RESEARCH ARTICLE

Editorial Process: Submission:08/05/2025 Acceptance:07/13/2026 Published:07/26/2026

Ribosomal Proteins in Blood Exosomes as Markers of an Unfavorable Prognosis in Patients with Breast Cancer

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Abstract

Introduction: Exosomes, extracellular vesicles (EVs) 30-150 nm in diameter, are known to mediate intercellular communication by transferring bioactive molecules that can reprogram recipient cell phenotypes. Our analysis of 5-year survival data from breast cancer patients (BCPs) revealed that the poorest outcomes were exclusively associated with conserved signature of three ribosomal proteins (RPs) - MRPL52, RPL28, and RPL40 (UBA52) - that were completely absent in healthy females (HFs) and better-prognosis cases. Strikingly, all patients with this exosomal RP profile succumbed to metastatic relapse within the observation period. **Methods:** While RPs are known components of tumor-derived exosomes, their absence in EVs from normal cells suggests cancer-specific sorting mechanisms. To investigate this, we characterized exosomal RP cargo from three breast cancer cell lines (MCF-7, BT-474, BT-549), finding cell-type-specific RP patterns that differed from the clinical BC signature. Transfection of BT-549 cells with FLAG-tagged RPS3 (an aggressive cancer-associated RP) induced dramatic changes in exosomal RP composition, including the appearance of RPL28 - matching our clinical findings. **Results:** Bioinformatic analysis revealed that the RPs identified in BCP blood exosomes (MRPL52, RPL28, RPL40, RPS6) are associated with poor prognosis across multiple cancers and participate in key oncogenic pathways. **Conclusion:** The concordance between our clinical and experimental data suggests that: (a) specific RPs are selectively packaged into exosomes under stress conditions; (b) this process may contribute to BC aggressiveness; (c) exosomal RP profiling could serve as a novel prognostic approach. These findings illuminate previously unrecognized roles of ribosomal components in cancer progression through EV-mediated communication.

Keywords: Exosomes- EVs- ribosomal proteins- triple-negative breast cancer- biomarkers- cancer progression

Asian Pac J Cancer Prev, **27 (7)**, 2497-2508

Introduction

It is well established that cells communicate with each other through extracellular vesicles (EVs), which transport a variety of biologically active molecules, ranging from small metabolites to proteins and their complexes with RNA [1, 2]. Among these EVs, exosomes -vesicles measuring between 30 and 150 nm - are the primary mediators of such intercellular communication. The transfer of specific molecular cargo from a donor cell to a recipient cell is believed to induce a programmed phenotypic change in the "message" [3-5].

By analyzing 5-year survival data from our previously published cohort of breast cancer patients (BCPs) [6], we identified that the poorest clinical outcomes were exclusively associated with the presence of three specific ribosomal proteins (RPs) (MRPL52, RPL28, and UBA52) within their exosomal cargo. There is a growing body of evidence indicating that RPs are components of exosomal cargo secreted by various malignant cells [7-13]. However, no data exist regarding the presence of RPs in exosomes derived from non-malignant cells [7]. This suggests that the inclusion of RPs in exosomes may be specifically associated with carcinogenesis. Furthermore, nearly all RPs have been implicated in cancer-related processes, raising the possibility that their intercellular transfer via exosomes contributes to cancer progression [7]. RPs are not merely structural components of the translational machinery. Numerous RPs possess extraribosomal functions unrelated to protein synthesis,

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acting independently of ribosomes (reviewed in [14–21]). These functions include regulation of DNA repair, apoptosis, cell cycle progression, and stress signaling. Nevertheless, the molecular mechanisms linking RP dysregulation to carcinogenesis remain incompletely understood.

Among RPs, RPS3/uS3 represents one of the most extensively characterized examples with experimentally validated links to malignancy. RPS3 exhibits multiple extraribosomal activities, including participation in base excision DNA repair (AP lyase activity), modulation of NF-κB signaling, and regulation of cell cycle progression [14, 17, 18]. Importantly, intercellular transfer of RPS3 via exosomes has been shown to induce phenotypic alterations in recipient cancer cells. Specifically, exosomal RPS3 derived from cisplatin-resistant gastric cancer cells conferred cisplatin resistance to sensitive cells through activation of the PI3K-Akt-cofilin-1 signaling pathway; direct overexpression of RPS3 produced a comparable effect [22]. These findings indicate that RP transfer through exosomes can influence malignant phenotypes and stress responses. Breast cancer represents a suitable model to investigate this phenomenon, as breast cancer cells actively secrete exosomes and RPS3 has been detected in exosomes derived from such cells [3, 8]. Therefore, to explore whether elevated intracellular levels of a stress-associated RP influence exosomal cargo composition, we increased the expression of RPS3 in the triple-negative breast cancer (TNBC) cell line BT-549 by introducing a plasmid encoding FLAG-tagged RPS3. We then analyzed the RP composition of exosomes secreted by these cells. Although RPS3 overexpression altered the RP cargo of exosomes, the exogenous protein itself was not incorporated into vesicles, suggesting an indirect effect on selective RP sorting.

Materials and Methods

Ethics statement

The study protocol was approved by the Ethics Committee of the Institute of Chemical Biology and Fundamental Medicine. Human samples were obtained according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from every female. Blood samples from healthy females (HFs, n = 21, age 38–65 years, and median age 56) were obtained from Novosibirsk Central Clinical Hospital. HFs did not have any female-related disorders (mastopathy, endometriosis, etc.) or malignant diseases. Blood samples from previously untreated BCPs (n = 23, age 31–78 years, and median age 58, Table 1) were obtained from Novosibirsk Regional Oncology Dispensary.

Cell lines

The human breast cancer cell lines BT-549 (triple-negative human breast cancer cells, ATCC® HTB-122™), MCF-7 (hormone receptor-positive breast cancer cells, ATCC® HTB-22™), and BT-474 (HER2-positive breast cancer cells, ATCC® HTB-20™) were cultured in the Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) (Thermo Fisher Scientific) and Penicillin-Streptomycin (100 μg/ml) (Thermo Fisher Scientific) in CO_2-incubator (5% CO_2) at 37°C up to 70-80% confluence.

