Imaging, Diagnosis, Prognosis

Clinical Cancer Research

Development of a Multiplexed Tumor-Associated Autoantibody-Based Blood Test for the Detection of Non–Small Cell Lung Cancer

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Abstract

Purpose: Non–small cell lung cancer (NSCLC) has an overall 5-year survival of <15%; however, the 5-year survival for stage I disease is over 50%. Unfortunately, 75% of NSCLC is diagnosed at an advanced stage not amenable to surgery. A convenient serum assay capable of unambiguously identifying patients with NSCLC may provide an ideal diagnostic measure to complement computed tomography–based screening protocols.

Experimental Design: Standard immunoproteomic method was used to assess differences in circulating autoantibodies among lung adenocarcinoma patients relative to cancer-free controls. Candidate autoantibodies identified by these discovery phase studies were translated into Luminex-based "direct-capture" immunobead assays along with 10 autoantigens with previously reported diagnostic value. These assays were then used to evaluate a second patient cohort composed of four discrete populations, including: 117 NSCLC (81 $T_{1\text{-}2}N_0M_0$ and 36 $T_{1\text{-}2}N_{1\text{-}2}M_0$), 30 chronic obstructive pulmonary disorder (COPD)/asthma, 13 nonmalignant lung nodule, and 31 "normal" controls. Multivariate statistical methods were then used to identify the optimal combination of biomarkers for classifying patient disease status and develop a convenient algorithm for this purpose.

Results: Our immunoproteomic-based biomarker discovery efforts yielded 16 autoantibodies differentially expressed in NSCLC versus control serum. Thirteen of the 25 analytes tested showed statistical significance (Mann-Whitney $P < 0.05$ and a receiver operator characteristic "area under the curve" over 0.65) when evaluated against a second patient cohort. Multivariate statistical analyses identified a six-biomarker panel with only a 7% misclassification rate.

Conclusions: We developed a six-autoantibody algorithm for detecting cases of NSCLC among several high-risk populations. Population-based validation studies are now required to assign the true value of this tool for identifying early-stage NSCLC. *Clin Cancer Res; 16(13); 3452–62. ©2010 AACR.*

Lung cancer remains the leading cause of cancer death nationwide, with ~160,000 deaths predicted in the United States in 2009 (1). Non–small cell lung cancer (NSCLC) accounts for 80% of all lung cancers and has a poor overall 5-year survival rate of ~15% (2–4). This low survival rate is largely attributed to the fact that >75% of patients present with local or distant metastasis at diagnosis, which are associated with a 21% and 3% 5-year survival, respectively (2, 3, 5). In contrast to these dismal figures, stage I disease is associated with a 50% to 67% 5-year survival but accounts for only 15% of patients (2). This suggests that if patients could be diagnosed at an earlier stage, when a complete surgical resection offers the greatest potential for a cure, the mortality associated with this disease could be reduced.

To that end, considerable efforts have been undertaken to produce an effective screening method for early NSCLC. In the 1950s, trials of chest X-rays alone or in combination with sputum analysis were completed. These studies showed no mortality advantage with the addition of the screen, and patients often became symptomatic and were diagnosed in the interval between screening exams (4, 6–8). In the early 1990s, projects such as the Early Lung Cancer Action Project began trials using helical computed tomography (CT) imaging as a new screening model. Besides showing increased sensitivity of chest CT over chest X-ray for detecting lung nodules, they showed an increase in the

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Translational Relevance

The best prospect for reducing lung cancer mortality is early detection, when surgery may be curative. Due to the typical asymptomatic nature of the early disease and a lack of effective screening methods for early detection, roughly 75% of non–small cell lung carcinoma (NSCLC) patients present with unresectable, incurable tumors. Recent advancements in low-dose spiral computed tomography (CT) technology may improve early detection, but its ability to reduce mortality from NSCLC has yet to be established. The high rate of false-positives triggering unnecessary surgeries or the need for serial measurements to improve specificity suggest that development of one or more economical serum tests to augment spiral CT would be very worthwhile. This study aims to develop a serum test that could be used as an initial diagnostic screen to assess NSCLC risk and potentially indicate who should have spiral CT done.

detection of early disease over previous chest X-ray screening trials and historical numbers without screening (9). These studies, however, were not prospective randomized controlled trials and did not confirm any survival benefit. Although these CT screening tests have the potential to diagnose early lung cancer in high-risk patients, they may require serial CT imaging and, therefore, repeated radiation to the chest, which has an associated risk of carcinogenesis (10) or may lead to biopsy or surgery of asymptomatic, benign disease. Sone et al. (11) showed that up to 30% of resected nodules found during CT screening for lung cancer were benign, as determined by histopathology.

In an effort to develop a clinically viable serum test to discriminate cases of NSCLC among those at "high risk" for NSCLC, we adopted a two-part strategy to both discover and validate novel serum biomarkers for this purpose. The first phase consisted of an immunoproteomic discovery strategy that compared the immunoreactivity of the control serum with serum from cases of lung adenocarcinoma using whole-cell lysates from an adenocarcinoma cell line as a source of candidate autoantigens. This was accomplished using two-dimensional Western blots followed by autoantigen identification through tandem mass spectrometry (MS/MS). The identity of these targets was then confirmed in a second two-dimensional Western blot using commercial antibodies for the identified autoantigens. After completion of this discovery effort, the second phase of experimentation was designed to evaluate the clinical relevance of these biomarkers across a large cohort of patients (consisting of cases of either pathologically confirmed NSCLC or cases grouped as high risk for NSCLC). For this objective, we selected the Luminex immunobead platform for this task because of its current presence in the clinical laboratory for transplant human leukocyte antigen (HLA) isotyping and the fact that multiple analytes can be evaluated concurrently with a very small quantity of serum. Multivariate statistical methods were then used to identify the most efficacious combination of biomarkers for distinguishing patient disease status (i.e., NSCLC versus non-NSCLC), which were then fashioned into a convenient classification algorithm. Our goal was to identify a clinically useful multianalyte autoantibody panel capable of discerning NSCLC from "non-NSCLC" populations.

