

Profiling Tumor-Associated Antibodies for Early Detection of Non-small Cell Lung Cancer

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Background: A blood test for non-small cell lung cancer (NSCLC) may be a valuable tool for use in a comprehensive lung cancer screening strategy. Here we report the potential of autoantibody profiling to detect early-stage and occult NSCLC.

Methods: T7-phage NSCLC cDNA libraries were screened with patient plasma to identify phage-expressed proteins recognized by tumor-associated antibodies. Two hundred twelve immunogenic phage-expressed proteins, identified from 4000 clones, were statistically ranked for their individual reactivity with 23 stage I cancer patient and 23 risk-matched control samples. All 46 samples were used as a training set to define a combination of markers that were best able to distinguish patient from control samples; this set of classifiers was then examined using leave-one-out cross-validation. Markers were then used to predict probability of disease in 102 samples from the Mayo Clinic CT Screening Trial (six prevalence cancer samples, 40 drawn 1 to 5 years before diagnosis, and 56 risk-matched controls).

Results: Measurements of the five most predictive antibody markers in 46 cases and controls were combined in a logistic regression model that yielded area under the receiver operating characteristics curve of 0.99; leave-one-out validation achieved 91.3% sensitivity and 91.3% specificity. In testing this marker set with samples from the Mayo Clinic Lung Screening Trial, we correctly predicted six of six prevalence cancers, 32 of 40 cancers from samples drawn 1 to 5 years before radiographic detection on incidence screening, and 49 of 56 risk-matched controls.

Conclusions: Antibody profiling may be a useful tool for early detection of NSCLC.

Key Words: Lung cancer, Early detection, Tumor-associated autoantibodies, Phage display, Biomarkers, Protein microarray.

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Lung cancer screening initiatives are based on the knowledge that only 25% of non-small cell lung cancer (NSCLC) is diagnosed at an early stage when curative surgical resection is still possible.¹ The use of solely age and smoking history as selection criteria in population-based computed tomography (CT) screening trials offers a low yield of cancer detection at significant cost.^{2–7} Moreover, the routine identification of indeterminate pulmonary nodules during CT screening often requires additional workup, magnifying the cost and adding potential morbidity from related interventional diagnostic procedures.

We have developed an assay for detecting NSCLC that could be a clinically valuable tool for early diagnosis, especially when used in concert with radiographic imaging and other screening modalities. In a previous report, we described the development of a diagnostic assay using tumor associated-antibodies as markers for NSCLC.⁸ Specifically, fluorescent microarray technology was adapted to the task of identifying phage-expressed NSCLC-associated proteins, used in turn to measure corresponding antibodies in blood. Combined measures of antibody reactivity to an arrayed panel of phage-expressed proteins were highly predictive of advanced stage NSCLC.⁸ Anticipating that antibody profiles may focus screening efforts by defining a population with high probability of disease, we determined the predictive accuracy of this approach for early-stage NSCLC and explored the potential to predict disease before a cancer could be detected on CT scan. We used 23 stage I NSCLC samples and 23 risk-matched controls to choose a set of markers, build a weighted statistical model, and determine the assay's predictive accuracy for early-stage NSCLC. Using an optimal combination of five markers determined above, we then assayed 102 samples from the Mayo Clinic Lung Screening Trial that included 56 noncancer samples, six prevalence cancers, and 40 cancer samples drawn 1 to 5 years before detection on incidence screening (Fig. 1).⁹

MATERIALS AND METHODS

Human Subjects

After informed consent, plasma samples were obtained from individuals with histology confirmed NSCLC at the University of Kentucky and Lexington Veterans Administration Medical Center. Noncancer controls were randomly chosen from 1520 subjects participating in the Mayo Clinic Lung Screening Trial. Briefly, individuals were eligible for