Prior to use, FBS was centrifuged at 100,000g for 2 h at 4°C to remove bovine EVs. The supernatant was collected and used to prepare bovine EV-depleted medium. Three days prior to cell harvesting, the culture medium was replaced with a depleted medium containing DMEM, a mixture of antibiotics and 10% FBS devoid of bovine EVs.

Exosomes isolation

After 48 h of cell cultivation conditioned medium was collected, and breast cancer cells were pelleted by centrifugation at ambient temperature for 5 min at 300 g. The supernatant was then centrifuged at 4°C for 20 min at 15,000g to remove cellular debris. To eliminate large EVs, the supernatant was filtered through 100 nm pore-size filters (Minisart high flow, 16553-K, Sartorius, Goettingen, Germany).

The filtrate was then centrifuged for 90 min at 100,000 g at 4°C. The pellets were sus-pended in 12 ml of PBS and centrifuged again for 90 min at 100,000 g at 4°C. This washing stage was repeated two times. Then, supernatant was removed, and pellets were resuspended in 300 μl of PBS, aliquoted, frozen in liquid nitrogen, and stored at -80°C.

For blood exosome isolation the protocol described previously was performed [6].

Table 1. BCP's characteristics

		N (%)
Tumor stage	T1-T2	20 (87%)
	T3-T4	3 (13%)
Nodal Status	N0	14 (62%)
	N1-N2	8 (34%)
	N3	1 (4%)
	M0	23 (100%)
Receptor status	Estrogen and Progesterone Positive	15 (65%)
	Estrogen and Progesterone Negative	5 (22%)
	Unknown	3 (13%)
HER2	Positive	15 (65%)
	Negative	5 (22%)
	Unknown	3 (13%)
Histologic Grade	II	16 (70%)
	III	2 (9%)
	Unknown	3 (13%)
Infiltrative ductal carcinoma		23 (100%)
Years without relapse	1	3 (13%)
	2	8 (35%)
	3	7 (30%)
	4	2 (8%)
	5+	3 (13%)
Total patients		23 (100%)

Transmission electron microscopy (TEM)

Morphology and membrane integrity of the isolated EVs was assessed by transmission electron microscopy (TEM) as described previously [23]. Specifically, 15 µl of exosomes were sorbed onto a copper grid coated with carbon-stabilized Formvar film and then 2% phosphotungstate acid was added. Exosomes were examined on a TEM (JEM 1400 (Jeol, Tokyo, Japan)) with a Veleta digital camera (EMS). Veleta (EMSIS, Muenster, Germany), vesicle sizes were estimated using iTEM software (EMSIS, Muenster, Germany).

Flow cytometry

The presence of CD9 and CD81 tetraspanins in the exosomal crown was confirmed by flow cytometry as described previously [3]. The MFI of stained exosomes was analyzed and compared to the isotype control (BD bioscience, Heidelberg, Germany). Each study was performed in triplicate. Flow cytometry was performed on the Cytoflex (Beckman Coulter, Bi-oBay, China), using CytExpert 2.0 Software.

Mass Spectrometry Analysis

For the identification of exosomal proteins by MALDI-TOF mass spectrometry, the proteins were separated using SDS-PAGE with 4.5% stacking gel and 10% resolving gel. Exosome samples were loaded in triplicate. Gel lanes were sliced into 0.5 cm strips from the start of the gel up to 23 cm of length and placed into 1.5 ml Eppendorf tubes. The gel pieces were washed twice by incubating with 200 µl of a solution containing 0.2 M (NH4)2CO_3 and 50% acetonitrile at 37°C for 30 min with shaking. The solution was then removed using a vacuum pump, and the gel pieces were dried by incubating in 100 µl of 100% acetonitrile for 10 min under vacuum.

After drying, 0.2 mM trypsin solution (T6567, Sigma, USA) in a mixture with 40 mM (NH4)2CO3, 5 µM CaCl2, was added to the gel pieces and incubated for 30 min at room temperature. Then, 60 µl of peptide extraction buffer (40 mM (NH4)2CO3, 5 µM CaCl2, 10% acetonitrile) was added, and the samples were incubated for 12 h at 37°C. Before spotting onto the MALDI-TOF mass-spectrometry target plate, the peptides from the resulting solutions were desalted using C-18 ZipTip columns (Millipore, Darmstadt, Germany). Each column was washed once with 10 µl of 100% acetonitrile, followed by 10 µl of 50% acetonitrile, and then three times with 10 µl of 0.1% trifluoroacetic acid. The sample was thoroughly pipetted before application. The column with the bound peptides was washed once with 10 µl of 0.1% trifluoroacetic acid. Next, 5 µl of a saturated solution of α-Cyano-4-Hydroxycinnamic Acid (HCCA) in 70% acetonitrile was added and then spotted onto the MTP 384 target plate ground steel T F. After crystallization, the target plate was loaded into the mass-spectrometer to obtain the peptide molecular weight spectrum.

Spectra were acquired using the following parameters: shots – 150, laser frequency – 66.7 Hz, laser attenuator offset – 85%, laser attenuator range – 21%, laser attenuator set – 5_ularge, laser focus offset – 0%, laser focus range – 100%, and laser focus value – 4%. The instrument was pre-calibrated for a mass range of 500-3800 Da. The obtained spectra were converted into mass values using flexAnalysis software. Subsequently, protein identification was performed using BioTools and Mascot Daemon software by searching the SwissProt database with the following search parameters: species – Homo sapiens, error tolerance – ±300 ppm, maximum number of missed cleavages – 2, fixed modifications – Propionamide (C), variable modifications – Oxidation (M), Phospho (ST); identification reliability not lower than 95%. Mass-spectra were registered at the Centre of collective usage "Mass-spectrometric studies" of Siberian Branch of Russian Academy of Sciences.

Coverage of about 10-20% of the protein sequence, with at least two peptides matching, was considered as reliable identification of minor proteins [24-26].

Plasmids

For production of the recombinant human RPS3, bearing a 3xFLAG epitope sequence at the N-terminus, plasmid pAG1-FLAG-uS3 bearing an insertion of the sequence coding for RPS3 with 3xFLAG peptide at the N-terminus was used. For the control experiments, plasmid pAG1 [27] without the insertion was applied [28].

