Proteomic Profiling of Du145 Cell Line Exosomes Identifies Icam1 as Aputative molecular Mechanism by Which Relb Promotes The proliferation, Migration, and Invasion of Prostate cancer Cells

Wenjing Li

First Affiliated Hospital of Soochow University

Jingjing Xu

First Affiliated Hospital of Soochow University

Li Cheng

Shanghai East Hospital

Lianjun Zhang

Chinese Academy of Medical Sciences and Peking Union Medical College

Qiang Shao

Suzhou Municipal Hospital

Feng Guo (✉ guofeng27@suda.edu.cn)

Nanjing Medical University Affiliated Suzhou Hospital <https://orcid.org/0000-0003-3543-3589>

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Abstract

Background: Prostate cancer (PC) is a serious health issue in men. Exosome plays essential roles in modulating the oncogenesis and progression of PC. RelB is highly expressed in PC and plays oncogenic parts in DU145 cells. We aim to uncover the protein panel of exosomes derived from RelB-knockdown DU145 cells (siRelB) as compared to control cells (sictrl) and explore a potential mechanism that RelB conferring the more aggressive phenotype to DU145 cells.

Methods: Exosomes derived from sictrl and siRelB were subjected to Liquid Chromatography-Mass Spectrometry for proteomics profiling. Label-free quantification strategy iBAQ (intensity-based absolute quantification) was used to quantify and Fold Change (FC) was calculated to identify the differentially expressed proteins (DEPs). The characterization of proteins was conducted by bioinformatics analysis. ICAM1 over-expressing DU145 cells (hICAM1) and control cells (hctrl) were established by transfection using Lipofectamine 2000. The cell growth, migration, and invasion capabilities were measured by the xCelligence real-time monitoring system. Annexin V/PI-staining was adopted to assess apoptosis. CCK-8 assay was applied for proliferation evaluation. Integrin β -1, MMP9, and uPA were detected by Western blot.

Results: 1259 exosomal proteins were identified, with 160 and 105 proteins unique to the siRelB and sictrl, respectively, while 994 proteins were present in both. We identified 137 upregulated and 55 downregulated proteins in siRelB. Gene Ontology (GO) analysis revealed that some DEPs had the cell adhesion molecular activity and participated in the cell adhesion process. Kyoto Encyclopedia of Genes and Genomes (KEGG) enriched that intercellular adhesion molecule-1 (ICAM1) was downregulated targeted by the NF- κ B signaling. The FC of exosomal ICAM1 was 2.136. The expression of ICAM1 was positively related to RelB in PC by UALCAN. ICAM1 was shown to be co-expressed with RelB by GeneMANIA. The protein abundance of exosomal ICAM1 was lower in siRelB. hICAM1 had enhanced abilities of proliferation, migration, and invasion, with higher expression of Integrin β -1 when compared to hctrl.

Conclusions: Our study identified 192 exosomal DEPs downstream of RelB in the DU145 cells. Exosomal ICAM1, conferring a more aggressive phenotype to DU145 cells, is a potential molecular mechanism modulating the tumorigenesis and progression of PC cells.

Background

It is estimated to have 191,930 new cases of prostate cancer (PC) in the USA in 2020. The predicted death caused by PC could be 33,330 [1]. Though great progress has been made in illuminating the roles of various factors during the tumorigenesis and progression process, the exact mechanism is not yet fully understood. Tumor microenvironment (TME), the “soil” on which tumor cells live, play a critical part in the development and progression of a tumor. The exchange of substances or messages between cells is an important form of communication between members of TME.

Released by cancer cells or other members in TME, exosomes, the vesicles endocytosing various biological molecules, such as protein, DNA fragment, mRNA and miRNA via lipid bilayer containing membrane protein, are the crucial mediator of intercellular communication, playing important roles in tumorigenesis and advancement of cancer cells [2-4]. Exosomes derived from DU145 prostatic cells fairly promote tumor cell proliferation and migration, and reduce apoptosis in LNCaP and RWPE-1 cells [5]. Exosomes secreted by metastases-initiating cells support continued cancer growth and progression through activating RANKL, c-Myc, and FOXM1, which create a more inclined metastasis microenvironment for PC [6]. It has been proved that tumor-derived exosomes can be uptaken by specific organs or cells, to create favorable TME for cancer metastasis. For example, miR-21, miR-141, and miR-375 enriched exosomes derived from PC play important roles in assisting tumor cells to acclimatize to the low-androgen environment of distant metastatic organs [7].

RelB is the primary member in the non-canonical Nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) pathway, participating in the development and progression of multiple tumors [8-10]. RelB has been shown to correlate with the tumorigenesis, development, and radio-sensitivity of PC. In PC tissues, the expression of RelB is highest among all the NF- κ B family members and directly related to the Gleason score, implying that RelB acted in the occurrence and development of PC. Suppressing RelB expression in malignant androgen-independent PC-3 cells greatly decreases the occurrence and proliferation of tumors [11]. Similarly, highly expressed RelB is associated with a more aggressive phenotype in androgen-independent DU145 cells. RelB-knockdown prominently attenuates the migration and invasion of the DU145 cells, because of the decrease of integrin β -1 [12]. RM1 cells transfected with the RelB-siRNA have an enhanced radio-sensitivity at various dosage points by clonogenic growth assay [13]. HZ08 could enhance the radio-sensitivity of PC cells through inhibiting PI3K/Akt/IKK α signaling pathway, leading to the down-regulation of MnSOD transcriptionally by keeping RelB from nuclear translocating [14].

NF- κ B has been shown to regulate the secretion of substance inside exosomes [15]. However, exosomes from the RelB-knockdown DU145 cells have not been addressed. It is important to explore the roles of key molecules regulated by RelB during the tumorigenesis and advance of PC cells. In this study, we have identified several candidate exosomal proteins downstream of RelB, using Mass Spectrometry (MS) technique. Of note, we found that exosomal ICAM1 expression might play important parts in modulating PC proliferation and aggressiveness, overexpression of ICAM1 will strengthen the abilities of cell proliferation, migration, and invasion of DU145 cells.

