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Original Article

Exosomes mediated pentose phosphate pathway in ovarian cancer metastasis: a proteomics analysis

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Abstract: Epithelial ovarian cancer is the most lethal gynecological malignancies for readily metastasis. Exosomes have played an influential role in carcinogenicity and cancer progression. Our aim is to discover exosome-related mechanisms in ovarian cancer progress and explore potential diagnostic biomarkers and therapeutic targets of ovarian cancer. We initially presented the proteomic profiles of exosomes derived from two late-stage ovarian cell lines, OVCA429 and HO8910PM. A total of 2940 exosomal proteins were recorded by MS. FunRich appropriately processed these exosomal proteins, manifesting some superiority in contrast to Blast2go. Moreover, we demonstrated the pentose phosphate pathway was a dominant mechanism in exosome mediated intracellular communication. Glucose-6-phosphate dehydrogenase, transketolase and transaldolase 1, three key enzymes regulated pentose phosphate pathway, were all marked in the same exosomal parts of proteins between two ovarian cell lines. Moreover, these key proteins might become diagnostic, prognostic biomarkers and therapeutic targets of ovarian cancer.

Keywords: Ovarian cancer, exosome, proteomics, Glucose-6-phosphate dehydrogenase (G6PD)

Introduction

Epithelial ovarian cancer (EOC) remains the most lethal gynecological cancer in the past 30 years, although cytoreductive surgery and chemotherapy have made some achievements [1]. The majority of victims succumb to metastasis, which is the most fearful aspect of epithelial ovarian cancer. Metastatic cancer cells along with their destinations, just like "seed and soil", are well interrelated and communicate with each other through a serious of changes in many proteins. Those proteins are involved in adhesion, angiogenesis, proliferation, metabolism and so on [2]. Potential biomarkers of proteins that could diagnose and treat EOC remain to be a promising strategy. Evaluation of serum CA125 and H4 levels are common clinical means for diagnosis and prognosis of EOC, however they are of relatively high false positive and false negative results [3]. Exosomes which contain amounts of proteins, functioning as cargo between cell and cell, manifest a substantial prospect for diagnosis and treatment of EOC.

Exosomes can serve as suitable biomarkers for diagnosis and treatment of malignancies. Firstly, cancer cells assemble and secrete more exosomes than normal cells. Secondly, exosomes are readily regenerated, nonliving and can be detected in human body fluids like urine, serum, saliva, ascites and so on [4]. Moreover, exosomes package and release unique oncogenic proteins, lipids, DNA and RNAs to modulate target cells depending on disease [5, 6]. Thus, identification of featured proteins in EOC-derived exosomes by MS provides significant potential for further discovery of biomarkers.

Because of the heterogeneous cellular origin of exosomes in body fluid, we extracted exosomes from the conditioned medium of epithelial ovarian cell lines, OVCA429 and HO8910PM [7, 8]. The two cells lines of EOC are late-stage serous epithelial ovarian cancer cells, with metastatic potential both in vitro and in vivo [9]. We initially detect their proteomic profiling and analyzed their exclusive proteins which may promote progress of EOC.

Materials and methods

Cell lines and cell culture

OVCA429 is a cell line originating from a patient with cisplatin-resistant, late-stage ovarian cancer and was purchased from the American Type Culture Collection (ATCC) [10]. HO8910PM is a highly metastatic human-originated ovarian carcinoma cell line. HO8910PM were purchased from Chinese Type Culture Collection in Wuhan University. Cell lines were cultured in RPMI 1640 medium (Gibco, USA) supplemented 10% (vol/vol) fetal bovine serum (Hyclone, USA). Cell lines were maintained in a humidified incubator with 5% CO_2 and 95% air at 37°C. All fetal bovine serum derived exosomes had been depleted by ultracentrifugation at 120,000 g overnight, and then filtrated through a 0.2 um filter (Millipore, USA).

