Cancer Letters 457 (2019) 142–150

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Malignant ascites-derived exosomes promote peritoneal tumor cell dissemination and reveal a distinct miRNA signature in advanced gastric cancer

Yanting Hu[a,1], Changsong Qi[a,1], Xiang Liu[b], Cheng Zhang[a], Jing Gao[a], Yi Wu[a], Jing Yang[a], Qian Zhao[a], Jian Li[a], Xiaojuan Wang[a,c,*], Lin Shen[a,**]

[a] Department of Gastrointestinal Oncology, Key Laboratory of Carcinogenesis and Translational Research Ministry of Education/Beijing, Peking University Cancer Hospital & Institute, Beijing, 100142, China
[b] Echo Biotech Co., Ltd, Beijing, China
[c] Department of Hepatobiliary and Pancreas Surgery, Beijing Tsinghua Changgung Hospital (BTCH), School of Clinical Medicine, Tsinghua University, Beijing, China

Keywords:
Gastric cancer
Peritoneal metastasis
Ascitic fluid
Exosomal miRNA

A B S T R A C T

Peritoneal dissemination (PD) is the most frequent metastasis with poor prognosis in patients with advanced gastric cancer (GC). However, the molecular mechanisms of PD remain poorly defined. Exosomes play a pivotal role in cancer progression. Thus, this study aims to investigate the effects of malignant ascites (MA)-derived exosomes from GC patients on tumor cells and to elucidate the underlying mechanism. *In vitro* and *in vivo* analysis showed that compared to exosome-depleted supernatants, exosomes from MA of GC patients promoted invasion of AGS cells by up-regulation of Epithelial-mesenchymal transition (EMT) signaling. In a mouse abdominal xenograft model, the median survival was shorter after MA-derived exosomes treatment than the control group (35.5 days versus 67 days, p = 0.0005). Moreover, 29 exosomal miRNAs from ascites were identified by high throughput sequencing among 8 paired GC patients before and after peritoneal chemotherapy and 3 individuals with non-malignant disease. In summary, MA-derived exosomes from patients with GC promote EMT signaling in GC cells and in mouse peritoneal tumor model. Differential exosomal miRNAs might be targeted therapeutically for inhibiting peritoneal metastasis, which provides new insights for the molecular mechanisms of PD in GC.

1. Introduction

Gastric cancer (GC) is the fifth most prevalent lethal malignancies worldwide and the third leading cause of cancer-related death [1]. Peritoneal dissemination (PD) is the most common metastasis from GC and this type of spread results in higher death rate compared to distant organ metastasis in GC [2,3]. Without treatment, the median survival of the GC patients with peritoneal metastases is 3–5 months with a 5-year survival rate of 2% [2]. For gastric cancer with PD, current treatment recommendations are poorly defined and remain unsatisfactory. Therefore, there is a need to reveal the underlying mechanisms of PD, as well as to develop more therapeutic targets for GC with PD.

Exosomes are membrane vesicles generated from the multivesicular endosomes, with diameters ranging in size between 30 and 100 nm [4,5]. Exosomes are released virtually in all types of cultured cells and are abundant in many kinds of body fluids, including plasma, saliva, urine and malignant effusions [6]. Extensive studies have shown that exosomes play a critical role in cell communication, transferring proteins, lipids, DNA and RNAs (microRNAs, mRNA) between cells [7]. Cancer-derived exosomes may promote metastasis by remodeling the tumor microenvironment and altering the extracellular matrix to attract more cancer cells to the niches [8–11]. Qu et al. demonstrated that gastric cancer-derived exosomes promoted peritoneal metastasis through disrupting mesothelial barrier and inducing peritoneal fibrosis

Abbreviations: GC, gastric cancer; PD, peritoneal dissemination; MA, malignant ascites; Exo, exosomes; EMT, Epithelial-mesenchymal transition; miRNAs, microRNAs

* Corresponding author. Department of Hepatobiliary and Pancreas Surgery, Beijing Tsinghua Changgung Hospital (BTCH), School of Clinical Medicine, Tsinghua University, Beijing, China.
** Corresponding author.
E-mail addresses: wxj0250@163.com (X. Wang), shenlin@bjmu.edu.cn (L. Shen).
[1] Yanting Hu and Changsong Qi contributed equally to this work.

https://doi.org/10.1016/j.canlet.2019.04.034
Received 22 October 2018; Received in revised form 9 April 2019; Accepted 26 April 2019

[12]. Exosomes isolated from malignant ascites in patients with GC could promote mesothelial cells (MCs) proliferation and convert MCs into carcinoma-associated fibroblasts (CAFs) thus contributing to peritoneal metastasis [13]. Thus, exosomes derived from cancer cells display crucial role in the formation of premetastatic environment during the process of gastric peritoneal metastasis. However, through which contents within the exosomes that play a regulatory role on peritoneal metastasis is still not clear.

Exosomes are found to be enriched in a subset of microRNAs (miRNAs) from their parental cells [14]. MiRNAs are ~22 nucleotides non-coding RNAs that regulate gene expression via posttranscriptional regulation as oncogenes or tumor suppressors [15]. MiRNAs has been shown to be deregulated in several process of cancers, suggesting potential candidates as diagnostic, prognostic and predictive biomarkers [16]. Previous studies have found several exosomal miRNA signatures as potential biomarkers for tumor types and identified specific exosomal miRNA clusters as potential candidates for diagnostic and prognostic biomarkers [17–19]. Several studies have identified distinct exosomal miRNA profiles in gastric cancer [20]. Serum exosomal miRNAs (miR-10b-5p, miR-132-3p, miR-185-5p, miR-195-5p, miR-20a-3p, miR-296-5p) were highly expressed in gastric cancer patients [21]. Wang et al. identified serum exosomal miR-19b-3p and miR-1106a-5p as potential diagnostic biomarkers for gastric cancer [22]. Tokuhisa et al. suggested that miR-21 and miR-1225-50 from peritoneal lavage may serve as novel potential diagnostic markers of peritoneal recurrence after gastric cancer resection [23]. However, the role of malignant ascites-derived exosomes in the process of gastric PD is not clear. And the molecular mechanisms by which exosomal communication impacts cancer cells are poorly defined.

In the current study, we investigated the role of malignant ascites-derived exosomes from the patients with GC and evaluated their invasive effects on gastric cancer cells in *in vitro* experiments and *in vivo* mouse model. We also assessed the exosomal miRNA profiles in ascites fluid from 8 individuals with GC and 3 individuals with non-malignant diseases. Differential miRNAs expression signatures were identified between non-malignant ascites and malignant ascites. Interestingly, the ascites-derived exosomes from individuals with GC had higher levels of miR-760, miR-6821-5p, miR-4745-5p, miR-200a-5p, miR-4741 and miR-320 compared to those from individuals with non-malignant diseases.

2. Material & methods

2.1. Biofluids preparation

Biofluids were obtained from Peking University Cancer Hospital. Ascites was obtained by celiac puncture and centrifuged at 2000g for 30 min. All samples were aliquoted and stored at −80 °C. All patients have written informed consent for their tissues to be used in research. This study was approved by the Ethics Committee of Peking University Cancer Hospital.

2.2. Cell lines and cell culture

The human GC cell line AGS was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). AGS cells were cultured in DMEM (11965-092) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 100 U/mL penicillin, and 100 μg/ml streptomycin (10378016, Life Technologies). To purify exosomes, conditioned medium was collected from AGS cells cultured for 48 h in DMEM supplemented with 10% exosome-depleted FBS (Life Technologies). All cultures were maintained at 37 °C with 5% CO_2.

2.3. Exosome isolation and quantification

Exosomes were isolated from the malignant ascites and non-malignant ascites by four successive centrifugation steps [24]. Briefly, ascites 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 (Optima MAX-XP, Beckman Coulter, rotor TLA 100.3). 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 suspended in PBS. The pellets were also dissolved in 1 ml Trizol LS reagent (Invitrogen, 10296010) for RNA isolation and 200 μl RIPA buffer (Beyotime, P0013C) for protein assay.

