Exosomal proteins as potential diagnostic markers in advanced non-small cell lung carcinoma

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Background: Lung cancer is one of the leading causes of cancer-related death. At the time of diagnosis, more than half of the patients will have disseminated disease and, yet, diagnosing can be challenging. New methods are desired to improve the diagnostic work-up. Exosomes are cell-derived vesicles displaying various proteins on their membrane surfaces. In addition, they are readily available in blood samples where they constitute potential biomarkers of human diseases, such as cancer. Here, we examine the potential of distinguishing non-small cell lung carcinoma (NSCLC) patients from control subjects based on the differential display of exosomal protein markers.

Methods: Plasma was isolated from 109 NSCLC patients with advanced stage (IIIA–IV) disease and 110 matched control subjects initially suspected of having cancer, but diagnosed to be cancer free. The Extracellular Vesicle Array (EV Array) was used to phenotype exosomes directly from the plasma samples. The array contained 37 antibodies targeting lung cancer-related proteins and was used to capture exosomes, which were visualised with a cocktail of biotin-conjugated CD9, CD63 and CD81 antibodies.

Results: The EV Array analysis was capable of detecting and phenotyping exosomes in all samples from only 10 μL of unpurified plasma. Multivariate analysis using the Random Forests method produced a combined 30-marker model separating the two patient groups with an area under the curve of 0.83, CI: 0.77–0.90. The 30-marker model has a sensitivity of 0.75 and a specificity of 0.76, and it classifies patients with 75.3% accuracy.

Conclusion: The EV Array technique is a simple, minimal-invasive tool with potential to identify lung cancer patients.

Keywords: lung cancer; EV Array; exosomes; extracellular vesicles; phenotyping; protein microarray; plasma; NSCLC

Multiple experiments have investigated and established that exosomes display various proteins on their membrane surface (6,7). Additionally, exosomes are readily available in the blood stream (8) and hereby constitute potential biomarkers of human diseases, such as cancer. Previous studies have identified plasma exosomes as useful markers in several cancer diseases including lung cancer (9–11). So far, a panel of 12 miRNAs and membrane proteins such as CD91, CD317 and EGFR have been suggested as potential exosomal markers of NSCLC (9,12,13). Nevertheless, studies concerning the diagnostic potential...
of exosomes in cancer patients have so far been complicated by high workload procedures when isolating and investigating plasma exosomes using ultracentrifugation. Furthermore, prior methodological constraints have enforced a single exosome-marker focus, which has had a negative bearing on sensitivity as well as the gain of new knowledge.

Protein microarrays are potent tools for investigating antigens or antibodies in samples such as plasma. In addition, they harbour an advantage over other methods by being able to detect multiple proteins simultaneously (14). Recently, it was shown that adjusted protein micro-arrays can capture extracellular vesicles fitting the description of exosomes (15), which here and in the following refers to vesicles expressing CD9, CD63 and/or CD81 and having a size of 30–100 nm.

The Extracellular Vesicle Array (EV Array) is a sandwich-ELISA-based method capturing exosomes using an antibody panel targeting the extracellular domain of selected membrane or membrane-associated proteins. The EV Array constitutes a fast, automated, economical and highly sensitive method for exploration of plasma exosomes while consuming as little as 10 µL sample.

Developing a plasma test for lung cancer detection is an attractive approach owed to the easy accessibility of blood samples and the minimal discomfort for the patients providing them. Reliable and easy performed diagnostic tools are particularly important in NSCLC patients, where effective minimal-invasive blood-based diagnostic methods are currently limited. The EV Array shows great potential as such a diagnostic tool. The aim of the present study was to explore the potential of plasma exosomes in diagnosing NSCLC, using a multi-marker approach based on multivariate analysis.

Material and methods

Patient samples

The plasma samples included in this study were collected from patients suspected of having lung cancer due to clinical symptoms. Patients were examined from May 2011 until January 2014 at the Department of Pulmonary Medicine, Aarhus University Hospital, Denmark. The outcome of the examination was undetermined at time of inclusion. One hundred and nine patients with NSCLC adenocarcinoma stage IIIa–IV were consecutively collected. A control group was made of 110 patients who were diagnosed to be cancer free based on computed axial tomography and, if relevant, negative biopsies. The only exclusion criterion was cancer. Since infection and inflammation are frequently observed amongst patients with lung cancer, acute or chronic infection or inflammation of any kind were not considered reason for exclusion. By doing so, a control group resembling the clinical situation in a diagnostic outpatient department could be made.