Exosome isolation and purification

Starved the cells when the cells reached 70%-80% confluence in their standard medium for 48 h. collected the condition media, centrifuged at 3,000 g for 15 min at 4°C to eliminate cells and cell debris. The supernatant was further centrifuged at 20,000 g, 30 min at 4°C. Then the exosomal pellets were acquired by ultracentrifugation at 120,000 g, 80 min at 4°C. Exosomes were washed in phosphate-buffered saline (PBS) and pelleted by ultracentrifugation at 120,000 g, 80 min at 4°C. The wash step was performed again. The final pellets of exosome were suspended in little volume of PBS and total protein concentration was detected by BCA Protein Assay Kit (Thermo, USA). After these preparations, exosomes were stored at -80°C refrigeration until use.

Western blotting

The pellets of each centrifugation including exosomes were lysed with IP lysis buffer (Beyotime, China) and mixed with Protease Inhibitor Mixture (Roche, Sweden). Cell lysates were separated under reducing condition by SDS-PAGE, and then immunoblotting was performed with corresponding primary antibodies: anti-TSG101 (Abcam, England) and secondary antibodies (Abcam, England). Quantified the binding secondary antibodies by Odyssey Imaging system (LI-COR Biosciences, USA).

Transmission electron microscopy

Exosomal pellets were fixed in 2.5% (w/v) glutaraldehyde in cacodylate buffer, PH 7.3 with 2% paraformaldehyde. After stained with 2% uranyl acetate, samples were observed by transmission electron microscope (Nanoport, USA) at 60 kV of acceleration voltage and recorded the images.

SDS-PAGE and in-gel digestion

Pellet of exosomes from each cell lines were electrophoresed on a 12% SDS-PAGE. Each lane was loaded 50 μg proteins. The gel with separated proteins were stained with Coomassie brilliant blue and divided into four slices. Procedure of digestion was performed followed standard protocols [1].

Mass spectrometry analysis

Separated the isolated peptides which were quantified by NanoDrop spectrophotometer (Thermo, USA). EASY-Spray column, 50 cm, was used to perform analytical chromatography. Each column was loaded 2 μg peptides. Mass spectrometry (MS) analyses were performed according to procedure [11].

Analysis of exosomal proteins

OVCA429 and HO8910PM are aggressive cell lines and readily to metastasis. FunRich (Functional Enrichment analysis tool) from ExoCarta (http://www.exocarta.org/), Blast2go (https://www.blast2go.com/) and David (http://david.abcc.ncifcrf.gov/) databases were used to perform analysis. The exosomal proteins from two ovarian cell lines were analyzed by these tools. Moreover, comparison between FunRich and Blast2go in analyzing the overlapped proteins was performed to explore the potential proteomic mechanisms.

Results

Characterization of ovarian cancer-originated exosomes

Western blotting and electron microscopy were performed to identify exosomes. In previous studies, TSG101 were confirmed as proteomic markers associated with exosomes [12]. In our assay, TSG101 manifested highly positive in

