Original Article

Exosomal AHSG in ovarian cancer ascites inhibits malignant progression of ovarian cancer by p53/FAK/Src signaling

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Background: The primary cause of mortality in patients with ovarian cancer (OC) is tumor metastasis. A comprehensive understanding of the mechanisms underlying metastasis in OC is essential for accurate prognosis prediction and the development of targeted therapeutic agents. Our findings indicate that alpha-2 Heremans Schmid glycoprotein (AHSG) is downregulated in OC exosomes. Consequently, the objective of this study was to identify novel prognostic markers and potential therapeutic targets for OC.

Methods: Exosomes derived from OC cells and patient ascites were purified and applied to OC cells to assess their migratory ability using wound-healing and transwell assays. AHSG expression was enhanced by overexpressing lentivirus, and the resulting exosomes were isolated and co-cultured with OC cells to verify their effect on the migration ability of OC.

Results: Exosomes in ovarian malignant ascites have been demonstrated to promote OC metastasis. However, our findings indicate that AHSG is down-regulated in OC tissues and ascites exosomes. Furthermore, overexpression of AHSG in OC cells has been shown to markedly decrease their migratory ability, as well as reduce the migratory ability of cancer cells after co-culture of its exosomes with cancer cells.

Conclusions: The low expression of AHSG in exosomes derived from OC tissues and ascites is associated with metastatic progression in OC patients. Additionally, cancer-derived AHSG can be transported to OC cells via exosomes, where it inhibits OC migration *in vitro* and *in vivo* by regulating the p53/FAK/Src signaling pathway. The present study demonstrated that AHSG, derived from cancer cells, exerts a negative regulatory effect on OC cell motility, migration, and metastasis. These findings suggest that AHSG is a potential candidate for OC treatment.

Keywords: Alpha-2 Heremans Schmid glycoprotein (AHSG); ascites; exosomes; migration; ovarian cancer (OC)

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Introduction

Ovarian cancer (OC) is one of the deadliest gynecological malignancies of the female reproductive system (1). The World Health Organization (WHO) classifies OC into three categories based on cell type: epithelial OC, ovarian mesenchymal cell carcinoma, and ovarian germ cell carcinoma (2). Of these, epithelial OC is the most common type in more than 90% of diagnosed ovarian malignancies (2). At the tissue level, epithelial OC can be divided into five main subtypes: clear cell, high-grade plasmacytoid, low-grade plasmacytoid, endometrioid, and mucinous (3). The mainstay of treatment for patients with advanced OC is tumor cytoreduction supplemented with platinum-based chemotherapy (4). However, despite this treatment, the overall survival of these patients remains low. Disease recurrence after multidrug resistance is the most important factor affecting their survival (5). Nevertheless, the primary cause of chemoresistance in OC is the elevated antioxidant capacity of OC cells. Consequently, the urgent need to identify new biomarkers and therapeutic targets to delay chemoresistance in OC is clear (6). OC has been thought to spread as tumor cells metastasize through the peritoneal fluid to the peritoneum and omentum, unlike most of the remaining malignancies that metastasize through the bloodstream (4). It is well known that the hallmark of OC is ascites and peritoneal fluid, which contains several components that can promote tumor metastasis as well as chemo-resistance, which can lead to morbidity and mortality in patients, and for which there is still a lack of research (7).

Exosomes are 40–200 nm extracellular vesicles containing cytophilic substances such as DNA, microRNA (miRNA), proteins, and lipids, and are widely found in various body fluids such as blood, ascites urine, lymph, bile, and urine (2). Compared with that of the ascites of nontumor patients, the concentration of exosomes in the ascites of malignant ascites is increased 3–4 times (8). Ascites-derived exosomes (ADEs) are considered to be associated with tumor burden, invasion and metastasis, and poor survival (9). According to recent studies, exosomes can facilitate cancer progression by mediating communication between tumor cells and surrounding cells. Malignant ADEs can promote OC metastasis *in vitro* and *in vivo*, which is based on epithelial-mesenchymal transformation (EMT) of OC cells (10). Malignant ADEs have been shown to stimulate peritoneal metastasis through transforming growth factor beta-1 (TGF-β1) induced mesenchymal transformation (11). These results suggest that ADEs play an important role in OC progression and are associated with poorer prognosis (12). Nonetheless, how OC cell-derived exosomal proteins promote OC metastasis needs to be further investigated.

Alpha-2 Heremans Schmid glycoprotein (AHSG), a negative acute-phase protein, is mainly produced by the liver and secreted into the blood. AHSG has been demonstrated to participate in various biological functions of the body, such as insulin resistance, calcification regulation, negative stress response and cancer progression (13). Under *in vitro* conditions, AHSG promotes cell adhesion and diffusion and increases aggression with the help of exosomes (14). AHSG plays different roles in different tumors. Previous reports have demonstrated that AHSG can promote the occurrence and progression of breast cancer, bladder cancer, and Lewis lung carcinoma (15-17). In contrast, AHSG, as a mimic of the TGF-β receptor, shows low expression in intestinal tumors and antagonizes intestinal tumorigenesis (18). However, the role of AHSG in the progression of OC has not been reported.

P53 (cellular tumor antigen p53) is a common mutated tumor suppressor gene in cancer that encodes the p53 protein (19). The p53 protein can be activated by different signaling pathways, such as DNA damage and hypoxia

Highlight box

Key findings

- A common feature of patients with advanced ovarian cancer (OC) is the accumulation of ascites in the abdominal cavity, where exosomes are present and can transmit messages. Our findings indicate that the exosome alpha-2 Heremans Schmid glycoprotein (AHSG) extracted from cancer cells negatively regulates OC cell motility, migration, and metastasis. The present study suggests that AHSG is a potential candidate for the treatment of OC.

What is known and what is new?

- Exosomes have been identified in OC ascites, and these exosomes have been demonstrated to promote the metastasis of OC.
- AHSG is down-regulated in ovarian tissue and ascites and has been demonstrated to inhibit the migration of OC cells. This provides a potential candidate for OC therapy.

What is the implication, and what should change now?

- Implications: the present study demonstrates that AHSG is expressed at lower levels in OC and correlates with prognosis. Furthermore, it provides an experimental rationale for AHSG to be a potential target for OC treatment.
- Actions needed: further studies are needed to elucidate the mechanisms in more detail and to validate these findings in the clinical setting, laying the foundation for the development of targeted therapies for OC.

pathways when cells are stimulated by internal and external factors (20). Therefore, the p53 protein plays an important role in a variety of biological processes, such as apoptosis, cell cycle arrest, senescence and cell metabolism (21). The p53 protein has also been reported to be widely involved in the regulation of cancer metastatic pathways, such as autophagy, invasion, angiogenesis, EMT, and anoikis (22). The p53 protein plays a carcinogenic role and enhances metastasis in multiple cancers, including gynecological tumors (23,24). Focal adhesion kinase (FAK) is a tyrosine kinase that is highly expressed in a variety of tumors and plays a kinase-dependent role in promoting tumor invasion and metastasis. The expression of FAK is proportional to the prognosis of patients (25). FAK can combine with Src to form a complex and participate in cancer progression (25). P53 and FAK promoters are reported to have binding sites. In prostate cancer, it has been found that interfering with p53 expression can activate the FAK/Src (proto-oncogene tyrosine-protein kinase Src) pathway and promote cancer metastasis (26).

