

Exosomal MMP-1 transfers metastasis potential in triple-negative breast cancer through PAR1-mediated EMT

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Research Article

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Abstract

Purpose

Triple-negative breast cancer (TNBC) is a subtype of breast cancer with high risk of distant metastasis, in which the intercellular communication between tumor cells also plays a role. Exosomes can be released by tumor cells and promote distant metastasis through intercellular communication or changes in tumor microenvironment, it is an optimized transportation facility for biologically active payloads. This was a hypothesis-generating research on role of exosomal payload in TNBC distant metastasis.

Methods

Exosomes isolated from supernatant of MDA-MB-231 and MDA-MB-231-HM (a highly pulmonary metastatic variant of parental MDA-MB-231 cells) were characterized. Transwell assay, wound healing and CCK-8 assay were employed to explore the effect of exosomal MMP-1 on the metastatic capability of TNBC cells *in vitro*. Human breast cancer lung metastasis model in nude mice was established to observe the effect of exosomal MMP-1 *in vivo*. Tissue microarray and blood samples of TNBC patients were applied to analyze the relevance between MMP-1 with metastasis.

Results

MDA-MB-231-HM cells secrete exosomes enriched MMP-1, which can be taken up and enhance invasion and migration activities of TNBC cells. After ingesting exosomes enriched with MMP-1, cells secrete more MMP-1, which may interact with membrane G protein receptor protease activated receptor 1 (PAR1), thereby initiating epithelial-mesenchymal transition (EMT) to enhance capability of migration and invasion. The expressions of MMP-1 and PAR1 in the metastases of the 231-HM-exo treated mice were both up-regulated. Clinically, the exosomal MMP-1 can be detected from serum of patients with metastasis at higher concentration than that in pre-operative patients. Moreover, in patients with multiple distant metastases, the level of exosomal MMP-1 is also higher than that in patients with single lesion.

Conclusion

MMP-1 from TNBC cells of high metastasis potential can promote the distant metastasis of transform those with low metastasis potential through PAR1-mediated EMT and is likely to be a potential molecular marker.

Background

Breast cancer is the most commonly diagnosed malignant tumor and the sixth cause of cancer-related deaths in women¹. In China, breast cancer is usually diagnosed at 45 to 55 years old, 15-20% of which is

triple-negative breast cancer (TNBC)². TNBC is a subtype of breast cancer with high malignancy, specifically referring to negativity of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2). TNBC is characterized by a high risk of recurrence and metastasis, a short progression-free survival (PFS) and overall survival (OS)³. TNBC also has a tendency to metastasize to different organs including the lung, liver, brain and bones, leading to different prognosis and response to treatment⁴. Regarding the mechanism of breast cancer metastasis, most prior studies have focused on the process of metastasis. The interaction between cancer cell clones was less to be explored in terms of teamwork in the formation of distant metastasis.

Exosome is a subtype of extracellular vesicles (EV) with a diameter of 30 to 150 nm, which can be secreted by almost all cells and detected in body fluids such as blood, saliva, cerebrospinal fluid, urine, semen and tumor microenvironment⁵. Exosomes carry proteins, lipids, mRNA and circRNA etc., which are considered to play an important role in tumor development such as tumor growth, invasion, metastasis, and regulation of tumor microenvironment⁶. There is increasing evidence showing that exosomes play an important role in tumor spread⁷ (for example, establishing a pre-metastasis niche⁸, promoting angiogenesis⁹, destroying the peritoneum¹⁰ or the blood-brain barrier¹¹), inducing drug resistance¹² and producing cancer-related fibroblast heterogeneity¹³. We speculate that cells with high metastasis ability can deliver metastasis-promoting substances to cells with normal metastasis ability through exosomes, initiate a series of downstream cascade reactions to enhance their metastasis ability. Our experiments in vitro showed that after co-cultivation of exosomes derived from classic TNBC breast cancer cells (MDA-MB-231) and exosomes derived from high lung metastasis breast cancer cells built in our laboratory (MDA-MB-231-HM)¹⁴⁻¹⁶, the invasion capability of MDA-MB-231 was significantly improved. Therefore, we speculate that the key protein carried in exosomes derived from highly metastatic cell lines is one of the relevant factors that promote invasion and distant metastasis. This study used mass spectrometry to screen the differential proteins in exosomes derived from the high lung-metastatic cell line MDA-MB-231-HM and the classic TNBC cell line MDA-MB-231, found MMP-1 to be the most different protein. MMPs are a family of zinc-dependent endopeptidases which are crucial to ECM degradation. Several MMPs have been detected in exosomes from tumor cells¹⁷. MT1-MMP existing in exosomes from tumor cells is involved in activation of proMMP-2 as well as degradation of ECM proteins^{18, 19}. It was also reported that combined expression of miR-21 and MMP-1 in urinary exosomes detects 95% of breast cancer²⁰. We suppose that MMP-1 may be a key protein to mediate the transfer of metastatic ability between tumor cells through exosomes.

Methods

Tissue samples

Tissue specimens (n=134) were collected from female patients with TNBC, defined as ER negative, PR negative, HER2 negative or 1+ or 2+ but fluorescence in situ hybridization (FISH) negative, who underwent operation of tumor in Fudan University Shanghai Cancer Center (Shanghai, China) between August of

2015 and May of 2016. After selection of formalin-fixed paraffin embedded (FFPE) tissue blocks of tumor sections, 5 tissue microarrays (TMA) blocks with 50 cores per TMA were constructed. Sections were cut at 4 μ M thick for immunohistochemistry. Clinical characteristics were obtained from the Electronic Medical Record System and telephone follow-up. The research protocol was approved by the Ethics Committee of the Fudan University Shanghai Cancer Center, and written informed consent was obtained from all patients prior to enrollment.

Immunohistochemical staining

Standard immunohistochemical procedures were carried out using anti-MMP-1(26585-1-AP, Proteintech), anti-PAR1(26366-1-AP, Proteintech) in human breast cancer TMAs and lung tumor sections of nude mice. The TMAs were digitized via whole-slide scanning with the Aperio T2 scanner (Aperio Technologies) and IOD values were assessed independently by two pathologists in from the Department of Pathology (Fudan University Shanghai Cancer Center); discrepancies were resolved by discussion until a consensus was reached. Lung tumor sections of nude mice were observed and imaged by microscope (OLYMPUS, BX43).

Cell lines and culture

MDA-MB-231, MDA-MB-468 and BT549 (human breast cancer cell line) were acquired from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). MDA-MB-231-HM cell line and was established from MDA-MB-231 using an in vivo stepwise selection scheme in Fudan University Shanghai Cancer Center (Shanghai, China), has a high potential for lung metastasis¹⁴. MDA-MB-231, MDA-MB-468 and MDA-MB-231-HM were grown in Leibovitz's L-15 medium, BT549 was cultured in RPMI 1640, containing 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 mg/ml) in incubator at 37°C (both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Tumor cell migration and invasion assays

24-well plates inserted with 8-mm pore size filters (Corning Life Sciences, Corning, NY, USA), for tumor cell invasion ability, the filter membranes were pre-coated with 20 μ l Matrigel (dilution, 1:5; BD Biosciences, Franklin Lakes, NJ, USA). Cells were seeded into the upper chambers, with L-15 containing 20% FBS in the lower chambers. Cells were incubated for 24-36h at 37°C, and cells that had invaded or migrated to the reverse side of the membrane were detected by staining with crystal violet, viewed and counted in at least five random fields under a light microscope (OLYMPUS, IX51).

Cell proliferation

A Cell Counting Kit-8 (PN812; Dojindo, Japan) was used for cell proliferation assays. Cells were seeded into a 96-well plate at a density of 2 \times 10³ cells per well in quintuplicate wells. At day 1-6 after culture, 10 μ l of CCK-8 solution was added to each well and incubated for 2h at 37°C, the absorbance of cells was

measured at a wavelength of 450nm for calculation of the optical density (OD) values (Synergy H1, Biotek).

Wound healing assay

Cells were seeded in 6-well plates and grew to almost complete confluence, then a 10 μ l pipette tip was used to scratch a gap on the cell layer. Cells were incubated in serum-free medium. The gap widths were measured and imaged under an inverted microscope (OLYMPUS, IX51) at 0h, 24h and 36h after scratching.

