Research Article

Cancer Prevention Research

Urinary Protein Biomarkers in the Early Detection of Lung Cancer

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Abstract

The early detection of lung cancer has the potential to greatly impact disease burden through the timely identification and treatment of affected individuals at a manageable stage of development. The insufficient specificity demonstrated by currently used screening and diagnostic techniques has led to intense investigation into biomarkers as diagnostic tools. Urine may represent a noninvasive alternative matrix for diagnostic biomarker development. We performed an analysis of 242 biomarkers in urines obtained from 83 patients with non–small cell lung carcinomas (NSCLC), 74 patients diagnosed with benign pulmonary conditions, and 77 healthy donors. A large number of significant alterations were observed between the NSCLC and control groups. A multivariate analysis identified a three-biomarker panel consisting of IGFBP-1, sIL-1Ra, CEACAM-1, which discriminated NSCLC from healthy controls with a sensitivity/specificity of 84/95 in an initial training set and 72/100 in an independent validation set. This panel performed well among multiple subtypes of NSCLC and early-stage disease but demonstrated only limited efficacy for the discrimination of NSCLC from benign controls and limited specificity for patients with several other cancers and tuberculosis. These findings demonstrate that urine biomarkers may provide screening and diagnostic properties which exceed those reported for serum biomarkers and approach a level necessary for further clinical development. *Cancer Prev Res; 8(2); 111–9.* *©2014 AACR.*

Introduction

Lung cancer is a devastating disease that accounts for more deaths in the United States annually than prostate and breast cancer combined (1). Effective methods of early detection could dramatically reduce disease mortality and greatly benefit overall public health. Non–small cell lung carcinomas (NSCLC) represent the vast majority of lung cancers, and although the overall five-year survival for patients with this diagnosis is a disappointing 15%, five-year survival for those patients diagnosed with stage IA NSCLC typically exceeds 60% (2). A number of techniques, including thoracic radiography, sputum cytology, and CT, are currently being evaluated as diagnostic tools for lung cancer. Although thoracic radiography and sputum cytology have failed to perform with adequate levels of sensitivity for early-stage disease in clinical trials [reviewed in Chanin and colleagues (3)], CT screening is now recommended for heavy smokers by the US Preventive Services Task Force (4). The limitations of CT scanning are also well documented, including the high identification rate of benign pulmonary nodules (5, 6). Such findings greatly reduce the specificity of CT, exacerbating the already high cost of the technology and leading to unnecessary patient anxiety and surveillance. Thus, it remains the need to identify additional effective methodologies.

Investigations about the use of biomarker measurements as early-detection tools for lung cancer have been conducted in serum, tissue, and sputum, with serum being the least invasive and, hence, most desirable testing matrix. Several serum biomarkers, including carcinoembryonic antigen (CEA), Cyfra 21-1, tissue polypeptide antigen (TPA), squamous cell carcinoma antigen, stem cell factor (SCF), GM-CSF, and VEGF, have demonstrated associations with NSCLC; however, each of these has failed to demonstrate the requisite sensitivity and specificity to warrant clinical development as diagnostic tools (7–11). A number of multianalyte panels comprised of both circulating proteins (12, 13) and tumor-associated autoantibodies (14, 15) have been evaluated with encouraging results. Recently, urine has been proposed as an alternative biofluid for analytical biomarker studies on the basis that the systemic information gained from such testing might be preserved while several of the limitations inherent to the use of blood could be eliminated. Urine is available in larger quantities than blood through less invasive means, allowing for repeated measurements aimed at patient surveillance or longitudinal studies. The urinary proteome is a direct product of renal filtration and consists of low molecular weight, soluble peptides that are highly amenable to proteomic analysis and may represent disease-specific cleavage processes. Renal filtration also results in a less complex matrix than that of blood, containing fewer factors known to interfere with biomarker assays (16). Studies have shown that this proteome is stable for hours at room temperature, days at 4°C, and years at −20°C (17).

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What remains in the development of urine-based analytical platforms is evidence that systemic disease-specific biomarkers are released into this biologic compartment in a manner which can be reliably measured and utilized for diagnostic means.

Effective biomarker-based diagnostic tools have the potential to serve as alternatives or adjuncts to CT scanning for lung cancer. Investigators participating in the National Lung Screening Trial, a randomized multicenter trial involving more than 53,000 current and former smokers, recently released findings indicating a 20% reduction in lung cancer death in individuals screened by low-dose helical CT versus standard chest X-ray (18). These encouraging findings illustrate the promise offered by CT-based screening if certain limitations inherent to the technique can be overcome. The FDA has specified criteria for effective screening in reference to the use of CT in pulmonary cancer. Among these criteria, proposed by investigators at the Cleveland Clinic (19), is the requirement that any screening test directed at a disease with a prevalence of 5% or less must detect preclinical disease with a sensitivity exceeding 95% when the specificity is less than or equal to 95%, and vice versa. Current estimates place the prevalence of lung cancer in high-risk groups at 1% to 3% (6, 20), well below the 5% threshold, whereas the overall sensitivity/specificity of CT screening in this setting was recently reported at 90/92.6 (21). Thus, a biomarker-based test providing improved levels of sensitivity/specificity may successfully augment the performance of CT and provide a basis for targeted screening of high-risk groups, such as smokers. Alternatively, biomarker panels may be used in the triage of patients at increased suspicion of lung cancer to facilitate the timely referral of patients more like to be diagnosed with malignancy and reduce the level of unnecessary testing and surgery currently being performed.

