RESEARCH ARTICLE

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Proteomic Profiling of Small Extracellular Vesicles Isolated from the Plasma of Vietnamese Patients with Non-Small Cell Lung Cancer Reveals Some Potential Biomarkers

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Abstract

Background: Considering the poor prognosis of non-small cell lung cancer (NSCLC), the objective of this study was to examine the potential of plasma-derived vesicles as a source of lung cancer-specific proteins. Extracellular vesicle (EV) cargos are specific to the source cells, hence they have the potential of being a source of cancer-specific proteins. **Methods:** The proteins differently expressed in cancer were determined and derived from EVs isolated from the plasma of NSCLC patients at the National Lung Hospital. To this end, purification was done using gel filtration chromatography and ultracentrifugation. In addition, nano liquid chromatography mass spectrometry (LC–MS/MS) was used for analyzing. **Results:** Fifty-seven EV-derived proteins related to NSCLC were highlighted in this research. Some of them have not been addressed before, such as EEF1A1 (elongation factor 1-α1), KPNB1 (Importin subunit beta 1), SRC (proto-oncogene tyrosine-protein kinase) and ACTC1 (actin, alpha cardiac muscle 1). This list was further confirmed through a comparison with ExoCarta and Vesiclepedia. **Conclusion:** This study is the first work to show the involvement of several novel proteins of small EV (EEF1A1, KPNB1, SRC, and ACTC1) in the progression of NSCLC. The results suggested that they could serve as novel biomarkers for non-small cell lung cancer in the future.

Keywords: Proteomics- exosomes- non-small cell lung cancer- NSCLC- plasma- extracellular vesicle- EV-small EV

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Introduction

Non-small cell lung cancer (NSCLC) constitutes 85 % of lung cancers (Evans 2013). The overall 5-year survival rate is only 10–15 % despite many efforts to improve the treatment. A major factor that contributes to poor prognosis is that most patients at the time of diagnosis are at an advanced stage of the disease (Siegel et al., 2018). Consequently, new approaches are required to identify new biomarkers and mechanisms of NSCLC that are susceptible to therapeutic inventions. Recent studies have supported the idea that extracellular vesicles (EVs) may provide a path toward this end.

The EVs are extremely diverse in origin, composition, morphology and possible functions. Recent studies have classified them into two groups based on their size, namely small EVs (< 200 nm in diameter) and medium and/or large EVs (> 200 nm in diameter). Moreover, intercellular cross-talk mediated by Evs in epithelial–mesenchymal transition associated with metastasis has opened a new door to the search for diagnostic and predictive approaches (Syn et al., 2016).

Small Evs are membrane-enclosed vesicular structures with a diameter ranging from 30 nm to 200 nm (Syn et al., 2016; Théry et al., 2018), mainly including exosomes and microvesicles (< 200 nm in diameter). Small Evs signal in three ways; first in juxtacrine fashion by activating the adjacent cells via the interactions between membrane proteins of small EVs to the target cells; second as paracrine agents following diffusion to neighbouring cells through the extracellular matrix; and third systemically as the result of transport to a distant location via circulation (Guo et al., 2019). They are widely detected in body fluids such as blood, urine, and saliva (Boukouris and Mathivanan, 2015).

The components of small EVs in NSCLC reflect the pathological processes associated with tumours. Moreover, small EVs are available in the plasma and serum, which give us an easily accessible and relatively non-invasive source of samples (Caby et al., 2005). The use of plasma-derived small Evs and their constituent markers in NSCLC have been studied by several studies

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(Lin and Yang, 2011; Poroyko et al., 2018; Nigita et al., 2018).

Among various approaches targeting small Evs, proteomic analysis can provide an insight into the early-stage or metastasis-associated proteins at the system level. This is shown in their applications in a number of malignancies (Bard et al., 2004). However, there are still unanswered questions about the specific markers from small EVs for NSCLC prognosis in Vietnamese patients. Therefore, in this study, we collected the plasma of 31 patients with NSCLC at four stages of the disease, along with 31 plasma samples from healthy donors as control subjects. The study revealed several prospective proteins as metastasis markers for NSCLC, some of which have not been addressed in prior studies on NSCLC, including SRC, EEF1A1, KPNB1, and ACTC1.

Materials and Methods

Patients and clinical samples

In total, 31 patients with NSCLC-including 16 men with a mean age of 58.7 years and 15 women with a mean age of 65.7 years-at the National Lung Hospital (Ha Noi, Viet Nam) took part in the study from June 2018 to December 2019. Based on the classification of tumour and metastasis, the patients were divided into two groups, 16 patients at stages I and II and the second group with 15 metastasis patients. All enrolled patients were diagnosed with primary adenocarcinoma.