Materials And Methods

Establishment of RelB-knockdown cell line

DU145 cell line was bought from ATCC (American Type Culture Collection). The cells passaged 2-5 times were used in our experiments. The cell line was certified using Short Tandem Repeat (STR) profiling (Shanghai Biowing Applied Biotechnology Company). All cell lines were routinely tested for mycoplasma. The RelB silenced DU145 cell line (siRelB) and control cell line (sictrl) had been established in a previous study [12]. Briefly, the shRNA-RelB (targeted gene 275-293: 5'-GCACAGATGAATTGGAGAT-3') was synthesized and cloned into the pSilencer3.1-H1-neo vector (cat no. 5770, Thermo Scientific™, USA). The pSilencer3.1-siRelB and the control plasmids were afterward transduced into cells by Lipofectamine 2000 (cat no. 12566014, Thermo Scientific™, USA) following the instructions. The cells were cultured in the presence of 400 ng/ μ l neomycin (cat no. E859-5G, Amresco) for about 15 days.

Cell culture and supernatant collection

DU145-siRelB and DU145-sictrl cells were maintained in RPMI-1640 media added with 10% exosome-depleted fetal bovine serum (EXO-FBS-50A-1, SBI, Japan), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂. After the cell confluence reached 85~95%, the culture medium was collected and stored at -80 °C after centrifugation at 500 g for 15 min.

Exosomes extraction and characterization

Exosomes from exo-free FBS supernatant of both siRelB and sictrl were isolated by ultracentrifugation. Firstly, dead cells and large cell debris were deposited by centrifugations at 1200 g for 20 min and 10,000 g for 20 min. Pellets from each centrifugation step were discarded. 100 ml medium was collected and ultracentrifuged at 100,000 g at 4 °C for 90 min (Optima XPN-100 ultracentrifuge, Beckman, USA). The sediment was resuspended in 1x PBS for further analysis. The morphological characteristics of exosomes were confirmed by transmission electron microscopy using the method described by Ying *et al* [16]. Particle size by NanoSight analysis was conducted to verify the uniformity of microvesicle extracted.

Exosome protein extraction, quantification, and immunoblotting

The protein of exosomes was extracted using a 5x RIPA buffer with 1 mM proteinase inhibitor. The mixture was vortex blended and cracked for 20 min on ice. The supernatant was collected after centrifugation at 10,000 g for 12 min. The BCA assay was used for measuring protein concentration (Thermo Fisher Scientific, USA). The absorbance at 562 nm was determined. We drew the standard curve according to the calibrated absorbance value and standard concentration. The concentration of protein samples was calculated based on the standard curve. 20 µg protein of exosomes were loaded on a 10% SDS gel for electrophoresis, followed by transfer to a polyvinylidene fluoride membrane. Primary antibodies used were rabbit monoclonal anti-human flotillin (cat no. 18634, Cell Signaling Technology, Germany), mouse monoclonal anti-human CD81, and mouse monoclonal anti-human CD63 (cat no. NB100-65805 and NBP2-42225, Novus Biologicals, USA). Goat anti-mouse IRDye 800cw secondary antibody and goat anti-rabbit IRDye 680 secondary antibody (cat no. 926-32210 and 926-32221, Li-COR Biosciences, USA) were used as secondary antibodies. The image was scanned by Odyssey's two-color infrared fluorescence imaging system (Li-COR Biosciences, USA).

Sample Preparation for Mass Spectrometry

The exosome samples were cracked in buffer with 8 M urea, 1% Triton X-100, 65 mM dithiothreitol (DTT, sigma, Germany), and 0.1% proteinase inhibitor (cocktail) using a high-intensity ultrasonic machine (JY99-IIDN, Scientz, China) on ice. Pellets were discarded after centrifugation at 5 000 g for 3 min at 4 °C. The pre-cooled 15% trichloroacetic acid was used to dissolve the protein for 2 h at -20 °C. Protein precipitation was obtained after centrifugation at 15 000 g for 10 min at 4 °C, followed by three washing steps with cold acetone, dried for 5 min and subsequently rehydrated in buffer containing 8 M urea, 100 mM triethylamine-carbonic acid buffer (TEAB, pH 8.0). The BCA assay was used to determine protein concentration. Add 10 mM DTT (Sigma, Germany) into 100 µg protein samples at 37 °C for 1 h, then 20 mM IAA (iodoacetamide, Sigma, Germany) were added for alkylation reduction at room temperature for 45 min. First enzymatic hydrolysis of protein samples was conducted at the present of 1 µg trypsin at 37 °C for 4 h, followed by second enzymatic digestion with another 1 µg trypsin at 37 °C for 12 h. Peptide mixture was acidized with 5% formic acid, demineralized using C18 spin columns, and eluted by 200 µl 50 mM NH₄HCO₃, then quantified and lyophilized.

Liquid Chromatography-Mass Spectrometry (LC-MS) Procedure

The procedure of LC-MS was referred to the instructions described by Zou *et al* [17]. The peptides were resuspended in 100 µl solvent A (A: water containing 0.1% formic acid; B: ACN containing 0.1% formic acid), separated by nanoLC and analyzed by on-line electrospray tandem mass spectrometry. The tests were conducted on a set of equipment including an EASY-nLC 1000 system (Thermo Fisher Scientific, Waltham, MA), an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA), and an online nano-electrospray ion source. 2 µl peptide sample was loaded onto the trap column (Thermo Scientific Acclaim PepMap C18, 100 µm x 2 cm), with a flow of 10 µl/min for 3 min and subsequently separated on the analytical column (Acclaim PepMap C18, 75 µm x 25 cm) with a linear gradient, from 2% B to 45% B in 180 min. The column was re-equilibrated at initial conditions for 5 min. The column flow rate was maintained at 300 nl/min. The electrospray voltage of 2.0 kV versus the inlet of the mass spectrometer was used.

