Gastric cancer-derived exosomes promote peritoneal metastasis by destroying the mesothelial barrier

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Abstract

An intact mesothelium serves as a protective barrier to inhibit peritoneal carcinomatosis. Cancer-derived exosomes can mediate directional tumor metastasis; however, little is known about whether gastric cancer-derived exosomes will destroy the mesothelial barrier and promote peritoneal dissemination. Here, we demonstrate that gastric cancer-derived exosomes facilitate peritoneal metastasis by causing mesothelial barrier disruption and peritoneal fibrosis. Injury of peritoneal mesothelial cells elicited by gastric cancer-derived exosomes is through concurrent apoptosis and mesothelial-to-mesenchymal transition (MMT). Additionally, upregulation of p-ERK in peritoneal mesothelial cells is primarily responsible for the MMT while contributing little to apoptosis. Together, these data support the concept that exosomes play a crucial role in remodeling the pre-metastatic microenvironment and identify a novel mechanism for peritoneal metastasis of gastric carcinoma.

Keywords

Gastric cancer
Exosome
Peritoneal metastasis
Mesothelial barrier destruction
Peritoneal mesothelial cell

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Introduction

Gastric cancer is one of the most frequently diagnosed cancers in China, with an estimated 679,100 new cases and the second highest mortality rate among all malignant tumors according to the latest annual statistics for 2015[1]. Metastasis is the main cause of the poor prognosis of gastric carcinoma because of the ease of peritoneal dissemination. The peritoneum is composed of a single layer of flat mesothelial cells and a thin sub-mesothelial connective tissue. An intact, confluent mesothelial layer acts as the first barrier against bacterial invasion and tumor attachment [2]. It has been proven that tumors implant more readily into damaged peritoneum than intact peritoneum [3-6]. Enhancement of the mesothelial barrier function effectively hinders cancer cell penetration into sub-mesothelial connective tissue [7,8]. Thus, an intact peritoneal mesothelium is important to prevent tumors from invading into sub-mesothelial compact zones.

Over the past few decades, studies of adverse factors that destroy the peritoneal mesothelial barrier have primarily centered on soluble cytokines and growth factors [9-12]. Exosomes are membrane-bound micro-vesicles secreted by different categories of cells into the extracellular space whose diameter ranges from 30 nm to 150 nm. Tumor-derived exosomes are rich in protein, mRNA, and microRNAs, and serve as important messengers of intercellular communication without the need for cell-cell contact [13,14]. Emerging evidence has suggested that exosomes can convey potential biologic information to nearby or distant places and create a pre-metastatic niche to determine organotropic metastasis [15-19]. However, the effects of cancer-derived exosomes on preparing a pre-metastatic environment in the peritoneum before peritoneal metastasis remain unknown. We hypothesized that gastric cancer-derived exosomes might destroy the mesothelial barrier by harming peritoneal mesothelial cells and further cause peritoneal fibrosis, which is a congenial pre-metastatic environment beneficial for peritoneal metastasis of gastric cancer.
It has been reported that peritoneal mesothelial cells undergo apoptosis and fall off the peritoneum due to pro-apoptotic stimulation [20-22]. Therefore, the confluent mesothelial layer collapses and exfoliation takes place. The damaged site of the peritoneum becomes a favorable microenvironment for single cancer cells or cancer cell clusters to adhere and colonize. In addition, peritoneal mesothelial cells can retract and transfer from epithelial-like morphology to spindle shapes (type II EMT) under different conditions, for example, under chronic inflammation conditions in cases of long-time peritoneal dialysis [23]. Type II EMT is associated with reparative fibrosis of normal epithelial cells in response to underlying injuries while Type III EMT refers to a phenotypic switch of neoplastic cells [24]. An EMT conversion confined to mesothelial cells has a more appropriate term: mesothelial-to-mesenchymal transition (MMT) [25]. The concept of MMT in peritoneal metastasis was firstly described in 2013 [26]. Recently, it has been described that tumor-derived exosomes could induce MMT [27,28]. Mesothelial cells lose apico-basal polarity, dissociate from each other, and finally invade into the deeper peritoneum or detach from the peritoneal surface after the MMT process. As a consequence, the continuous mesothelial layer is also destroyed and cancer cells gain more opportunity to invade the sub-mesothelial matrix. Therefore, we hypothesized that gastric cancer-produced exosomes could promote peritoneal mesothelial cell injury through apoptosis and an MMT process to destroy the mesothelial barrier.

