Development and Validation of a Plasma Biomarker Panel for Discerning Clinical Significance of Indeterminate Pulmonary Nodules

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Introduction: The recent findings of the National Lung Screening Trial showed 24.2% of individuals at *high risk* for lung cancer having one or more indeterminate nodules detected by low-dose computed tomography–based screening, 96.4% of which were eventually confirmed as false positives. These positive scans necessitate additional diagnostic procedures to establish a definitive diagnosis that adds cost and risk to the paradigm. A plasma test able to assign benign versus malignant pathology in high-risk patients would be an invaluable tool to complement low-dose computed tomography–based screening and promote its rapid implementation.

Methods: We evaluated 17 biomarkers, previously shown to have value in detecting lung cancer, against a discovery cohort, comprising benign ($n = 67$) cases and lung cancer ($n = 69$) cases. A Random Forest method based analysis was used to identify the optimal biomarker panel for assigning disease status, which was then validated against a cohort from the Mayo Clinic, comprising patients with benign ($n = 61$) or malignant ($n = 20$) indeterminate lung nodules.

Results: Our discovery efforts produced a seven-analyte plasma biomarker panel consisting of interleukin 6 (IL-6), IL-10, IL-1ra, sIL-2Rα, stromal cell-derived factor-1α+β, tumor necrosis factor α, and macrophage inflammatory protein 1 α. The sensitivity and specificity of our panel in our validation cohort is 95.0% and 23.3%, respectively. The validated negative predictive value of our panel was 93.8%.

Conclusion: We developed a seven-analyte plasma biomarker panel able to identify benign nodules, otherwise deemed indeterminate, with a high degree of accuracy. This panel may have clinical utility in risk-stratifying screen-detected lung nodules, decrease unnecessary follow-up imaging or invasive procedures, and potentially avoid unnecessary morbidity, mortality, and health care costs.

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The American Cancer Society estimates more than 226,160 new cases of lung cancer and approximately 160,340 lung cancer deaths in 2012, making it the most common cause of malignancy-related mortality in the United States.[1] Non–small-cell lung cancer (NSCLC) carries a dismal prognosis with a 5-year survival rate estimated to be less than 16%. Poor survival is partly because of the fact that 85% of lung cancer cases are diagnosed after metastatic disease progression, when curative treatment options are no longer available. Diagnosis of NSCLC before the development of extensive locoregional or distant metastases promises to improve 5-year survival rates by 60% to 80%.[2] Efforts have been made since the early 1970s to identify screening methods for early detection of NSCLC. Unfortunately, neither chest radiography nor sputum cytology proved to be effective.[3–6] Single-arm prospective uncontrolled studies on low-dose computed tomography (LDCT) of the chest screening yielded conflicting results.[1,7] Recently, the National Lung Screening Trial (NLST) demonstrated a 20% relative reduction in lung cancer mortality with LDCT screening of high-risk patients compared with annual chest radiograph with a median follow-up of 6.5 years.[8] High risk in patients was defined by this study to be individuals aged 55 to 74 years, having a smoking history of more than 30 pack-years, and having quit less than 15 years before randomization.

The approach to indeterminate screen-detected lung nodules in high-risk populations can represent significant challenges for the clinician. Data from the NLST showed that 24.2% of screening LDCT scans were positive, with 96.4% of these nodules determined as false positives.[8] An individual has an incidence of 33% of a false positive LDCT scan after two rounds of annual screening, and 7% of these individuals go through unnecessary invasive procedures to prove benign disease.[9] Avoidance of these unnecessary invasive procedures would benefit patient safety. The analysis for the

cost-effectiveness of LDCT screening has not been reported by the NLST at this time. However, estimates from other groups have concluded the added cost of LDCT screening for lung cancer to approximate $1.3 billion to $2.0 billion, annually.[9] In addition, it is estimated to cost $240,000 per one life saved by LDCT screening.[9] Decreasing the cost of LDCT screening by decreasing the need for subsequent invasive thoracic procedures or continued radiographic follow-up is paramount.

The potential of individual serum biomarkers to predict malignancy in indeterminate lung nodules has been researched and met with limited success. Published data on individual serum biomarkers, most notably cytokeratin 19 fragment 21.1, carcinoembryonic antigen, and tissue plasminogen activator in NSCLC show limited sensitivity and specificity, particularly in early-stage disease.[10,11] The objective of our study is to develop and validate a plasma biomarker panel with test characteristics compatible with its use as a companion test in high-risk patients with screening LDCT-detected indeterminate lung nodules.

