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Functional role of SIRT1-induced HMGB1 expression and acetylation in migration, invasion and angiogenesis of ovarian cancer

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Abstract. – OBJECTIVE: Ovarian cancer is a commonly occurred tumor in females. High motility group box-1 protein (HHMB1) is a chromosome-related protein with multiple functions. A recent study revealed critical roles of HMGB1 in occurrence and progression of ovarian cancer. Sirtuin 1 (SIRT1) is a recently identified novel molecule, which regulates acetylation of HMGB1. Whether SIRT1 is involved in migration, invasion or angiogenesis of ovarian cancer is unclear. This study aims to investigate the role of SIRT1-induced HMGB1 acetylation in migration, invasion, and angiogenesis in ovarian cancer.

PATIENTS AND METHODS: In ovarian cancer cell line, SIRT1 expression was potentiated. Western blot and immunofluorescence were used to measure HMGB1 expression, acetylation level, and nuclear translocation. Scratch assay and transwell chamber methods were used to examine cell migration and invasion potency. A mouse model with ovarian cancer cell transplantation was generated to measure induced nitric oxide synthase (iNOs) and CD105 expression.

RESULTS: Compared to adjacent tissues, ovarian cancer tissues had significantly decreased SIRT1 expression. In ovarian cancer cells, SIRT1 over-expression decreased HMGB1 and acetylation levels, and SIRT1 knockdown facilitated HMGB1 expression and acetylation. SIRT1 over-expression also suppressed nuclear translocation of HMGB1. Meanwhile, SIRT1 could suppress, migration and angiogenesis of ovarian cancer cells via HMGB1.

CONCLUSIONS: SIRT1 over-expression effectively inhibited HMGB1 expression and acetylation, thus inhibiting ovarian cancer migration, invasion and angiogenesis. HMGB1 modulated behaviors of ovarian cancer via SIRT1. Therefore, SIRT1 might work as a treatment target for managing ovarian cancer migration.

Key Words:
SIRT1, HMGB1, Ovarian cancer.

Introduction

Ovarian cancer is derived from female ovary tissues, and 90-95% of these cases belong to primary cancer[1]. Due to the lack of typical early symptoms and limited efficiency of screening, diagnosis is extremely difficult at early stage. Patient diagnosis is further compromised due to strong potency of metastasis at advanced stage. Therefore, the investigation of treatment approaches to inhibit migration or invasion of ovarian cancer cells and tumor angiogenesis presents high priority. Early study showed critical roles of high motility group box-1 protein (HHMB1) in ovarian cancer pathogenesis[2]. HMGB1 is a pluripotent chromosome related protein, which participates in various biological functions including nuclear DNA rearrangement, repair and transcription, thus playing important roles in maintaining chromosome stability[3]. HMGB1 is one highly conserved nuclear protein that can regulate gene transcription, and can maintain nuclear body structure[4]. In addition, HMGB1 can work as inflammatory cytokine to be related from necrosis cells or being actively secreted by stress cells[5]. A recent study showed that HMGB1 also worked as mediator playing important roles in multiple diseases. In lipid denaturation, inflammatory response, fibrosis and tumor occurrence, HMGB1 expression was significantly increased[6-8]. In recent years, HMGB1 over-expression has been reported

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yet, and has been shown to facilitated migration, invasion and angiogenesis of tumor cells[9].

Mammalian sirtuin 1 (SIRT1) is a NAD-dependent histone deacetylase, and plays critical roles in multiple physiological processes including gene transcription, cell aging, energy metabolism, oxidative stress and inflammatory response[10,11]. Other researches showed that certain microRNA can mediate migration and invasion behaviors of ovarian cancer cells via mediating SIRT1[12]. In recent years, SIRT1 is newly found to regulate acetylation and release of HMGB1. In pyaemia and fatty liver disease, SIRT1 expression was inhibited[13,14], thus elevating acetylation level of HMGB1 to potentiate its activity. However, whether SIRT1 participates in HMGB1-dependent migration, invasion and angiogenesis regulation in ovarian cancer is still unclear. Thus, we aimed to investigate the functional role of SRIT1-induced HMGB1 acetylation in migration, invasion and angiogenesis of ovarian cancer. We firstly utilized ovarian cancer cell line to up-regulate SIRT1 expression by cell transfection, followed by detection of HMGB1 expression, acetylation level change and nuclear translocation of HMGB1, to substantiate modulatory role of SIRT1 on HMGB1. We next examined the change on ovarian cancer cell line migration and invasion by HMGB1 acetylation mediated by SIRT1. Lastly, we introduced mouse model with ovarian cancer xenograft transplantation to examine the effect of SIRT1 mediated HMGB1 acetylation on ovarian cancer angiogenesis.