The Orbitrap Fusion mass spectrometer adopted a data-dependent mode for data collection by the alternative switch between Mass Scan (MS) and tandem MS (MS/MS) [18]. In MS scan, the ions with 350-1600 m/z and a charge state of >3 were chosen for deeper fragmentation in an orbitrap mass analyzer. The maximum ion introduction times were 50 ms for the full MS scan and 150 ms for MS/MS. The resolution were targeted as 120,000 (m/z, 200) for MS and 15,000 (m/z, 200) for MS/MS. The automatic gain control (AGC) were set as 5 x 10⁵ and 2 x 10⁵ for MS and MS/MS, respectively. Fragmentation was conducted to the ions in +2, +3, +4, or +5 charge state, with a minimum intensity threshold of 20,000. Precursor ions were fragmented in a mode of higher-energy collisional dissociation (HCD) at 30% of normalized energy in the fracture pool. One Microscan was recorded employing a dynamic exclusion of 48 sec all the time.

Identification of differentially expressed proteins (DEPs)

Raw MS data were analyzed by Maxquant database (version 1.5.3.30) and retrieved using the integrated Andromeda search engine with a search strategy of target- decoy. The peptide designation was conducted by running raw data against the SwissProt-Human data set (Release 2017-04-10) (total of 20259 entries). Trypsin (Promega, Madison, WI) was used for specific digestion, and a maximum of 2 missed cleavages was permitted. The mass tolerance for fragment ions and precursor ions were 0.02 Da and ±10 ppm, respectively. Carbamidomethylation of cysteine was set as a fixed modification. Protein N-terminal, O-GlcNAc protein modification, or lysine carbamidomethylation, methionine oxidization were set as variable modifications. False discovery rate (FDR)

thresholds set for peptide, protein, and modification sites were all 1%. For protein identification, minimum peptide length was set as 7 amino acids with fewest one match of unique peptide.

The label-free quantification strategy iBAQ (intensity-based absolute quantification) was applied to quantify. The raw data were further processed by Perseus software. Protein group LFQ intensities were log2 transformed. The missing values were replaced by random values in the Gaussian distribution below the median to simulate low abundance LFQ values. Fold change (FC) filtering and Student's *t*-test were performed to screen DEPs between two groups by using the R software. DEPs were identified if the minimum matched unique peptide ≥ 1 , with a threshold $p < 0.05$, absolute value of FC > 1.25 and the FDR correction ≤ 0.05 . Venn diagram, volcano plot, and hierarchical clustering were performed for the DEPs of the two groups.

Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment were implemented to understand the exact characters and functions of DEPs. A *p*-value of less than 0.05 was considered enriched significantly. Three biological repeats were performed in the two groups.

UALCAN database and GeneMANIA analysis

UALCAN is a web portal integrating and analyzing cancer data based on The Cancer Genome Atlas (TCGA) database, providing plots and graphs describing gene expression and clinical prognosis according to sample type, race, age, or other factors [19]. In the present study, we used UALCAN to interrogate the correlation between RelB and ICAM1 and the expression of the ICAM1 gene in PC patients. GeneMANIA is an online tool processing protein-protein interaction (PPI) network, and giving information about physical interaction, gene co-expression, gene co-location, gene enrichment analysis, and website prediction [20]. We predicted the interaction between RelB and ICAM1 and generated the networks through GeneMANIA.

Construction and verification of hICAM1 and hctrl cell lines

The ICAM1 overexpressed cell line (hICAM1) and control cell line (hctrl) were established by transfecting pCMV3-ICAM1-His plasmid (cat no. HG10346-CH, Sino Biological Inc., China) and negative control vector (cat no. CV015, Sino Biological Inc., China) into DU145 cells with Lipofectamine 2000 reagent (cat no. 12566014, Thermo Scientific™, USA). Cells were maintained in the culture medium containing 400 ng/μl hygromycin (Roche Diagnostics GmbH, Germany) for two weeks. RNA was extracted using TRIzol (cat. no. 15596-026, Invitrogen life technologies, USA) method according to the instruction. RT-qPCR was conducted to test the ICAM1 mRNA expression in different clones and exosomes, using the following primers; forward primer, 5'-TCCGGCGCCCAACGTGATTC-3', and reverse primer, 5'-CGGGGGCCATACAGGACACG-3'. The expression was normalized to β-actin (5'-GCTACGAGCTGCCTGACGG-3' for forward primer, and 5'-TGTTGGCGTACAGGTCTTTC-3' for reverse primer). ΔCt was the Ct difference between ICAM1 and β-actin of each sample. $2^{-\Delta Ct}$ was calculated to show the relative expression of ICAM1. Western blotting was applied to detecting the ICAM1 protein expression in cells or exosomes. Rabbit polyclonal anti-human ICAM1 (cat no. 4915, Cell Signaling Technology, Germany) was used as the primary antibody. The concentration of soluble ICAM1 (sICAM1) in exosome was measured using ELISA (cat no. DCD540, R & D systems, USA), and calculated based on the optical density (OD) at 450 nm. GraphPad Prism 6.0 was used to plot.

Description of the biological characters of cell lines

The cell growth, migration, and invasion activities were dynamically monitored by the xCelligence RTCA instrument (Roche) as described before [12]. In the cell growth assay, cells were planted at 7 000 per well. The monitoring was last for 80 h. A CIM 16-plate was used in cell migration assay, equipped with an upper and lower chamber. Cells were seeded at 40, 000 per well suspended in 30 μl pure RPMI-1640 in the upper chamber, while 170 μl complete media was loaded into the wells of the lower chamber. Wells on the upper chamber pre-coated with matrigel were used for the cell invasion assay. 60, 000 cells were seeded. The cell index was continuously monitored for 30 h for migration or invasion assay. CCK-8 assay (cat. no. ab228554, Abcam, England) was used to assess cell proliferation. Briefly, 6000 cells suspended in 100 μl culture medium were planted into 96-well plate with quintuplicate wells per sample. Three wells added with 100 μl culture medium were used as blank control. The 96-well plate was continued to incubate for 2 h after adding 10 μl CCK-8 reagent. The OD values at 450 nm were measured at 24 h, 48 h, and 72 h. AnnexinV and PI dyes were adopted to analyze the early apoptosis using flow cytometry. 1.5×10^5 cells suspended in 3,000 μl complete medium were planted into a 6-well plate with triplicate wells per sample. Cells were harvested and stained using annexin-V, PI, or both for 15 min in darkness according to the instructions (cat. no. 40302ES50, Yeasen Biotech, China). The early apoptosis proportion was analyzed at 24 h, 48 h, and 72 h. The protein level of metastasis-related proteins integrin β-1, MMP9 (cat no. 4706 and 13667, Cell Signaling Technology, Germany) and uPA (cat no. sc-59727, Santa Cruz Biotechnology, USA) were analyzed by western blotting. The image was scanned by Odyssey. The data were analyzed and plotted using GraphPad Prism 6.0.