Purification of exosomes from cell and human plasma

When cells grew at about 40–50% confluence, they were moved to medium containing exosome-depleted FBS, which was obtained by ultracentrifugation of standard FBS at 100,000g for 10h at 4°C followed by filtration through a 0.22 μ m vacuum filtration bottle. Supernatants were collected from 24-36h cell cultures. Exosomes were purified by differential centrifugation at 3,000g for 20 min to remove cell debris and dead cells. Vesicles were pelleted after centrifugation at 16,500g for 45 min and resuspended in PBS, then centrifuged at 100,000g for 2h at 4°C (Beckman Coulter, Optima XPN-100). After resuspending in an appropriate amount of PBS, the protein concentration was measured, recorded and stored for subsequent experiments.

For purification of circulating exosomes by differential centrifugation, blood from patients was centrifuged at 1,500g for 30min to obtain cell-free plasma. Plasma was centrifuged at 15,000g for 30min. The pelleted vesicles were suspended in PBS and then centrifuged at 100,000g for 2h at 4 °C.

Transmission electron microscopy (TEM) and Nanosight analysis (NTA) of exosomes

Purified exosomes were prepared on copper TEM grids (3.05mm; 200 mesh) by negative staining. 10 μ l sample was dropped on copper grids and incubated for 3mins. 10 μ l 2% uranyl acetate was pipetted on the grid and incubated for 10min. Excess solution was removed by with filter paper. Grid was stored in the dark grid box at RT until imaging. Imaging was conducted using a 120kV Biology Transmission Electron Microscope (Tecnai G2 SpiritBiotwin).

Nanoparticle tracking analysis was conducted by NanoSight NS300. Exosomes in 20 μ l PBS were diluted to 1000 μ l with DPBS, then loaded with a 1ml syringe to be tested. Three recordings of 30s at 37°C in camera were obtained and processed using NTA software.

Exosome labeling

Exosomes were resuspended in PBS and stained with DIO dye for 20 min at room temperature. The labeled exosomes were incubated with MDA-MB-231 cells pre-seeded on the small discs with medium containing exosome-depleted FBS for 24h at 37°C. Then the cells were washed three times in PBS-T, fixed with 4% paraformaldehyde for 15 min at room temperature, then washed 3 times with PBS-T. The cell

nuclei were counterstained with 5ug/ml DAPI (Cat.0100-20, SouthernBiotech) for 5 min. The uptake of the labeled exosomes by MDA-MB-231 cells was assessed using an inverted confocal microscope (Leica TCS SP8, Germany).

Enzyme-linked immunosorbent assay (ELISA)

100ul patient serum, exosomes purified from 250ul patient serum and 10ml cell culture media suspended in 100ul PBS were transferred to a clean tube. The concentrations of MMP-1 were determined using ELISA kits (Cat. EHC134, Quanticyto, Neobiscience, Inc, Shenzhen, China) according to the manufacturer's protocols.

Construction and transfection of MMP-1 short hairpin RNA (shRNA) lentivirus into tumor cells

An MMP-1 shRNA lentivirus was purchased (HanyinBiotech, Ltd., Shanghai, China) to knock down the expression of MMP-1 in MDA-MB-231-HM cells. The target sequences against MMP-1 were 5'-GCCTTCCAACCTCTGGAGTAAT-3' and 5'-GCTAGCTCAGGATGACATTGA-3' and 5'-GATTCTACATGCGCACAAATC-3'. MDA-MB-231-HM cells were plated into a 6-well plate, grown to reach 60-80% confluence and then transfected with viral supernatant diluted 1:1000 (lentivirus) in the presence of 10ug/ml polybrene for 24h, according to the manufacturer's protocol. Cells were selected with 3 g/ml puromycin for 7 days since 48h after transduction.

Western blot

Cell and exosome samples were lysed using RIPA buffer (Beyotime, Shanghai, China) containing protease and phosphatase inhibitors (Epizyme, Shanghai, China). The protein concentration was quantified using a BCA protein assay kit (Beyotime). Proteins were separated by 10% (gradient) SDS-PAGE (Epizyme) and transferred onto PVDF membranes (Amersham Hybond 0.45um; GE Healthcare). The membranes were blocked with 5% milk in trisbuffered saline-Tween (TBS-T) for 2h, then incubated with primary antibodies (anti-CD9(ab92726,Abcam), anti-CD63(ab134045,Abcam), anti-MMP-1(ab134184,Abcam), anti-PAR1(26366-1-AP,Proteintech), anti-Zeb-1(21544-1-AP,Proteintech), anti-Slug(12129-1-AP,Proteintech), anti-SNAI1(13099-1-AP,Proteintech), anti-E-cadherin(20874-1-AP,Proteintech), anti-Vimentin(10366-1-AP,Proteintech), anti-GAPDH(60004-1-Ig,Proteintech)) for 12h at 4°C. The membranes were washed with TBS-T for 3times, 15min per time, then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at RT. The proteins were visualized using the ECL western blotting substrate (cat. WBKLS0100, Millipore) and a Tanon-5200Multi device.

Lung colonization study

Six- to eight-week-old nude mice were injected in tail vein with 1×10^6 MDA-MB-231 cells. Cell-derived exosomes (15ug in 100ul PBS) were intravenous injected every other day for 2 weeks. The mice were euthanized six weeks after the cancer cell injection and their lungs were fixed, sectioned and analyzed for H&E quantify the metastatic tumor burden. Paraformaldehyde-fixed lungs were sectioned into 8-um-thick

sections, respectively, at 100µm intervals. Three large lung sections were stained with H&E and tumor nodules were counted and their area measured using the OlyVIA (Olympus) and ImageJ software. The number of the metastatic nodules were calculated by averaging data from individual sections.

Co-immunoprecipitation

Co-immunoprecipitation of MMP-1 or PAR1 from MDA-MB-231 cell lysates was carried out as follow. MMP-1 and PAR1 were immunoprecipitated using a rabbit anti-MMP-1 or anti-PAR1 antibody. Rabbit IgG were used as negative controls. Immune complexes were captured using the Protein A/G-agarose beads (LOT. 70474621, Roche), and immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting with rabbit monoclonal anti-MMP-1 antibody (26585-1-AP, Proteintech) or anti-PAR1 antibody (26366-1-AP, Proteintech). Immunoreactive bands were visualized using enhanced chemiluminescence.

Chemicals and inhibitors

Vorapaxar (HY-10119) was obtained from MedChemExpress.

Statistical analysis

All experiments in the study were performed in triplicate. Statistical analyses were performed with the SPSS v20.0 software (SPSS Inc., Chicago, IL, USA). Data are presented as the mean±standard deviation (SD). Quantitative data were compared with a two-tailed Student's t-test between groups and a one-way analysis of variance among multiple groups followed by Least Significant Difference post hoc test. Kaplan-Meier curves of disease-free survival were plotted and survival in the groups was compared by log-rank test. The significance levels of the data are denoted by * symbols as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$. $p < 0.05$ was considered to indicate a statistically significant difference.

Bioinformatics analysis

The association between MMP1 expression and prognosis in triple-negative breast cancer patients was analyzed by using the Kaplan Meier plotter, which is an online database that provides assessment of the effect of 54,675 genes on survival using 10,293 cancer samples, including 22,277 genes in 5,143 breast cancer samples (<http://kmplot.com/analysis/>).

Results

Isolation and verification of exosomes

In our previously study, MDA-MB-231-HM (MDA-231-HM), a highly pulmonary metastatic variant of parental MDA-MB-231 (MDA-231) cells, was derived within the presented model system by six cycles of the pulmonary metastasis implantation to the mammary fat pad (MFP)¹⁴, which was a valuable model system for the study of the molecular events underlying breast cancer metastasis^{15,16}. The differences in

pulmonary metastatic potential of the two cell lines make them valuable systems for understanding the molecular mechanisms underlying breast cancer metastasis. Transwell assay showed that migration and invasion capabilities of MDA-231-HM are stronger than MDA-231 cells (Figure 1A).

