ORIGINAL ARTICLE

Exosomal Proteins as Diagnostic Biomarkers in Lung Cancer

Birgitte Sandfeld-Paulsen, MD,[a,*] Kristine Raaby Jakobsen, MSc,[a,b] Rikke Bæk, MSc,[c] Birgitte Holst Folkersen, MD, PhD,[d] Torben Riis Rasmussen, MD, PhD,[d] Peter Meldgaard, MD, PhD,[e] Kim Varming, MD, PhD,[c] Malene Møller Jørgensen, MSc, PhD,[c] Boe Sandahl Sorensen, MSc, PhD[a]

[a]*Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark*
[b]*Department of Biomedicine, Aarhus University, Aarhus, Denmark*
[c]*Department of Immunology, Aalborg University Hospital, Aalborg, Denmark*
[d]*Department of Pulmonary Medicine, Aarhus University Hospital, Aarhus, Denmark*
[e]*Department of Oncology, Aarhus University Hospital, Aarhus, Denmark*

Received 12 April 2016; revised 18 May 2016; accepted 29 May 2016
Available online - 22 June 2016

ABSTRACT

Introduction: Exosomes have been suggested as promising biomarkers in NSCLC because they contain proteins from their originating cells and are readily available in plasma. In this study, we explored the potential of exosome protein profiling in diagnosing lung cancers of all stages and various histological subtypes in patients.

Methods: Plasma was isolated from 581 patients (431 with lung cancer and 150 controls). The extracellular vesicle array was used to phenotype exosomes. The extracellular vesicle array contained 49 antibodies for capturing exosomes. Subsequently, a cocktail of biotin-conjugated CD9, CD81, and CD63 antibodies was used to detect and visualize captured exosomes. Multimarker models were made by combining two or more markers. The optimal multimarker model was evaluated by area under the curve (AUC) and random forests analysis.

Results: The markers CD151, CD171, and tetraspanin 8 were the strongest separators of patients with cancer of all histological subtypes versus patients without cancer (CD151: AUC = 0.68, $p = 0.0002$; CD171: AUC = 0.60, $p = 0.0002$; and TSPAN8: AUC = 0.60, $p = 0.0002$). The multimarker models with the largest AUC in the cohort of patients with all lung cancer histological subtypes and in the cohort of patients with adenocarcinoma only covered 10 markers (all cancer: AUC = 0.74 [95% confidence interval: 0.70–0.80]; adenocarcinoma only: AUC = 0.76 [95% confidence interval: 0.70–0.83]). In squamous cell cancer and SCLC, multimarker models did not exceed CD151 as an individual marker in separating patients with cancer from controls.

Conclusion: We have demonstrated exosome protein profiling to be a promising diagnostic tool in lung cancer independently of stage and histological subtype. Multimarker models could make a fair separation of patients, demonstrating the perspectives of exosome protein profiling as a biomarker.

© 2016 International Association for the Study of Lung Cancer. Published by Elsevier Inc. All rights reserved.

Keywords: Lung cancer; Exosomes; Diagnostic; EV array

Introduction

Lung cancer is the main cause of cancer-related death worldwide.[1] Several factors are associated with the poor outcome of patients with lung cancer. One factor is the lack of effective therapies. Another is the late diagnosis, because survival rates drop dramatically from early to late stages.[2] Today, standard diagnostic procedures include computed axial tomography scan, positron emission tomography scan, endoscopic bronchial ultrasound–guided/esophagus ultrasound–guided transbronchial needle aspiration and/or transthoracic biopsy.[3] And yet, diagnosing can be challenging. The computed axial tomography scan is a very

*Corresponding author.

Disclosure: The authors declare no conflict of interest.

Address for correspondence: Birgitte Sandfeld-Paulsen, MD, Department of Clinical Biochemistry, Aarhus University Hospital, Nørrebrogade 44, Aarhus C 8000, Denmark. E-mail: birgne@rm.dk

© 2016 International Association for the Study of Lung Cancer. Published by Elsevier Inc. All rights reserved.

ISSN: 1556-0864
http://dx.doi.org/10.1016/j.jtho.2016.05.034

sensitive tool but it has a low specificity, giving a high number of false positives.[4] Cytological specimens are the most used diagnostic samples but they not always obtainable, just as there is a risk of complications after a transthoracic biopsy of up to 15%.[5] Noninvasive or minimally invasive supplements to the diagnostic workup in lung cancer are an unmet clinical need.

Exosomes are defined as small vesicles (diameter 30–100 nm) derived from the multivesicular bodies and released by exocytosis.[6,7] Exosomes can carry cargo from their originating cell as functional messenger RNA, microRNA, or proteins.[8,9] Exosomes are released from cells to their surroundings and may play a role in intercellular communication.[10] They can be purified from several body fluids and thus, the biomarker potential of exosomes is intriguing. In the blood of patients with cancer, exosomes are found in a higher concentration than in matched control patients.[11–14] As a diagnostic marker, exosomes have been evaluated in several cancers, such as melanoma and ovarian, prostate, and cervical cancers.[13,15–19] Only a few smaller studies have explored the potential of exosomes as diagnostic biomarkers in NSCLC adenocarcinoma (AC).[12,20,21] So far, membrane proteins such as CD91, CD317, and EGFR, as well as a panel of 12 microRNAs, have been shown to be potential exosomal markers of NSCLC.[9,12,20,22] In a feasibility study by our group, we found that an adjusted protein microarray, the extracellular vesicle (EV) array, could be used for profiling exosomes in advanced-stage NSCLC ACs on the basis of their surface proteins.[21] The EV array captures extracellular vesicles fitting the description of exosomes being vesicles expressing CD9, CD63, and/or CD81 and having a size of 30 nm to 100 nm. Though exosome protein profiling appears promising, whether it is a robust diagnostic tool in AC of all stages has yet to be evaluated, just as whether a lung cancer–specific profile can be found in other NSCLC histological subclasses or in SCLC is unsettled.