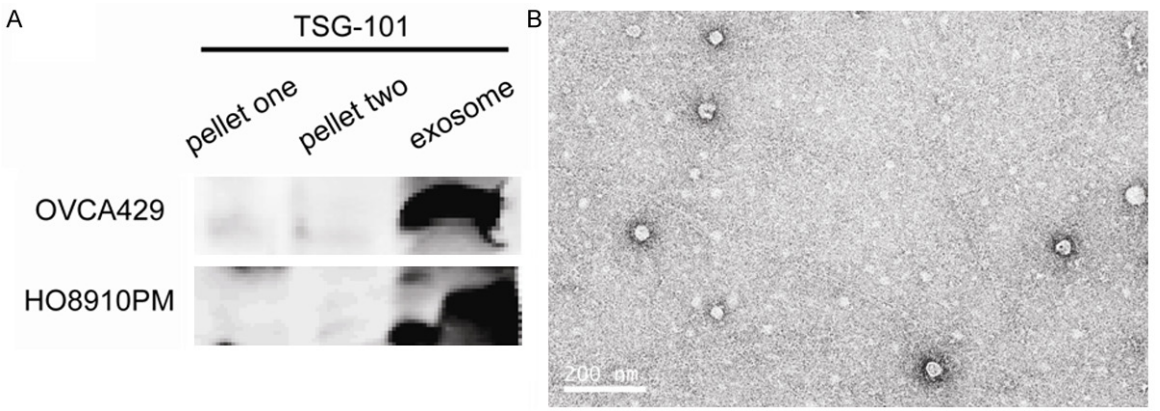

Figure 1. Validation of exosomes derived from late-stage ovarian cancer cell lines. A. Western blot showed that pellets of ultracentrifugation highly expressed of exosomal markers TSG-101. However, TST-101 hardly detect by western blot in the pellets of centrifugation. B. Images of exosomes recorded by transmission electron microscopy. These vesicles were intact, round and cap-shape which conform to characteristics of exosomes (scale bar: 100 nm).

exosomes, while pellets before ultracentrifugation is absent (**Figure 1A**). The size of ovarian cells derived vesicles ranged from 30 nm to 100 nm and their appearance were intact, round and saucer-like observed by electron microscopy (**Figure 1B**). These results satisfy the characteristics of exosomes.

Proteomic analysis of exosomes extracted from two ovarian cancer cell lines

Total proteins were determined by MS/MS and only those identified with an expected value of less than 0.05, a false discovery rate of 1% and two or more peptides were involved. Data resulted in recognizing 1483 exosomal proteins in HO8910PM and 1467 exosomal proteins in OVCA429 respectively (Supplementary Table 1). Among them, 835 proteins were highly overlapped (**Figure 2A**). The overlapped proteins might contain bioactive proteins contributing to progression of ovarian cancer. Thus, we further explored those same proteins in order to figure out their potential role as diagnostic, prognostic and therapeutic biomarker for late-stage ovarian cancer. Moreover, massive identified proteins were the same, which could ensure the successive steps of exosome extraction and purification were reliable. Those proteins in our assay also compared to previous proteins derived from ovarian cell lines recorded in the ExoCarta database. Furthermore, the 251 overlapped proteins were not kept in Exocarta, which demonstrated our original analysis on proteomic study of exosomes from high-grade ovarian cancer.

Annotation of identified exosomal proteins

As for FR data procession connected to Excarta database, we further used Blast2go to compare their gene ontology (GO) enrichment analysis. Multilevel choose level was set at level-2 in Blast2go. The overlapped proteomic comparison between OVCA429 and HO8910PM cell lines were performed. The cellular component (**Figure 2B**), biological process (**Figure 2C**) and molecular function (**Figure 2D**) classified by Blast2go were compared to FunRich (**Figure 3A-C**) respectively. However, FunRich also supported biological pathway (**Figure 3D**) and transcription factor (**Figure 4A**) in select interfaces. Results in FunRich were simple and also provided a comparison function between two selected data (**Figures 3E-H**, **4B**). Comparison between FunRich and Blast2go were listed in **Table 1**.

Exosome biogenesis was a well regulated procedure which contained four steps: initiation, endocytosis, formation of multivesicular bodies and secretion. A great deal of proteins, lipids, DNA and RNAs were packaged by exosomes [13]. These bioactive proteins were accounted for cell proliferation, movement, migration and adhesion which were induce tumor metastasis. For example, on the cellular component aspect, as expected, exosomes shift a lot of cytoplasm and nucleus proteins out of cell to communicate target cells. Exosomes purified from HO8910PM had been detected to contain more nuclear proteins than OVCA429. Moreover,