In addition, 31 subjects who visited the hospital for general medical examinations were recruited in this study as control. Fresh blood samples of all subjects were collected and centrifuged at 1500 x g for 10 minutes at room temperature to isolate the plasma. The plasma samples were then centrifuged at 10,000 x g for 10 min at 4^{o}C to remove the platelets. After isolation, the supernatant was stored at –25^{o}C for further analysis. Informed consent was obtained from all subjects involved in the study. The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the local ethical Committee of Vietnam National University, Hanoi, Vietnam (KLEPT.18.03).

Isolation of small extracellular vesicles from human plasma

The plasma samples were thawed on ice, pooled, centrifuged at 10000 x g for 10 min, and filtered through a 0.22-μm pore filter (Satorius, Germany) to remove the remaining platelets and apoptotic bodies. The filtration of 2-ml pooled plasma was then loaded onto a manually made size-exclusion chromatography (SEC) column packed with 10-ml Sepharose CL-4B (GE Healthcare, Uppsala, Sweden). The column was washed and equilibrated with filtered phosphate buffered saline (PBS, pH 7.4, 0.22-μm filter). The flow-through was collected in 30 fractions of 500 μl each using PBS as an elution buffer.

Dynamic light scattering (DLS)

The DLS analysis was carried out using Zetasizer Nano ZS with a 633-nm He–Ne laser (Malvern, Herrenberg, Germany). The measurements were performed at an angle of 173o with an automatic attenuator. A SEC fraction volume (40μL) was poured into the solvent-resistant cuvette. The temperature during the measurement was maintained at 25oC. The measurements were repeated five times for each sample and the final result was calculated as average.

Scanning Electron Microscopy (SEM)

The SEM imaging was developed using a 5-kV acceleration voltage as well as a secondary electron detector. The samples were first fixed with 2% (v/v) glutaraldehyde at 25^{o}C for 10 min before topographical images were captured using FE-SEM equipment (Nova NanoSEM 450, FEI, US) with several frames for variant observations and for later statistical analysis. A resolution of 256 × 256 pixels was applied throughout the experiment, with the scanning rate varied from 0.3 to 0.75 Hz. The images were analyzed using NanoScope Analysis 1.5 (Brucker Corporation).

Immunoblotting (Western blot)

Two markers including CD81 and CD63 were used to detect the presence of small EVs by immunoblotting. The selected fractions were separated on 10% (w/v) SDS-PAGE using TGX Stain-Free FastCast System (BioRad, USA), and then transferred to nitrocellulose membrane using a Turbo Trans Blot equipment (BioRad, USA) at 15V in 7 min. Five-percent (w/v) skimmed milk in TBS (50 mM Tris-Cl, pH 7.5, 150 mM NaCl) containing 0.1-% (v/v) Tween-20 (TBST) was used to block the membrane for 1 hour. The membrane was then incubated with the primary antibodies of CD81 and CD63 (Santa Cruz Biotechnology, USA) diluted 500 times in TBST overnight at 4oC. Then, the samples were washed three times using TBST, after which the membrane was incubated with anti-mouse IgGκ, horseradish peroxidase, and m-IgGκ BP-HRP (CM) (Santa Cruz Biotechnology, USA) at 10,000 times dilution in TBST for 1 hour at room temperature. Immunoreactivity was detected using Amersham ECL Advance Western Blotting Detection Kit. Furthermore, the protein bands were visualized on the ChemiDoc system (BioRad, USA).

Sample preparation, mass spectrometry, and interpretation of mass spectra

In-gel digestion

The SEC fractions 6 to 10 were ultracentrifuged at 120,000 x g at 4^{o}C for 120 min in an MLA-130 fixed-angle rotor (Beckman Coulter, US). The supernatant was discarded and the pooled small EV pellets were washed using PBS in a second ultracentrifugation. Fifteen-microgram of the protein of the washed small EV pellets was lysed in a non-reducing sample buffer (200-mM Tris-HCl, pH 6.8, 40-% (v/v) glycerol, 8-% (w/v) SDS, and 0.04-% (w/v) bromophenol blue) and boiled for 5 minutes at 95^{o}C before being loaded onto 15-% (w/v) SDS-PAGE which was run for 30 min at 100 V. The protein bands were stained using Coomassie and cut from the gel pieces. The gel pieces containing the proteins were treated with 25-mM ammonium bicarbonate and then with acetonitrile. One-mole dithiothreitol was added

to a final concentration of 10 mM and the samples were incubated at 60°C for 10 min. Afterward, carbamylation was achieved by adding 50-mM iodoacetamide to the mixture in the dark for 30 min at room temperature.