Urines obtained from patients with NSCLC, healthy controls, and individuals diagnosed with either benign lung abnormalities or pulmonary tuberculosis were evaluated using a large array of biomarker candidates. A bioinformatic analysis of the urine biomarker data identified a panel of three biomarkers capable of discriminating cases from controls with a high level of sensitivity and specificity.

Materials and Methods

Human urines

Urines collected from 83 patients diagnosed with NSCLC, 74 patients diagnosed with a variety of benign lung conditions, 28 patients diagnosed with pulmonary tuberculosis, and 77 healthy donors were obtained from Proteogenex Inc. (Table 1; Supplementary Table S1). All subjects were over the age of 18 and provided written informed consent. Pregnant women and subjects with a history of blood-borne illness were excluded. Urines were obtained before definitive treatment (surgery/chemotherapy/radiation) or on the day of surgery for patients with NSCLC and benign controls. Diagnoses for patients with NSCLC and benign controls were confirmed by clinical and pathologic evaluation. Some benign controls were symptomatic of lung disease at the time of collection. The most commonly reported symptoms were weakness, cough, fatigue, fever, chest pain, dyspnea, and blood in sputum. Healthy donors were cancer free and free of pulmonary illness at the time of donation. All urines were spot collected at the time of medical visit or donation. Specific collection times were not reported or utilized in the current analysis. Urines were frozen at -70°C or -80°C within one hour of collection and remained frozen until testing. Each urine sample was annotated with information about age, gender, ethnicity, histology (NSCLC, benign, pulmonary tuberculosis), stage (NSCLC), and smoking history. All patients and donors were Caucasian. The training and validation sets were received and tested separately.

Urines obtained from patients diagnosed with breast ($n = 25$) or prostate ($n = 25$) cancer were utilized in the analysis of cancer specificity for selected biomarker panels. Urines were obtained from patients diagnosed with various stages of disease and were collected by the Health Sciences Tissue Bank at the University of Pittsburgh Medical Center. All urines were collected and processed in the same manner as that described above.

Urine biomarker analysis

Urines were evaluated for levels of 242 cancer-related protein biomarkers (Table 2) using multiplexed bead-based immunoassays. The biomarker list was compiled based on a literature review of current proteins of interest within all fields related to lung cancer research. Biomarkers were selected from this list on the basis of suitable bead-based immunoassay availability. A total of 51 multiplexed panels were utilized in this study. Commercially available assays were obtained from Millipore, Novagen/EMD Chemicals, and R&D Systems and were performed according to the manufacturer instructions. The remaining multiplexed panels were developed according to strict quality control standards by the UPCI Luminex Core Facility (22) and were performed as described previously (23), with the exception that all urine samples were run undiluted. All biomarker testing were performed on undiluted samples immediately upon thawing without concentration of proteins or other pretest manipulation. Urines included in the training set were tested for the complete set of 242 analytes. Benign controls, patients with pulmonary tuberculosis, prostate cancer, and breast cancer were tested for a subset of 35 analytes. The selection of analytes for inclusion within this subset began with those biomarkers identified as most useful in the training set. A number of additional analytes of interest were also evaluated based on their inclusion in multiplexed panels utilized above. For the analysis involving patients with pulmonary tuberculosis, the training and validation sets were combined for the NSCLC and Healthy groups to increase the power of the study. Urine creatinine (UCr) levels were determined for each sample using the Creatinine Parameter Assay Kit (R&D Systems) and were used to evaluate the need for normalization of urine biomarker levels to account for variation in fluid intake.

Statistical analysis

Biomarker measurements among the NSCLC and control groups were evaluated by the Mann–Whitney nonparametric *U* test. An initial minimum level of significance of $P \leq 0.05$ was utilized. The FDR was controlled at 5% according to the method of Benjamini and Hochberg (24). Biomarker results were normalized according to UCr levels by dividing the fluorescence intensity level for each biomarker measurement by the UCr level expressed in mg/dL. Correlations in biomarker levels were examined using the Pearson test for correlation in Graphpad Prism.

Multivariate analysis

A Metropolis algorithm with Monte Carlo optimization (MMC) was used for the multivariate analysis of the biomarker results as described previously (25). Briefly, biomarker combinations of a

Table 1. Clinical characteristics of study population

	Training	Validation
Healthy		
n	49	28
Age range (median)	46–64 (57)	41–65 (56)
Male	28 (57%)	18 (64%)
Female	21 (43%)	10 (36%)
Smoking status		
Nonsmoker	16 (33%)	22 (79%)
Smoker	32 (65%)	6 (21%)
Unknown	1	0
Mean pack years	15	4
NSCLC		
n	54	29
Age range (median)	38–77 (65.5)	48–79 (64)
Male	40 (74%)	22 (76%)
Female	14 (26%)	7 (24%)
Smoking status		
Nonsmoker	10 (19%)	4 (14%)
Smoker	36 (67%)	23 (79%)
Unknown	8	2
Mean pack years	34	36
Adenocarcinoma	8 (15%)	8 (28%)
Squamous cell carcinoma	34 (63%)	14 (48%)
BAC	2 (3.5%)	2 (7%)
Undifferentiated	2 (3.5%)	0
NOS	8 (15%)	5 (17%)
Stage IA–IB	17 (31%)	10 (34%)
Stage IIA–IIB	12 (22%)	0
Stage IIIA–IIIB	14 (26%)	9 (31%)
Stage IV	4 (8%)	6 (21%)
Stage unknown	7 (13%)	4 (14%)
Benign		
n		74
Age range (median)		22–74 (52)
Male		48 (65%)
Female		26 (35%)
Histology[a]		
Benign neoplasm		52 (20%)
Cyst		8 (11%)
Other		10 (14%)
Unknown		4 (5%)
Symptomatic		47 (64%)
Asymptomatic		27 (36%)
Smoking status		
Nonsmoker		39 (53%)
Smoker		35 (47%)
Mean pack years		22
Pulmonary tuberculosis		
n		28
Age range (median)		22–61 (36)
Male		19 (68%)
Female		9 (32%)
Smoking status		
Smoker		27 (96%)
Nonsmoker		1 (4%)
Mean pack years		11
Histology		
Fibrous-cavernous		12 (43%)
Infiltrative		5 (18%)
Tuberculoma		7 (25%)
Focal		1 (4%)
Tuberculosis, NOS		3 (11%)
Symptomatic		18 (64%)
Asymptomatic		10 (36%)