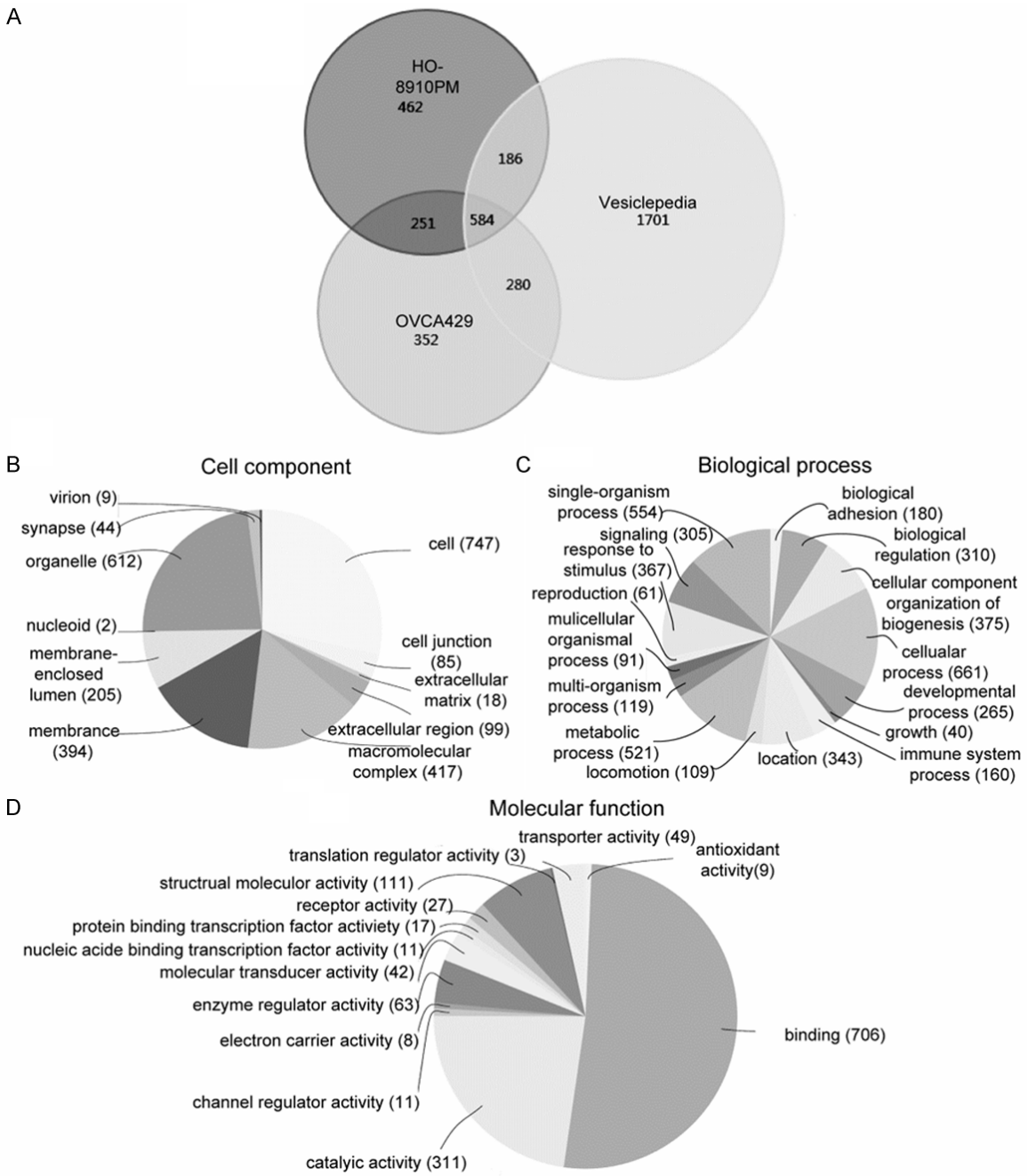

Figure 2. Analysis of exosommal proteins derived from late-stage ovarian cancer. (A) Three-way Venn diagram showing the overlap between exosomal proteins originated from HO8910PM and OVCA429 and Exocarta database. The number of proteins lies in three parts result in 584 proteins using FunRich. GO enrichment analysis of identified exosomal proteins by Blast2go. The cellular component (B), molecular function (C) and biological process (D) of overlap proteins were analyzed.

