Validation of a Multiprotein Plasma Classifier to Identify Benign Lung Nodules

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Introduction: Indeterminate pulmonary nodules (IPNs) lack clinical or radiographic features of benign etiologies and often undergo invasive procedures unnecessarily, suggesting potential roles for diagnostic adjuncts using molecular biomarkers. The primary objective was to validate a multivariate classifier that identifies likely benign lung nodules by assaying plasma protein expression levels, yielding a range of probability estimates based on high negative predictive values (NPVs) for patients with 8 to 30 mm IPNs.

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Lung nodules deemed indeterminate lack the features suggestive of benign etiologies¹ and present clinicians with a diagnostic conundrum. Patient and practitioner balance a desire for the certainty of a diagnosis against the tolerance for the unknown, while assessing the risk and yield of an invasive procedure and the likelihood of malignancy. Achieving an early diagnosis of cancer remains a clinical imperative² to improve the dismal 16% 5-year survival of nonsmall-cell lung cancer (NSCLC),³ and also to assuage the immediate concern and anxiety engendered among both patients and physicians upon the identification of such spots.⁴⁵ The use of computed tomography (CT) technology has grown annually with the number of nodules identified by chest CT scans approaching millions per year, most of which are benign.⁶ Patients with a nodule less than 8 mm in size or having benign radiographic features may be
managed expectantly by serial CT scan surveillance. However, those with larger nodules may embark on a diagnostic odyssey, including positron emission tomography (PET), transthoracic needle aspiration, bronchoscopy biopsy, and/or surgery. Therefore, innovative strategies to identify benign lung nodules may mitigate the diagnostic burden of those considered indeterminate, by providing complementary data for decision-making, minimizing surgical resection of benign processes, and managing more lung nodules by radiographic surveillance.

Extensive efforts to classify pulmonary nodules using molecular biomarkers, such as DNA, RNA, and proteins, have yielded novel insights into lung cancer pathogenesis, with most having been focused largely on identifying malignant rather than benign lung nodules. Proteins are attractive as biomarkers because they are the dynamic, functional molecules acting in cell communications, with those of greatest interest often being in low abundance in plasma or serum. Therefore, advances in bioinformatics are at the core of recent progress in the development of diagnostic biomarker classifiers. The current enthusiasm for introducing biomarkers into practice has also heightened expectations for rigor in their validation as diagnostic tools for a targeted or intended use population.

Our prior work applied multiple reaction monitoring mass spectrometry for the discovery and initial validation of a classifier incorporating plasma protein expression levels to differentiate benign and malignant pulmonary nodules with 90% negative predictive value (NPV). In this study, we performed a validation of a multiprotein plasma classifier that prioritizes the diagnostic parameters of sensitivity and NPV to identify likely benign lesions in patients presenting with 8 to 30 mm lung nodules.

**MATERIALS AND METHODS**

**Validation**

The study conforms to Institute of Medicine guidelines (Supplemental Table 4, Supplemental Digital Content, http://links.lww.com/JTO/A773) and the Standards for Reporting of Diagnostic Accuracy (STARD) criteria for reporting studies of diagnostic accuracy (Supplemental Table 5, Supplemental Digital Content, http://links.lww.com/JTO/A773). Protein expression analyses and computational procedures were performed in a clinical laboratory adhering to the Clinical Laboratory Improvement Amendments of 1988.

**Study Design and Oversight**

The overall objective was to validate the performance of an 11-protein classifier in identifying lung nodules with likely benign (i.e., nonmalignant) etiologies (Supplemental Materials, Supplemental Digital Content, http://links.lww.com/JTO/A773), yielding a range of probability estimates for use as a diagnostic adjunct in clinical assessments. A retrospective, case-control study utilized multiple reaction monitoring mass spectrometry to analyze archival plasma samples from subjects enrolled in clinical studies approved by the Ethics Review Board or Institutional Review Boards at multiple institutions, using a blinded data analysis strategy. Management of clinical data complied with the Health Insurance Portability and Accountability Act of 1996.