Phenotyping of extracellular vesicles using the EV Array

Production of microarrays

Microarray printing was performed on a SpotBot® Extreme Protein Edition Microarray Printer with a 946MP4 pin (ArrayIt Corporation, CA, USA). As a positive control 100 µg/mL of biotinylated human IgG was printed and PBS with 5% glycerol was used as negative control. Antibodies were printed on epoxy-coated slides (75.6 mm × 25.0 mm; SCHOTT Nexterion, DE), which were left to dry at room temperature overnight prior to further analysis.

Antibodies for production of the EV Array

A total of 37 anti-human antibodies were used and listed in the following with the corresponding clone, if available: CD146 (PI1H12), Flotillin-1, HB-EGF (4G10), HER3 (2F9), HER4 (H4.77.16), Hsp90 (IGF1), N-cadherin (8C11), p53 (pAb240), TAG72 (0.N.561), and TSG101 (5B7) from Abcam (MA, USA); SFTPD (VIF11) from Acris Antibodies GmbH (DE); EGFR and EGFRvIII from Antibodies-online.com (DE); CD63 (MEM-259) from Biolegend (CA, USA); HER2 (29D8) from Cell Signalling (MA, USA); CD9 and CD81 from LifeSpan Biosciences (WA, USA); SPA (6F10) from Novus Biologicals (CO, USA); CD13 (498001), CD14 (50040), CD142 (323514), CD151 (210127), CD206, CEACAM5 (487609), MUC1 (604804), TNF R1, TNF RII and Tspan8 (458811) from R&D Systems (MN, USA); AREG (S-13), EpCam (0.N.277), Mucin16 (X306), NY-ESO-1 (E978) and PLAP (88b) from Santa Cruz Biotechnology (CA, USA); CD171 from Sigma-Aldrich (MO, USA); c-MET (016) and PD-L1 from Sino Biological Inc. (China); CD163 (Mac2-158) from Trillium Diagnostics, LLC (ME, USA). All antibodies were diluted with PBS with 5% glycerol and printed in triplicates at 75–200 µg/mL.

Catching and visualization of exosomes

The EV Array was prepared as described by Jørgensen et al. (15) with modifications. In short, the microarray slides were initially blocked (50 mM ethanolamine, 100 mM Tris, 0.1% SDS, pH 9.0) prior to incubation with 10 µL plasma sample diluted (1:10) in wash-buffer (0.05% Tween®20 in PBS). The incubation was performed in Multi-Well Hybridization Cassettes (ArrayIt Corporation) at RT for 2 hours followed by overnight incubation at 4°C. Following a short wash, the slides were incubated with a cocktail of biotinylated detection antibodies (anti-human-CD9, -CD63 and -CD81, LifeSpan Biosciences, WA, USA) diluted 1:1,500 in wash-buffer. After a wash, a subsequent 30-minute incubation step with Cy5-labelled streptavidin (Life Technologies) diluted 1:1,500 in wash-buffer was carried out for detection. Prior to scanning, the slides were washed in wash-buffer, then in ultrapure/deionized water and finally dried using a Microarray High-Speed Centrifuge (ArrayIt Corporation). Scanning and spot detection was performed as previously described (15).
Data analysis

Graphs and statistics were carried out using GraphPad Prism (version 6.04, GraphPad Software, Inc., San Diego, CA, USA), Excel (version 2013, Microsoft, Redmond, WA, USA) and ROCCET (Version 03/17/2014, TMIC, AB, Canada). Heat maps and Hierarchical cluster analysis were produced using Genesis (version 1.7.6, IGB TU Graz, Graz, Austria).

Multivariant ROC analysis

Random Forests was used for classification and Monte-Carlo cross validation (MCCV) using balanced subsampling was employed to compute ROC curves and to calculate confidence intervals of their area under the curve (AUC). In each MCCV, two thirds (2/3) of the samples were used to evaluate the feature importance. The top important features were then used to build a classification model, which was validated on the 1/3 of the samples that were left out. Five hundred iterations were used to calculate performance of the model. Prior to analysis missing value estimation was performed by replacing missing values by “min.” A Top 20 of ratios (based on p-values) was computed and included into the analysis prior to data transformation and normalization by log2.