HO8910PM derived exosome-related transcriptional factor were slightly more than those from OVCA429. Therefore, we could deduce that many significant proteins in the nucleus were transported by exosomes to a distant niche that greatly improved cancer progression.

The overlapped exosomal proteins helped to reveal more information to study. As for the biological process, all the identified exosomal proteins were largely involved in signal transduction, protein metabolism, cell growth and/or maintenance and cell communication, while

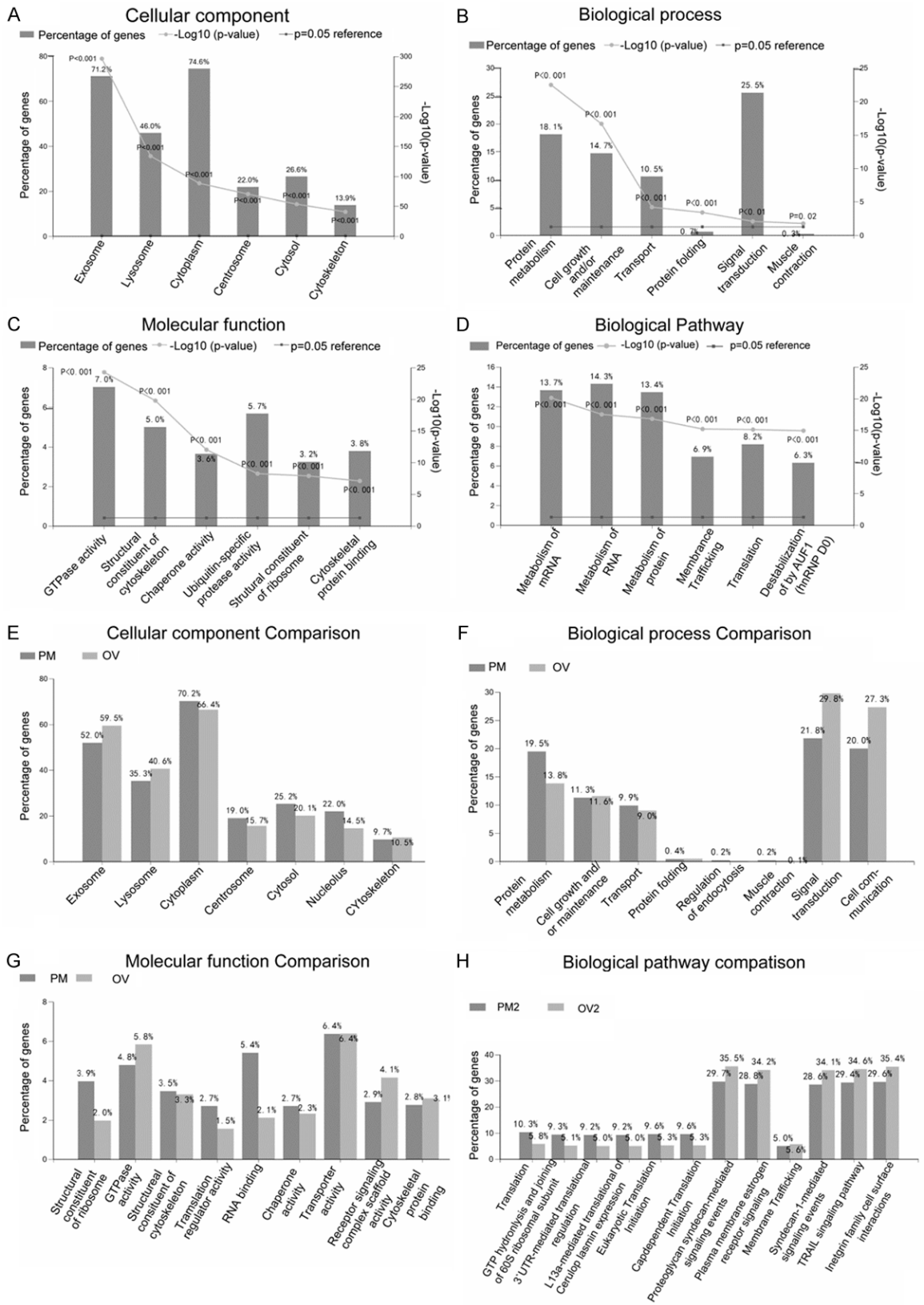

Figure 3. GO enrichment analysis of purified exosomes by FunRich. The cellular component (A, E), biological process (B, F), molecular function (C, G), and biological pathway (D, H) of HO8910PM, OVCA429 and overlapped proteins were compared, respectively. At biological aspects, metabolism of proteins and RNAs were outlined in the analysis of overlap exosomal proteins. PM refers to HO8910PM and OV refers to OVCA429 in the figure.

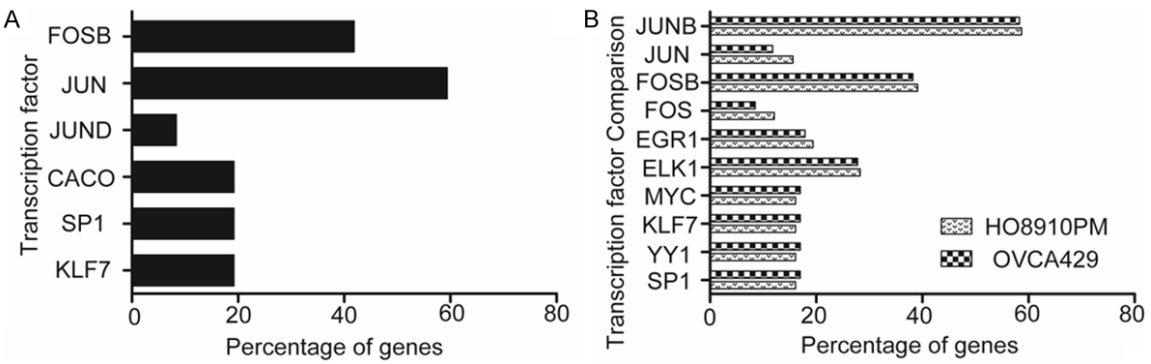

Figure 4. Transcription factor analysis by FunRich. A. SP1, KLF7, JUN, FOSB, JUND and CACD were significantly expressed among overlapped exosomal proteins (P<0.001). B. SP1, KLF7, EGR1 and ELK1 were the top four transcription factor by comparison between exosomal proteins originated from HO8910PM and OVCA429 respectively.

Table 1. The comparison of GO enrichment analysis between FunRich and Blast2go

	Advantages	Disadvantages
FunRich	Quickly; Include Exocarta data to compare; Provide transcription factor and biological pathway items; simply and intuitive; comparison function	Not specific enough
Blast2go	Specific and detailed; multilevel choose	Time consuming; not include Exocarta data

the overlapped proteins majored in those above processes expect for cell communication. In addition, on biological pathway analysis, the top three in overlapped proteins were metabolisms of mRNA, RNA and proteins, which greatly distinguish each group of proteins derived from cell lines (**Figure 3D**, **3H**).

KEGG pathway analysis of overlapped proteins

The overlapped exosomes derived from OVCA429 and HO8910PM may have contributed to the easily metastasized characteristic of high-grade EOC. Then we applied KEGG pathway to explore potential proteins as diagnostic and prognostic biomarkers and therapeutic targets (**Figure 5**). Regulation of cytoskeleton, tight junction, focal adhesion and gap junction were full proof for EOC metastatic. However, based on annotation results, we figured out metabolism reprogrammed played a dominant role in high-grade EOC-related exosomes. Among KEGG pathway results, we detected proteins related to pentose phosphate pathway (PPP), opening a significant key to further research (**Table 2**).

