Development of a serum biomarker panel predicting recurrence in stage I non–small cell lung cancer patients

Daniel Rinewalt, MD,[a] David D. Shersher, MD,[a] Shaun Daly, MD,[a] Cristina Fhied, MS,[b] Sanjib Basu, PhD,[c] Brett Mahon, MD,[d] Edward Hong, MD,[a] Gary Chmielewski, MD,[a] Michael J. Liptay, MD,[a] and Jeffrey A. Borgia, PhD[b,d]

Objective: Molecular diagnostics capable of prognosticating disease recurrence in stage I non–small cell lung cancer (NSCLC) patients have implications for improving survival. The objective of the present study was to develop a multianalyte serum algorithm predictive of disease recurrence in stage I NSCLC patients.

Methods: The Luminex immunobead platform was used to evaluate 43 biomarkers against 79 patients with resectable NSCLC, with the following cohorts represented: stage I (T_1-$T_2N_0M_0$) NSCLC without recurrence (n = 37), stage I (T_1-$T_2N_0M_0$) NSCLC with recurrence (n = 15), and node-positive (T_1-T_2N_1-N_2M_0) NSCLC (n = 27). Peripheral blood was collected before surgery, with all patients undergoing anatomic resection. Univariate statistical methods (receiver operating characteristics curves and log-rank test) were used to evaluate each biomarker with respect to recurrence and outcome. Multivariate statistical methods were used to develop a prognostic classification panel for disease recurrence.

Results: No relationship was found between recurrence and age, gender, smoking history, or histologic type. Analysis for all stage I patients revealed 28 biomarkers significant for recurrence. Of these, the log-rank test identified 10 biomarkers that were strongly ($P < .01$) prognostic for recurrence. The Random Forest algorithm created a 6-analyte panel for preoperative classification that accurately predicted recurrence in 77% of stage I patients tested, with a sensitivity of 74% and specificity of 79%.

Conclusions: We report the development of a serum biomarker algorithm capable of preoperatively predicting disease recurrence in stage I NSCLC patients. Refinement of this panel might stratify patients for adjuvant therapy or aggressive recurrence monitoring to improve survival. (J Thorac Cardiovasc Surg 2012;144:1344-51)

The American Cancer Society has estimates more than 226,160 new cases of lung cancer, along with approximately 160,340 deaths, in 2012, making it the most common cause of malignancy-related mortality in the United States.[1] Non–small cell lung cancer (NSCLC) is particularly lethal, because many patients present with regional (lymph node) or distant metastases, which has been associated with 24% and 4% 5-year survival, respectively.[1,2] In contrast, as much as 20% of patients with stage I disease will die of disease recurrence within 5 years of tumor resection. Recurrence in this group suggests that systemic tumor cell dissemination (locoregional or distant) had already occurred at surgery but was undetected by current clinical and pathologic staging practices.[3,4] This group of patients with occult ''micrometastatic'' disease will only receive postoperative surveillance as the standard of care, although these patients might experience substantial clinical benefit from more frequent postoperative surveillance and/or adjuvant systemic chemotherapy (similar to higher stage groups), if appropriate methods were available for definitive selection. The increased use of chest computed tomography (CT) screening protocols in the next several years is expected to shift NSCLC staging demographics toward stage I disease, given the preliminary data from the National Lung Screening Trial, demonstrating a 20% reduction in mortality from NSCLC with screening of high-risk patients relative to chest radiography.[5] Methods to definitively select stage I patients are urgently needed to help direct patients likely to benefit from adjuvant chemotherapy into prospective clinical trials and to assist with the management of the anticipated increased numbers of stage I NSCLC cases resulting from the implementation of low-dose CT screening protocols.

The objective of the present study was to develop a simple and cost-effective serum test that is prognostic for disease

From the Departments of Thoracic Surgery,[a] Biochemistry,[b] Preventative Medicine,[c] and Pathology,[d] Rush University Medical Center, Chicago, Ill.

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Address for reprints: Jeffrey A. Borgia, PhD, Departments of Biochemistry and Pathology, Rush University Medical Center, 554 Cohn Research Bldg, 1735 W. Harrison St, Chicago, IL 60612 (E-mail: Jeffrey_Borgia@Rush.edu).

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Abbreviations and Acronyms

CT = computed tomography
IGFBP = insulin-like growth factor binding protein
NSCLC = non–small cell lung cancer
OOB = out-of-bag
PDIA3 = protein disulfide isomerase 3
PET = positron emission tomography
TIMP = tissue inhibitor of metalloproteinase

recurrence in stage I NSCLC patients. For this effort, we reasoned that biomarkers we had reported in our previous studies[6-9] as efficacious for identifying patients with locoregional disease progression might also have utility in predicting recurrence in the stage I cohort. We based this hypothesis on the premise that occult micrometastases are the primary cause of disease recurrence in these cases, thereby providing a common mechanistic foundation to investigate. The resulting prognostic classification algorithm represents an exciting advancement in our ability to individualize treatment for stage I NSCLC patients.