Previous studies have reported that by promoting the metastasis and invasion of cancer cells, AHSG may serve as a regulator of many malignant tumors (15-17,27), but whether OC cell (SKOV3 and HO8910 cells)-derived AHSG can be released into the tumor microenvironment in a paracrine manner and consequently influence tumor progression have not been adequately investigated. In the current study, we demonstrated that ADEs were associated with OC ascites invasion and metastasis. In addition, AHSG protein expression was downregulated in epithelial OC tissues and exosomes, which plays an inhibitory role in the progression of OC. Critically, exosomes transferred cancer-derived AHSG to OC cells and inhibited OC migration by regulating p53/FAK/Src signaling. The study demonstrated the mechanism by which exosomes mediate communication through AHSG and confirmed the role of cancer-derived AHSG in tumor invasion and metastasis. Our study may therefore advance our understanding of OC and lead to more effective treatments for patients with OC at high risk of metastasis. We present this article in accordance with the ARRIVE and MDAR reporting checklists (available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-789/rc).

Methods

OC tissue specimens and ascites specimens

A protocol was prepared before the study without registration. Twenty-six OC ascites specimens and 20 benign disease ascites specimens were collected from patients who underwent surgery at Xuzhou Central Hospital from July 2021 to October 2022. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Xuzhou Central Hospital (Approval number: XZXY-LK-20220908-075). Informed consent was obtained from all individual participants. No patients had received radiotherapy or chemotherapy before the surgery, and all were pathologically diagnosed with OC (the included and excluded specimens are shown in Appendix 1). For removal of impurities and cellular components, the fresh ascites were centrifuged at 3,000 × g for 15 min at 4 ℃. The remaining sample was stored at −80 ℃ for subsequent experiments. Detailed information for this study population is presented in Table S1.

Cell culture and cell transfection

The human OC cell lines SKOV3 (kindly provided by Cancer Institute of Xuzhou Medical University) and HO8910 (kindly provided by Cancer Institute of Xuzhou Medical University) were cultured in RPMI 1640 medium (KeyGEN BioTECH, KGL1501-500, China) containing 1% penicillin-streptomycin (Beyotime, ST488S, China) and 10% fetal bovine serum (FBS, Biochannel, BC-SE-FBS07-500ml, China). The incubator was set at 37 ℃ with 5% CO_2. At a density of approximately 80–90% for OC cells, the cells were washed twice with phosphate buffer solution (PBS, Servicebio, G4202-500ML, China), and SKOV3 and HO8910 cells were harvested. Following the exclusion of exosomes from the serum, 0 cells were co-cultured with serum-free medium and 25 μg of exosomes or 25 μL of PBS was added to the medium. The cells were then cultured for 24 hours. After 24 hours of culture, SKOV3 and HO8910 cells were harvested for subsequent experiments.

For cell transfection, SKOV3 and HO8910 cells were inoculated in 24-well plates and subsequently transfected with a serum-free medium containing either the vector or AHSG-luc (Shanghai Gene Chemistry Co., Ltd., China) for 12 hours. The volume of lentivirus we used was based on the following formula: virus (μL) = cell number × multiplicity of infection/virus (stock solution) titer. After transfection, overexpressed cell lines were further selected with puromycin and verified by Western blots and quantitative real-time polymerase chain reaction (qRT-PCR). The transfection groups were as follows: SKOV3 (blank group), SKOV3-NC (negative control group), SKOV3-AHSG (AHSG

upregulated group), HO8910 (blank group), HO8910-NC (negative control group), and HO8910-AHSG (AHSG upregulated group).

Isolation of exosomes

The classic ultracentrifugation method was used to extract and purify exosomes. Ascites was collected and centrifuged at 3,000 ×g for 15 minutes, 11,000 ×g for 30 minutes and 12,000 ×g for 30 minutes to remove impurities and cellular components. The supernatant was collected and filtered through 0.45 μm (Merck Millipore Darmstadt, SLHUR33RB, Germany) and 0.22 μm filters (Merck Millipore Darmstadt, SLGPR33RB) and then, the exosomes were centrifuged twice by ultracentrifugation at 110,000 ×g for 70 minutes and dissolved in PBS.

Exosomes were isolated from the medium by ultracentrifugation. SKOV3 and HO8910 cells were cultured in a serum-free medium for 24 hours before the supernatant was collected. The supernatant was passed through a 0.22 mm filter (Merck Millipore, Darmstadt, Germany) to filter dead cells. Then, the supernatant was centrifuged at 10,000 ×g for 30 minutes. The subsequent procedures were the same as those used to extract exosomes from ascites.

Transmission electron microscopy

After ultracentrifugation, the exosomes were dissolved in 200 μL of PBS, and 50 μL was pipetted from the solution and dropped on copper mesh. The copper mesh was transferred to 3% pentanediol for 5 minutes. After the samples were washed 10 times with deionized water, they were exposed to 40 μL of phosphotungstic acid solution at a concentration of 4% for 30 s and then dried at room temperature (RT) for 30 minutes. The morphology and size of exosomes were observed by transmission electron microscopy (Tecnai G2 Spirit Twin, FEI Company, USA).

Dynamic light scattering analysis (DLA)

A laser nanoparticle size/potentiometer instrument (380ZLS, PSS, USA) was used to detect the size and concentration information of exosomes. First, the standard products were used to calibrate the machine, and then, the sample was loaded after passing the test.

Exosome uptake assay

For exosome uptake experiments, exosomes were labeled with the lipophilic dye Dil (Beyotime, C1990-1ml) and incubated at 37 ℃ for 20 minutes. Then, the labeled exosomes were cocultured with SKOV3 and HO8910 cells for 24 h. The nuclei were dyed with DAPI (Beyotime, P0131-5ml) after washing with PBS. Finally, the cells were imaged under a fluorescence microscope (Olympus, Kyoto, Japan).

Wound-healing assay

For the wound-healing assay, SKOV3 and HO8910 cells were placed in a 6-well plate (5×10^6 cells per well) and cultured in FBS until 90% confluence. Then, the wounds were scratched with a 200 μL pipette tip. Cells were cultured in a serum-free medium with or without 25 μg exosomes after washing exfoliated cells with PBS. The wound areas were observed at 0 and 24 h with a microscope (Olympus, Kyoto, Japan), and ImageJ software was used to analyze the data of the wound healing area.

Transwell assay

SKOV3 and HO8910 cells (1×10^5/200 μL) in FBS-free medium were added to the upper transwell chambers, and 600 μL of 10% FBS medium was added to the bottom wells. For exosomes, a serum-free medium with exosomes (25 μg) and PBS (25 μL) was added to the upper chamber. After incubation for 24 hours, the cells were fixed in 4% paraformaldehyde for 30 minutes, washed and stained with 0.1% crystal violet (Solarbio, G1062, China). Images were taken from five random fields (200× magnification), and the number of cells in each chamber was counted under a microscope (Olympus, Kyoto, Japan).

qRT-PCR

Total RNA was extracted from ovarian cells and tissues. Then, the RNA was converted to cDNA by HiScriptII Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China). Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Quantification of gene expression was performed on Light Cycle 96 software version 1.1 (Roche) with specific primers: AHSG forward,

CCCAGGGCTGATTTATAGACAA; AHSG reverse, GTTCAAGGTGTGTTTGTATCCC; GAPDH forward, AAGAAGGTGGTGAAGCAGGC; GAPDH reverse, TCCACCACCCAGTTGCTGTA.