Results

Exosomal markers can be detected in small amounts of plasma

The EV Array analysis was performed on 109 plasma samples from patients diagnosed with stage IIIa–IV NSCLC adenocarcinoma and on 110 plasma samples from patients without a cancer diagnosis (Table I). Even though only 10 μL unpurified plasma sample was loaded for each patient, sufficient signal for further analysis was obtained from all samples.

Thirty-seven different antibodies were used for the analysis of plasma exosomes. Some markers were previously used and optimised for EV Array analysis (15).

Table I. Selected baseline patient characteristics for the two study groups

| Characteristics | NSCLC group (N = 109) | Control group (N = 110) |
|-----------------|-----------------------|-------------------------|
| Age             | 45–88                 | 21–90                   |
| Median          | 66                    | 65                      |
| Gender          |                       |                         |
| Male            | 56 (51.4)             | 64 (58.2)               |
| Female          | 53 (48.6)             | 46 (41.8)               |
| Stage           |                       |                         |
| IIIa            | 28 (25.7)             |                         |
| IIla            | 20 (18.3)             |                         |
| IV              | 61 (56.0)             |                         |

In addition, antibodies targeting markers involved in NSCLC pathology were optimised for the EV Array analysis. For this purpose, members of the EGF system and selected markers of inflammation (CD163, CD206) (16,17) were selected (Table II).

Univariate analysis of exosomal markers

The univariate analysis of each marker using log2-transformed signal-values revealed generally a significantly higher marker-expression in control subjects compared to lung cancer subjects (Table II and Supplementary Fig. 1a and b).

Hierarchical cluster analysis for each of the groups depicted a high variety of expression among the individuals, which was also seen in a previously study of healthy donors (15). The hierarchical cluster analysis also showed co-variance among certain markers, for example CD9, CD81 and CD151 (marked with a box) and EGFRvIII, Flotilin-1, HER4, N-cadherin and CD163 (marked with a dashed box) (Fig. 1a and b). The control group had a significantly higher (p < 0.05) CD9, CD81 and CD151 expression when looking at log2-transformed data (Fig. 2a and Supplementary Fig. 1a). For the co-varying group consisting of EGFRvIII, Flotilin-1, HER4, N-cadherin and CD163, all markers were higher expressed in the control group, as well as when looking at log2-transformed data (Fig. 2b). As the cancerous patients showed a tendency to have a lower amount of positive antigens over all (Fig. 3a), a normalisation (calculation of the percentage) of the signals for each antigen to the total signal for each individual patient was performed (Fig. 3a). Taken together with the fact that the EV Array signal detection relies on CD9-, CD63- and/or CD81-antibody binding, the relative expression of these markers was examined further (Pie charts in Fig. 3a). When looking at the normalised data (Fig. 3b), the CD9, CD63 and CD81 percentage was significantly higher (p < 0.05) in the cancer group.

Normalised data for the co-varying group EGFRvIII, Flotilin-1, HER4, N-cadherin and CD163 was also investigated. For these markers the normalisation did not change the relations significantly (Fig. 3c).

Multivariate exosomal marker analysis as a diagnostic tool in NSCLC

To estimate the diagnostic value of each marker, ROC curves were generated. None of the individual markers alone gained an AUC indicating a diagnostic potential (Fig. 1a and b).

However, the diagnostic potential of a panel of markers was evaluated using multivariate analysis. For the multivariate analysis all log2-transformed data was included. The univariate analysis of the normalised data for CD9, CD63 and CD81 (Fig. 3b) showed a significant higher signal in the cancerous patients. Therefore, these normalised values were accepted in the multivariate analysis.
**Table II.** Description of the markers selected for the EV Array together with the outcome (p-value) of the non-parametric t-test comparing the log2 transformed data from the control group and the cancer group (Supplementary Fig. 1a and b)

