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Original Article

Proteomic identification of osteosarcoma-derived exosomes and their activation of pentose phosphate pathway

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Abstract: Osteosarcoma (OS) is the most common primary bone tumor in children and adolescents. Combined therapy has not improved the prognosis of osteosarcoma in the past five years, while new strategies need to be explored. We extracted serum exosomes from OS and healthy people. Contrasting to exosomes from healthy ones, those derived from patients suffering from OS significantly promoted the ability of adhesion, migration and viability of the MG63 in vitro study. Osteosarcoma-derived exosomes package proteins were mainly intracellular ones, while these proteins exclusively expressed glucose-6-phospate dehydrogenase (G6PD), phosphofructokinase, transaldolase 1 and transketolase that reprogrammed tumor metabolism and prompted progression of osteosarcoma. Western-blot assay confirmed osteosarcoma-derived exosomes contained generous G6PD than health people. In conclusion, serum exosomal proteins originated from osteosarcoma and healthy people were quite different from each other. Exosomes derived from osteosarcoma could promote tumor progression, for G6PD were largely packaged by serum exosomes. These special serum exosomes, which are abundant in G6PD, can be a promising target for diagnostic and therapeutic strategy for osteosarcoma.

Keywords: Osteosarcoma, exosome, glucose-6-phospate dehydrogenase, pentose phosphate pathway

Introduction

Osteosarcoma is the leading bone malignancy in adolescents. However, there exists a second incidence peak among individuals aged above 60 years. Despite the application of multimodal treatment strategies of osteosarcoma (OS), the overall five years survival rate for osteosarcoma is 68%. The age of the patient is related with the survival, older ones show the poorest survival. There is no significant difference between genders for osteosarcoma [1]. Apparently, we need a deep understanding of the biology of osteosarcoma to improve the outcome of suffered patients. While great endeavors have gone into the molecular genetics [2], one part that remains unknown is that of osteosarcoma-correlated exosomes.

Exosomes are small membrane vesicles (30-100 nm) secreted from endosomes by most cell types. Exosomes can be detected in most bodily fluids like serum, blood, amniotic fluid, saliva, ascites, urine and nasal secretion [3]. Almost all the cells have been shown to secrete exosomes, while cancer cells secrete greater amounts than normal cells [4, 5]. They contain a great deal of proteins, RNAs and lipids, which functioned as a mechanism through which secreted cells pass information to targeted cells [6, 7]. In correlation to neoplasms, there is obvious evidence that exosomes play a role in carcinogenesis by affecting biological processes like proliferation, metastasis, anchorage-independent growth, angiogenesis, immune system and so on [8]. Based on these mechanisms, some people argued that exosomes are involved in the whole progression of tumor metastasis. Targeted antigen localization to exosomes has proved to be an effective way to treat prostate cancer, which highlights the significance of full research of the proteins on exosomes [9]. However, little is known about sarcomas-related exosomes.

In our assay, we first identified exosomal proteins derived from serum of patients diagnosed with osteosarcoma and performed further in-depth proteomic analyses of their existence and function. The recognition of specific proteins on exosomes and their role of carcinogenesis could be helpful in exploring the possible diagnosis and treatment of osteosarcoma.

Materials and methods

Subjects

From February 2010 to December 2014, a total of 15 patients diagnosed with osteosarcoma in our hospital were recruited into study, and 15 healthy subjects served as controls. All the included patients were identified by percutaneous needle puncture pathology before any therapy and without other complications. Inclusion criteria for control group: subjects had no history of diseases, tumors or concomitant infection. The study was approved by ethics approval in our hospital and all the participants were assigned written informed consent.

Collection of clinical features

General information: age, gender; Medical history: smoking, drinking, dyslipidemia, diabetes mellitus, hypertension, and family history of tumor. Detection of blood pressure, temperature, heart rate, liver function, kidney function, glucose, electrolytes, lipids and routine blood test were performed.

Sample collection and processing

Venous blood of limosis was collected and allowed to stay for 60 min at room temperature. After centrifugation at 3,000 rpm for 10 min, the supernatant was collected and stored at -80°C for use.