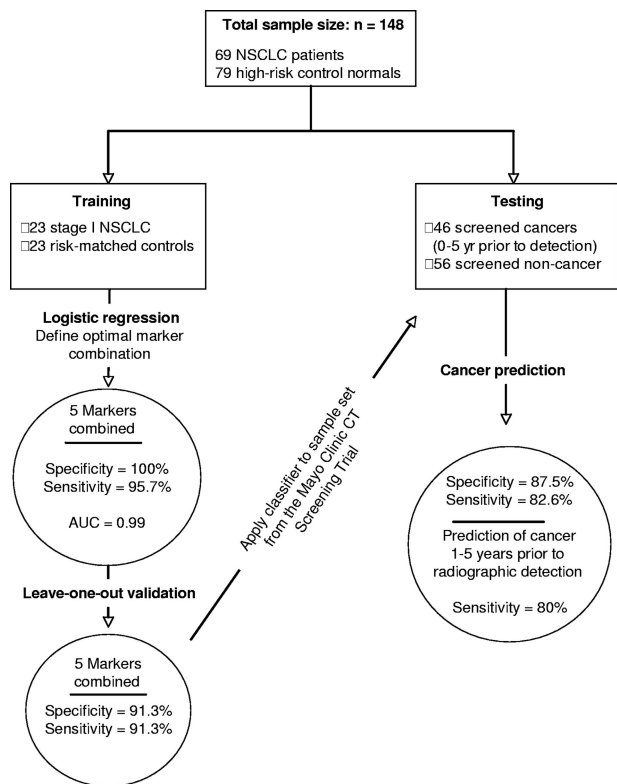


FIGURE 1. Experimental design. The flowchart delineates sample usage and designation to training and testing groups, as well as the statistical results of sequential evaluation.

the CT screening trial with a minimum 20 pack-year smoking history, age 50 to 75, and no other malignancy within 5 years of study entry.^{4,9} In addition to noncancer samples from the Mayo Lung Screening Trial, six stage I prevalence NSCLC samples and 40 prediagnosis samples were available for

analysis. Prediagnosis samples were drawn at study entry from subjects diagnosed with NSCLC incidence cancers on screening CT 1 to 5 years after sample donation. Serial blood samples were not drawn on these patients. Patient characteristics are shown in Table 1.

Phage Libraries, Biopanning, and High-Throughput Screening

One T7-phage NSCLC cDNA library was purchased (Novagen, Madison, WI) and a second was constructed from the adenocarcinoma cell line NCI-1650 using Novagen's OrientExpress cDNA Synthesis and Cloning systems.⁸ The libraries were biopanned with pooled plasma from 5 NSCLC patients (stage II–IV) and normal healthy donors to enrich the population of phage-expressed proteins recognized by tumor-associated antibodies as previously described.⁸ Phage lysates from the biopanned libraries were amplified and grown on LB-agar plates covered with 6% agarose for isolating individual phages. A colony-picking robot (Genetic QPix 2, Hampshire, UK) was used to pick 4000 individual colonies (2000 per library). The picked phages were reamplified in 96-well plates, grown until bacterial lysis, centrifuged, and 5 nL of phage containing supernatant from each well was then robotically spotted in duplicate onto FAST array slides (Schleicher and Schuell, Keene, NH) using an Affymetrix 417 Arrayer (Affymetrix, Santa Clara, CA).

Five individual NSCLC (stage III/IV) patient plasma samples not used in the biopan were used to identify immunogenic phage-expressed proteins from the screening slides of each library. Rabbit anti-T7 primary antibody (Jackson Immuno-Research, West Grove, PA) was used to detect T7 capsid proteins as a control for the amount of phage. Both plasma samples and anti-T7 antibodies were diluted 1:3000 with 1×TBS plus 0.1% Tween-20 (TBST) and incubated with the screening slides for 1 hour at room temperature. Slides were washed and then probed with Cy5-labeled anti-human and Cy3-labeled anti-rabbit secondary antibodies

TABLE 1. Characteristics of the Patient Samples^a

No. of cases	Age, y	Sex	No. of smokers	No. of controls ^a	Age, y	Sex	No. of smokers
Sample set A							
Ad = 7	51–79 (avg 65.1)	Male (17), female (6)	Active (20), former (3), never (0)	23	50–76 (avg 62.5)	Male (11), female (12)	Active (12), former (11), never (0)
Sq = 8							
NSCLC ^b = 8							
Total = 23							
Sample set B							
Prediagnosis ^c	51–80 (avg 63.6)	Male (20), female (26)	Active (24), former (22), never (0)	56	50–85 (avg 63.1)	Male (26), female (30)	Active (24), former (22), never (0)
0 y = 6							
1 y = 12							
2 y = 11							
3 y = 11							
4 y = 4							
5 y = 2							
Total = 46							