2.4. Electron microscopy

For transmission electron microscopy, freshly prepared exosomes were fixed by 1% glutaraldehyde for 2 h at room temperature. Then exosomes were loaded on carbon coated grids. Grids were then washed with PBS (membrane side faced down) and dried using a filter paper. For negative staining, exosome-grids were transferred to a 3% phosphotungstic acid solution (pH 7.0) for 10 min and then wicked off with filter paper. The microscopy images were captured by a Hitachi H-9000 transmission electron microscope at 300 kV with a slow-scan CCD camera.

2.5. Western blot analysis

Western blot was used to identify the surface markers of exosomes: CD81 (Santa cruz, 166028); CD63 (Abcam, ab59479); Tsg101 (Proteintech, 4497-1-AP), negative marker protein Calnexin (Cell Signaling, 2679S), and protein levels of E-cadherin (Cell Signaling, 3195); N-cadherin (Cell Signaling, 13116) and vimentin (Cell Signaling, 5741). Briefly, preparations were normalized for protein content and 5 × loading buffer was added and heated at 99 °C for 5 min. Then, the samples were loaded on 10% SDS- polyacrylamide gels and electrophoretic transferred to the nitrocellulose membranes at 300 mA for 2 h. The membrane was blocked in 5% non-fat milk and then incubated with the following primary antibody: anti-CD81 (1:500); anti-CD63 (1:500); anti- Tsg101 (1:1000); anti- Calnexin (1:2000); anti- E-cadherin (1:1000); anti- N-cadherin (1:1000); anti-vimentin (1:1000) at 4 °C overnight. After carefully washing with 1x Tris-buffered saline with tween-20, the membrane was incubated with secondary antibody (1:2000) at room temperature for 1 h. The protein band was detected using the chemiluminescence reagent ECL Select (GE Healthcare Life Sciences™) and images were acquired with Chemidoc™ gel imaging system (Bio-Rad).

2.6. Exosome labeling

To examine whether gastric cancer cell culture medium-derived or ascites-derived exosomes can be taken up by AGS, PKH26 (Sigma-Aldrich, PKH26GL) was used to label the exosomes [25]. The exosomes or PBS were stained with PKH26 dye in 400 μl 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 incubated with AGS cells with complete medium for 4 h 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 were counterstained with DAPI for 8 min and cell membrane were counterstained with PKH67 (Sigma-Aldrich, PKH67GL) for 5 min. The uptake of the labeled exosomes by AGS cells was assessed using an inverted confocal microscope.

2.7. Cell invasion assays

AGS gastric cancer cells were seeded in the upper chambers of 24-

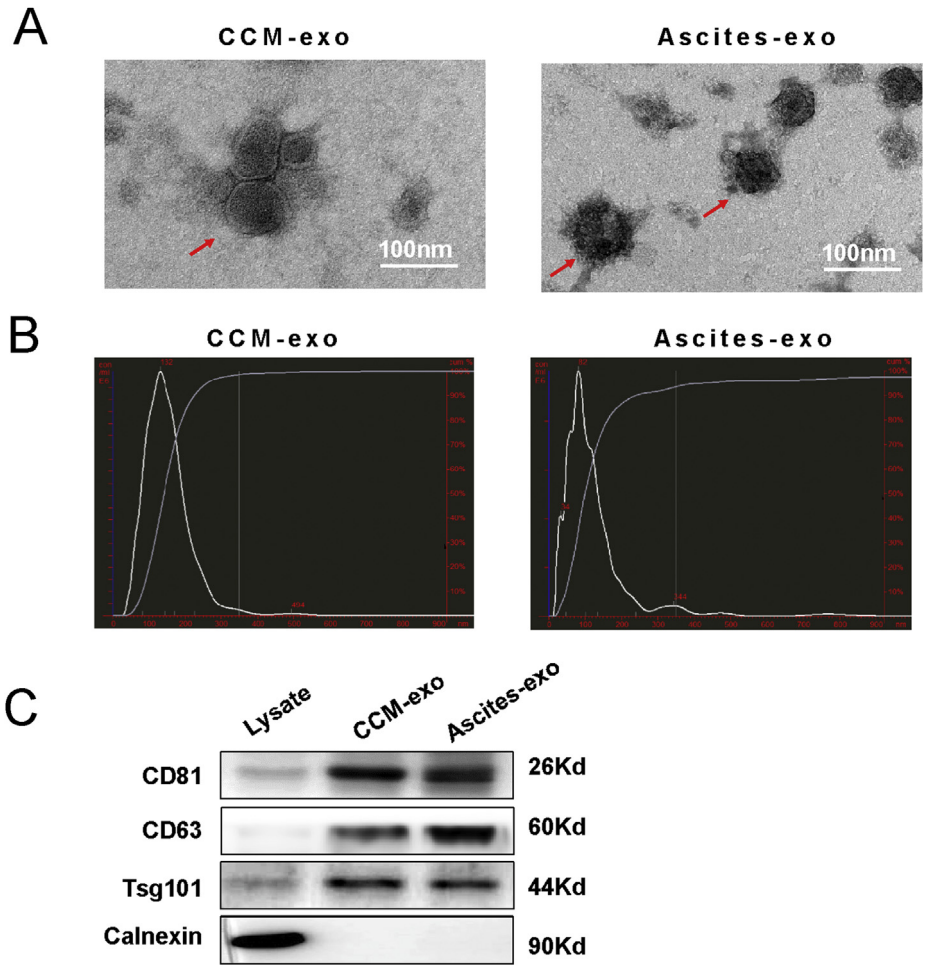

Fig. 1. Characterization of ascites and cell culture medias (CCM) derived exosomes. (**A**) Transmission electron microscopy images of exosomes from ascites and CCM. Magnification, × 200,000. Scale bar = 100 nm. (**B**) The particle size/concentration of exosomes from ascites and CCM were analyzed by NTA. (**C**) Western blot analyses of exosomal markers (CD81, CD63 and TSG101) of exosomes isolated by UC from ascites and CCM samples.

well transwell plates with pre-coated Matrigel (Corning, New York, NY) following the manufacturer's instructions for invasion assays. The lower chambers were filled with culture medium supplemented with 10% FBS. Gastric cancer cells-derived exosomes and ascites-derived exosomes quantified by microBCA were added to the upper chambers. The AGS and HGC27 gastric cancer cells were incubated with 50 μl of exosomes at 100 ng/μl and 400 ng/μl respectively. Exosomes-depleted supernatant was used as negative control. Invaded AGS cells (after incubation for 24 h) in the lower chambers were fixed and stained with crystal violet and counted under a microscope.

2.8. Establishment of peritoneal metastasis model in NOD/SCID mice

Six-week-old female nonobese diabetic/severe combined immune deficient (NOD/SCID) mice (Huafukang Bioscience, Beijing, China) were used in this study. All mice were maintained under pathogen-free condition. Mice were intraperitoneally injected with AGS cells (5×10^6/100 μl per mouse) plus PBS (100 μl), AGS cells (5×10^6/100 μl per mouse) plus exosome depleted ascites (100 μl Supernatant) and AGS cells plus malignant ascites-derived exosomes (100 μl AGS-exo) respectively (n = 6 in each group). After 2 days, mice received consecutively PBS, Supernatant, or exosomes (10 μg) i. p. with a volume of 100 μl once every other day for 3 times. Peritoneal tumor formation and animal survival were evaluated from the day of cancer cell injection until death. After 6 weeks, mice were sacrificed and the tumor weight, the number of metastatic nodules, the volume of ascites fluid were evaluated. The peritoneal nodules were isolated from the abdominal cavity and the tumors were weighed at the end of the experiment. Tumor tissues were processed for Western blot. All animal experiments were performed in accordance with the Animal Experimentation Ethics Committee of Peking University Cancer Hospital.

