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Exosomal proteins as prognostic biomarkers in non-small cell lung cancer

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ABSTRACT

Background: Use of exosomes as biomarkers in non-small cell lung cancer (NSCLC) is an intriguing approach in the liquid-biopsy era. Exosomes are nano-sized vesicles with membrane-bound proteins that reflect their originating cell. Prognostic biomarkers are needed to improve patient selection for optimal treatment. We here evaluate exosomes by protein phenotyping as a prognostic biomarker in NSCLC.

Methods: Exosomes from plasma of 276 NSCLC patients were phenotyped using the Extracellular Vesicle Array; 49 antibodies captured the proteins on the exosomes, and a cocktail of biotin-conjugated antibodies binding the general exosome markers CD9, CD81 and CD63 was used to visualise the captured exosomes. For each individual membrane-bound protein, results were analysed based on presence, in a concentration-dependent manner, and correlated to overall survival (OS).

Results: The 49 proteins attached to the exosomal membrane were evaluated. NY-ESO-1, EGFR, PLAP, EpCam and Alix had a significant concentration-dependent impact on inferior OS. Due to multiple testing, NY-ESO-1 was the only marker that maintained a significant impact on inferior survival (hazard rate (HR) 1.78 95% (1.78–2.44); $p = 0.0001$) after Bonferroni correction. Results were adjusted for clinico-pathological characteristics, stage, histology, age, sex and performance status.

Conclusion: We illustrate the promising aspects associated with the use of exosomal membrane-bound proteins as a biomarker and demonstrate that they are a strong prognostic biomarker in NSCLC.

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1. Introduction

Lung cancer is the leading cause of cancer-related death worldwide (Siegel et al., 2014). Despite improvements in both diagnostics and treatment, the overall mortality from lung cancer remains devastating with a 5-year survival below 15% (Siegel et al., 2014; The Danish National Lung, 2014). The use of prognostic biomarkers may optimise overall survival (OS) by helping identifying high-risk patients, thereby facilitating stratification into optimal treatment regimes. A large number of prognostic biomarkers have been suggested in lung cancer, but only a few have proven to be clinically relevant. The limited availability of relevant tumour tissue along with biopsy-associated risks and tumour heterogeneity have made the non-invasive and easily assessable liquid biopsies an intriguing source for anyone exploring new biomarkers.

Exosomes are small membrane vesicles (diameter 30 nm–100 nm) derived from the multi-vesicular body and released by exocytosis into the extracellular space constitutively or after cell activation (Harding et al., 1983; Pan and Johnstone, 1983). Exosomes can contain bioactive miRNA, mRNA or DNA from their originating cell protected by a lipid bilayer, and it has been suggested that they may play a role in intercellular communication (Valadi et al., 2007; Huang et al., 2013; Théry et al., 2009). In cancer, exosomes have been shown to be implicated in the crosstalk between tumour cells and normal cells thereby facilitating the malignant process (Roma-Rodrigues et al., 2014). Exosomes are readily available in body fluids and have therefore been evaluated as biomarkers in cancer (Taylor and Gercel-Taylor, 2008; Khan et al., 2014; Skog et al., 2008; Rabinowits et al., 2009). In lung cancer, several studies have found exosomes to be promising as diagnostic markers (Rabinowits et al., 2009; Yamashita et al., 2013; Li et al., 2011; Sandfeld-Paulsen et al., 2016). A few smaller studies have evaluated the prognostic perspectives of exosomal miRNA or extracellular vesicles in the bloodstream (Rabinowits et al., 2009; Fleitas et al., 2012; Hu et al., 2010), but the membrane-bound proteins on the exosomes have not previously been investigated in NSCLC. It has been suggested that exosomes are captured to modulate activity in the recipient cell, and it is likely that the membrane-bound proteins are important for this process.

The extravesicular (EV) Array is an adjusted protein microarray that captures extracellular vesicles fitting the description of exosomes, here and in the following defined as vesicles expressing CD9, CD63 and/or CD81 and with a size of 30 nm–100 nm (Jørgensen et al., 2013). The EV Array is a fast, automated, inexpensive and highly sensitive method that uses only a 10 μL sample. In a feasibility study by our group, we demonstrated that phenotyping of the membrane-bound proteins on the exosomes with the EV Array technique is a promising and applicable biomarker in NSCLC (Jakobsen et al., 2015). Similarly, we demonstrated the diagnostic potential of this approach (Sandfeld-Paulsen et al., 2016).