Cell transfection by plasmid DNA

Transfection of BT-549 cells with the plasmids pAG1and pAG1- FLAG-uS3 was carried out using GenJect39 reagent (Molecta, Russia). Cells were cultivated in 15 cm Petri dishes until 50% confluency and transfected according to the manufacturer's protocol with 20 µg of the plasmid and 30 µl GenJect39 reagent. For induction of the RPS3 protein synthesis, 2 µg/ml doxycycline (DOX) was added. Cells were cultured as described above during 48 h, then the cultivation medium was replaced with an exosome-depleted medium containing DMEM, a mixture of antibiotics, and 10% FBS free of bovine EVs. The conditioned culture medium after the incubation was further used for the isolation of exosomes.

Plasmid isolation

In 48 h after transfection, conditioned medium was collected and BT-549 cells were lysed with 0,6 % NP 40, 50 mM Tris HCl (pH 6.8), 0,15 M NaCl, 5 mM NaF, 1 mm PMSF, 2 mM EDTA, and 1mM Na3MoO4 for 10 min at 4°C (60 µl buffer/106 cells). Cell nuclei were pelleted by centrifugation (10,000 g, 20 min, room temperature) and resuspended in 100 µl of PBS. Plasmids pAG1 and pAG1-FLAG-uS3 were isolated from medium, membrane-cytosolic fraction and nuclei using the "DNA Isolation Kit" (BioSilica Ltd., Novosibirsk, Russia) according to the manufacturer protocols and concentrated by precipitation of DNA in acetone as triethylammonium salts and disolved in 20 µl of water [29].

PCR-analysis

The concentration of isolated plasmid DNA was measured by quantitative polymerase chain reaction (qPCR). The qPCR was performed in LightCycler 96 (Roche, France) with the application of a total reaction volume of 20 µl containing 1 µl of DNA diluted 1:100;

500 nM of 5'-gaggcctatataagcagagc-3' (forward) and 5'-tttgtccaaactcatcaatgtatc-3' (reverse) primers; 0.2 mM deoxynucleotide triphosphates, Taq polymerase buffer (containing 75 mM Tris-HCl, pH 8.8, 20 mM (NH4)2SO4, 0.01% Tween-20, 1.5 mM MgCl2), 3% DMSO, SYTO9 fluorescent dye (Thermo Fisher Scientific, USA) diluted 1:100 and 1 U of HS-Taq polymerase (Biolabmix, Russia). qPCR was performed under the following conditions: denaturation at 95 °C for 3 min, followed by 45 cycles at 95 °C for 10 s, 55 °C for 20 s and 72 °C for 85 s. Both plasmid DNAs were used as a standard for the calibration curves.

Western blotting

To test for the RPS3-FLAG production, 0.5 million transfected and cultivated as described above BT-549 cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 200 mM KCl, 15 mM MgCl2 and 1% Triton-X100 for 10 min on ice. The lysate was clarified by centrifuga-tion 5 min at 14,000 g. Total cells proteins were separated by SDS-PAGE with subsequent trans-fer onto a nitrocellulose membrane. The Western-blot identification of RPS3-FLAG protein was performed similarly to that described [28] by using mouse anti-FLAG M2 primary antibodies (# F1804 Sigma, USA) and rabbit primary antibodies against GAPDH (#60004-1-Ig, Proteintech, USA) were used as references. The blot was sequentially treated with corresponding peroxidase-conjugated secondary antibodies and developed with the SuperSignal West Femto Maximum Sensitivity Substrate Kit (Thermo Fisher Scientific) followed by exposure in the VersaDoc MP4000 system (Bio-Rad, USA).

To identify exosomal markers, exosomes from transfected cells were initially lysed in a buffer containing 62 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), and 40 mM di-thiothreitol for 10 min on ice, then heated at 95°C for 10 min and centrifuged at ambient temperature for 10 min at 10000 g. Exosomal proteins were separated by SDS-PAGE with subse-quent transfer onto a nitrocellulose membrane. The Western-blot identification of the exosomal markers was performed as described above using rabbit anti-CD9 (PAB097Hu01, Cloud-Clone Corp., USA) and anti-CD81 (PAB160Hu01, Cloud-Clone Corp., USA) primary antibodies.

Bioinformatics Analysis

Data from the SwissProt database were translated into the UniProt database for further analysis using the Retrieve/ID mapping platform (https://www.uniprot.org). Functional enrichment analysis of exosomal proteomes according to the Gene Ontology database was conducted using the STRING software (https://www.string-db.org/). Cellular localization, molecular functions, and involvement in biological processes were determined using the FunRich 3.1.3 software based on the Gene Ontology categories, namely Cellular Component, Molecular Function, and Biological Process. Involvement in biological pathways was assessed using the Reactome service (https://reactome.org/). For primary data processing and graph generation, Python libraries such as Pandas, Numpy, Matplotlib, and Seaborn were utilized.

Results

Characterization of exosomes

To characterize exosomes derived from conditioned medium of breast cancer cell lines and blood exosomes TEM and flow cytometry were employed. TEM analysis demonstrated the presence of well-defined, cup-shaped vesicles (40–95 nm) with low electron density and intact membranes in all samples. Vesicles smaller than 30 nm constituted no more than 13% of the total population (Figure 1).

Furthermore, we confirmed that the exosomes isolated from all sources exhibited typical exosomal protein markers, specifically the tetraspanins CD9 and CD81 (Figure 1). In summary, the data demonstrate that the small EVs isolated from conditioned medium possess all the characteristic features of exosomes.

Ribosomal cargo of breast cancer patients

Analysis of blood exosomes from 23 BCPs and 21 age-matched healthy controls, pro-vided in 2020 [6] revealed the presence of three ribosomal proteins - MRPL52, RPL28, and UBA52 -in the exosomal composition of 17 patients. Further investigation of 5-year survival rates demonstrated a strong correlation between the presence of these exosomal RPs and dis-ease recurrence (Spearman $r = 0.88$, $p = 0.001$) (Figure 2).

