The Protein Journal (2019) 38:586–597
https://doi.org/10.1007/s10930-019-09849-0

Characterization of Protein Profiling and mRNA Expression of LLC Exosomes

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Published online: 18 June 2019

Abstract

Circulating exosomes are promising biomarker source in various diseases. Exosomal constituents can stably exist in the circulating plasma and serum thus making them ideal biomarkers for a number of clinical applications. Exosomes can also mediate the occurrence of many types of diseases, including distal cancerous metastasis and tumour enlargement, through encapsulated proteins or RNAs, which regulate interactions among tissues. While performing these actions, exosomes show tissue specificity. However, the mechanism for such selection is not clear. For non-small cell lung cancer (NSCLC), molecular diagnostic markers and mechanisms of exosome-mediated tumour metastasis are not well understood. Therefore, in this study, we characterized LLC exosomal proteins and mRNAs by analysing their molecular profiles, laying a foundation for exploring diagnostic markers of lung cancer. Furthermore, the interactions between exosomal membrane proteins and their target proteins were analysed and revealed a possible tissue propensity of LLC cell-derived exosomes. These findings provide a theoretical basis for studying exosome-mediated tissue targeting and distal lung cancer metastasis.

Keywords LLC · Exosome · Proteomics · Transcriptomics · Target tissue analysis

1 Introduction

Exosomes are cell derived membrane vesicles (30 to 120 nm) that are formed through inward intracellular multivesicular endosome (MVE) membrane bulging and subsequent pinching off of small membranous vesicles within the MVE [1]. Exosomes have been widely reported to mediate local and systematic cell communications through transferring informations such as nucleotide fragments, mRNAs, miRNAs, and proteins [2, 3]. They have also been used as biomarkers for several clinical diagnostic and prognostic applications [4] based on their internal components, including miRNAs, mRNA, and proteins. For example,

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10930-019-09849-0) contains supplementary material, which is available to authorized users.

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MiRNA-1246, MiRNA-34a, MiR-29c have been reported to be potential biomarkers in oesophageal squamous cell carcinoma (ESCC) [5], Alzheimer's disease [6], prostate cancer [7] and fibrosis [8], respectively. Despite miRNAs, exosomal proteins and mRNAs can also be used as biomarkers. CNN2 protein, CD2AP mRNAs and hnRNPH1 mRNA have been reported to be novel biomarkers for hepatic fibrosis [9], kidney diseases [10], and hepatocellular carcinoma [11], respectively.

Exosomes are reported to be involved in many important physiological processes and play important roles on the pathogenesis of cancer including tumour growth, angiogenesis, immune escape and metastasis. Exosomes derived from prostatic cancer has been identified to have effects of reduced apoptosis, increased cancer cell proliferation [12]. Exosomes derived from Mesenchymal stem cells (MSCs) have blocking effects on tumour angiogenesis and reduced activity of VEGF and NF-κB [13]. In terms of exosomes' immune escape function, our lab early reported that tumour cell-derived miR-214 could promote tumour growth as well as host immune suppression by activating $CD4^+CD25^{high}Foxp3^+$ regulatory T cells (Treg) [14]. And Theresa et al. have given a good summary about many important roles played by exosomes along many aspects of tumour growth process [15].

On the other hand, exosomes have been suggested to have certain tendencies to particular tissues, and the preference of exosomes towards certain tissues plays important role in cancer metastases. For example, Helen et al. have identified an interaction between CD21 on B cells and the EBV glycoprotein gp30 on lymphoblastoid cell line (LCL1)-derived exosomes [16]. Integrins differ by their expression patterns are also suggested to be involved in organ-specific metastasis [17]. In addition, pancreatic cancer exosomes can promote cancerous metastasis to liver via building up the pre-metastatic niche [18–20]. All these examples of distant metastasis related to tumor derived exosomes (TDEs) suggests that the interaction between exosomal surface proteins and the target tissues play an important role during this process [21, 22]. Therefore, research investigating the natural targeting properties of exosomes is important for studying interactions among tissues.

In this study, we characterized the protein and mRNA profiles of NSCLC LLC cell-derived exosomes. The relevant functions and preliminary natural targets were analysed by applying Gene Ontology (GO) annotation and Kyoto Encyclopedia for Genes and Genomes (KEGG) pathway analysis. The protein–protein interaction (PPI) analysis was carried out by applying STRING® and the resultant PPI networks were further interpreted by Cytoscape software with nodes defined based on the degree value and the edge was defined based on the Edge Betweenness. This work provides a theoretical basis for studying the molecular profiles of exosomes in NSCLC and reveal potential biomarkers for diagnosis and treatment of NSCLC.

2 Materials and Methods

2.1 Cells and Cell Culture

LLC cells lines (ATCC) were used to isolate exosomes. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (ATCC 30-2002) containing 10% exosome-depleted fetal bovine serum (FBS) (ATCC 30-2020) and antibiotics (Gibco, CA, USA). Cell incubation performed at 37 °C in 5% CO_2 and final exosomes were depleted using a 0.22-μm filter and then ultra-centrifuged at 110,000*g* for 2 h.

2.2 Exosome Purification, Characterization and Analyses

LLC Cells were grown with FBS-free medium for 48 h and the supernatants were then collected for subsequent centrifugation steps. The centrifugation procedures were 300 g × 5 min, 3000 g × 30 min and 10,000 g × 60 min. Then, the enriched medium was treated by applying Exosome Isolation Kit (Invitrogen) to isolate exosomes from the prepared cell culture based on the manufacture's protocol [23, 24]. Exosomes were collected as pellets and subsequently re-suspended in phosphate-buffered saline (PBS). A 0.22-μm polyvinylidene fluoride (PVDF) membrane was used for clearing the purified exosome cell debris. At this stage, exosomes were ready for subsequently analysis including sequencing, western blot analysis and other biological studies.

