microRNA-4488 in extracellular vesicles derived from marrow mesenchymal stem cells suppresses ABHD8 to inhibit ovarian cancer progression

Lei Chang

Zhengzhou University First Affiliated Hospital

Junying Zhou

Zhengzhou University First Affiliated Hospital

Wanjia Tian

Zhengzhou University First Affiliated Hospital

Mengyu Chen

Zhengzhou University First Affiliated Hospital

Ruixia Guo

Zhengzhou University First Affiliated Hospital

Ningjing Lei (✉ leiningjing@hotmail.com)

Zhengzhou University <https://orcid.org/0000-0002-8615-4056>

Research

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Abstract

Background

Extracellular vesicle (EV) that delivered microRNAs (miRNAs) have been found as the important biomarkers participating in the pathological mechanism of ovarian cancer. Consequently, this study sought to examine the underlying mechanism of mesenchymal stem cell (MSC)-derived EVs containing miR-4488 in ovarian cancer.

Methods

The normal ovarian tissues and ovarian cancer tissues were extracted, and the information of MSC-EV miRNA was obtained by Bioinformatics analysis. RT-qPCR and western blot analysis were applied to detect miR-4488 and α/β -hydrolase domain-containing (ABHD)8 expression followed by determination of relationship between miR-4488 and ABHD8 by dual-luciferase reporter assay. After transfection with different plasmids and treatment with DMSO or GW4869 (inhibitor of EV), the regulatory roles of MSC-EV-miR-4488 in invasion, proliferation, apoptosis, and migration of cancer cells were explored. Besides, xenograft tumor in nude mice was conducted to explore the role of miR-4488 and ABHD8 in ovarian cancer *in vivo*.

Results

miR-4488 was poorly expressed and ABHD8 was highly expressed in ovarian cancer cells and tissues. ABHD8 was a target gene of miR-4488 while the knockdown of ABHD8 resulted in the suppression of proliferation, invasion, and migration while promoting the apoptosis of cancer cells. Functionally, MSC-EV-derived miR-4488 inhibited the expression of ABHD8. Additionally, miR-4488 over-expressed in MSC-EVs inhibited the cell proliferation, invasion, and migration through down-regulation of ABHD8 expression. At last, these *in vitro* findings were also confirmed *in vivo*.

Conclusion

To summarize, miR-4488 overexpressed in MSC-EVs suppressed ABHD8 expression to inhibit the cancer cell proliferation, invasion, and migration, thus suppressing ovarian cancer.

Background

Ovarian cancer is the second leading cause of death among women worldwide, and is made up of some distinct biological and molecular subtype properties (1). Patients with ovarian cancer may be diagnosed at late stage and suffer metastasis that is associated with poor prognosis (2). Although different kinds of strategies have been applied for ovarian cancer treatment, there is a shortage of the effective screening

strategy (3). Notably, mesenchymal stem cells (MSCs) have been reported to play the inhibitory role in the invasion of ovarian cancer (4). Therefore, this study attempts to investigate new therapeutic targets for ovarian cancer that may suppress the proliferation, invasion and migration of the ovarian cancer cells.

MSCs are able to repair the injured tissues through the paracrine activity, among which extracellular vesicles (EVs) were deemed as the process of reacted mechanism in MSCs (5). Various proteins, mRNAs and microRNAs (miRNAs) in MSC-EVs are found to be involved in diverse biological progression during tumorigenesis (6). It was reported that EV-miRNAs could regulate the immune alteration and the proliferation of cell in endometriosis (7). One of the miRNAs detected in EVs is miR-4488 (8), which is poorly expressed in patients diagnosed with stable Behcet's disease(9).

Previous studies have shown that the expression of ABHD8 is up-regulated in ovarian cancer (10), but the underlying mechanisms remains unclear. ABHD gene is connected with the superfamily of alpha/beta hydrolase (11). ABHD16A, which has the similar ingredient with ABHD8 as the member of ABHD protein family, plays a critical role in the regulation of metabolism and the development of disease occurrence (12). In the present study, we also found overexpression of α/β -hydrolase domain-containing 8 (ABHD8) in ovarian cancer cells and tissues compared with normal. We further investigated MSC-EVs and detected decreased expression of miR-4488 in ovarian cancer. The results showed that miR-4488 mediated cell activities by downregulating ABHD8. Taken together, we tried to figure out the combined regulatory roles of MSC-EV-miR-4488 and ABHD8 in ovarian cancer in this study.

Methods

Ethics statement

Written informed consent was obtained from all patients before the study. Study protocols were approved by Ethic Committee in First Affiliated Hospital of Zhengzhou University and based on the ethical principles for medical research involving human subjects of the Declaration of Helsinki. The animal experiments were performed by following the recommendations in the Guide for the Care and Use of Laboratory Animals. The protocol of animal experiments was approved by the Institutional Animal Care and Use Committee of First Affiliated Hospital of Zhengzhou University (No. 2019-KY-422).

Study subjects

All specimens were from paraffin samples (60 ovarian cancer tissues and 40 normal ovarian tissues) archived by the department of pathology of First Affiliated Hospital of Zhengzhou University. The patients with ovarian cancer received surgical treatment strictly in accordance with National Comprehensive Cancer Network (NCCN) ovarian cancer guidelines. None of the patients received the chemotherapy or radiation therapy before surgery. The 40 normal ovarian tissues were derived from normal ovarian tissue near ovarian cysts or serous cystadenomas.