**Study Inclusion and Exclusion Criteria**

The subject inclusion criteria were a minimum age of 40 years and any smoking history. The radiologic and pathologic criteria for lung nodule inclusion were a diameter between 8 to 30 mm, a histopathologic diagnosis of NSCLC or a benign process, or a clinical diagnosis of a benign etiology based on stability in size and appearance for 2 years after the baseline CT scan. The subject exclusion criteria included the lack of nodule size or histopathologic diagnosis, follow-up for less than 2 years, or a diagnosis of small-cell lung cancer. The subjects’ spirometry data and the global initiative for chronic obstructive lung disease criteria were used to define the presence and severity of chronic obstructive pulmonary disease (COPD). The cancer and benign subgroups were matched for age, gender, smoking history, and nodule size.

**Lung Nodule Protein Expression Classifier and Proteomic Analysis**

The classifier consists of five diagnostic and six normalization proteins (Table 1), which were fully defined, or “locked-down,” before sample analysis. The five diagnostic proteins were refined from the 13 proteins previously shown to discriminate benign and malignant lung nodules using stable isotope standards (Supplemental Materials, Supplemental Digital Content, http://links.lww.com/JTO/A773). The six normalization proteins were identified to reduce preanalytical and analytical variations in mass spectroscopic protein quantification. Plasma protein expression assays were performed as previously described using methods incorporating stable isotope standards (Supplemental Materials, Supplemental Digital Content, http://links.lww.com/JTO/A773).

**Data Analysis**

The first objective was to validate the overall performance of the classifier (Table 1) in identifying benign nodules, using the method of the partial area under the curve (pAUC). This objective required that the lower 95% confidence bound of the pAUC bounded by a sensitivity of 0.8 be higher than the corresponding pAUC of a nonperforming classifier. The second objective was to validate the performance of the classifier in identifying benign nodules at predefined reference values using binomial testing. This objective required that the lower 95% confidence bound of the fraction of benign samples among samples whose scores were less than or equal to the corresponding reference values be higher than the fraction of benign samples in the study. The fixed-sequence procedure was used to control the overall multitest error rate ($\alpha = 0.05$) in the study. Statistical analyses were performed using the Mann-Whitney and Fisher’s exact tests.

**RESULTS**

**Study Cohort**

Plasma specimens from 195 subjects with lung nodules at four institutions in different geographic regions of North America initially satisfied the study inclusion criteria, which included a minimum subject age of 40 years, but no stipulated smoking status or pack-year history. Thirty-two candidate
samples were excluded due to clinical criteria, and an additional 22 samples were excluded based on laboratory criteria. A total of 141 subjects satisfied all clinical and laboratory criteria, including 78 with cancer and 63 with benign diagnoses, demonstrated no overlap with those involved in the classifier's development, and were included in the data analysis (Fig. 1 and Table 2). There were no statistically significant differences in subject age, gender, and smoking history or lung nodule size between the cancer and benign groups.

Identification of Likely Benign Lung Nodules

The use of biomarkers for diagnostic purposes requires validation of the classifier's performance, including the definition of the clinically relevant performance range to impact decision-making. Based on the plasma measurements of five diagnostic and six normalization proteins, the protein expression classifier yields a score from 0 to 1, with each value associated with a sensitivity and a specificity, and lower scores toward the upper-right portion of the receiver operating characteristics (ROC) curve.