Sample set A: used for marker selection, statistical modeling, and accuracy prediction. All cancers were stage IA or IB non-small cell lung cancer. Sample set B: used for independent validation. ^a Of the controls, 34 of 79 had benign nodules (*n* = 11 sample set A, *n* = 22 sample set B). ^b Undifferentiated non-small cell lung cancer or inadequate tissue to characterize histology. ^c Adenocarcinoma, *n* = 23; bronchoalveolar cell carcinomas, *n* = 7; Squamous cell carcinoma, *n* = 13; non-small cell lung cancer, *n* = 5. Prevalence cancers (0 year) stage I = 6; incidence cancers (years 1–5) stage I = 25; stage II = 7; stage III = 6; stage IV = 2.

(Jackson Immuno-Research; 1:4000 each antibody in TBST) together for 1 hour at room temperature. Slides were washed again and then scanned using an Affymetrix 428 scanner. Images were analyzed using GenePix 5.0 software (Axon Instruments, Union City, CA). Phages with a Cy5/Cy3 signal ratio greater than 2 SDs from a linear regression were selected as candidates for use on a “diagnostic chip.”

Diagnostic Chip Design and Antibody Measurement

Two hundred twelve immunoreactive phages identified in the high-throughput screening above plus 120 “empty” T7 phages were combined, reamplified, and spotted in duplicate onto FAST slides as single diagnostic chips. Replicate chips were used to assay 23 stage I NSCLC and 23 risk-matched plasma samples using the protocol described for screening above. The median of Cy5 signal was normalized to the median of Cy3 signal (Cy5/Cy3 signal ratio) as the measurement of human antibody against a unique phage-expressed protein. Measurements were further normalized by subtracting background reactivity of plasma against empty T7 phage and dividing by the median of the T7 signal [(CY5/Cy3 of phage Cy5/Cy3 of T7)/Cy5/Cy3 of T7]. This methodology is quantitative, reproducible, and compensates for chip-to-chip variability, allowing comparison between samples.⁸

Statistical Analysis

The normalized Cy5/Cy3 ratio for each of the 212 phage-expressed proteins was independently analyzed for statistically significant differences between 23 patients and 23 control samples by *t* test using JMP statistical software (SAS, Inc., Cary, NC). The most predictive individual markers were checked for redundancy by polymerase chain reaction amplification using commercial T7-phage vector primers (Novagen) as previously described.⁸ Using a panel of nonredundant phage-expressed proteins, logistic regression analysis was performed to predict the probability that a sample was from an NSCLC patient. All 46 samples were used to build up classifiers that were able to distinguish patients from normal samples using individual or a combination of markers. Receiver operating characteristics (ROC) curves were generated to compare the predictive sensitivity, specificity, and the area under the curve (AUC). The classifiers were then examined using leave-one-out cross-validation within all the 46 samples.¹⁰ This set of classifiers was then used to predict the probability of disease in an independent set of 102 cases and risk-matched controls from Mayo Clinic Lung Screening Trial (Fig. 1). Relative effects of smoking and other nonmalignant lung disease were also assessed.

Sequence Identification of Phage-Expressed Proteins

When possible, phage identity was made based on significant nucleotide and translated nucleotide matches (bit score, *e* value, and percentage of sequence match) with a single gene in the GenBank database using BLASTN and BLASTX search engines.

