Exosomal PD-L1 Derived From Osteosarcoma is Associated With Pulmonary Metastasis for Patients With Osteosarcoma

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Research

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Abstract

Background

Recent studies indicated that exosomal programmed death-ligand 1 (PD-L1) derived from cancers could induce immunosuppression and tumor pathogenesis. However, it is unclear how the exosome influences the osteosarcoma (OS) progression and whether PD-L1 also exists in serum exosomes (Sr-exosomes) of patients with osteosarcoma.

Methods

We examined serum exosomes from 70 patients with osteosarcoma, 9 patients with benign tumors and 22 healthy donors. Osteosarcoma-derived exosomes and exosomal-PD-L1 were functionally evaluated using *in vivo* and *in vitro* models.

Results

The characteristics of serum exosomes of OS patients and OS cell line exosomes were confirmed by several methods. Bioinformatic analysis demonstrated that Sr-exosomes isolated from OS patients may involve in the important process of immune function and cancer pathogenesis for OS patients. Meanwhile, exosomes derived from OS cell line and serum of OS patients could induce osteosarcoma migration and invasion *in vitro*. Then, we confirmed PD-L1 existing in Sr-exosomes of patients with osteosarcoma and higher level of Sr-exosomal PD-L1 in OS patients compared to healthy donors and patients with benign tumor. Furthermore, exosomal-PD-L1 derived from osteosarcoma promoted the pulmonary metastasis, which was demonstrated in the osteosarcoma metastatic model.

Conclusion

We confirm that osteosarcoma releases the exosomes carrying PD-L1 on their surface and induces the pathogenesis of pulmonary metastasis for OS patients.

Background

Exosomes are secreted as the vesicles of a size range of 30 to 150 nm diameter, which are enclosed by the cell membrane. Inward budding of late endosomes develop into intracellular multivesicular endosomes and RNA, DNA, proteins and other small molecules are encapsulated into exosomes during the formation of exosome[1, 2]. Various types of cells such as immune cells, fibroblast and endothelial cells, release exosomes into the extracellular space and microenvironment, involving the tumor pathogenesis[3, 4]. Recent studies reported that tumor cell-derived exosomes stimulated cancer and sarcoma cell growth, survival, metastasis, angiogenesis and chemotherapy resistance[5–11]. Meanwhile,

for the immunosuppression, exosomes isolated from cancer cells can evade host immune surveillance, modulate tumor microenvironment and initiate the formation of premetastatic sites[12, 13].

As a major malignant bone tumor, osteosarcoma commonly occurs in children and young adults with metastasis occurring in approximately 15–20% of patients[5]. Neo-adjuvant chemotherapy has increased the 5-year survival of patients with localized tumors to 60–80%. Otherwise, patients with metastatic disease remain refractory to chemotherapy and have a 5-year survival of only 10–20%[5, 14, 15]. Thus, improvement for therapy of patients with pulmonary metastasis is a challenge for oncologists. Exosomes is reported to involve the process of pulmonary metastasis and can be utilized as a biomarker to monitor the tumor progression[16–18]. It has been reported that osteosarcoma has also been shown to generate exosomes, which may play an important role in tumor progression and metastasis[19–22]. Recent studies indicate that exosomal programmed death-ligand 1 (PD-L1) derived from cancers can induce immunosuppression and tumor pathogenesis[23–25]. However, it is unclear how the exosome influence the osteosarcoma progression and whether PD-L1 also exists in serum exosome of patients with osteosarcoma. Therefore, it is urgent to elucidate the exact mechanism of exosomes and exosomal PD-L1 in osteosarcoma pathogenesis.

Materials And Methods

Clinical characteristics of patients with osteosarcoma

The clinical cohort from the bone tumor center of Peking University People's Hospital included 70 patients with osteosarcoma and 9 patients with benign tumor. Patients were subjected to surgery between 2018 and 2019 at our tumor center. 22 healthy donors were included in the analysis. Fasting blood of 79 patients (70 osteosarcomas and 9 benign tumors) and 22 healthy donors was collected in plasma separator vacutainer tubes. All clinical samples were collected with written informed consent from patients and healthy donors, and the ethical approval was granted from the Committees for Ethical Review at our hospital. Clinical information included age, gender, side, location, tumor grade, treatment with neoadjuvant chemotherapy, pulmonary metastasis.

Cell Lines Culture

The human HOS, KHOS, U₂OS and 143B cell lines were preserved in our laboratory. The cells were repeatedly cultured in RPMI 1640 with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified cell incubator with an atmosphere of 5% CO₂ at 37 °C. Cells growing at an exponential rate were used for the experiments.

Generation Of Stable Rab27a And Pd-l1 Knockdown 143b Cells

Lentiviruses containing shRNA targeting human Rab27a or PD-L1 (Hanheng Biotechnology, Shanghai, China) were transfected into 143B cells to knock down endogenous Rab27a or PD-L1. Short hairpin RNAs (shRNAs) against human Rab27a (Top strand:

GATCCGCAAGCAATTGAGATGCTTCTGGATTCAAGAGATCC

AGAAGCATCTCAATTGCTTGGCTTTTTTG; Bottom strand: AATTCAAAAAAG
CCAAGCAATTGAGATGCTTCTGGATCTCTTGAATCCAGAAGCATCTCAATTGCTTGGCG) or human PD-L1 (Top strand: GATCCGCGAATTACTGTGAAAGTC

AATTTCAAGAGAATTGACTTTCACAGTAATTCGTTTTTTTG; Bottom strand:
AATTCAAAAACGAATTACTGTGAAAGTCAATTCTCTTGAAATTGACTTTCACAGTAATTCGCG) was packaged into lentiviral particles using 293T cells co-transfected with the viral packaging plasmids. Lentiviral supernatants were harvested 48 h after transfection. 143B cells were infected with filtered lentivirus and selected by 1 µg/ml puromycin.

Exosome Isolation

Cells were subsequently cultured with FBS-free DMEM medium for 72 h. Conditioned culture media was collected. Exosomes were isolated from the conditioned culture media by four successive centrifugation steps as the following steps. Culture media samples were centrifuged at 300 g for 10 min at 4 °C to discard floating cells. Supernatants were centrifuged at 800 g for 30 min, and 10,000 g for 30 min to further purify the ascites. Then 10 ml ascites was diluted in 10 mL PBS and filtered by a 0.22 µm filter. After that, the samples were centrifuged at 150,000 g for 2 h at 4 °C. The supernatant was discarded, and the exosome pellet was re-suspended in PBS. The samples were centrifuged at 150,000 × g 4 °C for 2 h in a last step. The final pellets of exosomes were stored at -20 °C.

For iodixanol density gradient centrifugation, exosomes harvested by differential centrifugation were loaded on top of a discontinuous iodixanol gradient (5%, 10%, 20% and 40%, made by diluting 60% OptiPrep aqueous iodixanol with 0.25 M sucrose in 10 mM Tris) and centrifuged at 100,000 g for 18 h at 4 °C (Beckman Coulter). The exosomes distributed at the density range between 1.13 and 1.19 g/ml, as previously demonstrated. The exosomes were further pelleted by ultracentrifugation at 100,000 g for 2 h at 4 °C[23, 24].