| Antigen          | Description                                      | P-value summary |
|------------------|--------------------------------------------------|-----------------|
| **Exosomal markers** |                                                  |                 |
| CD9              | Tetraspanin-family member (8)                    | *               |
| CD63             | Tetraspanin-family member (18)                   | ns              |
| CD81             | Tetraspanin-family member (18)                   | ****            |
| TSG101           | ESCRT complex member (19)                        | ****            |
| Hsp90            | Chaperone for EGFR (19,20)                       | **              |
| EpCam            | Marker of epithelial tumour-derived exosomes (9,21) | **              |
| **Cancer cell markers** |                                                |                 |
| PLAP             | Marker of seminomas and potential marker of NSCLC (22) | ***             |
| TAG72            | Marker of ovarian, colon and other cancers (23–25) | ****            |
| TSpan8           | Tetraspanin-family member, involved in tumour-angiogenesis (26) | ***             |
| NY-ESO-1         | Potential marker of NSCLC (27)                   | ***             |
| MUC16            | Potential marker of NSCLC (28,29)                | **              |
| MUC1             | Marker of prognosis and squamous carcinoma (30,31) | ****            |
| CEA              | Pre-treatment levels predict outcome of chemotherapy and erlotinib in NSCLC (32,33) | ns              |
| Flotillin-1      | Marker of metastasis and lung adenocarcinoma progression (34) | ***             |
| CD171            | Marker of different cancers including gynaecological cancers and small cell lung carcinoma (35) | ns              |
| CD151            | Prognostic marker of NSCLC/markers of adenocarcinomas (36,37) | *               |
| CD142            | Upregulated in NSCLC plasma (38)                 | ****            |
| CD146            | Potential prognostic marker of NSCLC (39,40)     | ***             |
| EGFR             | Oncogenic driver in NSCLC and target of clinical treatments (41) | ***             |
| HER2             | Overexpression correlates to benefit from EGFR inhibitors (42,43) | *               |
| HER3             | Associated with shorter PFS in melanoma (44)     | ns              |
| HER4             | Associated with shorter PFS in melanoma (44)     | ***             |
| AREG             | Membrane-bound ligand of EGFR previously found on exosomes (45) | **              |
| PDL-1            | Potential biomarker and treatment target in NSCLC (46,47) | ns              |
| MET              | Frequently overexpressed in NSCLC and correlated to EGFR-inhibitor resistance (48,49) | *               |
| HB-EGF           | Membrane-bound ligand of EGFR, previously found on exosomes (45,50) | **              |
| N-cadherin       | EMT marker and potential prognostic marker of NSCLC (51) | ****            |
| p53              | Tumour suppressor gene often low expression in NSCLC (52) | ns              |
| CD13             | Prognostic marker in NSCLC (53)                  | *               |
| EGFRvIII         | Truncated EGFR, oncogene in glioblastoma (54)    | ****            |
| **Other markers** |                                                  |                 |
| CD163            | Macrophage-derived inflammation marker involved in tumorigenesis (17,55) | ****            |
| CD206            | Mannose-receptor marker, marker of inflammation (16) | *               |
| CD14             | Macrophage-marker shown to be elevated in NSCLC (56) | *               |
| SFTPD            | Surfactant protein D, lung tissue marker (57)    | ns              |
| SP-A             | Surfactant protein A, lung tissue marker (57)    | *               |
| TNF R1           | Marker of inflammation, related to exosomes (58–60) | *               |
| TNF RII          | Marker of inflammation (59,60)                   | **              |

Unless otherwise stated the marker level was higher in the control group. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns = not significant; *elevated in cancer patients.

together with the individual relations between the analysed markers.

Figure 4 shows the ROC curves for the 3, 5, 10 and 30 best rated marker combinations. The 30-marker model has the largest AUC of 0.83, CI: 0.77–0.90, a sensitivity of 0.75 and a specificity of 0.76. This model classifies patients with 75.3% accuracy. No increase in AUC was observed when expanding the number of markers included beyond 30 (data not shown).

The importance of each marker or their individual relations for the model was assessed (Fig. 5) and assigned a value in the range 1–10. The most important marker was found to be CD81 followed by the relation between TAG72 and the normalised values of CD63. The top 10
Fig. 1. Hierarchical cluster analysis. Two groups of markers show co-variance both in the control group and in the cancer group (marked with boxes). a) Heat map illustration of all markers in the control group. b) Heat map illustration of all markers in the cancer group.

Fig. 2. EV Array signal intensities for selected antigens. a) The EV Array signal intensities for the exosomal markers CD9, CD63 and CD81 displayed in box plots. The co-variation of the signal intensities across the patient samples can be seen in Fig. 1 and Supplementary Fig. 2. b) Box plot of a group of antigens (Flotilin-1, HER4, EGFRvIII, N-Cadherin and CD163) showing a high degree of co-variation (see Fig. 1). *p < 0.05; **p < 0.001; ***p < 0.0001; ns = not significant.
ranking of the importance did not change between the models including 3, 5, 10 or 30 markers. Their respective coloured lines indicate the markers included in the individual models.