2.9. Small RNA library preparation and sequencing

Total RNA was extracted and purified from exosomes with miRNeasy® Mini kit (Qiagen, cat. No. 217004) according to the kit instruction. Then, sequencing libraries were generated using NEB Next Multiplex Small RNA Library Prep Set for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Libraries were sequenced using the Illumina HiSeq platform.

2.10. Differential expression analysis of miRNA

The sequencing reads were aligned to the miRbase and Human Genome (GRCh38). TPM was calculated based on the mapping results for each miRNA. The differential expression miRNA was generated with edgeR [26], only the miRNA with p-value < 0.05 and fold change ≥ 2 were selected as the candidate miRNA.

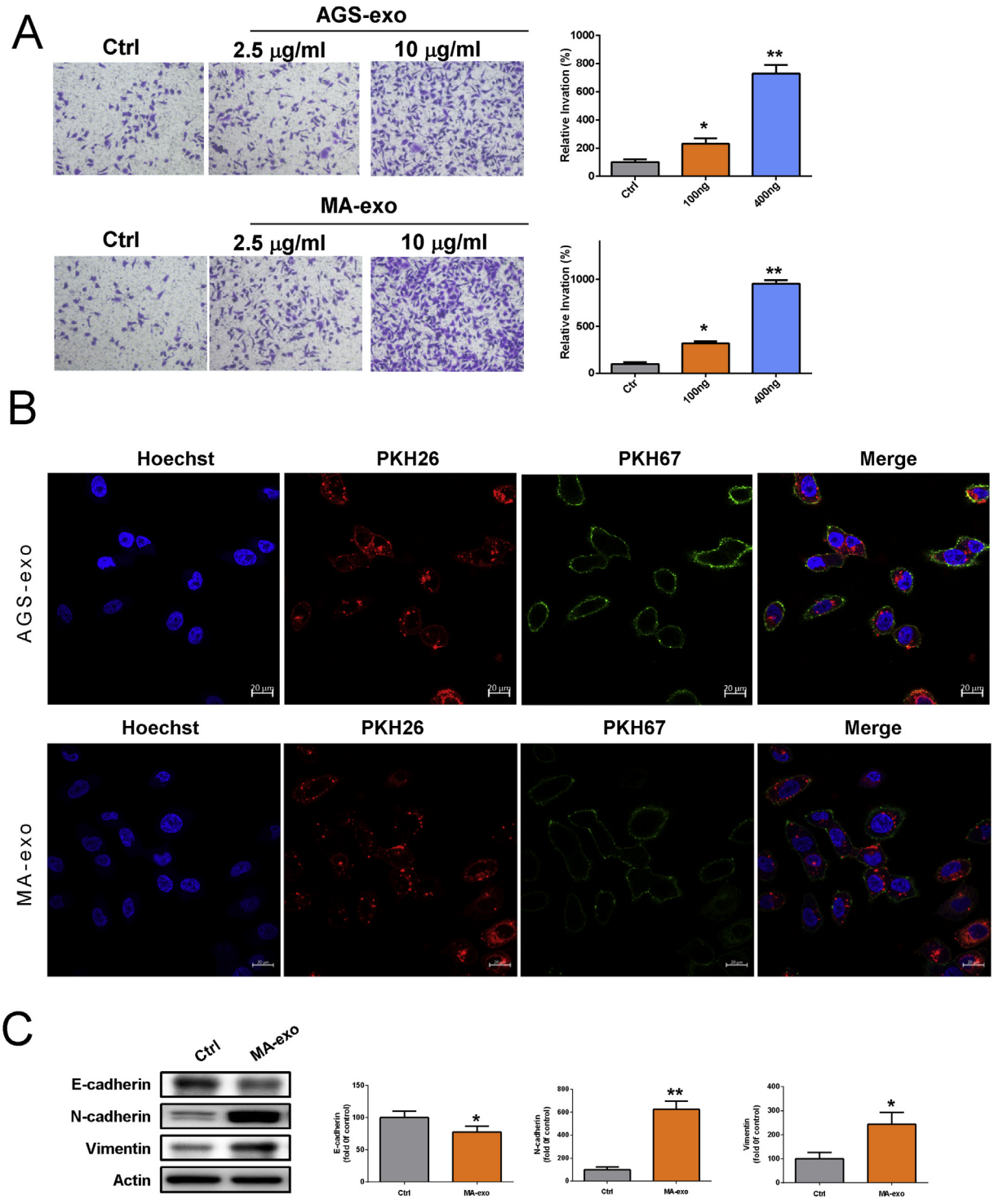

Fig. 2. Malignant ascites (MA)-derived Exosomes promoted GC cell migration *in vitro* (**A**) Transwell invasion assay for the invasive ability of AGS cells treated by various concentrations of exosomes from MA or CCM (2.5 μg/ml, 10 μg/ml). (**B**) The internalization of MA or CCM derived exosomes (PKH26-labeled, red fluorescence) by AGS cells (PKH67-labeled, green fluorescence) was examined by Confocal analysis. (**C**) Western blotting assays for the expression of EMT related markers in AGS cells treated with MA-derived exosomes. Data were presented as mean ± SD. All experiments were carried out in triplicates. *$p < 0.05$, **$p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.11. Target gene prediction of miRNA

The miRanda and RNAhybrid were used to predict the target genes of miRNA [27,28]. The genes appeared in the results of the two tools were taken as the target genes. The key parameters for miRanda is -sc 150 -en −30 -go −2.0 -ge −8.0, and RNAhybrid with key parameter -e −30.

2.12. Statistical analysis

Statistical analysis was carried out with two-tailed Student's t-test for methods comparison within each biofluid or Kolmogorov-Smirnov to compare particle size distribution. Only p value ≤ 0.05 were considered significant. Error bars in graphs represent standard deviation of the mean for three independent experiments. Analyses were performed