The samples were then subjected to digestion with trypsin (Promega, USA) at 37°C for 4 h. Cleavage was quenched with formic acid and the supernatant was analyzed directly without further processing.

Liquid chromatography (LC)–mass spectrometry (MS/MS) analysis

After digestion, half of each sample was subjected to nano LC–MS/MS using a Waters M-Class LC system interfaced using a ThermoFisher Fusion Lumos mass spectrometer. A trapping column and a 75-µm analytical column were packed with XSelect CSH C18 resin (Waters) and a 3.5-µm particle was used for the former and a 2.4-µm particle was used for the latter. Heat-up at 55°C using a column heater (Sonation) was used at the last step of column preparation.

Next, peptides were loaded onto a trapping column, followed by elution over a 75-µm analytical column at 350 nL/minute. The mass spectrometer was run in a data-dependent mode using an Orbitrap operated at 60 000 FWHM for MS and 15 000 FWHM for MS/MS at 3s per cycle.

Data processing for mass spectrometry

Mascot (Matrix Science, version 2.6.2) was used for data processing with Trypsin/P enzyme and SwissProt Human-010119_20190101 database. In terms of modifications, there were fixed modification of carbamidomethyl (C) (Cys, + 57.0215 Da) and variable modifications of oxidation (M) (Met, + 15.9950 Da), acetyl (N-term) (N-terminal, + 42.0106 Da), Gln-pyrbo-Glu (N-term Q, -17.0027), and deamidation (N/Q, +0.9840). Only the monoisotopic mass value was taken arrow into account. Ten ppm was adopted for peptide mass tolerance and 0.02 Da was adopted for fragmented mass tolerance. The maximum number of missed cleavages was two. Scaffold (version: Scaffold_4.10.0, Proteome Software) was then applied to the final Mascot DAT files for validation, since at least two unique peptides per protein were used for confirmation, filtering, and finally generation of a non-redundant list per sample. The data were filtered using 1% of proteins and peptides.

The final list of proteins from the MS/MS data were further processed using MaxQuant with an integrated Andromeda search engine (v.1.5.2.8) for functional annotation by Gene Ontology (GO). The search for the proteins was conducted against the Uniprot Human Database (released 2013_1 January 9th 2013).

Gene annotation and functional enrichment analysis

Gene Ontology (GO) annotation was carried out on the detected proteins of each group of samples in the Panther (Protein Analysis Through Evolutionary Relationships) database version 16 (Mi et al., 2021). The Retrieval of Interacting Genes/Proteins (STRING) database (Szklarczyk et al., 2019) was used to identify the protein–protein interaction (PPI) of proteins. High-through put functional enrichment analysis was conducted in FunRich (Pathan et al., 2015).

Results

Characterization of plasma small EVs in NSCLC patients and healthy individuals

After isolation of the small EVs, a series of standard experiments including DLS (dynamic light scattering), SEM (scanning electron microscopy), and western blot (Figure 1A) were carried out to detect and characterize the EV samples.

The results of protein quantification for 30 fractions obtained from the Sepharose CL-4B column (Figure 1B) showed an extremely low concentration of proteins in fractions 1 to 11. The amount of proteins slowly increased after the 12th fraction and the value reached its peak with the fraction 15; the peak value was maintained until the 23 rd fraction and then it declined to the baseline. This observation indicated that the fractions potentially rich in small EVs were the 6^{th} to 11^{th} (Figure 1B).

The experiments were then performed to measure the particle size distribution of the SEC fractions by DLS. The data demonstrated the existence of particles ranging from about 40 nm to 200 nm in diameter in the fractions 6 to 10 (Figure 1C), which was compatible with the size requirement of the Sepharose CL-4B beads. These sizes fit the expected range of diameter of human small EVs. There was only one peak found in this measurement, indicating the high purity of small EVs in the samples. The peak had an average size of particles of 75.35 nm, and 100 % of circular objects detected by DLS fell in this range. In addition, pictures from SEM analysis depicted the typical small EV-like round morphology (Figure 1D). The measurement of the particle size also affirmed the desirable diameter of the small EVs (Figure 1D) as the majority of particles were 70 to 110 nm in diameter.

