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Protein Profiling of Extracellular Vesicles Associated With Cisplatin Resistance in Lung Cancer

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Abstract. *Background/Aim: Extracellular vesicles (EVs) can mediate drug resistance within the tumor microenvironment by delivering bioactive molecules, including proteins. Here, we performed a comparative proteomic analysis of EVs secreted by A549 lung cancer cells and their cisplatin-resistant counterparts in order to identify proteins involved in drug resistance. Materials and Methods: Cells were co-cultivated using a transwell system to evaluate EV exchange. EVs were isolated by ultracentrifugation and analyzed using microscopy and nanoparticle tracking. EV proteome was analyzed by mass spectrometry. Results: EV-mediated communication was observed between co-cultured A549 and A549/CDDP cells. EVs isolated from both cells were mainly exosome-like structures. Extracellular matrix components, cell adhesion proteins, complement factors, histones, proteasome subunits and membrane transporters were found enriched in the EVs released by cisplatin-resistant cells. Conclusion: Proteins identified in this work may have a relevant role in modulating the chemosensitivity of the recipient cells and could represent useful biomarkers to monitor cisplatin response in lung cancer.*

Lung cancer is the most common cancer worldwide and the leading cause of cancer death in both men and women (1). Non-small cell lung cancer (NSCLC) is the most prevalent type of lung cancer, accounting for approximately 85% of all lung cancer cases (2). Most patients with NSCLC have non operable, advanced-stage disease at the time of diagnosis and platinum-based chemotherapy is the first-line treatment for these patients (3). However, the high incidence of tumor chemoresistance limits treatment success (4). Although numerous mechanisms have been associated with cisplatin (CDDP) resistance in lung cancer (5, 6), the cellular events within the tumor microenvironment that facilitate the acquisition and maintenance of chemoresistance are still poorly understood.

Recently, extracellular vesicles (EVs) have emerged as key players for cell-cell communication in the tumor microenvironment (7). EVs are a heterogeneous group of membrane-bound structures secreted by cells into the extracellular space. Based on their size and biogenesis, EVs can be broadly classified into two main categories: exosomes and microvesicles (8). Exosomes are small membrane vesicles (30-100 nm in diameter) formed as intraluminal vesicles within endosomal multivesicular bodies (MVBs), and secreted upon the fusion of MVBs with the plasma membrane. Microvesicles (MVs) range in size from 50 nm to 1000 nm in diameter, but the cancer-derived MV population termed oncosomes can be much larger (up to 10 μm) (9). MVs are generated by the outward budding and fission of the plasma membrane.

EVs mediate cellular crosstalk by transporting bioactive cargo between cells, including proteins, lipids, and nucleic acids (10). The EV cargo composition determine its biological effects and varies depending on the cell type and physiological/pathological state (11). In the tumor microenvironment, EVs have been shown to mediate several aspects of tumor biology, including chemoresistance (7). Recent studies have shown that EVs released by drug-resistant cancer cells have specific molecular cargo, which can confer drug resistance to recipient cells and regulate different aspects of tumor behavior (7, 12, 13). However, so far, few studies have been dedicated to characterizing the EV proteome from cancer cells with a drug resistance phenotype (14-17).

In this work, we performed a comparative proteomic analysis of EVs released by A549 lung cancer cells and their CDDP-resistant counterparts, A549/CDDP cells. The CDDP-resistance

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associated EV proteome was identified and its possible role in a scenario of EV-mediated chemoresistance was discussed.

Materials and Methods

Cell culture and chemoresistance induction. A549 cells (ATCC, CCL-185) were maintained in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37˚C in a humidified atmosphere of 5% CO_2. The CDDP-resistant subline A549/CDDP was developed using a stepwise drug treatment in which A549 parental cells (3×10^5) were continuously exposed to increasing concentrations of CDDP (0.1, 0.2, 0.3, 0.4 and 0.5 μM) for 72 h each. CDDP cytotoxicity (IC_{50}) was evaluated by sulforhodamine B (SRB) assay, as described previously (18). A549/CDDP cells were maintained in culture medium containing 0.5 μM of CDDP to ensure maintenance of the resistant phenotype. Cells were transferred to drug-free medium 3 days prior to the experiments.