Materials and Methods

Major Materials and Reagent

SIRT1, HMGB1, β-actin and induced nitric oxide synthase (iNOs) antibody were purchased from BioVision (K753-100, Mountain View, CA, USA). Horseradish peroxidase (HRP) labeled goat anti-rabbit secondary antibody and FITC-labeled goat anti-rabbit fluorescent secondary antibody were purchased from ZSJQ Biotech. (Beijing, China). Eosin, hematoxylin-staining solutions were purchased from Qiwu Biotech (Shanghai, China). Total protein extraction kit was purchased from Kaiji Biotech (Shanghai, China). Western blot lysis buffer and bicinchoninic acid (BCA) protein quantification kit were provided by Beyotime (Shanghai, China). Immunohistochemistry kit and diaminobenzidine (DAB) lysis buffer were purchased from ZSJQ Biotech. (Beijing, China).

Major Equipment

Ultrapure workstation was provided by Boxun (Changsha, China). Gel imaging system UVP Multispectral Imaging System (UVP, Sacramento, CA, USA). Model PS-9 semi-dry transfer electrophoresis was purchased from Jingmai (Nanjing, China). CO_2 chamber and Thermo-354 microplate reader were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Sample Collection

A total of 20 tumor tissue and adjacent tissues were collected from ovarian cancer patients (older than 18 years) that were recruited from Jinan Maternity and Child Care Hospital. Those patients with cognitive dysfunctions, tumor recurrence or other progressive disease, or with systemic infection and severe disorders were excluded. After informed consents were signed, ovarian cancer tissues and adjacent tissues (within 3 cm from cancer tissues) were collected during surgery and were immediately stored in liquid nitrogen for further assays. This study was approved by the Ethical Committee of Jinan Maternity and Child Care Hospital.

Cell Line and Culture

Ovarian cancer cell line Hey was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured within Dulbecco's modified eagle Medium (DMEM) containing 10% sterile fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY, USA) in a 37°C chamber with 5% CO_2.

Construction of SIRT1 Over-Expression Lentiviral Plasmid and siRNA Knockdown Plasmid and Cell Transfection

SRIT1 over-expression lentiviral plasmid and siRNA knockdown plasmid were designed and synthesized by Gimma (Shanghai, China). 24 h before transfection, cells were passed within 24-well plate until reaching 30-50% confluence. 1.25 μl small interfere RNA (siRNA) stock solution (20 μM) or over-expression plasmid (20 μM) was dissolved into 100 μL Option minima essential medium (Opti-MEM) medium as solution A. 1 μl Lipofectamine 2000 or Lipofectamine™ RNAiMAX was dissolved into Opti-MEM medium as solution B. After incubation for 5 min, solution A and B were mixed, and kept still for 20 min before adding culture plate. After 4 h incubation, DMEM medium containing 10% fetal bovine se-

rum (FBS) was used. SIRT1 expression was measured to calculate transfection efficiency.

Western Blot

12 h after cell transfection, culture medium was completely removed. Cells were washed in phosphate-buffered solution (PBS) for three times. 10 μl phenylmethanesulfonyl fluoride (PMSF, 100 mM, Amersham Biosciences, Little Chalfont, Buckinghamshire, England) were added into each 1 ml lysis buffer. Within 6-well plate, each well was added with 100 μl lysis buffer, and was processed on ice for 5-10 min. Cells were hanged on one side of the culture well by a swab, and cell debris and lysis buffer were removed into pre-cold Eppendorf (EP) tubes. Cell lysate was centrifuged at 12000 r/min for 5 min at 4°C. The supernatant was collected as total protein solution. Western blot was performed following previous literature[15]. Extracted total proteins were quantified by bicinchoninic acid (BCA) approach to unify concentrations. After adding load buffer, protein mixture was boiled for 5 min for complete denature. Loading samples were separated by electrophoresis in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) until targeted protein was separated with adjacent proteins. Separated protein samples were transferred to polyvinylidene difluoride (PVDF) membrane by 300 mA for 1 h. Rabbit anti-HMGB1, SIRT1, β-actin and iNOs antibody (1:1000 dilutions) were added for 4°C overnight incubation. The membrane was washed in Tris-buffered saline and Tween-20 (TBST-20) for three times, and horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:1000) was added for 2 h 37°C incubation. Chemiluminescence approach was employed for visualize protein bands.

Acetylation Assay for HMGB1

Expression level of acetylated HMGB1 was measured by immune-precipitation approach. Firstly, sufficient amounts of HMGB1 antibody were added into 200 μg proteins, and were incubated overnight at 4°C with gentle vortex. Immunocomplex was captured by adding 25 μl protein A+G agarose beads followed by 4°C gentle mixture for 3 h. The mixture was centrifuged at 1500 × for 5 min at 4°C. Precipitants were washed for three times in cold phosphate-buffered saline (PBS), and were resuspended in 1 × loading buffer, followed by 5 min boiling. Immunocomplex was then dissociated from beads. Supernatant was collected by centrifugation for Western blot.