Discussion

Exosomes act as substantial vectors between tumor cells and the microenvironment, resulting in tumor carcinogenesis and metastasis [14]. Melo et al. showed that exosomes excreted from sera of patients with breast cancer and breast cancer cell lines could educate normal epithelial cells transferred into tumor cells by a Dicer-dependent pattern [15]. Josson et al. firstly found that exosomal miRNA extracted from prostate stromal cells leading prostate cancer cells epithelial-mesenchymal transition [16]. It has been determined that, in fact, exosomes are essential to intitiate pre-metastatic niche as a companion for soluble factors [17, 18]. Moreover, exosomes are a stable and reliable diagnostic biomarker and therapeutic strategy for clinical diseases, especially cancer. The existed biomarkers for ovarian cancer include Claudin-4, MAGE3/6, TGF-beta 1, miR-21, 141, 200a, 200b and so on [19-21]. In therapeutic application of cancer therapy, exosomes have been serving as anti-cancer drug delivery vehicles, immunotherapy and removal targets [22-26]. Therefore, exploring proteins or nucleus acid packaged by exosomes manifest an influential potential for ovarian cancer research.

Considering the diversity of ovarian cancer, we chose HO8910PM and OVCA429 cell lines that have not been studied in this field in the previous study. In addition, these two cell lines were all established from late stage of human ovari-

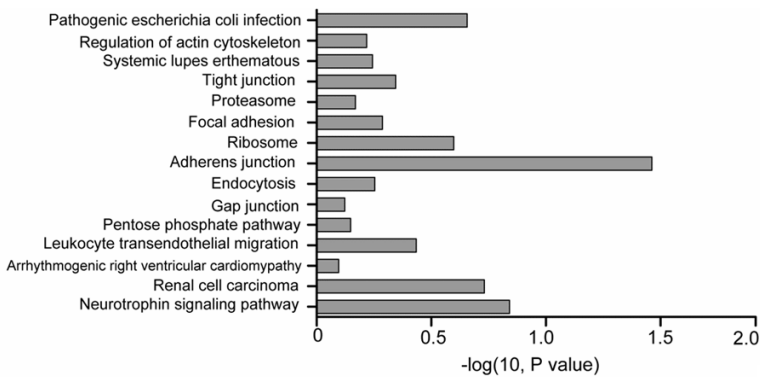

Figure 5. KEGG pathway analysis. The top fourteen pathways of overlapped proteins involved by David database.

Table 2. The overlap exosomal proteins detected in pentose phosphate pathway

Glucose-6-phosphate dehydrogenase
Hosphofructokinase, liver/platelet
Transaldolase 1
Transketolase
Aldolase A, fructose-bisphosphate
Glucose phosphate isomerase
Phosphoglucomutase 1/2

an cancer and their cell doubling time was about one and a half days, which was suitable for exosome study. Through differential ultracentrifugation to purified exosomes from cell lines, we attained high purity exosomes (**Figure 1**). However, this widely utilized method was time consuming, highly labor intensive and low yield [27]. So picking rapid proliferation cell lines was feasible.