EV uptake assay. Transwell co-cultures were performed in two distinct configurations: R/S, co-culture between A549/CDDP (upper compartment) and A549 (lower compartment) cells; and S/S, co-culture containing A549 cells in the upper and lower compartments. For that, parental A549 cells (1×10^5) were seeded onto glass coverslips in 24-well plates and allowed to adhere for 24 h. A549 or A549/CDDP cells labeled with Celltracker CM-DiI (Thermo Fisher Scientific) were plated at 1×10^5 on transwell inserts (1 μM pore size). Co-cultures were maintained in complete culture medium supplemented with 10% exosome-depleted FBS (System Bioscience, Palo Alto, CA, USA) for 24 h at 37˚C in 5% CO_2/air. Cells were fixed with 4% paraformaldehyde and nuclei were stained with DAPI. Cells were imaged using an Olympus FluoView 1000 confocal microscope. The experiments were performed in triplicate and co-cultures with no cell labeling were used as negative controls.

EV isolation and characterization. A549 and A549/CDDP cells were cultured in complete media until they reached 80-90% confluency, washed twice with PBS 1X pH 7.4 and then cultured for 48 h in serum-free media. EVs were isolated from culture supernatants by differential centrifugation/ultracentrifugation (Beckman Coulter, Brea, CA, USA, rotor 50.2 Ti) as previously described (19). EVs were resuspended in PBS and sample concentration was indirectly determined using Micro BCA protein assay (Thermo Fisher Scientific). For transmission electron microscopy (TEM) analysis, EVs were fixed with 2% paraformaldehyde, loaded on formvar/carbon-coated grids, rinsed with Milli-Q water, post-fixed with 2.5% glutaraldehyde and contrasted with 2% uranyl-acetate. Samples were viewed on a JEM-1200 EX-II electron microscope (JEOL, Tokyo, Japan) operated at 100 kV. Nanoparticle tracking analyses (NTA) were performed using ZetaView® (Particle Metrix, Meerbusch, Germany), which captures Brownian motion through a laser scattering microscope with a video camera. Videos were captured every 10 sec and each sample was measured at 11 different positions throughout the cell chamber, with two cycles of readings at each position. The hydrodynamic diameter of the vesicles was calculated using the Stokes-Einstein equation. For flow cytometry analysis, EVs were captured on anti-CD63-coated beads and detected with FITC anti-CD63 antibody. Samples were analyzed using a FACSCalibur (BD Biosciences, San Jose, CA, USA) flow cytometer. The presence of CD63 in the EV population was also assessed by western blot, as described previously (20).

Mass spectrometry analysis. EVs isolated from three independent cell cultures of each cell type were pooled to generate the A549 and A549/CDDP samples analyzed by MS. EVs were diluted in 25 mM ammonium bicarbonate pH 8.0 containing 0.5% w/v Rapigest (Waters, Milford, MA, USA) and heated at 100˚C for 5 min. Next, samples were processed and digested with trypsin following the RapiGest protocol. Peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a nanoACQUITY UPLC system coupled to a Xevo G2-XS Q-Tof mass spectrometer (Waters) with a low-flow probe at the source. The peptides were separated by analytical chromatography (Acquity UPLC BEH C18, 1.7μm, 2.1×50 mm, Waters) using a 7-85% water/ACN 0.1% formic acid linear gradient over 45 min. The MS survey scan was set to 0.5 s and recorded from 50 to 2000 m/z. MS/MS scans were acquired from 50 to 2000 m/z, and scan time was set to 1 s. Data were collected in the data-independent MSE mode of acquisition. Each sample was analyzed in triplicate.