Western blot analysis

Total protein was extracted with radioimmunoprecipitation assay (RIPA, Beyotime, P0013B) lysis buffer. Protein concentration was measured by the BCA protein assay kit (Beyotime, P0010) following the manufacturer's instructions. The same amount of protein sample was loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Servicebio, G2018) gels, transferred to a polyvinylidene fluoride (PVDF, Millipore, MA, USA) membrane pretreated with methanol and blocked with 3% BSA (Solarbio, A8020) at RT for 1 h. The membranes were reacted with primary antibodies at 4 ℃ overnight. The primary and secondary antibodies utilized are presented in Table S2. After reacting with the corresponding secondary antibodies at RT for 1 h, the membranes were imaged by a dual-color infrared laser imaging system (Odyssey® CLX, USA).

Immunohistochemical (IHC) staining

Paraffin-embedded sections of OC tissue and normal ovarian tissue were provided by Xuzhou Central Hospital. The anti-AHSG antibody was incubated with OC tissue and normal ovarian tissue sections overnight at 4 ℃. Then, the cells were stained with secondary antibodies for 10 minutes at RT. Finally, the cells were counterstained with 10% Mayer hematoxylin (Solarbio, G1080).

Enzyme-linked immunosorbent assay (ELISA)

The ELISA kit purchased from Wuhan Xinqidi Biotech Co. (Wuhan, China) was used to measure the level of AHSG in ascites samples. All samples were diluted with diluent reagent (1:2) before analysis. Optical density (OD) was read at 450 nm.

To measure AHSG levels in exosomes from ascites, we purchased an ELISA kit from Duoscientific Biotechnology Co., Ltd. (Hangzhou, China). The procedure was performed according to the instructions, and finally, the OD value was read at 450 nm.

Human AHSG protein data analysis

The UALCAN database (http://ualcan.path.uab.edu/) was used to detect the expression of AHSG protein in OC. To determine the prognostic value of AHSG in OC patients, we used Kaplan-Meier plotter (http://kmplot.com/analysis/) to investigate the prognostic value of AHSG in the OC dataset.

Analysis of tumorigenicity in nude mice

All animal experiments in this assay were authorized by the Ethics Committee of Xuzhou Medical University (approval number: 202208S085) in compliance with the institutional guidelines for the care and use of animals. To investigate the function of exosomal AHSG protein, we injected BALB/c nude mice with 1×10^7 SKOV3 cells. After tumor formation, 18 tumor-bearing mice were randomly divided into 3 groups (n=6): control, EXO-NC, and EXO-AHSG. Then, 25 μg exosomes were injected into the center of the tumor every 3 days. The tumor volume (mm^3) was calculated as (length × $width^2$)/2. After 3 weeks, all mice were sacrificed, and tumor bodies were removed and photographed.

Statistical analysis

All data were processed using SPSS 22.0 software. ImageJ software was used to process the gray value of protein bands and the wound healing area, while PicCnt software was used to count the number of migratory cells. Differences between the two groups were tested by Student's *t*-tests. Comparisons among multiple sets of data were performed using ANOVA. Overall survival (OS) and progression-free survival (PFS) survival curves were assessed by the Kaplan-Meier method. P values <0.05 were considered statistically significant.

Results

Exosomes found in the ascites of OC patients promote the metastasis of OC cells

Previous studies have shown that exosomes are highly enriched in malignant ascites of OC (9,28). Thus, we extracted exosomes from 26 OC patients and 20 benign ascites samples by ultracentrifugation. Transmission electron microscopy was used to observe the morphology

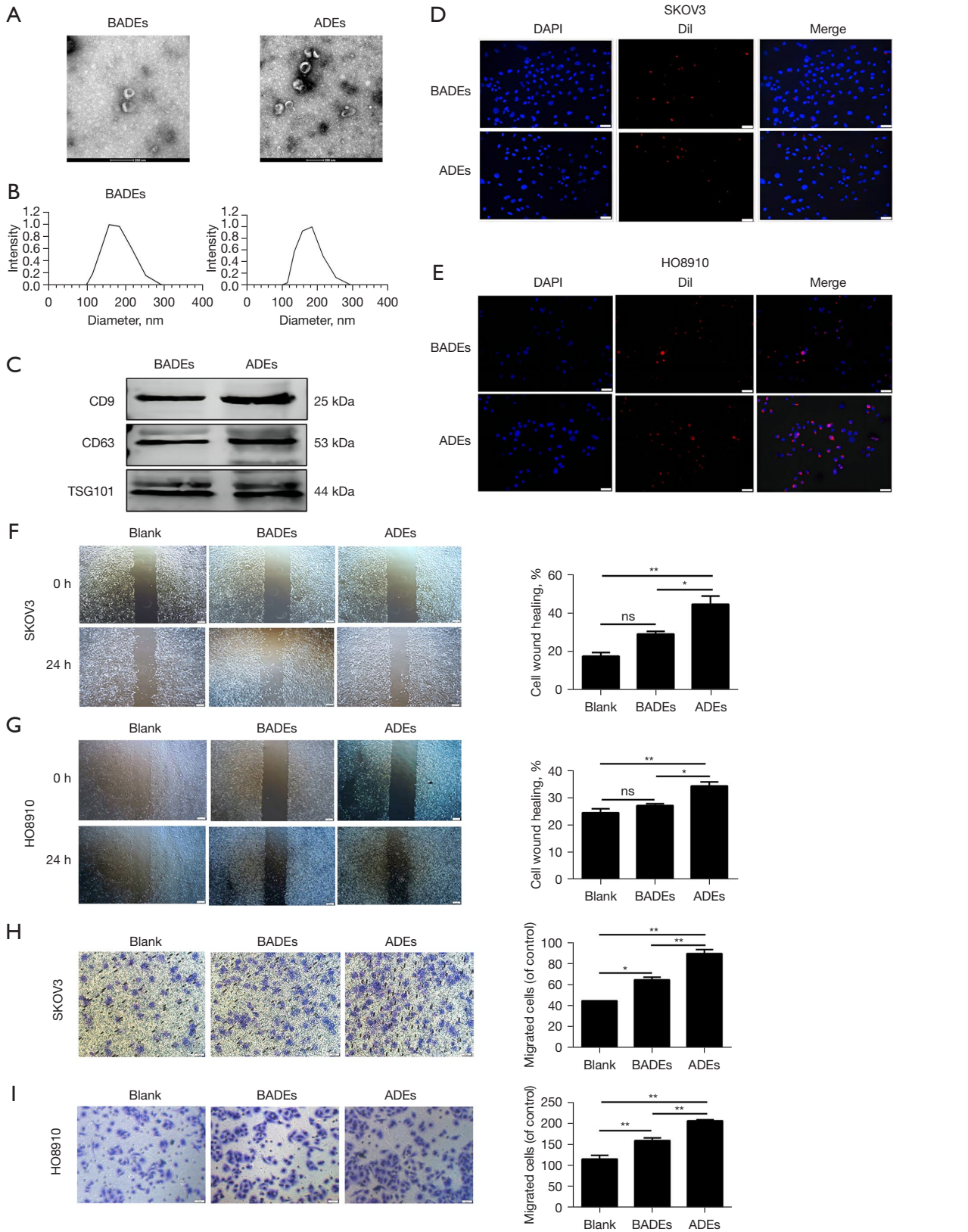

Figure 1 Exosomes derived from ovarian cancer ascites promote ovarian cancer cell migration. (A) The characteristics of exosomes were

determined by TEM in BADEs and ADEs. (B) The size of BADEs and ADEs were measured by DLS analysis. (C) The expression of CD9, CD63 and TSG101 was tested by the Western blot analysis of BADEs and ADEs. (D,E) Dil-labeled exosomes (red) were incubated with SKOV3 cells and HO8910 cells for 24 h observed by microscopy after staining of the nuclei (blue), Scale bar, 50 μm. (F,G) The migration of SKOV3 and HO8910 cells was induced with 1× PBS or exosomes in wound healing assays of the cells. Scale bar, 200 μm. (H,I) The migration of SKOV3 and HO8910 was induced with 1× PBS or exosomes in migration assays. Representative images were shown and migrated cells were counted. Crystal violet staining. Scale bar, 100 μm. Data are representative of at least three independent experiments and are presented as mean ± SEM. ns, no significance; *, P<0.05; **, P<0.01. TEM, transmission electron microscopy; DLS analysis, dynamic light scattering analysis; Dil, 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine; PBS, phosphate buffered saline; SEM, standard error of mean; ADE, ovarian cancer ascites-derived exosomes; BADE, benign ADE.