PATIENTS AND METHODS

Between 2004 and 2008, we enrolled 79 patients who were divided into the following cohorts: stage I (T_1-$T_2N_0M_0$) NSCLC without recurrence (n = 37), stage I (T_1-$T_2N_0M_0$) NSCLC with recurrence (n = 15), and node-positive (T_1-T_2N_1-N_2M_0) NSCLC (n = 27). All staging presented was from the pathologic evaluations. The patient inclusion criteria included NSCLC disease confined to the chest without evidence of distant metastases; no preoperative chemo- or radiotherapy within 1 year of our initial blood sampling; and a minimum of 2 years of clinical follow-up data. Patients who died perioperatively were excluded, because they would not have had sufficient follow-up to determine their recurrence status. Demographic information is listed in Table 1. All patient data were acquired with written formal consent and in absolute compliance with the institutional review board at Rush University Medical Center (Chicago, Ill).

Clinical workup at our institution for all NSCLC patients includes CT of the chest and/or abdomen and positron emission tomography (PET). Patients with evidence of possible mediastinal lymph node involvement were evaluated by mediastinoscopy before any additional surgical intervention. All patients with negative findings for mediastinal lymph node involvement on CT-PET scanning or with negative mediastinoscopy findings underwent complete anatomic resection with lymph node dissection of the hilar and/or mediastinal nodes. All intraoperative specimens were examined at surgery by pathologists within our institution to determine margin involvement and potential regional metastatic disease. Routine pathologic examination included hematoxylin and eosin permanent stains on all surgically removed tissue samples with immunohistochemical analysis performed as indicated to confirm disease origin. All samples denoted as lymph node negative had no evidence of metastases or micrometastases on complete pathologic evaluation.

Measurement of Serum Biomarker Concentrations

Serum was prepared using standard phlebotomy protocols from peripheral blood collected in red-top tubes immediately before pulmonary resection. All specimens were processed within 1 hour of collection, with 50 μL/mL added to the serum aliquots before archiving at –80°C. No specimen was subjected to more than 2 freeze–thaw cycles before immunoassay evaluation. Assays for insulin-like growth factor binding proteins (IGFBPs) 1 to 7, interferon-γ, CA19-9, cytokeratin 19 fragment 21-1, stem cell factor-1, stromal cell-derived factor-1α, monocyte colony-stimulating factor, interleukin-2Rα, tissue inhibitor of metalloproteinase (TIMP)-1, and IGF-1 were performed using Milliplex Map Kits (EMD-Millipore, Billerica, Mass). The assays used for the circulating autoantibody biomarkers were identical to our previously described methods.[6-9] All biomarker concentrations were calculated using a 5-parametric curve fit using xPONENT, version 4.03 (Luminex Corp, Austin, Tex) in a blinded fashion with measurement performed with the FlexMAP 3D system (Luminex Corp). Table 2 lists the 43 biomarkers evaluated in the present study.

The methods for candidate biomarker testing were identical to those previously reported.[6-9] In brief, we selected biomarkers for the present study using 2 methods. The first was from a review of the published data for biomarkers that had previously demonstrated efficacy for the early detection of lung cancer.[6] The second was based on previous efforts in our laboratory.[8] Specifically, we used Western blots prepared using lysates from a lung adenocarcinoma cell line (HCC827) that were then probed with pooled serum from 2 NSCLC patients groups, the first without nodal involvement and the second with node-positive disease. We compared the immunoblots and performed additional 2-dimensional gels to visualize proteins with a 10-fold difference in immunoreactivity for identification by mass spectrometry. These identified proteins were then selected for analysis in the present study.

Statistical Analysis

Descriptive statistics were obtained, and receiver operating characteristic curves (including area under the curve, specificity, and sensitivity) were used to assess the difference among the 43 individual biomarkers using SPSS statistical software, version 18.0 (SPSS, Chicago, Ill). Fisher's exact and log-rank tests were used to determine significance between the clinical factors (eg, age, gender, race, pack years of smoking exposure, and histologic type) and recurrence. The Kruskal-Wallis test was used to quantify the associations in biomarker concentrations between stage I (with and without recurrence) and positive cohorts, and the Mann-Whitney *U* and log-rank tests were used to determine the differences related to the interval to recurrence in stage I patients and overall survival. All *P* values reported are 2-sided. The optimal multivariate panel of biomarkers for predicting the clinical outcome was selected using variable selection algorithms performed within the Random Forest package, as previously described.[6] In brief, the Random Forest multivariate method grows numerous (1000 in the present study) cross-validated classification trees for a panel of biomarkers, with each tree used to predict group membership for each case. These are counted as the tree "votes" for that group. The forest chooses the group membership having the most votes for all the 1000 trees in the forest. Each such tree is grown by cross-validation, in which a training set (approximately two thirds of the values) is randomly selected from the full data, and each tree is grown on this training data to the largest extent possible (no pruning). The resultant tree is then used to predict the group membership for the remaining validation cases, termed an *out-of-bag* (OOB) prediction. This process is then repeated 1000 times (ie, another training set is randomly selected and a new tree is grown and used to perform another OOB prediction). The classification accuracy of the Random Forest is measured by the averaged error of the OOB predictions across the 1000 trees in the entire forest; this is termed the *OOB error rate.* The OOB error thus uses disjoint subsets of the data for model fitting and validation repeatedly. The reported sensitivity, specificity, and accuracy of the Random Forest method are determined from these OOB predictions from the trees, averaged over the 1000 trees in the forest.