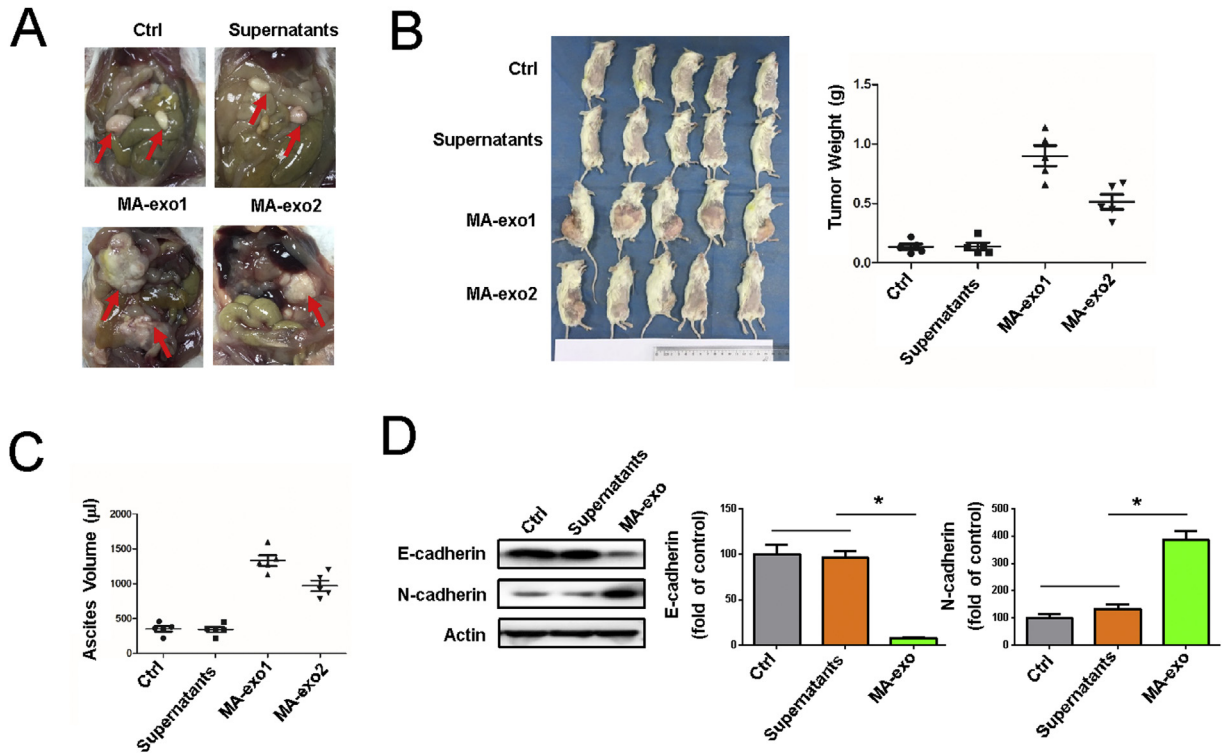

Fig. 3. Macroscopic observation of gastric intraperitoneal xenografts in NOD/SCID mice.(**A**) Macroscopic and representative images of mice intraperitoneal injected with AGS cells (Ctrl), AGS cells incubated with exosome-depleted supernatants (Supernatants) or incubated with MA-derived exosomes (MA-exo1,2) and sacrificed on the 42nd day. (**B**) Total weight of peritoneal tumor nodules in mice of Ctrl, Supernatants and MA-exo1,2 groups. (**C**) Total volume of peritoneal fluids in mice of Ctrl, Supernatants and MA-exo1,2 groups. (**D**) Western blotting assays for the expression of EMT related markers in peritoneal nodules of mice in Ctrl, Supernatants and MA-exo1,2 groups. Data were presented as mean ± SD. All experiments were carried out in triplicates. *$p < 0.01$.

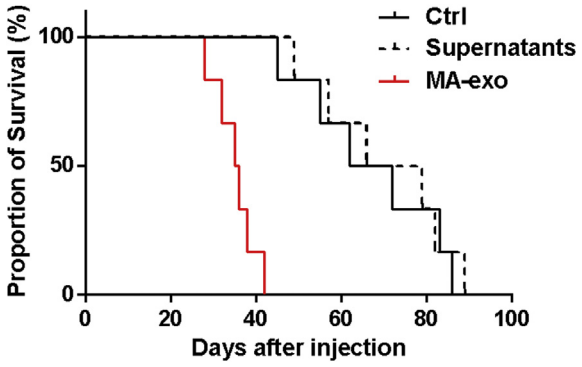

Fig. 4. Kaplan-Meier survival curves of NOD/SCID mice intraperitoneal injected by AGS cells with PBS, Supernatants or MA-derived Exosomes. Mice intraperitoneal injected by AGS cells (Ctrl), AGS cells incubated with exosome-depleted supernatants (Supernatants) or incubated with MA-derived exosomes (MA-exo) were monitored for survival over a period of longer than 70 days (median survival = 67, 72.5, and 35.5 days, respectively; Ctrl vs MA-exo p = 0.0005 and Supernatants vs MA-exo p = 0.0005) (n = 6 each group).

using GraphPad Prism 7.

3. Results

3.1. Characterization of exosomes from cell culture medium and ascites

The exosomes were purified from GC cells culture media and ascites by the ultracentrifugation method as previously reported [29]. An electron microscope was used to identify the biolayer, characteristic, cup-shaped morphology of purified exosomes (Fig. 1A). Nano Sight Technology showed that the size distribution of purified exosomes was between 30 and 100 nm (Fig. 1B). Moreover, Western blot analysis of protein extracted from AGS cells culture media (CCM) and from ascites revealed the presence of specific exosome markers (CD63, CD81, TSG101). The non-exosomal marker calnexin was absent in exosomes, although it was present in the parental cells (Fig. 1C). All together, these results confirmed that the main contents of the purified microvesicles were exosomes.

3.2. Malignant ascites–derived exosomes enhance invasion of GC cells

We also investigated the effect of exosomes isolated from malignant ascites (MA) on metastasis in *trans*-well invasion assay. The purified exosomes from AGS cells or from MA significantly increased cell invasion in a dose-dependent manner in AGS and HGC 27 cells compared to exosome-depleted liquid groups (Fig. 2A and S Fig. 1). We then detected the effect of the exosomes isolated from ascites of 8 paired patients with and without peritoneal perfusion. The results showed that the MA-derived exosomes from patient with peritoneal perfusion had lower inducing effect on cell invasion compared to the pre-peritoneal perfusion group (S Fig. 2). Cargo delivery involves the uptake and internalization of exosomes by the recipient cells. Here we determined if the exosomes were internalized or attached to the surface of recipient GC cells. AGS cells labeled with PKH67 (green) were treated with red PKH26-labeled exosomes isolated from AGS cells and from MA. Confocal microscopy analysis revealed the presence of PKH26 labeled exosomes from AGS cells or from MA exclusively in the cytoplasm of GC cells (Fig. 2B). These results indicate that GC cells can internalize exosomes purified from AGS cells or from MA. Epithelial-mesenchymal transition (EMT) is recognized as a driving force of cancer cell invasion and metastasis, a leading cause of cancer recurrence and cancer-related death [30]. Loss of epithelial markers (epithelial-cadherin, E-cadherin) and gain of mesenchymal cell markers (N-cadherin and vimentin) are

Table 1
Clinico-pathological characteristics of GC patients.

Number	Gender	Age	Tumor site	Lauren's classification	Peritoneal perfusion		Chemotherapy	
					Yes	No	Yes	No
G1/g1	Female	63	proximal	Diffuse	g1	G1	g1	G1
G2/g2	Female	34	proximal	Diffuse	g2	G2	g2	G2
G3/g3	Female	44	proximal	Diffuse	g3	G3	g3	G3
G4/g4	Female	52	proximal	Diffuse	g4	G4	g4	G4
G5/g5	Female	56	proximal	Diffuse	g5	G5	g5	G5
G6/g6	Female	61	proximal	Diffuse	g6	G6	g6	G6
G7/g7	Male	64	antral	Diffuse	g7	G7	g7	G7
G8/g8	Female	62	antral	Diffuse	g8	G8	g8	G8
S15	Male	71	antral	Diffuse	NA	S15	NA	S15
10–25	Male	61	antral	Diffuse	10–25	NA	10–25	NA

Table 2
Control subjects' demographic data and biochemical characteristics of ascitic fluids.

Characteristics	Case1 (N1)	Case2 (N2)	Case3 (N3)
Age	55	37	88
Gender	Female	Female	Female
Diagnosis	Liver cirrhosis	Liver cirrhosis	Liver cirrhosis
Colour	Clear/straw	Clear/straw	straw
Total cells/mm^3	1030	130	1650
LDH activity (U/L)	39	18	60
AFP level (ng/ml)	2.07	20.98	1.06

two critical steps in EMT [31]. In this respect, the expression levels of E-cadherin, N-cadherin and vimentin protein was measured upon treatment by the purified exosomes. As shown in Fig. 2C, a downregulation of E-cadherin and upregulation of N-cadherin and vimentin was observed in the cells treated by MA-derived exosomes. Hence, exosomes purified from malignant peritoneal fluid could modulate GC cell EMT thus increasing cancer cell migration and invasiveness. MA-derived exosomes had an important role in gastric peritoneal metastasis.