MS data analysis. MS data were processed and searched using ProteinLynx Global Server version 3.0.3 (PLGS 3.0.3, Waters). The searches were conducted against *Homo sapiens* protein sequences retrieved from the UniProtKB/Swiss-Prot database, with trypsin as the enzyme, maximum of one missed cleavage, fixed carbamidomethyl modification for cysteine residues, and oxidation of methionine as variable modification. Peptides and protein tolerance were set as automatic, minimum fragment ion per peptide as 2, minimum fragment ion per protein as 5, minimum peptide matches per proteins as 2 and the false discovery rate (FDR) as 4%. Only proteins identified in at least two out of three replicates were considered for qualitative and quantitative analysis in order to improve confidence. Label-free quantitation analysis was performed from peak intensity measurements (Hi3 method) (21) using PGLS ExpressionE algorithm. Data sets were normalized using the histone H3 (K7EK07) as reference, as this protein was detected in all samples and replicates with the lowest variance coefficient. Proteins were considered differentially expressed between samples if the regulation probability (*p*-value) determined by the PLGS quantification algorithm was below 0.05 or higher than 0.95.

Functional annotation and enrichment analysis. Protein functional annotation was performed using DAVID 6.8 (22). Homo sapiens was set as background for the enrichment analysis and functional annotation clustering was performed with classification stringency set to medium, similarity threshold of 0.5, multiple linkage threshold of 0.5, and an EASE enrichment threshold of 1.0. The *p*-value and the Benjamini-Hochberg FDR were used to determine significance of enrichment or overrepresentation of terms for each annotation.

Results

EV-mediated communication between A549 and A549/CDDP cells. CDDP-resistant subline A549/CDDP was generated from parental cell line A549 using a stepwise protocol of drug exposure. The CDDP cytotoxicity of each cell line was evaluated by SRB assay and A549/CDDP cells presented an

IC_{50} value about 4.5-fold higher (~9 μM) than that of the parental A549 cells (~2 μM) (Figure 1).

We have used a co-culture system to evaluate the EV-mediated communication between A549 and A549/CDDP cells. Co-cultures containing cells labeled with the fluorescent dye Celltracker CM-DiI in their upper compartments were used to monitor the EV transfer and internalization by the cells in the lower compartment using confocal microscopy. Recipient cells showed punctuate staining in their cytoplasm (mainly distributed in the perinuclear region), demonstrating that EV uptake and internalization occurred in both co-culture configurations analyzed (S/S and R/S) (Figure 2).

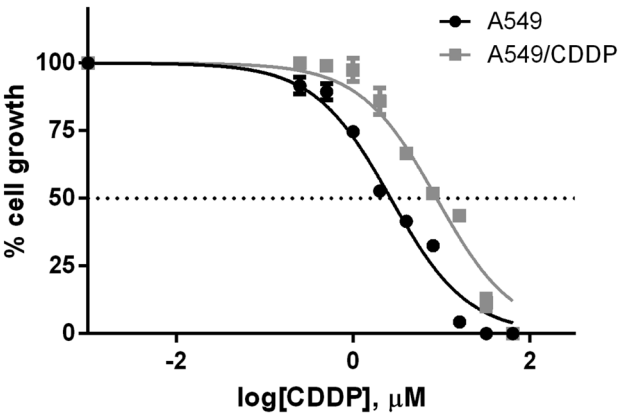

Figure 1. *Generation of CDDP-resistant A549/CDDP cells from A549 parental cells. IC_{50} values were calculated from dose-response curves of cells treated with increasing concentrations of CDDP.*

Characterization of EVs released by A549 and A549/CDDP cells. EVs released into the culture medium by A549 and A549/CDDP cells were isolated by differential centrifugation/ultracentrifugation and characterized by different techniques (Figure 3). Similar results were obtained for EVs isolated from A549 and A549/CDDP cells and, therefore, data presented in Figure 3 are representative from both cells.

The EVs visualized by TEM exhibited a rounded shape and variable size, with a predominant population of ~ 100 nm in diameter (Figure 3B). These results are in agreement with those obtained by NTA, which revealed particles ranged in size from 10 to 420 nm and a main population with 105 nm in diameter (Figure 3C). These results indicated that the EVs isolated from A549 and A549/CDDP cells were mainly exosome-like structures, although microvesicles have also been observed. The detection of the exosomal marker CD63 by flow cytometry and western blot (Figure 3D) confirmed the presence of exosome-like structures in the EVs isolated from A549 and A549/CDDP cells.