Results

RelB affected the proliferation, migration, and invasion of DU145 cells

In a previous study, we have demonstrated that the expression of RelB was higher in the DU145 cells when compared to the LNCaP and PC3 cell lines (Supplementary Figure 1A). We have also assessed the effect of RelB knockdown on the cell growth, migration, and invasion abilities of DU145 cells [12]. As the results showed (Supplementary Figure 1), the cell growth, migration, and invasion were markedly slower in siRelB cells than those in sictrl cells during the 72 h or 24 h persistent monitoring (all $p < 0.05$). The in vitro scratch assay revealed that siRelB cells migrated to the scratch center much slower than sictrl cells at 72 h. The protein level of integrin β-1 was decreased in RelB knockdown cells.

Quality assessment of exosomal protein samples

Transmission electron microscopy (Figure 1A) and NanoSight assay (Figure 1B) displayed that particles separated by ultracentrifugation contained tremendous DU145-derived EVs with a diameter of 30-150 nm. Immunoblotting revealed that exosomal samples were positive for protein markers CD63, flotillin, and CD81 (Figure 1C). To characterize the proteins in exosomes from sictrl and siRelB cells, proteomics profiling was performed by MS technique. Totally 1,259 proteins were identified through quantitative analysis. Results of consistency analysis between samples within the group showed that the R^2 among the three biological repeats were all above 0.99, indicating excellent consistency among the protein samples (Supplementary Figure 2). 89.4% (1,125/1,259) proteins were recorded in Exocarta database (Figure 1D). The protein samples had a better purity and were qualified for proteomic profiling.

Total proteome expression analysis

Among the 1,259 proteins, 994 proteins were present in both sictrl and siRelB cell lines. 105 proteins and 160 proteins were unique to sictrl and siRelB, respectively, as shown in the Venn diagram (Figure 2A). Among the common 994 proteins, the canonical exosome proteins could be identified [21], exemplified by heat shock proteins (HSP90 and HSP70), endosomal molecules (clathrin), cytoskeletal proteins (actin, tubulin, and myosin), multivesicular tetraspanins (CD63 and CD81), antigen presentation molecules (MHC class I), adhesion proteins (integrins), immunoglobulin family members (ICAM1/CD54), and membrane trafficking proteins (RAB proteins and annexins). Regardless of the different groups, the iBAQ averages of exosomal proteins of the six samples were analyzed. The percentage of protein was calculated by the mean iBAQ value of individual protein divided the sum of calibrated iBAQ values of 1,259 proteins in the proteinGroups. The top 25 proteins accounting for 56.21% of the total exosome proteome were summarized and the expression proportion of each protein in both groups was labeled in Figure 2B.

Recognition of differentially expressed proteins (DEPs) between sictrl and siRelB cells

FC was calculated based on protein expression ratio of siRelB to sictrl cells [$\text{mean (iBAQ_siRelB)} / \text{mean (iBAQ_sictrl)}$]. Basing on the following criteria, the absolute value of $FC > 1.25$, meanwhile $P \leq 0.05$ and FDR correction ≤ 0.05 , 192 DEPs were identified, showed by hierarchical clustering (Figure 3A). The logarithm of FC (expressed as logFC) and the minus logarithm of P -value (expressed as $-\log(P\text{-value})$) were used to perform the volcano plot. Compared with the sictrl cells, 137 proteins were upregulated and 55 proteins were downregulated in the siRelB cells. As the 13 proteins and 26 proteins solely expressed in sictrl and siRelB cells couldn't be shown in the volcano plot (Figure 3B), we performed another heat map analysis (Figure 3C).

Functional explore by GO enrichment of the DEPs

To further characterize the DEPs, we investigated the functions of PC derived exosomal proteins through GO analysis. All the proteins were inputted to DAVID for the enriched cellular component, biological process, and molecular function terms at a cutoff of $p < 0.05$. Microsoft Excel (version 2018) was used for plotting. The most populated exosomal proteins of the cellular component were cytoplasm, exosomes, and nucleus, the biological processes were mainly concentrated on signal transduction, protein metabolism, and cell communication; and the most dominant molecular functions were extracellular matrix structural constituent, chaperone activity and catalytic activity (Supplementary Figure 3A, 3B and 3C). Enrichment analysis of the 13 and 26 DEPs solely expressed in sictrl or siRelB were summarized in Table 1.

KEGG pathway analysis of the DEPs

Pathways enriched with the DEP were performed by the KEGG database. 9 pathways were enriched significantly based on the cutoff of $P\text{-value} < 0.05$ (Supplementary Figure 4A). Supplementary Table 1 summarized the DEPs involved in the individual pathways. Shown in the map of the Epstein-Barr virus infection pathway (Supplementary Figure 4B), transmembrane protein ICAM1, a molecule regulated by the non-canonical NF- κ B signaling, was downregulated in the siRelB cells (marked in a black rectangle).

Bioinformatics characters of differentially expressed exosomal ICAM1

Among the 192 DEPs, ICAM1 was downregulated in the absence of RelB, with an FC of 2.136, and $p\text{-value} < 0.001$. GO analysis displayed that, ICAM1 was mainly derived from the plasma membrane, cytoplasm, exosomes, and extracellular space. ICAM1 exerted predominantly the functions of cell adhesion molecule activity, cell communication, and signal transduction; and principally participated in cell communication and signal transduction processes. Importantly, exosomal ICAM1 was found to be regulated by NF- κ B signaling in the Epstein-Barr virus infection pathway by KEGG enrichment (Supplementary Figure 4B).