Characterization Of Purified Exosomes

For transmission electron microscopy (TEM) analysis, the exosome pellet resuspending solution was loaded onto formvar/carbon-coated EM grids, fixed with 2% paraformaldehyde (PFA), and stained with 1% aqueous uranyl acetate, then dried at room temperature. The samples were examined using a device TEM. For nanoparticle tracking analysis (NTA), vesicle enriched suspension with concentrations between 1×10^7 /mL and 1×10^9 /mL was examined using the ZetaView PMX 110 (Particle Metrix, Meerbusch,

Germany) equipped with a 405 nm laser to determine the size and quantity of particles isolated. A video of 60-sec duration was taken with a frame rate of 30 frames/sec, and particle movement was analyzed using NTA software.

Exosome Protein Quantification And Western Blot Analysis

The concentration of exosomal total protein was quantified by the bicinchoninic acid (BCA) assay (Thermo Fisher Inc.) using bovine serum albumin (BSA) as standard. The concentration of exosomal total protein in the serum was analyzed between six healthy donors and six patients with osteosarcoma. The pellets were also dissolved in 200 µL RIPA buffer for protein assay. The samples were individually homogenized in 5 mM Tris-HCl (4 mM EDTA, pH 7.4, containing 1 M pepstatin, 100 M leupeptin, 100 M phenylmethyl sulfonylfluoride, and 10 g/ml aprotinin) and cleared by centrifugation at 14,000 g for 10 min at 4 °C. Approximately 100 ug of protein were run on a discontinuous SDS-PAGE gel and transferred to a nitrocellulose membrane. The membranes were blocked with 5% skim milk in TBS containing 0.05% Tween 20 and were incubated with the following primary antibodies: (1) Mouse anti-CD9 (sc-13118, Santa Cruz, CA, USA), (2) Mouse anti-CD63 (sc-5275, Santa Cruz, CA, USA), (3) Mouse anti-TSG101 (sc-7964, Santa Cruz, CA, USA), (4) Mouse anti-Alix (sc-53540, Santa Cruz, CA, USA), (5) Mouse anti-calnexin (10427-2-AP, Promega, Madison, WI), (6) Mouse anti-PD-L1 (sc-293425, Santa Cruz, CA, USA) and Mouse anti-GAPDH (sc-47724, Santa Cruz, CA, USA). The optical density (OD) of the signals was quantified and expressed as the ratio of the tested proteins to GAPDH for analysis of protein in cells and to ALIX for analysis of protein in exosomes.

Comparision of RNA quantification in Sr-exosomes between patients with OS and healthy donors

Total RNA was isolated from serum exosomes of patients with osteosarcoma and healthy donors using TRIzol Reagent (Invitrogen) and the concentration of exosomal RNA in the serum was analyzed between six healthy donors and six patients with osteosarcoma.

Quantitative Pcr (qpcr)

Total RNA was isolated from 143B-WT and 143B-Rab27a-KD using TRIzol Reagent (Invitrogen), and reverse transcribed into first-strand complementary DNA (cDNA) with random primer with RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific). The samples were then analysed in an Applied Biosystems QuantStudio 3 Real-Time PCR system. GAPDH was used as an internal control.

Immunohistochemistry Analysis

Paraffin sections were incubated with the corresponding antibodies and stained with nonimmune serum in PBS instead of the primary antibody as the negative control. Based on the average percentage of positive cells calculated from at least 10 representative fields, positive staining was defined as a positive

cell percentage $\geq 10\%$. Staining intensity was classified as follows: 0, no staining or staining in $< 10\%$ of tumor cells; 1+, weak to moderate staining in 10 to 20% of tumor cells; 2+, strong staining in 10 to 20% of tumor cells or weak staining in 20 to 50% of tumor cells; 3+, moderate to strong staining in 20 to 50% of tumor cells or staining in 50% of tumor cells. The immunostaining assessment was conducted by two independent pathologists without any previous knowledge of the clinical characteristics and outcomes[26]. The primary antibodies were as follows: (1) Mouse anti-PD-L1 (sc-293425, Santa Cruz, CA, USA), (2) Rabbit monoclonal anti-E-cadherin (ab194982), (3) Rabbit polyclonal anti-N-cadherin (ab18203), (4) Rabbit monoclonal anti-vimentin (ab92547).

Immunogold Labeling

Fixed specimens at an optimal concentration were placed onto a 400 mesh carbon/formvar coated grids and allowed to absorb to the formvar for a minimum of 1 minute. The grids were placed into a blocking buffer for a block/permeabilization step for 1 hour. Without rinsing, the grids were immediately placed into the primary antibody at the appropriate dilution overnight at 4 °C (Mouse anti-PD-L1, sc-293425, Santa Cruz, CA, USA). As controls, some of the grids were not exposed to the primary antibody. The following day, all the grids were rinsed with PBS then floated on drops of the appropriate secondary antibody attached with 10 nm gold particles (AURION, Hatfield, PA) for two hours at room temperature. Grids were rinsed with PBS and were placed in 2.5% Glutaraldehyde in 0.1M Phosphate buffer for 15 min. After rinsing in PBS and distilled water the grids were allowed to dry and stained for contrast using uranyl acetate. The samples were viewed with a Tecnai Bio Twin transmission electron microscope (FEI, Hillsboro, OR) and images were taken with an AMT CCD Camera (Advanced Microscopy Techniques, Danvers, MA).

Bioinformatics analysis of Sr-exosomes from 20 OS patients and 6 healthy donors

Exosomal RNA was extracted using RNeasy® Mini kit (Qiagen, cat. No. 217004) according to the manufacturer's instructions. RNA quality was assessed by using UV 260/280. The pellets were also dissolved in 1 ml Trizol LS reagent (Invitrogen) for RNA isolation. Then, we used NGS to get sequencing data of mRNA in different Sr-exosomes from 20 patients with osteosarcoma and 6 healthy donors. These sequencing data were analyzed by bioinformatics method. Differentially expressed genes were identified based on RVM t test and false discovery rate (FDR) analysis. Differentially expressed genes with at least 1.2-fold change in either direction with $P < 0.05$ were considered to be up or downregulated. Based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, significantly changed pathways were identified. GO analysis was used to organize differentially expressed genes into hierarchical categories.

Microarray Analysis Of Exosomes Isolated From 143b And Uos

Exosomal RNA of 143B and U₂OS was extracted using Affymetrix Pico Kit and purified with an RNeasy mini kit (Qiagen GmbH, Hilden, Germany). cDNA was synthesized with One-Cycle Target Labeling and Control Reagents, and cRNA was created using a GeneChip IVT Labeling kit (both from Affymetrix; Thermo Fisher Scientific, Inc.). cRNA was fragmented, and then hybridized to an Affymetrix Human Clariom D Array (Affymetrix; Thermo Fisher Scientific, Inc). GeneChips were washed and stained in the Affymetrix Fluidics Station 450. All arrays were scanned using the Affymetrix GeneChip Command Console, which was installed in the GeneChip Scanner 3000 7G. The data were analysed with the Robust Multichip Analysis (RMA) algorithm using the Affymetrix default analysis settings and global scaling as a normalization method. Values presented are log₂ RMA signal intensities. Differentially expressed mRNA were identified using the Student's t-test for comparison of 143B (high metastatic ability) and U₂OS (low metastatic ability)[18]. Genes were considered differentially regulated between the two groups when there was > 2-fold difference in expression with $P < 0.05$. Differentially expressed mRNA with at least a 2-fold change in either the positive or negative direction were respectively considered up- or downregulated.