Materials and Methods

Patient populations

Proteomics discovery efforts. Two patient groups were enrolled in the "discovery" portion of this study, as defined in Table 1. The first group consisted of 10 patients with lung adenocarcinoma ($T_{1\text{-}2}N_0M_0$) that received complete anatomic resections with curative intent at the Rush University Medical Center, Chicago, IL. The second group that served as a control for the study was comprised of patients (n = 10) with nonneoplastic pulmonary disorders that were resected at Rush University Medical Center under suspicion of having NSCLC.

Algorithm development and validation cohort. Serum specimens from four patient populations (n = 196) were included in the Luminex validation study. See Table 1 for the basic patient demographics of these groups. The NSCLC group consists of 81 patients with early-stage ($T_{1\text{-}3}N_0M_0$) disease, 32 with locally advanced disease ($T_{1\text{-}3}N_{1\text{-}2}M_0$), and 4 with distant metastases. Criteria for study inclusion in the NSCLC cohort were broad (consisted of having a surgical resection and/or pathologic confirmation of NSCLC) and were not limited to any demographic or clinical factor. Patients treated with neoadjuvant chemoradiation or radiation therapy were excluded from the study. All patients in the NSCLC cohort underwent definitive resection of their primary tumor with systematic lymph node dissection, as we define elsewhere (12). All serum specimens from patients receiving anatomic resections were collected immediately before surgery and were, therefore, fasting.

The control cohort consisted of three separate groups: 31 volunteers seen in our Rheumatology Department as part of an osteoarthritis study (known hereafter as the "osteoarthritis" cohort) and had no history of lung disease or carcinomas of any type at the time of serum collection or in 3 years of clinical follow-up; deidentified COPD and asthma patients (n = 32 total) from Abbott Laboratories; and 16 patients that received anatomic resections at Rush University Medical Center for suspected NSCLC that, upon pathologic diagnosis, were diagnosed with nonneoplastic nodules (caseating granulomas, resolving pneumonias, etc.). The Abbott cohort was collected at the time of a bronchoscopy procedure (performed to investigate symptom escalation) and was fasting at the time of serum accrual. Patients participating in the osteoarthritis study had their serum collected as part of a normal office visit, and no requirement for fasting was made as part of this protocol.

Table 1. Basic patient demographics

	Discovery		Validation			
	Control	AdC	1	2	3	4
Age						
Range	56-83	65-86	46-85	49-92	47-80	39-84
Mean	69	72	68	68	61	63
Sex						
Male	7	6	52	16	8	5
Female	3	4	65	16	23	11
NSCLC						
Ia		2	28			
Ib		7	49			
IIa		1	2			
IIb			13			
IIIa			17			
IIIb			4			
IV			4			
Diagnosis						
Adenocarcinoma		10	63			
Adenosquamous			3			
Squamous			33			
Large Cell			2			
NSCLC-NOS			16			
Condition						
Asthma				6		
COPD				26		
Osteoarthritis					31	
Granuloma	6					8
Pneumonia	3					4
Benign cyst	1					1
Sarcoma						1
Lipoma						2

NOTE: The discovery cohort consisted of both control and lung adenocarcinoma (AdC) groups, whereas the validation cohort consisted of four groups that were as follows: 1, NSCLC patients; 2, COPD/asthma patients (Abbott cohort); 3, osteoarthritis control patients; and 4, resected patients with nonneoplastic nodules.

Proteomic discovery

Cell culture and preparation of cellular lysates. The human lung adenocarcinoma cell line HCC827 was obtained directly from the American Type Culture Collection expressly for the described studies, and all studies were done within 6 months of the original purchase date. Cell line authentications were done by the American Type Culture Collection (http://www.atcc.org/Science/tabid/203/Default.aspx). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C under a humidified atmosphere of 5% CO_2. All cells were kept within five passages total for the experiments described. Upon achieving 80% confluency, all cells were harvested and washed twice in PBS (pH 7.4). Cellular lysate were prepared by taking 1×10^7 cells in 500 μL of 1% NP40 diluted in TBS supplemented with complete mini protease inhibitor tablet (Roche Diagnostics). Cells were lysed for 30 minutes at 4°C, centrifuged for 10 minutes at 14,000 × *g*, and a protein concentration determined by the BCA method (Pierce).

Serum autoantigen profiling by two-dimensional Western blot analysis. A total of three gels were run simultaneously for the two-dimensional analysis; two gels were loaded with 100 μg of lysates from the HCC827 cell line and were subjected to Western blot analysis to identify differences in immunoreactivity between lung adenocarcinoma and control patient population, whereas one gel was loaded with 300 μg protein to visualize the protein pattern by gel staining. Whole-cell lysate were prepared for this analysis using a ProteoExtract protein precipitation kit (EMD Chemicals, Inc.). Isoelectric focusing was conducted using a Protean Isoelectric focusing cell (Bio-Rad) with the linear gradient program for 22,000 to 24,000 V-h and completed using otherwise standardized protocols recommended by

the apparatus manufacturer (Bio-Rad). After the two-dimensional gels were completed, they were either analyzed by two-dimensional Western blot analysis or stained with Gelcode Blue (Pierce Protein Research Products). For Western blot analysis, proteins from each gel were transferred onto a nitrocellulose membrane at 15 V, using standard "tank-transfer" protocols. After blocking with 1% bovine serum albumin (BSA) in PBS, membranes were incubated for 1 hour in a 1:500 dilution of pooled sera (10 specimens per group) from either the lung adenocarcinoma or control patient groups (n = 10 per group). The probed membranes were then washed with PBS and incubated with horseradish peroxidase–conjugated goat anti-human IgG (Jackson Laboratory) at a 1:100,000 dilution. Immunoblots were developed with the Enhanced ChemiLuminiscence system (GE Healthcare Bio-Sciences Corp.) and were documented on X-ray film. All gels and X-ray films were then scanned using a VersaDoc 4000 gel imaging system and compared using the PDQuest two-dimensional gel imaging software, version 8.0 (Bio-Rad). Replicate runs of the gels and immunoblots were done to ensure the reproducibility of observed patterns of immunoreactivity and the targets cored for protein identification.