2.3 RNA Isolation and Western Blot Analysis

LLC cell-derived exosomal RNA isolation procedures were carried out by using TRIzol LS Reagent (Invitrogen). Exosomes and proteins from LLC cells were quantified using antibodies against TSG101 MOUSE mAb (Santa Cruz sc-136111), CD63 Rabbit mAb (Santa Cruz sc-15363), CD9 Rabbit mAb (Santa Cruz sc-9148), Alix mouse mAb (Cell Signaling 2171s), GAPDH Mouse mAb (Santa Cruz sc-32233), KRAS Rabbit PAb (Proteintech 12063-1-AP), CDK1 Rabbit PAb (Proteintech 19532-1-AP), CDK3 Rabbit PAb (Proteintech 55103-1-AP).

2.4 LC–MS/MS and Data Analysis

The detailed methods of treatment and labelling of LLC exosomes, protein preparations and LC–MS/MS can be seen in our previous articles [25]. In terms of raw data analysis, Proteome Discoverer (Version 1.4.0.288, Thermo Fisher Scientific, Bremen, and Germany) with SEQUEST were

applied as the search engine. And the UniprotKB human database (downloaded on July 20, 2018) was applied for MS/MS spectra analysis. Modification related settings were cysteine carbamidomethylation for fixed modification. N-terminal acetylation and methionine oxidation were set as variable modifications. The peptide mass was set at 10 ppm, and the fragment mass tolerances was 20 mDa. A maximum of 2 missed cleavage sites was allowed. Filtration of a 1% false discovery rate were set for Peptide identifications.

2.5 Microarrays

Human genome expression analysis was performed by applying the Affymetrix GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, CA, USA) and the profiling analysis applied Affymetrix Expression Console software.

2.6 Functional Enrichment Analysis

Proteins identified in exosomes from either LLC or MIN6 cells were subjected to Gene Ontology (GO) and KEGG pathway analysis using Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources 6.7 (http://david.abcc.ncificrf.gov/) [26, 27]. The protein–protein interaction (PPI) network analysis was performed by applying the STRING® programme (10.5 version) [28]. The resultant PPI images were further imported into Cytoscape_v3.6.1 for better resolution based on adjustment of the node interaction degree and scores [29].

3 Results

3.1 Extraction and Characterization of Exosomes Derived from LLC

First, the LLC exosomes were purified by applying differential centrifugation methodologies and an Exosome Isolation Kit (Fig. 1a). Phenotypic features, including size and purity, were investigated using the TEM methodology (Fig. 1b). Western blot analysis proved that the exosome-specific proteins TSG101, CD63, CD9, and Alix were enriched in isolated exosomes but not found in cell lysates (Fig. 1c). Similarly, a high expression level of GAPDH was detected in cell lysates but not exosomes. Based on above results, it suggested that the exosome samples were well prepared and had a relatively high purity.

3.2 Proteomic Analysis of LLC Cell-Derived Exosomes

LC–MS/MS analysis was performed to examine the proteomic profiles of exosomes secreted by LLC cells. Two replicate samples of LLC cell-derived exosomes were studied and 1035 proteins were identified in common (S1 Table). Among these proteins, 120 proteins with an abundance value ≥ 2 were listed in Table 1. And 17 of 120 proteins with abundance value > 2 were presented in Fig. 2a. We analysed the commonly shared exosome-derived proteins using GO analysis of DAVID Bioinformatics

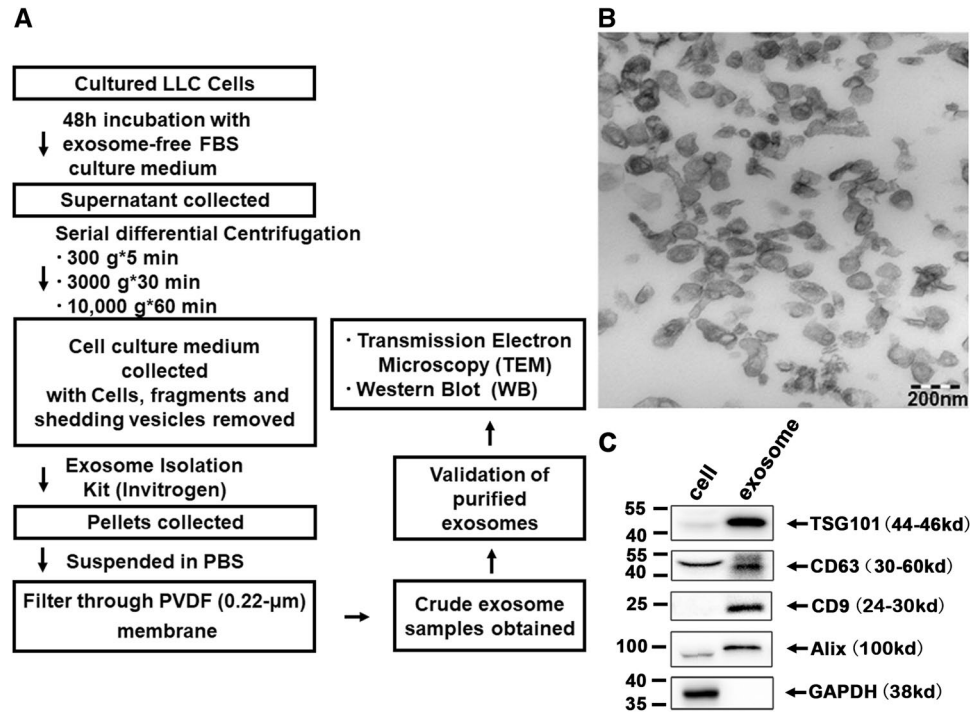


Fig. 1 Phenotypic characteristics of exosomes derived from LLC cells. **a** TEM image of exosomes isolated from the culture medium of LLC cells. **b** Western blot analysis of TSG101, CD63, CD9, and Alix protein levels in LLC cell-derived exosomes