Kaplan-Meier analysis demonstrated a complete separation of relapse-free survival curves between RP-positive and RP-negative BCPs. In the RP-positive group, relapse-free survival decreased from 0.82 at year 1 to 0.41 at year 2 and reached 0 by year 3, as all 17 RP-positive patients developed metastatic relapse within the observation period. In contrast, the RP-negative group retained a relapse-free survival probability of 0.83 at year 2 and 0.50 at year 5. Log-rank testing confirmed a statistically significant difference between groups ($\chi^2 = 11.02$, $p = 0.0009$). Notably, none of the HFs exhibited detectable levels of ribosomal pro-teins in their blood exosomes, indicating a cancer-specific signature within the present co-hort.

Ribosomal cargo of breast cancer cells exosomes

To compare the RP cargo of exosomes secreted by breast cancer cell lines MCF-7, BT-474, and BT-549, exosomal proteins were separated by SDS-PAGE. After cutting the gel into strips, in-gel trypsin digestion was performed, peptides were isolated from each strip and subjected to MALDI-TOF mass-spectrometry analysis for protein identification. Among the identified proteins, we analyzed universal major proteins and RPs. We revealed the presence of exosomal markers CD9, CD63, CD81, and MMP9, as well as 1, 3, and 6 RPs in exosomes secreted by MCF-7, BT-474, and BT-549, respectively (Supplementary Table 1). Notably, only one protein - RPL28 identified in the exosomes of MCF-7, BT-474, or BT-549 cell lines matched those previously detected in the blood exosomes of BCPs. Based on these observations, we decided to examine whether transfection of BT-549 cells with a plasmid encoding RPS3 protein, which has been implicated in potentially enhancing breast cancer

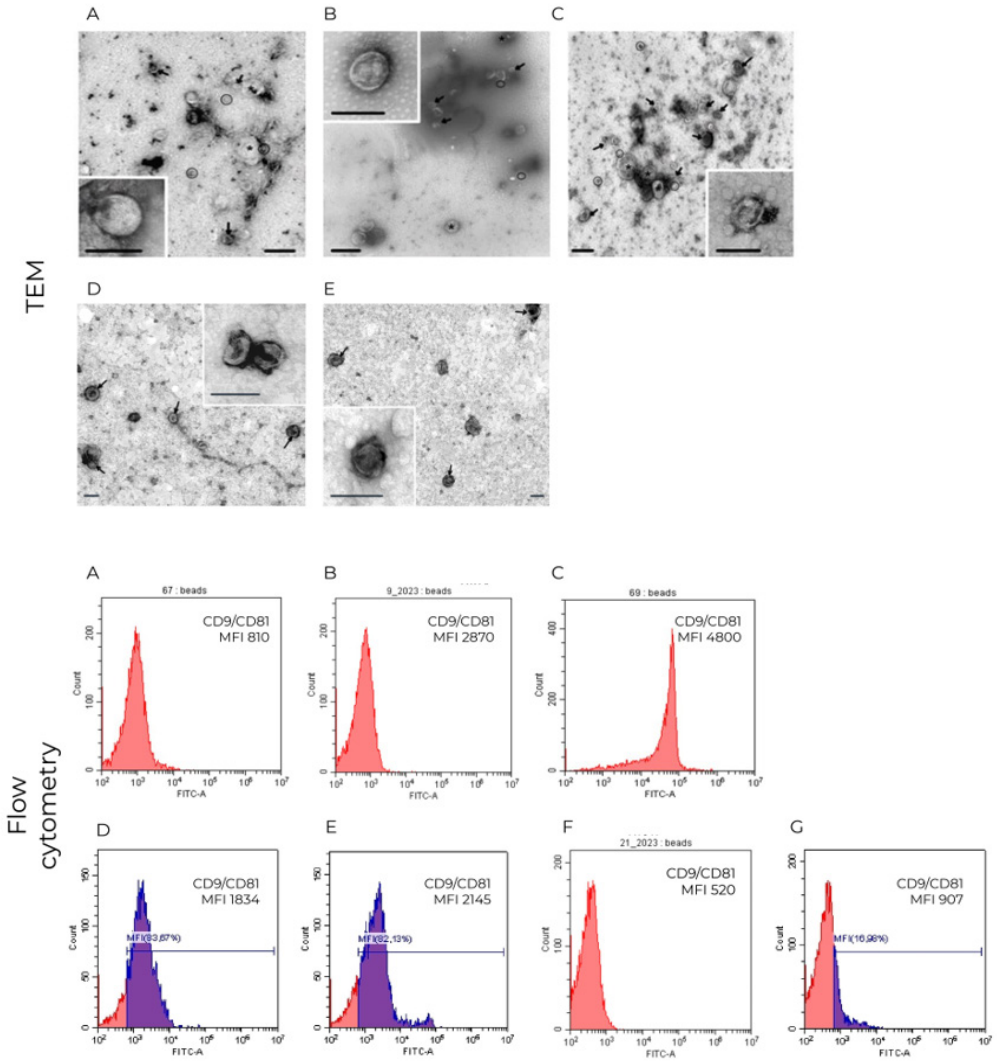

Figure 1. Identification of Exosomes Isolated from MCF-7 (A), BT-474 (B) and BT-549 (C), HFs (D), BCPs (E) conditioned medium by TEM and flow cytometry. Electron microscopy of exosomes were shown in top row. The length of the scale line corresponds to 100 nm, negative staining by phosphotungstate acid. Expression of CD81 on CD9-positive exosomes and isotype controls for cells (F) and blood (G) experiments were shown at bottom row. Representative me-dian fluorescence intensity (MFI) values are shown for flow cytometry.

aggressiveness, would lead to alterations in the exosomal protein profile, particularly with regard to the appearance of cancer-associated RPs.

Small EVs secreted by control and plasmid-transformed BT-549 cells do not differ in morphology and exosomal markers

It is known that in cells growing under normal conditions, RPs are mainly within ribosomes, the level of the ribosome-free RPs is low and increases upon a stress causing the stop of the ribosome biogenesis (for review see [30, 31]). Here, to increase the level of RPS3 in the BT-549 breast cancer cells, we transfected them with a plasmid containing a sequence encoding for the FLAG-tagged human RPS3 by a procedure analogous to that applied earlier for obtaining HEK293T cells producing FLAG-tagged RPS3 [27]. The distribution of the control (without insertion) plasmid and the plasmid encoding FLAG-tagged RPS3 among the conditioned medium, membrane-cytosol fraction, and nuclear fraction was assessed after isolation of DNA from the respective fractions by qPCR. With both kinds of the plasmids the distribution was similar and equal to approx. 5% in the nuclear fraction, 40% in the membrane-cytosol fraction and 55% in the conditioned medium.