It nevertheless remains unclear if phenotyping the membrane-bound proteins on exosomes can also be used to predict OS. Accordingly, the aim of this study was to evaluate the prognostic potential of membrane-bound proteins on exosomes in a non-selected prospective cohort of NSCLC of all stages and histologies.

2. Material and methods

2.1. Patients and plasma samples

A total of 304 NSCLC patients were recruited consecutively from a cohort of 1735 patients suspected of having lung cancer and referred to the Department of Pulmonary Medicine, Aarhus University Hospital, Denmark, from April 2011 to September 2014. Patient selection was described in an earlier study (Sandfeld-Paulsen et al., 2016). In brief, patients were included if they were suspected of having lung cancer due to clinical symptoms (n = 1735). Cancer of other origin was the only exclusion criterion (n = 301). In the present study, the inclusion criterion was pathologically verified NSCLC of any histologic subtype (n = 304). After quality control of the plasma samples, 276 patients were included in this study. Data on clinico-pathological characteristics histology, stage, WHO performance status (PS), age and sex were collected at the time of inclusion. Clinical and pathological characteristics are described in Table 1.

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

Blood samples (EDTA plasma) were collected at the first visit at the hospital before any diagnostic work-up was done. The blood samples were spun (1400 g for 15 min) to isolate plasma. After isolation, plasma was stored at −80 °C.

Table 1 – Patient characteristics. Clinical characteristics of the lung cancer cohort.

	NSCLC cohort (N = 276)
Sex (male:female)	145: 131
Age (median, range)	68.6 (39–89)
Histology, n (%)	
Adenocarcinoma	198 (72)
Squamous-cell carcinoma	69 (25)
Others	9 (3)
Stage, n (%)	
I	67 (24)
II	24 (9)
III	73 (26)
IV	112 (41)
PS, n (%)	
0	88 (32)
1	128 (46)
2	44 (16)
3	16 (6)
Smoking, n (%)	
Never	16 (6)
Former	159 (58)
Current	97 (35)
Missing	4 (1)

NSCLC: non-small cell lung cancer. PS: performance status.

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2.2. Production of EV microarray

Antibodies were printed on epoxy-coated slides (75.6 mm * 25.0 mm; SCHOTT Nexterion, DE) using a SpotBot® Extreme Protein Edition Microarray Printer with a 946MP4 pin (ArrayIt Corporation, CA, USA). Positive and negative controls were biotinylated human IgG (100 mg/mL) and PBS with 5% glycerol, respectively. After printing, the slides were left to dry overnight at room temperature. A total of 49 anti-human antibodies, listed in Table 2, were used for production of the EV Array. Antibodies were diluted with PBS with 5% glycerol and printed in triplicates at 75–200 mg/mL. The lung cancer-related antibodies were selected based on the literature and on our previous studies of the use of exosomal membrane-bound proteins as diagnostic biomarkers in lung cancer (Sandfeld-Paulsen et al., 2016).

2.3. Catching and visualising exosomes

Preparation of the EV Array was done as described by Jørgensen et al. (2013), i.e. catching and detection of the exosomes are performed in parallel in unpurified starting material. In short, blocking of the microarray slides (50 mM ethanolamine, 100 mM Tris, 0.1% SDS, pH 9.0) was performed prior to incubation with 10 μL plasma sample diluted (1:10) in wash-buffer (0.05% Tween®20 (Sigma–Aldrich, MO, USA) in PBS). Incubation of the microarray slides was performed in Multi-Well Hybridization Cassettes (ArrayIt Corporation) at room temperature for 2 h prior to overnight incubation at 4 °C. After washing, the slides were incubated with biotinylated detection antibodies (anti-human-CD9, -CD63 and -CD81, LifeSpan BioSciences, WA, USA) diluted 1:1500 in wash buffer. Following a second wash, the slides were incubated for 30 min with Cy5-labelled streptavidin (Life Technologies) diluted 1:1500 in wash buffer. Before detection, 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 (Jørgensen et al., 2013).

