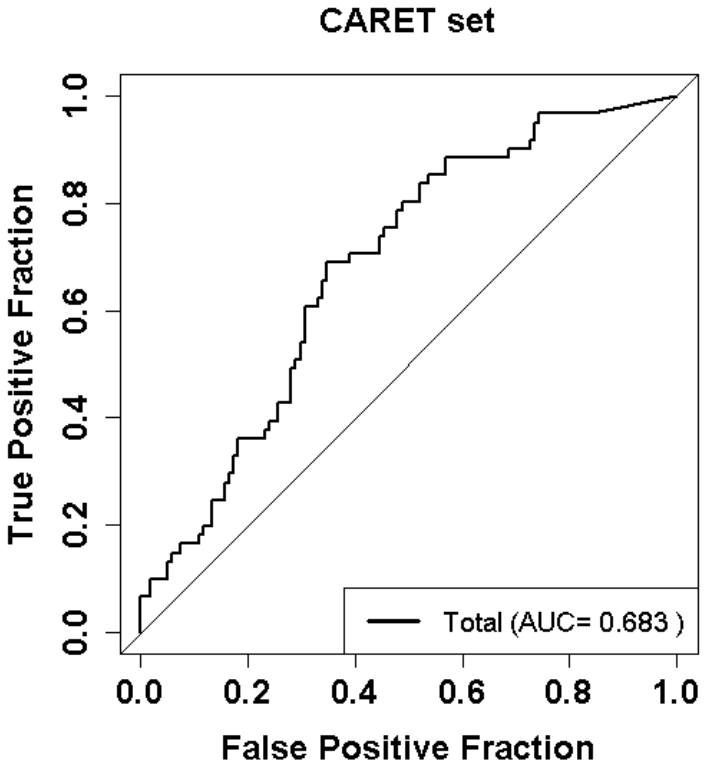


○ With Pro-SFTPB
△ Without Pro-SFTPB

Abbreviations: pro-SFTPB, pro-surfactant protein B.

Figure A5. ROC analysis of pro-SFTPB.

AUC, area under the curve.



Appendix Tables

Table A1. Clinical characteristics of subjects in the CARET set.

	Controls (%)	Cases (%)	P Value
Total	121 (100)	61 (100)	
Age (years)			
Mean	64.1	64.3	0.8254
SD	6.3	5.9	
Gender			
Female	32 (25.1)	16 (26.2)	1
Male	89 (74.9)	45 (73.8)	
Smoking (pack-year)			
Mean	46.9	58.7	< 0.0001
SD	16.9	22.0	
Asbestos exposure			
Yes	26 (21.5)	13 (21.3)	1
No	95 (78.5)	48 (78.7)	
BMI (kg/m^2)*			
Mean	26.7	26.8	0.8583
SD	5.2	5.7	
Stage			
I and II	-	11 (18.0)	
III and IV	-	40 (65.6)	
Unknown	-	10 (16.4)	
Histology			
Adenocarcinoma	-	26 (42.6)	
Squamous	-	17 (27.9)	
Other NSCLC	-	18 (29.5)	
Time span to diagnosis (months)			
Mean	-	6.2	
Range	-	0.9-12.4	

*BMI data for one control subject was not available.

Table A2. Demographics of newly diagnosed NSCLC set.

	Control (%)	NSCLC (%)
Total	38 (100)	28 (100)
Age (years)		
Mean	61.9	63.3
SD*	9.7	10.7
Gender		
Female	21 (55.3)	13 (46.4)
Male	17 (44.7)	15 (53.6)
Histology		
Adenocarcinoma	-	17 (60.7)
Squamous	-	11 (39.3)

* SD, standard deviation.