Protein profiles of EVs isolated from A549 and A549/CDDP cells. The protein cargo of EVs secreted by A549 and A549/CDDP cells was analyzed by LC-MS/MS. A viscous pellet was observed in highly concentrated samples of EVs from CDDP-resistant cells, differently from EV samples of A549 cells. For this reason, EV samples from A549/CDDP cells were divided into two fractions (pellet and supernatant), which were separately analyzed by MS. Results of the proteomic analyses are summarized in Figure 4A. Proteins identified in each sample are listed in the Supplementary Material. The identification of classic EV markers, such as Alix, CD9 and CD81, and the absence of contaminating proteins (calnexin, for example), demonstrates the efficiency of the protocol used for EV purification.

Quantitative analyses based on MS precursor intensity values were performed to identify differentially expressed proteins between samples. The protein profile specifically associated with EVs released from A549/CDDP cells was defined as the set of proteins found exclusively in the pellet and supernatant samples, plus the proteins identified as up-regulated in these samples compared to the sample of EVs from A549 cells. This CDDP-resistance associated EV proteome (62 proteins) is listed on Table I and includes extracellular matrix (ECM) components, cell adhesion proteins, complement factors, EV markers, nuclear proteins, proteasome subunits, and solute carrier (SLC) transporters. The functional annotation terms enriched in this protein set are presented in Figure 4B.

Discussion

CDDP resistance is a major clinical challenge for the treatment of advanced-stage lung cancer (5). Growing evidence indicates that EV-mediated intercellular communication plays essential roles in modulating tumor microenvironment and anti-cancer drug resistance (7, 23). We showed that EVs participate in the communication between CDDP-resistant and drug-sensitive A549 cells using a co-culture system with an EV-permissive 1.0-μm membrane. Although the *in vitro* system used is not able to reproduce the complex interactions that occur in the tumor microenvironment, we believe that it is more physiologically relevant than those using purified EV preparations alone, since co-culture allows dynamic bidirectional communication between cells and the exchange of soluble factors that can influence EV biogenesis, release and internalization (24, 25).

EVs can modulate the phenotype of target cells by transferring their bioactive cargo (10, 23). Multiple studies have shown that EV cargo reflects the phenotypic state of the cell of origin (11). For instance, EVs released by chemoresistant tumor cells contain molecules able to enhance drug export/detoxification or to induce survival signaling pathways in recipient cells, thus promoting resistance to anticancer drugs (7, 23, 26). Numerous studies have been dedicated to analyzing the EV-associated miRNAs and their participation in cancer