Examination of the content of the FLAG-labeled proteins in the samples prepared from the transfected BT-549 breast cancer cells showed the presence of a characteristic band corresponding to the product with the molecular weight expected for the FLAG-tagged RPS3, which was absent in the lane with the sample from control cells transfected with the same plasmid but without the inserted sequence coding for FLAG-RPS3 (Figure 3).

Exosomes secreted by BT-549 cells producing FLAG-labeled RPS3 and by the control cells transfected by pAG1 were isolated and characterized by TEM and exosomal markers. TEM revealed the presence of structured cup-shaped vesicles (40–100 nm) of low electron density with a preserved membrane in all samples representing exosomes. EVs with damaged membranes did not exceed

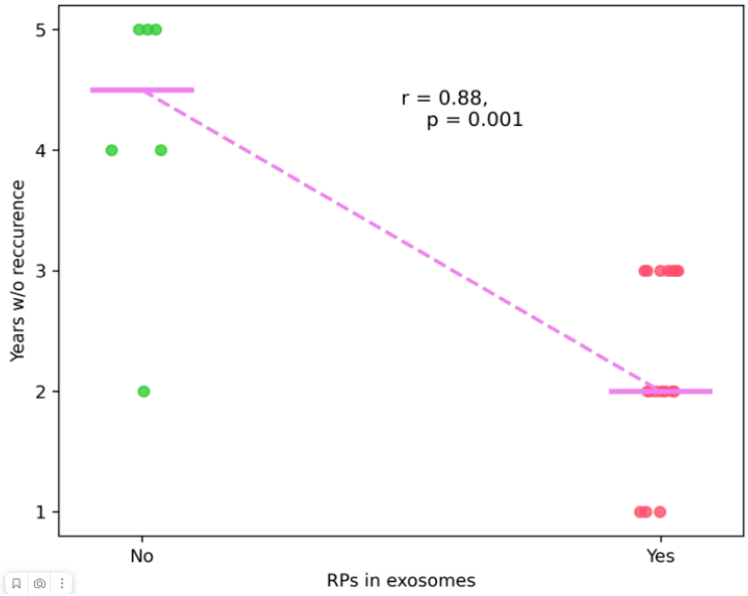

Figure 2. Scatterplot of Breast Cancer Relapse against Presence of RPs in Exosomes. Note: The solid line is the trend line, the dotted line is the 95% confidence interval.

12%, and the proportion of microvesicles (smaller than 30 nm) was no more than 20% (Figure 4A, B). We then confirmed that the exosomes isolated from both sources contained the typical exosome tetraspanin protein markers CD9 and CD81. Both proteins were detected in the content of the isolated vesicles by the Western blotting with specific antibodies (Figure 4C).

In general, features of exosomes secreted by the cells producing FLAG-RPS3 were similar to those from the control BT-549 cells.

Mass spectrometry analysis of the RP cargo from the control cells and cells overexpressing RPS3 revealed a difference in the representation of RPs

To analyze the RP cargo of exosomes secreted by non-transfected BT-549 cells and BT-549 cells with RPS3 overexpression, MALDI-TOF mass-spectrometry was used. With high reliability ($P < 0.05$) 6 and 4 RPs were identified, respectively. In addition, 4 major proteins – CD9, CD63, CD81 and MMP9 were common between both groups (Supplementary Table 1, Table 2). Of note,

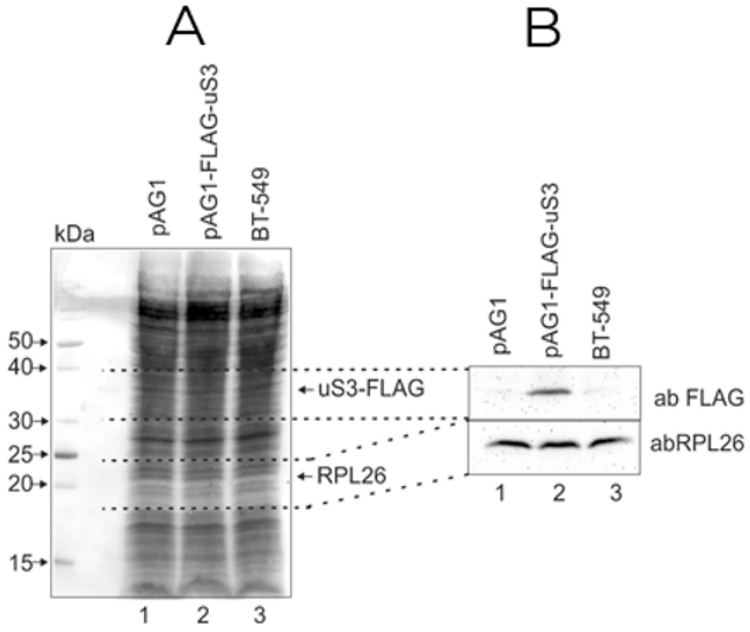

Figure 3. An Analysis of the Production of Target FLAG-tagged RPS3 in BT-549 Breast Cancer Cells by the Western Blotting with the Use of Specific Antibodies against FLAG-tag and against reference RP RPL26. Lanes 1 and 2 corresponds to the total protein from the cells transfected by the control plasmid pAG1 and with the insertion of the sequence coding for FLAG-RPS3/uS3, respectively (pAG1-FLAG-uS3); control lane 3 corresponds to the proteins from the untreated BT-549 breast cancer cells. A, a stained nitrocellulose membrane; B, Western blotting of the total protein samples from the cell lysates after SDS-PAGE and subsequent transfer onto a nitrocellulose membrane using specific antibodies. Prior to the Western blotting, the membrane was cut into pieces as shown by dotted lines.