The NPV was determined for each reference value by incorporating the prevalence of NSCLC in the target population with 8 to 30 mm lung nodules, as high NPVs are useful in identifying the absence of malignancy, e.g., benign nodules. Due to the lack of a consensus estimate of cancer prevalence for pulmonary nodules, a population-based weight-adjusted NSCLC prevalence of 23.1% was used, incorporating data from a national, multicenter chart review study of patients with indeterminate pulmonary nodules (IPNs, n = 377; unpublished data) and the National Lung Screening Trial (Supplemental Materials, Supplemental Digital Content, http://links.lww.com/JTO/A773). The NPV also increases from 84% at a reference value of 0.47 to 90% at a reference value of 0.36 (Fig. 2B), corresponding to sensitivity and specificity of 92% and 20%, respectively (Supplemental Figure 4, Supplemental Digital Content, http://links.lww.com/JTO/A773). The reference value-specific NPVs may be associated with potential false-negative results, possibly resulting in malignant nodules being misclassified as likely benign, as shown in Supplemental Table 7 (Supplemental Digital Content, http://links.lww.com/JTO/A773). No single or combination of clinical parameters was identified that distinguished a given lung nodule sample in the study cohort as a potential "false-negative" result, using either 0.47 or 0.36 as a reference value.

Classifier Independence of NSCLC Predictors

The cancer and benign groups were matched for age, pack-years, and nodule size, similar to what was done in the discovery and initial validation of the classifier proteins. This enabled further assessment of the relationship of the classifier score to these clinical parameters. With respect to age, smoking history pack-years, and nodule size, none of these parameters correlated with the classifier scores.
COPD Status and Classifier Scores

Because COPD is a major risk factor for lung cancer, the potential impact of a concurrent COPD diagnosis on classifier scores was determined. Spirometry data were available for 54 (45%) of the 121 subjects with a history of tobacco use, including 33 with a cancer diagnosis and 21 with a benign diagnosis (Fig. 3A). There was no difference between the classifier scores in subjects with malignant lung nodules without or with COPD (Fig. 3B). Similarly, there was no difference observed for classifier scores in subjects with benign lung nodules in the absence or presence of COPD.

| TABLE 2. Clinical Characteristics of Subjects and Lung Nodules |
|---------------------------------------------------------------|
| Validation Study (n=141)                                      |
| Characteristics          | Cancer | Benign | P Value |
|--------------------------|--------|--------|---------|
| Subjects                 | 78     | 63     | 0.85*   |
| Age (year)*              | 65 (59–72) | 65 (56–73) | 0.85*   |
| Gender                   |        |        | 0.18*   |
| Male                     | 35     | 36     |         |
| Female                   | 43     | 27     |         |
| Smoking history          |        |        |         |
| Status                   |        |        | 0.88*   |
| Never                   | 11     | 9      |         |
| Former                  | 48     | 41     |         |
| Current                 | 19     | 13     |         |
| Pack-year**              | 40 (30–60) | 30 (21–63) | 0.33*   |
| Lung nodules             |        |        |         |
| Size (mm)*               | 14 (12–16) | 15 (10–17) | 0.67*   |
| Source                   |        |        | 0.01*   |
| IUICPQ                   | 3      | 11     |         |
| Mayo Clinic              | 18     | 16     |         |
| New York                 | 32     | 13     |         |
| Vanderbilt               | 25     | 23     |         |
| Histopathology           |        |        |         |
| Benign diagnosis         |        |        |         |
| Granuloma                | —      | 27     |         |
| No malignancy            | —      | 10     |         |
| Hamartoma                | —      | 8      |         |
| Inflammation             | —      | 3      |         |
| Pneumonia                | —      | 2      |         |
| Scar                     | —      | 2      |         |
| CT surveillance          | —      | 4      |         |
| Other                    | —      | 7      |         |
| Cancer diagnosis         |        |        |         |
| Adenocarcinoma           | 67     | —      |         |
| Squamous cell            | 7      | —      |         |
| Large cell               | 2      | —      |         |
| Mixed                    | 2      | —      |         |

(Clinical Criteria, n=32
- age, n=5
- nodule size, n=5
- other cancer, n=4
- post-surgery, n=16
duplicate, n=3)

(Laboratory Criteria, n=22
depletion QC, n=11
quality control, n=1
analytical range, n=10)