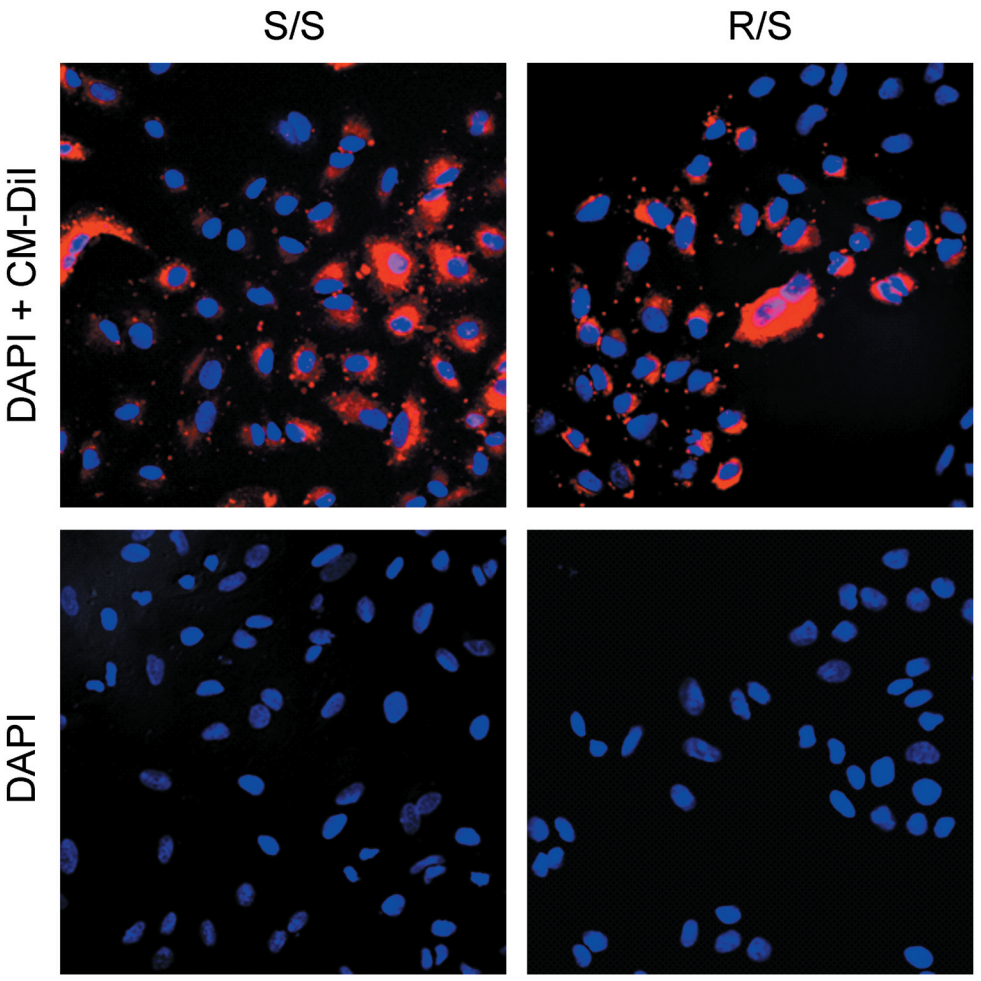

Figure 2. *EV-mediated communication between A549 and A549/CDDP cells. S/S and R/S co-cultures containing cells labeled with CM-DiI dye in the upper compartment. Images were z-stacks of optical sections obtained by confocal microscopy. Nuclei were stained with DAPI. Negative controls correspond to co-cultures excluding the cell labeling step.*

chemoresistance (12, 27), but few have focused on characterizing the EV proteome associated with drug resistance (14, 28). Of these, the majority targeted multidrug resistance proteins (MRPs) (29). Here, we performed a comparative proteomic analysis of the EVs secreted by A549 and A549/CDDP cells in order to identify proteins that may have a relevant role in CDDP resistance transfer in lung cancer cells.

Proteins shared by EVs derived from A549 and A549/CDDP cells include several of the top 100 EV markers described in Vesiclepedia and Exocarta databases. The identification of classical exosomal markers, such as Alix, CD9, CD63 and CD81, is in agreement with the results obtained in TEM and NTA experiments, which revealed a predominance of exosomes in the EV population secreted by A549 and A549/CDDP cells.

Proteins found enriched in EVs secreted by A549/CDDP cells comprise the EV proteome associated with CDDP resistance, which can modulate the chemosensitivity of the recipient cells in an EV-mediated cell-to-cell communication scenario. EVs can mediate drug resistance by transferring biological cargo able to improve drug detoxification, enhance cell viability and decrease apoptosis sensitivity in the recipient cells, thus leading to resistance to anti-cancer drugs (7, 23).

GSTP1, which participates in the detoxification of several anti-cancer drugs, was identified exclusively in the EVs released by A549/CDDP cells. In a previous study, GSTP1-containing exosomes were shown to be involved in chemoresistance transfer between cells (30). Proteins found enriched in the EVs secreted by A549/CDDP cells also include several proteasome subunits. The 20S proteasome core particle is a key protease of eukaryotic cells and its extracellular delivery mediated by EVs has been shown to improve cell survival within the tumor microenvironment (31). SLC