Discussion
Circulating exosomes are a promising biomarker source in various diseases. So far, only a limited number of studies have examined the diagnostic potential in relation
Until now, the studies have been limited to investigating exosomes captured by antibodies towards a single antigen, for example, EpCAM or CD9 (9,13). In spite of these constraints, promising results were obtained through these studies and a subset of exosomal miRNAs and the exosomal-bound CD91-protein was revealed as promising biomarkers. The present study examined plasma exosomes from 219 patients, initially suspected of having lung cancer, from a multi-marker aspect. Of these patients 109 where afterwards diagnosed with NSCLC. Investigation of 37 exosome capturing antibodies and the use of a cocktail of 3 different detecting antibodies against known exosome markers gave comprehensive information paving the way for multivariate analysis.

Our multivariate EV Array analysis results in a 30-marker panel classifying 75% of the patients correctly. This result is superior to exosomal CD91-expression alone (61%) as recently reported by Ueda et al. (13). Our data suggests that the EV Array analysis could be a valuable complementary tool in diagnosing NSCLC.

The univariate t-test analysis revealed higher exosomal marker abundance on the control patient exosomes compared to the cancer patient exosomes. This is in concordance with the recent study of serum samples from NSCLC patients and healthy blood donors performed by Ueda et al. (13). The decreased overall signal could be due to less compact exosomes in lung cancer patients, for example, fewer proteins per exosome. Presumably, this would result in fewer lung cancer exosomes being captured by the antibodies. Previously, differences in the

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**Fig. 4.** Multivariate analysis by Random Forests using the EV Array measurements of the exosomal antigens. Random Forests ROC curves generated by the cross validation performance. The area under curve (AUC) for top 3-, 5-, 10-, and 30-marker panels are given together with the 95% confidence interval.

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**Fig. 5.** Multivariate analysis by Random Forests using the EV Array measurements of the exosomal antigens. The mean average importance to the classification model using the 30-marker panel illustrated in Fig. 4 for each of the analysed exosomal antigens, the normalised values (indicated by ‘*’) and their internal relations (indicated by ‘/’). The top 10 ranking did not change between the models including 3-, 5-, 10- or 30-markers and the markers included in each model are visualised by the coloured lines. Colours refer to the number of variables showed in Fig. 4.

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protein load of the exosomes of melanoma patients have also been shown by Peinado et al. to have an influence on the survival rate (61). Another possibility is that exosomes from NSCLC patients express another subset of markers than the ones targeted in this analysis or that the exosomes carrying the cancerous markers are cleared by an activated immune system in the cancerous patients (62). Additional markers could be explored to optimise the EV Array and other detection antibodies could be evaluated for further investigation of this phenomenon.

The co-variation of exosomal markers constitutes an additional interesting finding. CD9 and CD81 remain well-described exosomal markers and may be expected to co-vary. Interestingly, CD63, another well-known marker, does not follow this pattern, which has also been described previously (15,63). The difference in expression of CD63 in relation to CD9 and CD81 highlights the advantage of detecting the signal with a cocktail of CD9, CD63 and CD81 antibodies in order to achieve sufficient information.

EGFRvIII together with Flotilin-1, HER4, N-cadherin and CD163 constituted an additional co-varying group (Fig. 1a and b). EGFRvIII is the truncated version of EGFR known from glioblastoma (54). Nevertheless, EGFR truncation is not normally observed in NSCLC and neither in normal tissue (64,65). An obvious first thought to explain the level of EGFRvIII signal in both control and cancer patients could point to an unspecific antibody binding to normal EGFR. However, since the patterns of these 2 markers (EGFR and EGFRvIII) vary from each other this explanation does not seem to be the case. Another explanation could be an unexplored tendency for exosomes to harbour truncated EGFR. This phenomenon should be explored further in order to assess the biological bearing of this truncated receptor.

As seen from the ranking of the 30 markers, their contribution differs in importance to the model. Increasing the average importance with new and more efficient markers could greatly strengthen the analysis and the diagnostic potential. Furthermore, as no increase in diagnostic potential was seen when including more markers, it is essential to substitute weak markers with better candidate markers in order to increase the average importance and thereby provide an even more valuable tool. In conclusion, the EV Array constitutes a broad spectra tool offering exhaustive information on exosomal content with a minimal of sample requirement, which can be optimized and adjusted to fit individual sets of samples.

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The authors have not received any funding or benefits from industry or elsewhere to conduct this study.

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