FIGURE 1. Flow chart of validation study plasma sample availability and exclusions based on clinical and laboratory criteria. The participating centers (n = 4) identified 195 plasma samples from 195 subjects initially satisfying the study inclusion criteria. After completion of clinical data monitoring, 32 candidate samples were excluded due to subject age less than 40 years; nodule size less than 8 mm or larger than 30 mm; nodule cancer pathology other than NSCLC; plasma sample collection after surgery; or provision of duplicate samples from the same subjects. An additional 22 samples were excluded after sample analysis based on laboratory criteria including depletion column quality control, mass spectrometry quality control, or a classifier result outside of the validated analytical range. A final total of 141 samples from 141 subjects satisfied the prespecified clinical and laboratory criteria, including 78 cancer and 63 benign samples, and were included in the data analysis.

Participating sites included the Institut Universitaire de Cardiologie et de Pneumologie de Québec (IUICPQ), the Mayo Clinic, New York University, and Vanderbilt University.

*Data shown are median values with quartile ranges indicated in parentheses.

Statistical analyses were performed using the Mann-Whitney test.

Statistical analyses were performed using Fisher’s exact test.

A never smoker is defined as an individual who has a lifetime history of smoking less than 100 cigarettes.

Pack-years are defined as the product of the total number of years of smoking and the average number of packs of cigarettes smoked daily among smokers only; one cancer sample and one benign sample were missing pack-year data.

Reported as no evidence of malignancy.

Reported as chronic inflammation or inflammatory changes.

Stable lung nodule on CT follow-up.

Other categories include bronchiolitis obliterans organizing pneumonia, chondroid lesion, emphysema, sclerosing hemangioma, interstitial pneumonia, intra-alveolar hemorrhage, and necrosis with hemosiderophages.

Incremental Diagnostic Value

To illustrate the potential added diagnostic value of the protein expression classifier to the clinical assessment of patients with lung nodules, a four-parameter clinical model
FIGURE 2. Protein expression classifier validation. The protein expression classifier yields a score between 0 and 1, with lower values associated with a higher probability of a benign etiology, based on the identification and quantification of specific plasma proteins (Supplemental Materials, Supplemental Digital Content, http://links.lww.com/JTO/A773). A, The statistical performance of the classifier was validated with a lower 95% confidence bound for the partial area under the curve (pAUC) of 0.026, which was greater than the targeted pAUC of 0.02; the performance of the classifier was validated at predefined reference values from 0.38 to 0.47. Shown are the receiver operating characteristic (ROC) curves based on the raw (circles) and fitted (grey line) data, along with the ROC curve associated with chance (grey dashed line). The maximal classifier score in the study for use as a reference value to assign a likely benign classification was 0.47, using the classifier’s partial area under the ROC curve (AUC) (shaded in grey). The associated sensitivity and specificity values (%) for the reference value of 0.47 are indicated (dashed lines). Similar data for reference values of 0.39 and 0.36, which correspond to NPVs of 87% and 90%, respectively, are indicated (dashed lines). B, A reference value is a specific score at or below which the classifier may assign a likely benign result to a given plasma specimen, based on the measured values of the classifier’s constituent proteins. Classifier scores between 0.18 and 0.47 may be used as a reference value. Shown are the positive and negative predictive values (PPV and NPV, respectively) for classifier reference values of 0.36, 0.39, and 0.47 using cancer prevalences of 15%, 20%, 23%, and 25%. The value of 0.47 is shown based on the prespecified criterion for validation of the classifier using the fitted sequence procedure; and the values of 0.36 and 0.39 are shown to illustrate a diagnostic performance of 90% NPV and an intermediate value, respectively.