of the exosomes, and the results showed that the exosomes presented a typical cup-shaped appearance (*Figure 1A*). DLS analysis showed that the exosome diameter pellets had diameters ranging from 132 to 216 nm (*Figure 1B*). All the above results were consistent with the typical characteristics of exosomes. Moreover, Western blot analysis showed positive expression of the exosome marker proteins CD9, CD63 and TSG101 in the extracted exosomes from ascites (*Figure 1C*). These results indicated that exosomes were successfully extracted from ascites by ultracentrifugation. We used Dil to label the exosomes, and then, the Dil-labeled exosomes were cocultured with SKOV3 and HO8910 cells for 24 hours. Subsequently, the effective uptake of exosomes by SKOV3 and HO8910 cells was observed under a fluorescence microscope. The results showed that SKOV3 and HO8910 cells could take up Dil-labeled exosomes from the medium (*Figure 1D*,*1E*).

To investigate the effects of OC ADEs and benign ascites-derived exosomes (BADEs) on the biological behavior of OC cells, we cocultured SKOV3 and HO8910 cells with exosomes obtained by ultracentrifugation, and then, we detected the wound healing area at 0 and 24 h. The outcomes indicated that the migration of SKOV3 and HO8910 cells in the ADE group was significantly higher than that in the BADE group (*Figure 1F*,*1G*). Furthermore, the migration of SKOV3 and HO8910 cells treated with ADEs was significantly increased compared with that of SKOV3 and HO8910 cells treated with BADEs in migration experiments (*Figure 1H*,*1I*). The above results suggested that the ADEs had a more significant ability to promote OC cells migration than the BADEs *in vitro*. ADEs can promote peritoneal metastasis of OC by delivering bioactive molecules to alter the properties of recipient target cells (8). This finding is consistent with our experimental results.

AHSG expression is downregulated in OC tissues and OC ADEs

The literature has already proven that the expression of AHSG protein is downregulated in colorectal cancer and lung adenocarcinoma but upregulated in head and neck squamous cell carcinoma and pancreatic cancer (29). To investigate the expression of the AHSG protein in OC, we first detected the AHSG protein through the UALCAN database, and the results indicated that the AHSG protein was downregulated in OC tissues (*Figure 2A*). Moreover, we searched for the protein in the Kaplan-Meier plotter database and found that AHSG protein was positively correlated with the prognosis of OC patients (*Figure 2B*,*2C*). To verify this finding, we collected OC tissue samples and normal ovarian tissue samples for the following experiments. The results of PCR and western blotting showed that the mRNA level (*Figure 2D*) and protein level (*Figure 2E*) of AHSG in cancer tissues were significantly lower than those in noncancer tissues. IHC also showed that the AHSG protein was downregulated in OC tissues (*Figure 2F*). Our outcomes were consistent with the database results. All the results suggest that AHSG protein expression is downregulated in OC. Moreover, we collected the abdominal effusion of OC patients and benign disease groups for ELISA detection. The results showed that there was no significant difference in the expression of AHSG protein between malignant ascites and benign ascites (*Figure 2G*). We also extracted exosomes from the same batch of ascitic samples by ultracentrifugation for ELISA detection. The results showed that the AHSG protein content in ADEs was lower than that in BADEs (*Figure 2H*). All of these data showed that the AHSG protein was downregulated in tumor tissues and ADEs.