In the present study, we assessed the effect of gastric cancer-derived exosomes on peritoneal microenvironment remodeling. Our experiments in vivo and in vitro confirmed that gastric cancer-derived exosomes promoted gastric cancer peritoneal carcinomatosis by breaching the mesothelial barrier and causing peritoneal fibrosis. Both apoptosis and MMT were possible mechanisms leading to injury of peritoneal mesothelial cells. In addition, activation of the MAPK/ERK pathway might be involved in this process.
Materials and methods

Cell lines and cell culture

Human gastric cell line MGC803 was obtained from the Type Culture Collection of the Chinese Academy of Sciences (China). Human peritoneal mesothelial cell line HMrSV5 was kindly donated by Prof. Youming Peng of the Second Hospital, Zhongnan University, Changsha, China and Prof. Pierre Ronco, Hospital Tenon, Paris, France. HMrSV5 cell line was originally established after retroviral transfection of primary culture of human peritoneal mesothelial cells with SV40 large-T antigen and exhibited major characteristics of normal peritoneal mesothelial cells [29]. They were maintained in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 mg/mL) in an atmosphere of 95% air and 5% CO2 at 37°C. All of the cells were sub-cultured every 2-3 days and harvested in the logarithmic phase of growth. HMrSV5 cells were washed with PBS twice before adding gastric cancer cells-derived exosomes in all tests.

Reagents and antibodies

Specific ERK1/2 inhibitor PD98059 was purchased from Promega (Madison, WI). Anti-Flotillin-1 monoclonal antibody was purchased from BD Biosciences Pharmingen (San Jose, CA). Anti-TSG101 and anti-CD9 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Fibronectin and anti-MMP2 antibody were purchased from Abcam (Cambridge, MA, USA). Polyclonal rabbit anti-human Collagen-I antibody and CD178 monoclonal antibody were purchased from wanleibio (Shenyang, China) and Invitrogen (California, USA) respectively. All of other antibodies used for western blotting were purchased from Cell Signaling Technology (Beverly, MA).
**Exosome purification**

MGC803 cells were cultured in RPMI-1640 supplemented with 10% FBS (depleted of bovine exosomes by overnight centrifugation at 100,000 g). Cultured conditioned medium was collected after 48 h and centrifuged for 15 min at 3000 g. Then, supernatant filtered through 0.22 µm pore filter to eliminate larger extracellular vesicles. Exosomes were isolated with two independent methods. For differential centrifugation, exosomes were pelleted by ultracentrifugation at 100,000 g for 70 min. In the other approach, exosomes were isolated with ExoQuick-TC™ (SBI) according to the manufacturer's instructions. Exosomes purified by ultracentrifugation were used for in vivo tests. Exosomes isolated with ExoQuick-TC™ (SBI) were used for other experiments. The pooled exosomes were re-suspended in PBS and stored at -80 °C. *Exosomes concentration was determined by protein quantification (Pierce BCA Protein Assay Kit, Thermo Scientific) according to the published literatures [30,31].*

**Electron microscopy**

On basis of the published literature of our laboratory, we made a little modification on transmission electron-microscopy of exosomes morphological observation [32]. Briefly, exosomes were fixed in 4% paraformaldehyde and then placed on electron-microscopy grids coated with formvar carbon, contrasted and embedded them in a mixture of uranyl acetate and methylcellulose. We viewed exosomes sample by a JEM-1200EX transmission electron microscope (JEOL, Japan). Exosome size was measured by the scale bar.

**Exosomes labeling**

Exosomes pooled from supernatant of MGC803 cells were re-suspended in PBS. Exosomes liquids and the same amount of PBS were labeled with DID dye (Invitrogen) at 37 °C for 20 min. Finally they were mixed with adequate PBS and centrifuged using 3 KDa MWCO (Millipore) to wash away uncombined DID dye for three times.