3.3. Malignant ascites–derived exosomes promote peritoneal metastasis of GC in AGS intraperitoneal metastatic xenograft mouse model

To assess the effects of exosomes on GC *in vivo*, the mouse model of peritoneal tumor cell dissemination was established. After 6 weeks of injection, mice were sacrificed. All the mice developed abdominal swelling with peritoneal tumors and bloody ascites. The tumor weight, the number of peritoneal nodules and the volume of ascites fluid were significantly increased in mice injected with AGS cells treated by MA-derived exosomes (MA-exo) compared to mice injected with AGS cells treated by exosomes-depleted supernatants (Supernatants) or AGS cells (Ctrl) alone ($p < 0.05$) (Fig. 3A -C). As shown in Supplementary Fig. 3, the nodules are difficult to get in control mice due to small tumor size. Also, the spread of tumors in the peritoneum can be observed and some of the nodules in a loosely coherent pattern were difficult to isolate. To verify the invasive effect of MA-derived exosomes on GC via EMT, the peritoneal nodules were harvested and the expression levels of markers of EMT signaling was determined. Consistent with the *in vitro* results, Western blot assay showed that the exosomes isolated from malignant peritoneal fluid, caused a significant decrease of E-cadherin, and a dramatic increase of N-cadherin (Fig. 3D). Meanwhile, mice intraperitoneal injected by AGS cells and MA-derived exosomes showed significantly shortened lifespans relative to those in the control groups with ($p = 0.0005$) median survival from 67 to 35.5 days (Fig. 4), whereas pretreatment of AGS cells with the supernatants or PBS did not have a significant effect on median survival. Taken together, these results demonstrated that the MA-derived exosomes from patients with GC promoted gastric peritoneal dissemination through aberrant EMT activation.

3.4. Malignant ascites–derived exosomes miRNA profiling

Ascites-derived exosomal miRNA expression profiles were determined for 8 paired gastric cancer patients (Table 1) with peritoneal dissemination before (G2 group) and after (G3 group) intraperitoneal chemotherapy. 3 patients (G1 group) with liver cirrhosis were used as non-malignant control (Table 2). The sequencing libraries were generated using NEB Next Multiplex Small RNA Library Prep Set for Illumina (NEB, USA) and sequenced using the Illumina HiSeq platform. Only miRNAs that were altered at least 2-fold were considered significant candidates. Using these criteria, we identified 100 miRNAs that were differentially contained in the MA-derived exosomes of GC samples (G2) relative to control subjects (G1) (Fig. 5A). Subsequently, 60 miRNAs were identified to be significantly different between G2 and G3 group. We also identified 29 miRNAs that were differentially expressed among G1, G2 and G3 groups (Fig. 5A). Fig. 5B shows hierarchical clustering analysis based on the identified 29 miRNAs among the three groups, suggesting specific differences in miRNA expression between GC patients with peritoneal dissemination before and after peritoneal chemotherapy. Notably, the levels of 5 of these miRNAs were down-regulated in MA-derived exosomes from GC patients before peritoneal chemotherapy and reversely up-regulated after peritoneal chemotherapy (Fig. 5B). Meanwhile, 22 miRNAs were up-regulated in MA-derived exosomes from GC patients before peritoneal chemotherapy and the upregulation were abolished in these patients after peritoneal chemotherapy (Fig. 5B).

4. Discussion

The prognosis of GC patients with peritoneal dissemination (PD) has remained poor [32]. Peritoneal metastasis is a multi-step process encompassing the (i) detachment of tumor cells from the primary site, (ii) survival in the microenvironment of the peritoneal cavity (iii) attachment of free cancer cells to mesothelial cells and subsequent tumor growth [3]. For gastric cancer with peritoneal metastases, current prevention or treatment recommendations includes neoadjuvant systemic chemotherapy, neoadjuvant intraperitoneal and systemic chemotherapy, cytoreductive surgery and perioperative chemotherapy [33]. However, the treatment strategies for GC patients with PD are still controversial and the clinical outcome remains unsatisfactory. Thus, understanding the molecular mechanisms of PD is critical for developing new therapies and improving the outcomes of GC patients. It is also important to identify specific prognostic factors that may be helpful in predicting the clinical outcome of treatment strategies in GC patients with PD.

In this study, we demonstrated, for the first time, the exosomes isolated from malignant ascites in patients with GC exhibited an invasive effect on gastric cancer cells *in vitro* and promoted GC cells

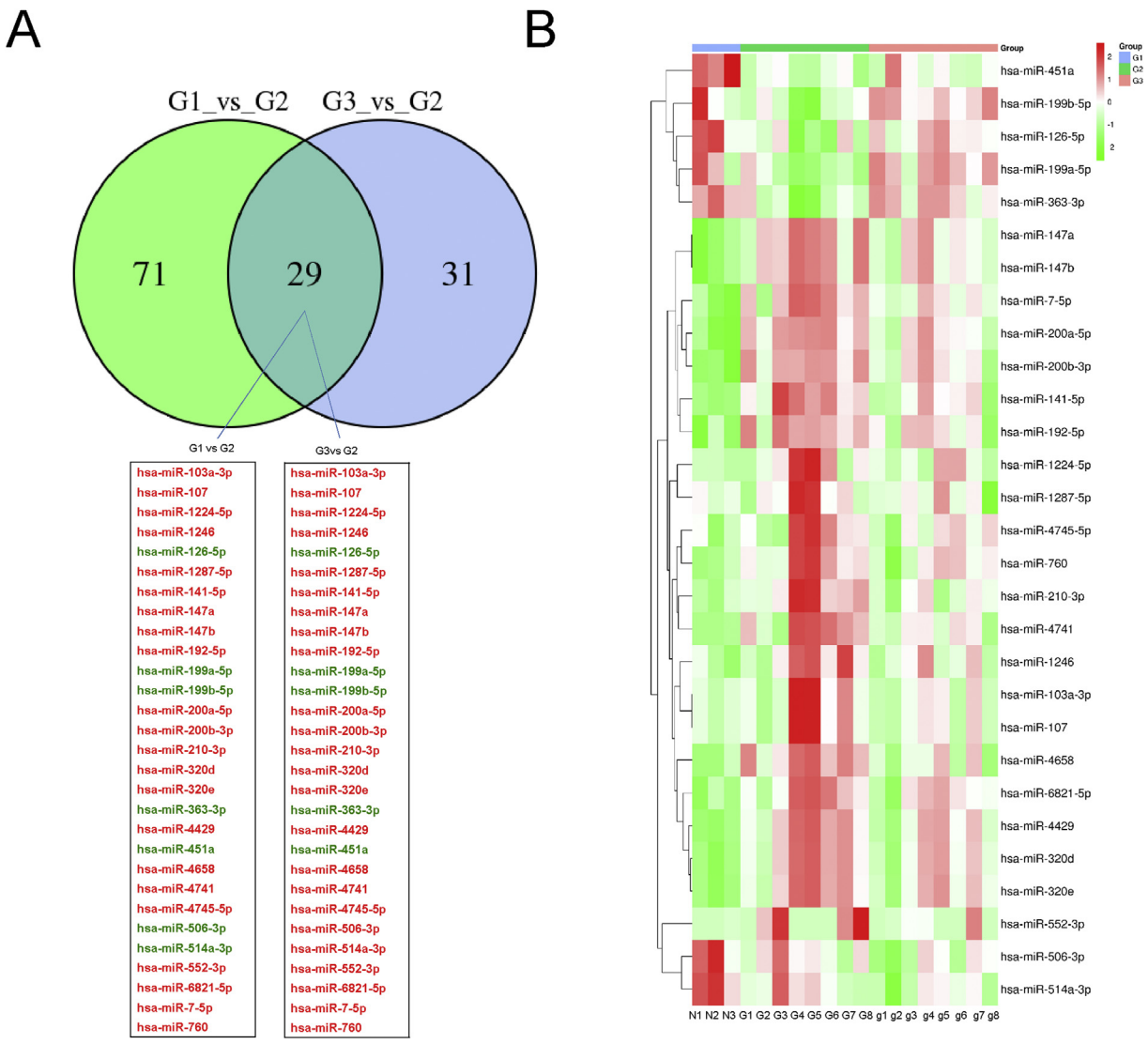

Fig. 5. Differential exosomal miRNA content in GC patients with Pre- or post-peritoneal chemotherapy and control subjects. (A) The Venn diagram shows the number miRNAs differentially expressed in ascites exosomes from control subjects (G1), GC patients before (G2) or after intraperitoneal chemotherapy for about 10 days (G3). The miRNAs in common are indicated below the diagram and colour-coded (red: elevated; green: decreased). (**B**) Heatmap of the per-row normalized expression levels of selected miRNAs differentially expressed in exosomes from GC and control subjects. G1, n = 3; G2, n = 8; G3, n = 8, using $P < 0.05$ (multiple *t*-test) and array threshold as cutoffs. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

disseminating in abdominal cavity *in vivo*. Our study shows that the exosomes can be consistently extracted from ascites and that exosomal miRNAs expression profile is specific in peritoneal fluid from GC patients with PD before or after peritoneal chemotherapy and from control subjects. As an efficient carrier of miRNA, exosome-based approach could be targeted for the treatment of GC with PD in the future.