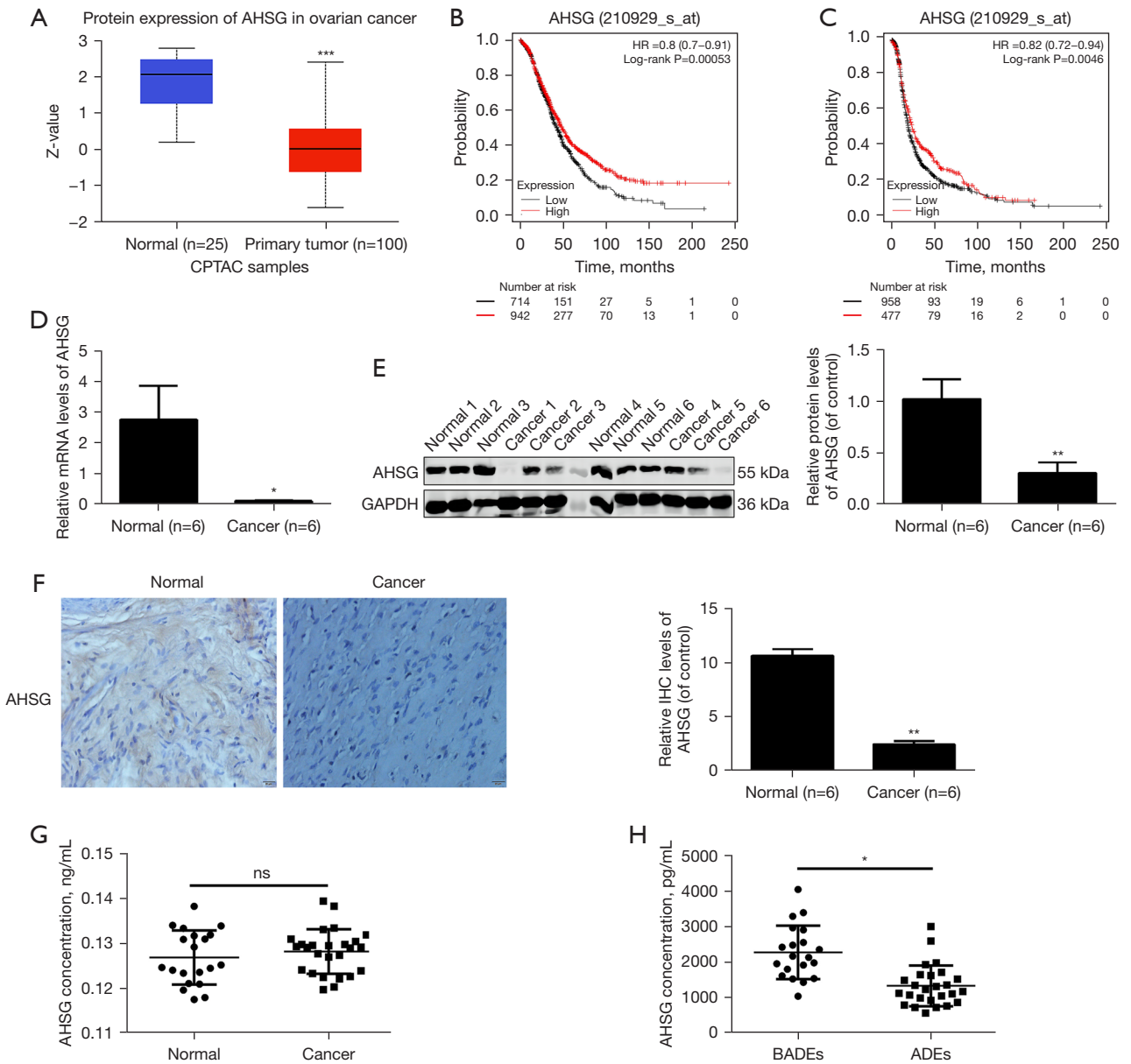

Figure 2 AHSG expression is downregulated in ovarian cancer tissues and ovarian cancer ascites-derived exosomes. (A) The UALCAN database was used to analyze the expression of AHSG in OC patients. (B,C) The K-M plotter database was used to analyze the prognosis of AHSG in OC patients (B: OS; C: PFS). (D) The expression of AHSG was analyzed by qPCR in the OC tissues and benign disease. (E) The expression of AHSG in ovarian tissues from OC patients and benign disease by Western blots. (F) IHC was used to detect the expression of AHSG in paraffin section of ovarian cancer. Original magnification: 400×. Normal group: ovarian tissue from benign disease patients, n=6; Cancer group, ovarian tissue from OC patients, n=6). (G,H) The expression of AHSG was analyzed by ELISAs of OC ascites and ascites-derived exosome. Data are representative of at least three independent experiments and are presented as mean ± SEM. ns, no significance; *, P<0.05; **, P<0.01; ***, P<0.001. OC, ovarian cancer; K-M, Kaplan-Meier; OS, overall survival; PFS, progression-free survival; HR, hazard ratio; qPCR, quantitative polymerase chain reaction; IHC, immunohistochemistry; SEM, standard error of mean; ELISA, enzyme-linked immunosorbent assay; ADE, ovarian cancer ascites-derived exosomes; BADE, benign ADE.

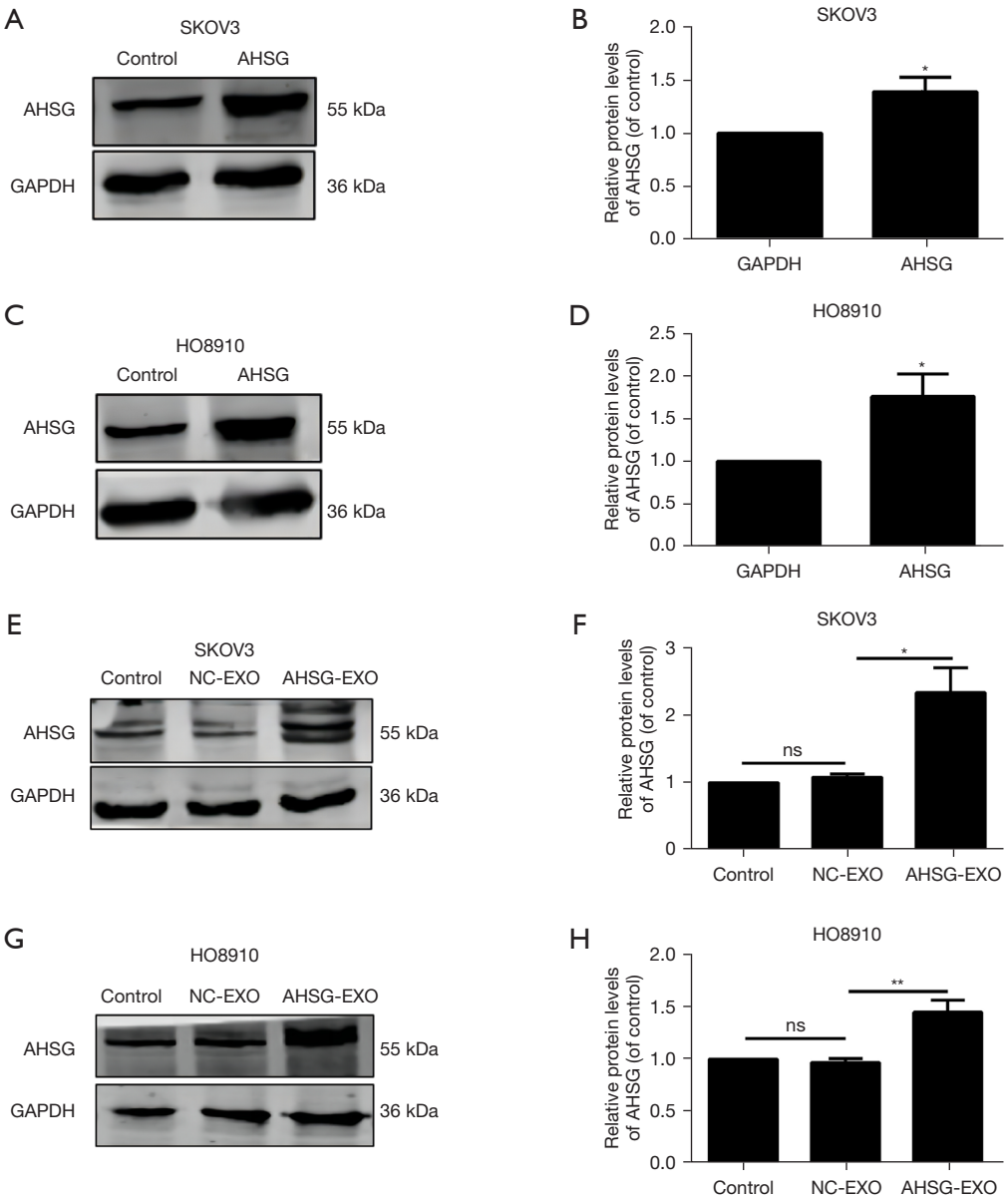

Figure 3 AHSG protein is transported by the exosomes from ovarian cancer cells. (A-D) Western blot was used to verify AHSG overexpression in SKOV3 and HO8910 cells. (E-H) Exosomal AHSG was added to SKOV3 and HO8910 cells, and Western blot analysis was used to detected the expression of AHSG protein in SKOV3 and HO8910 cells. Data are representative of at least three independent experiments and are presented as mean ± SEM; n=3. ns, no significance; *, P<0.05; **, P<0.01. SEM, standard error of mean.

AHSG protein is transported by exosomes from OC cells

We used the AHSG overexpression lentivirus to construct AHSG-overexpressing SKOV3 cells (AHSG-SKOV3) and HO8910 cells (AHSG-HO8910) and verified them by PCR and Western blot experiments. The results showed that the mRNA and protein levels of the AHSG protein were significantly increased in the AHSG-SKOV3 group and the AHSG-HO8910 group compared with the NC-AHSG group (*Figure 3A-3D*). Furthermore, we extracted exosomes from the culture medium of SKOV3-NC, HO8910-NC, AHSG-SKOV3, and HO8910-AHSG cells by ultracentrifugation and then detected the expression of AHSG in exosomes by western blotting. The results showed