Sample preparation for mass spectrometry. Differentially immunoreactive signals observed in the Western blots were cored from the Gelcode Blue stained two-dimensional gels for identification through MS/MS based on having a >10-fold difference in immunoreactivity. In-gel trypsin digestions were accomplished using methods specified by the Promega Corp. (http://www.promega.com/tbs/tb309/tb309.pdf). After digestion, the resultant peptides were concentrated and desalted using C_{18} ZipTip (Millipore) and spotted directly on the MALDI target plate. Recrystallized α-cyano-4-hydroxycinnamic acid (Protea Biosciences) suspended in 50% acetonitrile containing 0.1% trifluoroacetic acid at 5 mg/mL was added to each sample position (1 μL per sample) before analysis by MS/MS.

Mass spectrometric identification of proteins. Protein identification through mass spectrometry was done on a Shimadzu AXIMA Performance mass spectrometer (Shimadzu Biotech) in positive ion mode, optimized to the 700 to 4,000 m/z range. Data were acquired with 2,000 laser shots across each sample. A peptide mass fingerprint analysis was done using the monoisotopic peak list extracted by Mascot Distiller from the raw mass spectrometry files. Peptide matching and protein searches was accomplished against the National Center for Biotechnology Information and Swiss-Prot databases using the Mascot search engine (Matrix Science) with a mass tolerance set to 100 ppm and one missed cleavage with no modifications. In addition, three to five unique peptides were selected from the peptides observed in the mass spectrometry data to perform a MS/MS analysis. Protein identifications were accomplished by importing each peptide sequence tag (PKL) format file (generated by each MS/MS experiments) into the Mascot search engine and used a MS/MS tolerance of ± 0.3 Da to search the National Center for Biotechnology Information and the Swiss-Prot databases.

Criteria for assignment of protein identity consisted of the following parameters: MASCOT "MOWSE" scores of >67 (P = 0.05), sequence coverage of >30%, MS/MS data on a minimum of three unique tryptic peptides, and general agreement between predicted and observed mass/isoelectric point gel coordinate values. Further, two-dimensional Western blots were also done using commercially available monoclonal and polyclonal antibodies to confirm that the identified autoantigen correlated with the gel coordinates from which immunoreactivity was originally observed. Antibody sources were as follows: NY-ESO, survivin, recoverin, methylthioadenosine phosphorylase (MTAP), p53, peroxiredoxin, and triosephosphoisomerarase from Santa Cruz Biotechnology, Inc.; enolase 1 and glyceraldehyde-3-phosphate dehydrogenase from Abcam, Inc.; Annexin a1 from R&D systems; calponin 2 from Avia Systems Biology; hydroxysteroid (17-β) dehydrogenase and phosphoglycerate dehydrogenase from Sigma; and the remaining antibodies were from the Abnova Corp.

Serum test development

Recombinant proteins: sources and production. Recombinant proteins were obtained for each of the candidate autoantibodies identified in the proteomic discovery efforts having value for NSCLC detection (see Table 2) as well as for a second group of autoantibodies with documented value for our purpose, including NY-ESO (13–15), p53 (13, 16–21), peroxiredoxin (22, 23), triosephosphate isomerase (23), recoverin (24), 3-oxoacid CoA transferase (23), survivin (also known as BIRC5; ref. 25), c-Myc (13, 26), Annexin II (27), and ubiquillin (28). Commercial antibodies (as used above for target confirmation) were obtained for these targets to serve as positive controls during assay performance. A subset of the recombinant proteins (autoantigens) was custom prepared by our collaborators at Abnova Corp., as defined elsewhere (29, 30). These include α-enolase, glyoxalase domain containing 4, MTAP, phosphoglycerate mutase I, IMP-dehydrogenase II, triosephosphate isomerase, recoverin, phosphoglycerate dehydrogenase, erp-29, Annexin I, Annexin A1 (isoform CRA_b), hydroxysteroid-(17-β)-dehydrogenase 10 isoform 1, fumarate hydratase, heat shock protein-70 9B (mortalin-2), protein disulfide isomerase-associated 3 precursor, isocitrate dehydrogenase 1 isoform 1, calponin-2, c-Myc, Annexin II, 3-oxoacid CoA transferase, and GRP-78 precursor.

Custom Luminex immunobead assay development.

Microsphere-antigen coupling. The "direct capture" bead-based immunoassays were developed using protocols suggested by the Luminex Corp.'s suggested protocol (http://www.luminexcorp.com/support/protocols/protein.html). Briefly, between 5 and 10 μg of recombinant protein were conjugated with 5×10^6 SeroMAP microspheres (Luminex Corp.), each with a unique bead region identifier. This was accomplished by activating the microspheres suspended in a solution of sodium phosphate (pH 6.2), containing 5 mg/mL sulfo-NHS (Thermo Scientific) and 5 mg/mL 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Thermo Scientific). After a 20-minute incubation in the dark,