Here, we set out to evaluate protein profiling of exosomes in lung cancer of all stages and histological subclasses and to explore the diagnostic potential of this technique in a clinical setting.

Materials and Methods

Patients and Plasma Samples

Prospectively, 1739 patients referred to the Department of Pulmonary Medicine because of suspected lung cancer were recruited from April 2011 until September 2014. Patients with NSCLC and SCLC were defined the lung cancer cohort and were used in this study (Fig. 1). As a reference cohort, 150 patients who had clinical features (age, smoking history, and clinical symptoms, as well as symptoms leading to suspicion of lung cancer) resembling those of patients with lung cancer but did not actually have lung cancer were included consecutively. Patients with other kinds of cancer were excluded. At time of inclusion, clinicopathological characteristics were collected. Blood samples (ethylenediaminetetraacetic acid plasma) were collected before any diagnostic workup was done and used in this study. The blood samples were spun (1400 *g* for 15 minutes) to isolate plasma. After isolation, the plasma was stored at −80°C.

All patients gave informed written consent before inclusion, and the Central Denmark Region Committees on Biomedical Research Ethics approved the study (M-20100246).

Exosome Protein Profiling Using the EV Array

Production of the Microarray. Antibodies were printed on epoxy-coated slides (75.6 × 25.0 mm; SCHOTT Nexterion, Lyngby, Denmark) using a SpotBot Extreme Protein Edition Microarray Printer with a 946MP4 pin (ArrayIt Corporation, Sunnyvale, CA). Biotinylated human immunoglobulin G (100 mg/mL) was used as positive control and phosphate-buffered saline (PBS) with 5% glycerol was used as negative control. After printing, the slides were left to dry at room temperature overnight before further analysis.

Antibodies for Production of the EV Array. A total of 49 antihuman antibodies, including the following, were used with the corresponding clone, if available: carbonic anhydrase 9 (2D3), CD146 (P1H12), CD147, claudin1, flotillin-1, heparin binding EGF like growth factor (4G10), erb-b2 receptor tyrosine kinase 3 (2F9), erb-b2 receptor tyrosine kinase 4 (H4.77.16), heat shock protein 90 (IGF1), LDL receptor related protein 1 (8G1), N-cadherin (8C11), p53 (pAb240), sTn, tumor-associated glycoprotein 72 (0.N.561), and tumor susceptibility 101(5B7) (Abcam, Cambridge, MA); surfactant protein D (VIF11) (Acris Antibodies GmbH, Herford, Germany); EGFR and EGFRvIII (Antibodies-online.com); Alix (3A9) and CD63 (MEM-259) (Biolegend, San Diego, CA); erb-b2 receptor tyrosine kinase (29D8) (Cell Signaling Technology, Danvers, MA); CD9 and CD81 (LifeSpan BioSciences, Seattle, WA); surfactant protein antibody (6F10) (Novus Biologicals, Littleton, CO); carbonic anhydrase 12 (315), CD13 (498001), CD14 (50040), CD37 (424), CD82 (423), CD142 (323514), CD151 (210127), CD206, carcinoembryonic antigen (487609), mucin 1 cell surface associated (604804), tumor necrosis factor receptor 1, tumor necrosis factor receptor 2, and tetraspanin 8 (TSPAN8) (458811) (R&D Systems, Minneapolis, MN); amphiregulin (S-13), EpCam (0.N.277), 78 kDa glucose-regulated protein (N-20), Mucin16 (X306), New York esophageal squamous cell carcinoma-1 (E978), and leucyl cystidyl

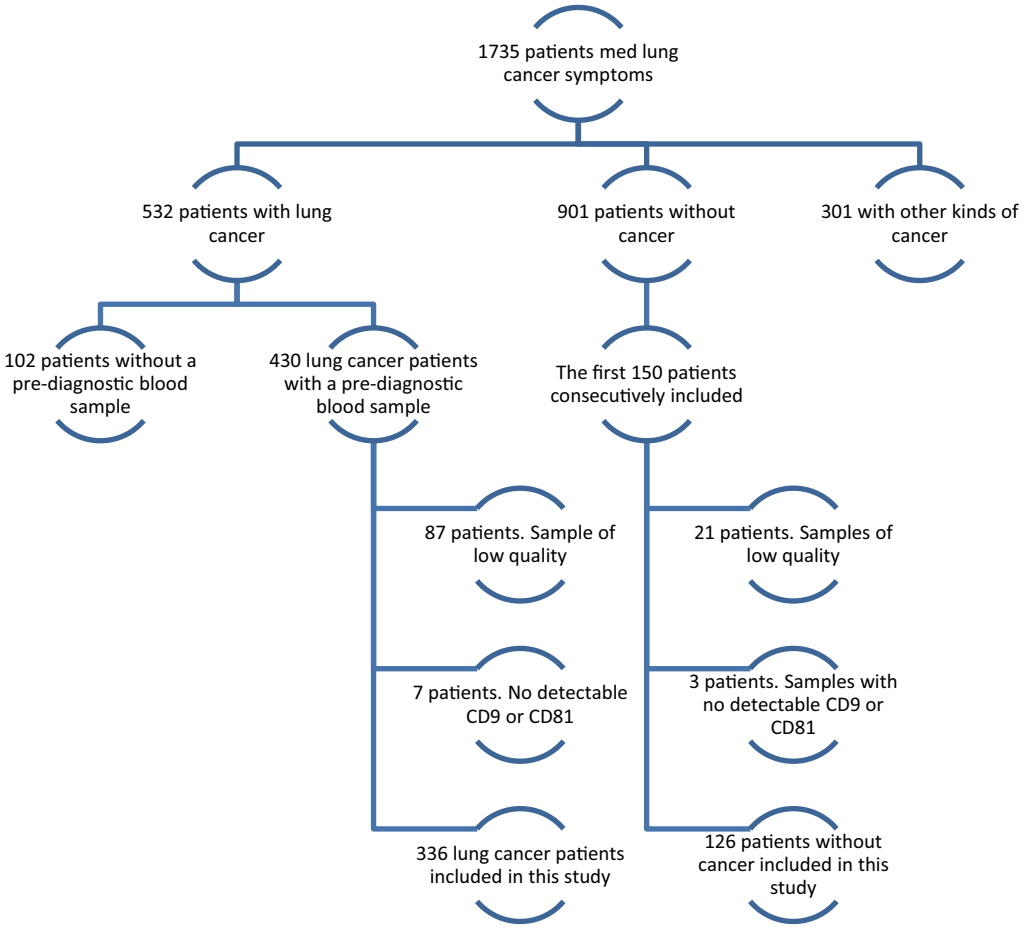

Figure 1. Flowchart describing the patient selection.