Table 1 120 Identified proteins in LLC cell line-derived exosomes

From	To	Species	Gene name
P11440	Cdk1	Mus musculus	Cell division cycle 2 homolog A (*S. pombe*)
Q9R190	Mta2	Mus musculus	Metastasis-associated gene family, member 2
P68510	Ywhah	Mus musculus	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide
P27048	Snrpb	Mus musculus	Small nuclear ribonucleoprotein B
P63101	Ywhaz	Mus musculus	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide
Q60605	Myl6	Mus musculus	Myosin, light polypeptide 6, alkali, smooth muscle and non-muscle
Q91YH5	Atl3	Mus musculus	Atlastin GTPase 3
P27601	Gna13	Mus musculus	Guanine nucleotide binding protein, alpha 13
P98063	Bmp1	Mus musculus	Bone morphogenetic protein 1
P61205	Arf3	Mus musculus	ADP-ribosylation factor 3
P63321	Rala	Mus musculus	v-ral simian leukemia viral oncogene homolog A (ras related)
Q08879	Fbln1	Mus musculus	Fibulin 1
Q60872	Eif1a	Mus musculus	Eukaryotic translation initiation factor 1A
Q7TMM9	Tubb2a	Mus musculus	Tubulin, beta 2A
P21107	Tpm3	Mus musculus	Tropomyosin 3, gamma
Q6P1F6	Ppp2r2a	Mus musculus	Protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), alpha isoform
P60335	Pcbp1	Mus musculus	Poly(rC) binding protein 1
Q3UYH7	Adrbk2	Mus musculus	Adrenergic receptor kinase, beta 2
Q61029	Tmpo	Mus musculus	Thymopoietin
P0C0S6	H2afz	Mus musculus	H2A histone family, member Z
P70336	Rock2	Mus musculus	Rho-associated coiled-coil containing protein kinase 2
P60843	Eif4a1	Mus musculus	Eukaryotic translation initiation factor 4A1
P61027	Rab10	Mus musculus	RAB10, member RAS oncogene family
Q00612	G6pdx	Mus musculus	Glucose-6-phosphate dehydrogenase X-linked
P61226	Rap2b	Mus musculus	RAP2B, member of RAS oncogene family
Q80U72	Scrib	Mus musculus	Scribbled homolog (Drosophila)
P68373	Tuba1c	Mus musculus	Tubulin, alpha 1C
P62827	Ran	Mus musculus	RAS-like, family 2, locus 9
P11499	Hsp90ab1	Mus musculus	Heat shock protein 90 alpha (cytosolic), class B member 1
P62259	Ywhae	Mus musculus	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide
Q8R1J9	Tor2a	Mus musculus	Torsin family 2, member A
Q8R050	Gspt1	Mus musculus	G1 to S phase transition 1
O88952	Lin7c	Mus musculus	lin-7 homolog C (C. elegans)
P57780	Actn4	Mus musculus	Actinin alpha 4
P19157	Gstp1	Mus musculus	Glutathione S-transferase, pi 1
Q3THE2	Myl12b	Mus musculus	Myosin, light chain 12B, regulatory
P10126	Eef1a1	Mus musculus	Eukaryotic translation elongation factor 1 alpha 1
Q61733	Mrps31	Mus musculus	Mitochondrial ribosomal protein S31
P62835	Rap1a	Mus musculus	RAS-related protein-1a
P10107	Anxa1	Mus musculus	Annexin A1
Q8VDD5	Myh9	Mus musculus	Myosin, heavy polypeptide 9, non-muscle
Q9R0K7	Atp2b2	Mus musculus	ATPase, Ca++ transporting, plasma membrane 2
Q9CS42	Prps2	Mus musculus	Phosphoribosyl pyrophosphate synthetase 2
P63094	Gnas	Mus musculus	GNAS (guanine nucleotide binding protein, alpha stimulating) complex locus
Q8CCK0	H2afy2	Mus musculus	H2A histone family, member Y2
P62492	Rab11a	Mus musculus	RAB11a, member RAS oncogene family
P61982	Ywhag	Mus musculus	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide
P62874	Gnb1	Mus musculus	Guanine nucleotide binding protein (G protein), beta 1
P39054	Dnm2	Mus musculus	Dynamin 2
Q61120	Shc3	Mus musculus	src homology 2 domain-containing transforming protein C3

Table 1 (continued)