Abbreviations: BAC, bronchioalveolar carcinoma; NOS, not otherwise specified.
[a]Complete breakdown of benign diagnoses provided in Supplemental Table S1.

predetermined size are randomly assembled. A scoring function (SF) is then calculated for each biomarker panel as a linear combination of biomarker concentrations multiplied by a coefficient for each biomarker assigned by Monte Carlo optimization. The resulting set of SFs for each biomarker combination are then evaluated for classification efficiency using 10% cross-validation. To avoid overfitting bias, our analysis was limited to panels consisting of two, three, or four biomarkers. Panels were evaluated based on sensitivity at predetermined specificity levels of 90% and 95%. A level of 95% specificity was chosen for the discrimination of patients with NSCLC from healthy controls to identify models capable of minimizing false-positive results. All multivariate analyses were restricted to the training set until the highest performing panels were identified. Urines in the validation set were then tested for each of the biomarkers included in the top performing panels, and the model was applied to the results. The predicted diagnosis for each sample, based on the derived scoring function, was compared with the actual diagnosis to evaluate the accuracy of the model. Receiver operating characteristic (ROC) curves were constructed from the algorithm results, and the area under the ROC curve (AUC) was determined using GraphPad Prism. Because of the concern that discrepancies in smoking history between the case and control groups may contribute to biomarker panel performance, the MMC algorithm was applied to the biomarker results in a separate analysis that included pack years (PY) as an independent variable.

Results

Urine biomarker analysis

When the biomarker measurements were analyzed with the FDR controlled at 5%, 37 biomarkers were found to be significantly altered between the patients with NSCLC and healthy controls with a maximum *P* value of 0.0037 (Table 3). Ferritin and C-reactive protein (CRP) were the most significantly altered biomarkers in this analysis, demonstrating elevated levels in patients with NSCLC, followed by thrombospondin, which was significantly decreased in the NSCLC group. CEACAM-1 was also among the most significantly altered biomarkers, demonstrating elevated levels in patients with NSCLC. Among all of the significantly altered biomarkers, roughly half were observed to be increased in the NSCLC group, whereas half were decreased. When the NSCLC and benign groups were compared, only 3 biomarkers were observed to be significantly altered (Table 3). CEACAM-1 was the most significantly altered biomarker in this analysis, followed by ferritin and CRP. For each of these biomarkers, levels were significantly higher in NSCLC cases in comparison with the benign controls. The distributions of the most significantly altered biomarkers in the NSCLC, benign, and healthy groups are presented in Fig. 1A.

The analysis of UCr-normalized data resulted in alterations among the majority of individual biomarker *P* values; however, the relative significance of each biomarker was largely unaffected. The vast majority of biomarkers demonstrated an increase in significance. CRP remained the most significantly altered biomarker in both the NSCLC versus healthy and NSCLC versus benign comparisons. Ferritin and CEACAM-1 also remained among the most highly altered biomarkers; however, the significance of thrombospondin was markedly reduced. *P* value alterations tended to be most severe among biomarkers of lesser significance (data not shown).

The urines of patients with pulmonary tuberculosis were evaluated for a subset of 35 biomarkers and compared with the other