Western blot analysis was conducted to detect CD63 and CD81 and two exclusive proteins found on the membrane of all small EVs (Figure 1E). The signals of CD81 were observed in fractions 6 to 9; these signals were strongest in fraction 7; however, weaker in fraction 8 (Figure 1E). The CD63 was detected in three fractions, namely 7, 8 and 9 (Figure 1E), and as seen for CD81, the potent signal was spotted in fraction 7, which became slightly weaker in fraction 8 and quite faded in 9 (Figure 1E). These results proved the appearance of small EVs in the fractions collected after the gel filtration.

Profile of proteome in plasma small EVs of NSCLC patients

The LC–MS/MS analysis indicated 243 proteins in small EVs derived from the non-metastatic stages of I–II NSCLC samples. There were 294 proteins in the small EVs isolated from metastatic stages III–IV NSCLC patients. In the control group, a total of 214 proteins were detected. The data collected from mass spectrometry analysis is provided in Supplementary data.

The proteins derived from the small EVs of the control and patient groups were compared with the proteins in the ExoCarta (Keerthikumar et al., 2016) and Vesiclepedia

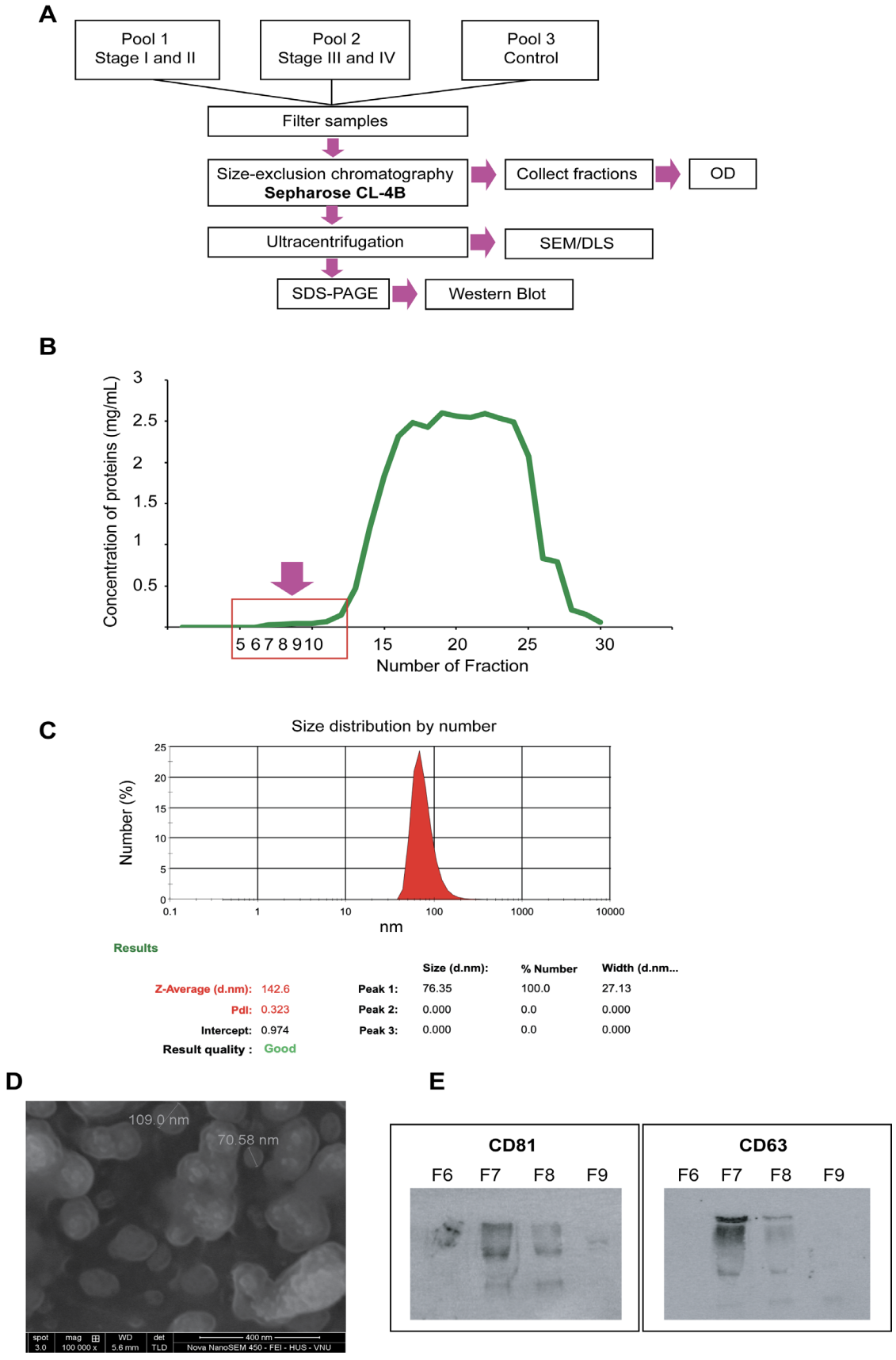

Figure 1. Analysis of Plasma Small EVs from NSCLC Patients and Healthy Individuals. The validation of plasma small EVs was carried out in pooled samples of plasma from patients at stages I–II and III–IV NSCLC, as well as plasma from healthy individuals. A. Workflow of characterisation of plasma EVs B. Isolation of small EVs from plasma proteins using a Sepharose CL-4B column. The concentration of protein (mg/ml) of 30 fractions collected from the column (500 µl per fraction). C. DLS analysis of plasma small EVs of NSCLC. The x-axis presents the diameter size in nm. The y-axis shows the percentage of particles. D. Topographical imaging by SEM of small EVs purified from the plasma of NSCLC and control groups. Scale bar = 400 nm. E. Western blot of two small EV markers CD81 and CD63. Fractions 6 to 9 were found to contain small EVs.