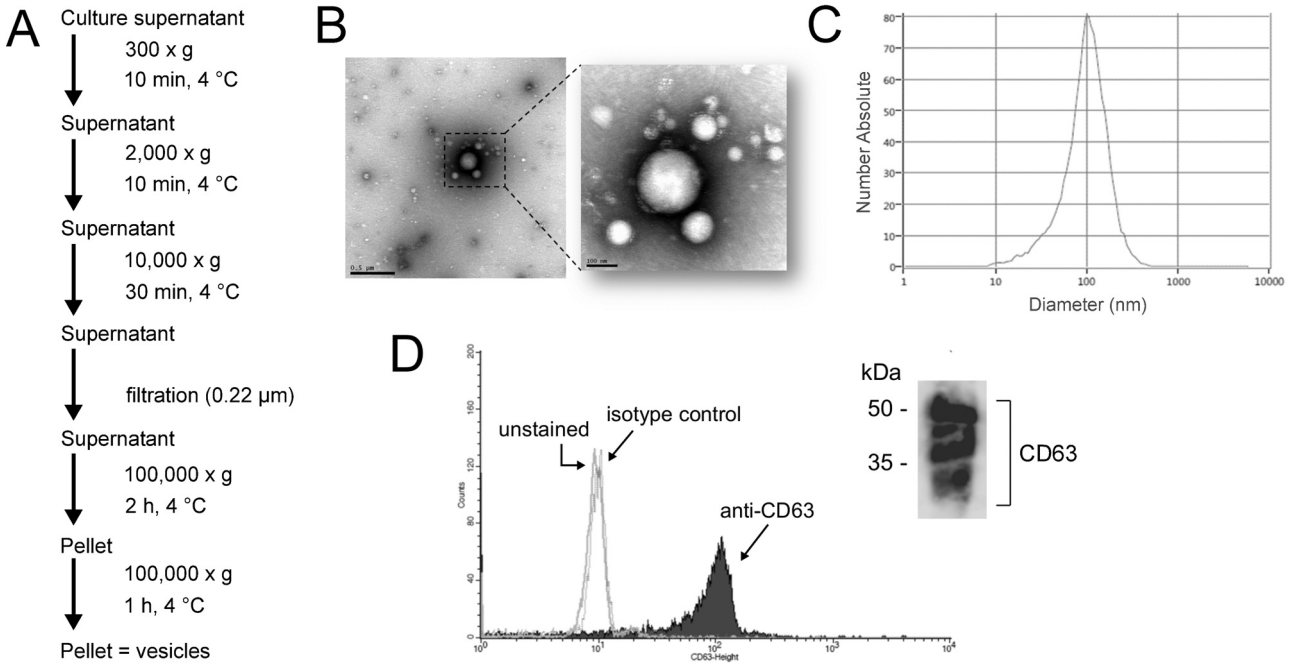

Figure 3. *Isolation and characterization of EVs released by A549 and A549/CDDP cells. A) Schematic representation of the differential centrifugation/ultracentrifugation method used for EV isolation. B) TEM images of EVs (scale bar of 0.5 µM); (inset) Close-up view of a group of vesicles (scale bar of 100 nm). C) NTA profile showing the EV particle-size distribution. D) CD63 detection by flow cytometry and western blot. Data shown are representative from both cells.*