Table 2. Complete list of multiplexed analytes

Inflammation	6Ckine[a], Adiponectin[a], BCA-1[a], BLC/CXCL13[a], CCL14α/HCC-1[a], CCL19/MIP3β[a], CCL20/MIP3α[a], CD137/4-1BB[a], CD30[a], CD40L[a], CTACK[a], CXCL11/I-TAC[a], CXCL6/GCP2[a], CXCL7/NAP2[a], CXCL9/MIG[a], ENA-78[a], Eotaxin 1, 3[a,e], Flt-3L[a], Fractalkine[a], G-CSF[a], GM-CSF[a], gp130[a], I-309[a], IL1β, 6, 8, 11, 16, 20, 21, 23, 28α, 29, 33[a,e], IL1RI, 1RII, 2Rα, 4R, 6R[a,e], INFβ[a], INFω[a], IP-10[a], LIF[a], MCP-1, 2, 4[a,e], M-CSF[a], MIF[a], MIP-1δ, 4[a,e], MPO[b], NGAL[a], OC[a], OPG[a], OPN[a], OSN[a], Perforin[a], RAGE[a], RANKL[a], RANTES[a], SCF[a], SDF-1α+β[a], TARC[a], TNF α, β[a,e], TNF RI, RII[a,e], TSLP[a], XCL1/Lymphotactin[a]
Growth/ angiogenesis	Amphiregulin[b], Ang-2[c], angiogenin[b], angiostatin[c], ANGPTL 3, 4, 6[a,e], BDNF[a], Betacellulin[b], CD-105[c], CNTF[a], EGF[b], EGFR[b], endostatin[c], epiregulin[b], ErbB2[c], FGF-19, 21, 23[a,e], FGF-β[b], HB-EGF[b], HGF[a], IGF-1R[b], IGFBP-1-7[a,e], NGF[a], PDGF-AA, BB, AA/BB[a,e], TGFα[a], Thrombomodulin[b], Thrombospondin[c], VEGF[b], VEGFR 1-3[a,e]
Tumor markers	AFP[a], CA-125[c], CA15-3[c], CA19-9[c], CA72-4[c], CEA[c], Cytokeratin19[c], EPCAM[c], fPSA[c], HE4[c], HER-2[b], Mammaglobin[c], Mesothelin[c], tPSA[c], SCC[c]
Endocrine	ACTH[a], alpha MSH[a], Cortisol[a], ERα[b], FSH[a], GH[a], LH[a], Melatonin[a], NT-Pro-BNP[b], Orexin A[a], Oxytocin[a], progesterone receptor[b], Prolactin[a], PTH[a], TSH[a]
Metabolism	AGRP[a], Amylin (total)[a], Apo AI, AII, B, CII, CIII, E[a,e], C-Peptide[a], FABP1[a], Ghrelin[a], GIP[a], GLP-1[a], Glucagon[a], H-FABP[b], Insulin[a], Leptin[a], MDA-LDL[b], PP[a], PYY[a], TPO[a]
Serological	α2-Macroglobulin[a], β2-Microglobulin[a], Complement H, C3, C4[a,e], CRP[a], Ferritin[a], Fetuin-A[b], HSA[a], PAI-1[b], Prealbumin[a], SAA[a], SAP[a], vWF[b]
Proteases/PIs	α1-Antitrypsin[a], CathepsinD[a], Kallikrein10[c], MMP-1-3, 7–9, 12, 13[d,e], TIMP 1-4[d,e], tPAI-1 (total)[a], uPA[b]
Apoptosis	Bcl-2[c], Granzyme A, B[a,e], Hif1α[c], sFAS[a], sFASL[a], TRAIL[a]
Adhesion	ALCAM[c], CEACAM-1, 6[c,e], E-Cadherin[b], E-Selectin[a], Fibronectin[a], ICAM-1[a], NCAM[a], PIGF[b], Tenascin C[b], VCAM-1[a]
Other	β-Endorphin[a], Calbindin[b], Clusterin[a], CystatinC[a], DKK-1[a], EN-RAGE[b], GSTα[b], GSTπ[b], HSP27 (Total)[b], HSP60[b], HSP70[b], HSP90α[b], Involucrin[a], Keratin-1, 10, 11[a], Keratin-6[a], KIM-1[a], LOX-1[b], Lp(a)[b], LPS[a], MICA[c], Neurotensin[a], NSE[c], Oncostatin[c], PBEF[c], PEDF[a], pHSP27 (Ser78/Ser82)[b], Renin[a], Substance P[a], TFF-3[a], TgII[c], THP[b]

[a]Millipore; [b]Novagen/EMD; [c]UPCI Luminex Core Facility; [d]R&D Systems (immunoassay suppliers); [e]family members measured separately.

experimental groups (Supplementary Table S2). Of the 35 evaluated biomarkers, 21 were observed to be significantly altered in the comparison of patients with pulmonary tuberculosis and healthy controls. In the comparison of patients with pulmonary tuberculosis and NSCLC, 14 biomarkers were observed to be significantly altered. A separate set of 12 biomarkers was observed to be significantly altered in the comparison of the pulmonary tuberculosis and benign groups.

We noted a high degree of variability in the proportion of smokers and nonsmokers among the NSCLC and control groups and among the training and validation sets (Table 1). To address this, urines from the training set were combined and redistributed on the basis of smoking status. Subjects of unknown smoking history were excluded. Each of the urine biomarkers listed in Table 3 was examined in these two groups as described in Materials and Methods. After controlling the FDR at 5%, none of the tested biomarkers were found to differ significantly between smokers and nonsmokers. The effect of gender on urine biomarker levels was examined in the same manner. A total of 21 biomarkers were observed to differ significantly among males and females. Age was evaluated as a potential confounder using the Pearson test of correlation. Six biomarkers were observed to correlate with age ($P < 0.05$). The complete list of biomarkers significantly associated or correlated with smoking status, gender, and age is presented in Supplementary Table S3.

Biomarker panel analysis

When the MMC algorithm was applied to the training set data, the top performing two-biomarker combinations identified each included IGFBP-1 and either Kal10, ferritin, or sIL-1Ra. Each of these combinations outperformed the best individual markers in terms of sensitivity; however, ROC AUC values were comparable. The top performing three-biomarker panels each included combinations of IGFBP-1, ferritin, CEACAM-1, and/or sIL-1Ra and each provided similar performance in the training set. Each of these panels provided notably elevated levels of sensitivity in comparison with the two-biomarker combinations and individual markers and AUC values which were similar or modestly increased in some cases. The top performing four-biomarker combinations each included the combination of IGFBP-1, ferritin, CEACAM, and either ALCAM or HE4. Both panels performed nearly identically in the training set and provided notable improvements in sensitivity and AUC over the smaller panels and individual markers. Each of the top performing two-, three-, and four-biomarker panels were further evaluated for performance in the discrimination of NSCLC and benign controls. The complete results of this analysis are presented in Table 4. Overall, performance was marked diminished in this analysis in comparison with the discrimination of NSCLC and healthy controls.