Exosome labeling and osteosarcoma cell uptake in vitro

To examine whether Sr-exosomes derived from the patient with osteosarcoma can be taken up by osteosarcoma cell lines, 143B and U₂OS, PKH26 (Sigma-Aldrich, PKH26GL) was used to label exosomes. The exosomes or PBS were stained with PKH26 dye in 400 μ l of Diluent C for 4 min at room temperature. Then an equal volume of 1% BSA were used to stop the labeling reaction, after which they were washed with PBS and ultracentrifuged again. The labeled exosomes or PBS were respectively incubated with 143B and U₂OS cells with complete medium for four hours at 37 °C in an atmosphere of 5% CO₂. Then the cells were washed three times in PBS to eliminate the influence of serum exosomes. The cell nuclei was counterstained with DAPI for 8 min and cell membrane were counterstained with CFSE (Sigma-Aldrich) for 5 min. The uptake of the labeled exosomes by 143B and U₂OS cells was assessed using an inverted confocal microscope.

Cell Counting Kit 8 Assay And Transwell Assay

For the assessment of serum exosomes of patients with OS on 143B and U₂OS cell proliferation, cells were seeded at a density of 2000 cells per well in 96-well plates. The cells were treated with different exosome concentration of 10, 20, 30, 40 μ g/ml for 24, 48 and 72 hours. Cell proliferation was analyzed using Cell Counting Kit 8 (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol.

For the evaluation of serum exosomes of patients with OS and healthy donors on 143B and U₂OS migration and invasion, cells (6×10^4) were respectively seeded into the non-coated upper chamber of transwell plates (8 mm pore size; Corning) for a migration assay and into matrigel coated upper chamber (BD Bioscience, 354234) for an invasion assay with 50 μ g/ml exosomes. For the evaluation of exosomes derived from osteosarcoma on 143B and U₂OS migration and invasion, the same procedure as above

mentioned was performed with 10 ug/ml and 50 ug/ml. After culturing for 24 h, cells were fixed with methanol and stained with a 0.1% crystal violet solution. Migrated cell populations were evaluated in five fields per well under a microscope.

Wound-healing Assay

Confluent 143B cell monolayers were scratched with a sterile 100- μ l pipette tip and treated with or without GW4869 at a dose of 1ug/ml. Cell migration was monitored for 24 and 48 hours under a microscope. The widths of the 'wound' (scratched areas) were measured and the proportion of wound healing was calculated by the following formula: $100\% - (\text{width after 24 h} / \text{width at the beginning}) \times 100\%$.

ELISA analysis of Sr-exosomal PD-L1 expression for patients with OS

For detection of PD-L1 on exosomes, the serum from patients was analyzed by ELISA. Exosomes were isolated as above mentioned and then resuspended in 100 ul PBS. 50 ul suspense was analyzed as the schematic diagram shown in Figure-4N. Equilibrate all materials and prepared reagents to room temperature prior to use. All standards, controls and samples were analyzed in duplicate. The protocol was followed as the instruction (ab214565, Human PD-L1 SimpleStep ELISA® Kit). Calculate the average absorbance value for the blank control (zero) standards. Subtract the average blank control standard absorbance value from all other absorbance values. Create a standard curve by plotting the average blank control subtracted absorbance value for each standard concentration (y-axis) against the target protein concentration (x-axis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve. Determine the concentration of the target protein in the sample by interpolating the blank control subtracted absorbance values against the standard curve. Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample. Highest standard should be further diluted and reanalyzed. Similarly, samples which measure at an absorbance values less than that of the lowest standard should be retested in a less dilute form.

Pulmonary Metastatic Model Study

All protocols were approved by the animal care committee of the Peking University Health Science Center. We have minimized the number of animals used and their suffering.

Experiment NO 1 *in vivo*, to evaluate the effect of 143B-derived exosomes and inhibitor of exosome secretion GW4869 on pulmonary metastasis, 143B cells were injected into tail vein of six-week-old female BALB/c nude mice (Vitalriver, Beijing, China) in a volume of 10 μ L (50,000 cells) as the primary injection. Administration of PBS, GW4869 (at a dose of 2.5 mg/kg, from day 3 to day 21, once three days, eight times in total. Sigma-Aldrich, cat. D1692) and 143B-derived exosome (at a dose of 10ug per mouse, from day 3 to day 21 once three days, eight times in total) as secondary injection were performed after primary

injection. Mice were euthanized at four weeks after the secondary injection. Experiment NO 2 *in vivo*, the model and treatment were followed as the flowchart indicated in Figure-6H. The mice were sacrificed at four weeks after secondary injection. The lung specimen were stored at -80°C for experiments.

Haematoxylin And Eosin Staining

Mice were sacrificed at four weeks (Experiment NO 1 and 2 *in vivo*) after secondary injection and the lung tissue were dissected. Osteosarcoma specimen of patients were stored at -80°C after resection. The tissue was fixed in 10% formalin at room temperature overnight and consequentially dehydrated in 70% ethanol. Samples were mounted in paraffin blocks and sectioned at 5 mm of thickness. Slides were baked at 60°C for 15 minutes, then treated with Xylene Substitute (Citrisolv) (Fisher chemical cat. x3p-1GAL) then rehydrated and stained with Hematoxylin, followed with washes with running water. An incubation with Acid Alcohol (Sigma, cat. A3429) was performed, followed with washes with running water. The samples were then stained with eosin (Thermo Scientific) then dehydrated with ethanol followed by Xylene Substitute (Citrisolv). Samples were mounted with Permount (Fisher Scientific, cat. SP15-500). Slides were submitted for pathologic evaluation. Pathologist was blinded to the sample.

The mouse weight was recored for analysis and lungs were collected after H&E staining. Metastasis was measured by visually counting the number of metastatic nodules, maximum diameter per metastatic nodule and cross-section area per metastatic nodule in the entire mouse lung section for each mouse, using Nikon NIS-Elements software (Nikon Corporation Instruments, Tokyo, Japan).

Results

Confirming the characteristics of serum exosomes (Sr-Exo) of OS patients and OS cell line exosomes

The plan for analysis of patients with OS and benign tumor and healthy donors was shown in the flowchart (Figure-1A). The exosome fraction was characterized by transmission electron microscopy (TEM) and nanoparticle tracking system for morphology, concentration and size, as well as by western blot analysis for exosome associated biomarkers. As nanoparticle tracking analysis was shown in Figure-1E & Figure-2A, the size distribution of these Sr-Exo of OS patients and OS cell line-derived exosomes were mostly around 100 nm. TEM showed that the extracellular vesicle (EV) fraction, which was derived from OS patient serum and osteosarcoma cells, contained typical cup-shaped vesicles which was exosomes (Figure-1C, 2B). Exosomes from 143B and U₂OS cell lines and OS patient serum are positive for the representative biomarkers (i.e. Tsg101, Alix, CD63 and CD9) in western blot analysis that are among the top 100 most frequently detected exosomal proteins (shown at website, www.exocarta.org) (Figure-1D, 2C). In summary, the above mentioned experiments confirmed EV extracted from the cell line media and clinical samples as the exosome.