All patients were 29 to 69 years old, among which the median age of patients with benign tumors and with the ovarian cancer tumors (diameter 5–18 cm and median diameter 11 cm) was 48 years and 51 years respectively. There were 37 cases of serous (61.67%), 23 cases of mucinous (38.33%) and 25 cases of lymph node metastasis (41.67%). According to the clinical staging and pathological classification criteria from International Union of Obstetrics and Gynecology (FIGO) in 2000, there were 13 cases at stage I, 21 cases at stage II, and 26 cases at stage III while based on the histological grades, there were 11 cases at stage G1, 27 cases at stage G2, and 22 cases at stage G3. All specimens were independently diagnosed by more than two pathologists in line with the World Health Organization (WHO) histological classification criteria. The clinicopathological information was obtained from the medical records of patient.

Bioinformatics analysis

The forward miRNA of ABHD8 was predicted by TargetScan database (http://www.targetscan.org/vert_71/), miRDB database (<http://mirdb.org/index.html>), and miRSearch database (<https://www.exiqon.com/miRSearch>). The information of miRNA in EV derived from MSCs was obtained by EV miRNA database (<http://bioinfo.life.hust.edu.cn/EVmiRNA#!/>). The intersection depended on the above results.

Immunohistochemical (IHC) assay

The normal ovarian and ovarian cancer tissues were fixed with neutral formaldehyde, embedded in paraffin, and made into sections (4 μ M). After dewaxing with xylene in order for 20 min each time, the sections were also hydrated in different gradients of alcohol for 2 min in sequence, and then placed in ultrapure water for use. Subsequently, the sections were subjected to antigen retrieval for 2 min, and were naturally cooled at room temperature. The sections were cultured with avidin for 10 min and washed with phosphate buffered saline (PBS) for 3 times (each for 5 min). After PBS washing for 3 times, the sections were blocked with 5% goat serum for 10 min and incubated with the diluted rabbit monoclonal antibody ABHD8 (HPA037658, 1: 200, ATLAS ANTIBODIES, Sweden) at 4 °C overnight. After that, the sections were cultured with secondary biotin-labeled goat anti-rabbit immunoglobulin (IgG) (ab150077, 1: 1500, Abcam, Cambridge, UK) for 10 min, incubated with streptavidin-peroxidase solution at 37 °C for 15 min, and then cultured with freshly prepared 3,3'-diaminobenzidine (DAB) solution. The sections were observed under the microscope and after 30 s of color development. Additionally, the sections were counter-stained with hematoxylin, and returned to blue for 30 min through the rinse of running water. Following after, the sections were dehydrated with different concentrations of alcohol (each for 2 min), cleared in xylene, and sealed with neutral gum with the staining effect observed under a microscope. Each section was observed from 5 randomly selected fields (100 cells/each). The expression of ABHD8 was scored semi-quantitatively according to the percentage of cell staining and the intensity of staining.

Cell culture

The human ovary-related cell lines Caov-3, SKOV3, OVCAR-3, and human ovarian surface epithelial cell line IOSE-80 (American Type Culture Collection cell bank) were cultured in a saturated humidity incubator

at 37 °C, 5% CO₂. The culture medium was renewed every 2 to 3 days.

Plasmid construction and cell transfection

ABHD8 and miR-4488 sequences from National Center of Biotechnology Information (NCBI) were applied to construct over-expression plasmid (pcDNA3.1-ABHD8), knockdown plasmid (pLKO.1-shABHD8) and knockout plasmid (pX459-ABHD8-sgRNA). The cells were respectively transfected with short hairpin RNA (shRNA)-negative controls (NC), pLKO.1-shABHD8, NC-mimic, miR-4488 mimic, NC-inhibitor, miR-4488 inhibitor, pcDNA 3.1-NC, pcDNA3.1-ABHD8, miR-4488-inhibitor, pLKO.1-shABHD8, pX459-ABHD8-sgRNA, EVs-miR-4488, EVs-NC mimic, dimethylsulfoxide (DMSO), and GW4869 inhibitors (EVs inhibition) plasmids. After that, the selected cells at passage 3 were placed in a 24-well plate the day before the experiment. The initial cell concentration was 1.5×10^5 cells / well and the cells were cultured to the monolayer. When reaching 80% cell density, cell transfection was performed by following the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, California, USA). The used transfection sequences are displayed in Supplementary Table 1.

Dual luciferase reporter assay

The target gene of ABHD8 and miR-4488 was analyzed by bioinformatics prediction websites (<http://www.rna-society.org/raid/index.html> and <http://www.microrna.org/microrna/home>). Luciferase assay was used to investigate whether miR-4488 interacted with ABHD8. According to the binding sequence between ABHD8 mRNA 3'UTR and miR-4488, the target and the mutant sequences were designed and synthesized respectively. XbaI and NotI restriction sites were added to the two ends of the sequence during the synthesized process. Next, the synthesized fragments were cloned into PUC vectors respectively. After identifying the positive clone and re-combinant plasmid by DNA sequencing method, the fragments were sub-cloned into the psiCHECK-2 vectors and then transformed into E. coli DH5α cells for amplify the plasmids. Above recombinant plasmids were extracted following the instructions of the Omega (D1100-50T, Solarbio, Beijing, China) plasmid mini-extraction kit. The cells were inoculated into a 6-well plate at 2×10^5 per well. The four recombinant vector plasmids were co-transfected into 293T cells (ATCC cell bank) with miR-4488 mimics or miR-4488 NC for 48 h. The luciferase activity change of ABHD8 3'UTR effected by miR-4488 in cells was detected according to dual Luciferase reporting system (E1910). The luciferase activity was measured by Glomax20/20 luminometer (Promega, Madison, WI, USA).