2.4. Data preparation

Each antibody was printed in triplicate and the mean value was used to estimate signal intensity. To exclude samples of low quality, the positive-to-negative ratio was calculated: (positive − negative)/positive. If the positive-to-negative ratio was below 0.98, the sample was excluded. To evaluate each triplicate, the coefficient of variance (CV) was calculated. If the CV was above 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). Since CD63 was expressed in only 192 out of 276 samples, CD63 was not considered valid as a normalisation factor. Both CD9 and CD81 were expressed in all 276 samples, and the geometric mean of the two was used as normalisation factor. For each antibody, the signal intensity was divided by the geometric mean of CD9 and CD81 to evaluate the protein density on the exosomes.

Table 2 – Antigens evaluated as exosomal membrane-bound proteins. Each antigen is normalised to the geometric mean of CD9 and CD81.

	Antigen	Median	Range	Fraction % (n = 276)
Exosomal markers	CD9	1.22	0.45–6.84	100
	CD63	0.02	0.00–0.99	70
	CD81	0.82	0.15–2.24	100
	CD82	0.58	0.17–2.40	100
	CD37	0.00	0.00–1.36	44
	TSG101	0.00	0.00–1.36	38
	Alix	0.09	0.00–3.22	75
	Hsp90	0.01	0.00–1.03	55
Cancer cell markers	EpCam	0.03	0.00–6.76	65
	PLAP	0.02	0.00–4.85	59
	TAG72	0.01	0.00–1.21	60
	Tspan8	0.11	0.00–1.95	96
	NY-ESO-1	0.00	0.00–4.08	50
	MUC16	0.00	0.00–0.17	6
	MUC1	0.08	0.00–1.06	80
	CEA	0.02	0.00–1.67	66
	Flotilin-1	0.00	0.00–1.67	35
	CD171	0.02	0.00–1.03	75
	CD151	0.58	0.00–4.43	97
	CD142	0.04	0.00–2.08	74
	CD146	0.14	0.00–7.94	86
	EGFR	0.02	0.00–1.04	72
	HER2	0.00	0.00–1.19	50
	HER3	0.00	0.00–2.25	24
	HER4	0.03	0.00–1.41	59
	AREG	0.05	0.00–2.97	75
	PDL-1	0.00	0.00–0.57	29
	cMET	0.00	0.00–0.21	22
	HB-EGF	0.01	0.00–2.67	59
	N-cadherin	0.00	0.00–0.90	46
	p53	0.01	0.00–1.94	57
	CD13	0.01	0.00–4.03	54
	EGFRvIII	0.00	0.00–0.78	38
	CA-IX	0.00	0.00–1.84	24
	CA-XII	0.02	0.00–1.36	62
	CD56	0.00	0.00–0.75	31
	CD147	0.00	0.00–0.70	35
	LRP1	0.20	0.00–10.98	83
	CD276	0.02	0.00–1.03	59
	Claudin1	0.00	0.00–1.39	31
	sTn	0.01	0.00–6.60	53
	GRP78	0.01	0.00–2.63	57
Other	CD163	0.01	0.00–1.09	55
	CD206	0.05	0.00–12.80	71
	CD14	0.00	0.00–0.84	44
	SFTPD	0.04	0.00–4.67	60
	SP-A	0.18	0.00–8.16	83
	TNF RI	0.07	0.00–2.21	80
	TNF RII	0.00	0.00–2.05	37

Median value and range of each antigen are presented, along with the fraction (%) of the 276 patients whit measurable levels of the given the protein.

2.5. Statistical analysis

All p-values presented are two-sided. OS was defined as death from any cause and calculated from the date of diagnosis to death or last day of follow-up (May 1, 2016), whichever came

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first. OS was estimated by the Kaplan–Meier method and compared using the log-rank test. Uni- and multivariate hazard ratios (HRs) were estimated by Cox proportional hazard analyses. HR is presented with a 95% confidence interval (CI). The proportional hazard assumption was assessed graphically by plotting Schoenfeld residuals and log–log plots. The estimated HR was evaluated by 1000 bootstraps to assess the robustness of the estimates. In case of biased data due to e.g. limited sample size, bootstrapping offers a validation by providing an alternative to the traditional parametric estimates, namely the bias-corrected confidence intervals (BCIs) (Hayes, 2009). Only results with a BCI not including 1 were considered valid. Bonferroni correction was applied and only p-values below 0.001 were considered truly significant ($\alpha = 0.05/49$ exosomal markers $= 0.001$). Statistical analyses and graphs were made in STATA version 13.0 (StataCorp LP, Texas). Heat maps and hierarchical cluster analysis were produced using Genesis (version 1.7.6, IGB TU Graz, Graz, Austria).