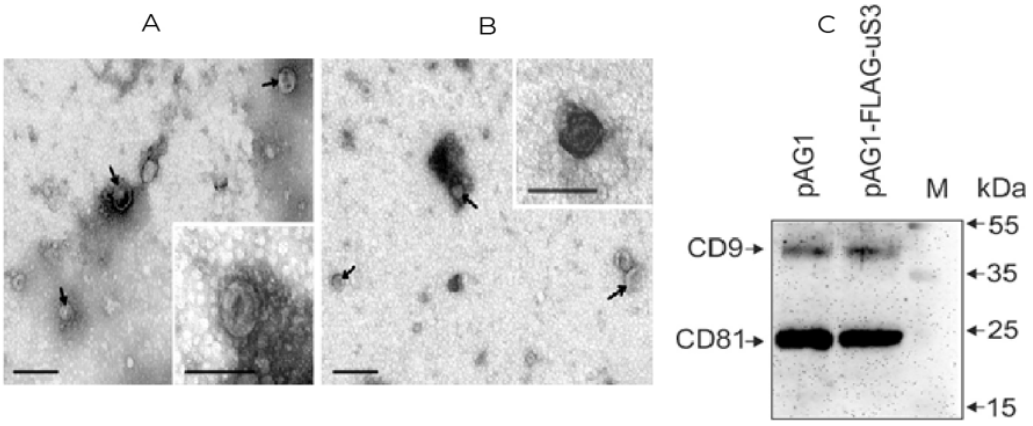

Figure 4. Identification of Exosomes Isolated from Conditioned Medium of BT-549 Cells Trans-fected by the FLAG-RPS3-coding Plasmid and BT-549 Cells Transfected by the Control Plasmid pAG1 by TEM and Western Blotting. Total view of micrographs of exosome preparations ob-tained from cells transfected by control plasmid pAG1 (A) and cells transfected by the FLAG- RPS3-coding plasmid (B). Inserts show exosomes. Scale bars correspond to 100 nm, negative staining by phosphotungstate acid. Western blotting examination of the specific protein markers in exosomes secreted by BT-549 breast carcinoma cells transfected by control pAG1 or pAG1-FLAG-uS3 plasmids with the use of antibodies against the corresponding proteins (C). The sample applied for each lane contained total protein obtained from exosomes derived from ap-prox. 3 million cells.

all but one (MRP-L42) of identified RPs in exosomes were previously discovered in other studies, using mass-spectrometry and annotated in the Vesiclepedia database (http://www.microvesicles.org/).

One can see that exosomes secreted by the BT-549 breast carcinoma cells producing FLAG-tagged RPS3, and by the control ones share the same set of major protein components including typical exosome markers (Supplementary Table 1, Table 2). On the other hand, the sets of RPs in exosomes from the FLAG-RPS3-producing cells dramatically differs from those obtained with the control cells. Moreover, these sets even do not overlap, and the most unexpectedly, RPS3 was not found in neither sets. Importantly, following transfection we detected RPL28 in

Table 2. Mass-spectrometry Identification of Major Exosomal Proteins and RPs Secreted by pro-ducing RPS3 BT-549 Cells in Exosomes

Proteins in exosomes	Gene name / Protein name	Uniprot ID	Mol weight, Da	Identified peptides	Coverage, %
Universal proteins	CD9	P21926	25416	YLLFGFNFIFWLAGIAVLAIGLWLRFDSQTKSIFEQETNNNNSSFYTGVY ILIGAGALMMLVGFLGCCGAVQESQCMLGLFFGFLLVIFAIEIAAAIWGY SHKDEVIKEVQEFYKDTYNKLKTKDEPQR MIFGMIFSMILCCAIRRNREMV	66%
	CD63	P08962	23042	MSRGLQLLLLSCAYSLAPATPEVK LLEGGEERMETPQEDHLRGQHYHQKGQNGSFDAPNERPYSLKIR KYRAEIVLLLALVIFYLTLIIFTCK AGMERAFLPVTSPNK	49%
	CD81	P60033	25809	MGVEGCTKCIKYLLFVFNFVFWLAGGVILGVALWLR IQESQCLLGTFFTCLVILFACEVAAGIWGFVNKDQIAKDVKQFY- DQALQQAVVDDDANNAKAVVKTFHETLDCCGSSTLTALTTSVLK KIDDLFSG KLYLIGIAA	55%
	MMP9	P14780	78458	MSLWQPLVLVLLLGCCFAAPR VAEMRGESKSLGPALLLLQKQLSLPETGELDSATLKAMRTPR EHGDGYP DGLLAHAFPPGPGIQGDAHFDDDELWSLGKGVVVPTRFGNAD- GAACHFPFIFEGR SDGLPWCSTTANYDTDDRFGFCPSER ADSTVMGGNSAGELCVFPFTFLGKEYST	59%
RPs	RPL28/eL28	P46779	15809	ATLSSIR SQKPVMVK	14.90%
	RPL29/eL29	P47914	17812	KLDRLAYIAHPK RTQAPTK	12.60%
	RM20/ MRPL20	Q9BYC9	17645	CQVELNR SLAALASR	14.90%
	RPS6/eS6	P62753	28876	KLNISFPAGCQK LSSLRASTSK	11.40%

Table 3. Bioinformatics Analysis of the Biological Pathways in which the RPS6/eS6 Protein is Involved*

Biological pathways	P-value
Formation of the ternary complex, and subsequently, the 43S complex	0.0222
Ribosomal scanning and start codon recognition	0.0255
mTORC1-mediated signalling	**0.0052**
S6K1-mediated signalling	**0.0052**
mTOR signalling	**0.0128**
PKB-mediated events	**0.0132**
PI3K Cascade	**0.0180**
IRS-mediated signalling	**0.0241**
Translation initiation complex formation	0.0255
Activation of the mRNA upon binding of the cap-binding complex and eIFs, and subsequent binding to 43S	0.0260
Insulin receptor signalling cascade	0.0264

* The non-ribosomal functions of the RPS6/eS6 protein are indicated in bold.

exosomes from FLAG-RPS3-producing BT-549 cells - the same RP we previously identified in blood exosomes from TNBC patients. This finding provides substantial support for our hypothesis regarding the association of this RP with malignant cell behavior. Note that in RPS3/uS3 protein was detected in exosomes from two breast cancer cell lines (4T1, and 4T1.2) and was not detected in exosomes from two other breast cancer cell lines (67NR, 66cl4) [8]. Given that our work and the cited work [8] used the same technique for obtaining exosomes, it can be assumed that the presence of RPs in exosomes depends on the type of cell line mimicking a particular cancer type.