3-(4, 5)-dimethylthiahiazo (-z-y1)-3,5-di-phenyltetrazoliumromide (MTT) assay

The ovarian cancer cells in the logarithmic growth after transfection were collected, detached into a cell suspension, and adjusted to 4×10^3 cells per-well (150 μL). The pre-mixed cells (50 μL) from each experimental group were cultured in a 96-well plate for incubation in an incubator at 37 °C, 5% CO₂. After that, 20 μL of MTT (Sigma, San Francisco, CA, USA) was added to each well for further culture in an incubator for 4 h. The violet-red crystals in the plates were seen under the microscope. Following after, the

medium was removed and the cells in each well were incubated with 150 μ L DMSO for 15 min. The optical density (OD) value at 490 nm was measured by a microplate reader (Becton, Dickinson and Company, Franklin lakes, New Jersey, USA). The OD₄₉₀ value in each well was recorded for 2 consecutive days with OD value as the number of cells.

Transwell assay

The cells after transfection were starved in serum-free medium for 24 h, detached, washed with 1 $\times$ PBS for three times, and re-suspended with serum-free medium Opti-MEM (31985062, Gibco, Gaithers burg, MD, USA) containing 10 g/L bovine serum albumin (BSA) with the cell density adjusted to 2.5×10^4 cells/mL. Subsequently, the Transwell chamber was placed in a 24-well plate, and the upper chamber surface at the bottom membrane of the Transwell chamber was coated with Matrigel (40111ES08, Yeasen Biotech Co., Ltd, Shanghai, China) 1: 8 dilution, which was incubated for 1 h to solidify. After routine digestion, the cells were rinsed with 1 $\times$ PBS twice, re-suspend in RPMI 1640 medium and adjusted to 1×10^5 cells/mL. Next, the cell suspension (100 μ L) was added to the upper chamber of the Transwell chamber with Matrigel gel (Becton, Dickinson and Company, Franklin lakes, New Jersey, USA), while 600 μ L RPMI 1640 medium (Gibco Company, NY, USA) containing 20% fetal bovine serum (FBS) was added to the lower chamber for culture in a 37 $^{\circ}$ C, 5% CO₂ incubator for 24 h. Following after, the Transwell chamber was taken out, the non-invasive cells on the inside of upper chamber were wiped with a cotton swab, and then the remaining cells were fixed with 4% paraformaldehyde for 15 min, stained with 0.5% crystal violet solution (made with methanol) for 15 min and washed 3 times with PBS. A total of 5 fields were randomly selected for photographing under the inverted microscope (XDS-800D, Caikon, Shanghai, China). The trans-membrane cells were counted and there were 3 replicates in each group at repeated 3 times for the average.

Scratch healing assay

The ovarian cancer cells and human ovarian surface epithelial cells after transfection were collected, and the cell suspension was inoculated into a 6-well plate at a cell density of 1×10^5 cells/well. When the cell confluence reached 90%, 200 μ L of sterile Tip was utilized to equally make 4 horizontal and vertical scratches at the bottom of each well. Next, with the removal of original culture solution, the cells in each well were added with 2 mL culture solution containing 10% serum and then placed in the incubator. The photographs were taken under an inverted microscope at 0 and 24 h. The average value of cell counts in 5 fields was taken for each sample and the width of multiple scratch points was measured. Additionally, the scratch healing rate in each group was calculated [(scratch width at 0 h - scratch width at 24 h)/scratch width at 0 h $\times$ 100%]. The average was taken by repeated 3 times to compare the cell migration ability in each group.

Flow cytometry

The cells in the culture plate were gently rinsed with 2 mL of PBS solution after 48 h of transfection and cultured with 0.5 mL of 0.25% trypsin until the cells began to fall off the wall of the culture plate observed under the microscope. The cells were completely fallen off by continuously triturate. Then the cells were

re-suspended gently in the culture medium to make the cell density of about 1×10^6 cells/mL. Next, cell suspension (0.5 mL) was transferred from the cell culture plate (5×10^5 cells) to a clean centrifuge tube. The cells were stained, re-suspended with 0.5 mL pre-chilled $1 \times$ binding buffer, added with 5 μ L Annexin V-fluorescein isothiocyanate (FITC) and 10 μ L propidium iodide (PI) for incubation for 15 min under conditions void of light. Subsequently, the cells were determined and analyzed by flow cytometer (Becton, Dickinson and Company, Franklin lakes, New Jersey, USA).

Immunofluorescence (IF) assay

The subcellular localization of ABHD8 in ovarian cancer cells and human ovarian surface epithelial cell was analyzed. After 24 h of inoculation, the cells were washed with pre-chilled PBS 3 times, fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton 100 cells for 15 min. After PBS washing for 3 times, the cells were blocked with 5% BSA for 1 h and incubated with 2% BSA-contained ABHD8 antibody (HPA037658, 1: 200, ATLAS ANTIBODIES, Sweden) for 2 h. Next, the cells were then added with fluorescent secondary antibody containing 2% serum for culture for 1 h, washed with PBS for 3 times, and stained with diluted Hoechst33342 (C0030, Solarbio, Beijing, China) for 15 min. Afterward, the cells were washed with PBS 3 times, added with 100 μ L PBS, and incubated under conditions void of light at 4 °C. The fluorescence images were recorded by a laser confocal scanning microscope (FV1000, Olympus, Tokyo, Japan).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