3. Results

3.1. *Exosomal membrane and membrane-associated proteins*

Hierarchical cluster analysis showed much expression variety among the patients without favouring any of the clinical characteristics of histology, stage, age or sex. Co-expression of several of the exosomal membrane-bound proteins was observed (Figure 1 and Figure S1).

3.2. *Survival analyses*

To explore exosomal membrane-bound proteins as prognostic biomarkers in NSCLC, the presence of the individual marker was evaluated. Each marker was dichotomised based on its detection or non-detection. The impact of each marker on OS was estimated by univariate and multivariate analysis adjusting for the clinico-pathological characteristics of age, sex, PS, histology and stage (Table 3). In total, 4 markers showed a prognostic potential ($p \leq 0.05$), all leading to improved survival. To test the robustness of the estimates, 1000 bootstraps were performed which confirmed the 4 markers' impact on OS ($p < 0.05$) (CD171, Flotilin1, HER3, GRP78) (Table 3). Nevertheless, according to the Bonferroni correction ($p < 0.05/49 = p < 0.001$), only one marker could truly predict OS; CD171 (HR 0.56 95%CI (0.41–0.79); $p = 0.001$). This marker was found in 75% of the patients. Univariate survival curves of CD171 are depicted in Figure S2. Hierarchical cluster analysis showed covariance between the four markers (Figure 1).

Since some of the markers were present in the majority of the samples, the presence of the marker alone was not an adequate approach. Therefore, the level of the individual marker was evaluated as a prognostic marker. As splitting the group based on observed concentration would introduce bias, the level was evaluated as a continuous variable. Each marker's effect on OS was estimated by univariate and multivariate analyses adjusting for the clinico-pathological characteristics of age, sex, PS, histology and stage (Table 4). In total, seven markers had a significant prognostic potential ($p \leq 0.05$) with increasing level. The bootstrap algorithm was performed which left three markers with a significant prediction of an inferior OS (NY-ESO-1, EGFR and PLAP) (Table 4). The Bonferroni correction was applied to minimise the risk of accepting false positives. Although the p-value was not <0.001, NY-ESO-1 showed a strong association (HR 1.53 95% (1.13–2.10); $p = 0.007$).

As described in Table 1, some of the markers were only quantifiable in a subset of patients. The large number of patients with no measurable level of a given marker introduces skewness to the dataset. This could potentially lead to underestimation of the impact on OS by any given marker. To further explore the exosomal surface proteins as prognostic markers, each marker was evaluated as a continuous variable, but only in the patients in whom the protein was measurable (levels above 0). The results are presented in Table 5. As expected, the number of markers with a significant impact on OS increased. But most importantly, all of the three initially selected markers maintained their effect on OS. In total, nine markers had a significant impact on OS ($p \leq 0.05$). After the bootstrap algorithm was performed, five markers maintained their prognostic potential, predicting inferior survival (NY-ESO-1, EGFR, PLAP, EpCam and Alix) (Table 5). Nevertheless, according to the Bonferroni correction ($p < 0.001$), only NY-ESO-1 was a strong prognostic biomarker of OS (HR 1.78 95% (1.78–2.44); $p = 0.0001$). The hierarchical cluster analysis showed covariance of NY-ESO-1, PLAP, EpCam and Alix. In contrast, EGFR was not a covariate (Figure 1).

4. Discussion

Much recent research in exosomes suggests that they may serve as cancer biomarkers. Their use as diagnostic biomarkers has been investigated among others in lung cancer (Taylor and Gercel-Taylor, 2008; Skog et al., 2008; Rabinowits et al., 2009; Sandfeld-Paulsen et al., 2016; Khan et al., 2012). However, the use of exosomes as prognostic markers has been evaluated in only a few small studies focused on extracellular vesicles or exosomal miRNA (Rabinowits et al., 2009; Fleitas et al., 2012; Hu et al., 2010). It is very likely that exosomal surface proteins reflect the pathological processes in NSCLC, but no studies have previously investigated the prognostic potential of exosomal membrane-bound proteins.