We purified exosomes from supernatants of MDA-231 and MDA-231-HM cells by differential centrifugation^{21,22}, and verified them by Transmission electron microscopy (TEM) (Figure 1B), Western blot (Figure 1C) and nanoparticle tracking analysis (NTA) (Figure 1D). After co-cultivating MDA-231 cells with 231-HM-exo for 24h, the exosomes were observed by confocal microscopy close to the nucleus, indicating that the exosomes have been taken up by MDA-231 cells (Figure 1E).

MMP-1 in exosomes mediates the transmission of metastasis capability

MDA-231 cells were pretreated with PBS, 231-exo and 231-HM-exo for 24h and then analyzed by Transwell migration and invasion assay. The results showed that the migration and invasion ability of MDA-231 cells co-cultured with 231-HM-exo was significantly improved while the invasion ability of MDA-231 cells co-cultured with 231-exo was improved (Figure 2A). We assume that 231-HM-exo was taken up by MDA-231 cells, releasing its cargo to increase the content of protein or RNA, which directly or indirectly activated downstream signaling pathways and improved the invasion and migration capability of MDA-231 cells *in vitro*. In order to find the key protein transferred by exosomes that changes the ability of invasion and migration capability, we screened the differential proteins between 231-exo and 231-HM-exo by mass spectrometry. From the heat map we found that MMP-1 is the protein with the most significant difference in expression (Figure 2B).

MMP-1 belongs to matrix metalloproteinase (MMP) family, which includes a series of zinc- and calcium-dependent endopeptidases. MMPs are of crucial importance for invasive cancer cells to break extracellular matrix (ECM) barriers and start the metastatic cascade^{23,24}. MMP1 has also been shown to be related to invasive phenotype, metastasis and response to chemotherapy in human breast cancer, and is related to poor prognosis²⁵⁻²⁷. We verified the results of mass spectrometry by western blot of MMP-1 in MDA-231, MDA-231-HM, 231-exo and 231-HM-exo and saw significant difference in both cells and exosomes (Figure 2C).

Targeted knockdown of MMP-1 RNA inhibits metastasis through exosomes

We used lentivirus packaged short-hairpin RNA (shRNA) to infect MDA-231-HM to knockdown MMP-1. We selected 231-HM-shRNA3, whose MMP-1 in the exosome was significantly downregulated, hereinafter referred to as MDA-231-HM-shMMP1 (Figure 3A, B). In order to rule out the change of proliferation capability caused by MMP-1, which may interfere the results of migration and invasion assays, we used CCK-8 kit to analyze the proliferation of MDA-231, MDA-231-HM, MDA-231-HM Negative Control (MDA-231-HM-NC), and MDA-231-HM-shMMP1. Results showed no correlation between MMP-1 expression and cell proliferation (Figure 3C). Both Transwell and wound healing assay showed that migration and invasion activity of MDA-231-HM was significantly weakened after targeted knockdown of MMP-1 (Figure 3D, E).

After observing the differences in migration and invasion ability of cells, we focused on cell-to-cell transmission of MMP-1 through exosomes. Transwell assay showed that co-cultivation of MDA-231 with 231-HM-shMMP1-exo for 24h significantly reduced the migration and invasion of 231 cells co-cultured with 231-HM-exo. Considering the heterogeneity of TNBC, we added MDA-MB-468 (MDA-468) and BT549 to repeat the above experiment and the results were similar, proving that the role of MMP-1 in exosomes is not limited to MDA-231 cell line (Figure 3F, G).

MMP-1 binding to PAR1 promotes the metastasis of TNBC possibly through EMT

The function of MMP-1 on ECM degradation has been reported^{23,24}, which cannot fully explain the enhanced invasion and migration activity both. MMP-1/PAR1 as a key signal to activate downstream signaling pathways to facilitate vascular intravasation and metastatic dissemination has also been proposed in several tumors²⁸⁻³⁰. Protease-activated receptor 1 (PAR1) is a G protein-coupled receptor that is classically activated by the serine protease thrombin cleavage of the N-terminal outer domain, can also be directly activated by MMP-1 and MMP-9³¹. PAR1 is also considered as an independent factor for the poor prognosis of tumors and a potential therapeutic target³².

We found that after ingestion of exosomes with different levels of MMP-1, the level of free MMP-1 secreted by MDA-231 cells into the supernatant was positively correlated with the level of MMP-1 in exosomes (Figure 4A). Then, we demonstrated through co-immunoprecipitation that MMP-1 can directly interact with PAR1 to stimulate downstream metastasis related pathways (Figure 4B). The PAR1 irreversible antagonist, Vorapaxar, was added at a concentration gradient to the medium of 231 cells co-cultured with 231-HM-exo and could effectively reverse the effect of 231-HM-exo. Vorapaxar inhibited 58% and 79% of the migration, 45% and 57% of the invasion at 75 μ M and 100 μ M, respectively (Figure 4C). This strongly explains that MMP-1 can not only degrade collagen to promote invasion, but mainly rely on PAR1 to exert its invasion effect. Inhibiting PAR1 can greatly inhibit this acquired capability of invasion. In order to eliminate that the change in cell proliferation ability brought by PAR1 may interfere the results of migration and invasion assays, we first used CCK-8 assay to detect the proliferation of MDA-231 cells co-cultured with 231-HM-exo and Vorapaxar. Results showed that cell proliferation was independent of 231-HM-exo and Vorapaxar (Figure 4D).

According to previous reports, exosome-associated MMPs are involved in the processes of EMT in some tumors³³⁻³⁵. The decrease in keratin filaments and the decrease of E-cadherin, as well as the increase of vimentin, fibronectin, N-cadherin, α -SMA and various proteases, resulted in the loss of polarity and acquisition of the mesenchymal phenotype^{36,37}. We speculated that PAR1 was activated by MMP-1 and mediated epithelial-mesenchymal transition (EMT) which enhanced the ability of metastasis in TNBC. MDA-231 cells were treated with PBS, 231-HM-exo, 231-HM-shMMP1-exo and sufficient Vorapaxar and Western blot was used to detect the expression of EMT biomarkers. The down-regulation of E-cadherin and up-regulation of ZEB-1, Vimentin and Slug in MDA-231 cells co-cultured with 231-HM-exo suggested mesenchymal phenotype, while in 231-HM-exo and Vorapaxar group, E-cadherin was up-regulated, ZEB-1, Vimentin and Slug were significantly down-regulated, suggesting epithelial phenotype. It can be seen that

the EMT process promoted by 231-HM-exo can be reversed by PAR1 antagonist (Figure 4E). Based on the results of *in vitro* experiments, we propose that after 231-HM-exo is ingested by targeted cells, the secretion of MMP-1 increases, which can directly degrade ECM, and may possibly also cleave and activate PAR1-mediated EMT to promote cell migration and invasion.

Exosomal MMP-1 mediates the transmission of metastasis capability *in vivo*.

To verify the metastasis ability of MDA-231 and MDA-231-HM cells *in vivo*, we established a human breast cancer lung metastasis model in nude mice. We observed that mice injected with MDA-231-HM cells (n=3) had significantly more lung metastases than MDA-231 cells (n=3, $p<0.05$) (Figure 5A), which means that the metastatic ability of MDA-231-HM *in vivo* is much stronger than that of MDA-231 cells.

To determine the effect of MMP-1 in exosomes on the transfer ability of MDA-231 cells *in vivo*, We further constructed nude mouse lung metastasis model with MDA-231 cells and treated it with PBS, 231-exo, 231-HM-exo and 231-HM-shMMP1-exo every other day for 2 weeks (Figure 5B). Metastatic nodules in mice treated with 231HM-exo (n=9) was significantly more than that with PBS (n=9) and 231-exo (n=9) ($p<0.001$), and which were reduced by 231-HM-shMMP1-exo (n=9) ($p<0.005$) (Figure 5C-E). A hypothesis consistent with results *in vitro* can be drawn, exosomes with high levels of MMP-1 can promote metastasis ability of MDA-231 *in vivo*, which would be weakened by reducing MMP-1 in exosomes.

Conducting immunohistochemical staining for MMP-1 and PAR1 on lung lesions of nude mice, we saw the expression of MMP-1 and PAR1 in lung lesions of mice treated with 231-HM-exo increased, while the expression of 231-HM-shMMP1-exo treated group decreased (Figure 5F).