aminopeptidase (8B6) (Santa Cruz Biotechnologies, Dallas, TX); CD171 Sigma-Aldrich (St. Louis, MO, USA); c cellular mesenchymal-to-epithelial transition factor (016) and programmed death ligand 1 (Sino Biological Inc., Beijing, People's Republic of China); CD163 (Mac2-158) (Trillium Diagnostics, Bangor, ME); CD56(BD Biosciences, San Jose, CA); and CD276 (Sdix, Newark, DE).

All antibodies were diluted with PBS with 5% glycerol and printed in triplicates at 200 mg/mL.

Exosome Catching and Visualization. Preparation of the EV array was done as described by Jørgensen et al.[23] In short, the microarray slides were blocked (50 mM ethanolamine, 100 mM Tris, and 0.1% sodium dodecyl sulfate [pH 9.0]) before incubation with 10 μL unpurified plasma sample diluted (1:10) in wash buffer (0.05% Tween20 [Sigma-Aldrich] in PBS). The microarray slides were incubated in Multi-Well Hybridization Cassettes (ArrayIt Corporation) at room temperature for 2 hours followed by overnight incubation at 4°C. After a wash, the slides were incubated with biotinylated detection antibodies (antihuman CD9, antihuman CD63, and antihuman CD81, LifeSpan BioSciences) diluted 1:1500 in wash buffer. After washing, incubation for 30 min with cyanine 5–labeled streptavidin (Life Technologies, Carlsbad, CA) diluted 1:1500 in wash buffer was carried out for detection. Before scanning, the slides were washed in wash buffer followed by MilliQ water and dried using a Microarray High-Speed Centrifuge (ArrayIt Corporation). Scanning and spot detection were performed as previously described.[23]

Statistics

Data. Each antibody was printed in triplicate and the mean of the total signal was used to estimate the signal intensity. To exclude samples of low quality, the positive-to-negative ratio was calculated as (Positive – Negative)/Positive. If the positive-to-negative ratio was less than 0.98, the sample was excluded. To evaluate each triplicate, the coefficient of variance was calculated. If the coefficient of variance was greater than 0.3, the triplicate was excluded. The signal intensity of a given antibody was calculated by subtracting the mean of the negative triplicate (PBS spot). To evaluate the protein density on the exosomes, a normalization of each spot signal was made. The capture antibodies, CD9, CD81, and CD63, were evaluated as normalization factors. Because CD63

Table 1. Patient's Clinical and Pathological Characteristics

Characteristic	Cancer (n = 336)				No Cancer (n = 126)	p Value
	Adenocarcinoma (n = 199)	Squamous Cell (n = 71)	SCLC (n = 57)	Other (n = 9)		
Sex (males to females)	91:108	52:19	29:28	3:6	61:65	0.52
Median age (range)	67.7 (39-89)	69.6 (40-86)	68.6 (52-83)	63.0 (22-90)	64.5 (22-90)	0.0001
Smoking, n (%)[a]						
Former	127 (64)	49 (69)	36 (63)	1 (11)	86 (68)	0.37
Active	68 (34)	21 (30)	20 (35)	8 (89)	39 (31)	
Missing	3 (2)	1 (1)	1 (2)	0 (0)	1 (1)	
Stage, n (%)						
I	44 (22)	22 (31)	3 (5)	4 (44)		
II	11 (5)	11 (15)	3 (5)	1 (11)		
III	47 (24)	26 (35)	13 (23)	1 (11)		
IV	97 (49)	12 (17)	38 (67)	3 (33)		

Note: The *p* value refers to chi-square or Student's *t* test testing homogeneity of sex, age, or smoking status.
[a]Smoking status is based on self-reported information.

was expressed in only 379 of 472 samples, it was not considered valid as a normalization factor. CD9 and CD81 were expressed in 468 and 466 samples, respectively. The geometric mean of the two was used as normalization factor. For each antibody, the signal intensity was divided by the geometric mean of CD9 and CD81 before further analysis. Before area under the curve (AUC) analysis, data were log2-transformed.

Statistical Analysis. Clinical characteristics were compared by the Pearson chi-square test or Student's *t* test. Differences between groups were assessed by the Mann-Whitney rank sum test. All *p* values were two sided. To adjust for multiple comparisons, Bonferroni correction was applied and only *p* values less than 0.001 were considered significant ($\alpha = 0.05/49$ exosomal markers $= 0.001$). Missing values were replaced by the minimum value. Univariate receiver operating characteristic (ROC) and AUC were estimated, and a 95% confidence interval (CI) was calculated using 500 bootstrapping. In multivariate analysis, random forests was used for classification, ROC curves and AUC was made by Monte Carlo-cross validation by using balanced subsampling. Two-thirds of the samples were used as a test cohort and validated in the last third of the samples. To avoid positive overestimations, the procedure was repeated 500 times. AUC and ROC curves were compared by the algorithm developed by Hanley et al.[24] Statistical analyses were carried out in STATA software, version 13 (StataCorp, Dallas, TX), and ROCCET software, version 3.0 (TMIC, Edmonton, Alberta, Canada).[25]

Results

Patient Characteristics

In this study, 581 patients were included and 463 were eligible for analysis: 336 with lung cancer and 127 without cancer (see Fig. 1). Their clinical and pathological characteristics are described in Table 1. There was a similar distribution of clinical characteristics in the two groups; however, patients with advanced-stage squamous cell carcinoma (SCC) were underrepresented, with only 17% stage IV patients as compared with 45% expected.[2]