Each of the biomarkers panels identified using the MMC algorithm and listed in Table 4 was further evaluated for disease selectivity in patients diagnosed with a variety of benign pulmonary lesions, pulmonary pulmonary tuberculosis, prostate cancer, and breast cancer (Table 5). Several trends were observed in this analysis. The inclusion of Kal10 in two-biomarker combinations resulted in a high degree of disease selectivity; however, this marker was not found to be useful in the classification of NSCLC versus healthy in larger panels. Ferritin was included in many of the high performing panels; however, this marker was associated with poor disease selectivity. A general trend was noted wherein selectivity diminished as panel size increased; therefore, although the identified four-biomarker panels offered the highest cancer classification performance, disease selectivity was notably poor. On the basis of these findings, we concluded that the three-biomarker panel comprised of IGFBP-1, sIL-1Ra, and CEACAM-1 provided the most efficacious combination of cancer classification and disease selectivity.

The panel of IGFBP-1, sIL-1Ra, and CEACAM-1 was further evaluated in the validation set where it provided a sensitivity of 72% at a specificity of 100% (Table 4). The two alternative three-biomarker panels listed in Table 4 were also evaluated in the validation set based on their similar performance characteristics in the training set and among benign controls. In the validation set, the panel of IGFBP-1, sIL-1Ra, and CEACAM-1 provided the highest AUC level. ROC curves demonstrating the overall performance of each panel in the training and validation sets as well as in

Table 3. Urine biomarker levels in patients with NSCLC and controls

		Median urine levels				
Marker	**Units**	**Healthy**	**Benign**	**NSCLC**	**NSCLC vs. healthy[a]**	**NSCLC vs. benign[a]**
Ferritin	pg/mL	2,257	5,916	15,444	7.8×10^{-10}	1.9×10^{-5}
CRP	pg/mL	143	171	1,265	1.7×10^{-9}	2.4×10^{-5}
Thrombospondin	pg/mL	27,771	5,983	2,347	7.0×10^{-8}	NS
CEACAM-1	pg/mL	3,199	4,504	8,112	2.2×10^{-7}	1.7×10^{-5}
FGF-21	pg/mL	8.61	3.69	3.43	2.8×10^{-6}	NS
sCD30	pg/mL	5.39	6.39	6.56	4.4×10^{-6}	NS
Lp(a)	pg/mL	0.773	0.979	0.915	5.0×10^{-6}	NS
TSH	uIU/mL	0.087	0.138	0.197	8.0×10^{-6}	NS
PDGF-AA	pg/mL	442	309	149	1.1×10^{-5}	NS
HE4	pg/mL	59,738	102,729	210,120	1.6×10^{-5}	NS
MIF	pg/mL	632	282	171	1.9×10^{-5}	NS
FSH	mIU/mL	4.55	10.5	12.9	1.9×10^{-5}	NS
PBEF	pg/mL	66,259	54,502	31,156	9.0×10^{-5}	NS
Involucrin	pg/mL	2,213	597	408	1.1×10^{-4}	NS
OPG	pg/mL	54.5	90.6	83.9	1.4×10^{-4}	NS
Endostatin	pg/mL	1,060	544	395	2.3×10^{-4}	NS
sIL-1Ra	pg/mL	130	58.6	20.1	2.5×10^{-4}	NS
TNFB	pg/mL	0.713	0.674	0.640	2.6×10^{-4}	NS
LPS	pg/mL	24.5	20.1	19.8	3.7×10^{-4}	NS
Complement C3	ng/mL	1.43	0.833	0.280	5.0×10^{-4}	NS
IGFBP-1	pg/mL	79.4	220	232	8.3×10^{-4}	NS
C-Peptide	pg/mL	13,935	11,462	11,373	1.1×10^{-3}	NS
IL6	pg/mL	1.04	3.67	2.30	1.2×10^{-3}	NS
Fibronectin	pg/mL	3,581	2,735	2,047	1.3×10^{-3}	NS
Kallikrein10	pg/mL	3,058	2,182	1,415	1.4×10^{-3}	NS
AFP	pg/mL	31.3	28.4	23.2	1.5×10^{-3}	NS
sCD137/4-1BB	pg/mL	9.23	16.1	14.9	1.7×10^{-3}	NS
G-CSF	pg/mL	0.292	0.316	0.251	1.7×10^{-3}	NS
TIMP-1	pg/mL	7.60	24.6	21.3	1.8×10^{-3}	NS
MMP-13	pg/mL	9.30	10.2	10.8	1.9×10^{-3}	NS
NGAL	pg/mL	2,353	3,105	3,446	2.0×10^{-3}	NS
Clusterin	pg/mL	87,916	152,174	154,297	3.0×10^{-3}	NS
HSP70	pg/mL	130	78.0	65.2	3.0×10^{-3}	NS
PDGF-AB/BB	pg/mL	11.0	11.3	11.7	3.2×10^{-3}	NS
MMP-12	pg/mL	1.81	1.81	1.94	3.2×10^{-3}	NS
LIF	pg/mL	8.81	9.64	9.54	3.6×10^{-3}	NS
CA72-4	U/mL	0.244	0.244	0.263	3.7×10^{-3}	NS

Abbreviation: NS, not significant.