RESULTS

High Throughput Screening and Marker Selection

The diagnostic chip, composed of 212 immunoreactive phage-expressed proteins plus controls, was derived from two T7-phage NSCLC cDNA libraries that were screened with NSCLC patient plasmas. Because anomalous proteins are generally accumulated and not lost during tumor progression, screening was performed with stage II to IV samples, rationally assuming that more advanced stage samples would provide a broad range and robust source of tumor-associated antibodies generated in the early life of the tumor.¹¹ The relative predictive value of each phage clone for early-stage lung cancer was suggested by statistical differences in the mean normalized signal from 23 early-stage patients compared to 23 high-risk controls (Fig. 2). Forty-nine of these 212 candidate markers showed statistically significant differences between cases and control ($p < 0.05$), 33 of which had $p < 0.01$; 13 markers offering the highest level of discrimination ($p = 1.9 \times 10^{-11}$ to $p = 0.00007$) were further evaluated. Sequence analysis revealed several duplicates and triplicates among those 13 phage-expressed “capture” proteins. Redundant clones were eliminated, and the antibody reactivity to the five most reactive unique phage-expressed proteins was analyzed individually as well as a combination to achieve the most optimal predictive accuracy (maximal discrimination among these 46 samples, Fig. 2).

Statistical Modeling and Assay Prediction Accuracy

Logistic regression was used to calculate the sensitivity and specificity of individual and combinations of multiple markers. The AUC ROC curve for each individual marker, achieved by assaying all the 46 samples to estimate predictive ability, ranged from 0.74 to 0.95; combinations of five markers indicated significant ability to distinguish early-stage patient samples from risk-matched controls (AUC = 0.99) (Fig. 2). The computed sensitivity and specificity using leave-one-out cross-validation were 91.3% and 91.3%, respectively (Table 2). We were unable to accurately distinguish histologic type of tumor in this sample set.

A sample cohort from the Mayo Clinic CT Screening trial that included 46 samples drawn 0 to 5 years before diagnosis (six prevalence cancers and 40 precancer samples) and 56 risk-matched samples from the screened population was then analyzed as an independent data set (Fig. 1). We were able to accurately classify 49 of 56 noncancer samples, six of six cancer samples drawn at the time of radiographic detection on a screening CT, nine of 12 samples drawn 1 year before diagnosis, eight of 11 drawn 2 years before, 10 of 11 drawn 3 years before, four of four drawn 4 years before diagnosis, and one of two drawn 5 years before diagnosis, corresponding to 87.5% specificity and 82.6% sensitivity (Table 3). Three of the eight precancer samples incorrectly classified were bronchoalveolar cell histology.

In the testing sets, we correctly classified six of six noncancer controls with a clinical diagnosis of chronic obstructive pulmonary disease, one individual with sarcoidosis,