Table 2. Results from proteomic identification of antigens

Spot no.	Antigen	Accession no.	MOWSE Scores	Molecular weight (kDa) exp./obs.	Sequence coverage (%)	Total number of peptides assigned
1	α-Enolase	GI: 450357	228	50/47	64	20
2	Glyoxalase domain containing 4	GI: 16198390	112	40/33	44	12
3	Glyceraldyhyde-3-phosphate dehydrogenase	GI: 31645	211	40/36	52	13
4	MTAP	GI: 847724	148	35/31	74	15
5	Phosphoglycerate mutase 1	GI:130348	193	36/28	20	11*
6	IMP-dehydrogenase 2	GI: 6693016	78	64/56	31	19
7	Phosphoglycerate dehydrogenase	GI: 23308577	103	64/57	37	16
8	Endoplasmic reticulum protein 29 (erp 29)	GI: 5803013	68	36/29	45	11
9	Annexin A1, isoform CRA_b	GI: 19582952	113	40/40	44	13
10	Annexin 1	GI: 4502101	116	40/38	46	14
11	α-enolase	GI: 450357	71	50/47	30	10
12	Hydrosteroid (17-β) dehydrogenase 10	GI: 4758504	84	27/27	37	10
13	Fumarate hydratase	GI: 19743875	83	64/54	50	10
14	Isocitrate dehydrogenase 10 isoform 1	GI: 28178825	134	50/46	40	17
15	Calponin 2 isoform b	GI: 41327730	90	40/29	37	11
16	Heat shock 70-kDa protein 9B (mortalin-2)	GI: 21040386	94	55/50	20	10*
17	α-Enolase	GI: 450357	104	50/47	51	16
18	Protein disulfide isomerase-associated 3	GI: 21361657	232	62/55	49	21
19	Enolase 1 variant	GI: 62896593	90	50/47	30	11
20	GRP78 precursor	GI: 386758	183	64/72	44	27
21	α-enolase	GI: 450357	95	50/47	43	14

Abbreviations: exp. - expected; obs. - observed.

*Denotes protein identities assigned with sequence coverage of <30%, but strong MS/MS data on ≥5 unique peptides to support this assignment.

the microspheres were washed and resuspended in 50 mmol/L MES (pH 5.0), and the appropriate volume of recombinant protein added. The beads were then incubated at ambient temperature in the dark with continuous mixing for 2 hours. Following incubation, the microspheres were then washed twice with PBS containing 0.1% BSA and 0.2% Tween 20, and stored in the same buffer at 4°C.

Microsphere validation. Commercial antibodies (as defined above) were obtained for all candidate autoantigens to serve as a positive control during the direct capture assays. All antigen-coupled microspheres were subjected to individual validation per procedures recommended by the Luminex Corp. Briefly, serial dilutions of each protein-specific antibody (ranging from 4 to 0.0625 μg/mL in PBS, 1% BSA) were incubated for 2 hours with 5,000 antigen-conjugated microspheres per well in a 1.2-μm polyvinylidene difluoride filter 96-well microtiter plate (Millipore). Following washing with PBS/1% BSA, the immobilized autoantibody complex was incubated with 4 to 6 μg/mL of the biotin-conjugated, anti-human polyclonal antibody (Sigma-Aldrich Co.) for 1 hour with constant agitation. Finally, after two washes (as before), the complex was incubated with 4 to 6 μg/mL R-phycoerythrin–conjugated streptavidin (Thermo Scientific) for 45 minutes with constant agitation. The resulting complex was again washed and resuspended in PBS/1% BSA before being read on our Luminex 100 bioanalyzer that uses the IS 2.3 software (Luminex Corp.). Performance characteristics were then established for each assay, including range, %CV, sensitivity, and specificity, all with in SPSS v15.0.

Following successful individual assay validation, multiplex validation was done using a modified "leave one out" protocol from Luminex Corp. The protein-coupled microspheres were grouped into sets containing four to six

autoantibody assays per panel selected based on having low protein sequence homology across the group as to avoid cross-reactivity. The median fluorescence intensities (MFI) of each microsphere set (performed as above) were compared with the panel values to confirm that the multiplexed protein-microsphere sets did not have positive interference with one another. Finally, serial dilutions of three stock serum specimens were also used to evaluate the individual microspheres for cross-reactivity.

Serum analysis using validated microsphere sets. Once multiplexing validation was completed, the resulting five distinct combinations of autoantigen-microsphere panels were used to evaluate the patient serum specimens (n = 196) for autoantibody levels. For this assessment, all serum was typically diluted 1:20 using PBS containing 1% BSA, and assays were otherwise done as described above. The reported median fluorescence intensity values, which correlate to the concentration of a given autoantibody in the serum, were scaled relative to the MFI values obtained using available monoclonal or polyclonal antibodies as standards. The actual MFI value used for scaling was selected on the basis of being closest to the median MFI value for the entirety of the patient cohort. These are called "MFI^{SCALED}" values in the remainder of the article.

Univariate statistics. Descriptive statistics, receiver operator characteristic (ROC) parameters [including "area under the curve" (AUC), specificity, and sensitivity], and P values from Mann-Whitney U test with two comparator groups were obtained for the individual analytes using the SPSS statistical software version 15.0 (SPSS), as we previously defined (12).

Multivariate analysis. An optimized multivariate panel of autoantibody biomarkers was selected from the data resulting from evaluation of the patient cohorts using the Random Forests package in R (31, 32) as previously described (12). The optimal panel of biomarkers resulting from the random Forest variable selection process described above was then used by a Classification and Regression Tree (CART) algorithm to model a specific classification system for identifying a patient's cancer status (NSCLC versus non-NSCLC). This analysis was done using the RPART package of the R statistical software suite (33). From the final classification tree, we are able to calculate standard test performance characteristic values (i.e., misclassification error and ROC curve parameters).