Exosomal Markers

Rank Sum. Univariate analysis of each marker was performed by comparing the reference cohort with the entire lung cancer cohort and with the AC, SCC, and SCLC subgroups, respectively. When the entire cancer cohort was compared with the reference cohort, several markers were deregulated in the patients with cancer compared with in those without cancer. After Bonferroni correction, however, only three markers were found to be significantly up-regulated in the cancer cohort ($p \leq 0.001$): CD151 ($p < 0.00001$), CD171 ($p = 0.0003$), and TSPAN8 ($p = 0.001$) (Table 2 and Fig. 2). CD151, CD171, and TSPAN8 were also up-regulated in the AC subgroup, although for TSPAN8 the upregulation was not significant ($p = 0.002$) as opposed to for CD151 ($p = 0.00001$) and CD171 ($p = 0.00001$). In SCC, CD151 and TSPAN8 were the only markers with a significant upregulation in the cancer group: CD151 ($p < 0.00001$) and TSPAN8 ($p = 0.0005$), whereas in SCLC, CD151 was the only marker significantly up-regulated ($p < 0.00001$) (see Fig. 2).

To evaluate whether the individual markers were affected by increasing stage, each group of patients with cancer was repeatedly split into two, comparing high stages to low stages (I versus II, III, and IV; I, II versus III, IV, and I, II, and III versus IV) (Table 3). In AC and SCLC, no marker was significantly increased or decreased with

Table 2. Exosomal Proteins as Individual Diagnostic Biomarkers

	All Cancer			AC			SCC			SCLC		
Markers	*p* Value[a]	AUC	*p* Value[b]	*p* Value[a]	AUC	*p* Value[b]	*p* Value[a]	AUC	*p* Value[b]	*p* Value[a]	AUC	*p* Value[b]
CD151	0.00001	0.68	0.0002	0.00001	0.68	0.0008	0.00001	0.69	0.001	0.00001	0.71	0.0002
CD171	0.0003	0.61	0.0002	0.00001	0.63	0.00002	ns	0.57	ns	ns	0.54	ns
TSPAN8	0.001	0.60	0.0002	0.002	0.60	0.0004	0.0005	0.61	0.003	ns	0.58	ns
HER2	0.003	0.58	0.003	0.006	0.58	0.006	0.02	0.59	0.04	ns	0.57	ns
Flotilin[c]	0.004	0.58	0.003	0.003	0.59	0.002	ns	0.54	ns	0.03	0.59	0.05
SFTPD	0.008	0.58	0.004	0.02	0.57	0.007	0.05	0.58	ns	ns	0.57	ns
NY-ESO-1[c]	0.009	0.57	0.01	0.01	0.58	0.02	ns	0.56	ns	ns	0.58	0.09[d]
CD9	0.01	0.57	ns	ns	0.56	ns	0.005	0.61	ns	ns	0.57	ns
CD81[c]	0.01	0.57	ns	ns	0.56	ns	0.005	0.57	ns	ns	0.57	ns
CD82[c]	0.02	0.57	ns	0.005	0.59	0.009	ns	0.57	ns	ns	0.50	ns
Mucin16[c]	0.02	0.53	0.03	0.01	0.54	0.02	ns	0.52	ns	ns	0.54	ns
EGFRvIII	0.03	0.56	0.05	ns	0.55	ns	ns	0.56	ns	ns	0.57	ns
PLAP[c]	0.03	0.56	ns	0.03	0.57	0.05	ns	0.53	ns	ns	0.55	ns
CD142	ns	0.55	0.03	ns	0.54	ns	0.02	0.60	0.008	ns	0.55	ns
CD206	ns	0.55	ns	0.04	0.57	ns	ns	0.52	ns	ns	0.52	ns
CEA	ns	0.55	ns	ns	0.54	ns	0.009	0.60	0.03	ns	0.50	ns
CAIX	ns	0.52	ns	ns	0.53	ns	ns	0.52	ns	ns	0.55	0.09[d]
CD56	ns	0.52	ns	ns	0.50	ns	ns	0.53	ns	ns	0.55	0.01

Note: Markers with an AUC *p* value less than 0.05 were included in building a multimarker model.
[a]Outcome of the nonparametric *t* test comparing the groups with and without cancer.
[b]Outcome of the receiver operating characteristic curve analysis comparing the cancer groups with the reference group without cancer.
[c]Elevated in patients without cancer.
[d]Included in building a multimarker-model in SCLC.
AC, adenocarcinoma; SCC, squamous cell carcinoma; ns, not significant (p>0.05); TSPAN8, tetraspanin 8; HER2, erb-b2 receptor tyrosine kinase 2; SFTPD, surfactant protein D; NY-ESO-1, New York esophageal squamous cell-1; EGFRvIII, type III EGFR mutation; PLAP, leucyl cystidyl aminopeptidase; CEA, carcinoembryonic antigen; CAIX, carbonic anhydrase 9.

advancing stage. In SCC, carbonic anhydrase 9 was significantly higher in stage I compared with in higher stages and a trend toward a down-regulation with increasing stage was shown when stage I and II were compared with stage III and IV. CD9 and CD81 were lower in the 12 patients with stage IV compared with in the 58 patients with lower stages, but this difference was not significant ($p = 0.003$). Furthermore, no difference was seen upon comparison of high and low stage with groups of equal numbers (stages I and II versus stages III and IV or stage I versus stages II, III, and IV). For tumor-associated glycoprotein 72, a strong tendency of lower levels in the low stages was found in AC upon comparison of stage I, II versus stage III, IV. Although not as strong, this tendency was confirmed upon comparison stage I versus stage II, III and IV.