To investigate the relationship between RelB gene and ICAM1 gene, we viewed the correlation item of RelB gene in PC on UALCAN website. According to the TCGA database, among the top 24 genes positively correlated with RelB, ICAM1 ranked second, with a Pearson correlation coefficient as 0.71 (Figures 4A and 4B). When compared to the normal samples ($n=52$), the expression of RelB and ICAM1 in tumor samples ($n=497$) were both significantly higher ($p=9.0237E-03$ and $9.9639E-04$, respectively) (Figures 4C and 4D). The interaction network generated by the GeneMANIA web portal indicated that RelB interacted with ICAM1 mainly in a pattern of co-expression, or mediated by RELA, NFKB2, NFKB1, MST1R, REL, NFKB1A, CXCL-13, ICAM3 and ITGAM in a small part (Figure 4E).

Establishment of ICAM1-overexpressing DU145 cell line

Western blotting displayed that the protein level of RelB was markedly decreased after the transfection of shRNA-RelB (Figure 5A). Compared with the sictrl cells, ICAM1 protein was also significantly down-regulated in the siRelB cells (Figure 5A). We further verified that the concentration of sICAM1 and protein expression of ICAM1 in exosomes derived from the siRelB cells were both significantly lower than those from the sictrl cells (Figure 5D and 5E). To explore the effect of ICAM1 on DU145, we established ICAM1-overexpressing and control cell lines by plasmid transfection. As shown in Figures 6B and 6C, the expression of ICAM1 in clone 2 was significantly higher than that in other clones and control cells both at mRNA and protein levels, indicating that the ICAM1-

overexpressing cell line (clone-2, named hICAM1) and control (named hctrl) were successfully established. hICAM1 had an increased concentration of sICAM1, as well as protein and mRNA levels of exosomal ICAM1. The siRelB cells had the lowest expression level of ICAM1 ($p < 0.05$; Figures 5D-5F).

Carcinogenic characteristics of ICAM1 overexpressed DU145 cell line

xCELLigence real-time cell analysis system showed that the cell index of the hICAM1 cell was slightly higher than that of the control cell, and the difference was statistically significant after culturing for 56 h or more (Figure 6A). CCK-8 assay indicated that the viability of hICAM1 was mildly superior to hctrl ($p < 0.05$; Figure 6B). ICAM1 overexpression had no significant influence on the apoptosis of DU145 (Figure 6C). The proportions of early apoptotic cells in hctrl group were 1.203 ± 0.05 (%), 1.240 ± 0.03 (%) and 0.687 ± 0.04 (%) at 24 h, 48 h and 72 h; while those in hICAM1 group were 1.037 ± 0.04 (%), 1.240 ± 0.03 (%) and 0.657 ± 0.04 (%). We also found that the hICAM1 cells migrated and invaded markedly faster than the hctrl cells during the 24-h' successive monitoring (Figures 6D-6E).

To deeper understand the mechanism that ICAM1 overexpression strengthened the aggressiveness of DU145, the expression of metastasis-related proteins, such as integrin β -1, MMP9 (matrix metalloproteinase 9), and uPA (urokinase-type plasminogen activator) were determined by western blotting. Figure 7F displayed that the expression of integrin β -1 was increased in the hICAM1 cells. No significant differences were observed in the expression of MMP9 and uPA.

Thus, ICAM1 overexpression significantly reinforced the proliferation, migration, and invasion abilities of DU145 cells, indicating a more aggressive phenotype. The upregulated integrin β -1 participated in migration and invasion activities.

Discussion

It remains largely unclear how exosomes from tumor microenvironment regulate PC oncogenesis and progression. Previous studies suggest that RelB was positively correlated with the proliferation, migration, and invasion properties of the DU145 prostatic cell line. The silence of RelB leads to reduced cell growth, migration, and invasion abilities of DU145 cells. To this end, we have characterized the exosomal protein profile of RelB-knockdown cells. We discovered that 55 proteins were downregulated and 137 proteins were upregulated in the presence of RelB-knockdown, and the downregulated exosomal ICAM1 maybe a putative target of RelB in the regulation of PC proliferation and metastasis.

Secreted microvesicles were separated from DU145 conditioned media by ultracentrifugation. We verified those free vesicles as exosomes by electron microscopy, NanoSight analysis, and examination of the exosomal protein markers CD63, flotillin, and CD81. Based on the LC-MS technique and bioinformatics analysis, we characterized the proteins regulated by exosomes derived from the RelB knockdown DU145 cell line for the first time. 1,259 proteins were identified and 89.4% proteins can be retrieved in the Exocarta database. The canonical proteins in exosomes could be identified, such as heat shock proteins, cytoskeletal proteins, endosomal molecules, multivesicular tetraspanins, antigen presentation molecules, adhesion proteins, immunoglobulin family members, and so on [21]. Moreover, exosomal proteins specific for DU145 cells were detected, exemplified by integrin family members, CD276, ANXA2, CLSTN1, FASN, and FLNC [7]. These results demonstrated that exosomes from PC DU145 cells not only have similar features as exosomes from other cell types but also have specific characteristics.

According to the screening criteria, 192 DEPs were identified. Except for the 39 DEPs presenting in only one cell line, the FC of 153 DEPs varied from 1.286 to 47.986. The GO grouping system classified the proteins basing on their functional properties. Most of the DEPs originate from the cytoplasm, exosomes, and nucleus, manifesting the common biological processes, including signaling pathways, metabolism regulation, cell-cell interaction, cell-matrix interaction, and so on. Under the terms of biological process and molecular function of GO, as well as KEGG analysis, some DEPs were involved in cell-cell adhesion, cell adhesion molecule activity, and focal adhesion. Among the DEPs, ICAM1 attracted our attention. Further, we obtained important data showing the tight correlation between RelB and ICAM1 from the UALCAN website. Co-expression was the main pattern RelB interacting with ICAM1 based on the GeneMANIA tool. A kappa B-site had been identified in the promoter of the ICAM1 gene [22]. In summary, we speculated that ICAM1 was a target downstream of RelB.