Plasma exosomal MMP-1 level is elevated in patients with shorter DFS.

A total of 134 surgical tissues from TNBC patients from August 2015 to May 2016 were collected and made into tissue microarrays (TMA). The characteristics of patients at baseline are shown in Table 1. The patients were followed up to 2020.8, median follow-up time was 56.8 months. MMP-1 expression was determined by immunohistochemistry and a quarter one fourth of the OD value of MMP-1 (OD=4.009) was chosen as a cut-off value to define 'high' and 'low' expression (Figure 6A). In the univariate Kaplan-Meier analysis, TNBC showing high expression of MMP-1 had a significantly shorter disease-free survival (DFS) (logrank $p=0.028$) (Figure 6B). Survival curve retrieved from Kaplan-Meier plotter showed that high level MMP-1 expression is associated with significantly worse DFS (logrank $p=6e-13$) in TNBC patients (Figure 6C).

Blood samples from patients at baseline of pre-surgery (n=18) and initial metastasis (n=30), baseline characteristics are shown in Table 2. The MMP-1 expression level in serum and exosomes purified from serum was detected by ELISA. We found that MMP-1 expression in initial metastasis group was significantly higher than that in pre-surgery group, whether in serum ($p<0.0001$) or exosomes ($p<0.0001$). It was confirmed that the MMP-1 carried in exosomes was related to the recurrence and systemic metastasis of TNBC (Figure 6D, E).

Furthermore, we distinguished patients with single metastatic site (n=13) and multiple metastatic sites (n=17) in the initial metastasis group to research the correlation between MMP-1 and systemic metastasis. The concentration of MMP-1 in the serum has no statistical difference (p=0.1488) while in exosomes, MMP-1 detected in patients with multiple metastases was significantly higher than that in single metastatic site (p<0.01), which suggests that for patients with distant recurrence and metastasis, MMP-1 detected in exosomes would be more sensitive than in serum (Figure 6F, G).

Interestingly, we collected blood samples from several patients (n=11) before and after first-line chemotherapy and tumor response was evaluated in accordance with the Response Evaluation Criteria in Solid Tumors (RECIST 1.1) guidelines by computed tomography scanning or magnetic resonance imaging after 2 cycles³⁸. We found that MMP-1 level in exosomes was also related to response to chemotherapy. The expression of MMP-1 in exosomes of patients with partial response (PR)(n=4) decreased while increased in patients with progressive disease (PD)(n=3). In patients with stable disease (SD)(n=4), the expression of MMP-1 in exosomes both increased and decreased, which suggests that MMP-1 in exosomes even has the potential to be an indicator of therapeutic efficacy (Figure 6H).

Discussion

Many reports demonstrated that exosomes can mediate tumor metastasis by horizontal transfer of bioactive molecules to recipient cells^{8, 11, 38-40}. In this study, we first reported that TNBC cells with high metastasis potential transform those with low metastasis potential via exosomal MMP-1.

Exosomes have been reported to take part in promoting tumor metastasis and drug resistance^{41, 42}. Exosomes potentially promote tumor development by regulating biological functions, including angiogenesis, immunity, vascular leakiness, and reprogramming recipient cells to construct pre-metastatic niche (PMN) and metastasis⁴³⁻⁴⁵. To study the role of exosomes in tumor metastasis, we extracted, verified and observed the exosomes taken up by MDA-231 cells. Then mass spectrometry was applied for the most different expressed protein between high and low lung metastasis cell-derived exosomes, which is MMP-1.

Historically, MMPs have been studied for a long time in cancer biology, and accumulated evidence showed that MMPs are related to progression, metastasis, treatment and prognosis of cancer⁴⁶⁻⁴⁸. As reported, MMPs mediate the degradation of ECM and basement membrane (BM) at all stages of cancer progression, thereby promoting the development of the surrounding microenvironment and distant metastasis^{13,28}. However, the fact that MMP-1 is transmitted between cells through exosomes to enhance metastasis ability of low-metastatic cells has not yet been reported.

Unsurprisingly, our study found that knocking down the MMP-1 of MDA-231-HM with shRNA reduced MMP-1 in MDA-231-HM-secreted exosomes. Hereafter, we observed that exosomes with reduced MMP-1 content actually affected the invasion and migration ability of MDA-231 *in vitro* and *in vivo*. Further, we explored the mechanism of the influence of exosomal MMP-1. The most widely known function of MMP-1

is to degrade collagen I, II and III in the ECM, but it cannot fully explain the enhanced activity of both invasion and migration. In addition to degrading collagen, MMP-1 can also directly PAR1 by cutting the extracellular N-terminus of PAR1²⁶. PAR1 is a G protein-coupled receptor that is classically activated by the serine protease thrombin cleavage of the N-terminal outer domain. PAR1 activated by MMP-1 provides a link between the extracellular proteolytic activity important for ECM remodeling and the signal transduction leading to cell migration and invasion³¹. It was reported that MMP-1 and MMP-13 cut the N-terminal outer domain of PAR1 at non-canonical sites, activating the G-protein signaling pathway³⁹. In breast cancer cells, thrombin and MMP-1 activated PAR1-dependent phospho-Akt signals⁴⁶. In our study, immunoprecipitation assay showed that MMP-1 directly interacts with PAR1. The PAR1 antagonist, Vorapaxar can also significantly reverse the up-regulated metastatic capability of MDA-231 after co-culture with 231-HM-exo at a certain concentration. It can also be seen that the EMT process promoted by 231-HM-exo can be reversed by PAR1 antagonist. We took the lung tissues for immunohistochemistry and found that the expression of MMP-1 and PAR1 were higher in the 231-HM-exo-treated group, whereas those were down-regulated with 231-HM-shMMP1-exo. Therefore, it is concluded that free MMP-1 in the supernatant, which sources may be from released MMP-1 from internalized exosomes and production by initial low metastasis capacity MDA-231 cells, binds to the membrane surface receptor PAR1 protein and then promotes the metastasis of the triple-negative breast cancer possibly through EMT.

By searching the Kaplan-Meier database, we observed that the expression level of MMP-1 is related to DFS of TNBC, and this finding was then confirmed by TMA of surgical tissue samples of TNBC patients in our center. There was also significantly up-regulated level of MMP-1 in serum and exosomes in patients with metastasis. Moreover, whereas there was no statistical difference in serum MMP-1 expression in patients with solitary lesion and multiple distant metastasis, MMP-1 in exosomes produced statistical differences, indicating that exosomes may be more sensitive and accurate in predicting and evaluating distant metastases. Furthermore, comparing exosomal MMP-1 level before and after first-line chemotherapy in several patients showed that MMP-1 alteration is associated with the efficacy, indicating implication in predicting the efficacy of chemotherapy.

There are still some limitations in this study. In terms of experimental data, first of all, due to the limitations of laboratory facilities, we were unable to record the trajectory of cell migration and draw trajectory diagrams to visually display and compare the metastatic ability of cells. Secondly, in the study of the relationship between the changes in the level of MMP-1 in exosomes and the efficacy of first-line chemotherapy in clinical patients, the number of blood samples was too small to draw a statistical conclusion. In terms of conclusion, the mechanism of EMT caused by the interaction between PAR1 and MMP-1 has not been directly clarified through experiments. We could only suppose and prove that the cells go through EMT by western blot assay for changes in EMT-related markers before and after the treatment of exosomes. Finally, the research is currently aimed at TNBC, and ER-positive and HER-2-positive breast cancer cell lines may be included for further study.

Conclusions

In summary, exosomes secreted by cells of high metastasis ability enriched in MMP-1 can transform cells with low metastasis ability in TNBC into more malignant cells, which is possibly mediated EMT via interaction with PAR1. Clinically, higher MMP-1 level in exosomes in patient's blood indicates more chance of occurrence of distant metastasis, along with a certain potential for the evaluation of therapeutic effects.

Abbreviations

TNBC, Triple-negative breast cancer; EV, extracellular vesicles; MMP-1, matrix metalloproteinase-1; PAR1, protease-activated receptor 1; ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor-2; PFS, progression-free survival; OS, overall survival; FFPE, formalin-fixed paraffin embedded; TMA, tissue microarrays; OD, optical density; TEM, Transmission electron microscopy; NTA, Nanosight analysis; MFP, mammary fat pad; shRNA, short-hairpin RNA; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; DFS, disease-free survival; RECIST 1.1, Response Evaluation Criteria in Solid Tumors; PR, partial response; PD, progressive disease; SD, stable disease; PMN, pre-metastatic niche.