Bioinformatic analysis global mRNA expression of Sr-exosome in osteosarcoma patients and healthy donors

This study was designed to progress the global mRNA screening included the serum samples from 20 osteosarcoma patients and 6 healthy volunteers. The clinical characteristics of 20 patients with OS was shown in Table-1. Our research provided a new insight into RNA sequence in exosomes and may make a little contribution to the understanding of exosomal mRNA composition in patients with OS. We found parts of differential expression of RNAs, significant KEGG pathways and GO enrichment in exosomes between patients with OS and healthy donors.

Volcano plots of the differentially expressed genes in the Sr-exosome of six healthy donors and twenty patients with OS, $\log_2$ fold change > 1 with statistical significance ($P < 0.05$) detected by RNA-seq using NGS. Significantly upregulated and downregulated genes were colored in red and green, respectively. X axis: $\log_2$ fold change of gene expression. Y axis: statistical significance of the differential expression in the scale of $-\log_{10}$ (p value) (Figure-1F).

Bioinformatic analysis revealed 248 mRNAs as exclusively significant expression in the Sr-exosomes derived from OS patient than that from the healthy donors (Table-S2). Heatmap of differential mRNA expression showed 75 Sr-exosomal mRNAs were identified to be highly expressed in OS patients compared to healthy donors and 173 Sr-exosomal mRNAs were low expression in patients with OS compared with healthy donors (Figure.1I). KEGG pathways analysis revealed that which was enriched in mRNA. The number of according mRNAs was labeled for each pathway. It revealed that several mRNAs in Sr-exosome derived from OS patients were enriched in the cancer-associated and immune-associated pathways. The cancer-associated pathways included FOXO signaling, Jak-STAT signaling, MAPK signaling, PI3K-Akt signaling, Ras signaling and TNF signaling pathways. Meanwhile, the cancer-associated and immune-associated pathways contained antigen processing and presentation, B cell receptor signaling, chemokine signaling, Fc-gammaR-mediated cytotoxicity and T cell receptor signaling pathways (Figure.1H). Differentially expressed mRNAs in Sr-exosome of six donors and twenty OS patients involved in pathways related to cancer and ones related to immune function were plotted in a Venn diagram. Co-expression genes included IL7R, PAK2, NFKBIA, PF4V1, PIK3R5, TNFAIP3, MCL1, CCL14, TNFRSF1B, IL4R, WAS, CSF3R, TCF7, PXN, GRB2 (Fig. 1G, Table.S3&4). These results illustrated that Sr-exosomal mRNAs derived from OS patients may participate in the osteosarcoma pathogenesis, especially the immune process in the microenvironment.

We also found the different expressed mRNAs in the Sr-exosome between OS patients and healthy donors, likely FLOT2, is related to the vesicle genesis. Venn diagram showed FLOT2 as the co-expression genes between Sr-exosome DEG (different expression genes, OS patients vs healthy donors) and gene of associating with exosome biogenesis (Figure-1J). A list of genes identified from the literature with known function in exosome biogenesis[27]. Reviewing the recent literature, we have listed genes with known functions in vesicular trafficking and secretion, like the ESCRT components TSG101, MVB12, VPS36 and PDCD6IP (ALIX), and of typical extracellular vesicle markers (e.g., FLOT1, FLOT2 and the tetraspanins

CD9, CD63 and CD81) was assembled (Table. S1). GO analysis demonstrated that Sr-exosomes of OS patients involved in the process of immune function during osteosarcoma pathogenesis, which was pointed by arrowhead shown in Figure-1K.

Exosomes derived from OS cell line and patient serum induces osteosarcoma migration and invasion

We first tested if exosomes derived from OS cell line and patient serum were internalized to the recipient 143B and U₂OS cells. 143B and U₂OS cells respectively labeled with CFSE (green) were treated with red PKH26-labeled exosomes isolated from 143B cell line and patient serum. Immunofluorescent microscopy analysis revealed the presence of PKH26 labeled exosomes from 143B cells or patient serum exclusively in the cytoplasm of 143B and U₂OS cells, especially around the perinuclear region (Figure-2D). Thus, the recipient cells were inundated with the exogenous exosomes. These results indicated that osteosarcoma cells can internalize exosomes derived from osteosarcoma cell.

Cells viability were examined by using Sr-exosomes derived from OS patient and healthy donor in different concentration to treat 143B and U₂OS cells for 24, 48 and 72 hours. Sr-exosomes derived from OS patients didn't affect the viability at low and high doses of exosomes compared to ones derived from healthy donors (Figure-2E, $P > 0.05$). Next, we determined the effect of exosomes isolated from patient/healthy donor serum and OS cell line on the migration and invasion of 143B and U₂OS. Firstly, we detected the effect of the exosomes isolated from serum of 3 OS patients on the osteosarcoma cell lines. The purified Sr-Exo from the OS patient significantly increased cell migration and invasion compared to Sr-Exo of the healthy donor (Figure-2F, $P < 0.05$). Then likewise, the purified exosomes from the media of 143B cells statistically induced cell migration and invasion in a dose-dependent manner for 143B and U₂OS cells compared to control (exosome-depleted liquid group) (Figure-2G, $P < 0.05$).

Osteosarcoma exosome promotes pulmonary metastasis and pharmacological inhibitor of exosome secretion GW4869 decreases OS metastasis in metastatic mouse model

To reveal the effect of GW4869, a small molecule of exosome secretion inhibitor, on the migration of 143B cells *in vitro*, it showed that GW4869 could not influence the migration of 143B cells (Figure-3A-C). Whereas nanoparticle tracking analysis revealed that the concentration of exosomes produced by 143B cells treated with GW4869 was lower than that of control group (Figure-3D). Next, we determined the effects of exosome derived from osteosarcoma cells and GW4869 on OS metastasis in the metastatic model (Figure-3E). Gross specimens of lungs in three groups was shown in Figure-3F. Treatment of 143B tumor-bearing mice with GW4869 significantly reduced pulmonary metastasis nodules and metastatic lesion area *in vivo* compared to control group ($P < 0.05$). Meanwhile, injection with exosomes derived from 143B could increase metastatic lesion nodules and area compared to control and GW4869 treated groups ($P < 0.05$) (Figure-3G, J & K). However, we didn't find the significant difference of maximum diameter among GW4869, exosome derived from osteosarcoma cells and control (Figure-3I). Furthermore, the upregulation of E-cadherin and downregulation of N-cadherin and vimentin was observed in the metastatic lesion treated by GW4869 and the inverse condition occurred in 143B

exosomes treated group (Figure-3L). It illustrated exosomes derived from OS could stimulate the tumor to disseminate to the lung and GW4869 can be used to inhibit the secretion of osteosarcoma cells and other supporting cells in the microenvironment. Thus it can be a new targeted therapy to reduce the pulmonary metastasis in the future.