ICAM1 was downregulated by 2.136 fold in exosomes derived from the siRelB cells. ICAM1 was annotated to origin from the plasma membrane and exosomes, was of transmembrane structure with an extracellular domain, belonging to the immunoglobulins adhesive molecules family. Exosomal ICAM1 was found to be regulated by the NF- κ B signaling through KEGG enrichment. In the previous study, we have discovered that the mRNA expression of ICAM1 in the siRelB cells was lower than that in the sictrl cells by high throughput screening of differentially expressed mRNA (data not published). We had also verified the lower protein expression of ICAM1 or exosomal ICAM1 in RelB-knockdown DU145 cells. The TCGA dataset indicated that both of ICAM1 and RelB expressed higher in tumor samples when compared to normal samples. Thus, we proposed that ICAM1 was positively regulated by RelB.

ICAM1 is a multifunctional transmembrane glycoprotein, regulated by multipath such as PKC, MAP, and NF- κ B signal pathways, and exerted positive or negative functions in cancer development. Compared to non-metastatic PC cell line P69, miR-296-3p was much higher in highly metastatic cell line M12, by targeting and inhibiting the expression of ICAM1 [23]. Biological studies showed that cells with higher expression of ICAM1 had enhanced abilities of cell proliferation, migration, and invasion. Importantly, ICAM1 existed both on plasma membranes and in exosomes, contributing together to the phenotypical heterogeneity. Yu et al found that the migration of prostate cancer cells was enhanced after bradykinin treatment by increasing ICAM1 expression, though signal transduction pathways induced by B2 receptor, AP-115, Akt, and PI3K [24]. Proteomic analysis of exosomes from nasopharyngeal carcinoma cells identified that the expression of ICAM1 was upregulated, which played a critical role in exosomes-induced angiogenesis. It has also been demonstrated that the internalization of exosomes could result in the alteration of ICAM1 expression in recipient HUVECs [25].

To further investigate the mechanism of strengthened migration and invasion upon ectopic expression of ICAM1 in DU145 cells, we detected the protein levels of integrin β -1, MMP9, and uPA. The protein level of integrin β -1 was up-regulated in ICAM1-overexpressing DU145 cells. In our previous study, we have verified that the RelB-knockdown suppressed the migration and invasion abilities of DU145 cells, likely due to the downregulated integrin β -1 [12]. Taken together,

integrin β -1 was likely involved in regulating the migration and invasion properties of DU145 cells, which was affected by the RelB-ICAM1 axis. Integrin β -1, a member of heterodimeric transmembrane cell surface receptors, plays a key role in regulating cell metastatic growth. Activation of β 1 integrin and the autophosphorylation induced by integrin of focal adhesion kinase in PCa cells correlated with metastatic potential in vivo [26]. Trop-2, a transmembrane molecule, could enhance the migratory and metastatic abilities of PC cells by up-regulating integrin β -1-dependent cell adhesion to fibronectin and signaling [27]. MMP9, a major component of the basement membrane, is a pivotal enzyme for degrading type IV collagen. uPA can degrade the components of the ECM, including laminin, fibronectin, and collagen. MMP9 and uPA had been reported to contribute to the metastasis of PC [28, 29]. However, ICAM1 had no effect on the protein expressions of MMP9 and uPA in DU145 cells in our study.

Conclusions

Our study identified 192 exosomal DEPs due to lacking RelB in DU145 cells. The RelB-knockdown led to an abundant reduction of exosomal ICAM1. ICAM1 was demonstrated to enhance the proliferation, migration, and invasion abilities of DU145 cells. Exosomal ICAM1 might be an important factor in facilitating PC progression. Here, we also provided rich proteomics data containing numerous individual proteins that were worth in-depth studies. Clinical samples were in need to validate the correlation between ICAM1 expression and other clinical indexes in PC.

Abbreviations

PC: prostate cancer; LC-MS: liquid chromatography-mass spectrometry; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; DEPs: differentially expressed proteins; ICAM1: intercellular adhesion molecule-1; NF- κ B: nuclear factor κ -light-chain-enhancer of activated B cells; TME: tumor microenvironment; MS: mass spectrometry; ATCC: American Type Culture Collection; STR: short tandem repeat; FBS: fetal bovine serum; CD: clusters of differentiation; DTT: dithiothreitol; TEAB: triethylamine-carbonic acid buffer; IAA: iodoacetamide; AGC: automatic gain control; HCD: higher-energy collisional dissociation; FDR: false discovery rate; iBAQ: intensity-based absolute quantification; FC: fold change; LFQ: label-free quantification; TCGA: the cancer genome atlas; PPI: protein-protein interaction; sICAM1: soluble ICAM1; CCK-8: cell counting kit-8; OD: optical density; MMP9: matrix metalloproteinase 9; uPA: urokinase plasminogen activator; HSP90: chaperones heat shock protein 90; HSP70: chaperones heat shock protein 70.

Declarations

Ethical approval

All procedures performed in studies involving human participants were following the Ethics Committee of Nanjing Medical University Affiliated Suzhou Hospital.

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Not applicable.

Conflict of interest statement

The authors have declared no financial/commercial conflicts of interest.

Data availability statement

The data used to support the findings of this study are included within the article, or available from the corresponding author upon request.

Consent for publication

All authors consent for publication.

Authors' contributions

FG and QS designed the research. WJL and JJX performed the research and analyzed the data. LC and LJZ supported for the bioanalysis of raw data. FG and WJL wrote the paper. All authors have read and approved the final version of this manuscript.