Sets of RPs in exosomes from control cells and FLAG-RPS3-producing cells were analyzed using FunRich 3.13. Among the latter, only RPS6 was found to have non-ribosomal functions (Table 3) associated with cellular stress. Thus, the appearance of RPS6 in exosomes after the cell transfection appears to be a signal to other cells about cellular stress associated with the elevated level of ribosome-free RPS3.

Discussion

Our results regarding the identification of RPs in exosomes from BCPs, control breast cancer cells, and RPS3-overexpressing cells highlight three principal observations. First, under conditions of excessive RPS3 production mimicking ribosomal stress the RP composition of BT-549-derived exosomes changes substantially. Second, the overexpressed RPS3 itself is not incorporated into exosomes, indicating that intracellular abundance does not directly determine vesicular loading. Third, the appearance of RPL28 in exosomes following RPS3 overexpression, paralleling its detection in patient-derived exosomes, suggests a potential link between ribosomal stress and selective RP export associated with tumor aggressiveness.

The clinical cohort included patients with heterogeneous receptor status (ER/PR-positive, HER2-positive, and receptor-negative tumors), reflecting the biological diversity of breast cancer. Within the limitations imposed by cohort size, the exosomal RP-positive phenotype was observed across molecular subtypes, suggesting that the detected signature may reflect a stress-associated tumor state rather than strict subtype identity. However, subtype-adjusted regression analysis was not feasible due to the limited number of events. Therefore, independent validation in larger, molecularly stratified cohorts will be necessary to determine whether the prognostic significance of exosomal RPs is maintained after adjustment for tumor stage, grade, and receptor status. With regard to previously published data, high-throughput proteomic analyses have extensively characterized EV cargo from breast cancer cell lines and patient-derived samples. For example, Gangoda et al. reported proteomic signatures of exosomes secreted by breast cancer cell lines with varying metastatic potential, identifying RPs among vesicular components but without linking them to patient survival or subtype-specific prognosis [8]. Thus, while RPs have been repeatedly detected in EVs derived from breast cancer models and other malignancies, previous studies have largely addressed their presence or functional implications in vitro rather than their association with long-term clinical outcome. Importantly, to our knowledge, no prior publication has directly linked the presence of RPs in circulating exosomes to a specific molecular subtype of breast cancer (luminal, HER2-positive, or triple-negative) or demonstrated subtype-specific prognostic stratification based on exosomal ribosomal cargo. Therefore, the present findings extend existing EV proteomics research by introducing a clinically correlated RP-based prognostic stratification framework that appears independent of classical receptor-defined subtype classification, although confirmation in larger subtype-stratified cohorts remains necessary.

Current knowledge regarding the individual RPs identified in this study as passengers of exosomes from patient's blood, breast cancer cells with and without modeled ribosomal stress? In exosomes from the control breast cancer BT-549 cells, we found RPs from both small (RPS2/uS5 and RPS27/eS27) and large (RPL13A/uL13 and RPL36/eL36) subunits of cytoplasmic ribosomes and two RPs from large mitochondrial ribosomal subunit, RM16/MRP-L16 and RM42/MRP-L42 (Supplementary Table 1).

One of the found cytoplasmic RPs, RPL13A/uL13,

has well-known extra-ribosomal properties relating to its participation in the immune signaling at inflammation. In particular, this protein can be phosphorylated by a specific kinase activated by interferon gamma (IFN-γ) at inflammation, dissociate from the ribosome and participate in the assembly of the Gamma-Activated Inhibitor of Translation (GAIT) complex. This complex binds to the GAIT elements present in the 3′ UTR of mRNAs encoding proteins implicated in the inflammation and thereby inhibits their translation [32].

For other cytoplasmic RPs from the exosomal cargo of the control BT-549 cells, well-recognizable extraribosomal properties are not known; there are only some data on their association with carcinogenesis. So, RPL36/eL36 is down-regulated in hepatocellular carcinoma cells [33], while RPS2/uS5, oppositely, is up-regulated at prostate cancer [34]. As for RPS27/eS27, it has various types of association with cancer depending on the type of malignant cells. Its homolog RPS27L/eS27-like (96% homology to RPS27) in colon cancer and osteosarcoma cells is implicated in the p53 pathway, where it is an MDM2 substrate and competes with p53 for MDM2 binding [35]. RPS27L is down-regulated in colorectal cancer cells [36], whereas in prostate cancer cells, RPS27 is up-regulated [37]. For all that, molecular mechanisms underlying the cancer-associated features of the above RPs are unknown and none of these proteins, to our knowledge, have been reported to be associated with breast cancer (however, the latter does not reflect the lack of association of these RPs with this cancer type and could be due to insufficient studies in the field).

Analogously, both mitochondrial RPs RM16/MRP-L16 and RM42/MRP-L42 found here in the cargo of the control BT-549 cells are associated with some cancers. So, MRP-L42 is significantly up-regulated in the glioma tissues as well as in the lung adenocarcinoma tissues and cell lines [38], and the increased level of the protein is remarkably related to poor prognosis for patients [39]. Expression level of MRP-L16 is also associated with various types of cancer, namely, with colorectal cancer [40], hepatocellular carcinoma and [41], notably, with breast cancer [42]. In the latter case, the low level of MRP-L16 significantly indicated poor prognosis in breast cancer patients, pointing to potential anti-tumor properties of this protein in this type of cancer, the feature opposite those of MRP-L16 in other above-mentioned tumors and of other above-discussed RPs. Summarizing information on RPs – passengers of exosomes from the control breast carcinoma cells, we would like to conclude that there are no specific structural features and/or functions shared by these RPs, and none of these RPs are associated with signaling pathways relating to stress.