In a prospective cohort counting 276 non-selected NSCLC patients of all stages, we evaluated 49 exosomal membrane-bound proteins and found that nine markers have a potential as prognostic markers in NSCLC. We demonstrated that NY-ESO-1, EGFR and PLAP are prognostic markers of inferior survival with increasing concentration level. For some of the markers, we observed a large number of patients with no detectable expression of the given marker. It is a well-known fact that NSCLC is a heterogeneous disease with different driver mechanisms, and in most patients with NSCLC the specific driving mechanism remains unknown. The diversity in expression levels of the exosomal membrane-bound proteins may reflect this heterogeneity.

The large number of patients with no detectable expression of a given marker could weaken the determination of the impact of that marker on survival. We therefore evaluated the diagnostic potential of the individual marker only if it

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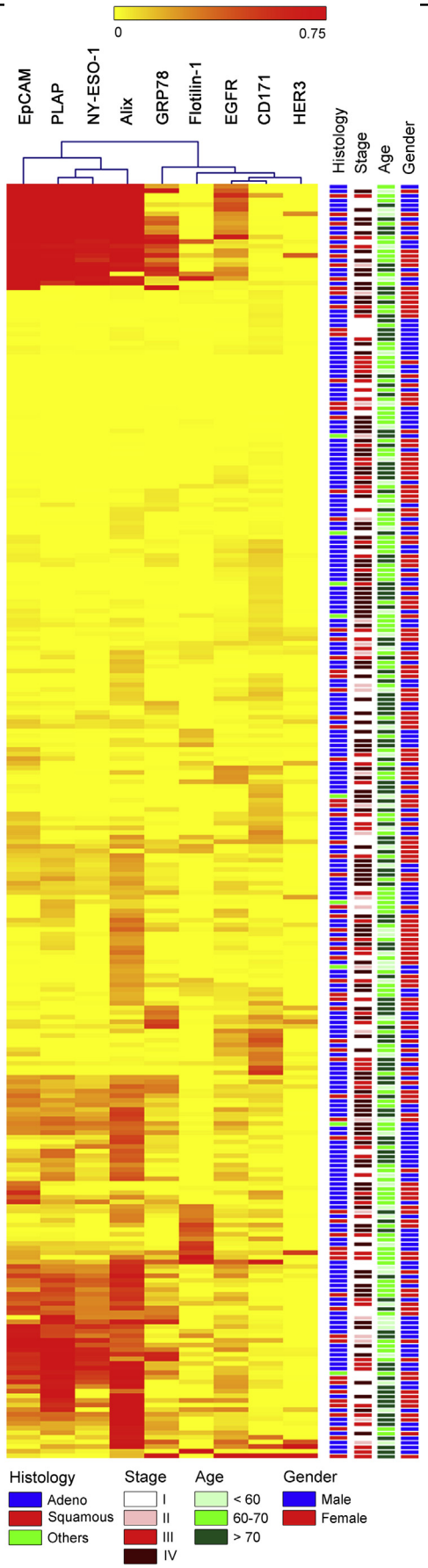

was present. As expected, this added EpCam and Alix to the group of markers with a potential impact on OS. However, in the present dataset, only NY-ESO-1 remained significant after the Bonferroni correction was applied. NY-ESO-1 (New York Esophageal squamous cell carcinoma-1) is a testis cancer antigen expressed in many solid cancers (Esfandiary and Ghafouri-Fard, 2015). Studies have evaluated the gene expression of NY-ESO-1 in NSCLC and found that it is present in 15–30% of lung cancer samples. However, our results were conflicting when NY-ESO-1 was correlated with OS (Gjerstorff et al., 2013; Grah et al., 2014; Konishi et al., 2004). In the present study, we found exosomal NY-ESO-1 in 50% of the patients. No previous studies have examined exosomal membrane-bound proteins as prognostic markers in NSCLC. Exosomal EGFR is one of the few membrane-bound proteins that have been evaluated in NSCLC. Yamashita et al. (2013) detected the presence of exosomal EGFR in five out of nine plasma samples from NSCLC patients, but none in nine healthy controls. Similarly, in tumour biopsies, Huang et al. (2013) found exosomal EGFR in 80% of patients with NSCLC, but only in 2% of patients with chronic obstructive lung disease. This is in concordance with our findings where exosomal EGFR was detected in the plasma of 72% of the patients. The study of the presence of the exosomal membrane-bound proteins as prognostic biomarkers suggested that four markers indicated superior OS. Nevertheless, when the Bonferroni correction was applied, only the presence of CD171 remained a marker of superior OS. This was unexpected since CD171 evaluated by immunohistochemistry in tumour samples has been associated with metastatic behaviour and a poor prognosis (Tischler et al., 2011). However, it remains unknown whether the impact of CD171 on OS measured in the exosomes can, if fact, be equivalent to CD171 measured in the actual tumour. Another explanation could be related to the method used. In the present study, we used the EV Array, an immunoaffinity-based isolation method that captures extracellular vesicles fitting the description of exosomes (Jørgensen et al., 2013). The EV Array is a sandwich-based ELISA modality including capture and detection antibodies. The detection antibodies ensure that the proteins caught are detected only if they are in conjugation with the exosomal markers CD9, CD81 or CD63, ensuring that other plasma proteins will not be detected. Defining exosomes using surface proteins, we challenge the fact that exosomes all have one “household” surface protein that is ubiquitously expressed. Therefore, the International Society for Extracellular Vesicles (ISEV) recommends that when defining exosomes, three or more exosomal markers should be used to optimise the number of exosomes caught (Lötvall et al., 2014). As described previously, extracellular vesicles isolated by the EV Array fit the description of exosomes in size and density (Jørgensen et al., 2013). Nevertheless, it is possible that not all exosomes express CD9, CD81 or CD63 in which case our isolation procedure would miss a subset of exosomes that expresses none of these markers. In the present study, CD63 was expressed only in 70% of the samples. We therefore question the use of CD63 as a standard EV marker, which is supported