DISCUSSION

Although most lung nodules are benign,5 the decision to pursue serial CT scan surveillance is often difficult for those characterized as indeterminate (Supplemental Table 3, Supplemental Digital Content, http://links.lww.com/JTO/A773).1 To address the need for diagnostic adjuncts to the clinical predictors of malignancy, our prior work identified a panel of plasma proteins that discriminates benign from malignant lung nodules based on high sensitivity and high NPV and involves molecular pathways implicated in lung cancer. This study demonstrates successful validation of a protein expression classifier using an independent plasma sample set, yielding a range of NPVs to estimate the probability that a patient’s lung nodule is due to a benign, i.e., nonmalignant, etiology.

By incorporating the expression values of 11 plasma proteins quantified by mass spectrometry, the classifier yields a score that may be translated into a probability that an IPN is benign. Such a probability may be useful to discriminate nodules that are benign from those that are indeterminate at the time of initial assessment.1 The classifier includes five diagnostic proteins that play roles in diverse signaling pathways implicated in homeostasis and lung cancer pathogenesis. Expression of fructose-1,6-bisphosphate aldolase, an enzyme regulating diverse cellular functions, is upregulated in adenocarcinoma tissues and correlates with the metastatic potential of squamous cell carcinoma.37,38 Collagen alpha-1 (XVIII) chain is an extracellular matrix protein constituent of vascular and epithelial basement membranes whose expression is strongly associated with poor outcomes in NSCLC.39 Downregulation of the expression of ferritin light chain identified in the early stages of squamous cell carcinoma suggests its potential as a biomarker for early diagnosis.40 Tissue expression of galectin-3-binding protein, which is implicated in angiogenesis and cell adhesion, motility and invasion, correlates with poor survival rates in lung cancer patients.41,42 Thrombospondin-1 is an endogenous angiogenesis inhibitor previously implicated as a circulating diagnostic biomarker discriminatory for lung cancer.43,44 The 141 validation study plasma samples analyzed, and the 247 patient samples in our prior study, were representative of the
classifier’s target population. Achieving the first-validation objective based on the partial AUC of the ROC curve enabled optimization of the classifier’s sensitivity, and achieving the second-validation objective defined the range of classifier scores used to derive the NPVs, based on the associated sensitivity and a weight-adjusted estimate of NSCLC prevalence for the target population. The importance of cancer prevalence on NPV was demonstrated by comparing the classifier NPVs based on the observed prevalence in the study cohort to the weight-adjusted prevalence estimated for the target population (Supplemental Fig. 4, Supplemental Digital Content, http://links.lww.com/JTO/A773). This study’s cancer prevalence of 55% is artifactually high given the case-control study design and is not representative of the cancer prevalence in the overall population of patients with IPNs. By contrast, use of the 23% cancer prevalence estimate for the target population allowed the calculation of NPVs (for the range of classifier reference values) that would more likely be observed in clinical practice.

The potential impact of lower or higher prevalence of NSCLC on the protein expression classifier is shown in Figure 2B, demonstrating that a lower cancer prevalence increases the classifier’s NPV at each reference value, whereas a higher cancer prevalence decreases its performance. By contrast, the corresponding low positive predictive values based on the associated specificities were anticipated due to the prioritization of identifying benign rather than malignant nodules. Thus, the proteomic analysis of a patient’s plasma based on the classifier may be used to derive a probability, ranging from an NPV of 84% that a given individual’s lung nodule is likely due to a benign etiology.

Several aspects of the data suggest that the protein expression classifier may be a useful diagnostic adjunct to the current paradigm for assessing indeterminate lung nodules. There was no correlation between classifier scores and the clinical parameters of age, smoking history pack-years, or nodule size for this cohort, which was also previously