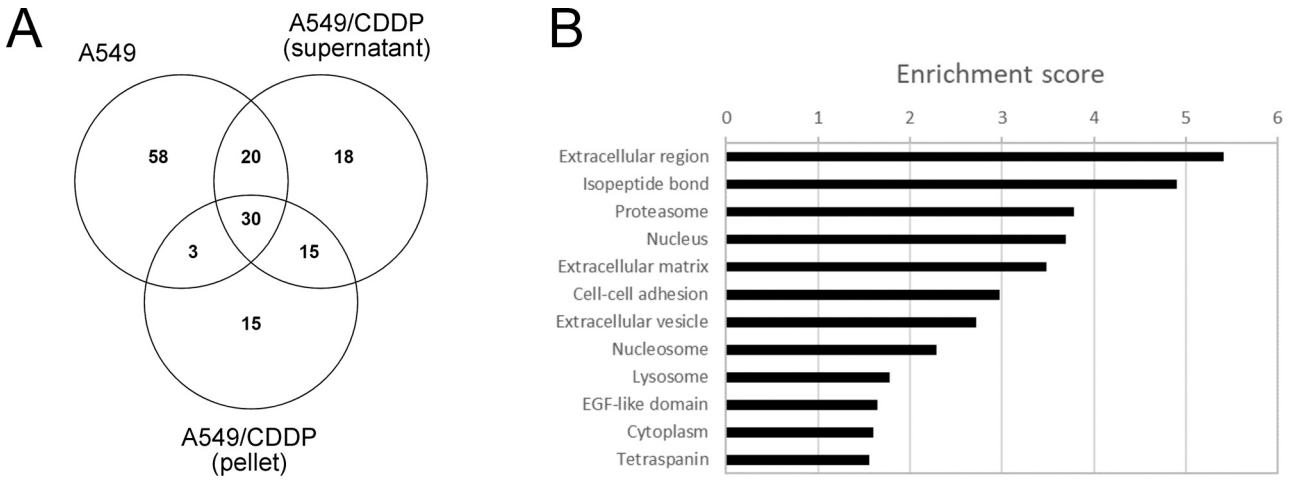

Figure 4. *Overview of proteomic analysis of EVs secreted by A549 and A549/CDDP cells. A) Venn diagram of proteins identified by LC-MS/MS. B) Gene annotation terms over-represented in EV proteome associated with CDDP resistance. The x axis is the enrichment score acquired from DAVID functional annotation tool.*

transporters found in the EV proteome associated with CDDP resistance may also play a relevant role in the growth of the recipient tumor cells, since the supply of these proteins *via* EVs could help to cope with their high demand for amino acids and glucose (32, 33).

Surprisingly, we found complement factors in the EVs secreted by A549/CDDP cells. Complement proteins are synthesized and secreted by alveolar type II cells, such as A549, during lung inflammation and infection (34). Our results suggest that these proteins play a role in chemotherapy response as well.

Table I. *EV proteome associated with CDDP resistance in A549 cells*.

Accession number	Gene name	Description
A0A024R4M0	*RPS9*	40S ribosomal protein S9
F5GZS6	*SLC3A2*	4F2 cell-surface antigen heavy chain
P52209	*PGD*	6-phosphogluconate dehydrogenase_decarboxylating
P60709	*ACTB*	Actin_ cytoplasmic 1
O00468	*AGRN*	Agrin
P06280	*GLA*	Alpha-galactosidase A
P04083	*ANXA1*	Annexin A1
O75531	*BANF1*	Barrier-to-autointegration factor
P98160	*HSPG2*	Basement membrane-specific heparan sulfate proteoglycan core protein
P16278	*GLB1*	Beta-galactosidase
O43570	*CA12*	Carbonic anhydrase 12
X6R5C5	*CTSA*	Carboxypeptidase
Q9H444	*CHMP4B*	Charged multivesicular body protein 4b
O43809	*NUDT21*	Cleavage and polyadenylation specificity factor subunit 5
P39060	*COL18A1*	Collagen alpha-1(XVIII) chain
P01024	*C3*	Complement C3
B1AP13	*CD55*	Complement decay-accelerating factor
K7ERG9	*CFD*	Complement factor D
P27105	*STOM*	Erythrocyte band 7 integral membrane protein
P02792	*FTL*	Ferritin light chain
P02671	*FGA*	Fibrinogen alpha chain
P02675	*FGB*	Fibrinogen beta chain
P02679	*FGG*	Fibrinogen gamma chain
P09211	*GSTP1*	Glutathione S-transferase P
Q9UBI6	*GNG12*	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12
P16403	*HIST1H1C*	Histone H1.2
P10412	*HIST1H1E*	Histone H1.4
C9J0D1	*H2AFV*	Histone H2A
H0YFX9	*H2AFJ*	Histone H2A (Fragment)
P0C0S5	*H2AFZ*	Histone H2A.Z
Q71DI3	*HIST2H3A*	Histone H3.2
P13645	*KRT10*	Keratin_ type I cytoskeletal 10
P04264	*KRT1*	Keratin_ type II cytoskeletal 1
O15230	*LAMA5*	Laminin subunit alpha-5
G3XAI2	*LAMB1*	Laminin subunit beta-1
P11047	*LAMC1*	Laminin subunit gamma-1
P00338	*LDHA*	L-lactate dehydrogenase A chain
H3BRK3	*NQO1*	NAD(P)H dehydrogenase [quinone] 1
Q15758	*SLC1A5*	Neutral amino acid transporter B(0)
Q14112	*NID2*	Nidogen-2
Q13219	*PAPPA*	Pappalysin-1
P62937	*PPIA*	Peptidyl-prolyl cis-trans isomerase A
H7BYW6	*PDGFA*	Platelet-derived growth factor subunit A (Fragment)
Q02809	*PLOD1*	Procollagen-lysine_2-oxoglutarate 5-dioxygenase 1
I3L3D5	*PFN1*	Profilin (Fragment)
Q8WUM4	*PDCD6IP*	Programmed cell death 6-interacting protein
H0YN18	*PSMA4*	Proteasome subunit alpha type-4
P60900	*PSMA6*	Proteasome subunit alpha type-6
P20618	*PSMB1*	Proteasome subunit beta type-1
P49721	*PSMB2*	Proteasome subunit beta type-2
P49720	*PSMB3*	Proteasome subunit beta type-3
Q8NFJ5	*GPRC5A*	Retinoic acid-induced protein 3
P11166	*SLC2A1*	Solute carrier family 2_ facilitated glucose transporter member 1
P52823	*STC1*	Stanniocalcin-1
G5EA09	*SDCBP*	Syndecan binding protein (Syntenin)_ isoform CRA_a
A6NNI4	*CD9*	Tetraspanin
J3KQ42	*TSPAN4*	Tetraspanin
H0YDJ9	*CD81*	Tetraspanin (Fragment)
Q8WUA8	*TSKU*	Tsukushin
Q5JP53	*TUBB*	Tubulin beta chain
P04004	*VTN*	Vitronectin
B1AHC9	*XRCC6*	X-ray repair cross-complementing protein 6