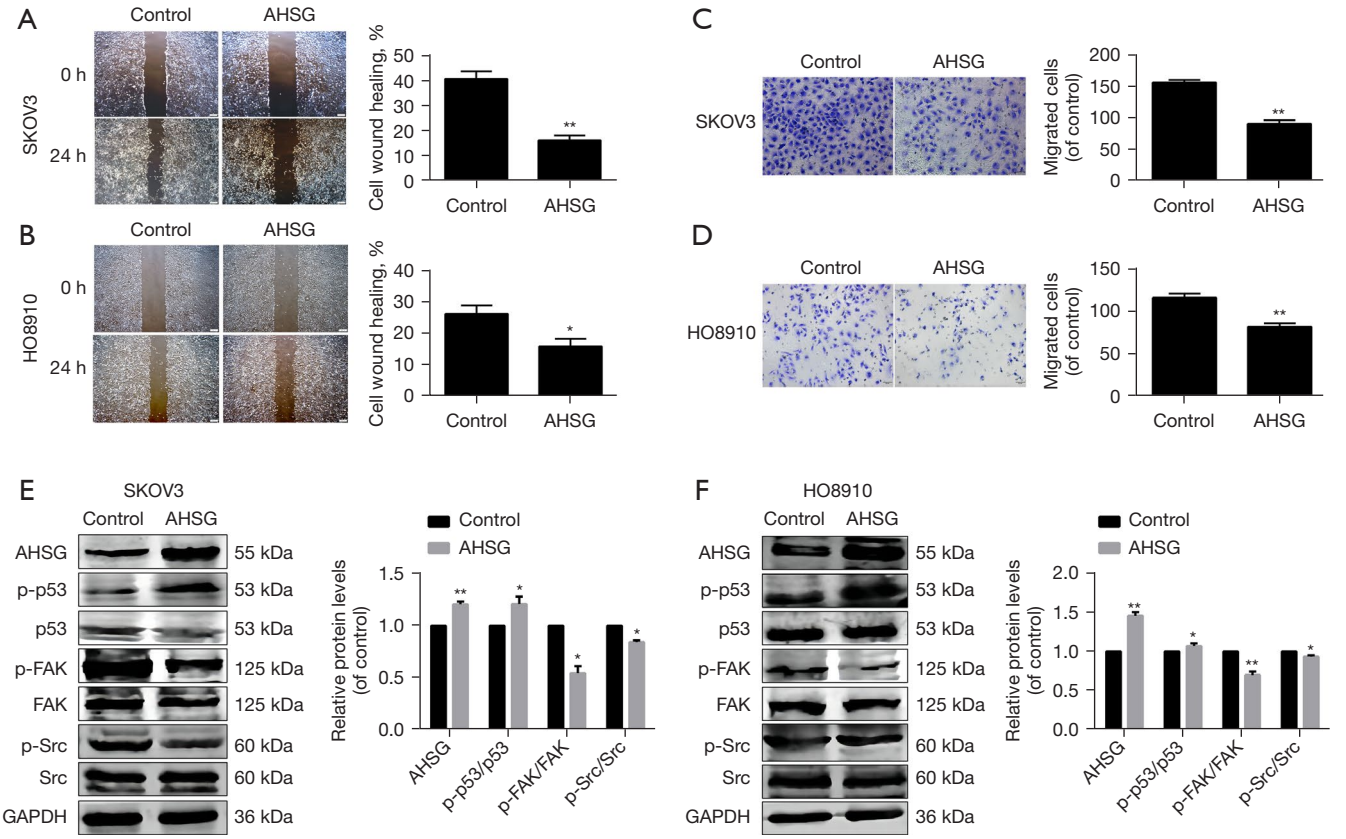

Figure 4 AHSG inhibits the migration of ovarian cancer cells through p53/FAK/Src signaling pathway. (A,B) The migratory abilities of transfected SKOV3 and HO8910 cells were determined by wound healing assays. Scale bar, 200 μm. (C,D) The migratory abilities of transfected SKOV3 and HO8910 cells were determined by cell migration assays. Representative images were shown and migrated cells were counted. Crystal violet staining. Scale bar, 100 μm. (E,F) Detection of the protein levels of AHSG, p53, p-p53, FAK, p-FAK, Src and p-Src in SKOV3 and HO8910 cells after upregulation of AHSG. Data are representative of at least three independent experiments and are presented as mean±SEM; n=3. *, P<0.05, **, P<0.01, compared to the control group. p53, cellular tumor antigen p53; p-p53, activated p53; FAK, focal adhesion kinase; p-FAK, phosphorylated focal adhesion kinase; Src, Proto-oncogene tyrosine-protein kinase Src; p-Src, phosphorylated Proto-oncogene tyrosine-protein kinase Src; SEM, standard error of mean.

that compared with that in the NC group, the expression level of AHSG in the exosomes extracted from the culture medium of the AHSG-SKOV3 group and the AHSG-HO8910 group was significantly increased (*Figure 3E-3H*). These results suggest that the AHSG protein can be secreted into exosomes by SKOV3 and HO8910 cells.

AHSG inhibits the migration of SKOV3 and HO8910 cells through the p53/FAK/Src signaling pathway

Tumor migration behavior is a crucial link in the malignant progression of OC. We first used the wound-healing assay to investigate the effect of AHSG on the migration of SKOV3 and HO8910 cells. As shown in *Figure 4A,4B*, the migration of the AHSG overexpression group was significantly decreased compared with that of the NC group. Furthermore, we used a migration experiment to verify the migration of SKOV3 and HO8910 cells. The results showed that the migration of the AHSG overexpression group was significantly decreased compared with that of the NC group (*Figure 4C,4D*).

The p53/FAK/Src signaling pathway is closely related to proliferation, differentiation and metastasis (30). We used bioinformatics analysis to analyze the correlation between AHSG and p53, and the results showed that p53 and AHSG presented a positive correlation (Figure S1). Western blotting was used to detect the protein expression level of the p53/FAK/Src signaling pathway in SKOV3 and