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Western blotting

Cell samples or exosomes were solubilized in 1% Triton lysis buffer on ice and quantified according to the Coomassie blue G250 staining technique. Following this, all the samples were added 3× sampling buffer and boiled at 95 °C for 5 min. Then lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electronically transferred to poly vinylidene difluoride (PVDF) membranes. After blocking with 5% skimmed milk in TBST, the blots were probed with the indicated primary antibodies at 4 °C overnight and reacted with horseradish-peroxidase-conjugated secondary antibodies as indicated for 30 min at room temperature. Finally, the target bands of proteins were detected with enhanced chemiluminescence reagent (SuperSignal Western Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA). Band intensity analysis was finished by Image J software.

Flow cytometry

HMrSV5 cells were seeded at 2×10⁵/well in six-well plates and then exposed to exosomes stimulation. Cells of each well were collected and incubated with 5 µl Annexin V and 10 µl propidium iodide for 10 min and 5 min respectively in the dark. After being washed, the collected samples were tested and analyzed eventually by flow cytometry.

MTT assay

HMrSV5 cells were seeded in 96-well plates at a density of 4000/well and treated with different concentrations of exosomes. Subsequently, 20 µl of MTT (5 mg/ml) was added to each well and incubated for 4 h at 37 °C. Finally, 200 µl of DMSO was added to solubilize the crystals and the optical density was measured at 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).
**Histological appearance of peritoneum and peritoneal metastasis model**

Six weeks-year-old BALB/c male nude mice were selected and randomly assigned to two groups (n=5 or 6 in each group). Mice in the experimental group and control group were treated with MGC803 exosomes (50µg/0.5ml PBS) and sterile PBS respectively by intraperitoneal injections on days 1, 3 and 5. After one week, all of the mice were sacrificed and the mice peritoneum was collected for following scientific research. To examine morphologic changes of the peritoneum, hematoxylin and eosin (H&E) was stained and observed by light microscopy. In peritoneal metastasis model, MGC803 cells (5×10^6/0.5ml PBS) were injected into abdominal cavity on days 5 followed by intraperitoneal inoculation of exosomes on days 1, 3 and 5 as above mentioned. Four weeks later, these mice were sacrificed and total weight of metastatic nodules was evaluated in control and experimental group. Animal tests were performed in accordance with China Medical University Animal Ethics Committee.

**In situ cell apoptosis detection**

The obtained peritoneum was further used to perform TUNEL assay according to the manufacturer's instructions using in situ cell death detection kit, POD (wanleibio, Shenyang, China). In brief, the peritoneal sections were deparaffinized, rehydrated and incubated with 1× proteinase K for 15 min at room temperature in turn. After rinsing with PBS, they were blocked with 3% H2O2 for 10 min. Then these slides were incubated with TUNEL reaction buffer solution at 37 ºC for 60-90 min after PBS washing. Next, tissue sections were incubated with POD converter for 30 min followed by DAB and hematoxylin staining. The sections were finally cleared with xylene and mounted with neutral resins.

**Immunofluorescence**

For the paraffin embedding sections, they were firstly deparaffinized and rehydrated. For cells, they were firstly seeded, treated in Lab-Tek chamber slides (Nunc S/A, Polylabo, Strasbourg, France), and fixed in 3.3% paraformaldehyde for 20

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min. Then both paraffin embedding sections and slides were permeabilized with 0.2% Triton X-100 for 5 min and blocked with 5% bovine serum albumin (BSA). For double staining, they were primed with anti-ZO-1 mouse antibody and anti-Vimentin rabbit antibody overnight at 4°C. Alexa Fluor 568-conjugated goat anti-mouse IgG or Alexa Fluor 488-conjugated goat anti-rabbit IgG were mixed up in 1% BSA on the next day. Cells were incubated with the blocking solution for 1 h at room temperature in the dark followed by 4′6′-diamidino-2-phenylindole (DAPI) 5 min of nuclei staining. Finally, they were mounted and analyzed by laser confocal scanning microscope (TCS SP2/AOBS, LEICA, Germany).

Statistical analysis

All data presented in the study are expressed as the mean ± standard deviation. Representative results were from at least three independent experiments. Differences between treated and control groups were evaluated by the Student’s t-test. P<0.05 was considered to be statistically significant.