Separation and identification of exosomes

Each group of serum samples were mixed and centrifuged at 3,000 rpm for 15 min. The supernatant was transferred to an aseptic tube, followed by addition of ExoQuick Exosome Precipitation Solution (SBI, Japan). Incubation period was 30 min. Following addition of ExoQuick/supernatant, centrifugation was performed at 1500 rpm for 30 min. The supernatant was removed, and centrifugation was performed again for 5 min at 1500 rpm to remove residual ExoQuick solution. Protein lysis buffer was added, and BCA method was employed to determine the protein concentration of exosomes. Western blot assay was performed to detect the CD63 expression.

Cell lines and cell culture

MG-63 (human osteosarcoma cell lines) purchased from Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco-modified Eagle's medium (Hyclone, Utach, USA) supplemented with 10% fetal bovine serum (Hyclone, Utach, USA). All cells were cultured with 5% CO_2 and 95% air at 37°C in a humidified incubator.

Transmission electron microscopy

We distinguished exosomes respectively between serum of healthy and patients diagnosed of osteosarcoma. Samples were fixed by 2.5% glutaraldehyde incacodylatd buffer and stained with 0.75% uranyl formate. Transmission electron microscope (FEI NanoPort, China) was applied at 60 kV acceleration voltage and images were saved.

Western blotting assay

Exosomes were lysed with IP lysis buffer (Beyotime, China) accompanied with Protease Inhibitor Mixture (Roche, Switzerland). Electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gel separated lysates. The separated lysates were transferred onto nitrocellulose membranes and blocked in 5% BSA supplemented with 0.1% Tween. After blocking, anti-CD63 and anti-G6PD (rabbit anti-human polyclonal antibody, Abcam, San Diego, USA) of primary antibodies were incubated with membranes overnight at 4°C. Another incubated with secondary antibody (goat anti-rabbit, Abcam, San Diego, USA) for 20min, Odyssey Infrared Imaging System (Li-COR, USA) was used to visualize with IRDye 800CW-labeled (Li-COR Biosciences, Lincoln, NE, USA). ImageJ software (NIH, Bethesda, MD, USA) quantified the relative densitometry.

Cell viability assay

Cell Counting Kit-8 (CCK8, Dojindo, Japan) were used to measure cell viability. MG-63 treated with 10 μg/ml exosomes respectively derived

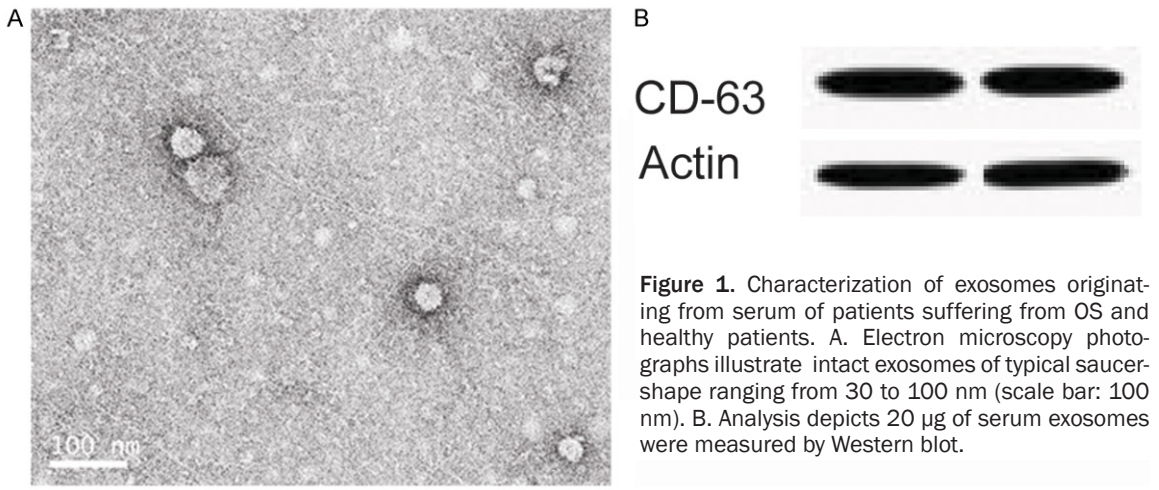

Figure 1. Characterization of exosomes originating from serum of patients suffering from OS and healthy patients. A. Electron microscopy photographs illustrate intact exosomes of typical saucer-shape ranging from 30 to 100 nm (scale bar: 100 nm). B. Analysis depicts 20 μg of serum exosomes were measured by Western blot.