Pd-l1 Is Located At Surface Of Osteosarcoma Exosomes

Although PD-L1 is well known to be upregulated in many tumor tissues, such as melanoma, breast cancer and lung carcinoma, few studies have been focused on osteosarcoma, especially circulatory PD-L1 delivered by exosomes. We first revealed the level of serum exosome-delivered PD-L1 for OS patients and confirmed the existence of PD-L1 at the surface of exosomes, which derived from osteosarcoma cell and serum of patients with OS.

As shown in Figure-4A-B, the positivity rate of PD-L1 in osteosarcoma specimen was 35.5% (22/62) and the positivity rate was not so high as melanoma, breast cancer and lung carcinoma, which was reported in our previous study[26]. Haematoxylin and eosin staining revealed the tumor had destruction of cortical bone and invasion of the surrounding soft tissue could be seen. The tumor region was composed of spindle cells, which showed the irregular trabecular bone (Figure-4C). Western blot analysis was performed on four osteosarcoma cell lines for total protein and it indicated that HOS, KHOS, 143B and U₂OS cell lines showed stable PD-L1 protein expression (Figure-4E). Due to stable PD-L1 expression of OS cell lines, we defined the PD-L1 expression level of U₂OS as the reference and the Sr-exosomal-delivered PD-L1 expression of OS patients were compared to U₂OS for western blot analysis except for the regular other exosome markers.

Higher level of Sr-exosomal PD-L1 in OS patients compared to healthy donors and patients with benign tumor

In order to reveal whether several parameters of exosomes can distinguish OS patients from healthy donors, we performed the following analysis. We compared the particle concentration of Sr-exosomes between OS patients and healthy donors and the result showed there was no significant difference between them by using the nanoparticle tracking system (Figure-4J, K). We found there was no or only marginal difference in the concentration of serum exosomes or the total protein or mRNA level in these exosomes (Figure-4L, M).

The analysis of density gradient centrifugation experiment confirmed that PD-L1 existed in Sr-exosomes derived from OS patients (Figure-4I). Western blot analysis showed Sr-exosomal PD-L1 expression of OS patients was significantly higher than that of healthy donors and patients with benign tumors ($P < 0.05$) (Figure-4F, G). By immunogold labeling for Sr-exosomal PD-L1, we detected PD-L1 at the surface of exosome derived from osteosarcoma cells and serum of healthy donors and patients with osteosarcoma and benign tumor, whereas PD-L1 positive exosomes from serum of healthy donors and patients with benign tumor were fewer than that from patients with osteosarcoma (Figure-4D, H). Next, we evaluated

whether Sr-exosomal PD-L1 from patients with OS was higher than that of healthy donors and patients with benign tumor. Schematic of ELISA to measure PD-L1 concentration at the surface of exosomes isolated from healthy donors and patients with benign tumor and osteosarcoma was shown in Figure-4N. We found that the level of Sr-exosomal PD-L1 from 50 OS patients was significantly higher than levels noted from healthy donors and patients with benign tumor ($P < 0.05$; Figure-4O). Receiver operating characteristic curve (ROC) indicated that Sr-exosomal PD-L1 showed an AUC of 0.695 (95% CI: 0.577–0.814) ($P = 0.006$, Figure-4P). The result of ROC indicated a strong correlation between Sr-exosomal PD-L1 and osteosarcoma in ELISA analysis. These results encouraged us to perform further analysis to potentially reveal on the mechanism of Sr-exosome, which was derived from OS, especially PD-L1 contained in Sr-exosomes, for osteosarcoma pathogenesis and metastasis.

Change of Sr-exosomal PD-L1 for OS patients between pre-treatment and post-surgery

In light of the highly distinguishing patients with osteosarcoma from healthy donors and patients with benign tumor, we next evaluated whether Sr-exosomal PD-L1 expression could identify patients with pulmonary metastasis from ones without metastasis at diagnosis. The radiological images of four OS patients with and without pulmonary metastasis were shown in Figure-5A. Western blot analysis revealed the presence of PD-L1 in Sr-exosome of patients with osteosarcoma at diagnosis, whereas there was no significant difference of its level in exosome derived from patients with metastasis compared to those without metastasis ($P > 0.05$, Figure-5B, C). The biochemical parameters had also poor ability in diagnosing patients with pulmonary metastasis from ones without metastasis, except serum alkaline phosphatase (ALP) level (Figure-5D-G). Furthermore, Sr-exosomal PD-L1 level, which was tested with both ELISA and western blot analysis, had no significant correlation with the biochemical parameters, which was showed in Supplementary Figure-S1.

The change of Sr-exosomal PD-L1 expression during time course was next evaluated between pre-treatment and post-surgery. We found that the level of Sr-exosomal PD-L1 expression which was analyzed by ELISA test, was significantly downregulated after neoadjuvant chemotherapy (Figure-5J). Western blot analysis also revealed Sr-exosomal PD-L1 expression was significantly lower, whereas some patients didn't show obvious decrease of PD-L1 expression (eg, Patient 1) (Figure-5I). The disease was stable in the lung assessment for Patient 2 and 3 during the follow-up. The radiological images detected the pulmonary metastasis after neo-chemotherapy for Patient 1 (Figure-5H). It illustrated that the change of Sr-exosomal PD-L1 expression in OS patients was related to the disease progression during the follow-up.

In order to reveal the influence of Sr-exosomes isolated from OS patients on the PD-L1 expression of osteosarcoma cells, we performed the western blot analysis. The clinical information of three OS patients, whose Sr-exosomes were used as was shown in Figure-5K. We found there was a trend that Sr-exosomes derived from OS patients can upregulate PD-L1 expression of osteosarcoma cells (Figure-5L, M, N).

Microarray analysis of exosomal mRNA derived from 143B and U₂OS

Endo-Munoz et al. revealed 143B cell line spontaneously formed pulmonary metastasis while U₂OS cell line didn't after mice were injected with OS cells and tumor growth was followed for 4–6 weeks[18]. Based on this study, we set 143B as a higher invasive cell line and U₂OS as a lower one. We found 5792 mRNA upregulated and 983 mRNA downregulated of 143B compared to U₂OS (Figure-6B).

Establishment of co-expression network among Sr-exosomal different expressed mRNA between 20 OS patients and 6 healthy donors

Based on the bioinformatic mRNA DEG data of serum exosomes between 20 OS patients and 6 healthy donors, we established the co-expression network among the different expression genes, which had PD-L1 as the core gene (Figure-6A). From the co-expression network, we found the some genes, likely ALPL and C5AR1 which were associated with tumor invasion and metastasis shown in Venn diagram, to be close to core gene PD-L1 (Figure-6A, B). It reveal that several genes in exosomes derived from OS cells may be related to exosomal PD-L1. Furthermore, exosomal PD-L1 played an important role in osteosarcoma pathogenesis, with KEGG analysis showing Fc gammaR-mediated phagocytosis, cell adhesion, TNF signaling pathways involvement in the progression of osteosarcoma and biological process including neutrophil activation involved in immune response, neutrophil activation, neutrophil mediated immunity, leukocyte cell-cell adhesion, T cell activation and positive regulation of leukocyte cell-cell adhesion (Figure-6C). And then, co-expression network, which have PD-L1 centered, can be divided into three function modules. One of three function modules was associated with PD-L1, which had more biological function of cell-cell adhesion (Figure-6D).