From	To	Species	Gene name
P18760	Cfl1	Mus musculus	Cofilin 1, non-muscle
P63017	Hspa8	Mus musculus	Heat shock protein 8
P55012	Slc12a2	Mus musculus	Solute carrier family 12, member 2
O35914	Bnc1	Mus musculus	Basonuclin 1
P09411	Pgk1	Mus musculus	Phosphoglycerate kinase 1
P35922	Fmr1	Mus musculus	Fragile X mental retardation syndrome 1 homolog
P63242	Eif5a	Mus musculus	Eukaryotic translation initiation factor 5A
P63268	Actg2	Mus musculus	Actin, gamma 2, smooth muscle, enteric
P55258	Rab8a	Mus musculus	RAB8A, member RAS oncogene family
P05213	Tuba1b	Mus musculus	Tubulin, alpha 1B
P21995	Emb	Mus musculus	Embigin
P83887	Tubg1	Mus musculus	Tubulin, gamma 1
Q62095	Ddx3y	Mus musculus	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked
P63037	Dnaja1	Mus musculus	DnaJ (Hsp40) homolog, subfamily A, member 1
Q8VDN2	Atp1a1	Mus musculus	ATPase, Na+/K+ transporting, alpha 1 polypeptide
O35737	Hnrnph1	Mus musculus	Heterogeneous nuclear ribonucleoprotein H1
Q9Z2U0	Psma7	Mus musculus	Proteasome (prosome, macropain) subunit, alpha type 7
P32883	Kras	Mus musculus	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
Q7TT50	Cdc42bpb	Mus musculus	CDC42 binding protein kinase beta
P97298	Serpinf1	Mus musculus	Serine (or cysteine) peptidase inhibitor, clade F, member 1
Q7TPR4	Actn1	Mus musculus	Actinin, alpha 1
Q9JI58	Raet1d	Mus musculus	Retinoic acid early transcript 1, delta
Q5SQX6	Cyfip2	Mus musculus	Cytoplasmic FMR1 interacting protein 2
Q08093	Cnn2	Mus musculus	Calponin 2
Q04736	Yes1	Mus musculus	Yamaguchi sarcoma viral (v-yes) oncogene homolog 1
P07901	Hsp90aa1	Mus musculus	Heat shock protein 90, alpha (cytosolic), class A member 1
P31324	Prkar2b	Mus musculus	Protein kinase, cAMP dependent regulatory, type II beta
P60710	Actb	Mus musculus	Actin, beta
P01899	H2-D1	Mus musculus	Histocompatibility 2, D region locus 1
Q9D8B3	Chmp4b	Mus musculus	Chromatin modifying protein 4B
Q91V24	Abca7	Mus musculus	ATP-binding cassette, sub-family A (ABC1), member 7
Q07133	Hist1h1t	Mus musculus	Histone cluster 1, H1t
Q8BIK4	Dock9	Mus musculus	Dedicator of cytokinesis 9
P28862	Mmp3	Mus musculus	Matrix metallopeptidase 3
Q61147	Cp	Mus musculus	Ceruloplasmin
Q9Z0N1	Eif2s3x	Mus musculus	Eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked
P05064	Aldoa	Mus musculus	Aldolase A, fructose-bisphosphate
P62137	Ppp1ca	Mus musculus	Protein phosphatase 1, catalytic subunit, alpha isoform
Q8BT60	Cpne3	Mus musculus	Copine III
Q80SW1	Ahcyl1	Mus musculus	*S*-Adenosylhomocysteine hydrolase-like 1
P59108	Cpne2	Mus musculus	Copine II
Q6PHZ2	Camk2d	Mus musculus	Calcium/calmodulin-dependent protein kinase II, delta
Q9QXS1	Plec	Mus musculus	Plectin 1
Q922D8	Mthfd1	Mus musculus	Methylenetetrahydrofolate dehydrogenase (NADP + dependent)
Q9JKB1	Uchl3	Mus musculus	Ubiquitin carboxyl-terminal esterase L3 (ubiquitin thiolesterase)
P61079	Ube2d3	Mus musculus	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)
P68254	Ywhaq	Mus musculus	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide
P62960	Ybx1	Mus musculus	Nuclease sensitive element binding protein 1 (Y box protein 1)
Q6PHN9	Rab35	Mus musculus	RAB35, member RAS oncogene family
Q9ESE1	Lrba	Mus musculus	LPS-responsive beige-like anchor

Table 1 (continued)

From	To	Species	Gene name
Q9CQI7	Snrpb2	Mus musculus	U2 small nuclear ribonucleoprotein B
P62715	Ppp2cb	Mus musculus	Protein phosphatase 2a, catalytic subunit, beta isoform
Q61235	Sntb2	Mus musculus	Syntrophin, basic 2
P17182	Eno1	Mus musculus	Enolase 1, alpha non-neuron
Q80YP0	Cdk3	Mus musculus	Cyclin-dependent kinase 3
P10853	Hist1h2bf	Mus musculus	Histone cluster 1, H2bf
P20152	Vim	Mus musculus	Vimentin
Q62159	Rhoc	Mus musculus	ras homolog gene family, member C
P46467	Vps4b	Mus musculus	Vacuolar protein sorting 4b (yeast)
Q3UX61	Naa11	Mus musculus	ARD1 homolog B (*S. cerevisiae*)
Q9CQW9	Ifitm3	Mus musculus	Interferon induced transmembrane protein 3
Q80UM3	Naa15	Mus musculus	NMDA receptor-regulated gene 1
Q8CI94	Pygb	Mus musculus	Brain glycogen phosphorylase
P08752	Gnai2	Mus musculus	Guanine nucleotide binding protein (G protein), alpha inhibiting 2
P35441	Thbs1	Mus musculus	Thrombospondin 1
Q9JI11	Stk4	Mus musculus	Serine/threonine kinase 4
Q8BVP5	Csnk1g2	Mus musculus	Casein kinase 1, gamma 2
O88551	Cldn1	Mus musculus	Claudin 1
P68372	Tubb4b	Mus musculus	Tubulin beta-2C
Q99JY9	Actr3	Mus musculus	ARP3 actin-related protein 3 homolog (yeast)

The expression levels of proteins in list were ≥ 2

Resources 6.7 software. Proteins were categorized by protein class, cellular component, molecular function, and biological process. The relative annotations represent specific functional enrichment of proteins (with data presentation of $-\log_{10} P$ value). And a high protein categorization value (%) refers to a greater functional enrichment. The protein classes of LLC cell-derived exosome were mostly associated with nucleic acid binding (21%) and hydrolase (15%) (Fig. 2b). Of the cellular component classifications, cell part (43%), organelle (27%), and macromolecular complex (22%) accounted for the majority of the contents (Fig. 2c). In terms of molecular function, the identified proteins showed high percentages of catalytic activity (40%) and binding (38%), which suggested that the LLC cell-derived exosomes possessed these two essential molecular functions (Fig. 2d). The biological processes of LLC cell-derived exosomal proteins were mainly concerned with cellular process (32%), metabolic process (25%), cellular component organization and biogenesis (10%) (Fig. 2e). On the other hand, a KEGG pathway identified through the DAVID Bioinformatics Resource. As shown in Fig. 2e, there were significant numbers of enriched proteins associated with the ribosome (119.4) and proteasome (59.34), and weaker associations with aminoacyl-tRNA biosynthesis (21.69), spliceosome (19.25), ECM-receptor interaction (18.06), and focal adhesion (15.40). Taken together, the LLC cell-derived exosomes may suggest to play functional roles involving synthesis, processing and degradation of DNA, RNA and proteins. In addition, they were involved in the maintenance of cell or tissue structure, function and carrying signals for cellular ECM. The identified pathway information was consistent with the biological process analysed above.