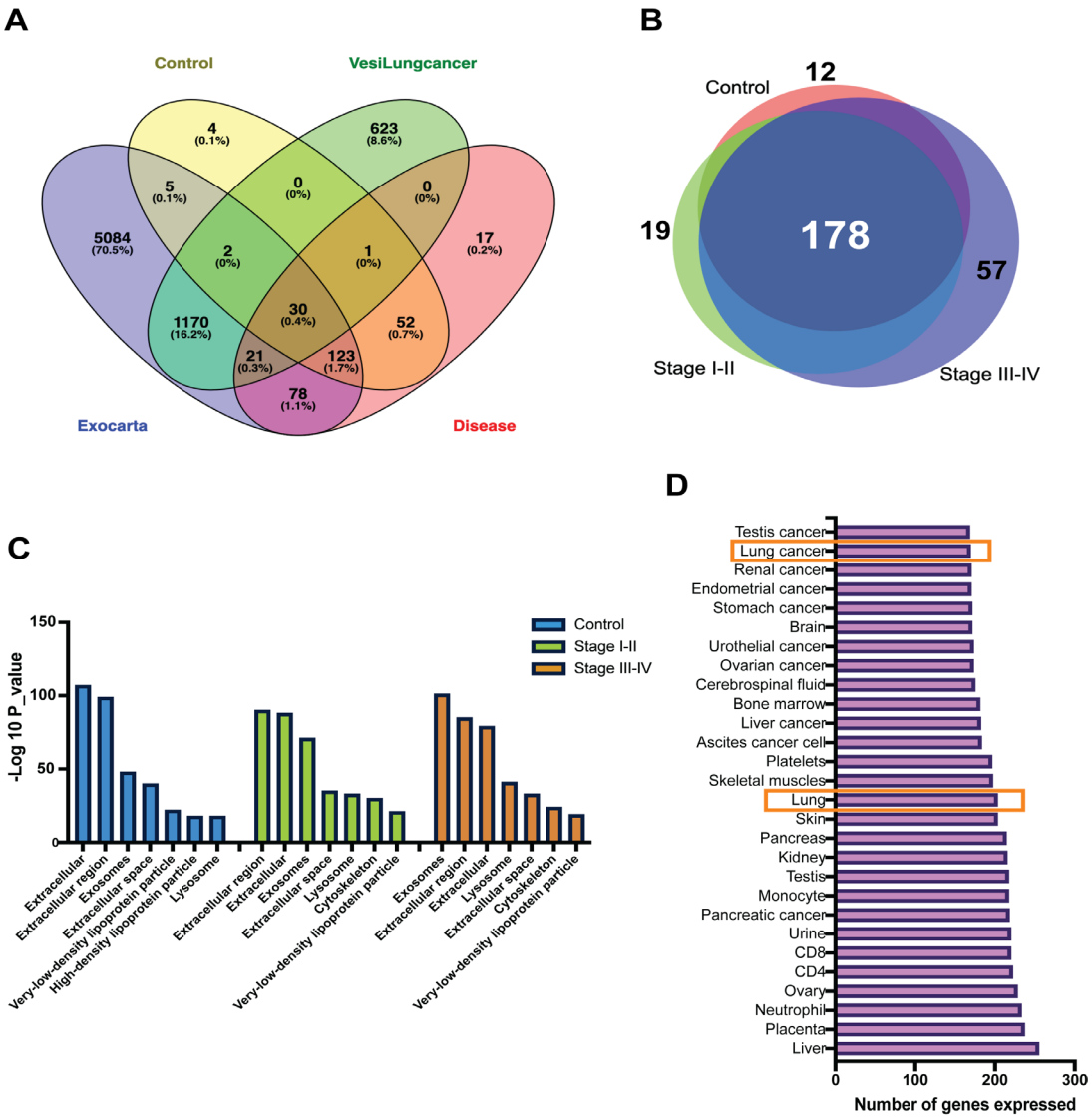

Figure 2. Bioinformatic Analysis of Small EV-Derived Proteins. A. Venn diagram of the overlap among EVs in the plasma of the control, disease group, Exocarta database and Vesiclepedia database. B. The number of proteins specific to each group or shared by two groups but not a third. C. Enrichment of EV proteins from the 3 plasma pools in the cellular components (Funrich analysis) D. Tissue derivation of small EV proteins.

(Pathan et al., 2019) databases using the proteins from Vesiclepedia selected as those identified in lung cancer cell lines. In total, 160/214 proteins were found in the control group and 252/322 proteins were found in the disease group (Figure 2A, Supplementary data) overlapping with proteins from ExoCarta. That is, the majority of proteins found in this study were located in small EVs. The overlap between proteins found in plasma EVs of NSCLC patients and the proteins in EVs from lung cancer cell lines in Vesiclepedia was only 52/322 (Figure 2A, Supplementary data).

Since the EVs isolated from plasma had specific features of the original tissue, in this case the lung, the tissue expression mapping was conducted with Funrich software. It was clear from the data that there was high enrichment of lung-derived and lung cancer-related proteins (Figure 2D). Moreover, various proteins were detected from other types of cancers found in other studies, such as pancreatic and liver cancers (Figure 2D).

Compared to the NSCLC group, there were 12 proteins with specifically more expressed in the samples from the healthy group. Nineteen proteins were detected only in stages I–II of NSCLC, while 57 were found solely in stages III–IV (Figure 2B, Supplementary data).

The Gene Ontology (GO) analysis of proteins found in the three groups, when compared to the total set of proteins identified in this study, demonstrated a clear enrichment in the extracellular region and exosomes (Figure 2C). In the case of EV proteins isolated from the plasma of NSCLC patients, even the majority of proteins were from exosomes (Figure 2C).

Protein-protein interaction (PPI) network of 57 different proteins between metastasis conditions and others identified in small EVs from NSCLC patients

There were 57 proteins detected by LC–MS/MS in small EVs isolated from NSCLC patients with metastasis (Figure 2B). These proteins were subjected to STRING to construct the network of metastatic-exclusive proteins (Figure 3) and depict how they may lead to the migration