AUC. To test whether the individual markers were able to separate the groups with the different cancers from the group without cancer, ROC curves and AUC were calculated for each marker individually (see Table 2). In the entire cancer cohort, CD151, CD171, and TSPAN8 could significantly separate the groups (CD151: AUC = 0.68, $p = 0.0002$; CD171: AUC = 0.60, $p = 0.0002$; TSPAN8: AUC = 0.60, $p = 0.0002$). In the AC subgroup, CD171 and TSPAN8 were able to separate the groups (CD171: AUC = 0.63, $p = 0.00002$; TSPAN8: AUC = 0.60, $p = 0.001$), whereas CD151 showed a trend toward significant separation (AUC = 0.67, $p = 0.006$). In the SCC and SCLC groups, CD151 was the only single marker that significantly separated the groups (SCC: AUC = 0.69, $p < 0.0005$: SCLC: AUC = 0.71, $p = 0.0001$).

Multimarker Models. Multimarker models were made to evaluate whether a combination of markers could optimize the separation of the groups with cancer versus the group without cancer. On the basis of the AUC, markers with an individual ability to separate the groups ($p < 0.05$) were included (Table 2). In the entire cancer cohort as well as in the AC subgroup, 10 markers were included (Fig. 3*A* and *B*). The model with the largest AUC included all 10 markers (all cancers: AUC = 0.74 [95% CI: 0.70–0.80]; AC: AUC = 0.76 [95% CI: 0.70–0.83]). The accuracy of placing the patients in the correct group was 72% for the AC subgroup (sensitivity 0.72, specificity 0.72) and 68% for the entire cancer cohort (sensitivity 0.71, specificity 0.69). For AC, an increase in AUC with increasing number of markers was observed (Fig. 3*C*). Although the 10-marker model had the largest AUC, the difference between the eight-, nine-, and 10-marker models was not significant, indicating that the

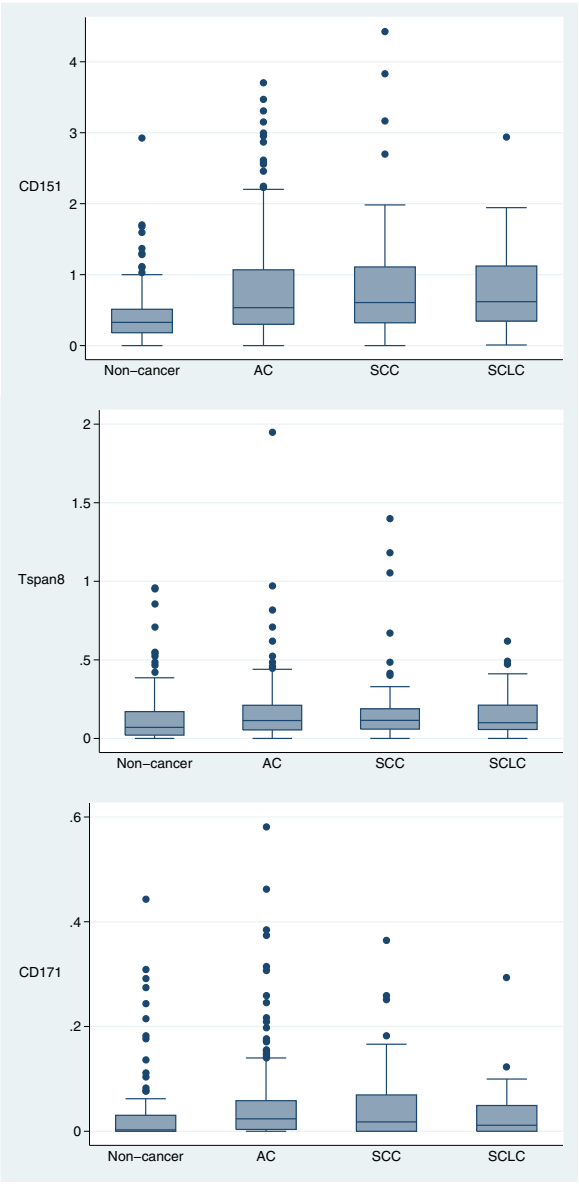

Figure 2. Extracellular vesicle array signal intensities as box plots for the antigens CD151, tetraspanin 8 (TSPAN8), and CD171 in the groups with and without cancer. AC, adenocarcinoma; SCC, squamous cell carcinoma.

eight-marker model may be as strong a diagnostic tool as the 10-marker model.

In SCC, on the basis of the markers' individual ability to separate cancer from noncancer, only five markers were included in the analyses (see Fig. 3*C*). Although minimally, as was seen in AC, the AUC increased with increasing numbers of markers, yet the difference between the AUS in the two-marker model and the five-marker model was not significant ($p = 0.25$). The AUC of the five-marker model was only just exceeding the AUC of CD151 as an individual marker (five markers: AUC = 0.70 [95% CI: 0.62–0.78]; CD151: AUC = 0.69 [95% CI 0.62–0.76]), with no difference between the two ($p = 0.94$). In SCLC, only two markers had an effect when analyzed individually in separating cancer from noncancer. Therefore, additional markers were included ($p < 0.10$); this allowed a total of five markers to be included in the model. As opposed to what was observed in AC and SCC, the AUC did not increase with the number of markers (Fig. 3*D*). In fact, none of the multimarker models exceeded CD151 as an individual diagnostic marker in SCLC (five-marker model: AUC = 0.65 [95% CI: 0.55–0.73], accuracy 60%; CD151: AUC = 0.71 [95% CI: 0.62–0.79)], accuracy 64% [sensitivity 0.60, specificity 0.75]). In all cancer groups, the importance of each marker was estimated for every multimarker model. The most important marker was CD151, with an average importance markedly exceeding that of all other markers (see Fig. 3*A*–*D*). Lastly, to test whether the multimarker models could be further optimized, the inclusion of markers with no significant AUC when analyzed individually was tested. This demonstrated that there was no additional effect by including further markers (data not shown).