The PPI network of LLC cell-derived exosomal proteins revealed 158 nodes (proteins with detected number ≥ 1 were selected) and 970 edges (Fig. 2g) (S2 Table). About 50% of proteins showed significant high degree values. Actin (ACTB) is crucial for several biological functions including cell motility, synaptic vesicle endocytosis, postsynaptic actin cytoskeleton organization, regulation of protein localization to plasma membrane. Heat shock protein HSP 90-beta (HSP90AB1) is generally involved in protein folding. And it is involved in the process of cell maturation, structural maintenance and regulation of target proteins. Cyclin-dependent kinase 1 (CDK1) is a protein kinase family and its function is tightly connected with the regulation of cell cycle control. In order to validate the potentiality of exosomal proteins to be biomarkers, we selected a group of proteins with high expression values, including CDK1, CDK3, PPP1CA, CDC42 and KRAS, Alix here were reference genes. And western blot analysis were performed (Fig. 2h). The levels of exosomal proteins of LLC cell line were compared with that of CT26 and Hepa1-6. Results show that CDK1 and CDK3 are more abundant in LLC exosomes and KRAS was

abundant in exosomes of all these three types of cancer cells, while PPP1CA and CDC42 were not detected.

3.3 Conjoint Analysis of the mRNA and Protein Profiles of LLC-Derived Exosomes

In addition to the proteomic analysis of LLC-cell derived exosomes, the transcriptomics were analysed. Two replicas of the exosome samples were analysed based on the whole-genome expression arrays, and 2531 co-expression signals were identified (Fig. 3a) (S3 Table). Molecular functional studies of these mRNA transcripts revealed that among seven kinds of molecular functions, catalytic activity (45%) accounted for the largest part, followed by binding (37%), and transport activity (10%) (Fig. 3b). The intersection analysis among LLC exosomal proteins and mRNA transcripts showed 557 genes in common (Fig. 3c). Additionally, the KEGG pathway analysis showed that these genes were mostly related to oxidative phosphorylation (59.64), Huntington's disease (46.48), Parkinson's disease (32.10), and Alzheimer's disease (31.59), which was similar to the molecular function analysis (Fig. 3d). Taking this into consideration, the data suggested that both RNA and proteins identified in the exosomes were translated or not had no significant correlations. For PPI of LLC exosomal mRNAs, top 100 of 2531 mRNAs were selected for PPI analysis and 92 nodes and 2849 edges identified (Fig. 3e) (S4 Table). Majorities of mRNAs with high degree values were translated to ribosomal proteins, such as Rps3a1, RPL41, RPL29, RPL17A. These proteins were either structural constituents or functional participants of ribosomes. There were UBA52 and UBB which were ubiquitin related proteins and involved in post translational modification and ubiquitin dependent translation. On the other hand, Eukaryotic translation elongation factor (EEF1A1) as its name indicates, it played major role in mRNA binding, receptor tyrosine kinase binding, translation initiation factor activity. Nascent polypeptide-associated complex subunit alpha (NACA) has function of preventing inappropriate targeting of non-secretory polypeptides to the ER.

3.4 Target Tissue Analysis of LLC Exosomes

Exosomes mediated tissue targeting transport possibly by interacting with relevant receptors through surface membrane proteins. Therefore, it was necessary to identify proteins that were able to mediate exosome transport, which was very important for studying the functions and mechanisms of the exosomes. A total of 1035 proteins derived from LLC exosomes were further analysed for KEGG pathway. The signaling pathways involved in cell interaction were selected for protein interaction studies, including ECM-receptor interaction (Fig. 4a), gap junction (Fig. 4b), focal adhesion (Fig. 4c), adherens junction (Fig. 4d) and tight junction (Fig. 4e), and the details were listed in supplemental Table S5. The exosome proteins are labelled with stars in the figure, proteins present on the membrane interacting with the proteins labelled with a star on the opposite site were considered to be its target protein.

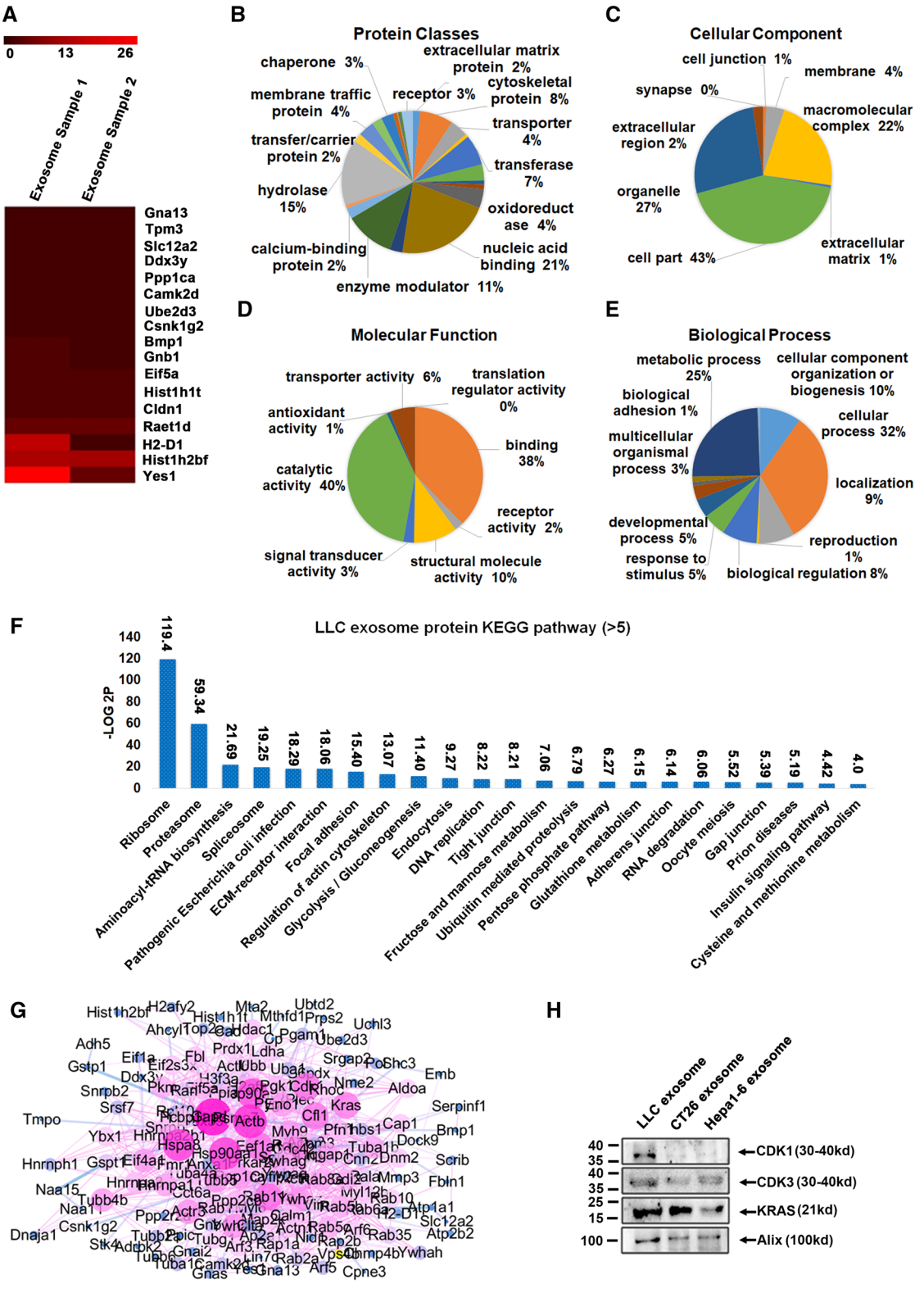
To exert further efforts on the distribution of these target proteins, we applied the UP-TISSUE tool from the DAVID Bioinformatics Resource to investigate the tissue distribution of our protein targets of interest. The target proteins of LLC cell-derived exosomes were mainly associated with brain, mammary gland, bone marrow, placenta, lung, heart and liver (Fig. 4f) (S6 Table).