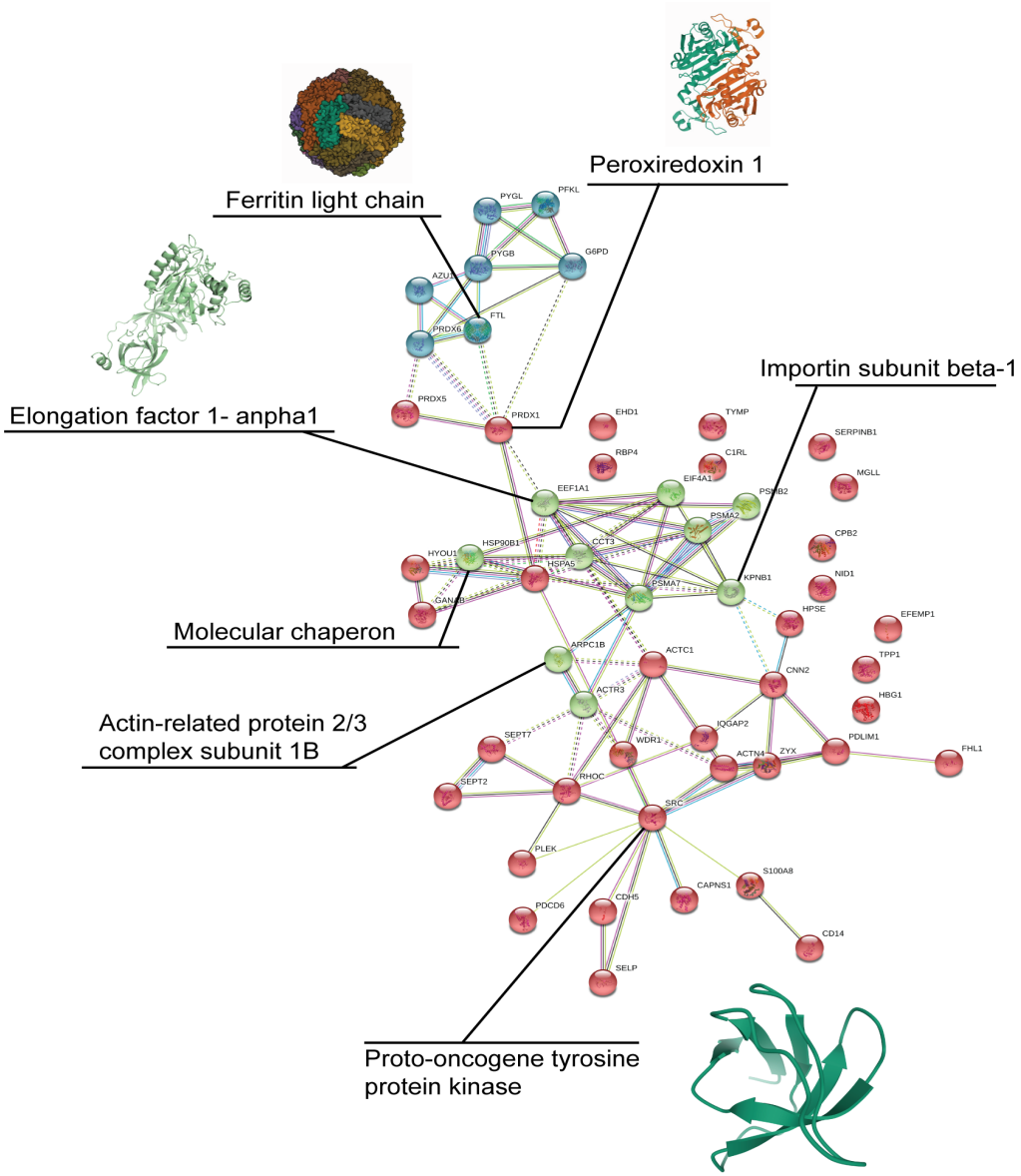

Figure 3. Network of Metastatic-Exclusive Proteins. Fifty-seven highly expressed proteins identified in small EVs from NSCLC patients at stage III–IV were subjected to STRING (Szklarczyk et al., 2019).

of tumour cells to other organs.

The topology of the network revealed several hub nodes, viz. SRC (proto-oncogene tyrosine-protein kinase), FTL (Ferritin light chain), EEF1A1 (elongation factor 1- α1), ARPC1B (Actin-related protein 2/3 complex subunit 1B), KPNB1 (Importin subunit beta 1), HSP90B1 (molecular chaperone), PRDX1 (Peroxiredoxin-1), ACTR3 (Actin-related protein 3), ACTC1 (Actin, alpha cardiac muscle 1), ACTN4 (Alpha-actinin-4), and HSPA5 (glucose-regulated protein) (Figure 3). All of these proteins have already been already confirmed by ExoCarta (Keerthikumar et al., 2016), thereby reinforcing our claim that our proteins originated from small EVs.

Discussion

The proteomic profile of small EVs in NSCLC revealed several possible candidates as prognosis biomarkers of the cancer. Initially, the small EVs were successfully isolated from the plasma of NSCLC patients using two main techniques: size-exclusion chromatography and ultracentrifugation. Several studies have used only one of the SEC (Karimi et al., 2018) and ultracentrifugation (Chiasserini et al., 2014; Li et al., 2011) to separate small EVs from other cellular components; however, the current study combined these two techniques. All crucial properties of small EVs were affirmed, including size characterization by DLS, shape identification by SEM, and protein markers by Western blot.

The PPI network of the proteins involved in the late stage revealed crucial information about the hub proteins, which could serve as potential drug targets for the treatment of cancer in the future. The hub proteins have been discussed for their involvement in tumour progression and their potential as a target of anticancer drugs; however, most of them have not received any attention in the NSCLC research.