After transfection for 24 h, the total RNA was extracted by TRIZOL (Invitrogen, Carlsbad, California, USA). ABHD8 and miR-4488 primers were designed and synthesized by Invitrogen company (Supplementary Table 2). RNA was reversely transcribed into cDNA using TaqMan™ MicroRNA Reverse Transcription kits (4366596, Invitrogen, Carlsbad, California, USA) and Takara-PrimeSc-pt™ RT reagent Kit (D2680A, Takara, Dalian, Liaoning, China). With GAPDH and U6 as internal controls (Invitrogen, Carlsbad, California, USA), qPCR kit (DRR820A, TAKARA, Dalian, Liaoning, China) was used to configure the PCR reaction system to 25 μ L. The PCR reaction solution was reacted on a real-time fluorescence quantitative PCR instrument (ABI, Waltham, Massachusetts, USA). The PCR reaction conditions were 95 °C for 5 min, 95 °C for 10 s, 58 °C for 30 s, 72 °C for 20 s and 78 °C for 20 s, with a total of 40 cycles. The final data were analyzed using the $2^{-\Delta\Delta C_t}$ method.

Preparation and identification of EVs

The existing study demonstrated that human MSCs were isolated from umbilical cord explant cultures (13). The cells were cultured in α MEM (Sigma Chemie GmbH, Steinheim, Germany) supplemented with 10% allogeneic human AB serum, and then incubated in 100 U/mL penicillin, 100 μ g/mL streptomycin, and 2 mM L-glutamine (Sigma, St. Louis, MO, USA). After treatment in Accutase (Sigma, St. Louis, MO, USA) at 37 °C for 3 min, MSCs were subjected to the subculture. The next step was that the isolated human umbilical vein-derived endothelial cells (HuVEC) were cultured with the supplement

mixturesupplement mixture of endothelial cell growth medium (PromoCell GmbH, Heidelberg, Germany) in endothelial cell basal medium MV2 (PromoCell GmbH, Heidelberg, Germany). The following cells were dissolved with trypsin / Ethylene Diamine Tetraacetic Acid (EDTA) solution (Sigma, St. Louis, MO, USA) at 37 °C for 10 minutes to be the subculture of HuVEC. After that, the confluent cell cultures (approximately 2×10^6 cells) were cultured with suitable dilution (1: 700) of solvent (50.17% ethanol = 0.07% ethanol final concentration) at a density of 1.4×10^4 cells/cm². After that, the cells were washed with serum-free medium and incubated with serum-free medium for 24 h. While removing the culture medium, the supernatant was centrifuged by four times of centrifugation (at 360 g for 10 min, at2000 g for 10 min, at 10000 g for 30 min, and at 100,0000 g for 70 min). The precipitated cell-derived EVs were resuspended in 50 µL PBS and stored at -80 °C. Bicinchoninic acid (BCA) method was utilized to measure the protein mass of the EV preparations, 10 µg EV protein was separated on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel. The protein was transferred to nitrocellulose membrane (GE Healthcare Lifescience, Freiburg, Germany) after semi-dry blotting (PeqLab, Erlangen, Germany), which was remained at 1.5 mA / cm² for 1 h. Then, the blots were incubated with mouse monoclonal antibody CD63 (clone MX-49.129.5) (Santa Cruz, Heidelberg, Germany) at a dilution of 1: 200. The EV transmission electron microscopy (TEM) samples were prepared following the instructions of manufacturer (EV-TEM-easy kit; 101Bio, Palo Alto, CA, USA). Besides, EV particles were observed by Hitachi H7600 electron microscope (Hitachi, Tokyo, Japan) at an acceleration voltage of 100 kv. For the experiment of EVs transfer co-culture, EVs stored at -80 °C were cultured with the medium used for the cell assay for 24 to 48 hours. The EVs-free and cell-attached EVs were removed through the wash of PBS 5 times. The detached cells treated by trypsin were applied to detect the contained RNA extracted by TRIZOL through the assay of RT-qPCR.

Western blot analysis

The total cell protein was extracted and the protein concentration was determined by BCA protein concentration assay kit (P0011, Beyotime, Beijing, China). A total of 40 µg protein loading was extracted, subjected to electrophoretic separation with 10% SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride (PVDF) membrane which was blocked with Tween 20 (TBST) solution containing 5% BSA at room temperature. Following after, the membrane was respectively cultured with diluted primary antibody rabbit polyclonal antibody ABHD8 (HPA037658, 1: 200, ATLAS ANTIBODIES, Sweden), Bax (1: 2000, ab32503, Abcam, Cambridge, UK), B-cell lymphoma 2 (Bcl-2) (1: 2000, ab32124, Abcam, Cambridge, UK), Cleaved-caspase3 (1: 100, ab4051, Abcam, Cambridge, UK), CyclinD1 (1: 2000, ab134175, Abcam, Cambridge, UK), and GADPH (1: 3000, ab37168, Abcam, Cambridge, UK) at 4 °C overnight. Additionally, the membrane was re-probed with secondary antibody to goat anti-rabbit IgG antibody (SA00001-2, Proteintech Group, Wuhan, China) at room temperature for 2 h, developed by an enhanced chemiluminescence (ECL) method after washing with TBST three times.