| A | Cancer | Benign |
|---|---|---|
| **Gender** | n | Median | Interquartile Range | p-value | n | Median | Interquartile Range | p-value |
| Male | 35 | 0.554 | (0.474, 0.666) | 0.527* | 36 | 0.487 | (0.342, 0.618) | 0.868* |
| Female | 43 | 0.519 | (0.428, 0.672) | | 27 | 0.468 | (0.386, 0.540) | |
| **Smoking Status** | | | | | | | | |
| Never | | | | | | | | |
| Former | 48 | 0.542 | (0.471, 0.668) | 0.623** | 41 | 0.519 | (0.384, 0.643) | 0.064** |
| Current | 19 | 0.506 | (0.464, 0.638) | | 13 | 0.428 | (0.371, 0.540) | |
| **COPD** | | | | | | | | |
| Yes | 12 | 0.572 | (0.506, 0.658) | 0.410* | 6 | 0.554 | (0.386, 0.650) | 0.756* |
| No | 21 | 0.516 | (0.458, 0.665) | | 15 | 0.565 | (0.452, 0.676) | |
| **GOLD§** | | | | | | | | |
| No COPD | 21 | 0.516 | (0.458, 0.665) | 0.484** | 15 | 0.565 | (0.452, 0.676) | 0.484** |
| 1 | 4 | 0.540 | (0.435, 0.621) | | 2 | 0.689 | (0.524, 0.854) | |
| 2 | 7 | 0.571 | (0.519, 0.659) | | 4 | 0.485 | (0.374, 0.617) | |
| 3 | 1 | 0.714 | (0.714, 0.714) | | 0 | | | |
| 4 | 0 | | | | | | | |

**FIGURE 3.** Distribution of classifier scores between cancer and benign histopathology by tobacco use and chronic obstructive pulmonary disease (COPD). A. Shown are the correlations of classifier scores by subject gender, tobacco use history, and COPD subgrouped by the global initiative for chronic obstructive lung disease (GOLD) classification system for malignant and benign lung nodules. (Statistical analyses were performed using either the Mann-Whitney test* or the Kruskal-Wallis test.** Data provided only for those nonsmoking subjects satisfying the GOLD definition of COPD.§) B. Box plots of classifier scores for subjects without and with COPD in association with lung nodules, with either a diagnosis of NSCLC (cancer) or a benign etiology (benign), demonstrate no statistically significant impact of COPD on classifier scores.
The potential limitations of this study derive from specifics of the experimental design relating to the classifier performance priorities and molecular biomarkers. As illustrated by the classifier ROC curve and the associated AUC and pAUC, the developmental prioritization of sensitivity allowed us to develop a classifier with high NPVs, which allows for the accurate identification of a likely benign lung nodule. However, the emphasis on sensitivity and high NPV throughout the discovery and validation of the classifier's constituent proteins yielded a specificity ranging from 48% to 20% for NPVs ranging from 84% to 90%, respectively, demonstrating that the classifier may identify approximately half of the lung nodules that are benign. Therefore, a higher probability of an accurate assignment of a nodule as likely benign is accompanied by a lower proportion of nodules with that probability. Clinicians may need to consider this performance compromise for the classifier, not only in comparison with similar issues with other diagnostic modalities, but also with respect to their potential morbidities.

In summary, this work validates the performance of a proteomic classifier that identifies likely benign lung nodules.
with a high NPV, in patients with a minimum age of 40 who present with a lung nodule 8 to 30 mm in diameter. The data suggest that the classifier may serve as a noninvasive, objective, biology-based, and quantitative diagnostic adjunct to current modalities, such as PET scan, that may help decrease the number of lung nodules categorized as indeterminate and facilitate their diagnostic triage to surveillance imaging. Incorporation of the classifier result early during the initial clinical assessment may provide assurance about the likelihood of a benign etiology, with further reassurance provided by radiographic stability on subsequent chest CT scans. The noninvasive advantage of a blood-based molecular diagnostic test for lung nodules may preclude a diagnostic odyssey of successively more invasive procedures, reassure patients and physicians about surveillance decisions, decrease concern and anxiety about malignancy, and mitigate the morbidity and costs associated with biopsies and surgery that unfortunately often lead to benign diagnoses. 1

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