from normal people and osteosarcoma. Absorbance at 450 nm was measured after 0, 24, 48, 72 h incubation.

Cell adhesion assay

Cells were harvested and transferred to collagen IV at 0.5×10^5 cells/ml in the presence of serum exosomes at 10 μg/ml derived from patients suffered from OS and health, and then incubated for 2 h. Microscopy and absorbance at 450 nm was applied to detect the bounded cells after the non-adherent cells were removed.

Cell migration assay

Being starved overnight, MG-63 was harvested. Harvested MG-63 resuspend at 0.8×10^4 cells/ml with serum-free medium and 10 μg/ml exosomes purified respectively from normal people and osteosarcoma. Added the mixture onto the top chambers of 8 um pore cell culture (Millipore, Massachusetts, USA) for 24 h. The migrated cells affixed to bottom membranes were fixed, stained with crystal violet. Every five stochastic fields were calculated under microscope (Olympus, Japan).

Differential proteomic analysis of serum exosome

We collected exosomes, which were derived from serum of normal people and patients that suffered from osteosarcoma. According to manual, analyzed proteins of them through proteomic mass spectrometry analysis, respectively. Identified the differential proteins expressed only in osteosarcoma, then analyzed ontology and pathway of them by Blast2go (https://www.blast2go.com/) and David (http://david.abcc.ncifcrf.gov/). These website allow us arrange proteins to facilitate deep-going analysis.

Statistical analysis

All data have been calculated as the mean ± standard deviation (X ± SD) and student's t-test performed for analyzed statistical significance between groups with SPSS version 21.0. Differences with *P* values (*) < 0.05 are referred to a significant level.

Results

Clinical features were matched in two groups

The age (13.25 ± 3.46 years), gender and medical history were matched between two groups. The performed routine test results were normal.

Serum exosomes were typical

Exosomes purified from serum manifests unique round, saucer-like shapes under electron microscopy, which range from 30-100 nm (**Figure 1A**). Extracted exosomes had been illustrated by highly CD63-positive which was used as exosome marker (**Figure 1B**).

Serum exosomes promote the adhesion and migration of MG-63

MG-63 cell lines are suitable for research of osteosarcoma proliferation and metastasis