RNA pull-down assay

The ovarian cancer cells transfected with wild type (WT)-ABHD8 and mutant type (MUT)-ABHD8 were treated with biotin-labeled miR-4488 (50 nM/each). After transfection 48 h, the collected cells were washed with PBS and lyzed with specific cell lysate (Ambion, Austin, Texas, USA) for 10 min. A total of

50 mL sample cell lysate was sub-packaged. The residual lysate was incubated with M-280 streptavidin magnetic beads (Sigma, St. Louis, MO, USA) pre-coated RNase-free and yeast tRNA (Sigma, St. Louis, MO, USA) for 3 h at 4 °C, which was washed with cold lysate twice, with low salt buffer three times and with high salt buffer once. The antagonist ABHD8 probe was considered as the negative control. Total RNA was extracted with Trizol, and the expression of miR-4488 was detected by RT-qPCR.

Xenograft tumor in nude mice

A total of 24 healthy female nude mice aged 6–8 weeks (bought from Vital River, Beijing, China) were maintained at the animal facility in Henan Academy of Medical and Pharmaceutical Sciences under pathogen-free conditions according to institutional guidelines. Animal protocols were reviewed and approved by the Henan Academy of Medical and Pharmaceutical Sciences and Use Committee. The experiment was started one week after adaptive feeding. The health status of nude mice was observed before the experiment. Nude mice were subcutaneously injected with constructed vector-NC, ABHD8-vector, sh-ABHD8, and sh-NC (1×10^9 pfu/100 μ L) plasmids. To research the effects of MSC-EV-miR-4488 on tumor formation in nude mice, the mice were subcutaneously injected EV-NC mimic and EV-miR-4488 mimic. After feeding for 6 weeks, the mice were euthanized, and the size and weight of tumor were measured.

Statistical analysis

All data were analyzed by SPSS 21.0 software (IBM, Armonk, NY, USA) with $p < 0.05$ as a level of statistically significance. Measurement data were presented as the mean $\pm$ standard deviation. Differences between two groups were compared by unpaired t test, while differences among multiple groups were determined by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons posttest. Besides, the comparison of the data in each group at different time points was performed using repeated measures ANOVA, followed by Bonferroni test.

Results

Potential targeting relationship between MSC-EV-miR-4488 and ABHD8 in ovarian cancer

The current study reported that down-regulated ABHD8 was related to ovarian cancer with lower risk (10), but the mechanism of ABHD8 in ovarian cancer was still unclear. To further investigate the forward regulation mechanism of ABHD8, the forward miRNA that regulated ABHD8 was predicted by database such as TargetScan (Fig. 1A). Meanwhile, miRNA information of MSC-EVs was obtained by EV-miRNA database. As shown in Fig. 1B, the achieved intersection caused by EV-miRNA and prediction results was only one miRNA, miR-4488. Coherently, MSC-EVs probably carried miR-4488 to target to ABHD8, thereby regulating the development of ovarian cancer.

ABHD8 is highly expressed and miR-4488 is poorly expressed in ovarian cancer tissues and cells

The expression of ABHD8 in ovarian cancer and normal ovarian tissues was detected by IHC. The results demonstrated that the positive expression of ABHD8 in ovarian cancer tissues was higher than normal ovarian tissues (Fig. 2A&B; $p < 0.05$). The expression of ABHD8 and miR-4488 detected by RT-qPCR in ovarian cancer and normal ovarian tissues identified that compared to normal ovarian tissues, ABHD8 was highly expressed and miR-4488 was poorly expressed in ovarian cancer tissues (Fig. 2C; all $p < 0.05$).

The expression of ABHD8 and miR-4488 in purchased ovarian cancer cell lines Caov-3, SKOV3 and OVCAR-3 were determined by RT-qPCR. As shown in Fig. 2D, ABHD8 was higher and miR-4488 was lower in all three OC cell lines compared to normal ovarian epithelial cell line (IOSE-80). To research the effects of ABHD8 and miR-4488 on ovarian cancer cells, Caov-3 cell lines were used in subsequent experiments, which showed the greatest changes among the three. Taken together, ABHD8 was up-regulated while miR-4488 was down-regulated in ovarian cancer.

Down-regulation of ABHD8 inhibits the proliferation, migration, and invasion but promotes the apoptosis of ovarian cancer cells

In the following, the effect of ABHD8 on ovarian cancer cell functions was explored with transfection of pcDNA3.1-ABHD8 or pLKO.1-shABHD8. Flow cytometry and MTT demonstrated that pcDNA3.1-ABHD8 plasmid transfection led to decreased apoptosis rate and increased proliferation in Caov-3 cells while pLKO.1-shABHD8 plasmid led to opposite trends (Fig. 3A-C; $p < 0.05$). Additionally, as shown in Fig. 3D, western blot analysis revealed that pcDNA3.1-ABHD8 plasmid transfection led to reduction in the expression of Bax and Cleaved-caspase3 protein but elevation in the expression of Bcl-2 and Cyclin D1 protein (all $p < 0.05$). However, the above expression showed opposite trends after pLKO.1-shABHD8 plasmid transfection (all $p < 0.05$). Additionally, there were no significant differences in these indicators between the un-treated cells and cells transfected with both pLKO.1-shABHD8 and miR-4488 mimic (all $p > 0.05$). Scratch healing and Transwell assay demonstrated that pcDNA3.1-ABHD8 plasmid transfection led to increased cell migration and invasion in Caov-3 cells while pLKO.1-shABHD8 plasmid led to the opposite trends (Fig. 4A-D; $p < 0.05$). Above results demonstrated that knockdown of ABHD8 significantly inhibited the cell proliferation, migration, and invasion as well as promoted the cell apoptosis in ovarian cancer.