Influence of knockdown of Rab27A and PD-L1 on 143B cell

Knockdown of 143B Rab27A (143B-Rab27A-KD) by CRISPER-Cas9 were confirmed by qPCR and western blot analysis (Figure-7A, B). Transmission electron microscopy (TEM) analysis showed there was fewer exosomes in the supernate isolated from 143B-Rab27A-KD and it also proved the knockdown validity of regenerated cell line (Figure-7C). 143B-Rab27A-KD proliferated slowly than 143B-Rab27A-WT in Cell Counting Kit 8 assay and maybe it illustrated that knockdown of Rab27A influenced not only on the exosome secretion, but also 143B cell proliferation (Figure-7D). Meanwhile, knockdown of 143B PD-L1 (143B-PD-L1-KD) by CRISPER-Cas9 were also confirmed by qPCR and western blot analysis (Figure-7A, B). We found less PD-L1 existed on the surface of exosomes isolated from 143B-PD-L1-KD compared to 143B-PD-L1-WT by immunogold labeling for PD-L1 (Figure-7E). Otherwise, knockdown of PD-L1 didn't affect the proliferation of osteosarcoma cell by Cell Counting Kit 8 assay (Figure-7F).

Exosomal Pd-l1 Facilitates Osteosarcoma Pulmonary Metastasis

To investigate the effect of osteosarcoma-derived exosomal PD-L1 on the pulmonary metastasis *in vivo*, we performed different 143B metastatic models in nude mice as the flowchart and the respective treatments (Figure-7H). Importantly, we revealed that knockdown of Rab27A, which mediated the release of exosomes, blocked exosomal PD-L1 secretion (Figure-7G). We injected 143B-WT and 143B-Rab27a-KD into tail vein as the primary injection to acquire Control, 143B-WT and 143B-Rab27a-KD metastatic model. Then, injection of PBS, 143B-WT-Exo and 143B-PD-L1-KD-Exo as the secondary injection to reveal the effect of different exosomes on the pulmonary metastasis (Figure-7H).

Based on the analysis of metastatic models, we can evaluate the effect of osteosarcoma-derived exosome on the pulmonary metastasis among A, B and C groups. Likewise, the assessment of exosomal PD-L1 on the influence of lung metastasis can be achieved among C, D and E groups. Secretion of exosomes derived from osteosarcoma was decreased in Group C due to knockdown of Rab27a (Figure-7C) and then we found the number of pulmonary metastasis nodules and maximum diameter of metastatic lesion in Group C were significantly depressed compared to Group B ($P < 0.05$) (Figure-7J-L). Furthermore, the formation of pulmonary metastasis nodules in Group E, for which injection of exosomes derived from parental 143B-PD-L1-KD cells was performed, was obviously inhibited compared to that Group D. Injection of exosomes derived from 143B-WT and 143B-PD-L1-KD in Group D and E can induce the progression of pulmonary metastasis compared to Group C (Figure-7M-O).

Discussion

Our study is aiming to illuminate how the exosome influence the osteosarcoma progression and whether PD-L1 also exists in serum exosome of patients with osteosarcoma. Exosomes act as messengers in most cancers, which eventually leads to angiogenesis, immune suppression and tumor metastasis[23, 24]. Several studies reported that exosomes isolated from osteosarcoma were associated with the tumor pathogenesis and illustrated many mechanisms of metastatic progression in their studies[28–31]. Meanwhile, recent research indicated that exosomal PD-L1 derived from cancers can induce immunosuppression and tumor pathogenesis[23, 24, 32].

In the present study, bioinformatic analysis demonstrated that Sr-exosomes isolated from OS patients may involve important process of immune function and cancer pathogenesis. Furthermore, it showed 248 mRNAs as exclusively significant expression in the Sr-exosomes derived from OS patient compared to that from healthy donors and several mRNAs were enriched in cancer and immune associated pathways, which can be drawn the conclusion from KEGG analysis. We also found the different expressed mRNAs in the Sr-exosome between OS patients and healthy donors, likely FLOT2. FLOT2 is considered as the exosome marker and related to the vesicle biogenesis[33]. These results illustrated that Sr-exosomal mRNAs derived from OS patients may participate in the osteosarcoma pathogenesis, especially the immune process in the microenvironment. Interestingly, PD-L1 in Sr-exosomes of OS patients was significantly higher than that of patients with benign and healthy donors. This result confirmed the exosomes and exosomal PD-L1 may be related to the pathogenesis of osteosarcoma from the clinical perspective. Afterwards, based on the bioinformatic mRNA DEG data of serum exosome between 20 OS

patients and 6 healthy donors, we established the co-expression network among the different expression genes, which had PD-L1 as the core gene. From the co-expression network, we found some genes, likely ALPL and C5AR1 which were associated with osteosarcoma invasion and metastasis shown in Venn diagram, to be close to core gene PD-L1. It reveals that several genes in exosomes derived from OS cells may be related to exosomal PD-L1. Furthermore, exosomal PD-L1 played an important role in osteosarcoma pathogenesis, with KEGG and GO analysis which was shown in Figure-1H, K and Figure-6C, D.

In the present study, we found Sr-exosomes isolated from OS patients could induce the migration and invasion of osteosarcoma *in vitro*. We reckoned that Sr-exosomes derived from patients with OS included other origins such as supporting cells (i.g. fibroblast and endothelial cells), immune cells and bone marrow-derived mesenchymal stem cells (BMSCs), except osteosarcoma. However, the complexity of Sr-exosomes isolated from OS patients can't explain the effect of osteosarcoma derived exosomes on the tumor pathogenesis. Qin et al. MSC-derived exosomal miRNA as a mediator of the exosomal effects on osteosarcoma enhanced the progression of osteosarcoma[3]. Based on above mentioned reason, we isolated exosomes from 143B cells and interacted with osteosarcoma cells *in vitro*. Afterwards, it demonstrated that exosomes, which was derived from 143B cells, can stimulate the migration and invasion of osteosarcoma cells, which was in concordance with the manifestation of Sr-exosomes of OS patients.

It has been reported that osteosarcomas may communicate with their environment by secretion of soluble proteins and generating exosomes[34–38]. Studies of tumor-derived exosomes may reveal possible new important targets for cancer therapy, as well as perhaps yield tumor antigens that are diagnostic or prognostic for tumor aggressiveness[24, 25]. However, specific isolation of exosomes from the serum of cancer patients remains a challenge due to the lack of specific markers that can be used to identify and distinguish cancer exosomes from exosomes produced by other cells. In the literature, Melo reported a cell surface proteoglycan, glypican-1 (GPC1), specifically enriched on cancer cell-derived exosomes, with high sensitivity and specificity, to detect early stages of pancreas cancer[33]. However, lack of good biomarker for sarcoma lead to us to find out a better approach to screen the osteosarcoma prognosis.