4 Discussion

Exosomes play vital roles in the pathogenesis of diseases, especially in the occurrence, angiogenesis and metastasis of cancer. They are naturally secreted exocrine bodies with characteristics of low immunogenicity and high efficiency. Compared with traditional drug delivery methods, including virus carriers and nano-particles, exosomes tend to have advantages of easy release, avoiding immune clearance, and high working efficiency. Therefore, exosomes have been broadly studied as drug delivery cargo in vivo.

The molecular composition of exosomes are rich in different molecules including mRNAs, microRNAs, and proteins. Many studies have found out that they are functionally important in applied as diagnostic markers [30], mediating cell–cell interaction and natural targeting. For example in HCC, exosomal IncRNA-RP11-513I15.6, miR-1262, and RAB11A have been suggested as potential diagnostic factors [31]. Exosomes derived from fibroblast are suggested to promote breast cancer cell metastasis through activating Wnt-planar cell polarity (PCP) signaling [32]. Secreted exosomal miR-150 has ability to promote angiogenesis in both vitro and vivo [33]. What's more, exosome integrins $\alpha_6\beta_1$ and $\alpha_v\beta_5$ were suggested to tightly associate with the cancerous metastasis of bone-tropic tumour to lung and liver, respectively [17]. Therefore, studies of the molecular composition of exosomes are very important to further clarify the function and use of exosomes as tools to treat diseases.

Lung cancer is a kind of highly malignant cancer and endangers human health due to its increased mortality and morbidity. In order to explore the role of lung cancer derived exosomes during the process of cancerous pathogenesis and metastasis, it is necessary to study the function of lung cancer-secreted endocrine bodies. Among genes identified from LLC cell derived proteins and mRNAs, only few proteins have been reported including FN1 [22] as a diagnostic marker for gastric cancer and FASN [34] as a candidate biomarker for prostate cancer. Since exosome are potential



diagnostic markers for lung cancer, its surface proteins have attracted much attentions. Several lung cancer derived exosomal proteins have been reported as diagnostic markers for lung cancer, such as LRG1 [35], EGFR [36], CD9, CD63, CD81 [37], GRB2, SRC [38], CD151, CD171, tetraspanin8 [39], TCF21 [40], NY-ESO-1, PLAP, EpCam, Alix [41] and Tim-3 and Galectin-9 (Exo-T/G) [42]. In our study, majorities of identified proteins are not being reported yet, and

◂**Fig. 2** Proteomic analysis of LLC cell-derived exosomes. A heat map of 17 proteins with expression value > 2 derived from LLC exosome (**a**). A total of 1035 proteins were co-expressed among three samples. The proteins in LLC cell-derived exosomes were analysed by GO **b** protein classes, **c** cellular component, **d** molecular function, **e** biological process annotation, and **f** KEGG pathway **g** PPI network of proteins derived from LLC exosomes. Only nodes with interactions were shown and the network edges were calculated based on confidence and the rest settings were default including the minimum medium confidence of 0.4, and seven active interaction sources. Each node represents a protein and nodes with red colour are proteins with high degrees. Lines represent protein–protein associations. For sold line with thick patterns are associations with highest edge confidence. **h** Western blot analysis of CDK1, CDK3, KRAS and Alix protein levels in LLC, CT26 and Hepa1-6 cell-derived exosomes

we strongly believe that these proteins have great potentials to be diagnostic markers for lung cancer. ACTB, HSPA8, HSP90AB1, KRAS, CDK1, CFL1, RAB11A, YWHAE, CDC42, CLDN1, ACTR3, TUBB4B, EIF4A1, PPP1CA, TUBB1, and ENO1 are proteins which is highly correlated with lung cancer. Among these proteins, our results find that CDK1 and CKD3 are enriched specifically in exosomes derived from lung cancer cell line, suggesting that they may serve as potential markers for lung cancer. Intriguingly, KRAS was somehow globally abundant in exosomes derived from cancer cells, indicating that it may be a common indicator of cancer.