Declarations

Funding

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

YH ZHU and ZH TAO have contributed equally to this work and share first authorship.

XC Hu and T Li conceived the study and gathered together the team. YH Zhu performed the experiments. YH Zhu and ZH Tao contributed to experimental development control and validation, as well as to data analysis. T Li, Y Chen, SC Lin and MY Zhu provided resources for study development and data acquisition. YH Zhu prepared the figures and the original draft. All authors provided critical feedback and reviewed and edited the manuscript. XC Hu and T Li obtained the funding for the study accomplishment.

Ethics approval

This study was approved by the Ethics Committee of Shanghai Cancer Center of Fudan University.

Consent to participate

All patients signed the informed consent.

Consent for publication

All authors are consent for publication.

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Tables

Table 1. Patient Characteristics at baseline (N=134)

Characteristics	Pts (N=134)
	N (%)
Age (Median, range)	52, 24-82
< 40 years	18 (13.43%)
≥ 40 years	116(86.56%)
Menstruation status	
Post-menopausal	86(64.18%)
Pre-menopausal	48(35.82%)
Pathological tumor size	
≤2 cm	32(23.88%)
2-5 cm	97(72.39%)
>5 cm	5(3.73%)
Grade	
I	10(7.46%)
II	41(30.60%)
III	83(61.94%)
Ki-67	
≤15%	7(5.22%)
16-30%	31(23.13%)
> 30%	96(71.64%)

Nodal status	
0 positive nodes	74(55.22%)
1-3 positive nodes	40(29.85%)
≥4 positive nodes	20(1.54%)
Prior Adjuvant Chemotherapy	
Anthracyclines	3(2.24%)
Taxanes	15(11.19%)
Both	88(65.67%)
Unknown	28(20.90%)

Table 2 Patient Characteristics at baseline (N=48)

Characteristics	Initial metastasis (N=30)	Post-operation (N=18)
	N (%)	N (%)
Age (Median, range)	50 [31-66]	51, 31-68
< 40 years	7 (23.3%)	3 (16.7%)
≥ 40 years	23 (76.7%)	15 (83.3%)
Menstruation status		
Post-menopausal	20 (66.7%)	5 (27.8%)
Pre-menopausal	10 (33.3%)	13 (72.2%)
ECOG performance status		
0	0 (0)	0 (0)
≥1	30 (100%)	18 (100%)
Neoadjuvant Chemotherapy		
Yes	3 (10%)	0 (0)
No	27 (90%)	18 (100%)
Pathological tumor size		
0 to ≤2 cm	14 (46.7%)	12 (66.7%)
2 to ≤5 cm	13 (43.4%)	5 (27.8%)
>5 cm	3 (10%)	1 (5.56%)
Number of metastatic sites		
<3	17 (56.7%)	-
≥3	13 (43.3%)	-
Metastatic sites		
Lymph nodes	19 (63.3%)	-
Liver	3 (10%)	-
Bone	9 (30%)	-
Lung	18 (60%)	-
Chest wall	6 (20%)	-
Pleura	2 (6.67%)	-

Others	0 (0)	-
Visceral metastasis		
Yes	24 (80%)	-
No	6 (20)	-
Disease-free survival		
> 12 months	26(86.7%)	-
≤ 12 months	4(13.3%)	-

ECOG, Eastern Cooperative Oncology Group;

Figures

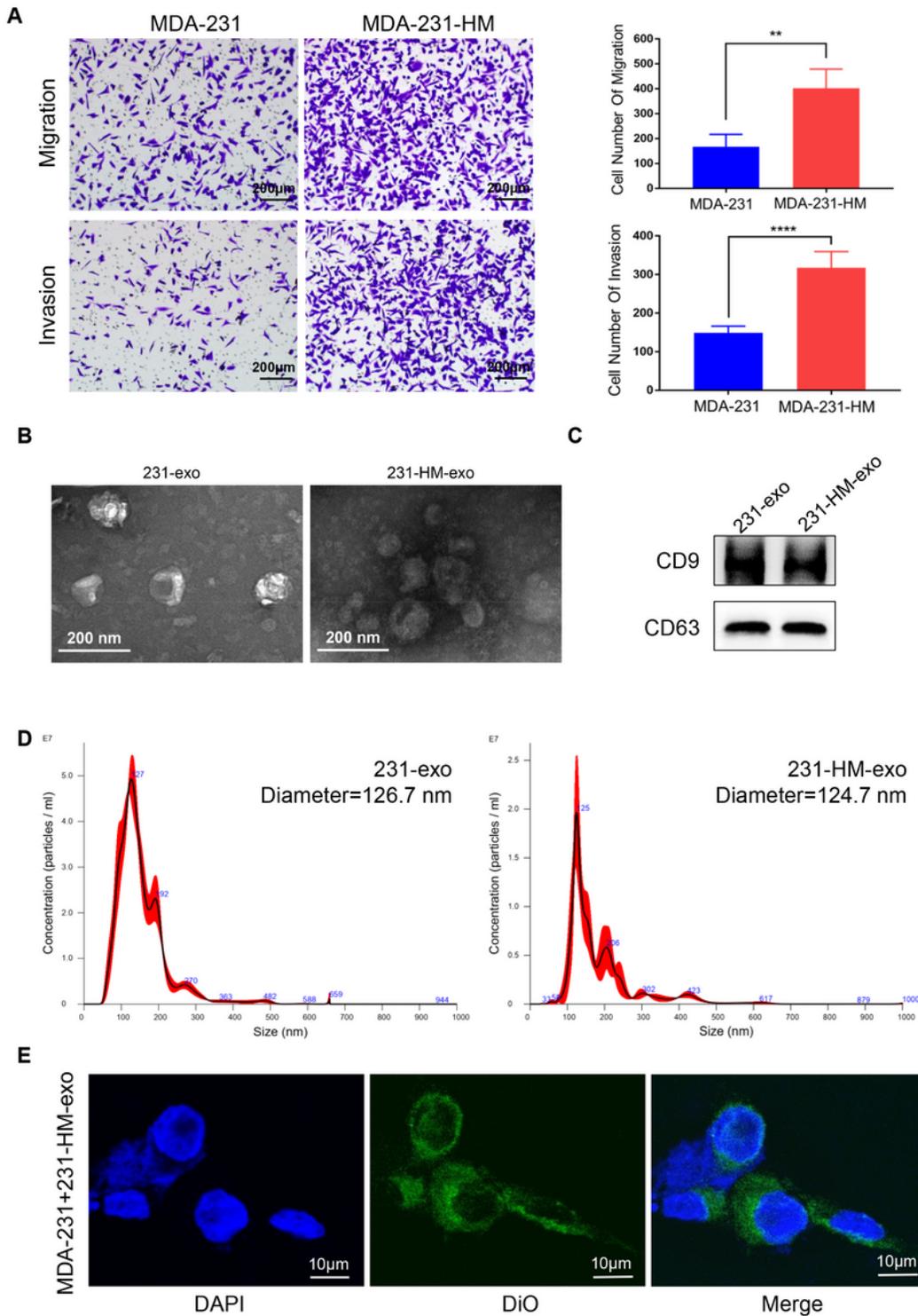


Figure 1

Isolation and verification of exosomes. (A). Migration and invasion assay of MDA-231 cells and MDA-231-HM cells. Scale bar: 200 μ m. (B). Transmission electron microscopy image of 231-exo and 231-HM-exo. Scale bar: 100 nm. (C). Western blot analysis of exosome markers in 231-exo and 231-HM-exo. (D). Nanoparticle analysis of 231-exo and 231-HM-exo, confirming the expected size range of 30-150 nm in diameter. (E). Immunofluorescence images of DiO-labeled 231-HM-exo (green) ingested by MDA-231 cells

(blue) after co-culture for 24h. Scale bar: 10 μ m. Representative images were presented from three independent experiments. Data are shown as the mean \pm SD. N.S., no significance, * $p < 0.05$, ** $p < 0.01$.