Figure 1 – Hierarchical cluster analysis. Heat map illustration of the nine markers that have an impact on overall survival along with clinical characteristics (histology, stage, age and sex).

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Table 3 – Membrane-bound exosomal proteins as prognostic biomarkers in NSCLC based on the presence of each marker.

Marker	Univariate			Multivariate				Fraction (%) (n = 276)[b]
	HR	95%CI	p	HR	95%CI	p	Bootstrap	
CD171	0.71	0.52–0.98	0.04	0.56	0.41–0.79	0.001	a	75
Flotilin1	0.73	0.53–0.99	0.04	0.63	0.46–0.86	0.004	a	35
HER3	0.68	0.48–0.96	0.03	0.61	0.43–0.87	0.006	a	24
GRP78	0.75	0.56–0.99	0.05	0.69	0.51–0.91	0.01	a	57
cMET	1.11	0.79–1.57	0.53	1.41	1.00–2.01	0.05	–	22
sTn	1.66	1.06–2.59	0.03	1.51	0.93–2.43	0.07	–	53
Tspan8	2.37	0.97–5.76	0.06	2.22	0.91–5.47	0.08	–	96

Only markers with a trending or significant ($p \leq 0.10$) impact on OS are presented.
a Multivariate results verified by bootstrapping.
b Number of patients with the individual marker present as a fraction of the total number of patients.

Table 4 – Membrane-bound proteins as prognostic biomarkers based on the expression level.

Marker	Univariate			Multivariate			
	HR	95%CI	p	HR[a]	95%CI	p	Bootstrap
NY-ESO-1	1.11	0.83–1.47	0.49	1.53	1.13–2.10	0.007	b
EpCam	1.03	0.83–1.28	0.80	1.31	1.04–1.65	0.02	–
CAIX	2.81	1.00–7.84	0.05	2.84	1.18–6.81	0.02	–
CD13	1.93	1.11–3.37	0.02	1.81	1.09–3.03	0.02	–
EGFR	1.21	0.25–5.79	0.81	4.43	1.18–16.68	0.03	b
PLAP	1.01	0.80–1.29	0.91	1.28	1.02–1.60	0.03	b
CD276	1.53	0.33–7.10	0.59	4.11	1.00–17.05	0.05	–
AREG	1.58	0.92–2.71	0.09	1.55	0.94–2.54	0.08	–
HER2	1.16	0.29–4.61	0.83	3.28	0.88–12.18	0.08	–

a Hazard ratio adjusted for stage, histology, PS, age and sex.
b Multivariate results verified by bootstrapping. Only markers with a significant or a trending impact on OS ($p < 0.10$) are included.

by earlier studies using the EV Array (Jørgensen et al., 2013; Baek et al., 2016) and other techniques (He et al., 2014; Basu and Bhattacharyya, 2014; Franquesa et al., 2014). It is possible that exosomes from cancer cells express other proteins than those explored in the present study.