Our results identified that LLC exosomal proteins and mRNAs mainly possess catalytic and binding activity. And the protein KEGG pathways were dominantly ribosome and proteasome-related. This may suggest that LLC cell-derived exosomes could interact with the targeted cells and thus mediate the regulatory effects caused by LLC cells. Therefore, analysis of these results offers an innovative research

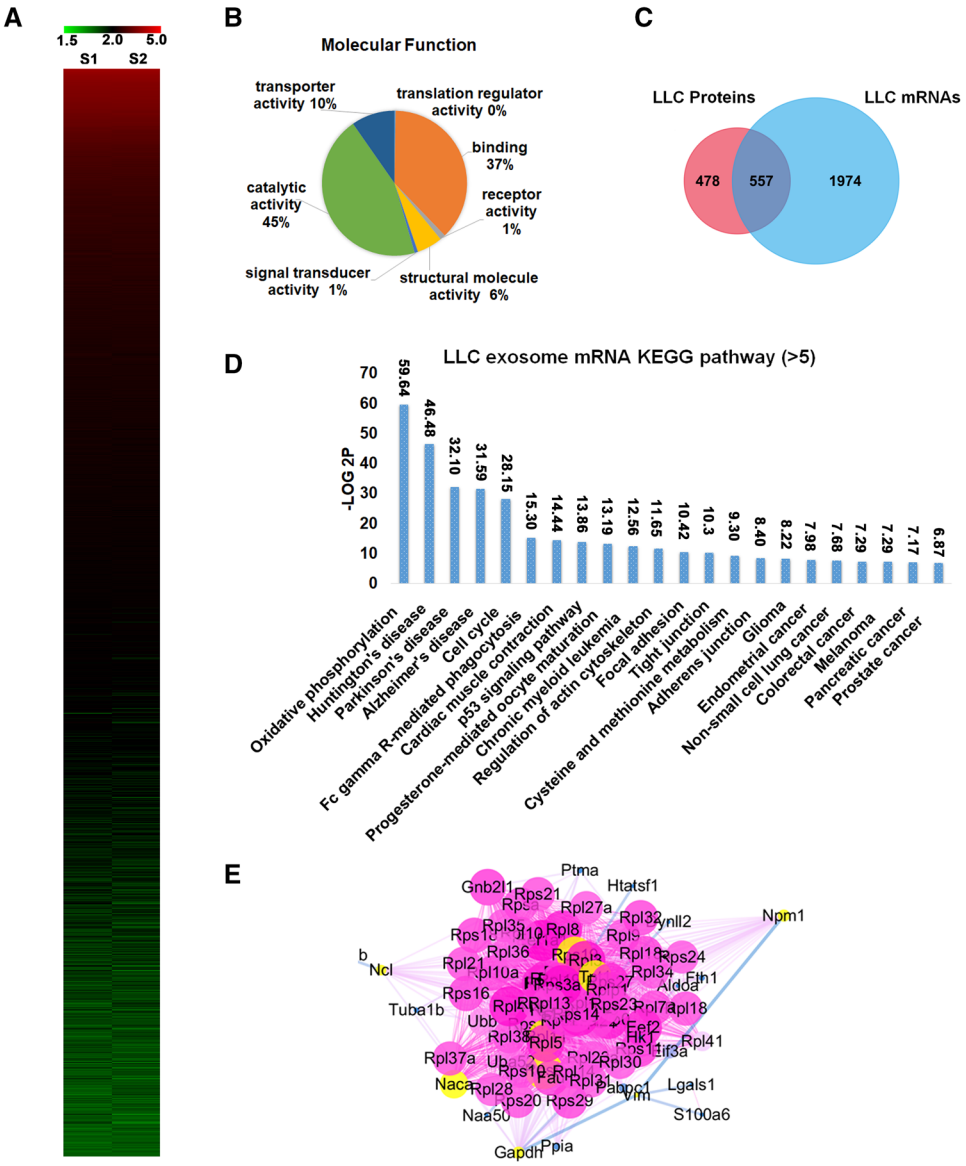


Fig. 3 Conjoint analysis of the mRNA and protein profiles of LLC cell-derived exosomes. **a** A heat map of 2531 mRNAs derived from LLC exosomes. **b** Molecular functional classification of 2531 mRNAs derived LLC exosomes. **c** Venn diagram of LLC exosomal mRNAs and proteins. 557 genes were shared between proteins (pink) and mRNA (blue) s. **d** KEGG pathway analysis of LLC exosomal mRNAs. **e** PPI network of mRNAs derived from LLC exosomes. Only nodes with interactions were shown and the network edges were calculated based on confidence and the rest settings were default including the minimum medium confidence of 0.4, and seven active interaction sources. Each node represents a protein and nodes with red colour are proteins with high degrees. Lines represent protein–protein associations. For sold line with thick patterns are associations with highest edge confidence