ExoCarta is a free web-based database that catalogs lipids, RNA and proteins. It is easy to operate and provides FR software to analyze your findings about exosomes [28]. By using FR, a total of 2950 proteins detected by MS were quickly processed in GO enrichment. However, many research results did not upload to ExoCarta, and could explain why we found 814 exosomal proteins that were unreported in the ExoCarta database (**Figure 2A**). These results emphasized the innovation and significance of our assay as well. On the other hand, we used Blast2go as a contrasting way to perform GO enrichment analysis on the overlap detected proteins. Blast2go is a controversial bioinformatics solution for functional annotation and analyses of annotation data. **Table 1** showed that FR might be simpler and more convenient compared to Blast2go in exosomal proteins analysis. First of all, using FR, the procession time only took seconds, but using Blast2go, we consumed more than one day. Secondly, FR directly creates a Venn diagram in contrast to exosome datasbase ignoring download exosomal files. Thirdly, by contrast to Blast2go, FR holds an equally high quality and suitable for exosomal protein analysis. Take cellular composition for example, FR classifications include exosome, lysosome, while when using Blast2go these locations are absent. There are two main roles of lysosomes in life of exosomes: one is to degrade exosomes, the other one is help exosomes to fuse target cells' plasma membrane that make communicate feasible [29-31]. As for biological process, the description in FR was simpler and more basic than that in Blast2go. Cellular process is listed first in Blast2go with comprehensive indicated, while the first process in FR is protein metabolism which seemed more specific. In addition, transcription factors such as SP1, JUN, FOSB, KLF7 and so on were outlined in our study by FunRich analysis. These transcription factors would be a great help to further study for exosomes' role in EOC.

Through comparison of proteins derived from HO8910PM and OVCA429, we illustrated the pentose phosphate pathway was crucial for exosome regulated ovarian cancer survival, proliferation and metastasis. The main reasons were: in cellular component and biological processes, two high-grade ovarian cancer cells derived exosomes packaged lots of nucleus proteins and took part in metabolism; in biological pathway and KEGG pathway analysis, PPP manifests a significant role and function that is influentially involved in metabolic transformation that accounts for tumor development [32, 33]. Metabolic transformation is the key and first step for cancer evolvement, and is influenced by many oncogenes and tumor suppressor genes. Reprogrammed metabolism is essential for cancer cells to survive in microen-

vironment of metastatic sites and optimize their growth ability [34, 35]. Apart for providing energy, activation of PPP results in resisting oxidant injury that benefits tumor survival [36]. The most important role of PPP is to divert glucose from energy generation to ribose-5-phosphate (R5P) and namide adenine dinudeotide phosphate (NADPH) production. R5P is an influential precursor regulated nucleotide biosynthesis, while NADPH is major in lipid and DNA biosynthesis and oxidation resistance through reduction of glutathione. Glucose-6-phosphate dehydrogenase (G6PD) acts as the first and rate-limiting enzyme, while transketolase (TKT) and transaldolase 1 (TALDO1) play the other two key roles in PPP. Their activation leads to glucose transfer to non-oxidative branch and produces lots of NADPH and R5P [37]. In our study, G6PD, TKT, together with TALDO1 were all included in the overlapped exosomes with significant value. G6PD, TKT and TALDO1 were proved to be important regulated molecules in cancer. Li et al. indicated that G6PD influenced tumor proliferation and antioxidative capacity. Tumor cells with G6PD knockdown reduced growth and increased susceptibility to stress of oxidant [38]. Wang et al. showed that elevated G6PD expression was closely related to poor prognosis of human gastric cancer [39]. Hu et al. pointed out that G6PD via the STAT-3/5 pathway promotes tumor apoptosis and proliferation [40]. Ricciardelli et al. found that TKT played an influential role in promoting ovarian cancer cell growth and metastasis, which was consistent with our study's result [41]. Moreover, Basta et al. found that genetic polymorphisms of TALDO1 were associated with head and neck carcinomas [42].

In conclusion, we have successfully performed analysis of high quality exosomal proteins from two late-stage ovarian cancer cells, unlike any other proteomic analysis ever performed. FR has its own priority to perform the exosomal GO analysis, which would feasible and lucid to achieve our purpose. The common exosomal proteins in metastatic ovarian cancer lines reveal G6PD, transketolase and transaldolase 1 in pentose phosphate pathway would act as a diagnostic, poor prognostic and therapeutic target of late-stage ovarian cancer. Further studies should validate these candidates and explore the potential mechanisms behind them.

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Disclosure of conflict of interest

None.

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