Results

Characterization and internalization of gastric cancer-derived exosomes

We isolated and purified exosomes from the MGC803 cell culture supernatant. Then the obtained exosomes were visualized under transmission electron microscopy, shown in Fig. 1A. They have a characteristic ovoid or round shape that was limited by a bilayer membrane and the size distribution was mainly between 30 nm and 100 nm. We detected exosome markers Flotillin-1, CD9, and TSG101 by immunoblotting as identified in the ExoCarta database [33]. The intracellular endoplasmic reticulum protein “Calreticulin” as a negative control was confirmed absent in the exosomes but present in the primary gastric cell lysates when loading the same amounts of input protein. To determine whether exosomes could be taken up by target cells, we labeled them with DiD dye (red). Fig. 1C showed that exosomes marked with red dye were efficiently taken up by mesothelial cells and dispersed in the cytoplasm, while no fluorescence was present in the control group. Judging from these
results, we successfully extracted gastric cancer cell-secreted exosomes from the cell culture medium and confirmed uptake of exosomes by peritoneal mesothelial cells.

**Gastric cancer-derived exosomes destroyed mesothelial barrier and increased peritoneal metastasis**

In order to determine whether gastric cancer-derived exosomes could destroy the mesothelial barrier *in vivo*, morphologic changes of mice peritoneum were analyzed through H&E staining. In PBS-treated mice, the mesothelial cell monolayer covering the peritoneum surface was intact while the vast majority of the peritoneum was damaged in the experimental group, leaving sub-mesothelial areas exposed to the abdominal cavity to different extents. Fig. 2A shows a typical change. We could also see substantially thickened connective tissue in the exosomes-treated mice (Fig. 2B). This suggested that gastric cancer-derived exosomes played a special role in peritoneum modification.

Since an adaptive pre-metastatic environment is quite helpful for subsequent metastasis, we further explored whether these pathological alterations were associated with peritoneal carcinomatosis. After pretreatment with MGC803 cells-secreted exosomes as described in the Materials and Methods, MGC 803 cells were then injected into the abdominal cavity of nude mice. The total weight of the tumor nodules in the exosomes-treated group increased significantly compared to PBS-treated mice (Fig. 2C). Our findings indicated that gastric cancer-derived exosomes might accelerate peritoneal metastasis of gastric cancer by disrupting the mesothelial barrier and building a fibrotic niche.

**Induction of apoptosis by gastric cancer-derived exosomes**

Our and other previous findings have revealed that tumor-produced exosomes are able to induce T lymphocyte apoptosis to eliminate immune barriers to tumor progression [32,34]. And malignant extracellular vesicles were confirmed to facilitate peritoneal dissemination in ovarian cancer by induction of apoptosis in mesothelial cells [35]. Here we aimed to probe into potential cytotoxic effects of gastric cancer.
exosomes on peritoneal mesothelial cells. HMrSV5 cells were stimulated by MGC803 cells-secreted exosomes at the indicated concentrations for 0, 6, and 12 h. Assessment of cell viability by MTT assay suggested that exosomes induced mesothelial cell damage in a time- and dose-dependent manner (Fig. 3A). Furthermore, we conducted western blotting to examine the levels of proteins related to apoptosis. After treatment with exosomes for 6 h, cleavage of PARP and caspases-3 was detected (Fig. 3B). To quantify the percentage of apoptotic mesothelial cells, Annexin V-PI double staining was carried out. The total early and late apoptosis rates of HMrSV5 cells exposed to 200 and 400 µg/ml exosomes increased by 8.3% and 13.8% respectively (Fig. 3C). In order to explore whether early apoptosis increased by shortening treatment duration, we detected apoptosis rate of HMrSV5 cells at 3 h. The result was shown in Supplementary Fig.1. Besides, MGC803 cells-secreted exosomes purified by ultracentrifugation could exert their pro-apoptotic effects on HMrSV5 cells, too (Supplementary Fig.2A). We also confirmed that exosomes-derived from another two gastric cancer cell lines BGC823 and SGC7901 were also able to induce apoptosis of peritoneal mesothelial cells while exosomes-derived from non-cancer cell HMrSV5 did not show the similar effects with cancer-derived exosomes (Supplementary Fig.3). These results indicated that gastric cancer-derived exosomes possess the ability to induce apoptosis of peritoneal mesothelial cells.