Our results provided clear visual evidence of PD-L1 existence at the surface of exosome produced by osteosarcoma cells using electron transmission microscopy for immunogold labeling. Western blot analysis also showed that these exosomes expressed typical exosomal biomarkers, which represented components of the endosomal sorting complex required for transport (ESCRT). Furthermore, we can use Sr-exosomal PD-L1 to differentiate OS patients from patients with benign tumor and healthy people, with ROC analysis revealing an AUC of 0.695. This finding illustrated that Sr-exosomal PD-L1 can be used as the important biomarker to monitor the progression of OS patients.

Several studies revealed the presence of PD-L1 in exosomes isolated from blood samples of patients with cancer, and the level of PD-L1 correlates with pathological features of these patients[23, 24, 39, 40]. Chen

et al. reported metastatic melanomas released exosomes, delivering PD-L1 on their surface. And furthermore, stimulation with interferon- γ increased the amount of PD-L1 on these vesicles, which suppressed the function of CD8 T cells and facilitated tumour growth. It also showed that in patients with metastatic melanoma, the level of circulating exosomal PD-L1 positively correlates with that of IFN- γ , and varied during the course of anti-PD-1 therapy. The number of tumour-infiltrating CD8 T lymphocytes (TILs) decreased significantly after the injection of exosomes. B16-F10 exosomes also decreased the proportion of proliferating PD-1 + CD8 T cells in both spleen and lymph nodes, suggesting that exosomal PD-L1 suppresses anti-tumour immunity systemically[24]. Troyer et al. reported the negative effect of OS exosomes on CD4 + and CD8 + T cell proliferation was much greater than that of osteoblast derived exosomes[41]. In the present study, we showed that in the metastatic model, mice injected with 143B-Rab27a-KD, which failed to secrete more exosomes, had abundant metastatic lesions in the lung compared to ones received injection of wild type 143B osteosarcoma cell. It also illustrated that exosomes derived from osteosarcoma facilitated the progression of pulmonary metastasis. Afterwards, we found that injection of exosomes derived from parental 143B-PD-L1-KD cells was obviously inhibited compared to that receiving injection of exosomes derived from 143B-WT in the metastatic model. These results showed that the exosomal-PD-L1 can stimulate the pulmonary metastasis in the osteosarcoma metastatic model.

Personalized medicine strategies are widely advocated for the treatment of osteosarcoma patients with pulmonary progression[42–46]. However, we are currently faced with rigorous problem due to lack of excellent therapy which can control the tumor development [47–50]. Detecting a good personalized medicine strategy is promising for osteosarcoma patients. Liu et al. reported A485, a p300/CBP inhibitor abrogated immuno process and decreased the secretion of exosomal PD-L1 by blocking the transcription of CD274, which combined with the anti-PD-L1 antibody to reactivate T cells function for tumor attack[25]. Thus, suppression of exosomal PD-L1 may be a promising personalized therapy. It has been reported that GW4869 could decrease the secretion of tumor cell exosome, which inhibit the cell growth [51]. Although we can not observe the significantly inhibiting effect of GW4869 on the osteosarcoma cell growth *in vitro* in the present study, inhibition of exosome secretion by GW4869 could suppress the pulmonary metastasis *in vivo* study. It may be related to the effect of inhibiting the exosome secretion of supporting cells (i.g. endothelial and fibroblast cells, which support metastatic osteosarcoma cell in the lung metastatic lesion) in the microenvironment for OS patients.

There were several limitations in the present study. It has been reported that one of the mechanisms of tumor metastatic progression is the exosome associated with immune suppression[12, 52, 53]. However, it was not evaluated in this study how exosomes derived from osteosarcoma interact with the immune cells. We found there was no significant difference of Sr-exosomal PD-L1 level in patients with metastasis compared to those without metastasis. This phenomenon may be related to the small samples and patients with the pulmonary metastasis being at the early stage, which didn't have enough tumor burden of metastatic lesions. Thus, in the future study, we need to collect more samples to reveal the objective results.

Conclusion

In conclusion, to our knowledge, we confirm for the first time that osteosarcoma releases extracellular vesicles, mostly in the form of exosomes, that carry PD-L1 on their surface and induces the osteosarcoma pathogenesis.

Abbreviations

OS, Osteosarcoma; Sr-exosome or Sr-Exo, Exosome derived from serum; PD-L1, Programmed death-ligand 1; TEM, Transmission electron microscopy; EV, Extracellular vesicle.

Declarations

Ethical Approval and Consent to participate

This study was reviewed and approved by the Ethics Committee of Peking University People's Hospital and all the participants signed an informed consent form.

Consent for publication

All the patients involved in our study obtained written consent for publication.

Availability of data and materials

The datasets used and/or analyzed during the current study are available within the manuscript and its supplementary information files.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

WJ and WXF carried out the experiment, performed the analysis and drafted the manuscript. ZHL and SX collected the samples and carried out the experiment, RTT and HY collected data and performed the statistical analysis, ZRX and ZBX participated in its design. GW designed the study and reviewed the article. All authors have been actively involved in the drafting and critical revision of the manuscript, and each provided final approval of the version to be published.

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Tables

Table-1 Clinicopathologic characteristics of 20 patients with osteosarcoma, receiving Sr-exosomalRNAseq in this study

No	ID	Gender	Age	Side	Location	Enneking stage	Pathological type
Case-1	A35-07	F	13	R	distal femur	IIB	osteoblastic
Case-2	A35-08	M	16	R	proximal tibia	IIB	osteoblastic
Case-3	A35-09	M	7	R	distal femur	IIB	telangiectatic
Case-4	A35-10	F	17	L	proximal tibia	IIB	osteoblastic
Case-5	A35-11	F	10	R	distal femur	IIB	osteoblastic
Case-6	A35-12	M	9	R	proximal tibia	IIA	osteoblastic
Case-7	A35-13	M	16	R	distal femur	IIA	chonblastic
Case-8	A35-14	F	15	L	proximal tibia	IIB	osteoblastic
Case-9	A35-15	M	15	R	distal femur	IIB	chonblastic
Case-10	A35-16	M	16	L	proximal humerus	IIB	chonblastic
Case-11	A35-17	M	16	R	distal femur	IIB	osteoblastic
Case-12	A35-18	F	17	R	proximal tibia	IIA	osteoblastic
Case-13	A35-19	M	33	R	proximal femur	III	osteoblastic
Case-14	A35-20	F	34	L	proximal tibia	III	osteoblastic
Case-15	A35-21	M	10	R	proximal tibia	III	osteoblastic
Case-16	A35-22	M	14	L	distal femur	III	osteoblastic
Case-17	A35-23	M	13	L	distal femur	III	osteoblastic
Case-18	A35-24	F	12	R	proximal tibia	IIB	osteoblastic
Case-	A35-	M	35	L	distal femur	IIB	chonblastic

19	25						
Case-20	A35-26	M	19	R	proximal tibia	IIA	chonblastic

Figures

Figure 1

Bioinformatic analysis global mRNA expression of Sr-exosome in 20 osteosarcoma patients and 6 healthy donors. A, A flowchart showing the plan for analysis of healthy donors and patients with OS and benign tumor. B, Clinical characteristics of OS patients, which received Sr-exosome RNA sequence. C, Electron microscopy shows vesicles of 30–100 nm diameter corresponding to exosomes. D, Western blot analysis for the representative biomarkers of exosomes. E, Nanoparticle tracking system for

concentration and size of exosomes. F, Volcano plots of the differentially expressed genes in Sr-exosomes of six healthy donors and twenty patients with OS. G, Venn diagram showing co-expression genes of differentially expressed mRNAs in Sr-exosome of six donors and twenty OS patients between pathways related to cancer and ones related to immune function. H, KEGG pathways analysis revealing the enrichment in Sr-exosomal mRNA between OS patients and healthy donors. I, Hierarchical clustered heatmap of differentially expressed genes in Sr-exosomes of six donors and twenty patients with OS. J, Venn diagram showing FLOT2 as the co-expression genes between Sr-exosome DEG (different expression genes, OS patients vs healthy donors) and gene of associating with exosome biogenesis. K, GO analysis demonstrating Sr-exosomes of OS patients involvement in the process of immune function.