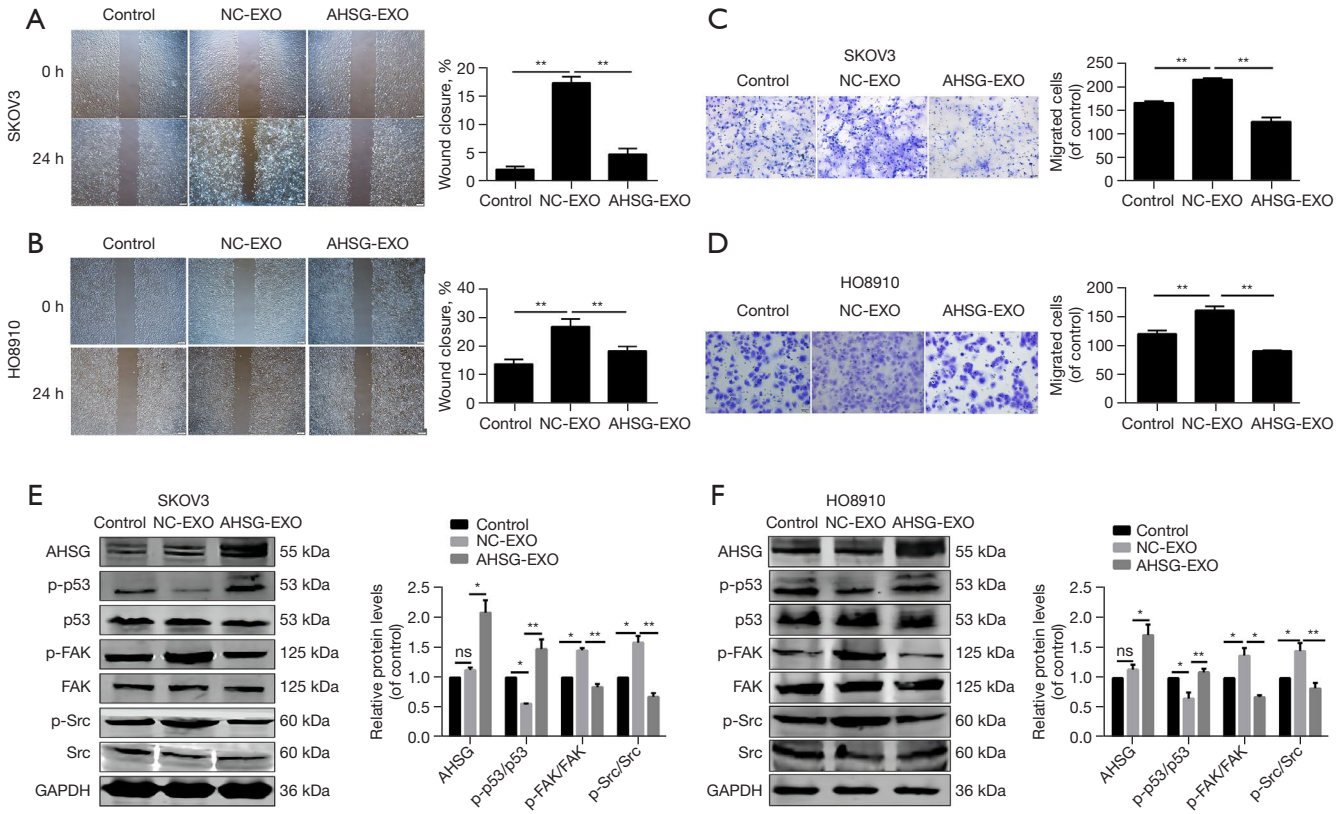

Figure 5 Exosomal AHSG protein inhibits the migration of ovarian cancer cells through the p53/FAK/Src signaling pathway. (A,B) The SKOV3 and HO8910 cells were incubated with exosomes, and then, cell migration was determined by wound healing assays. Scale bar, 200 μm. (C,D) The SKOV3 and HO8910 cells were incubated with exosomes, and then, cell migration was determined by wound healing assays. Representative images were shown and migrated cells were counted. Crystal violet staining; scale bar, 100 μm. (E,F) The SKOV3 and HO8910 cells were incubated with exosomes for 24 h, and the protein levels of p53, p-p53, FAK, p-FAK, Src and p-Src were detected by Western blots. Data are representative of at least three independent experiments and are presented as mean ± SEM; n=3. ns, no significance; *, P<0.05; **, P<0.01. p53, cellular tumor antigen p53; p-p53, activated p53; FAK, focal adhesion kinase; p-FAK, phosphorylated focal adhesion kinase; Src, Proto-oncogene tyrosine-protein kinase Src; p-Src, phosphorylated Proto-oncogene tyrosine-protein kinase Src; SEM, standard error of mean; OCCs, ovarian cancer cells.

HO8910 cells when we upregulated AHSG expression. As shown in *Figure 4E,4F*, the expression level of p-p53 (activated p53) protein was significantly increased, while the expression levels of phosphorylated focal adhesion kinase (p-FAK) and p-Src were significantly decreased when AHSG was overexpressed. These results suggest that AHSG may inhibit the migration of SKOV3 and HO8910 cells by regulating the p53/FAK/Src signaling pathway.

Exosomal AHSG protein inhibits SKOV3 and HO8910 cell migration through the p53/FAK/Src signaling pathway

To examine the biological role of exosomal AHSG protein, we cocultured OC cells with exosomes extracted from the culture medium supernatant from the NC group (NC-EXO) and the AHSG overexpression group (AHSG-EXO). The wound healing assay showed that the migration of the AHSG-EXO group was significantly decreased compared with that of the control group and NC-EXO group (*Figure 5A,5B*). Furthermore, compared with that of the control group and NC-EXO group, the migration of SKOV3 and HO8910 cells treated with AHSG-EXOs was significantly reduced. All the data suggested that the exosomal AHSG protein could inhibit the migration of SKOV3 and HO8910 cells (*Figure 5C,5D*). These results suggest that the exosomal protein AHSG can significantly inhibit the migration of

SKOV3 and HO8910 cells.

We cocultured SKOV3 and HO8910 cells with NC-EXOs and AHSG-EXOs for 24 h. Then, we detected the effects of exosomal AHSG protein on p53, FAK and Src proteins in SKOV3 and HO8910 cells by Western blotting. The expression level of p-p53 protein in the OC cells treated with AHSG-EXOs was significantly increased, while the expression levels of p-FAK and p-Src were significantly decreased. These results suggested that exosomal AHSG protein can be taken up by SKOV3 and HO8910 cells to promote the activation of p53 and inhibit the phosphorylation of FAK/Src (*Figure 5E,5F*). Therefore, we suggest that exosomal AHSG protein could inhibit the migration of SKOV3 and HO8910 cells through the p53/FAK/Src signaling pathway.

Exosomal AHSG protein inhibits the progression of OC cells in vivo

Next, we evaluated the effect of exosomal AHSG protein on OC development *in vivo*. Tumorigenicity analysis was performed in nude mice subcutaneously injected with SKOV3 cells, which were injected with PBS, NC-EXOs and AHSG-EXOs, and in the tumor center every 3 days. The results showed that the exosomal AHSG protein could significantly inhibit the growth of tumors *in vivo*, as demonstrated by tumor size, tumor weight, and tumor volume (*Figure 6A-6D*). These results indicated that exosomal AHSG protein could inhibit OC growth. In addition, we performed IHC analysis of p-p53, p-FAK and p-Src in tumor tissues and verified that exosomal AHSG significantly increased the expression of p-p53 and decreased the expression of p-FAK and p-Src in tumor tissues (*Figure 6E*). We also determined the expression of the metastatic markers vascular endothelial growth factor receptor (VEGFR), matrix metallopeptidase-2 (MMP2) and matrix metallopeptidase-9 (MMP9) in the tumor tissue, and the results showed that VEGFR, MMP2, and MMP9 were downregulated in the tumor tissues injected with AHSG-Exos (*Figure 6F,6G*). All the results suggested that exosomal AHSG could inhibit the tumor progression of OC *in vivo*.

Discussion

OC is the most common cancer of the female reproductive system and has the highest mortality rate among gynecological malignancies in women (31). Metastasis is an important factor affecting the prognosis of OC patients. Fifty-eight percent of OCs have metastases at diagnosis, and the 5-year survival rate is only 30%, compared with the 5-year survival rate for locally nonspread OC, which is 93% (32). Therefore, it is important to explore the mechanism of OC metastasis to improve the survival rate and prognosis of OC patients.

Metastasis is a key link in tumor progression and the most common cause of cancer recurrence and death (33). Most women with epithelial OC present with peritoneal metastases accompanied by the accumulation of ascites at the time of diagnosis, which is a metastatic pattern specific to OC (34). Malignant ascites comprise a unique tumor microenvironment that is highly enriched in exosomes (28). Moreover, many studies have shown that exosomes secreted by tumor cells can regulate tumor growth, metastasis, angiogenesis and drug resistance (35-37). These molecules can also monitor the progression of the disease and be used as a diagnostic marker for the disease (38).

As a phosphorylated glycoprotein, AHSG participates in the normal physiological activities of the body and regulates various cancer processes (14). We found that the presence of exosomal AHSG contributes to the adhesion of cells to various extracellular matrices, and these exosomes are also responsible for premetastatic niche formation and mediate growth and motility (14). We also found that AHSG can transmit adhesion, motility, invasion, and growth signals in tumor cells via activated exosomes in astroglioblastoma (39). However, the ability of exosomal AHSG to affect OC migration has not been reported. Through ELISAs, we found that AHSG was enriched in ascitic exosomes and showed low expression in ADEs (*Figure 2H*). Our results show that AHSG can be transferred to receptor cells through exosomes and inhibits the migration of OC cells *in vitro* (*Figure 5A-5D*).