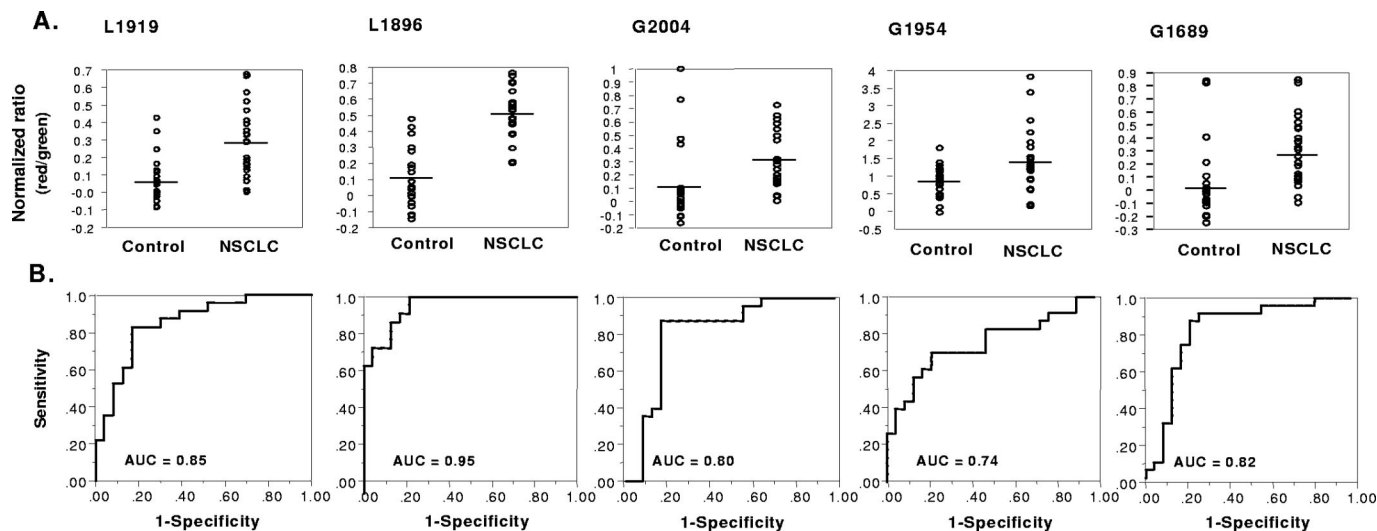


FIGURE 2. Statistical analysis of the five individual markers in the training group. A: Dot plots show the reactivity of 23 stage I non-small cell lung cancer (NSCLC) patients and 23 risk-matched control samples to phage-expressed proteins L1919, L1896, G2004, G1954, and G1689. The horizontal lines within the plots indicate the mean for each group. B: The corresponding receiver operating characteristics curves and the value of the area under the curve (AUC) are shown for each individual marker.

TABLE 2. Logistic Regression and Leave-One-Out Validation in Training Group

Phage clone	Training ^a			Validation ^b	
	AUC	Specificity, %	Sensitivity, %	Specificity, %	Sensitivity, %
L1919	0.85	82.6	78.3	82.6	60.9
L1896	0.95	87	87	87	87
G2004	0.80	82.6	65.2	82.6	65.2
G1954	0.74	82.6	87	73.9	69.6
G1689	0.82	82.6	65.2	82.6	65.2
5 Combined	0.99	100	95.7	91.3	91.3

AUC, area under the receiver operating characteristics curve. ^aTraining Set consisted of 23 high-risk normal and 23 NSCLC stage-one patient samples. ^bLeave-one-out validation: prediction of single sample based on 45 cases and controls.

and one individual with an interval diagnosis of breast cancer. In the latter independent testing set, two individuals with localized prostate cancer were also correctly classified as normal. One individual with a previous diagnosis of breast cancer (>5 years before) was classified as noncancer, but a second was classified as cancer. Thirty-four of 79 noncancer subjects had benign nodules detected on screening CT scans (Table 1). History of active versus former smoking did not appear to affect the predictive accuracy of the test. There was also no association of assay sensitivity with time to diagnosis.

Sequence Analysis of Phage-Expressed Proteins

Although the identity of the phage-expressed proteins is not critical for use in a diagnostic assay, the nucleotide sequences of the five predictive phage-expressed proteins were compared to the GenBank database to obtain possible identities and also compared to those that were highly pre-

dictive of advanced stage lung cancer.⁸ Nucleotide sequences obtained from the five clones used in the final predictive model showed great homology to paxillin, SEC15L2, BAC clone RP11-499F19, XRCC5, and MALAT1. The first three were identified in previous work as immunoreactive with plasma from patients with advanced-stage lung cancers.⁸ XRCC5 is a DNA repair gene overexpressed in some lung cancers.¹² Anomalous activity and aberrant expression of paxillin, a focal adhesion protein, has been associated with an aggressive metastatic phenotype in lung cancer and other malignancies.¹³⁻¹⁷ SEC15L2 is an intracellular trafficking protein that does not have any known malignant association, although its physiologic function suggests that it could have a role in the malignant phenotype.¹⁸ MALAT1 is a regulatory ncRNA known to be anomalously expressed in lung cancer.^{19,20} The BAC clone has no known associated protein function.