Figure 2

Effect of exosomes isolated from serum of OS patients and osteosarcoma cells on osteosarcoma migration and invasion. A, Nanoparticle tracking system for concentration and size of exosomes derived from medium of 143B and U2OS. B, Electron microscopy showing exosomes isolated from medium of

143B and U2OS. C, Western blot analysis for the representative biomarkers of exosomes. D, Immunofluorescent analysis detecting internalization of exosomes from 143B cells or OS patient serum to the recipient 143B and U2OS cells. E, Treatment of 143B and U2OS cells for 24, 48 and 72 hours using different concentration of Sr-exosomes derived from OS patient and healthy donor. F, Effect of exosomes isolated from patient/healthy donor serum and OS cell line on the migration of 143B and U2OS in vitro. G, Influence of exosomes isolated from patient/healthy donor serum and OS cell line on the invasion of 143B and U2OS in vitro.

Figure 3

Effect of exosome secretion inhibitor GW4869 on osteosarcoma progression. A-C, Effect of the small molecule of exosome secretion inhibitor GW4869, on the migration of 143B cells in vitro. D, Nanoparticle tracking analysis showing inhibition of exosome secretion by utilizing GW4869. E, A flowchart showing the plan to test GW4869 on pulmonary metastasis nodules and metastatic lesion area in vivo. F, Gross specimens of lungs in three groups. G, Haematoxylin and eosin staining to show the metastatic lesions in the lung of three groups. H, Weight change of mice in three groups. I-K, Maximum diameter, number and areas of metastatic lesion nodules in three groups. L, Upregulation of E-cadherin and downregulation of N-cadherin and vimentin was observed in the metastatic lesion treated by GW4869.

Figure 4

Higher level of Sr-exosomal PD-L1 in OS patients compared to healthy donors and patients with benign tumor. A-B, Positivity rate of PD-L1 in osteosarcoma specimen by immunohistochemistry analysis. C, Pathological changes in the osteosarcoma of patients by haematoxylin and eosin staining. D, A representative TEM image of exosomes immunogold-labelled with anti-PD-L1 antibodies isolated from HOS, KHOS, 143B and U2OS. E, Western blot analysis was performed on four osteosarcoma cell lines to test PD-L1 expression of osteosarcoma cells and exosomes. F-G, Western blot analysis showing Sr-exosomal PD-L1 expression of healthy donors and patients with OS and benign tumors. H, A representative TEM image of Sr-exosomes immunogold-labelled with anti-PD-L1 antibodies derived from healthy donors and patients with osteosarcoma and benign tumor. I, Density gradient centrifugation confirming that PD-L1 existing in Sr-exosomes derived from OS patients. J-K, Analysis of particle concentration of Sr-exosomes between OS patients and healthy donors by nanoparticle tracking system. L, Concentration of RNA level in Sr-exosomes between patients with OS and healthy donors. M, Concentration of total protein level in Sr-exosomes between patients with OS and healthy donors. N, Schematic of ELISA to measure PD-L1 concentration in exosomes derived from healthy donors and

patients with osteosarcoma and benign tumor. O, Levels of PD-L1 in exosomes derived from serum of healthy donors and patients with osteosarcoma and benign tumors as assayed by ELISA. P, ROC indicated that Sr-exosomal PD-L1 showed an AUC of 0.695.

Figure 5

Change of Sr-exosomal-PD-L1 of OS patients during follow-up. A, Radiological images of four OS patients with and without pulmonary metastasis. B-C, Western blot analysis revealing no significant difference of Sr-exosomal PD-L1 level in exosome derived from patients with metastasis compared to those without metastasis. D-G, The biochemical parameters of OS patients with and without pulmonary metastasis. H, Follow-up for patient 1 with disease progression and patient 2 with stable disease. I, Sr-exosomal PD-L1 expression by western blot analysis for patients 1, 2 and 3 during the follow-up. J, Level of Sr-exosomal PD-L1 expression before and after neoadjuvant chemotherapy. K-N, Influence of Sr-exosome isolated from OS patients on the osteosarcoma cells.

Figure 6

Establishment of co-expression network among Sr-exosomal different expressed mRNA between 20 OS patients and 6 healthy donors. A, Establishment of co-expression network among the different expression genes, which had PD-L1 as the core gene. B, Venn diagram demonstrating several genes (ALPL and C5AR1), which were associated with invasion and metastasis, to be close to core gene PD-L1 in the co-expression network. C, Important role of exosomal PD-L1 involved in osteosarcoma pathogenesis by KEGG pathways and biological process analysis. D, Three function modules showing in the Bioinformatic analysis of co-expression network.

Figure 7

Influence of exosomal-PD-L1 on disease progression of osteosarcoma. A-B, Confirmation of knockdown of 143B Rab27A (143B-Rab27A-KD) and 143B PD-L1 (143B-PD-L1-KD) by CRISPER-Cas9 by qPCR and western blot analysis. C, Representative transmission electron microscopy images showing there was fewer exosomes in the supernate isolated from 143B-Rab27A-KD. D, Cell Counting Kit 8 assay for proliferation of 143B-WT and 143B-Rab27A-KD. E, Comparison of exosomal PD-L1 expression isolated

from 143B-PD-L1-KD and 143B-PD-L1-WT by immunogold labeling. F, Cell Counting Kit 8 assay for proliferation of 143B-WT and 143B-PD-L1-KD. G, Exosomal PD-L1 expression for three osteosarcoma cell lines, including 143B-WT, 143B-Rab27A-KD and 143B-PD-L1-KD. H, A flowchart showing the plan to investigate the effect of osteosarcoma-derived exosomal PD-L1 on the pulmonary metastasis in vivo. I, Gross specimens of lungs in five groups. J, Representative HE images showing the metastasis of Rab27a knockdown tumors in BALB/C mice after indicated treatments, respective injection of PBS, exosomes derived from 143B-WT and 143B-Rab27a in Groups A, B and C. K-L, Maximum diameter of metastatic lesion and number of pulmonary metastasis nodules in three groups. M, Effect of exosomes derived from 143B-PD-L1 knockdown in 143B-Rab27a- knockdown metastatic models. N-O, Maximum diameter of metastatic lesion and number of pulmonary metastasis nodules in C, D and E groups. Experiments were performed using 5 mice for each group. The weights of mice in five groups with different indicated treatments (n =5 mice per group). Data represent mean $\pm$ SD.

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