Results

Serum autoantigen profiling by two-dimensional Western blot analysis

To identify candidate tumor-associated autoantigens differently represented in our two patient groups, we resolved our HCC827 cellular lysates through two-dimensional Western blots, with each immunoblot probed individually with pooled sera from the control and adenocarcinoma patient groups (n = 10 per group; see Table 1 for patient characteristics). Figure 1A shows a representative Coomassie-stained two-dimensional gel of the proteins extracted from the lung adenocarcinoma cell line HCC827 with the differences in immunoreactivity to patient sera shown in Fig. 1B and C. A total of 21 spots were selected for identification through MS/MS based on possessing a >10-fold difference in immunoreactivity.

Protein identification through MS/MS

Candidate autoantigens recovered from the two-dimensional gels were analyzed on our Shimadzu AXIMA Performance mass spectrometer to establish protein identity through standard in-gel digestion methods. A peptide fingerprint analysis coupled with MS/MS experiments was used to determine the identity of the selected autoantigens and are presented in Table 2. Each target identified by this analysis correlated highly to the predicted gel coordinates (both isoelectric point and apparent molecular mass) from which it was originally excised. Intriguingly, the MS/MS data for the spot numbers 12 and 18 identified

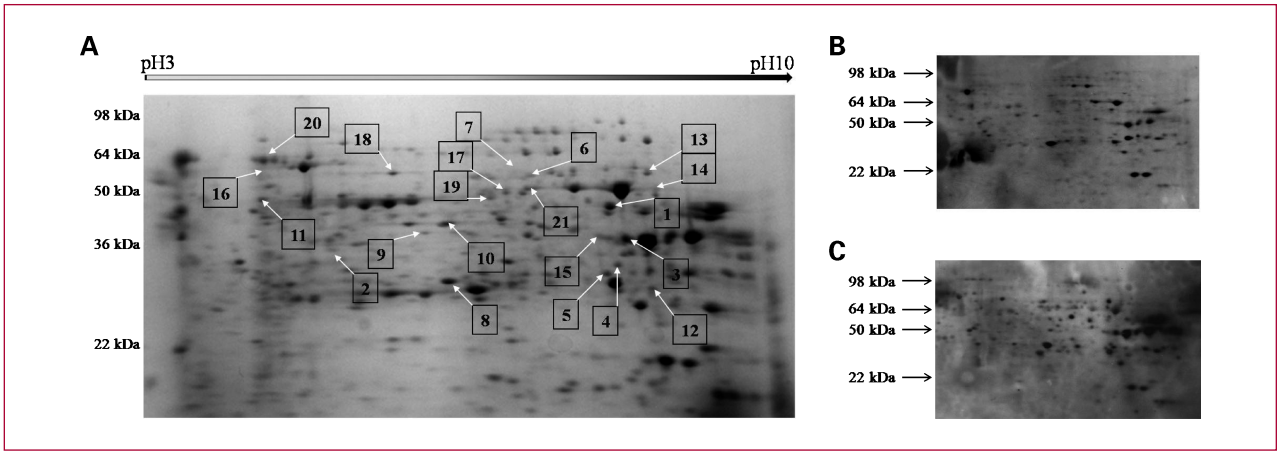

Fig. 1. Representative two-dimensional protein gel of HCC827 cellular lysates with accompanying immunoblots. Coomassie-stained two-dimensional gel for proteins isolated from HCC827 cellular lysates (A), with matched immunoblots from the control (B) and stage I adenocarcinoma (C) cohorts.

two proteins for each spot, namely 3-hydroxyacyl-CoA dehydrogenase type 2 and hydrosteroid (17-β) dehydrogenase 10 isoform 1 for spot 12, and ER-60 protease and protein disulfide isomerase-associated 3 precursor for spot 18. A protein-protein BLAST on the National Center for Biotechnology Information Web site showed that each of these pairs shared 100% sequence homology. Also of interest is the observation that isoforms of α-enolase were identified in five different positions (spots 1, 11, 17, 19, and 21) from this analysis, whereas Annexin A1 was identified in two positions (spots 9 and 10). These results were confirmed through two-dimensional Western blots using commercially obtained polyclonal and monoclonal antibodies against each candidate autoantigen. Further, these studies also showed that each gel coordinate contained an immunoreactive protein that corresponded to that shown in Fig. 1 (data not shown).

Autoantigen validation against a second patient cohort

In our effort to come closer to a clinically usable early detection panel for the detection of NSCLC, 15 of the 16 distinct autoantigens identified in our immunoproteomic analysis were translated into Luminex-based immunobead serum assays. The glyoxalase domain containing 4 was not validated by Luminex assays due to a lack of readily attainable recombinant protein for assay development, although it is currently in development. In addition, 10 promising markers (NY-ESO, p53, peroxiredoxin, triosephosphate isomerase, recoverin, 3-oxoacid CoA transferase, survivin, c-Myc, Annexin II, and ubiquillin) found in the literature were also translated into Luminex-based immunobead assays by our group. With this, a total of 25 custom immunobead assays were used to evaluate our 117 NSCLC patient serum and 79 control patient serum for circulating autoantibodies specific for NSCLC status. The demographic and clinical characteristics of these cohorts are defined in Table 1.

Of the 15 autoantibodies (identified by this report) that were evaluated, 7 were found to be significantly elevated (AUC of >0.60 and a Mann-Whitney *U* value of <0.05) in NSCLC patients relative to the control population. These include inosine-5-monophosphate dehydrogenase (IMPDH), fumarate hydratase, α-enolase, endoplasmic reticulum protein 29, Annexin 1, hydrosteroid 17-β dehydrogenase, and MTAP. Of those evaluated from the literature, we found Annexin II to be the most promising as a biomarker, possessing an AUC of 0.683 and $P < 0.001$. Ubiquilin, c-Myc, NY-ESO, 3-oxoacid CoA transferase, and p53 also found to be significant in the AUC and Mann-Whitney two-sided *P* value. All other analytes failed to meet statistical significance. Table 3 lists the individual test performance characteristics for the tests against the autoantigens.