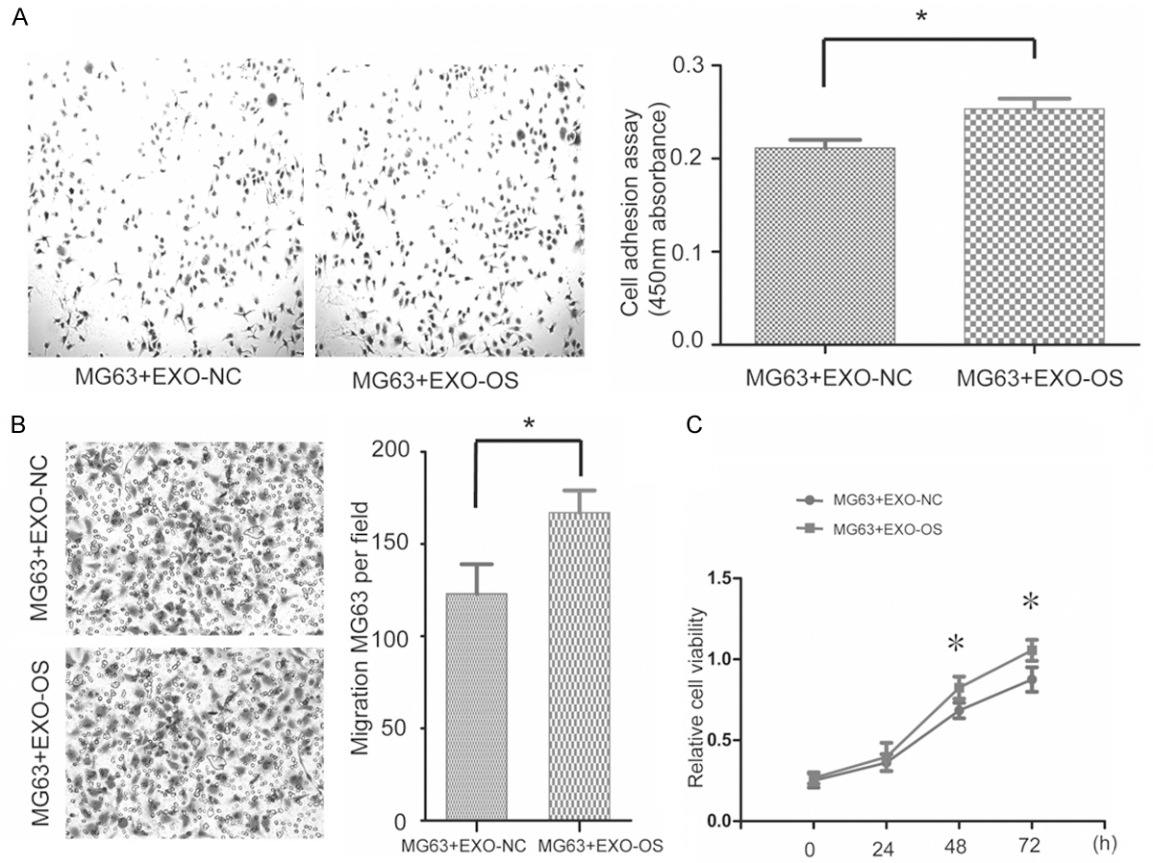

Figure 2. Serum exosomes extracted from patients suffering from OS and healthy patients, respectively, influence viability, adhesion and migration ability of MG63 (OS cell lines) in vitro. A. The relative ability of adhesion of MG63 is measured after incubated with 10 μg/ml of serum exosomes derived from patients diagnosed with OS and healthy patients, respectively. B. At the concentration of serum exosomes of 10 μg/ml derived from two groups, the relative migration ability of MG63 is measured after 24 h incubation. C. After incubated with serum exosomes of 10 μg/ml purified from patients of OS and health, respectively, the relative viability of MG63 have been calculated at 0 h, 24 h, 48 h and 72 h. Quantification analysis data are signified as means ± standard deviation (SD), n=3; *P < 0.05, **P < 0.01, ***P < 0.001.

research [10]. We focused on exosomal function on proliferation, adhesion and migration of osteosarcoma for its poor prognosis. We illustrated that serum exosomes extracted from osteosarcoma could significantly prompt the adhesion (**Figure 2A**), migration (**Figure 2B**) and viability ability of MG-63 (**Figure 2C**) when contrasted to the controls.

Differential proteomic analysis of serum exosomes that exist only in patients

Mass spectrometry analysis discerned 233 differential proteins only expressed in patients, while 409 proteins were in common and another 130 proteins existed in healthy (**Figure 3A**). We further illustrated these exclusive proteins in patients as follows: in cellular component, most proteins comprised cell and intracellular component, only 27 proteins belonged to extracellular (**Figure 3B**); in molecular function, the most clusters recognized were binding and catalytic activity (**Figure 3C**); in biological process, cellular process, metabolic process and single-organism process were the greatest expressed (**Figure 3D**).