Discussion

Exosomes play an important role in intercellular communication and in cancer; exosomes has been associated with growth and survival of tumor cells, preparation of a premetastatic niche, drug resistance, and angiogenesis.[26–29] Exosomes contain enriched amounts of cell-specific markers, especially those of endosomal origin such as the tetraspanins CD9, CD81, and CD63, and therefore, attention has been focused on exosomes as possible biomarkers in cancer. In AC, studies have shown exosomes in the blood to be a potential diagnostic tool.[9,12,20,22] In a feasibility study, we explored use of the EV array in which catching and detection of the exosomes is performed in parallel in unpurified starting material. In a selected group of patients with advanced-stage AC, we found the EV array to be promising as a diagnostic tool.[21] In this prospective study, we evaluated exosome protein profiling in patients in whom lung cancer was suspected on the basis of their clinical symptoms. This allowed us to include nonselected patients with lung cancer not only from the group with AC but also from all subgroups and stages. Furthermore, patients with no cancer but with other lung symptomatic diseases were used as a control group, as a result of which we are the first to evaluate exosomes in a setting that truly reflects the clinic. Most importantly, we found significantly higher levels of the tetraspanins CD151 and TSPAN8 and the cell adhesion molecule CD171 in cancer than in noncancer. However, in SCLC, only CD151 was significantly up-regulated in patients with cancer. Our results are consistent with earlier studies in which CD151, TSPAN8, and CD171

Table 3. Variation in Exosomal Proteins by Stage

	Adenocarcinoma			Squamous			SCLC
Markers	I, II, III vs. IV	I, II vs. III, IV	I vs. II, III, IV	I, II, III vs. IV	I, II vs. III, IV	I vs. II, III, IV	I, II, III vs. IV
Low (n) vs. high (n)	104 vs. 100	72 vs. 132	61 vs. 143	58 vs. 12	33 vs. 37	22 vs. 48	17 vs. 38
CD151	ns	ns	ns	ns	ns	[a]	ns
CD171	[a]	ns	ns	ns	ns	ns	ns
HER2	[a]	ns	ns	ns	ns	ns	ns
CD9	ns	ns	ns	[b]	ns	ns	ns
CD81	ns	ns	ns	[b]	ns	ns	ns
CD82	ns	[a]	ns	ns	ns	ns	ns
Mucin16	[a]	ns	ns	ns	ns	ns	ns
CEA	ns	ns	ns	ns	ns	[a]	ns
CD37	ns	[a]	[a]	ns	ns	ns	ns
Claudin1	[a]	[a]	ns	ns	ns	ns	ns
CAIX	ns	[a]	ns	ns	ns	ns	ns
CAXII	ns	ns	ns	ns	[a]	[c]	ns
TSG101	ns	[a]	ns	ns	ns	ns	[a]
HER3	ns	ns	ns	ns	ns	ns	[a]
TNFRI	ns	ns	ns	ns	ns	[a]	ns
p53	ns	ns	ns	ns	ns	[a]	[a]
TAG72	[a]	[b]	ns	ns	ns	[a]	ns

Note: High versus low stages compared by Wilcoxon rank sum test. Because of low number of patients with limited disease in SCLC, a comparison of stages I, II, and III versus stage IV is the only one demonstrated.
[a] $p < 0.05$.
[b] $p < 0.005$.
[c] $p < 0.001$.
ns, not significant ($p > 0.05$); HER2, erb-b2 receptor tyrosine kinase 2; CEA, carcinoembryonic antigen; CAIX, carbonic anhydrase 9; CAXII, carbonic anhydrase 12; TSG101, tumor susceptibility 101; HER3, erb-b2 receptor tyrosine kinase 3; TNFRI, TNF receptor superfamily member 1A; p 53, tumor protein p53; TAG72, tumor-associated glycoprotein 72.

were evaluated in NSCLC tumor samples by immunohistochemistry.[30–32] Here, high levels of CD151 were associated with aggressiveness of the cancer. Furthermore, in vitro studies have found exosomal CD151 along with exosomal TSPAN8 to be closely correlated with initiation of metastatic behavior owing to their ability to modulate the extracellular matrix in addition to modulating or stabilizing associated molecules.[33] CD171 has been associated with epithelial-mesenchymal transition, metastasis, and poor prognosis in patients with lung cancer.[32,34] In a study by Ueda et al.,[22] exosomal protein profiling was evaluated as a diagnostic tool in serum from 165 patients with lung cancer (119 with AC and 46 with SCC) compared with in serum from 64 normal controls and 29 patients with interstitial pneumonia. As opposed to our findings, they found CD91 (LRP1) to be a diagnostic marker of AC but not SCC. The difference could be explained by the choice of controls because we did not include healthy controls in our study.

To our surprise, the vast majority of the exosomal proteins did not differ between stages. Our findings could be explained by the aggressiveness of lung cancer, in which 5-year survival rates in the low stages are as low as 50%,[2] indicating an aggressive cancer already in the low stages. It is likely that exosomes are released to promote tumor growth, survival, and preparation of premetastatic niches independently of stage. In their study, Ueda et al.[22] did not find any difference in CD91 between stages either. In SCC, CD9 and CD81 are not significantly down-regulated in stage IV compared with in stages I to III, although because of the low number of patients with advanced stage in our study, it is questionable whether this tendency is valid in a stage-balanced SCC cohort. Furthermore, when the SCC cohort was split in all other combinations (stage I and II versus stage III and IV or stage I versus stage II, III, and IV) there were no tendencies of difference between groups.