When we collected clinical ascites samples, we found that most of the ascites were from high-grade serous OC, a subtype of OC that often has *TP53* mutations (40). This gene can encode the p53 protein, which is extensively involved in the progression of OC. We found a positive correlation between AHSG and p53 by searching the UALCAN database (*Figure 4E*), so we hypothesized that AHSG regulates OC progression through p53 signaling. It has been reported that the p53 protein can promote proliferation and metastasis through the FAK/Src pathway in lung adenocarcinoma. This molecule plays the same role in prostate cancer (35). FAK is a nonreceptor tyrosine kinase involved in signaling pathways related to proliferation, migration and angiogenesis (40). When activated, FAK can bind to Src family proteins to

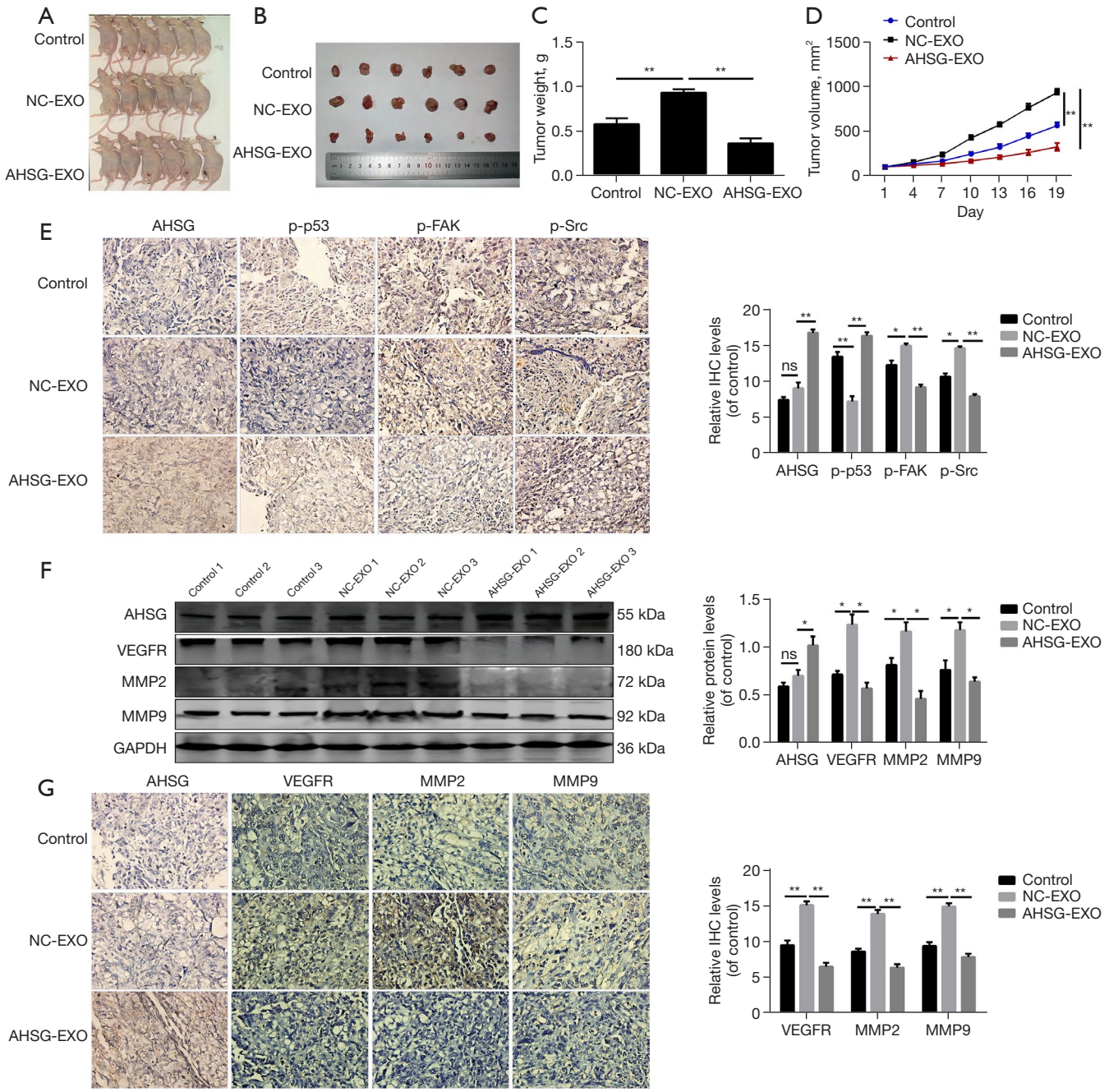

Figure 6 Exosomal AHSG protein inhibits the progression of ovarian cancer cells *in vivo*. (A) Image for analysis of tumorigenicity in nude mice. (B) Representative images of dissected tumors from nude mice were shown. (C) The average tumor weight was calculated and presented. (D) The average tumor volume was calculated and presented. (E) The expression of AHSG, p-p53, p-FAK and p-Src was measured by IHC in the tumor tissues of the mice. Magnification: ×40. (F) Western blotting was used to detect the protein expression of metastasis markers VEGFR, MMP2 and MMP9 in tumor tissues of the mice. (G) The expression of VEGFR, MMP2 and MMP9 was measured by IHC in the tumor tissues of the mice. Scale bar, 20 μm. Data are representative of at least three independent experiments and are presented as mean ± SEM; n=3. ns, no significance; *, P<0.05; **, P<0.01. p-p53, activated p53; p-FAK, phosphorylated focal adhesion kinase; p-Src, phosphorylated Proto-oncogene tyrosine-protein kinase Src; IHC, immunohistochemistry; VEGFR, vascular endothelial growth factor receptor; MMP2, matrix metallopeptidase-2; MMP9, matrix metallopeptidase-9; IHC, immunohistochemistry; SEM, standard error of mean.

form the FAK-Src complex. The combination of the FAK-Src complex can initiate a variety of downstream pathways to promote cancer metastasis (41). In this study, we found that both exosomal AHSG and AHSG proteins inhibited the progression of OC through the p53/FAK/Src signaling pathway (*Figures 4E,4F,5E,5F*). This finding suggests that exosomal AHSG and AHSG proteins may act to inhibit OC migration through a similar mechanism. *In vivo* experiments also confirmed our conclusion (*Figure 6A-6E*). In addition, the expression levels of the metastatic markers VEGFR, MMP2 and MMP9 were determined, which further confirmed that exosomal AHSG could inhibit OC cell migration (*Figure 6F,6G*). However, these results only preliminarily suggest that exosomal AHSG can inhibit the progression of OC, which still needs to be verified by further experiments.

Conclusions

In conclusion, our findings indicate that AHSG expression levels were low in OC tissue samples. Accordingly, we postulate that it is associated with OC metastasis. Therefore, low levels of the exosomal protein AHSG were detected in ascites samples from OC patients. The exosomal AHSG protein was demonstrated to inhibit OC metastasis at the cellular and exosomal levels, respectively, through wound healing and permeabilization assays. With regard to the mechanism, it is proposed that exosomal protein AHSG exerts an inhibitory effect on OC cell metastasis via the p53/FAK/Src pathway. Furthermore, *in vivo* experiments demonstrated that exosomal protein AHSG impeded the metastasis of OC.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE and MDAR reporting checklists. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-789/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-789/dss

Peer Review File: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-789/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-789/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The clinical study was conducted according to the principles expressed in the Declaration of Helsinki (as revised in 2013) and was approved by the Research Ethics Committee of Xuzhou Central Hospital (Approval number: XZXY-LK-20220908-075). Informed consent was obtained from all individual participants. The animal study was approved by the Research Ethics Committee of Xuzhou Medical University (Approval number: 202208S085) in compliance with the institutional guidelines for the care and use of animals. The maximal tumor size/burden was not exceeded the permissible tolerance in institutional guidelines.

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