With the Random Forest multivariate analysis, we identified a panel of six analytes [IMPDH, phosphoglycerate mutase, ubiquillin, Annexin I, Annexin II, and heat shock protein 70-9B (HSP70-9B)] that was the optimal combination of analytes for distinguishing NSCLC patients from the cancer-free controls (with an observed ROC parameter of AUC of 93.4%). Boxplots for these six analytes are shown in Fig. 2 with the cohorts partitioned. No correlation with age, gender, or smoking status was observed in any of these six analytes that were stronger than those observed according to disease status. A CART analysis was then fashioned from these analytes and used to define a specific algorithm for classifying patients according to NSCLC disease status (see Fig. 3). This algorithm provided "excellent" ROC parameters for the overall study, including an AUC of 0.964, a sensitivity of 94.8%, and a specificity of 91.1%. The overall misclassification rate was 7% against the entire patient population ($n = 196$). The general agreement of the results yielded by the two multivariate algorithms (i.e., random forest and CART) serves as an ad hoc "internal confirmation" for the specific six analytes selected by each method and generates confidence in our methods.

Table 3. ROC AUC and Mann-Whitney *P* values for each individual analyte

Analyte	Mann-Whitney *P* (two sided)	ROC AUC
IMPDH	<0.001	0.739
Annexin II	<0.001	0.693
Fumarate hydratase	<0.001	0.678
Ubiquilin	<0.001	0.677
c-MYC	<0.001	0.652
α-Enolase	0.0013	0.635
NY-ESO	0.0016	0.633
p53	0.0023	0.628
Endoplasmic reticulum protein 29 (erp 29)	0.013	0.604
3-Oxoacid CoA Transferase	0.014	0.604
MTAP	0.018	0.6
Annexin I	0.019	0.599
Hydroxysteroid (17-β) dehydrogenase 10	0.026	0.594
Heat shock protein 70-kDa protein 9B (mortalin-2)	0.088	0.572
phosphoglycerate mutase	0.09	0.571
Recoverin	0.177	0.557
Heat shock protein 5 (GRP78 precursor)	0.186	0.556
Peroxiredoxin	0.202	0.554
Calponin 2	0.204	0.554
Phosphoglycerate dehydrogenase	0.336	0.541
Protein disulfide isomerase 3	0.33	0.541
Glyceraldehyde-3-phosphate dehydrogenase	0.366	0.538
Triosephosphate isomerase	0.361	0.538
Survivin	0.625	0.521
Isocitrate dehydrogenase	0.636	0.52

Discussion

The development of an economical serum test to augment spiral CT-based screening protocols may help address some of the limitations of this paradigm, including the high rate of false positives that trigger unnecessary surgery and the need for serial measurements to improve specificity. This study aimed to develop a serum test that could be used to discern NSCLC patients from cancer-free controls and may provide a means to indicate who should have spiral CT done.

Since the 1970s, multiple groups have attempted to harness circulating tumor-specific autoantibodies as diagnostic biomarkers for a variety of cancers, including NSCLC. This idea largely stems from the popular notion that although a cancer is still asymptomatic and in its early stages, autoantibodies produced in response to cancer-specific antigens may be detectable due to signal amplification from the humoral immune response. To date, numerous autoantibody targets have been evaluated for their value in early detection of lung cancer or for prognostication (13, 16, 19, 22, 27, 34–39). Despite the promise these studies have shown in this direction, little has been done outside the initial discovery efforts to validate these findings and to translate these promising antibodies into a clinically usable test.

The present study identified and validated a large number of potential tumor autoantibodies for NSCLC. Three of these autoantibodies (MTAP, fumarate hydratase, and the endoplasmic reticulum protein 29) represent new autoantigen targets for distinguishing NSCLC from control populations. Interestingly, although MTAP antibodies have not been previously reported in the literature, Watanabe et al. (40) showed by immunohistochemistry that NSCLC tissue has decreased protein levels of MTAP relative to control groups. The lower protein content in NSCLC cells may represent an adaptive response to the autoantibody elevation found in our study. Of the analytes previously seen in the literature, IMPDH, Annexin II (16, 34), ubiquilin (28), c-Myc (26), and α-enolase (23, 35) showed the most promise (AUC > 0.63) for application in the early diagnosis of NSCLC. Interestingly, although p53 autoantibodies have been found to have numerous associations with regard to NSCLC, including the correlation with response to chemotherapy (20), with overall prognosis (21), and with serologic detection of disease (13, 16, 36, 39), this marker was eliminated in the first iteration of the Random Forest multivariate statistical test. This is despite possessing excellent significance (ROC curve AUC = 0.628, $P = 0.0023$) in the univariate analysis.

The six-analyte blood test that resulted from the present study (consisting of IMPDH, phosphoglycerate mutase, ubiquillin, Annexin I, Annexin II, and HSP70-9B) possessed excellent test performance characteristics when tested against our 196 patient cohort that was composed of four clinically distinct groups, with only 13 patients misclassified overall. Within the classification errors we encountered, we observed a 4% false-positive rate in the non-NSCLC cohorts, which includes 2 patients from the Abbott cohort (6% of group; 1 asthma, 1 COPD),