[a]*P* value calculated by the Mann–Whitney *U* test with FDR controlled at 5%.

the benign controls are presented in Fig. 1B. The selected panel was further evaluated for the ability to correctly classify specific subgroups of patients with NSCLC and benign controls (Supplementary Table S4). At a specificity of 95%, the panel correctly classified 69% of adenocarcinomas, 88% of squamous cell carcinomas, and 75% of bronchioalveolar carcinomas from the pool of healthy controls. This panel also correctly identified 23 of 27 (85%) early-stage (stage 1A/1B) cancers. To further evaluate the impact of smoking history on our analysis, the panel was applied separately to smokers and nonsmokers within the NSCLC group and found to perform moderately better (83% vs. 71% accuracy) among smokers. This panel also correctly identified 94% of male cases and 100% of female cases. The panel correctly classified 62% of the benign controls and performed slightly better among symptomatic individuals within that group in comparison with asymptomatic individuals.

The MMC algorithm was also used to identify multimarker panels specifically able to discriminate patients with NSCLC from Benign controls and patients with pulmonary tuberculosis from healthy controls. The results of this analysis are presented in Supplementary Table S5. For the discrimination of patients with NSCLC from benign controls, the two most useful individual biomarkers were CRP and MCP-2. The combination of these two biomarkers was identified by both algorithms as the top performing two-biomarker panel and provided significant improvement over either biomarker alone. The addition of a third biomarker (sIL-1Ra, TNFα) to the CRP/MCP-2 combination resulted in further improvements in performance; however, the performance characteristics of the two resulting three-biomarker panels were nearly identical. Several four-biomarker panels were evaluated which each included the combination of CRP, MCP-1, and TNFα; however, each resulted in only a modest improvement in sensitivity. CRP was the highest performing individual biomarker for the discrimination of patients with pulmonary tuberculosis from healthy controls. The combinations of CRP/MCP-2 and cortisol/MCP-2 performed equally well and offered significant improvement over CRP alone. The combination of cortisol, MCP-2, and IL10 was identified as the best three-biomarker panel; however, this panel did not demonstrate any improvement over the two-biomarker combinations.

When PY was included as an independent variable in the multivariate analysis of NSCLC cases and healthy controls, it was not included among the 12 highest performing two-, three-, or four-biomarker panels identified. In the comparison of NSCLC and benign controls, PY was found to factor prominently among the two-biomarker combinations and was included in 4 of the top

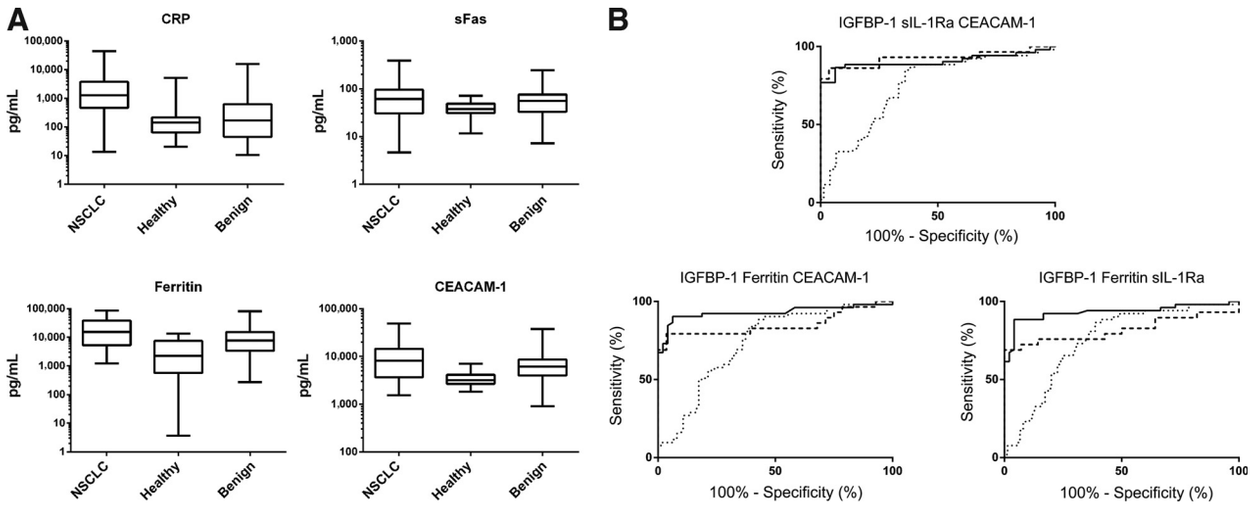

Figure 1.

Top performing urine biomarkers and multimarker panel. A, box and whisker plots of top performing urine biomarkers. Levels of 235 urine biomarkers were measured in patients with NSCLC ($n = 54$), healthy ($n = 49$), and benign ($n = 74$) controls. Biomarkers demonstrating the strongest performance in the classification of cases versus controls are shown. Boxes represent median with 25th and 75th percentiles. Whiskers represent maximum and minimum values. B, ROC analysis of selected multimarker panels. Performance of the top three 3-biomarker combinations in the classification of patients with NSCLC from healthy and benign controls. Solid line, NSCLC vs. healthy training set; dashed line, NSCLC vs. healthy validation set; dotted line, NSCLC vs. benign.

10 identified combinations (Supplementary Table S5). PY was not included among the 12 highest performing three- or four-biomarker combinations.