MSC-EV-miR-4488 interacts with ABHD8

ABHD8 was enriched in the nucleus using IF assay (Fig. 5A). Through the analysis of online software, there was the specific binding region between the ABHD8 gene and the miR-4488 sequences that ABHD8 was the targeted RNA of miR-4488 (Fig. 5B). TEM detection showed that spherical EVs were isolated from MSC-EVs, and western blot analysis confirmed the presence of transmembrane protein (CD63) in MSC-EVs but no Calnexin, suggesting the successful extraction of EV (Fig. 5C). The expression of miR-4488

and ABHD8 after transfection of miR-4488 mimic plasmids was detected. The results demonstrated that the expression of miR-4488 was significantly higher while ABHD8 expression was significantly decreased in IOSE-80 and Caov-3 cells transfected with miR-4488 mimic and NC-mimic plasmids ($p < 0.01$) (Fig. 5D). The expression of miR-4488 and ABHD8 after transfection of EVs-miR-4488 was detected. As shown in Fig. 5E, compared to IOSE-80 and Caov-3 cells co-cultured with EVs-NC-mimic or DMSO, the expression of miR-4488 was elevated while ABHD8 expression was decreased after co-cultured with EVs-miR-4488 or GW4869, respectively (all $p < 0.01$). Dual-luciferase reporter gene experiment detection identified that MSC-EV-miR-4488 bound to ABHD8, and could not bind to ABHD8-MUT (Fig. 5F). Coherently, over-expression of miR-4488 was specifically targeted to ABHD8, suggesting that MSC-EV-miR-4488 competitively bound to ABDH8.

MSC-EV-miR-4488 suppresses ovarian cancer cell proliferation, migration, and invasion by specifically targeting ABHD8

Moreover, the effect of miR-4488 on ovarian cancer cell functions was explored with transfection of miR-4488 mimic, miR-4488 inhibitor, pLKO.1-shABHD8, EVs-miR-4488 or GW4869. Flow cytometry, MTT, scratch healing assay and Transwell assay demonstrated that miR-4488 mimic, EVs-miR-4488 or pLKO.1-shABHD8 plasmid transfection led to increased apoptosis rate as well as decreased proliferation, invasion, and migration while miR-4488 inhibitor or GW4869 led to the opposite trend in Caov-3 cells (all $p < 0.05$). Subsequently, miR-4488 inhibitor plasmid or GW4869 transfection led to elevated Bcl-2 and Cyclin D1 protein, and decreased Cleaved-caspase3 and Bax protein while miR-4488 mimic, EVs-miR-4488, or pLKO.1-shABHD8 resulted in the opposite trends (all $p < 0.05$) (Fig. 6A-D and 7A&B). Taken together, MSC-EV-miR-4488 significantly inhibited the proliferation, migration, and invasion but increased the apoptosis of ovarian cancer cells.

MSC-EV-miR-4488 inhibits tumorigenesis of ovarian cancer cells in vivo through down-regulation of ABHD8

The results from xenograft tumor in nude mice demonstrated compared to the control nude mice, mice in the vector and sh-NC groups showed no significant change in expression of ABHD8 and miR-4488 as well as in tumor growth speed and weight; compared with the sh-NC group, mice in the sh-ABHD8 group had decreased ABHD8 expression, growth speed of subcutaneous tumor and tumor weight but no change in miR-4488 expression; the expression of ABHD8 elevated, the growth speed of subcutaneous tumor accelerated and tumor weight increased in nude mice injected with pcDNA3.1-ABHD8 plasmids, but there was no change in miR-4488 expression compared to the nude mice injected with vector (Fig. 8A, C, D, E). The results demonstrated that ABHD8 significantly increased the rate of tumor formation of ovarian cancer cells in mice. Moreover, after the additional treatment of EVs in mice, we found that there was no difference in expression of ABHD8 and miR-4488 as well as in tumor growth speed and weight in nude mice injected with EV-NC mimic plasmids compared to the control nude mice, while the growth speed was slower and the tumor weight was reduced, the expression of miR-4488 increased and ABHD8 decreased in nude mice injected with EV-miR-4488 mimic plasmids than in nude mice injected with EV-NC

mimic (Fig. 8B-E; $p < 0.05$). Taken together, miR-4488 up-regulated in MSC-EVs interacting with down-regulation of ABHD8 suppressed the growth of tumors in ovarian cancer *in vivo*.

Discussion

Ovarian cancer, as the second lethal gynecologic cancer in women on a global scale, is hard to diagnose in the early stage (1). As the epithelial malignancy, the patients with ovarian cancer show poor prognosis and are short of the effective treatment (14). Especially, the treatment of surgery and chemotherapy could improve survival rate of patients, but 5-year survival rate still keeps the trend of low at 45% (15).

Exosomes are also referred to as extracellular vesicles, mediating the noninvasive diagnosis in ovarian cancer because extracellular vesicles deliver such as RNA and protein (16). Peculiarly, exosome-derived miRNAs have been demonstrated to exert potential functions in ovarian cancer (17). Therefore, this study attempted to explore miR-4488 in MSC-EVs inhibited the proliferation, invasion and migration of ovarian cancer cells by down-regulating ABHD8, thus preventing the development of ovarian cancer.