Previous researches have demonstrated that the exosomal miRNAs may be used as biomarkers for cancer diagnosis, obtained via non-invasive tumor biopsies. Liu et al. reported that miR-21 and miR-146a were associated with high levels of exosomes isolated from cervicovaginal lavage specimens in cervical cancer [34]. Motohiko et al. collected exosomes from malignant ascites and peritoneal lavage fluid and analyzed exosomal miRNA expression to detect peritoneal dissemination-related miRNA species [23]. MiR-1225-5p, miR-320c, miR-1202, miR-1207-5p, and miR-4270 were identified with high expression in malignant ascites. Here in our study, we further analyzed the expression profiles of exosomal miRNAs in the ascites of patients with non-malignant disease and patients with GC before and after intraperitoneal chemotherapy by RNA sequencing to identify candidate miRNA biomarkers for the prediction of peritoneal metastasis and treatment targets in GC. Additionally, tukey's honest significant difference was used to further investigate the different expression of each miRNA in different ascites-derived exosomes from control subjects and from GC patients with PD before or after peritoneal chemotherapy. The analysis results revealed that there were 100 differentially expressed miRNAs between G1 and G2, 60 differentially expressed miRNAs between G3 and G2. The Venn of exosomal miRNAs expression overlaps in ascites from different groups was shown in Fig. 5A. In accordance with previous findings, we also found miR-320c was upregulated in malignant ascites [23]. In addition to miR-320c, miR-320a, miR-320b and miR-196, were also found to be upregulated in malignant ascites from GC patients (G1 vs G2; G3 vs G2) (Fig. 5A, S Table ss 1 and 2). Accumulating evidences suggests that miR-196 is overexpressed in several types of cancer especially in digestive tract cancer tissues and associated with aggressive clinicopathological status [35,36]. Tsai MM et al. reported that miR-196a/-196b induce cell metastasis through the negative regulation of radixin in human gastric cancer [37]. This may indicate that malignant ascites-derived exosomes containing miR-196a/-196b create the premetastatic niche in the peritoneum, which then supports peritoneal invasion in patients with GC.

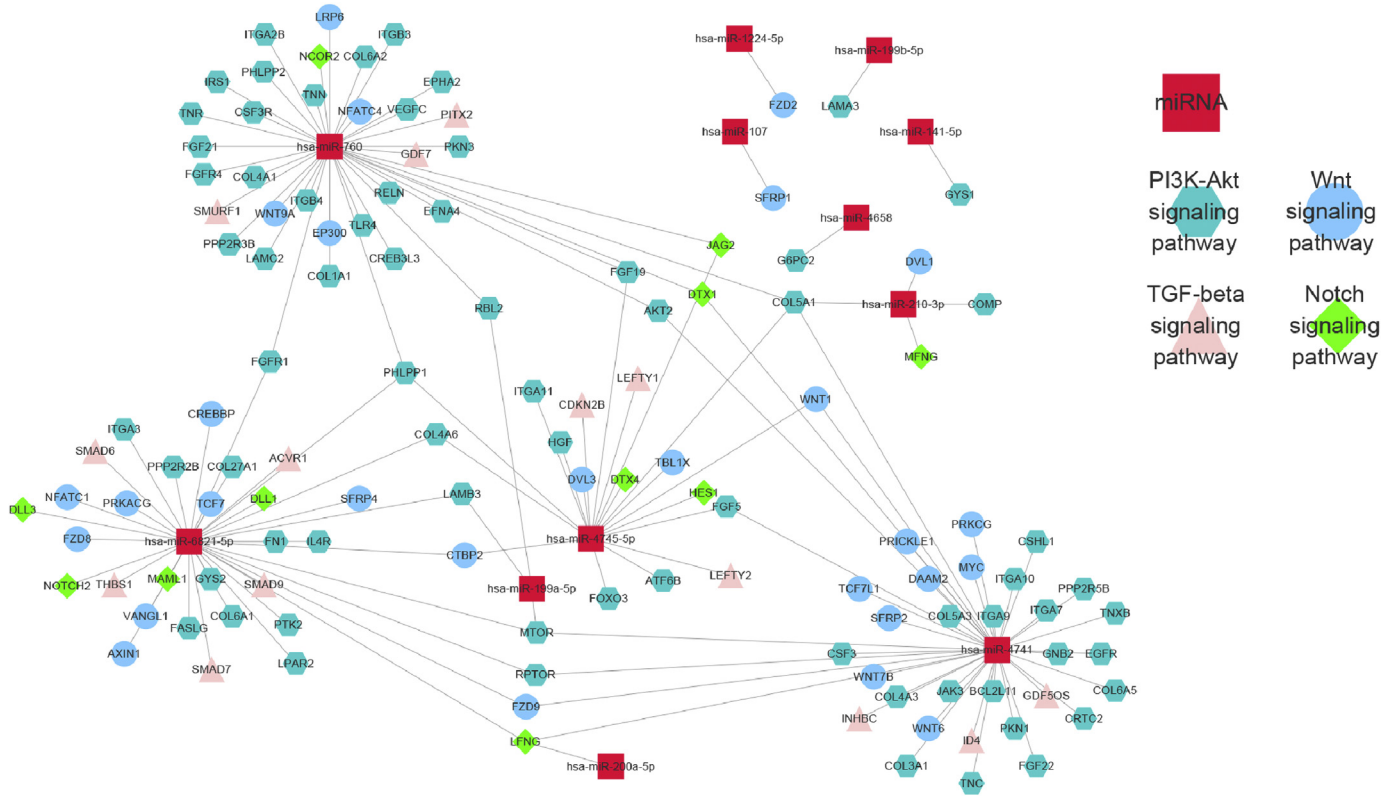

Fig. 6. Competing RNA network in GC patients with peritoneal dissemination. The competing RNA network was based on miRNA-mRNA. The network consisted 12 miRNAs correlated with 4 signaling pathways including PI3K-AKT, Wnt, TGF-β and Notch signaling pathway.

Extensive studies indicate that exosomes participate in the peritoneal dissemination of gastric cancer through promoting the proliferation and survival of gastric cancer cells via PI3K-AKT, NF-κB, Hedgehog signaling and HIF-1α pathways, assisting gastric cancer cells in adhering to the peritoneum and inducing angiogenesis via transportation of pro-angiogenic molecules including epidermal growth factor receptor (mRNA) [38–41]. Based on our *in vitro* and *in vivo* experiments, we have found the inducing effect of MA-derived exosomes in cell invasion by EMT signaling in gastric cancer cells. Transcription program switching in EMT is regulated by signaling pathways such as transforming growth factor β (TGF-β), Wnt–β-catenin, Notch and receptor tyrosine kinases [42]. To determine the function of miRNAs-RNA interactions, the miRanda and RNAhybrid were used to predict the target genes of 12 miRNAs such as miR-760, miR-6821-5p, miR-4745-5p, miR-200a-5p, miR-199a-5p and miR-4741. Through target gene prediction, the 12 afore mentioned miRNAs and the target genes were collected (Fig. 6). Furthermore, miRNA target analysis revealed that genes involved in TGF-β, Wnt, Notch and PI3K-AKT signaling pathways were found to have an important role in GC patients with peritoneal metastasis. These RNA interactions could provide a novel perspective of the mechanisms of peritoneal dissemination in gastric cancer.