Author details

¹Department of Oncology, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215006. ²Department of Clinical Laboratory, Nanjing Medical University Affiliated Suzhou Hospital, Suzhou, Jiangsu 215002. ³Department of Clinical Laboratory, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215006. ⁴Department of Oncology, Shanghai East Hospital, Tongji University School of Medicine, Shanghai, 200123. ⁵Suzhou Institute of Systems Medicine, Chinese Academy of Medical Sciences & Peking Union Medical College, Suzhou, Jiangsu 215123. ⁶Department of Urology, Nanjing

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Tables

Table 1
Enrichment analysis of top 39 of 192 DEPs in siRelB vs sictrl

ID	Gene Name	Full Name	siRelB	sictrl	GO analysis			Expression in Prostate cancer	COSMI (prosta
					Cellular component	Molecular function	Biological process		
O15460	P4HA2	Prolyl 4-hydroxylase subunit alpha-2	+		Endoplasmic reticulum	Catalytic activity;	Metabolism;Energy pathways;Peptide metabolism;	No	Yes
O60271	SPAG9	C-Jun-amino-terminal kinase-interacting protein 4	+		Plasma membrane; Integral to membrane; Nucleus; Cytoplasm; Exosomes; Lysosome	unknown	Cell growth and/or maintenance	No	Yes
O75674	TOM1L1	TOM1-like protein 1	+		Golgi apparatus; Cytosol; Cytoplasm; Lysosome; Endosome	Receptor signaling complex scaffold activity	Cell communication; Signal transduction	Yes	Yes
O76071	CIAO1	Probable cytosolic iron-sulfur protein assembly protein CIAO1	+		Nucleus; Cytoplasm; MMXD complex	Transcription regulator activity	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	Yes	No
P02788	LTF	Lactotransferrin	+		Plasma membrane; Golgi aparatus; Nucleus; Nucleolus; Cytoplasm; Exosomes; Lysosome; Endoplasmic reticulum; Secretory granule; Extracellular; Stored secretory; others granule;	Transporter activity	Transport	Yes	Yes
P10768	ESD	S-formylglutathione hydrolase	+		Cytoplasm; Exosomes; Lysosome; Cytoplasmic membrane-bounded vesicle	Hydrolase activity	Metabolism; Energy pathways;	No	No
P15924	DSP	Desmoplakin	+		Plasma membrane; Nucleus; Nucleolus; Cytoplasm; Exosomes; Lysosome; Extracellular; Cytoskeleton; Cornified envelope	Structural constituent of cytoskeleton	Cell growth and/or maintenance	No	Yes
P17936	IGFBP3	Insulin-like growth factor-binding protein 3	+		Plasma membrane; Nucleus; Cytoplasm; Extracellular; Extracellular region; Extracellular space;	Growth factor binding	Cell communication; Signal transduction; Apoptosis	Yes	Yes
P27694	RPA1	Replication protein A 70 kDa DNA-binding subunit	+		Nucleus; Actin cytoskeleton; Cytoplasm; PML body; Nucleoplasm; Centrosome; DNA replication factor A complex;	DNA binding	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	Yes	Yes

P28070	PSMB4	Proteasome subunit beta type-4	+	Nucleus; Cytoplasm; Exosomes; Lysosome; Centrosome; Proteasome complex; Intermediate filament cytoskeleton;	Ubiquitin-specific protease activity	Protein metabolism	Yes	No
P28074	PSMB5	Proteasome subunit beta type-5	+	Nucleus; Cytosol; Cytoplasm; Exosomes; Proteasome complex;	Ubiquitin-specific protease activity	Protein metabolism	No	No
P34096	RNASE4	Ribonuclease 4	+	Secretory granule; Extracellular; others	Ribonuclease activity	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	No	No
P50579	METAP2	Methionine aminopeptidase 2	+	Cytoplasm; Exosomes; Lysosome; Cytoplasmic membrane-bounded vesicle	Translation regulator activity	Protein metabolism	Yes	Yes
P53999	SUB1	Activated RNA polymerase II transcriptional coactivator p15	+	Nucleus; Nucleolus; Mitochondrion; Cytoplasm; Exosomes; Centrosome; Transcription factor complex;	Transcription factor activity	Protein metabolism	Yes	Yes
P62834	RAP1A	Ras-related protein Rap-1A	+	Plasma membrane; Nucleus; Mitochondrion; Cytosol; Cytoplasm; Exosomes; Perinuclear region; Lysosome; Late endosome;	GTPase activity	Cell communication; Signal transduction	Yes	No
Q06124	PTPN11	Tyrosine-protein phosphatase non-receptor type 11	+	Plasma membrane; Cytosol; Cytoplasm;	Protein tyrosine phosphatase activity	Cell communication; Signal transduction	Yes	No
Q08J23	NSUN2	tRNA (cytosine(34)-C(5))-methyltransferase	+	Nucleus; Nucleolus; Cytoplasm;	RNA methyltransferase activity	unknown	No	No
Q13564	NAE1	NEDD8-activating enzyme E1 regulatory subunit	+	Cytoplasm; Lysosome; Insoluble fraction	Receptor signaling complex scaffold activity	Cell communication; Signal transduction	No	No
Q14160	SCRIB	Protein scribble homolog	+	Plasma membrane; Cytoplasm; Exosomes; Cell-cell junction; Cell junction; Cell-cell adherens junction; Synapse; Cell leading edge; Postsynaptic membrane; Presynaptic membrane; Basolateral membrane; Scrib-APC-beta-catenin complex;	Ubiquitin-specific protease activity	Protein metabolism	No	Yes
Q15257	PTPA	Serine/threonine-protein phosphatase 2A activator	+	unknown	unknown	unknown	No	No
Q5T749	KPRP	Keratinocyte proline-rich protein	+	Exosomes; Cytoskeleton;	unknown	Cell differentiation	No	Yes