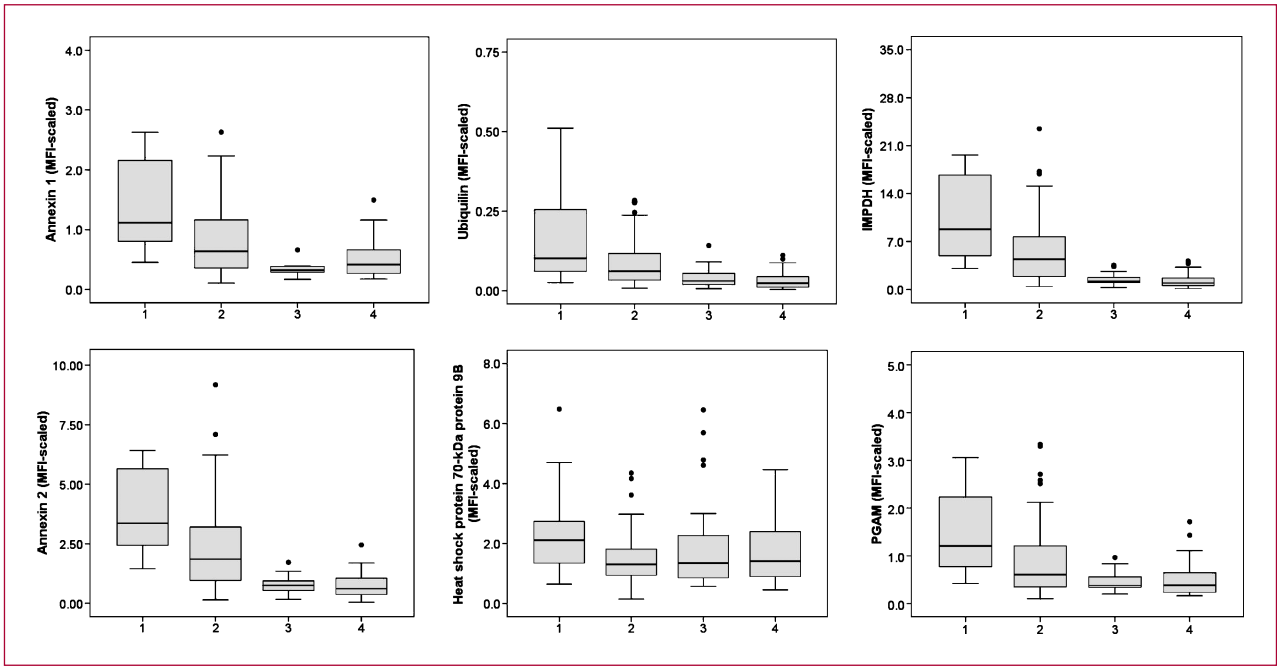

Fig. 2. Representative boxplots for the six analytes selected by the Random Forest algorithm. Distributions of circulating autoantibody levels with cohorts separated as follows: 1, NSCLC patients; 2, COPD/asthma patients; 3, "cancer-free" control patients; and 4, resected patients with nonneoplastic nodules. MFI-scaled, MFI values scaled to a standard concentration of appropriate commercial antibody; PGAM, phosphoglycerate mutase.

1 patient from the "osteoarthritis" cohort (3% of group), and 4 patients from the cohort that had resected "nonneoplastic" nodules (25% of group; 2 pneumonia, 1 pneumonitis, and 1 granulomas). No clinical features helped explain the high rate of misclassification within any of these groups. These findings support the idea that at this time, the algorithm may be well suited to help indicate which symptomatic patients should have further diagnostic evaluations done. Further validation will need to be done to determine the performance characteristics against an appropriate cohort of asymptomatic patients. The high rate of misclassifications within the resected nonneoplastic group may be partially accounted for by the fact that specific inflammatory conditions, such as interstitial lung disease, COPD, and asthma, has been reported to induce the production of autoantibodies (41–45) that may be common to the autoantibodies found in patients with NSCLC. However, whether these autoantibodies are produced before or during carcinogenesis is not known at this time and it may follow that a subset of these targets may provide a means to early disease detection. This subject will be further pursued in future studies. It should also be noted that the number of patients in this resected nonneoplastic patient cohort was relatively low (16 total), making it difficult to extrapolate the significance of this general finding. In addition, given this entire cohort was incorrectly resected for suspected NSCLC, this group may be the most challenging to discern by any current diagnostic method. Even so, we successfully classified 75% of these patients, suggesting there may be value to this approach that may complement current