We have demonstrated with multimarker models that exosome protein profiles are able to detect patients with lung cancer with an accuracy of 68% (72% in the case of AC and 64% in the case of SCC). For AC, the separation with an AUC of 0.76 is considered fair and shows that the EV array could have potential as a supplement in the clinic. Because the AUC increased with increasing number of markers, it may be possible to enhance the AUC by

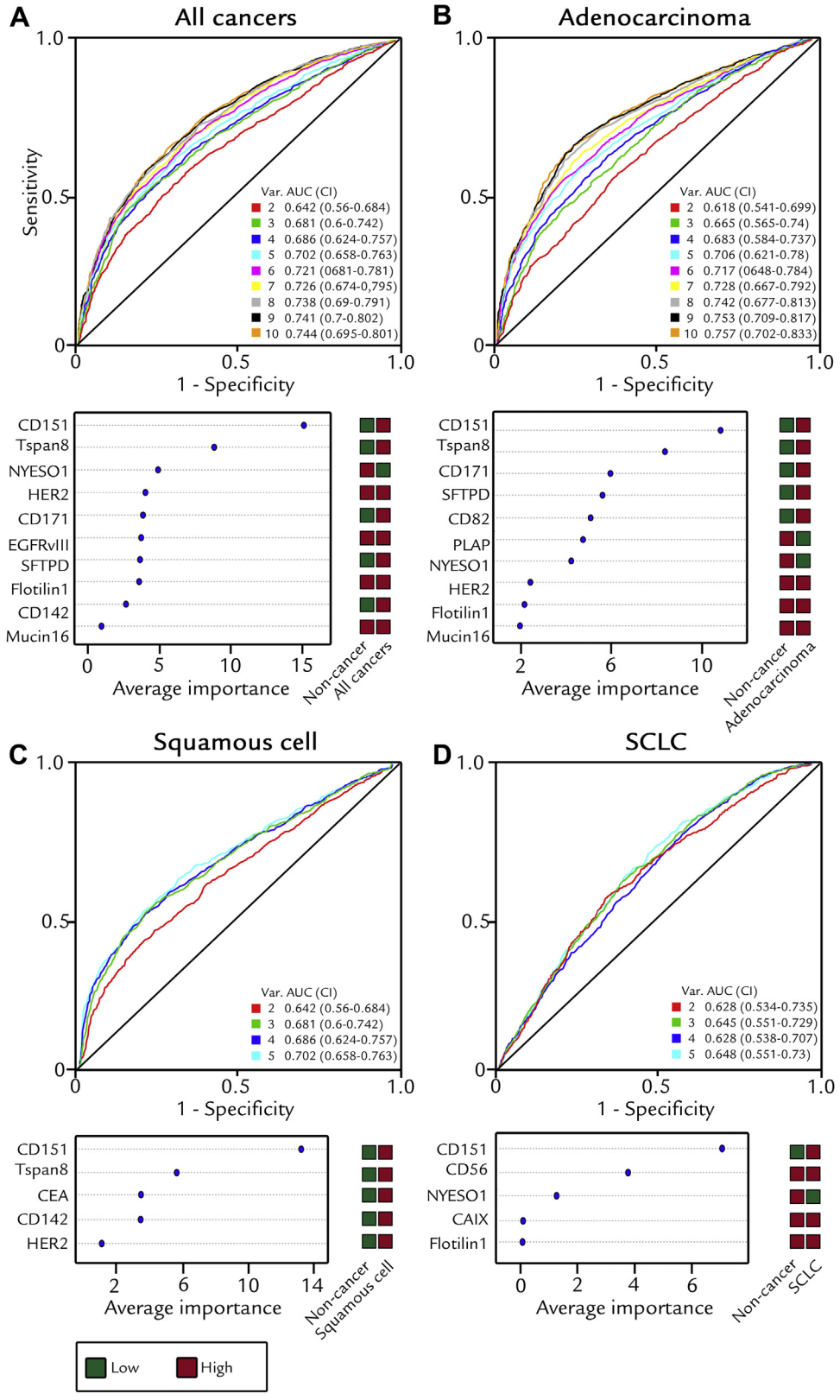

Figure 3. Multivariate analysis by random forest. Receiver operating characteristic curves generated by the cross-validation performance. The area under the curve (AUC) for the number of markers is given with the 95% confidence interval (CI) for the entire cancer cohort (*A*) and for each cancer subgroup (*B*, Adenocarcinoma; *C*, Squamous Cell; *D*, Small cell carcinoma). Average importance is depicted for the markers included in multimarker models. TSPAN8, tetraspanin 8; NY-ESO-1, New York esophageal squamous cell-1; HER2, erb-b2 receptor tyrosine kinase 2; EGFRvIII, type III EGFR mutation; SFTPD, surfactant protein D; PLAP, leucyl cystidyl aminopeptidase; CEA, carcinoembryonic antigen; CAIX, carbonic anhydrase 9.

inclusion of additional markers in the EV array. In SCLC and SCC, the multimarker models did not exceed the separation of patients by CD151. The antibodies chosen for this study could explain this lack of efficiency because they may not represent the molecular mechanisms in SCC and SCLC as well as they do in AC. Furthermore, in vitro studies in SCLC have shown CD9 to be absent or highly reduced in 17 different SCLC cell lines,[35] just as a smaller study found CD9 to be absent in tumor samples from 16 chemonaive patients with SCLC.[36] Because CD9 is a part of our exosome capture cocktail as well as our normalization factor, we could risk missing tumor-derived exosomes in SCLC.

In conclusion, here have demonstrated exosome protein profiling by the EV array in a prospective study including patients with symptoms of lung cancer. We found CD151, TSPAN8, and CD171 to be highly expressed in exosomes from patients with cancer compared with in those from patients without cancer. In a multimarker model including 10 exosomal markers, we were able to make a fair separation of AC and noncancer, demonstrating the promising perspectives of exosome protein profiling as a biomarker. This needs to be validated in an independent cohort. Further optimization of the EV array to include SCC- and SCLC-related markers is needed to fully incorporate exosome protein profiling in a diagnostic setting.

Acknowledgments

The authors are thankful to Birgith West Mortensen and Lene Dabelstein Pedersen for laboratory help and to Ninna Aggerholm-Pedersen for statistical help.