Q8NBJS	COLGALT1	Procollagen galactosyltransferase 1	+		Exosomes;	unknown	unknown	No	Yes
Q8IUR6	CREBRF	CREB3 regulatory factor	+		Nucleus;	DNA binding	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	No	Yes
Q8NFZ4	NLGN2	Neurologin-2	+		Plasma membrane; Membrane; Postsynaptic membrane;	Binding	Cell communication; Signal transduction	No	No
Q99574	SERPINI1	Neuroserpin	+		Cytoplasmic vesicle	Protease inhibitor activity	Protein metabolism	Yes	Yes
Q9HB63	NTN4	Netrin-4	+		Extracellular;	Extracellular matrix structural constituent	unknown	No	Yes
O15118	NPC1	Niemann-Pick C1 protein	+		Plasma membrane; Integral to membrane; Integral to plasma membrane; Perinuclear region of cytoplasm; Cytoplasm; Exosomes; Lysosome; Endoplasmic reticulum; Membrane; Nuclear envelope;	Receptor activity	Cell growth and/or maintenance	Yes	Yes
P23588	EIF4B	Eukaryotic translation initiation factor 4B	+		Cytosol; Ribosome; Cytoplasm; Eukaryotic translation initiation factor 4F complex	Translation regulator activity	Protein metabolism	Yes	No
P52888	THOP1	Thimet oligopeptidase	+		Plasma membrane; Cytoplasm; Extracellular;	Metallopeptidase activity	Protein metabolism	Yes	No
P61221	ABCE1	ATP-binding cassette sub-family E member 1	+		Mitochondrion; Cytoplasm; Centrosome;	Transporter activity	Transport	Yes	Yes
P62081	RPS7	40S ribosomal protein S7	+		Ribonucleoprotein complex; Nucleolus; Cytosolic small ribosomal subunit; Cytosol; Ribosome; Exosomes; Lysosome; Centrosome;	Structural constituent of ribosome	Protein metabolism	No	Yes
P98095	FBLN2	Fibulin-2	+		Extracellular; Proteinaceous extracellular matrix;	Extracellular matrix structural constituent	Cell growth and/or maintenance	No	Yes
Q6UVK1	CSPG4	Chondroitin sulfate proteoglycan 4	+		Plasma membrane; Integral to plasma membrane; Exosomes; Cell surface; Membrane;	Protein binding	Cell communication; Signal transduction	Yes	Yes
Q9BR76	CORO1B	Coronin-1B	+		Nucleus; Cytoplasm; Exosomes; Cytoskeleton;	unknown	Signal transduction	Yes	No
Q9NY26	SLC39A1	Zinc transporter ZIP1	+		Plasma membrane; Integral to	Auxiliary transport protein activity	Transport	Yes	Yes

				membrane; Golgi apparatus; Membrane fraction; Lysosome; Endoplasmic reticulum;				
Q9UNS2	COPS3	COP9 signalosome complex subunit 3	+	Cytoplasm; Lysosome; Insoluble fraction	Transcription regulator activity	Cell communication; Signal transduction	Yes	Yes
P01857	IGHG1	Immunoglobulin heavy constant gamma 1	+	unknown	unknown	unknown	No	No
P04818	TYMS	Thymidylate synthase	+	Nucleus; Nucleolus; Mitochondrion; Cytoplasm;	Ligase activity	Metabolism; Energy pathways	Yes	No
P51693	APLP1	Amyloid-like protein 1	+	Plasma membrane; Golgi apparatus; Perinuclear region of cytoplasm; Cytoplasm; Perinuclear region; Endoplasmic reticulum; Extracellular; Basement membrane;	Transcription regulator activity	Cell communication; Signal transduction; Synapse organization and biogenesis	Yes	Yes
Note: "+" represents the upregulated expression.								

Figures

Figure 1

Quality control of exosome samples. (A) Electron microscopy analysis of exosomes secreted by DU145 cells. Scale bar, 200 nm. The exosome was indicated by the arrow. (B) The particle size of the vesicles secreted was measured by NanoSight analysis. (C) Exosome protein markers CD63, flotillin, and CD81 were measured by western blot analysis. (D) Record of the exosomal proteins secreted by DU145 in the ExoCarta database.

Figure 2

Total proteome expression analysis. (A) Venn diagram of proteins in siRelB versus siCtrl exosomes. (B) The percentages of the top 25 exosomal proteins in siCtrl and siRelB cells, respectively.

Figure 3

DEPs expression analysis. (A) 192 DEPs expression analysis by hierarchical clustering. (B) 153 DEPs expression analysis by volcano plot. Red and green dots indicated up-regulated and down-regulated proteins, respectively. (C) Heat map analysis of 39 DEPs expressed only in one type of cell.

Figure 4

The correlation between RelB gene and ICAM1 gene in PC (UALCAN). (A) Heatmap showing the top 24 genes positively correlated with RelB in PC. The expression level of genes was shown as log2(TPM+ 1). (B) Scatter plot depicting the correlation between RelB and ICAM1 by Pearson correlation analysis, the Pearson cc was 0.71.(C) The comparison of RelB expression in normal samples and tumor samples by UALCAN database ($p=9.023700E-03$). (D) The comparison of ICAM1 expression in normal samples and tumor samples by UALCAN database ($p=9.963900E-04$). (E) The interaction network between RelB and ICAM1 in PC (GeneMANIA).

Figure 5

RelB and ICAM1 expression in DU145 cells or exosomes. (A) Protein expression of RelB and ICAM1 in siCtrl and siRelB by western blotting. (B) The relative expression of ICAM1 mRNA in hCtrl and different ICAM1-overexpressing clones by RT-qPCR. (C) Protein expression of ICAM1 in hCtrl and different clones by western blotting. (D) The concentration of sICAM1, (E) protein, and (F) mRNA expression of ICAM1 in exosomes derived from siCtrl, siRelB, hCtrl, and hICAM1 cell lines by ELISA, western blotting, and qRT-PCR.

Figure 6

Carcinogenic characteristics of ICAM1 overexpressing DU145 cell line. (A) Cell growth assessment by the xCelligence RTCA instrument. (B) Cells viability evaluation by CCK-8 assay. (C) Early apoptotic cell proportion assessed by annexin V/PI staining. (D) Migration and (E) Invasion abilities assessed by the xCelligence RTCA instrument. (F) Protein expressions of integrin β -1, MMP9, and uPA in hctrl and hICAM1 by western blotting.

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