Analysis of exclusive serum exosomal proteins in patients

Analysis of exclusive serum exosomal proteins in patients by KEGG revealed four important pathways for promotion of osteosarcoma. These pathways were proteasome, ribosome,

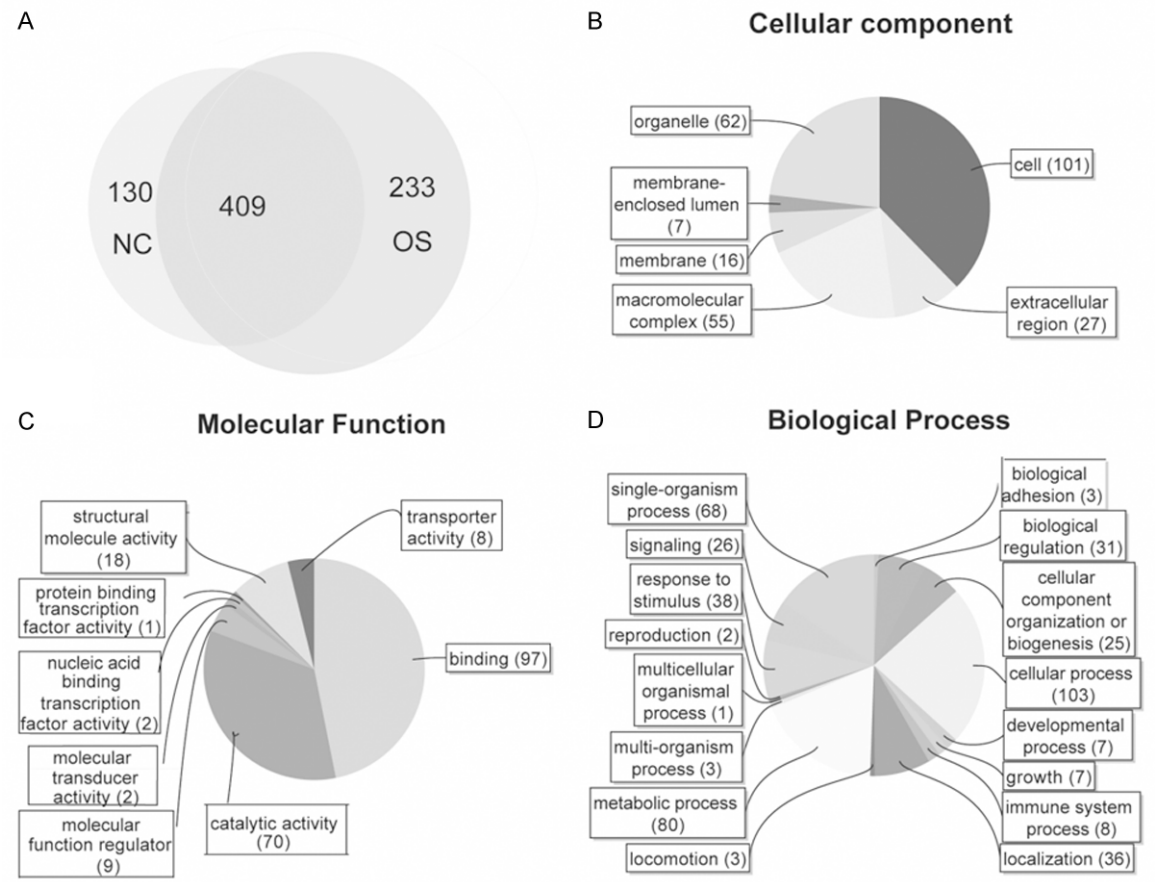

Figure 3. Differential proteomic analysis of serum exosomes that exist only in patients. (A) The diagram shows the distribution of common and is unique proteins listed by mass spectrometry analysis. Exosomal proteins extracted from exosomes that unique in patient of OS are classified using Blast2go dataset based on (B) cellular component, (C) molecular function, (D) biological process.