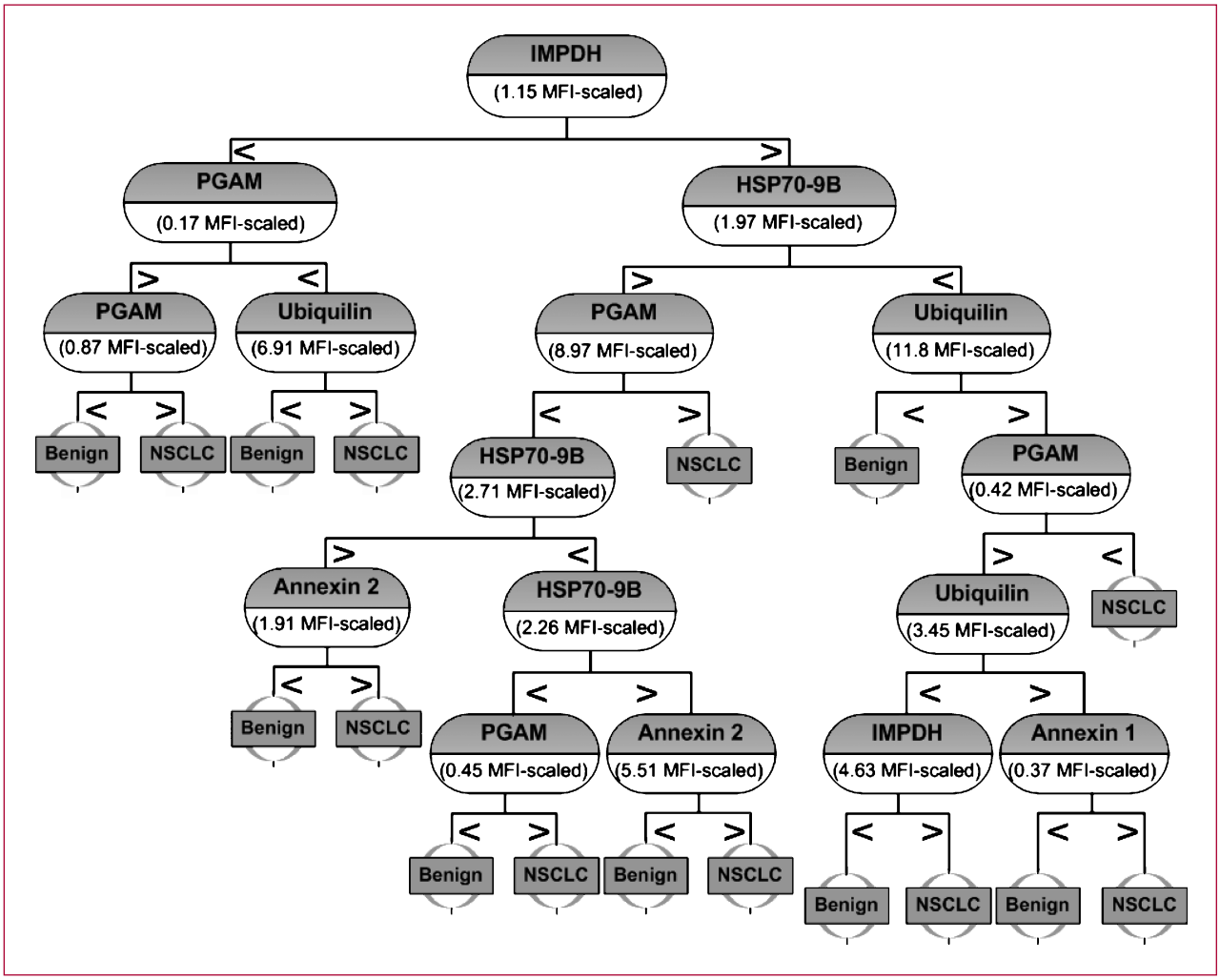

Fig. 3. CART-based algorithm for the six-autoantibody panel. CART for predicting if a patient is positive for NSCLC. Briefly, the algorithm represents a series of binary "*if-then*" decision rules that are used to split the data into separate branches of the tree. Each node of the tree displays the analyte being considered and the threshold concentrations used to partition the patient groups. Additional classifications continue along each arm of the split in which it is indicated whether the measured value is either less than or equal to, or exceeding the indicated threshold cutoff value. The diagnosis by this decision tree is listed at each terminal node, with each final arm on the left indicating cases predicted as "benign" and each final arm on the right predicted as "NSCLC." Abbreviations: MFI-scaled, MFI values scaled to a standard concentration of appropriate commercial antibody; PGAM, phosphoglycerate mutase; HSP70-9B, heat shock protein 70-kDa protein 9B (mortalin-2).

clinical and radiographic diagnostic criteria. Perhaps of more concern is the observed 5% (3.1% overall) false-negative rate (6 of 117) observed in the NSCLC cohort. Although these misclassifications were not clustered around a single subgroup or clinical parameter, there was a higher incidence (four total) of patients with poorly differentiated tumors within this category. The relevance of this observation is still under investigation. Also of interest is that these misclassifications were not limited to stage IA patients (two $T_1N_0M_0$, four $T_2N_0M_0$), possibly indicating that errors were not due to test sensitivity. Along these lines was the finding that the histology of these misclassifications was equally distributed between squamous cell carcinoma and adenocarcinoma despite the fact that our test was originally directed exclusively against adenocarcinoma specimens. This suggests the targets identified were general to the production of autoantibodies for NSCLC. Interestingly, all squamous cell carcinoma patients possessed tumors with poor differentiation upon pathologic examination. This is particularly important because the numbers of patients with non-adenocarcinoma histology is substantial (~40%) in the general population of NSCLC patients.

Multianalyte autoantibody panels have previously been reported by other investigators studying NSCLC, but, to the best of our knowledge, our panel has test performance characteristics for detection of NSCLC superior to any serum test reported. In addition, several of the multianalyte panels proposed have very small patient populations relative to that reported here. Nakanishi et al. (23) in 2006 used a technique similar to our own protein discovery effort to compare adenocarcinoma with tuberculosis and healthy controls. Although their patient sample was very small (5 cases of adenocarcinoma, 10 tuberculosis, and 10 controls), they found several of the same autoantibody targets as in our discovery effort (α-enolase, IMPDH, PHGDH, glyceraldehyde-3-phosphate dehydrogenase), with two of their targets, IMPDH and α-enolase, showing high individual significance in our validation test. In a separate study with a broader patient population, Zhong et al. (39) found an AUC of 0.73 for a three-autoantibody panel with laminin receptor 1, Annexin A1, and 14-3-3 θ. Although these individual analytes may be useful in a future panel, their combined panel has a lower AUC than our top five analytes individually (IMPDH, Annexin A2, fumarate hydratase, ubiquilin, and c-MYC) and a much lower ROC curve parameters than our final panel. Until recently, the most sensitive screening test is one by Zhong et al. (39) who used protein microarrays to profile tumor-associated antibodies in NSCLC and reported five tumor antigens that yielded a specificity and sensitivity over 90% in the prediction of patients' samples. Finally, a recent report by Leidinger et al. (46) produced a test performance and classification accuracy rates similar to that reported here (sensitivity of 97.8%, a specificity of 85.7%, and an accuracy of 94.6%.) for distinguishing stage I NSCLC (n = 29) from controls (n = 80 high risk patients and 26 with non-tumor lung pathologies) using an array of peptide antigens. However, this autoantibody-based signature was based on 1,827 peptide clones that would make this approach cost prohibitive in serving as a method to screen large numbers of patients for lung cancer.

Our observations suggest that further study of this six-autoantibody panel is warranted. We hope to validate our current panel's ability to detect early-stage disease in patient cohorts from multi-institutional studies (46). If validated, this panel could have clinical and economical implications for lung cancer screening.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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