DISCUSSION

Although blood tests for lung cancer have multiple uses, specific application to early diagnosis is highly compelling for its potential impact on disease outcomes.^{21,22} In this report, we explore the potential of autoantibody profiling as a tool to complement radiographic screening for lung cancer. Serial CT screening is highly sensitive for lung cancer, but criticisms arise from the high cost and the low 64% specificity.^{2-7,23,24} Moreover, the routine identification of indeterminate pulmonary nodules during radiographic imaging frequently leads to expensive workup and potentially harmful intervention, including major surgery.²⁴ Currently, age and smoking history are the only two risk factors that have been used as selection criteria by the large screening studies, and so far an appropriate subpopulation has not been defined.^{2-7,23,24} A blood test that could detect radiographically apparent cancers (>0.5

TABLE 3. Predictive Accuracy Using Five Combined Biomarkers^a in Testing Set

	Noncancer high risk	Cancer (n = 46)					
		Years to cancer					
		0	1	2	3	4	5
No. classified correctly/total	49/56 ^b	6/6	9/12 ^c	8/11 ^c	10/11 ^c	4/4	1/2 ^c
Histology (no.)							
Adeno carcinoma		4	7	4	3	3	0
Squamous		2	3	4	3	1	0
NSCLC		0	1	3	0	0	1
Bronchoalveolar		0	1	0	5	0	1
Stage (no.)							
I		6	9	7	9	1	2
II		0	2	1	1	2	0
III		0	1	1	1	1	0
IV		0	0	2	0	0	0

^aMarkers used in this validation were the same five used in the training group. ^bIncluded 22 of 56 with benign pulmonary nodules, eight individuals with obstructive lung disease, two individuals with localized prostate cancer, and two individuals with remote diagnosis of breast cancer. ^cIncorrectly classified: 1 year, one stage I bronchoalveolar carcinoma; one stage I adenocarcinoma; one stage I squamous cell carcinoma; 2 years, two stage I squamous cell carcinomas; one stage I undifferentiated non-small cell lung cancer; 3 years, one stage I bronchoalveolar carcinoma; 5 years, one stage I bronchoalveolar carcinoma.

cm) and occult disease or premalignancy (below the limit of radiographic detection) would define individuals for whom screening is most warranted.

We have previously described methodology to efficiently screen T7-phage NSCLC cDNA libraries for multiple phage-expressed proteins recognized by antibodies in NSCLC patient plasma. We showed that these proteins could be displayed in an array fashion and used to measure multiple antibodies simultaneously, the combination of which has excellent ability to discriminate advanced stage NSCLC from control samples.⁸ With interest in improving early detection for lung cancer, we now investigate the ability of antibody profiling to distinguish stage I lung cancer and occult disease from risk-matched control samples.

We statistically identified a five-antibody marker combination from an arrayed panel of 212 immunoreactive phage-expressed proteins that achieved excellent discrimination between 23 stage I NSCLC and 23 risk-matched control samples. The 91% predictive accuracy obtained in a leave-one-out validation of these 46 samples is consistent with similarly high levels achieved with autoantibody profiling for prostate cancer recently described by Wang et al.²⁵ The potential of this approach to complement radiographic screening for lung cancer can be recognized in subsequent validation where combined measures of these five antibody markers correctly predicted 49 of 56 noncancer samples from the Mayo Clinic Lung Screening Trial, as well as six of six prevalence cancers and 32 of 40 incidence cancers from blood drawn 1 to 5 years before radiographic detection, corresponding to 87.5% specificity and 82.6% sensitivity.

Of the 40 pre-NSCLC diagnosis samples from the Mayo Screening trial, it is impossible to determine which were from individuals with established (occult) cancers and which were from individuals with dysplastic lesions yet to undergo malignant transformation. Based on doubling times, a lung cancer can be present 3 to 5 years before reaching the conventional size limits of radiographic detection (0.5

mm).^{22,23,26,27} We also know that premalignant lesions and associated molecular abnormalities can be present for decades before malignant transformation.^{22,26–30} It is therefore not unexpected that premalignant lesions can be distinguished from normal epithelium by histologic, genetic, and proteomic analysis,^{26–33} and thus not surprising that autoantibodies are generated to anomalous proteins. Importantly, three of these five early-stage markers also had high predictive value in advanced cancer,⁸ consistent with our knowledge that anomalous protein expression is often conserved during tumor progression and supporting the association of these markers with established NSCLC.^{8,11,26,27} Notably, none of the markers, alone or in combination, showed specificity for a unique histologic subtype of NSCLC, although three of the seven BACs in the case sample cohort were incorrectly classified as normal (three of eight precancer samples misclassified). With the exception of BAC, an indolent cancer that may not induce as robust an antibody response as other histologic types of NSCLC, we assume that our markers are variably expressed in malignant/premalignant lesions of the lung. The presence of benign pulmonary nodules and other nonmalignant lung disease (e.g., obstructive pulmonary disease) did not appear to alter marker specificity.