**Induction of MMT by gastric cancer-derived exosomes**

As mentioned before, we speculated that the MMT process may be another form of exosomes-induced damage of peritoneal cells. After 6 h incubation with MGC803 cells-secreted exosomes, a distinct alteration in cellular morphology was observed in HMrSV5 cells (Fig. 4A). HMrSV5 cells cultured in the absence of exosomes showed a typical polygonal and cobblestone-like appearance. MGC803 cells-secreted exosomes purified by ultracentrifugation could induce morphological transformation of HMrSV5 cells, too (Supplementary Fig.2B). We also observed morphological change in peritoneal mesothelial cells followed treatment of exosomes from other gastric
cancer cell lines including BGC823 and SGC7901 while HMrSV5 cells-secreted exosomes did not show the similar effects with cancer-derived exosomes(Supplementary Fig.4). To better characterize exosomes-induced MMT, MMT related protein expression was then evaluated and displayed in Fig. 4B. The level of Zo-1 decreased; meanwhile, mesenchymal indicator Vimentin and stromal components such as Fibronectin and Collagen-I increased to a great extent followed exosomes treatment. We further detected MMT marker of HMrSV5 cells after exposed to MGC803-derived exosomes for 24h and 48h. The result revealed that downregulation of Zo-1 and upregulation of Vimentin still existed and showed the same tendency as 6 h (Supplementary Fig.5). Immunofluorescence staining assay also demonstrated the MMT process (Fig. 4C). On the whole, these results revealed that gastric cancer-derived exosomes could induce MMT of peritoneal mesothelial cells.

**Gastric cancer-derived exosomes promoted mesothelial cell injury by apoptosis and MMT in vivo**

We verified in vivo that apoptosis and the MMT process were two mechanisms for exosomes-induced injury in peritoneal mesothelial cells. We observed brown positive tunnel staining of mesothelial cells (the arrows) shown in Fig. 5A and this suggested that some of the mesothelial cells underwent apoptosis followed treatment with MGC803 exosomes. In addition, we observed Zo-1 downregulation and Vimentin upregulation in some areas of the peritoneum where mesothelial cells had not been completely stripped off the peritoneum (Fig. 5B). These findings confirm that both apoptosis and MMT contribute to mesothelial cell injury induced by gastric cancer-derived exosomes. We also tried to investigate the apoptosis and MMT reason. The further research showed that gastric cancer-derived exosomes contained MMP2 and FasL protein (soluble form: 26 KD). As reported, MMP was associated with EMT in tubular epithelial cells [36] and breast cancer cells [37], while FasL was related to apoptosis in mesothelial cells [20] and Jurkat T cells [32]. It indicated that gastric
cancer-derived exosomes might induce MMT by expression of MMP2 and induce apoptosis by FasL (Supplementary Fig.6).

Roles of ERK pathway activation in mesothelial cell injury induced by gastric cancer-derived exosomes

In order to further investigate the responsible mechanisms of mesothelial cell apoptosis and MMT triggered by gastric cancer-derived exosomes, we measured the activation of ERK1/2 signaling pathways. As shown in Fig. 6A, the phosphorylation level of ERK was obviously increased in response to MGC803 exosomes and the upregulated p-ERK was reversed when they were pretreated with ERK inhibitor PD98059 (25 µM). For the purpose of figuring out whether the ERK pathway participated in the exosomes-induced apoptosis, we used flow cytometry for a quantitative assessment of HMrSV5 cell apoptosis. In contrast to cells only receiving exosomes, the apoptosis of cells pretreated with PD98059 was not blocked (Fig. 6B). However, p-ERK activation was clearly necessary for the MMT process (Fig. 6C & D). Inhibition of the ERK1/2 pathway not only partially suppressed the cell morphological changes but also blocked the MMT marker changes. These results suggested that apoptosis and MMT synchronously occurred through independent biological courses even though they are receiving a single stimulus at one time.