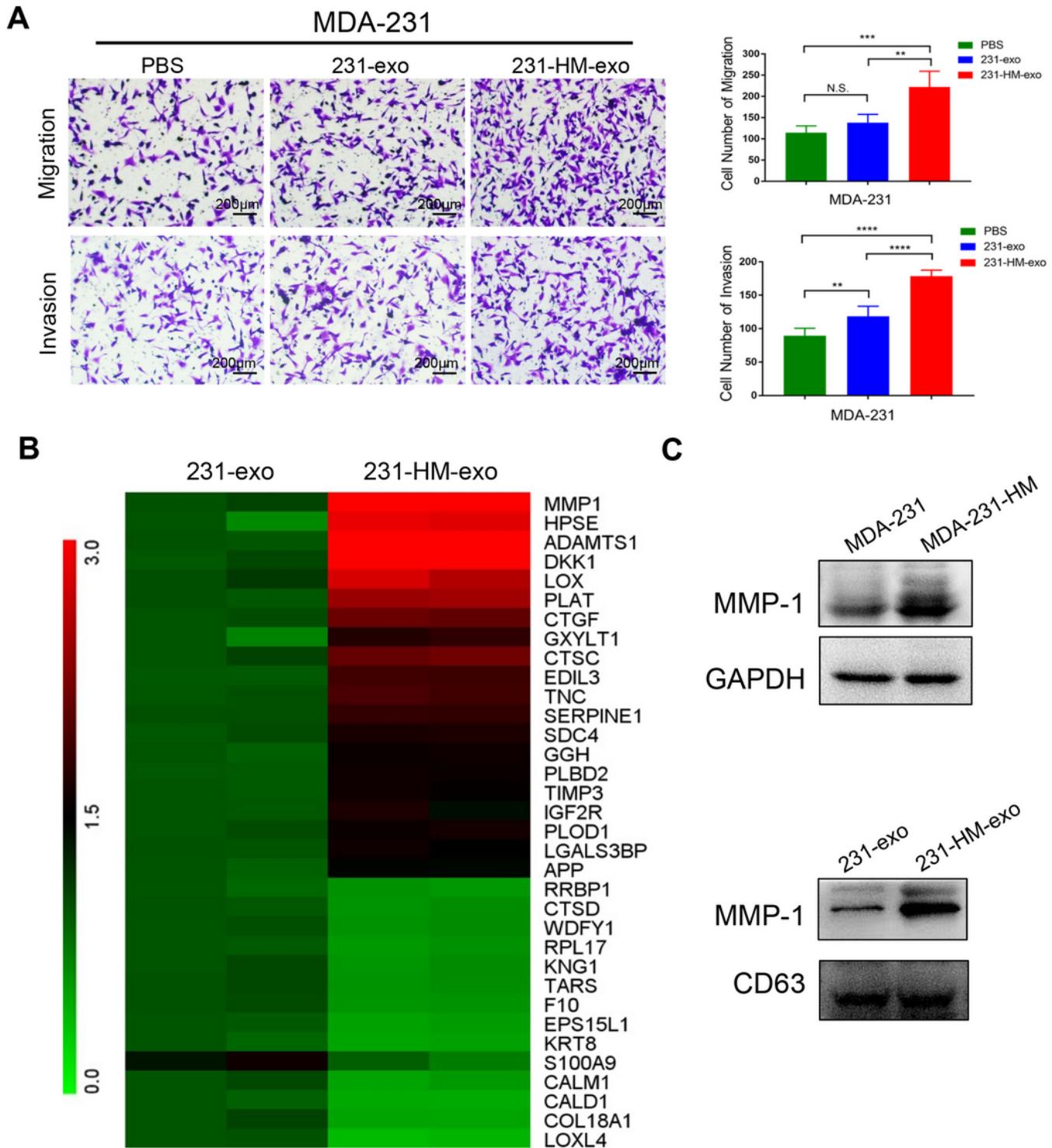


Figure 2

MMP-1 was upregulated in 231-HM-exo. (A). Transwell assay of MDA-231 cells pretreated with PBS, 231-exo and 231-HM-exo. Scale bar: 200 μ m. (B). Heat map showing the relative expression of proteins in 231-exo and 231-HM-exo. (C). Western blot analysis of MMP-1 in MDA-231, MDA-231-HM cells, 231-exo and

231-HM-exo. Representative images were presented from three independent experiments. Data are shown as the mean \pm SD. N.S., no significance, * $p < 0.05$, ** $p < 0.01$.

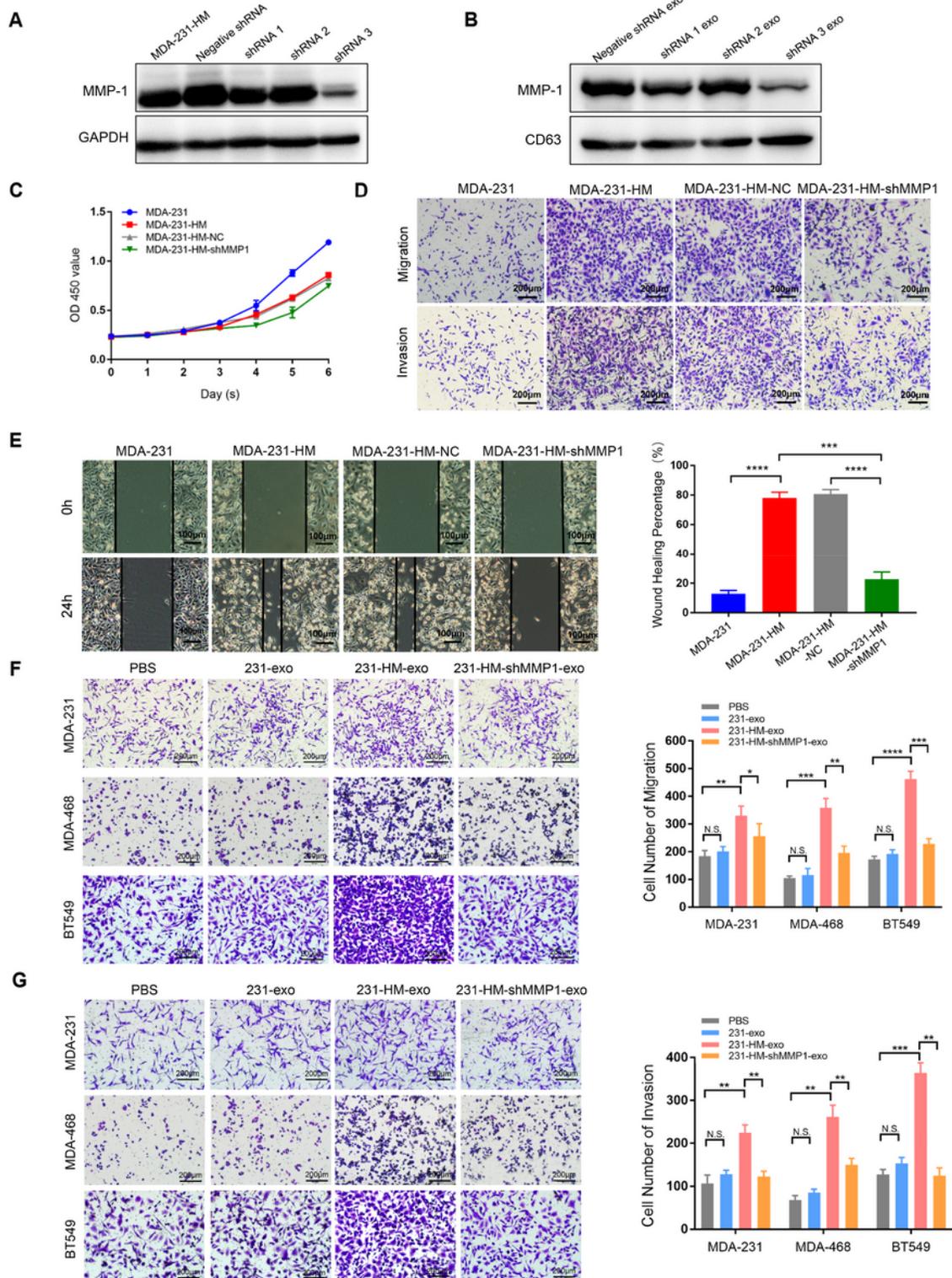


Figure 3

Targeted knockdown of MMP-1 RNA inhibits exosomes-mediated metastasis in vitro. (A). Downregulation of MMP-1 in MDA-231-HM cells by short-heparin RNA. (B). Western blot analysis of MMP-1 in exosomes purified from MMP-1-shRNA-treated 231HM cells. (C). CCK-8 assay of MDA-231, MDA-231-HM, MDA-231-