In the present study, we found that malignant ascites-derived exosomes promoted the invasion of gastric cancer cells by inducing EMT signaling, however, through which miRNAs in this process is unknown. We have observed the phenotype shift of AGS cells to an elongated, mesenchymal cell after the treatment by malignant ascites-derived exosomes as shown in Fig. 2B and S Fig. 1B. We will interrogate the changes of molecular markers in EMT signaling such as E-cadherin, N-cadherin and vimentin upon treatment of MA-derived exosomes by immunocyto- and histochemistry in AGS cells and the peritoneal tumors induced by MA-derived exosomes in NOD-SCID mice in our future study. Accumulating evidences suggests that exosomes induce carcinoma-associated fibroblasts transition in peritoneal mesothelial cells (MCs). Here, our study lacks the experiments to investigate the effect of exosomes on MCs. Another limitation for this study is, that we have not yet validated the predicted exosomal miRNAs in large cohort of GC patients with PD.

In conclusion, we demonstrated in this study that the malignant ascites from GC patients released functional exosomes into the peritoneal cavity, and these exosomes were internalized by the circulating gastric cancer cells and induced peritoneal dissemination via the abnormal activation of EMT signaling pathway. Malignant ascites-derived exosomes exhibit aberrant miRNA expression patterns and that exosomal miRNAs might be critical mediators of PD in gastric cancer. Further elucidation of the molecular mechanism underlying PD is essential for developing novel treatments and improving the outcome of GC patients with PD.

Conflicts of interest

The authors declare no competing financial interests.

Funding

The work was supported by the National Key R&D Program of China (2017YFC1308900); the National Natural Science Foundation of China (No.81872341).

Author's contributions

YTH, CSQ, CZH, JG, YW and XJW performed the experiments; CSQ, JY, QZ and JL provided material support; XL performed the bioinformatic analysis; XJW and LS designed the project and wrote the paper; LS supervised the research.

Ethics approval and consent to participate

All the patients were informed of sample collection and usage. The tissue samples were collected and used in accordance with approval by the Clinical Research Ethics Committee of Peking University Cancer Hospital and Institute.

Acknowledgements

We would like to thank Mr. Guanyi Kong (Echo Biotech Co., Ltd, Beijing, China) for providing bioinformatic assistance during the preparation of this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.04.034.