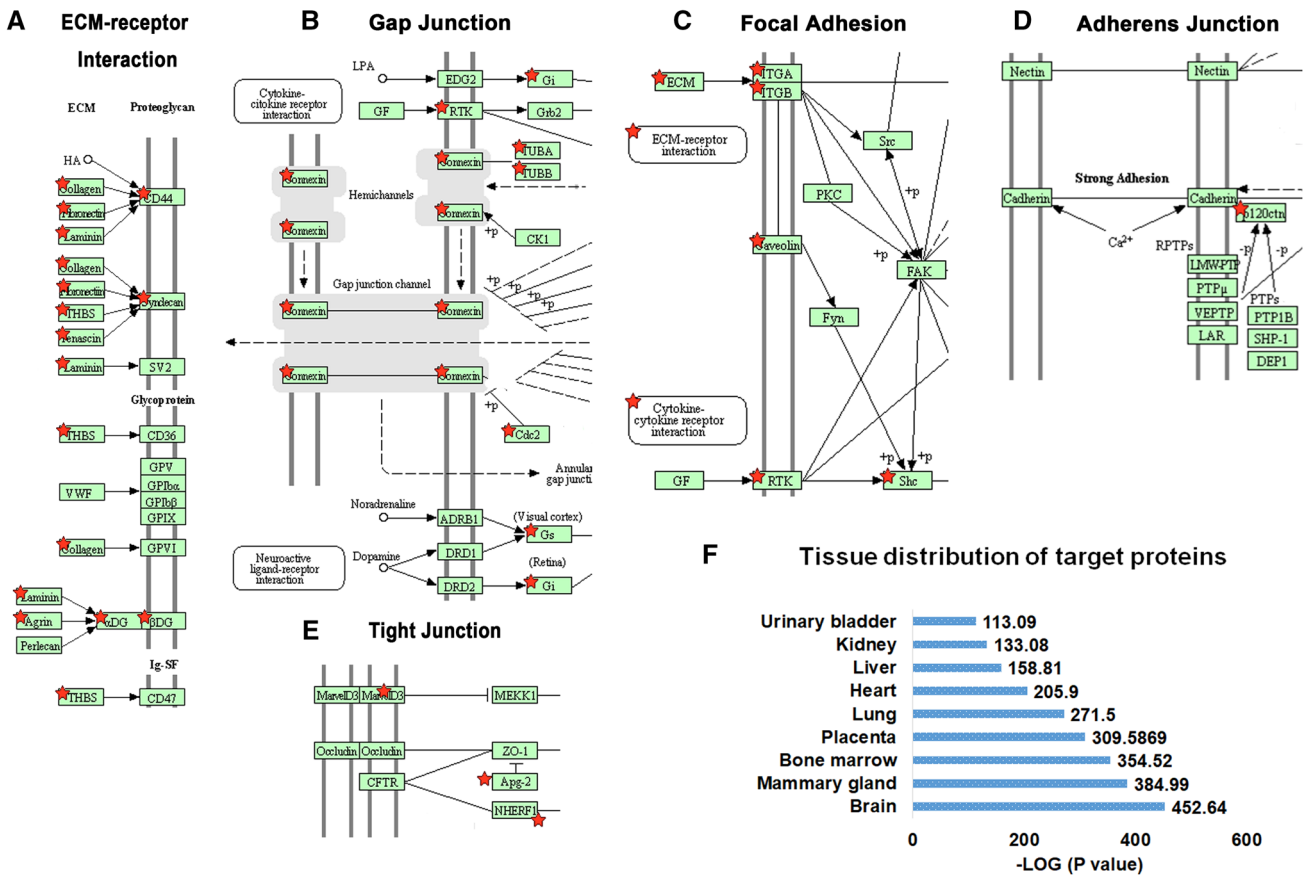


Fig. 4 Functional analysis and target tissue prediction of LLC exosomal proteins. Analysis of the specific interactions of specific membrane binding proteins derived from LLC cell-derived exosomes, respectively. The functional annotation charts obtained from DAVID Bioinformatics Resources 6.7, in terms of ECM-receptor interaction (**a**), gap junction (**b**), focal adhesion (**c**), and adherens junction (**d**), tight junction (**e**), target tissue analysis (**f**). Proteins marked with stars are the proteins derived from LLC exosomes

direction for studying the regulatory effects of cancer tissues to their target tissues via exosomes.

Additionally, exosomes tend to have preferences for their target tissues. They play important role in the aspects of target tissues selection, regulatory effects of lung cancer on target tissues and metastasis. Thus, we further examined the natural targeting characteristics of LLC cell-derived exosomes. Previous studies have reported that lung cancer is easily metastasized to the brain [43], liver [44], lymph node [45], and bone [46]. In this study, the target proteins of the proteins derived from LLC exosomes are dominantly associated with brain, mammary gland, bone marrow, placenta, heart and liver, which are consistent with earlier reports. Based on our results, recognition of LLC exosomal CD47 and ECM protein THBS1/THBS2 may lead to exosome targeting to brain and therefore promote brain metastasis. Exosomal surface DAG1 binds to LAMA4 from ECM may facilitate exosome targeting to mammary gland, further leading to lung cancer mammary gland metastasis. In addition, our finding identified that recognition of exosomal surface ITGA5 and ECM COL5A2 could cause lung cancer bone marrow metastasis. In terms of the metastasis to liver, interactions between COL1A1/COL1A2 and SDC1 could cause the metastasis transfer to liver.

These studies provide a theoretical basis for investigating exosomal mediated distal metastasis. Moreover, certain exosomes containing highly expressed proteins and mRNAs could serve as potential prognostic and diagnostic biomarkers for lung cancer. Of course, these predicted results need to be confirmed by a large number of experiments. And these results provided new research directions for study of the natural targeting of exosomes and the metastasis of lung cancer mediated by exosomes.

Moreover, these data provide a basis for the selection of LLC cell exosomes as drug delivery system. As important drug delivery system, exosomes have been extensively studied for its roles in the pathogenesis of diseases, especially in the development, angiogenesis and metastasis of cancers. The molecules delivered by exosomes, such as proteins, mRNAs and miRNAs, are involved in these processes. And these disease-promoting molecules will have side effects on patients when exosomes were used as drug delivery vehicles. A comprehensive analysis of the components of LLC exosomes will

provide a better understanding for biology of LLC cell-derived exosomes. And the same time, by studying the natural targeting of exosomes, drugs can be delivered to the target tissues more effectively.

In conclusion, this study examined the expression profiles of proteins and mRNAs of exosomes derived from LLC cells. And these results provide a theoretical basis for investigating the function of lung cancer cells secreted exosomes on the processes of lung cancer. Moreover, certain exosomes containing highly expressed proteins and mRNAs could serve as potential prognostic and diagnostic biomarkers for lung cancer. And the predicted results need to be confirmed by further experiments.

Acknowledgements We thank for Pei He and Shibei Wang collecting exosomes of LLC cells and running assays for following bioinformatics investigations in this study. We thanks Yujing Zhang designing and supervising this work.

Funding This work was supported by grants from the National Natural Science Foundation of China (No. 31771666, 31741066, 31200969) and the Industrialization of Mongolian Medicine of "Prairie Talents" engineering (NEI ZU TONG ZI No. 2015-56).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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