HM-NC and MDA-231-HM-shMMP1 cells. (D). Transwell migration and invasion assay of MDA-231, MDA-231-HM, MDA-231-HM-NC and MDA-231-HM-shMMP-1 cells. Scale bar: 200 μ m. (E). Wound healing assay of MDA-231, MDA-231-HM, MDA-231-HM-NC and MDA-231-HM-shMMP1 cells. Scale bar: 100 μ m. (F). Migration assay of MDA-231, MDA-468 and BT549 cells co-cultured with PBS, 231-HM-exo, 231-HM-NC-exo and 231HM-shMMP-1 for 24h. Scale bar: 200 μ m. (G). Invasion assay of MDA-231, MDA-468 and BT549 cells co-cultured with PBS, 231-HM-exo, 231-HM-NC-exo and 231HM-shMMP1-exo for 24h. Scale bar: 200 μ m. Data are shown as the mean \pm SD. N.S., no significance, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

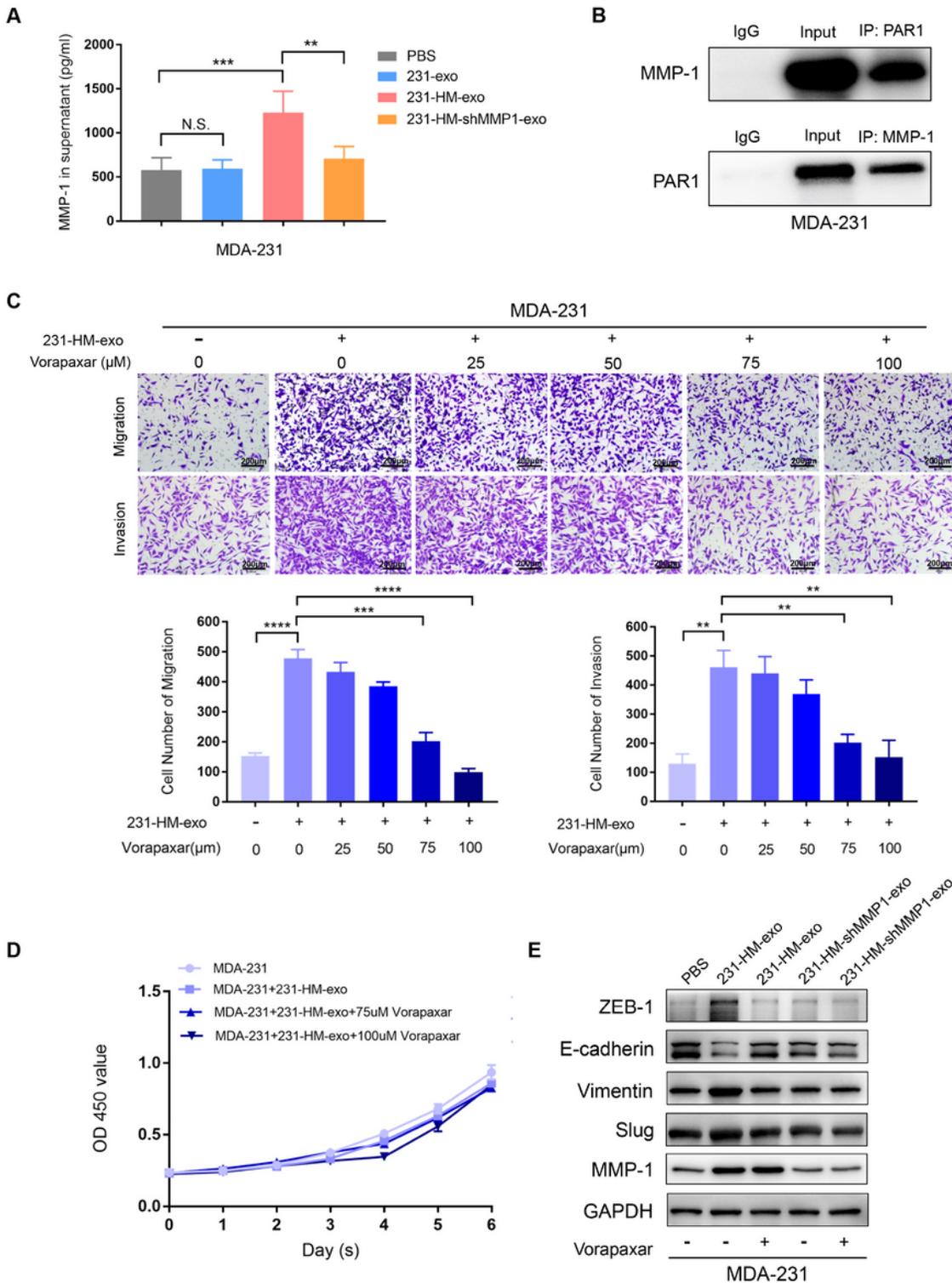


Figure 4

MMP-1 binding to PAR1 promotes the metastasis of TNBC possibly through EMT. (A). Quantification of MMP-1 in supernatants of 231 cells after pre-treated with PBS, 231-exo, 231-HM-exo and 231-HM-shMMP1-exo, measured by ELISA. (B). Whole-cell lysates from activated 231 cells were subjected to immunoprecipitation with anti-MMP-1 and immunoblotted for PAR1, then with anti-PAR1 and immunoblotted for MMP-1. (C). Transwell migration and invasion assay of MDA-231 cells treated with

231-HM-exo and Vorapaxar (irreversible PAR1 antagonist) at different concentration. Scale bar: 200 μ m. (D). CCK-8 assay of MDA-231 cells co-cultured with 231-HM-exo and Vorapaxar at certain concentration. (E). Western blot analysis of MMP-1 and EMT-related biomarkers in MDA-231 cells co-cultured with PBS, 231-exo, 231-HM-exo and 231-HM-shMMP1-exo. Representative images were presented from three independent experiments. Data are shown as the mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