Discussion

Several urine biomarkers, including CRP, ferritin, and sFas, were found to perform particularly well on an individual basis in the discrimination of NSCLC from healthy controls, but demonstrated limitations as multimarker panel components. This was not surprising, particularly with regard to CRP and ferritin, as these factors represent acute phase reactants likely to be associated with disease in a nonspecific manner. Two recent studies that utilized subject cohorts nested within the large prospective Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial concluded that elevated serum levels of CRP, alone or in combination with IL6, were associated with a greater risk of lung cancer in prediagnostic samples (26, 27). In those studies, risk increased steadily with serum CRP levels and differed markedly among current and former smokers. Notably, the combination of CRP and IL6, measured in urine in the current study, was not effective in the discrimination of NSCLC from healthy controls (55% sensitivity at 95% specificity). Another recent study found increased ferritin levels in the sera of patients with NSCLC along with elevated expression in tumor samples (28). In that study, the authors concluded that the elevated serum ferritin levels were likely the result of inflammation and oxidative stress rather than body iron overload. This conclusion is in agreement with our finding that ferritin was considerably nonselective in the comparison of NSCLC with other disease states. Several *in vitro* studies in lung cancer cell lines have implicated sFas in tumor progression, immune evasion, response to chemotherapy, and metastasis (29–32). Although sFas was included in several preliminary multimarker panels, these panels were not among those selected for highest performance.

The urine biomarkers CEACAM-1, sIL-1Ra, and IGFBP-1 were selected for inclusion in our optimally performing panel. The adhesion molecular CEACAM-1 has been previously shown to mediate the formation of a proangiogenic tumor microenvironment that supports tumor vessel maturation in a transgenic

Table 4. Multimarker panel performance in the classification of patients with NSCLC from healthy and benign controls

	NSCLC vs. healthy (training)			NSCLC vs. benign			NSCLC vs. healthy (validation)		
	SN	SP	AUC (95% CI)	SN	SP	AUC (95% CI)	SN	SP	AUC (95% CI)
CRP	57	95	0.931 (0.885–0.978)	39	90	0.753 (0.654–0.852)			
sFas	57	95	0.917 (0.861–0.973)	4	90	0.578 (0.414–0.642)			
Ferritin	62	95	0.856 (0.785–0.926)	32	90	0.713 (0.623–0.803)			
CEACAM-1	63	95	0.801 (0.708–0.893)	22	90	0.685 (0.590–0.780)			
IGFBP-1 Kal 10	82	95	0.873 (0.795–0.952)	82	58	0.737 (0.645–0.829)			
IGFBP-1 Ferritin	78	95	0.902 (0.837–0.968)	78	58	0.727 (0.638–0.816)			
IGFBP-1 sIL-1Ra	75	95	0.872 (0.797–0.947)	75	65	0.720 (0.631–0.810)			
IGFBP-1 Ferritin CEACAM-1	86	95	0.930 (0.873–0.988)	86	57	0.736 (0.649–0.824)	79	92	0.850 (0.738–0.962)
IGFBP-1 sIL-1Ra CEACAM-1	84	95	0.905 (0.837–0.974)	84	62	0.745 (0.657–0.832)	72	100	0.926 (0.848–1.00)
IGFBP-1 Ferritin sIL-1Ra	88	95	0.932 (0.877–0.987)	88	57	0.751 (0.665–0.836)	72	85	0.819 (0.697–0.940)
IGFBP-1 Ferritin CEACAM-1 ALCAM	92	95	0.951 (0.902–0.999)	92	50	0.743 (0.657–0.829)			
IGFBP-1 Ferritin CEACAM-1 HE4	92	95	0.954 (0.906–1.00)	92	49	0.756 (0.672–0.841)			

Abbreviations: SN, sensitivity; SP, specificity.

Table 5. Performance of multimarker panels in non-NSCLC conditions

	Correctly classified as non-NSCLC (%)[a]			
	Benign lung	TB	Prostate cancer	Breast cancer
IGFBP-1 Kal 10	58	50	88	64
IGFBP-1 Ferritin	58	25	24	40
IGFBP-1 sIL-1Ra	65	58	76	72
IGFBP-1 Ferritin CEACAM-1	57	20	16	36
IGFBP-1 sIL-1Ra CEACAM-1	62	33	68	60
IGFBP-1 Ferritin sIL-1Ra	57	25	28	44
IGFBP-1 Ferritin CEACAM-1 ALCAM	50	20	28	44
IGFBP-1 Ferritin CEACAM-1 HE4	49	20	16	16

Abbreviation: TB, pulmonary tuberculosis.

[a]Performance at 95% specificity for NSCLC versus healthy controls.

mouse model (33). A separate study found CEACAM-1 expression in 81.3% of primary tumors from patients with NSCLC with preserved expression in lymph node and hematogenous metastases which was negatively correlated with overall and progression-free survival (34). An association between NSCLC and serum CEACAM-1 levels has been reported recently (35). To the best of our knowledge, the current study is the first to report such an association regarding urine CEACAM-1 levels. The anti-inflammatory cytokine sIL-1Ra was found to be associated with increased lung cancer risk in a prospective analysis of patients enrolled in the PLCO trial (36). This cytokine was also included in two multimarker panels capable of risk stratifying screen detected pulmonary nodules and discriminating patients with NSCLC from a group of high-risk individuals in separate studies (37, 38). A specific role or association for IGFBP-1 in NSCLC has yet to be described, although expression of this marker has been described in NSCLC cell lines on a limited basis (39, 40).

Our group previously reported on the use of serum biomarkers as screening tools for NSCLC in a similarly designed study (41), and a comparison of this and the current studies provides several noteworthy observations. Macrophage migration inhibitory factor (MIF) was an essential component of the top biomarker panels in the previous serum study; however, although it was found to be significantly altered, it was not included in the top performing panels in the current study. In the previous study, MIF was significantly elevated in the sera of patients with NSCLC with respect to controls, whereas in the current study involving urine, the opposite trend was observed. The former observation is consistent with the emerging role of MIF in NSCLC as an autocrine/paracrine driver of tumor development. The present finding in urine may be indicative of increased retention of MIF by diseased tissues or of some mechanistic alteration in the renal filtration of MIF. A similar observation was made for sIL-1Ra, which was also found to demonstrate opposing trends in serum and urine. Several additional biomarkers including IGBPB-1, thrombospondin, HE4, and Complement C3 all displayed consistent trends in alterations between the two studies. Thus, the relationship between serum and urine biomarkers exhibits considerable complexity, which appears to be incompletely explained by glomerular filtration.