Recent evidence suggests that nuclear proteins found in EVs, such as histones, may be involved in a survival mechanism based on the loading of harmful DNA into exosomes in order to maintain cellular homeostasis during chemotherapy (35). Histones were found to be enriched in the EVs from A549/CDDP cells and we can speculate that the viscous pellet observed in this sample could be related to a greater amount of DNA.

EVs can also carry proteins able to activate pro-survival signaling pathways in the recipient cells, such as the PI3K/AKT pathway (7). Platelet-derived growth factor (PDGF) and its receptors (PDGFR) regulate a variety of biological processes, including cellular growth, cell survival and angiogenesis (36). PDGFR expression is very common in lung tumors and its inhibition potentiated CDDP cytotoxicity in NSCLC cell lines (37). Therefore, PDGFA identified in the EVs secreted by A549/CDDP cells could activate downstream effectors such as PI3K in the recipient cells, thereby inducing resistance to CDDP. The identification of ECM and adhesion proteins in EVs secreted by A549/CDDP cells suggests a major role for ECM cell adhesion in A549 cell chemoresistance. ECM components serve as ligands for cell surface receptors such as integrins that activate a range of signaling pathways, including PI3K/AKT, thus preventing drug-induced apoptosis (38).

Overall, our comparative proteomic analysis allowed the identification of the EV proteome associated with CDDP resistance in A549 cells. The identified proteins may represent pro-survival factors able to enhance drug detoxification and cell viability, thus promoting chemoresistance in the recipient cells. In addition, EV proteins identified here can be explored as potential non-invasive biomarkers to monitor CDDP response in lung cancer.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

Study conception and design, H.B. and K.M.M.; data acquisition, H.B., N.A.C., C.S.D., and E.D.S.; data analysis and interpretation, H.B., N.A.C., C.S.D., E.D.S., and K.M.M.; resources, H.B.F., A.Z. and K.M.M.; drafting of the manuscript: H.B. and K.M.M.; critical revision: H.B.F and A.Z.

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Supplementary Material

Supplementary Material are available at http://www.cbiot.ufrgs.br/professores/zaha/

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