Among four RPs identified in the cargo of the exosomes secreted by RPS3/uS3-producing cells (Table 3, association with cancers has been reported for two of them, RPL29/eL29 and RPS6/eS6. The former is found to be associated with pancreatic cancer and gingival squamous cancer [43, 44], whereas the latter – with three types of cancers (gastric [45], pancreatic and non-small cell lung cancer) [46, 47]. The most important and interesting thing for these RPs is that one of them, RPS6/eS6, has a bunch of well described extraribosomal functions, the majority of which directly concern the participation of this RP in various signaling pathways related to stress (Table 3). Several serine residues of RPS6 are targets for numerous kinases including S6 kinases 1-2 (S6K1-2), protein kinase A (PKA), p90 ribosomal S6 kinases 1-4 (RSK1-4), casein kinase 1 (CK1), and death-associated protein kinase (DAPK) that are involved in many basic cell response and regulation processes including those listed in Table 3 [47-51]. Many pathological stimuli can induce sequential phosphorylation of RPS6 by these kinases, thus implicating this ribosomal protein in a variety of biological pathways also related also to carcinogenesis. It should also be emphasized that the biological activity of RPS6 is largely determined by its phosphorylation status, which is regulated by S6 kinases downstream of PI3K/AKT/mTOR signaling [49, 52]. Phosphorylated RPS6 has been associated with tumor progression and unfavorable clinical outcomes in several malignancies, including breast cancer [47, 53]. The present proteomic approach does not allow discrimination between phosphorylated and non-phosphorylated forms of RPS6 in exosomes. Determining whether exosomal RPS6 is present in its activated phosphorylated form would provide stronger evidence linking ribosomal stress to PI3K/AKT/mTOR pathway activation and represents an important direction for future studies. For instance, RPS6 is implicated in the PI3K/AKT/mTORC1 signaling pathways, which are activated by its phosphorylation in many cancers [52] and which was shown to inhibit p53-mediated tumor suppression [46, 54]. Higher risk of recurrence in patients with early-stage breast cancer is associated with elevated levels of phosphorylated RPS6 due to activation of the PI3K pathway [52].

Taken together, the emergence of RPS6/eS6 in exosomes secreted by BT-549 cells overexpressing RPS3 may reflect a stress-responsive remodeling of exosomal cargo. Given the established involvement of RPS6 in PI3K/AKT/mTOR signaling and its phosphorylation-dependent role in tumor progression [47–53], its selective export under these conditions may represent a mechanism of stress signal propagation between tumor cells. Although the phosphorylation status of exosomal RPS6 was not assessed here, this possibility warrants further investigation.

The RPs of BCPs' blood exosomes identified in our study (RPL28, MRPL52, and UBA52) exhibit well-documented associations with malignant processes across various cancer types. Database analysis (Human Protein Atlas; https://www.proteinatlas.org/) reveals these proteins are consistently expressed in multiple malignancies, including breast cancer: of particular significance is RPL28, which we detected both in BCP-derived exosomes and in our ribosomal stress model. This protein has been independently validated as a negative prognostic marker in prostate and colorectal adenocarcinomas, suggesting its potential role in tumor progression beyond breast cancer. The consistent appearance of RPL28 in both clinical samples and in exosomes derived from RPS3-overexpressing BT-549 cells deserves particular attention. RP dysregulation has been linked to proliferative and

invasive tumor phenotypes [45, 37], and ribosomal stress is known to induce translational reprogramming associated with tumor survival and progression [31, 33]. Thus, selective export of RPL28 into exosomes may reflect stress-associated ribosomal remodeling in aggressive tumors, although its direct functional role in recipient cells remains to be clarified. MRPL52 demonstrates elevated expression patterns in invasive breast carcinoma specimens; the ubiquitin-ribosomal fusion protein UBA52 shows dual oncogenic relevance: it could serve as an adverse prognostic indicator in hepatocellular carcinoma due to the HPA and has been previously reported in gastric cancer-derived exosomes [55], indicating its possible involvement in exosome-mediated tumor progression pathways.

The present study has several limitations that should be acknowledged. First, the clinical cohort was relatively small (n = 23). Second, the proteomic analysis was qualitative rather than quantitative; therefore, differences in abundance of RPs could not be assessed. Finally, functional assays evaluating the impact of exosome-mediated transfer of specific RPs on recipient cell behavior were beyond the scope of this work. Future studies should address whether exosomal RPL28 or RPS6 modulate migration, epithelial–mesenchymal transition, stemness markers, or drug resistance in recipient breast epithelial or stromal cells.

The concordance between clinical observations and the experimental ribosomal stress model strengthens the hypothesis that selective RP sorting into exosomes may represent a biologically meaningful process in tumor progression. In particular, the recurrent detection of RPL28 in both patient-derived and stress-modeled exosomes suggests its potential role as a marker of aggressive tumor behavior.

Concluding remarks

Our findings demonstrate that intracellular protein overexpression does not necessarily lead to its incorporation into exosomes, highlighting important considerations for developing exosome-based therapeutic delivery systems. The study reveals several critical insights:

• Ribosomal stress induced by RPS3 overexpression fundamentally alters the RP composition of exosomes without incorporating the overexpressed protein itself;

• RPS6 emerges as a specific marker of ribosomal stress in exosomal cargo and most significantly, we identified RPL28 in exosomes from both our cellular model and BCP blood samples, along with other cancer-associated RPs (MRPL52, UBA52) that show established correlations with tumor aggressiveness across multiple malignancies. These observations raise important mechanistic questions about how overproduced RPs influence the selective packaging of other RPs into exosomes while excluding themselves and whether the consistent appearance of specific RPs (particularly RPL28) in both experimental and clinical exosomes reflects their functional role in cancer progression. The identification of stress-induced RP signatures in malignant cell exosomes, mirroring those found in patient samples, provides a valuable framework for future investigations into exosome-mediated stress responses in cancer. Furthermore, our results suggest that monitoring exosomal RP profiles could serve as a novel approach for detecting ribosomal stress conditions in tumors, with potential diagnostic and therapeutic implications.

Author Contribution Statement

Conceptualization, D.G. and S.T.; methodology, A.Sh., E.B., L.Y., A.G. and D.S.; data curation, A. Ch.; writing original draft preparation, A.Sh., D.G. and S.T.; writing review and editing, A.Ch., S.T.; supervision, S.T. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for profit sectors.

Approval

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Local Ethics Committee of the Institute of Chemical Biology and Fundamental Medicine. Informed consent was obtained from all subjects involved in the study.

Conflict of Interest

The authors declare no conflicts of interest.

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