The use of spot-collected urines in the current study raises questions regarding the temporal reproducibility of biomarker measurements within individuals. The current study was not designed to evaluate this; however, this question was addressed recently (42). In that study, variations in biomarker measurements were assessed in individuals on an intra- and interday basis. The level of variability was typically on the order of 25% to 100% and differed considerably among biomarkers. The degree to which this variability corrected upon creatinine normalization also varied considerably among evaluated biomarkers, and in several cases, creatinine normalization resulted in an increase in variability. Although each of the biomarkers reported in that analysis was included in the current study, the biomarkers included in the IGFBP-1, sIL-1Ra, CEACAM-1 panel were not evaluated in the previous study. However, we would reasonably expect each of these biomarkers to display similar ranges of variability and further note that the magnitude of differences among cases and controls reported in the current analysis far exceeds the previously reported range of variability within individuals. Our observations regarding creatinine normalization appear to support this notion, as the most highly altered biomarkers remained highly significant after normalization, whereas biomarkers of lesser significance were affected more profoundly.

Our validated panel was only moderately selective in the discrimination of NSCLC from several other disease conditions, including pulmonary tuberculosis, breast cancer, and prostate cancer, indicating a potential limitation to this type of screening approach. Our analysis also consistently demonstrated a higher degree of biomarker alteration and discriminatory performance when NSCLC cases were compared with healthy versus benign controls. We, therefore, conclude that nonspecific pathologic biomarker responses are contributing to our findings. Thus, further studies of this type will be necessary to identify additional biomarkers specific for NSCLC to expand the clinical utility of urine biomarker panels.

The IGFBP-1, sIL-1Ra, CEACAM-1 panel correctly identified 62% of the benign controls and performed slightly better in symptomatic versus asymptomatic individuals. The utility of a biomarker-based test as a tool for the differential diagnosis in the primary care setting would be defined by its ability to stratify patients based on lung cancer risk to aid in referral decisions. The prevalence of lung cancer among symptomatic individuals referred for additional testing varies considerably based on patient demographics, the training of the attending physician, the availability of necessary medical equipment, and facilities and other factors (43). If this prevalence is estimated to range from 5% to 30%, the panel would provide a positive-predictive value (PPV) ranging from 10% to 49% and a negative-predictive value ranging from 99% to 91%. The best alternative panels listed in Supplementary Table S5 provide a somewhat higher PPV range (27%–88%) and lower NPV range (98%–88%). Clearly, an improvement in PPV will be necessary to prevent a high level of unnecessary referrals and the attendant cost and patient anxiety. However, it is also clear based on the demonstrated NPVs that if implemented properly, biomarker tests of this type may provide means of ruling out unnecessary referrals among individuals who

would have been referred for additional testing according to current guidelines.

The classification efficiency achieved by the panel of IGFBP-1, sIL-1Ra, CEACAM-1 in our study compares favorably with a number of recent findings in serum involving protein biomarkers, microRNAs, proteomic profiles, and autoantibodies (13–15, 38, 44). The performance of this panel also exceeds that of our recently reported serum biomarker panel consisting of MIF, prolactin, and thrombospondin, which provided a sensitivity/specificity of 74/90 in a study of 164 patients with NSCLC and healthy controls (41). The evaluation of candidate biomarker panels in patients with early-stage cancer is likely the most critical aspect in panel development, given the potential to produce a stage shift among cancer diagnoses and improvements in survival trends. In the current study, the panel of IGFBP-1, sIL-1Ra, CEACAM-1 performed well among the patients with early-stage NSCLC included. The limited number of early-stage cancers included in our report does represent a limitation of the study; however, it is important to note that early-stage cancers represented a full one third of all cases evaluated, and the proportion of early-stage disease included in the validation set was higher than that of the training set. We noted a difference in the distribution of smokers and nonsmokers in our training and validation sets. However, based on our analysis of individual biomarkers and biomarker panels with respect to smoking history, the altered distribution does not appear to account for the difference in performance. Our analysis does indicate that smoking history factored significantly into efforts to identify biomarker panels useful in the comparison of NSCLC and benign controls, and this supports our overall assessment that the urine biomarkers included in this study were not highly effective in the discrimination of NSCLC from benign controls.

The performance of the IGFBP-1, sIL-1Ra, CEACAM-1 panel among NSCLC cases and healthy controls appears promising given the high levels of sensitivity and specificity achieved and the reproducibility of these characteristics in the validation set. The identified urine panel was accurate in each of the major NSCLC subtypes and among early-stage disease, but was limited in the discrimination of NSCLC from benign controls, patients with tuberculosis, and other cancers. Further evaluation of this panel and other candidate urine biomarker panels should focus more heavily on early-stage disease to confirm the performance reported here. The evaluation of urines obtained before diagnosis of NSCLC through prospective studies would further expand the translatability of these findings.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B.M. Nolen, A. Lomakin, A.E. Lokshin
Writing, review, and/or revision of the manuscript: B.M. Nolen, A.E. Lokshin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Marrangoni, A.E. Lokshin
Study supervision: A.E. Lokshin

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