Initially, we identified that MSC-EVs contained miR-4488 by database in ovarian cancer. A previous study has reported that MSCs are utilized to treat the tumor disease such as ovarian cancer (18). It has been reported that EVs inhibit the CD4 and CD8 T-cell proliferation in ovarian cancer (19). In addition, EVs that carry the MMP1 mRNA have the capable to induce the apoptosis of mesothelial cells in ovarian cancer (20). Notably, EVs was detected to contain miR-4488 (8).

Subsequently, we identified that miR-4488 was poorly expressed in ovarian cancer, and miR-4488 in EVs derived from MSCs suppressed the cancer cell proliferation and invasion but promoted the apoptosis in ovarian cancer. miR-4488 is poorly expressed in Adeno-associated virus cells (21). Previous study indicates miR-1180 in BMSCs promotes the expression of Wnt signaling pathway to induce the cell glycolysis and chemoresistance in ovarian cancer, and down-regulated miR-1180 in BMSCs suppresses the cell proliferation in ovarian cancer (22, 23). miR-644-5p over-expressed in BMSC-EVs protects the ovarian granulosa cells by targeting p53, contributing to recover the function of ovarian (24). Up-regulated miR-30b-3p inhibits the OVCAR3 cell proliferation, migration, and invasion while enhancing the apoptosis in ovarian cancer (25).

Furthermore, we detected that ABHD8 was a target gene of miR-4488 by dual-luciferase reporter gene assay, and ABHD8 was up-regulated in ovarian cancer. ABHD5 is highly expressed in endometrial cancer tissues, and down-regulated ABHD5 suppresses the HEC-1A cell proliferation and invasion in endometrial cancer (26), which further supported our results. Then inhibition of ABHD8 was confirmed to suppress the proliferation and migration of ovarian cancer cells in our study. A previous study demonstrated that the down-regulation of ABHD4 suppresses the OVCAR3 ovarian cancer cells (27). ABHD2 knockdown accompanied by shRNA could induce the apoptosis of fibroblasts with A23187 (28). Moreover, ABHD5 as one of α/β -hydrolase members, and the protein-related factor of triacylglycerol hydrolase activity stimulated by ATGL is consider as the suppressor factor in colorectal cancer (29, 30). ABHD5 suppress the migration of colorectal cancer in xenograft models (31). Additionally, EVs from hAMSC-CM promoted

the expression of Bax, Caspase9, and Caspase3 while suppressing the expression of Bcl-2 in ovarian cancer (32). Down-regulated ABHD15 results in the apoptosis of cells accompanied by elevated Bax protein and reduced Bcl-2 protein (33). Moreover, the interaction between miRNA and ABHD8 and their functional mechanisms in ovarian cancer have been addressed in our study. Specifically, over-expressed miR-4488 in MSC-EVs inhibited ABHD8 expression to inhibit the invasion, proliferation and migration as well as promoted the apoptosis of ovarian cancer cells. Importantly, one recent study concerning the interaction between miR-485 and ABHD2 demonstrates that ABHD2 is a potential target of miR-485 which both exerts functions in embryonic carcinoma cell differentiation (34).

Conclusions

In general, the main findings obtained from the current study identified the regulatory role of miR-4488 in MSC-EVs interacting with ABHD8 in ovarian cancer. MSC-EVs-derived miR-4488 suppressed the proliferation, invasion, and migration of ovarian cancer cells by suppressing ABHD8, thus repressing the development and progression of ovarian cancer. These findings suggested a MSC-EVs-derived miRNA-based therapeutic target for the treatment of ovarian cancer.

Abbreviations

miRNAs: microRNAs; EVs: extracellular vesicles; MSC: mesenchymal stem cell; ABHD8: α/β -hydrolase domain-containing 8; IHC: Immunohistochemistry; MTT: 3-(4,5)-dimethylthiazolium (-z-y1)-3,5-di-phenyltetrazolium bromide; IF: Immunofluorescence; RT-qPCR: reverse transcription quantitative polymerase chain reaction

Declarations

Ethics approval and consent to participate

The study about was specimen and clinical data approved by the Ethics Committee of First Affiliated Hospital of Zhengzhou University, with the following ethics batch number: 2019-KY-422. All procedures about nude mouse xenograft model were performed in accordance with the guidelines for laboratory animal ethics issued by Henan Academy of Medical and Pharmaceutical Sciences and Use Committee.

Consent for publication

Not applicable.

Availability of data and materials

Data and materials in the current study are available from the corresponding author on reasonable request.

Competing interests

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Authors' contributions

LC and NL conducted scientific direction; JZ and WT performed the experiments; LC contributed to drafting the manuscript; NL revised the manuscript. MC contributed to the results; RG contributed to analyzing the data.

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Figures

A

Position 132-139 of ABHD8 3' UTR	5' ...GCCAGGUCAGGGAGA-CGCCCCCA...	8mer	-0.44	98	0.44	0	N/A
hsa-miR-4488	3' GCGGCCUCGGGCGGGGGA						

B

Figure 1

MSC-EV-miR-4488 is specifically targeted to ABHD8 in ovarian cancer. (A) The intersection of database prediction results and EVmiRNA (Ellipse: the prediction results of one database; middle part: the intersection of four sets of data). (B) The binding site of ABHD8 and miR-4488.