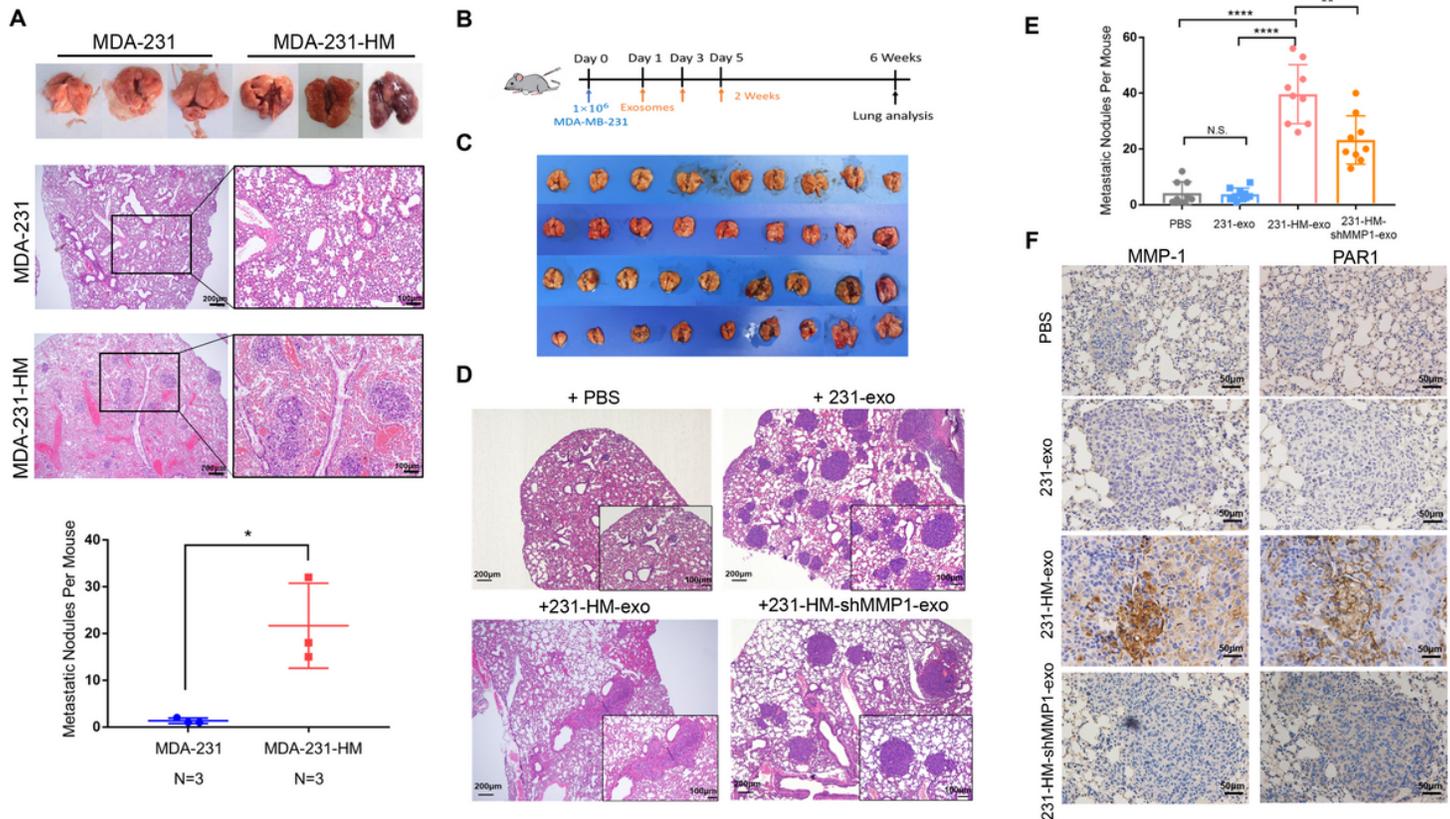


Figure 5

Exosomal MMP-1 mediates the transmission of metastasis capability in vivo. (A). Representative lung tissues and H&E images from lung sections of mice injected with 1×10^6 231 and 231HM cells in the tail vein ($n=3$ in each group). Scale bar: 200 μ m (left), 100 μ m (right). Number of metastatic lung nodules per mouse as determined by sum of three coronal sections per mouse. (B). Schematic diagram of lung metastasis model in nude mice. (C-D). Representative lung tissues(C) and H&E images(D) from lung sections of mice injected with 1×10^6 MDA-231 cells and then treated with PBS, 231-exo, 231-HM-exo and 231-HM-shMMP1-exo ($n=9$ in each group). (E). Number of metastatic lung nodules per mouse as determined by sum of three coronal sections per mouse. (F). Representative images for immunohistochemistry of MMP-1 and PAR1 in lung sections of nude mouse lung metastasis model. Scale bar: 50 μ m. Representative images were presented from three independent experiments. Data are shown as the mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

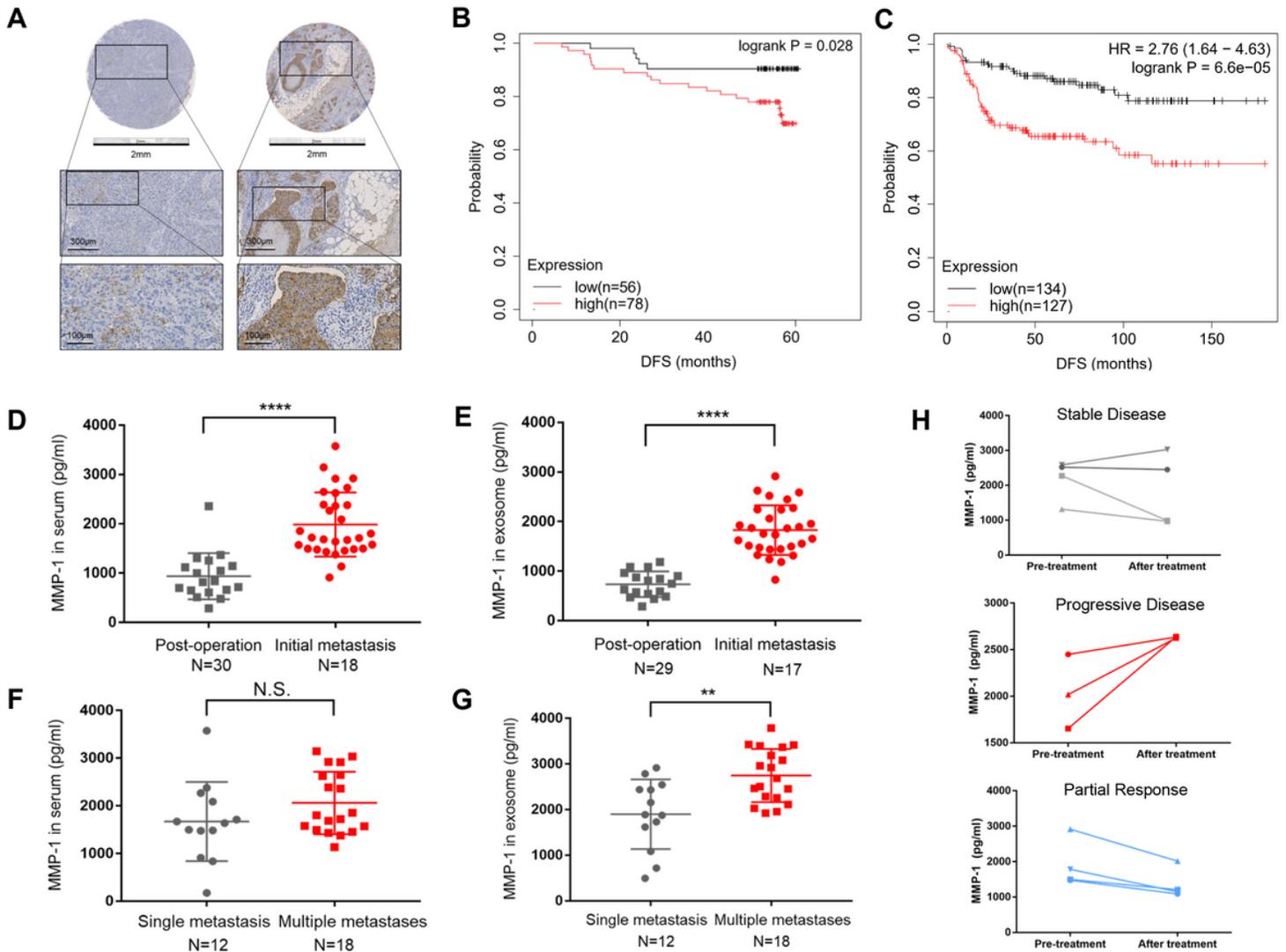


Figure 6

Plasma exosomal MMP-1 level is elevated in patients with shorter DFS. (A). Representative IHC stained images of breast cancer tissues of patients in Shanghai Cancer Center from Aug 2015 to Aug 2020 (n=134), referring high (n=78) and low (n=56) expression of MMP-1. (B). Kaplan-Meier survival analysis showing the relationship between MMP-1 expression and DFS of breast cancer patients (n=134). (C). The DFS curves of TNBC patients with high (n=134) and low (n=127) MMP-1 expression. Data was retrieved from the Kaplan Meier plotter (<http://kmplot.com/analysis/>). (D-E). Quantification of MMP-1 in serum and exosomes isolated from the serum of breast cancer patients before surgery (n=18) and first diagnosed as metastasis (n=30), measured by ELISA. (F-G). Quantification of MMP-1 in serum and exosomes isolated from the serum of breast cancer patients with single metastatic site (n=13) and multiple metastatic sites (n=17), measured by ELISA. (H). Quantification of MMP-1 in exosomes isolated from the serum of breast cancer patients (n=11) before and after first-line chemotherapy, measured by ELISA. Efficacy has been assessed as 'stable disease' (n=4), 'progressive disease' (n=3) and 'partial response' (n=4), respectively. Data are shown as the mean \pm SD. N.S., no significance, *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001.