It was found that Src was located at the centre of one cluster in the metastatic-exclusive network (Figure 3). The Src is a tyrosine kinase well-known for its significance

in sarcoma biology (Chen et al., 2015). Studies have proposed several mechanisms of Src activation in human tumour progression including domain interaction and structural remodeling under the control of many activators and upstream kinases and phosphatases (Sen and Johnson, 2011). In the case of NSCLC, a reciprocal regulation of c-Src and STAT3 activation was demonstrated (Byers et al., 2009) when the depletion of c-Src by siRNA led to an increase in pSTAT in the models of NSCLC cell lines and murine xenograft. Currently, some inhibitors of Src are under clinical trials for the treatment of various cancers (Chen et al., 2015). This body of evidence supports our observation of the core function of Src in the network. The future studies may investigate other interactors of Src found in this research.

One of the central hubs of the metastasis network was eEF1A1 (Figure 3). It belongs to the eukaryotic elongation factor family that regulates protein synthesis, translation machinery, cell proliferation, and apoptosis (Caraglia et al., 2000). This protein is pleiotropic and overexpressed in several cancers, such as hepatocellular carcinoma (HCC) (Chen et al., 2018). We found no study on the role of eEF1A1 in NSCLC; however, Li et al. (2011) reported the elevation of eEF1A2 expression in this cancer compared to the control groups (Kawamura et al., 2014). Indeed, the connections between eEF1, EGFR, and SRC family kinase were reported in small EVs derived from the cell lines of colorectal cancer (Beckler et al., 2013).

The KPNB1 has been widely studied for many cancers using models of cell lines, including colorectal cancer (Beckler et al., 2013), ovarian cancer (Liang et al., 2013), and NSCLC (Sekimoto et al., 2017). Based on the model of adenocarcinoma cell lines, KPNB1 worked with polo-like kinase 1 (PLK1) to regulate cell proliferation and apoptosis (Sekimoto et al., 2017). No study has ever addressed its expression in the tissue or plasma of NSCLC patients.

The ACTC1 was highly expressed in metastasis but not in the other two conditions. It is a crucial protein involved in apoptosis, ATP binding, and myosin binding (Bookwalter and Trybus, 2006). This protein is differently expressed in NSCLC cell lines under treatment with paclitaxel and docetaxel, indicating the complexity of its function during the progression of NSCLC and its impact on drug response (Che et al., 2013). In addition, the change in the expression of Actc1 genes has been reported in a study on lung tumorigenesis using an Akt knockout mouse model (Franks et al., 2016). Despite the fact that several studies have been carried out based on the cancer cell line model, this protein has not been reported in small EVs from the plasma of NSCLC patients.

Compared to other large-scale proteomics studies, our work was restricted in terms of the number of patients (only 31 cases). However, with the clear division of them into two groups of stages I and II, the finding of the biomarker for metastasis is still reliable and valuable. In addition, the usage of pooled samples as a representative for patients and control groups is undeniably one of limitations of this study which would be later validated by detecting some of the listed potential proteins using Western blot or ELISA.

Our study found for the first time the presence of these proteins in small EVs isolated from the plasma of NSCLC patients. These findings are in line with those of other studies on the role of these proteins in the development and metastasis of cancer. Many of these studies did not focus on NSCLC, leaving room for further research on the underlying pathways and biological processes of these proteins in tumour conditions. The mechanism of the function of these elements in cancer needs to be further investigated to confirm their actual capability of becoming the biomarker for NSCLC. Therefore, future works can focus on the presence and concentration of some of the found proteins including EEF1A1, KPNB1, SRC, and ACTC1 that can be identified using Western Blot or ELISA. The sensitivity and specificity of each are the upcoming parameters to be collected to establish the accuracy of their usage as a biomarker.

Author Contribution Statement

Thao Phuong Bui: Formal analysis, Methodology, Data Curation, Validation, Visualization, Writing - Original Draft; Phuong Lan Le: Resources, Funding acquisition, Writing - Review & Editing; Linh Thi Tu Nguyen: Methodology, Project administration, Funding acquisition, Supervision, Writing - Review & Editing; Le Trung Tho: Resources, Project administration; Thai Hong Trinh: Conceptualization and design, Methodology, Project administration, Funding acquisition, Supervision, Writing - Review & Editing.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of Vietnam National University (Hanoi, Vietnam) grant KLEPT.18.03, with an approval number 2436/QĐ-ĐHQGHN, signed on 20 July 2018.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest

The authors declare no competing interests.

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