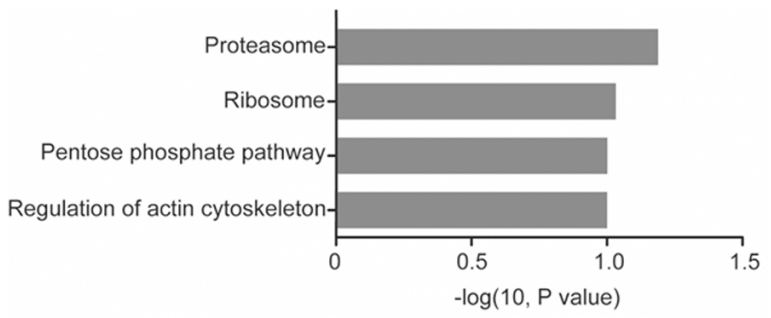

Figure 4. KEGG pathway analysis. Serum exosomal proteins exist only in patient of OS analyzed their KEGG pathway by David dataset.

pantose phosphate pathway and regulation of actin cytoskeleton (**Figure 4**). The specific proteins that are involved in the four pathways mentioned above were listed in **Table 1**. Furthermore, we confirmed G6PD expressed significantly more in serum exosomes derived from OS than those from controls by western-blot assay (**Figure 5**).

Discussion

We exclusively explored the role of serum exosomes on progression of osteosarcoma. Osteosarcoma is a highly aggressive tumor that is the most common primary malignant bone tumor. Hanahan et al. proposed that six biological capabilities are acquired in the multistep progression of human tumors. These are resisting cell death, evading growth suppressors, sustaining proliferative signaling, inducing angiogenesis, activating invasion, metastasis and enabling replicative immortality [11]. The

Table 1. The specific proteins exclusively expressed in serum exosomes of OS that are involved in KEGG pathway

Pathway	DAVID Gene Name
Proteasome	Proteasome 26S subunit, non-atpase, 1
	Proteasome 26S subunit, non-atpase, 7
	Proteasome 26S subunit, non-atpase, 8
	Proteasome subunit, alpha type, 4
	Proteasome subunit, beta type, 1
	Proteasome subunit, beta type, 5
	Proteasome subunit, beta type, 6
	Proteasome subunit, beta type, 9
Ribosome	Ribosomal protein L6 pseudogene 27; 19; 10
	Ribosomal protein S10; ribosomal protein S10 pseudogene 4; 11; 22; 7; 13
	Ribosomal protein S16 pseudogene 1; 10; ribosomal protein S16
	Ribosomal protein S18 pseudogene 12; 5; ribosomal protein S18
	Ribosomal protein S25 pseudogene 8; ribosomal protein S25
	Ribosomal protein S28 pseudogene 6; 9; ribosomal protein S28
	Ribosomal protein SA pseudogene 9; 8; 58; 19; 18; 15; 61; 29; 12
	Ribosomal protein, large, P1
	Ribosomal protein, large, P2 pseudogene 3; ribosomal protein, large, P2
Pentose phosphate pathway	Glucose-6-phosphate dehydrogenase
	Phosphofructokinase, liver
	Transaldolase 1
	Transketolase
Regulation of actin cytoskeleton	CD14 molecule
	IQ motif containing gtpase activating protein 1
	Cell division cycle 42; cell division cycle 42 pseudogene 2
	Guanine nucleotide binding protein, gamma 12
	Integrin, alpha 2
	Integrin, alpha V
	Myosin, light chain 12A, regulatory, non-sarcomeric
	Ras-related C3 botulinum toxin substrate 2
	Vinculin

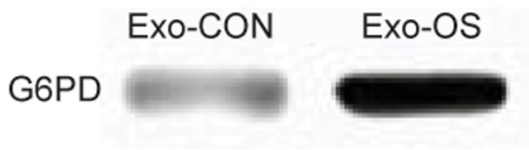

Figure 5. Analysis show 20 μg serum exosomal proteins originated from OS and controls respectively, were measured by Western blot.

confirmed exosomal biological function emerging in all the multistep mentioned above, such as angiogenesis [12], proliferation [7], resisting cell death [13], activates invasion and metastasis [12, 14]. Vast advancements have been made on discerning diagnostic or therapeutic biomarkers for osteosarcoma, including regulation of microRNA, chromosomal translocations, but seldom progress related to exosomes [15]. In our study, serum exosomes extracted from patients who suffered from osteosarcoma had acquired an increased ability of adhesion, migration and viability in contrast to health. In addition, we investigated the differential expressed proteins in osteosarcoma and health in order to discern their potential mechanism.