References

[1] J. Ferlay, I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D.M. Parkin, D. Forman, F. Bray, Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012, Int. J. Cancer 136 (2015) E359–E386.
[2] I. Thomassen, Y.R. van Gestel, B. van Ramshorst, M.D. Luyer, K. Bosscha, S.W. Nienhuijs, V.E. Lemmens, I.H. de Hingh, Peritoneal carcinomatosis of gastric origin: a population-based study on incidence, survival and risk factors, Int. J. Cancer 134 (2014) 622–628.
[3] M. Kanda, Y. Kodera, Molecular mechanisms of peritoneal dissemination in gastric cancer, World J. Gastroenterol. 22 (2016) 6829–6840.
[4] R. Bhome, F. Del Vecchio, G.H. Lee, M.D. Bullock, J.N. Primrose, A.E. Sayan, A.H. Mirnezami, Exosomal microRNAs (exomiRs): small molecules with a big role in cancer, Cancer Lett. 420 (2018) 228–235.
[5] K. Trajkovic, C. Hsu, S. Chiantia, L. Rajendran, D. Wenzel, F. Wieland, P. Schwille, B. Brugger, M. Simons, Ceramide triggers budding of exosome vesicles into multivesicular endosomes, Science 319 (2008) 1244–1247.
[6] A.V. Vlassov, S. Magdaleno, R. Setterquist, R. Conrad, Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials, Biochim. Biophys. Acta 1820 (2012) 940–948.
[7] H. Valadi, K. Ekstrom, A. Bossios, M. Sjostrand, J.J. Lee, J.O. Lotvall, Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells, Nat. Cell Biol. 9 (2007) 654–659.
[8] M.Y. Fong, W. Zhou, L. Liu, A.Y. Alontaga, M. Chandra, J. Ashby, A. Chow, S.T. O'Connor, S. Li, A.R. Chin, G. Somlo, M. Palomares, Z. Li, J.R. Tremblay, A. Tsuyada, G. Sun, M.A. Reid, X. Wu, P. Swiderski, X. Ren, Y. Shi, M. Kong, W. Zhong, Y. Chen, S.E. Wang, Breast-cancer-secreted miR-122 reprograms glucose metabolism in premetastatic niche to promote metastasis, Nat. Cell Biol. 17 (2015) 183–194.
[9] A. Hoshino, B. Costa-Silva, T.L. Shen, G. Rodrigues, A. Hashimoto, M. Tesic Mark, H. Molina, S. Kohsaka, A. Di Giannatale, S. Ceder, S. Singh, C. Williams, N. Soplop, K. Uryu, L. Pharmer, T. King, L. Bojmar, A.E. Davies, Y. Ararso, T. Zhang, H. Zhang, J. Hernandez, J.M. Weiss, V.D. Dumont-Cole, K. Kramer, L.H. Wexler, A. Narendran, G.K. Schwartz, J.H. Healey, P. Sandstrom, K.J. Labori, E.H. Kure, P.M. Grandgenett, M.A. Hollingsworth, M. de Sousa, S. Kaur, M. Jain, K. Mallya, S.K. Batra, W.R. Jarnagin, M.S. Brady, O. Fodstad, V. Muller, K. Pantel, A.J. Minn, M.J. Bissell, B.A. Garcia, Y. Kang, V.K. Rajasekhar, C.M. Ghajar, I. Matei, H. Peinado, J. Bromberg, D. Lyden, Tumour exosome integrins determine organotropic metastasis, Nature 527 (2015) 329–335.
[10] B. Costa-Silva, N.M. Aiello, A.J. Ocean, S. Singh, H. Zhang, B.K. Thakur, A. Becker, A. Hoshino, M.T. Mark, H. Molina, J. Xiang, T. Zhang, T.M. Theilen, G. Garcia-Santos, C. Williams, Y. Ararso, Y. Huang, G. Rodrigues, T.L. Shen, K.J. Labori, I.M. Lothe, E.H. Kure, J. Hernandez, A. Doussot, S.H. Ebbesen, P.M. Grandgenett, M.A. Hollingsworth, M. Jain, K. Mallya, S.K. Batra, W.R. Jarnagin, R.E. Schwartz, I. Matei, H. Peinado, B.Z. Stanger, J. Bromberg, D. Lyden, Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver, Nat. Cell Biol. 17 (2015) 816–826.
[11] C. Grange, M. Tapparo, F. Collino, L. Vitillo, C. Damasco, M.C. Deregibus, C. Tetta, B. Bussolati, G. Camussi, Microvesicles released from human renal cancer stem cells stimulate angiogenesis and formation of lung premetastatic niche, Cancer Res. 71 (2011) 5346–5356.
[12] G. Deng, J. Qu, Y. Zhang, X. Che, Y. Cheng, Y. Fan, S. Zhang, D. Na, Y. Liu, X. Qu, Gastric cancer-derived exosomes promote peritoneal metastasis by destroying the mesothelial barrier, FEBS Lett. 591 (2017) 2167–2179.
[13] Z.P. Sun, A.Q. Li, W.H. Jia, S. Ye, G. Van Eps, J.M. Yu, W.J. Yang, MicroRNA expression profiling in exosomes derived from gastric cancer stem-like cells, Oncotarget 8 (2017) 93839–93855.
[14] L. Cheng, R.A. Sharples, B.J. Scicluna, A.F. Hill, Exosomes provide a protective and enriched source of miRNA for biomarker profiling compared to intracellular and cell-free blood, J. Extracell. Vesicles 3 (2014).
[15] D.P. Bartel, MicroRNAs: target recognition and regulatory functions, Cell 136 (2009) 215–233.
[16] V.S. Nair, L.S. Maeda, J.P. Ioannidis, Clinical outcome prediction by microRNAs in human cancer: a systematic review, J. Natl. Cancer Inst. 104 (2012) 528–540.
[17] T. Matsumura, K. Sugimachi, H. Iinuma, Y. Takahashi, J. Kurashige, G. Sawada, M. Ueda, R. Uchi, H. Ueo, Y. Takano, Y. Shinden, H. Eguchi, H. Yamamoto, Y. Doki, M. Mori, T. Ochiya, K. Mimori, Exosomal microRNA in serum is a novel biomarker of recurrence in human colorectal cancer, Br. J. Canc. 113 (2015) 275–281.
[18] S.B. Ye, H. Zhang, T.T. Cai, Y.N. Liu, J.J. Ni, J. He, J.Y. Peng, Q.Y. Chen, H.Y. Mo, C. Jun, X.S. Zhang, Y.X. Zeng, J. Li, Exosomal miR-24-3p impedes T-cell function by targeting FGF11 and serves as a potential prognostic biomarker for nasopharyngeal carcinoma, J. Pathol. 240 (2016) 329–340.
[19] J. Nilsson, J. Skog, A. Nordstrand, V. Baranov, L. Mincheva-Nilsson, X.O. Breakefield, A. Widmark, Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer, Br. J. Canc. 100 (2009) 1603–1607.
[20] K.B. Chen, J. Chen, X.L. Jin, Y. Huang, Q.M. Su, L. Chen, Exosome-mediated peritoneal dissemination in gastric cancer and its clinical applications, Biomedical reports 8 (2018) 503–509.
[21] L. Pan, W. Liang, M. Fu, Z.H. Huang, X. Li, W. Zhang, P. Zhang, H. Qian, P.C. Jiang, W.R. Xu, X. Zhang, Exosomes-mediated transfer of long noncoding RNA ZFAS1 promotes gastric cancer progression, J. Cancer Res. Clin. Oncol. 143 (2017) 991–1004.
[22] N. Wang, L. Wang, Y. Yang, L. Gong, B. Xiao, X. Liu, A serum exosomal microRNA panel as a potential biomarker test for gastric cancer, Biochem. Biophys. Res. Commun. 493 (2017) 1322–1328.
[23] M. Tokuhisa, Y. Ichikawa, N. Kosaka, T. Ochiya, M. Yashiro, K. Hirakawa, T. Kosaka, H. Makino, H. Akiyama, C. Kunisaki, I. Endo, Exosomal miRNAs from peritoneum lavage fluid as potential prognostic biomarkers of peritoneal metastasis in gastric cancer, PLoS One 10 (2015) e0130472.
[24] P. Peng, Y. Yan, S. Keng, Exosomes in the ascites of ovarian cancer patients: origin and effects on anti-tumor immunity, Oncol. Rep. 25 (2011) 749–762.
[25] C.A. Franzen, P.E. Simms, A.F. Van Huis, K.E. Foreman, P.C. Kuo, G.N. Gupta, Characterization of uptake and internalization of exosomes by bladder cancer cells, BioMed Res. Int. 2014 (2014) 619829.
[26] M.D. Robinson, D.J. McCarthy, G.K. Smyth, edgeR: a Bioconductor package for differential expression analysis of digital gene expression data, Bioinformatics 26 (2010) 139–140.
[27] D. Betel, M. Wilson, A. Gabow, D.S. Marks, C. Sander, The microRNA.org resource: targets and expression, Nucleic Acids Res. 36 (2008) D149–D153.
[28] M. Rehmsmeier, P. Steffen, M. Hochsmann, R. Giegerich, Fast and effective prediction of microRNA/target duplexes, Rna 10 (2004) 1507–1517.
[29] S.A. Melo, L.B. Luecke, C. Kahlert, A.F. Fernandez, S.T. Gammon, J. Kaye, V.S. LeBleu, E.A. Mittendorf, J. Weitz, N. Rahbari, C. Reissfelder, C. Pilarsky, M.F. Fraga, D. Piwnica-Worms, R. Kalluri, Glypican-1 identifies cancer exosomes and detects early pancreatic cancer, Nature 523 (2015) 177–182.
[30] L. Huang, R.L. Wu, A.M. Xu, Epithelial-mesenchymal transition in gastric cancer, Am. J. Tourism Res. 7 (2015) 2141–2158.
[31] N.H. Myong, Loss of E-cadherin and acquisition of vimentin in epithelial-mesenchymal transition are noble indicators of uterine cervix cancer progression, Korean J. Pathol. 46 (2012) 341–348.
[32] L. Shen, Y.S. Shan, H.M. Hu, T.J. Price, B. Sirohi, K.H. Yeh, Y.H. Yang, T. Sano, H.K. Yang, X. Zhang, S.R. Park, M. Fujii, Y.K. Kang, L.T. Chen, Management of gastric cancer in Asia: resource-stratified guidelines, the Lancet, Oncology 14 (2013) e535–547.
[33] O. Glehen, F. Mohamed, F.N. Gilly, Peritoneal carcinomatosis from digestive tract cancer: new management by cytoreductive surgery and intraperitoneal chemohyperthermia, the Lancet, Oncology 5 (2004) 219–228.
[34] J. Liu, H. Sun, X. Wang, Q. Yu, S. Li, X. Yu, W. Gong, Increased exosomal microRNA-21 and microRNA-146a levels in the cervicovaginal lavage specimens of patients with cervical cancer, Int. J. Mol. Sci. 15 (2014) 758–773.
[35] C. Chen, Y. Zhang, L. Zhang, S.M. Weakley, Q. Yao, MicroRNA-196: critical roles and clinical applications in development and cancer, J. Cell Mol. Med. 15 (2011) 14–23.
[36] Y.C. Lu, J.T. Chang, E.C. Chan, Y.K. Chao, T.S. Yeh, J.S. Chen, A.J. Cheng, miR-196, an emerging cancer biomarker for digestive tract cancers, J. Cancer 7 (2016) 650–655.
[37] M.M. Tsai, C.S. Wang, C.Y. Tsai, C.Y. Chen, H.C. Chi, Y.H. Tseng, P.J. Chung, Y.H. Lin, I.H. Chung, C.Y. Chen, K.H. Lin, MicroRNA-196a/-196b promote cell metastasis via negative regulation of radixin in human gastric cancer, Cancer Lett. 351 (2014) 222–231.
[38] J. Qi, Y. Zhou, Z. Jiao, X. Wang, Y. Zhao, Y. Li, H. Chen, L. Yang, H. Zhu, Y. Li, Exosomes derived from human bone marrow mesenchymal stem cells promote tumor growth through Hedgehog signaling pathway, Cell. Physiol. Biochem. : Int. J. Exp. Cell. Physiol. Biochem. Pharmacol. 42 (2017) 2242–2254.
[39] T. Wang, D.M. Gilkes, N. Takano, L. Xiang, W. Luo, C.J. Bishop, P. Chaturvedi, J.J. Green, G.L. Semenza, Hypoxia-inducible factors and RAB22A mediate formation of microvesicles that stimulate breast cancer invasion and metastasis, Proc. Natl. Acad. Sci. U.S.A. 111 (2014) E3234–E3242.
[40] T. Arita, D. Ichikawa, H. Konishi, S. Komatsu, A. Shiozaki, S. Ogino, Y. Fujita, H. Hiramoto, J. Hamada, K. Shoda, T. Kosuga, H. Fujiwara, K. Okamoto, E. Otsuji, Tumor exosome-mediated promotion of adhesion to mesothelial cells in gastric cancer cells, Oncotarget 7 (2016) 56855–56863.
[41] T.B. Steinbichler, J. Dudas, H. Riechelmann, Skvortsova II, The role of exosomes in cancer metastasis, Semin. Canc. Biol. 44 (2017) 170–181.
[42] D.M. Gonzalez, D. Medici, Signaling mechanisms of the epithelial-mesenchymal transition, Sci. Signal. 7 (2014) re8.