In most studies, exosomes are isolated by time- and material-consuming ultracentrifugation in combination with a sucrose gradient, which effectively ensures a high number of exosomes based on density. However, the risk of aggregation and rupture of EVs is considerable which is not the case with immunoaffinity-based isolation methods (Taylor and

Table 5 – Exosomal surface proteins as prognostic biomarkers.

Marker	Univariate			Multivariate				n[c]
	HR	95%CI	p	HR[a]	95%CI	p	Bootstrap	
NY-ESO-1	1.19	0.89–1.59	0.25	1.78	1.78–2.44	0.0001	b	138
HER3[d]	3.98	1.50–10.52	0.005	3.93	1.52–10.13	0.004	–	65
CAIX[d]	4.72	1.61–13.84	0.05	5.05	1.81–14.06	0.002	–	67
EpCam	1.07	0.86–1.32	0.80	1.37	1.09–1.72	0.007	b	180
CD13	1.80	1.00–3.25	0.02	1.81	1.08–3.03	0.02	–	150
PLAP	1.07	0.83–1.35	0.63	1.33	1.06–1.67	0.02	b	163
CD276	1.90	0.32–11.33	0.59	6.54	1.35–31.60	0.02	–	163
EGFR	1.18	0.21–6.67	0.81	4.76	1.17–19.39	0.03	b	200
Alix	1.17	0.86–1.61	0.83	1.39	1.02–1.90	0.04	b	207
HER2	1.16	0.28–4.91	0.83	3.51	0.89–13.84	0.07	–	137

Each marker evaluated in patients with measurable levels of the individual marker.
a Hazard ratio adjusted for stage, histology, PS, age and sex.
b Multivariate results verified by bootstrapping. Only markers with a significant or a trending impact on OS ($p < 0.10$) are included.
c Number of patients included.
d Due to low number of events, only stage, PS and histology was included in the multivariate analysis.

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Shah, 2015). The EV Array approach uses as little as 10 μL unpurified plasma and allows for a large number of proteins to be tracked in parallel, which lowers the workload and heightens the clinical applicability.

To our knowledge, this is the first study to evaluate exosomal membrane-bound proteins in a large NSCLC cohort. In a previous study (Sandfeld-Paulsen et al., 2016), we evaluated the diagnostic potential of the exosomal membrane-bound proteins and found that the variation between stages was non-significant, which could reflect the aggressive nature of NSCLC independently of stage. Among the exosomal membrane-bound proteins identified as potential prognostic markers in the present study, especially CD171, but also NY-ESO-1, PLAP and Flotilin1 had some potential as diagnostic markers. Nevertheless, a clinically relevant approach required a combination of 10 markers to ensure a fair discrimination between cancer and non-cancer patients. The prospective nature of our cohort is a major strength of the study. We included patients and collected blood samples before knowing whether the patient had cancer or not, and before any diagnostic procedures were conducted. Using a non-selected cohort raises the validity of the study because it reflects the real-life situation in the clinic.

We identified nine individual markers with a significant ($p < 0.05$) prognostic potential. Since this study tested 49 different markers, the risk of multiple testing is present. Because of the results of the Bonferroni correction, we therefore cannot conclude on more than one marker. However, since all nine biomarkers seem to affect survival to some extent, our findings would benefit from further verification in a new set of clinical samples. We speculate that in such a validation cohort, combining the markers into a single measure could enhance the prognostic potential in NSCLC. Furthermore, since the membrane-bound proteins are a reflection of the cancer and can be obtained from a simple blood sample, the potential of using this tool for continuous monitoration of cancer development is promising. Thus, our findings in lung cancer call for studies exploring whether the observed association with lung cancer can be extended to other cancers, i.e. whether we are faced with a general cancer phenomenon. Additionally, functional investigations are needed to better understand the biological mechanisms of the exosomal membrane-bound proteins in lung cancer and cancer in general.

5. Conclusion

Exploration of exosomal membrane-bound proteins as prognostic markers in NSCLC revealed a total of nine markers with potential impact on OS that were independent of known clinico-pathological factors. Nevertheless, due to the limitations of the dataset, only NY-ESO-1 was found to be a truly significant prognostic biomarker of inferior survival. In patients with NSCLC in whom NY-ESO-1 is found, a different treatment strategy may be needed and exosomal membrane-bound protein determination may therefore be a strong tool in the clinic. However promising, our results need to be validated in an independent cohort.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2016.10.003.

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