References

1. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin*. 64:9–29.
2. Dansk Lunge Cancer Gruppe [Danish National Lung Cancer Group]. Årsrapport 2014 [in Danish]. 2014. http://lungecancer.dk/documents/ED76D14E-3915-4D9C-9033-55D1B03402FB.pdf. Accessed April 1, 2016.
3. Vansteenkiste J, Crinò L, Dooms C, et al. 2nd ESMO Consensus Conference on Lung Cancer: early-stage non-small-cell lung cancer consensus on diagnosis, treatment and follow-up. *Ann Oncol*. 2014;25:1462–1474.
4. MacMahon H, Austin JHM, Gamsu G, et al. Guidelines for management of small pulmonary nodules detected on CT scans: a statement from the Fleischner Society. *Radiology*. 2005;237:395–400.
5. Wiener RS, Schwartz LM, Woloshin S, Welch HG. Population-based risk for complications after transthoracic needle lung biopsy of a pulmonary nodule: an analysis of discharge records. *Ann Intern Med*. 2011;155:137–144.
6. Harding C, Heuser J, Stahl P. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J Cell Biol*. 1983;97:329–339.
7. Pan BT, Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell*. 1983;33:967–978.
8. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol*. 2007;9:654–659.
9. Huang S-H, Li Y, Zhang J, Rong J, Ye S. Epidermal growth factor receptor-containing exosomes induce tumor-specific regulatory T cells. *Cancer Invest*. 2013;31:330–335.
10. Théry C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol*. 2009;9:581–593.
11. Peinado H, Alečković M, Lavotshkin S, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat Med*. 2012;18:883–891.
12. Rabinowits G, Gerçel-Taylor C, Day JM, Taylor DD, Kloecker GH. Exosomal microRNA: a diagnostic marker for lung cancer. *Clin Lung Cancer*. 2009;10:42–46.
13. Taylor DD, Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecol Oncol*. 2008;110:13–21.
14. Khan S, Bennit HF, Turay D, et al. Early diagnostic value of survivin and its alternative splice variants in breast cancer. *BMC Cancer*. 2014;14:176.
15. Skog J, Würdinger T, van Rijn S, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol*. 2008;10:1470–1476.
16. Logozzi M, De Milito A, Lugini L, et al. High Levels of Exosomes Expressing CD63 and Caveolin-1 in Plasma of Melanoma Patients. Cao Y, ed. *PLoS One*. 2009;4:e5219.
17. Liu J, Sun H, Wang X, et al. Increased exosomal microRNA-21 and microRNA-146a levels in the cervicovaginal lavage specimens of patients with cervical cancer. *Int J Mol Sci*. 2014;15:758–773.
18. Khan S, Jutzy JMS, Valenzuela MMA, et al. Plasma-derived exosomal survivin, a plausible biomarker for early detection of prostate cancer. *PLoS One*. 2012;7:e46737.
19. Gabriel K, Ingram A, Austin R, et al. Regulation of the tumor suppressor PTEN through exosomes: a diagnostic potential for prostate cancer. *PLoS One*. 2013;8:e70047.
20. Li Y, Zhang Y, Qiu F, Qiu Z. Proteomic identification of exosomal LRG1: a potential urinary biomarker for detecting NSCLC. *Electrophoresis*. 2011;32:1976–1983.
21. Jakobsen KR, Paulsen BS, Bæk R, Varming K, Sorensen BS, Jørgensen MM. Exosomal proteins as potential diagnostic markers in advanced non-small cell lung carcinoma. *J Extracell Vesicles*. 2015;4:26659.
22. Ueda K, Ishikawa N, Tatsuguchi A, Saichi N, Fujii R, Nakagawa H. Antibody-coupled monolithic silica microtips for highthroughput molecular profiling of circulating exosomes. *Sci Rep*. 2014;4:6232.
23. Jørgensen M, Bæk R, Pedersen S, Søndergaard EKL, Kristensen SR, Varming K. Extracellular vesicle (EV) array:

microarray capturing of exosomes and other extracellular vesicles for multiplexed phenotyping. *J Extracell Vesicles*. 2013;2. http://www.journalofextracellularvesicles.net/index.php/jev/article/view/20920/28067. Accessed December 16, 2015.
24. Hanley JA, McNeil BJ. The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology*. 1982;143:29–36.
25. Xia J, Sinelnikov IV, Han B, Wishart DS. MetaboAnalyst 3.0—making metabolomics more meaningful. *Nucleic Acids Res*. 2015;43:W251–W257.
26. Thuma F, Zöller M. Outsmart tumor exosomes to steal the cancer initiating cell its niche. *Semin Cancer Biol*. 2014;28:39–50.
27. Peinado H, Lavotshkin S, Lyden D. The secreted factors responsible for pre-metastatic niche formation: old sayings and new thoughts. *Semin Cancer Biol*. 2011;21:139–146.
28. Sceneay J, Smyth MJ, Möller A. The pre-metastatic niche: finding common ground. *Cancer Metastasis Rev*. 2013;32:449–464.
29. Fontana S, Simona F, Saieva L, et al. Contribution of proteomics to understanding the role of tumor-derived exosomes in cancer progression: state of the art and new perspectives. *Proteomics*. 2013;13:1581–1594.
30. Tokuhara T, Hasegawa H, Hattori N, et al. Clinical significance of CD151 gene expression in non-small cell lung cancer. *Clin Cancer Res*. 2001;7:4109–4114.
31. Kwon MJ, Seo J, Kim YJ, et al. Prognostic significance of CD151 overexpression in non-small cell lung cancer. *Lung Cancer*. 2013;81:109–116.
32. Tischler V, Pfeifer M, Hausladen S, et al. L1CAM protein expression is associated with poor prognosis in non-small cell lung cancer. *Mol Cancer*. 2011;10:127.
33. Yue S, Mu W, Zöller M. Tspan8 and CD151 promote metastasis by distinct mechanisms. *Eur J Cancer*. 2013;49:2934–2948.
34. Hai J, Zhu C-Q, Bandarchi B, et al. L1 cell adhesion molecule promotes tumorigenicity and metastatic potential in non-small cell lung cancer. *Clin Cancer Res*. 2012;18:1914–1924.
35. Funakoshi T, Tachibana I, Hoshida Y, et al. Expression of tetraspanins in human lung cancer cells: frequent downregulation of CD9 and its contribution to cell motility in small cell lung cancer. *Oncogene*. 2003;22:674–687.
36. Kohmo S, Kijima T, Otani Y, et al. Cell surface tetraspanin CD9 mediates chemoresistance in small cell lung cancer. *Cancer Res*. 2010;70:8025–8035.