Cells exchange materials by various paracrine and endocrine mechanisms and this influential intracellular communication can be regulated by exosomes. Advances in mass spectrometric quantitative techniques and proteomics datas-

ets have made the identification of proteins in disease and health become possible in the last two decades, which greatly prompted progression of the biomarker study field [16]. We characterized significant proteomic components of serum exosomes of osteosarcoma to reveal intracellular exchange and potential mechanism. The content of exosomes differed a lot dependent on pre-conditioning and cell types [17, 18]. This is the original study on complete molecular and biochemical characterization of exosomes derived from serum of osteosarcoma.

Through analyzing differential expressed serum exosomal proteins between patients and controls, we discovered most proteins were intracellular components. The component revealed that biological exosomes packaged and protect intracellular proteins to communication target cells, to promote tumor metastasis. While at the molecular function aspect, these exclusive expressed proteins in osteosarcoma were mainly related to binding, catalytic activity and structural molecular activity, which closely referred to adhesion and migration [19]. At the biological process aspect, most proteins mentioned above were related to package, location to facilitate exosomal communication between cells [3].

Using KEGG pathway analysis, it was clearer to understand the mechanism of exosomal proteins in osteosarcoma [20]. Proteasome and ribosome are interacted with exosomes in physiology or pathogenic status. Proteasome appear the similar shape of exosome and evolved as nano-compartments for degradation of macromolecules [21]. Proteasome also coregulated synthesis and degradation of proteins and RNAs with exosomes and ribosome [22]. To our interest, pantose phosphate pathway and regulation of actin cytoskeleton were outlined the special influence of serum exosomal proteins of osteosarcoma.

We confirmed that G6PD were largely delivered by exosomes in OS. The original finding could explain OS progress promoted by exosomes. “Metabolic transformation” phenomenon is crucial for cancer cells’ survival and metastasis [23, 24]. Pantose phosphate pathway (PPP) could provide NAPDH and ribose-5-phosphate to biosynthesis of glucose and DNA, which become a key driver of tumor proliferation and metastasis [25]. Pantose phosphate pathway also play a anti-oxidant role and regulated by many oncogenes and tumor suppressor genes, such as p53, PTEN, CREM, p63, Myc, AKT, AMPK, PI3K , LKB1/AMPK, mTORC1, K-ras and so on [26-28]. In our study, glucose-6-phospate dehydrogenase (G6PD), phosphofructokinase, transaldolase 1, transketolase were typically detected in OS patient rather than health. G6PD, as the first and key rate-limiting enzyme in PPP, has a profound impact on cancer cell growth [29]. G6PD directly controls the flux of PPP, and the activity of G6PD leads to an increasing biosynthesis of DNA and lipids, which are both essential for cell proliferation and division. Therefore, we initially inferred one important exosomal function of OS. Exosomes packaged G6PD secreted from OS cells, circulated to metastasis, activated PPP and helped to reprogram in tumor energetic metabolism resulting in tumor progression.

Based on our unique result of exosomes-related metabolic function, we may figure out new therapeutic field of exosomes. Clinical studies and research have proven exosomes to be a robust and accessible source of biomarkers for early diagnosis of tumor [30]. The advantages of exosomes lie in their nonliving and ability to be easily recovered from human fluids [31]. CD63, CD81, TSG101, Alix and Aquaporin-5 were traditional exosomal proteins, providing a possible form of immune therapy [32]. Exosomes extracted from tumors could carry tumor antigens and present them to immune cells, inducing an anti-tumor immune response, leading to the death of tumor cells [33, 34]. Most importantly, exosomes may serve as a key cell-free vaccine to overcome tumors [35, 36]. Targeting exosomes that had large amount of G6PD could distinguish tumor or normal cells origin, further inhibiting tumor progression. Therefore, exosomes are substantial current strategies for diagnostic or therapeutic targets worth being introduced into clinical treatment against tumors.

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Disclosure of conflict of interest

None.

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