Discussion

According to the “seed and soil” theory proposed by Stephen Paget, cancer cells(“seed”) only metastasize to favorable environments (“soil”) where they can survive and proliferate rapidly [38]. In the last few years, exosomes have attracted a lot of attention in the field of tumor directional metastasis and they are thought of as pioneers to modify the “soil” to prepare it for the cancer cells. As reported recently, exosomes produced by various cancers such as kidney cancer, melanoma, pancreatic cancer, and breast cancer are able to reconstruct specific pre-metastatic environments and guide organ-oriented metastases [15-17,39]. Pancreatic
cancer-derived exosomes can induce liver to form a fibrotic environment and promoted pancreatic cancer liver metastasis, and breast cancer derived-exosomes can destroy the vascular endothelial barrier and promote tumor metastasis to the liver, lung, and brain. We speculated that gastric cancer-derived exosomes may play a similar role in establishment of a peritoneal pre-metastatic niche. Our animal experiment proved that gastric cancer cells-secreted exosomes ruined the integrated mesothelial monolayer, and caused extensive matrix deposition under the sub-mesothelial compact zone. Previous researches showed that areas of traumatized peritoneal mesothelium with widespread matrix deposition acted as “soil” for peritoneal carcinomatosis of gastric cancer [40]. We further demonstrated that such adaptive changes in peritoneum induced by gastric cancer-derived exosomes promoted peritoneal metastasis. This suggests that gastric cancer-derived exosomes probably play an important role in modification of the pre-metastatic environment and facilitation of peritoneal metastasis.

Apoptosis or MMT are two critical events contributing to mesothelial cell injury while carcinoid injury to the mesothelium could initiate progression of peritoneal fibrosis. Additionally, previous studies confirmed that lethal or pro-fibrotic factors released by malignant tumor cells constantly accumulated in the peritoneal cavity and induced mesothelial cell injury as well as peritoneal fibrosis [41,42]. In vitro, we demonstrated that gastric cancer-derived exosomes induced co-instantaneous apoptosis and MMT of peritoneal mesothelial cells. During this process, peritoneal mesothelial cells retracted and underwent tremendous morphological changes, leaving large bare areas between residual cells. Our in vivo experiment also verified that gastric cancer-derived exosomes induced apoptosis and MMT of peritoneal mesothelial cells accompanied by peritoneal clearance. This suggests that gastric cancer-derived exosomes promote peritoneal mesothelial cell injury though apoptosis and MMT and further leads to mesothelial barrier destruction.

Our further research showed that gastric cancer-derived exosomes contained MMP2 and FasL protein. As reported, MMP was associated with EMT in cancer cells.
[37], while FasL was related to apoptosis in mesothelial cells [20]. Besides, TGFβ1 was once reported to be able to trigger both EMT and apoptosis in epithelial cells and mesothelial cells [21,43-45]. Also, TGF-β-mediated MMT seemed to be crucial to form a suitable metastatic niche in peritoneal metastasis [46]. It was interestingly that TGFβ1 was found to be present in cancer exosomes and can exert its biological function [47,48]. Thus, we hypothesize that MMP2, FasL and TGFβ1 all may account for exosomes-induced mesothelial cells injury.

Our next meaningful finding was MAPK/ERK activation after exosomes-induced damage of peritoneal mesothelial cells. The MAPK/ERK pathway widely participates in cell proliferation, differentiation, migration, apoptosis, and senescence [49]. Although ERK pathway activation promotes cellular survival in most cases, there are some articles stating that the phosphorylation of ERK1/2 has a positive relationship with apoptosis [50,51]. Additionally, ERK pathway has been previously described to participate in peritoneal MMT [52]. In the present study, we found that PD98059 successfully reversed the MMT process of mesothelial cells, but failed to ameliorate exosomes-induced apoptosis. These results indicate that during the process of exosomes-induced cell death and phenotype transition, the ERK pathway might exert different roles. Additional studies are necessary to identify the critical signaling pathway responsible for apoptosis of peritoneal mesothelial cells.

In conclusion, gastric cancer cell-derived exosomes induced injury of peritoneal mesothelial cells through apoptosis and MMT, resulting in mesothelial barrier destruction and peritoneal fibrosis. Our study shows that gastric cancer-derived exosomes can facilitate peritoneal dissemination and has identified a novel mechanism for gastric cancer peritoneal metastasis.

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**Author contributions**

GD and JQ designed the research, performed experiments and wrote the manuscript. ZY and XC evaluated the data and provided technical assistance.

YC, YF and SZ coordinated the work. DN provided the HMrSV5 cell lines. XQ and YL conceived and supervised the study.

**References**


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