PATIENTS AND METHODS

Patient Cohorts

Between 2004 and 2010, we enrolled 136 patients at Rush University Medical Center (RUMC) and divided them into the following cohorts: (a) pathologically diagnosed lymph-node negative lung cancer (n = 69) and (b) benign disease (n = 67). All stage classifications were according to the 7th edition criteria[12,13] and were pathologically confirmed. Patients with benign resected disease (n = 35) were diagnosed with granulomatous inflammation (n = 21), nonspecific inflammatory changes (n = 9), and lung infections (n = 5). The remaining patients in our benign cohort were part of an internal screening program with LDCT-determined benign disease (n = 32). Inclusion criteria for individuals enrolled in our screening program were ages of 50 years or more, or smoking history of more than 20 pack-years. All participants were followed with annual LDCT and remained cancer-free for a minimum 2-year follow-up. Demographic information for these patient groups are contained in Table 1. Our validation population (n = 81) consisted of the following cohorts received from our collaboration with the Mayo Clinic: (a) pathologically diagnosed lymph-node negative lung cancer (n = 20) and (b) benign disease (n = 61). Demographic information is contained in Table 2. All patient data was acquired with written informed consent and in absolute compliance with the Institutional Review Board at either Rush University Medical Center or the Mayo Clinic.

TABLE 1. Demographics for the Discovery Population from Rush University Medical Center

	Benign Screening	Benign Resected	Lung Cancer
Sex			
Male	14 (44)	19 (54)	29 (42)
Female	18 (56)	16 (46)	40 (58)
Age, yr			
Median	61	65	67
Range	51–82	20–80	48–83
Smoking history, pack-years			
Median	36	1	35
Nonsmoker	06–26	0–60	0–120
Nodule size, mm			
Median	4	14	18
Range	2–17	4–75	7–175
TNM			
$T_1N_0M_0$			51
$T_2N_0M_0$			13
$T_3N_0M_0$			02
$T_4N_0M_0$			00
Histologic diagnosis			
Adenocarcinoma			49 (72)
Squamous cell			10 (14)
Neuroendocrine	0		10 (14)
Benign diagnosis			
Granuloma		21 (60)	
Inflammation		09 (26)	
Infection		05 (14)	

Measurement of Plasma Biomarker Concentrations

Plasma was prepared using standard phlebotomy protocols from peripheral blood collected in yellow-top tubes either immediately before an anatomical resection, or in conjunction with a lung cancer screening trial. No specimen was subjected to more than two freeze and thaw cycles.[14–17] Seventeen candidate biomarkers were used for discovery based on previously shown success in differentiating NSCLC from benign disease.[15] Assays for CA-125, cytokeratin 19 fragment 21-1, osteopontin, stromal cell-derived factor -1(α+β) (Millipore, Billerica, MA) were measured as a four-plex assay kit; interleukin-1 receptor antagonist (IL-1ra), $sIL\text{-}2R_{\alpha}$, IL-6, IL-10, Eotaxin, monocyte chemotactic protein 1, macrophage inflammatory protein-1α, tumor necrosis factor alpha (TNF-α) (Millipore) as an eight-plex assay kit; and sE-Selectin, sICAM-1 (Millipore) as a two-plex assay kit. The remaining plasma biomarkers (Millipore) were run individually and consisted of soluble epidermal growth factor receptor (sEGFR) matrix metalloproteinase 2, and C-reactive protein. All biomarker concentrations were calculated with a five-parametric curve fit, using xPONENT v4.0.3 (Luminex Corp., Austin, TX) in a blinded fashion, using data collected on a Luminex FlexMAP 3D system. Table 3 lists the 17 biomarkers evaluated in this study.

Statistical Methods

Methods for candidate biomarker testing were consistent with those previously reported by our group.[14–17] Descriptive statistics were obtained along with receiver operator characteristics parameters (including *area under the curve* [AUC]) to assess the performance of the 17 individual candidate biomarkers, using SPSS 18.0 for Windows (SPSS Inc., Chicago, IL). The Mann–Whitney rank sum test was used to evaluate differences in biomarker concentrations. A threshold for