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Figure 2

ABHD8 is highly expressed and miR-4488 is poorly expressed in ovarian cancer tissues and cells. (A & B) The expression of ABHD8 protein in ovarian cancer and normal ovarian tissues (400 ×) and its corresponding histogram by IHC. * $p < 0.05$ vs. the normal ovarian tissues. © The relative expression of ABHD8 and miR-4488 measured by RT-qPCR in ovarian cancer and normal ovarian tissues. * $p < 0.05$ vs. the normal ovarian tissues. (D) The relative expression of ABHD8 and miR-4488 in ovarian cancer cell lines Caov-3, SKOV3 and OVCAR-3 as well as human ovarian surface epithelial cell IOSE-80 detected by RT-qPCR. * $p < 0.05$ vs. IOSE-80 cells. Measurement data were presented as the mean $\pm$ standard deviation. Differences between two groups were compared by unpaired t test, while differences among multiple groups were determined by one-way analysis of variance, followed by Tukey's multiple comparisons posttest.

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Down-regulation of ABHD8 inhibits the proliferation and promotes the apoptosis of ovarian cancer cells. (A & B) The apoptosis of Caov-3 cells transfected with different plasmids detected by flow cytometry and its corresponding histogram. * $p < 0.05$ vs. The control cells; # $p < 0.05$ vs. Caov-3 cells transfected with shRNA-NC plasmids; \$ $p < 0.05$ vs. Caov-3 cells transfected with pLKO.1-shABHD8 plasmids. (C) OD value in Caov-3 cells at 0 h, 24 h and 48 h detected by MTT method. * $p < 0.05$ vs. the control cells; # $p < 0.05$ vs. cells transfected with shRNA-NC plasmids. (D) The expression of Bax, activated Cleaved-caspase3, Bcl-2, and Cyclin D1 in cells after transfection determined by western blot analysis. Measurement data were presented as the mean $\pm$ standard deviation. Differences between two groups were compared by unpaired t test, while differences among multiple groups were determined by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons posttest. Besides, the comparison of OD

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Figure 4

Over-expressed ABHD8 promotes the migration and invasion ability of cancer cells in ovarian cancer. (A & B) The migration of Caov-3 cells in each group tested by scratch healing assay and its corresponding histogram. (C & D) The invasion of Caov-3 cells in each group tested by Transwell assay (400 ×) and its corresponding histogram. * $p < 0.05$ vs. Caov-3 cells treated with vector; # $p < 0.05$ vs. Caov-3 cells transfected with shRNA-NC plasmids. Measurement data were presented as the mean $\pm$ standard deviation. Differences among multiple groups were determined by one-way analysis of variance, followed by Tukey's multiple comparisons posttest. The experiment was repeated three times.

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Figure 5

MSC-EV-miR-4488 interacts with ABHD8. (A) The position of ABHD8 in OVCAR-3, IOSE-80, SKOV3 and Caov-3 cells detected by IF assay (400 ×) (red: cell membrane; green: ABHD8). (B) The binding site of ABHD8 and miR-4488 predicted by online software. * $p < 0.05$ vs. IgG treatment. (C) The isolation of spherical EVs from MSC-EVs determined by TEM and the presence of CD63 and Calnexin in MSC-EVs tested by western blot analysis. (D) The expression of miR-4488 and ABHD8 in IOSE-80 and Caov-3 cells after transfection with miR-4488-mimic. (E) The expression of miR-4488 and ABHD8 in IOSE-80 and Caov-3 cells co-cultured with EVs-NC-mimic, EVs-miR-4488, DMSO, and GW4869. (F) The binding of EVs-miR-4488 with ABHD8, ABHD8-MUT detected by dual luciferase reporter gene experiment. * $p < 0.05$ vs. NC mimic treatment for comparing miR-4488 expression; # $p < 0.05$ vs. NC mimic treatment for comparing ABHD8 expression; ** $p < 0.01$ vs. NC mimic treatment for comparing miR-4488 expression; ## $p < 0.01$ vs. NC mimic treatment for comparing ABHD8 expression. Measurement data were presented as the mean $\pm$ standard deviation. Differences between two groups were compared by unpaired t test. The experiment was repeated three times.

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Figure 7

miR-4488 over-expressed in MSC-EVs suppresses the cell invasion and migration by specifically targeting ABHD8 in ovarian cancer. (A) The migration of Caov-3 cells transfected with different plasmids detected by scratch healing assay. (B) The invasion of Caov-3 cells transfected with different plasmids detected by Transwell assay. * $p < 0.05$ vs. Caov-3 cells transfected with NC mimic or GW4869; # $p < 0.05$ vs. Caov-3 cells transfected with NC inhibitor; \$ $p < 0.05$ vs. Caov-3 cells transfected with shRNA NC plasmids. Measurement data were presented as the mean $\pm$ standard deviation. Differences among multiple groups were determined by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons posttest. The experiment was repeated three times.

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Figure 8

MSC-EV-miR-4488 inhibits tumorigenesis of ovarian cancer cells in vivo through down-regulation of ABHD8. (A) ABHD8 and miR-4488 expression detected after interfering with ABHD8. (B) ABHD8 and miR-4488 expression determined after treating with MSC-EV-miR-4488. (C) The growth curve of tumor after treating with ABHD8 expression and MSC-EV-miR-4488; (D) Representative images of tumor after treating with ABHD8 expression and MSC-EV-miR-4488; (E) The final histogram of tumor mass obtained after the intervention with ABHD8 and MSC-EV-miR-4488. * $p < 0.05$ vs. Nude mice injected with vector and EVs-NC mimic; # $p < 0.05$ vs. Nude mice injected with shRNA-NC plasmids. Measurement data were presented as the mean $\pm$ standard deviation. Differences among multiple groups were determined by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons posttest. Besides, the comparison of tumor volume in each group at different time points was performed using repeated measures ANOVA, followed by Bonferroni test ($n = 6$).

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