The specific role of these five proteins in tumor development or progression is unknown. Further investigation could reveal pathophysiologic importance, although it should be emphasized that an extended nucleotide homology of the phage-expressed protein to a known sequence may be entirely irrelevant to the epitope recognized by the autoantibody. Any of these five phage-expressed proteins (100–175 amino acids in length) may include short stretches of amino acids belonging to different antigenic parent proteins, even though the complete sequence does not bear significant homology to that protein, so-called mimotopes.²⁵ Although we have found that a statistical combination of these five proteins provides a very high level of discrimination, we have not exhaustively screened these tumor libraries for immunogenic proteins.

Further, we have used highly stringent statistical methods for selecting and combining antibody measures. Importantly, if additional predictive accuracy is required for clinical implementation, the methods described here allow us to incorporate additional markers in a single assay to accommodate disease heterogeneity and also allow further adjustment of sensitivity and specificity by statistically weighting various markers.

Although an ideal screening test would have high specificity and sensitivity that approaches 100%, perfect prediction is not expected.²¹ Of some potential relevance for use of this specific five-antibody marker set for screening is that the eight of 40 occult cancers were incorrectly classified as normal. Similar to screening for prostate cancer, breast, and colon cancer (PSA and digital rectal examinations, mammograms and clinical breast examinations, stool guaiac and colonoscopy), no single test is likely to be a practical and comprehensive *independent* screening modality for lung cancer screening. In context, the initial report of the Mayo Clinic Lung Screening Trial by Swensen et al.³⁴ described 35 cases of NSCLC diagnosed by CT alone, one NSCLC detected by sputum cytologic examination alone, and one stage IV NSCLC clinically detected between annual screening scans, corresponding to a 94.5% sensitivity of CT scanning alone. Further, retrospective review after the first annual incidence scan revealed that small pulmonary nodules were missed on 26% of the prevalence scans, consistent with significant false-negative rates reported in other CT screening trials.^{34,35} The diameter of the retrospectively identified nodules was <4 mm in 231 participants (62% of those 375 participants), 4 to 7 mm in 137 (37%), and 8 to 20 mm in six (2%). As such, the 82.6% sensitivity of autoantibody profiling for NSCLC compares quite favorably to that of CT screening alone, by comparison may perform especially well for small tumors, and represents an unparalleled advance in the detection of occult disease. Moreover, the 87.5% specificity of this assay well exceeds that of CT scanning, which becomes increasingly more important as the percentage of benign pulmonary nodules increases in the at-risk population, rising to levels of 69% of participants in the Mayo Clinic Screening Trial.^{9,22} The data do, however, suggest these markers may not be very sensitive for the BAC histologic subtype.

These data suggest that antibody profiling could be a powerful tool for early detection when incorporated into a comprehensive screening strategy.^{26,35,36} Admittedly, implementation of autoantibody profiling as a screening tool will require rigorous validation. Problematically, the number of clinical blood samples from individuals with occult disease or premalignancy available from investigational screening trials is exceedingly small. Further validation of these results may best be accomplished with analysis of archived samples from other completed or ongoing CT screening trials or with samples collected in studies investigating autofluorescence bronchoscopy. It will also be important to more fully evaluate the specificity of antibody signatures in patients with a variety of benign lung disease and autoimmune disease and to define the specificity for lung versus other cancers.³⁶ It is also rational to test these markers with small-cell lung cancer to

determine the predictive accuracy for this lung cancer variant. Although not specifically explored in this study, application to the closely related clinical problem of distinguishing benign from malignant nodules identified on CT screening is also logical. The ultimate validation of antibody profiling will be accomplished in a prospective lung cancer screening trial that will then be followed by careful population-based analysis to define an optimal screening approach for lung cancer.³⁶ Although a blood test for lung cancer could significantly alter the current screening paradigm, the impact on disease-specific mortality will need to be proven.

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