Immunofluorescence

Cultured cells were inoculated into 6-well plate. 24 h after transfection, cells were fixed within 10% formaldehyde for 24 h, and were blocked in normal goat serum for 20 min. Primary antibody of HMGB1 or CD105 (1: 100) was added for 4°C overnight incubation. On the next day, cells were incubated for 30 min at room temperature. After PBS washing, fluorescein isothiocyanate (FITC) labeled secondary antibody was added for dark incubation, and cells were washed in 4',6-diamidino-2-phenylindole (DAPI) staining buffer for 10 min. After washing, cells were observed under an inverted microscope.

Cell Migration Assay

All cells were seeded in 6-well plate until fully attached growth and transfection. 12 h later, normal medium was added. When cells reached 90% confluence, 200 μl pipette tip were used to draw parallel scratch lines in the middle of plate bottom. Scratched cells were washed away and the plate was observed under an inverted microscope. Pictures were taken at certain positions with equal width of scratch lines across different wells. 12 h later, images were taken at the same plate for observing cell migration conditions.

Transwell Assay for Cell Invasion Potency

Transwell chamber and assay apparatus were pre-cold at 4°C fridge one night before assay. Extracellular matrix (ECM) gel was thawed at 4°C fridge. Transwell chamber was placed into 24-well plate, which was laid flat in iced box. ECM gel was diluted and added into transwell chamber (50 μl per well), which was incubated at 37°C for 4 h still incubation. Residual liquid was removed and the chamber was air-dried for further use. Cholangiocarcinoma cells were harvested for 12 h, and were then prepared into cell suspensions by adding trypsin. After adjusting cell concentration, all groups of cells were added into the upper chamber, whilst lower chamber was filled with culture medium containing 10% fetal bovine serum (FBS). The experiment consisted of normal control group, SIRT1 knockdown group and SIRT1 over-expression group, each of which contained 3 replicated wells. After adding liquids, transwell chambers were extracted at specific time points, and were stained by 0.1% crystal violet, and were observed under an inverted microscope. A total of five fields were samples from upper, lower, left, right and middle sites for enumeration.

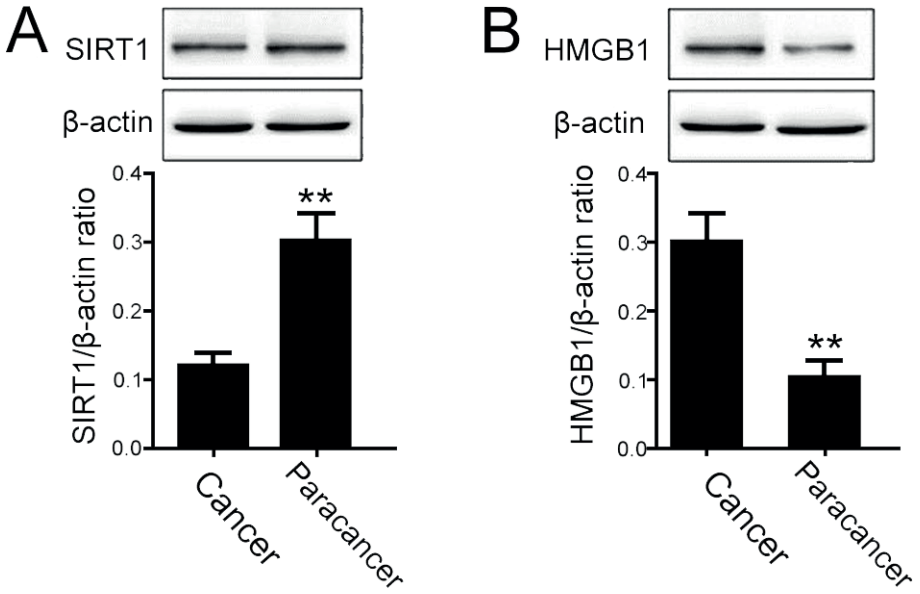

Figure 1. SIRT1 and HMGB1 expression and acetylation in ovarian cancer tissues. (A) SIRT1 expression level. (B) HMGB1 expression level. $^{**}p<0.05$ compared to tumor adjacent tissues.

Animals and Grouping

Male and female BALB/c nude mice were provided by Vital River Lab Animal Technology Co. Ltd. (Beijing, China). Mice were randomly divided into three groups (n=10), including ovarian cancer group, SIRT1-inhibited ad SIRT-1 over-expression group. All mice were fed on specific pathology free (SPF) barrier animal house, with aging between 4 and 8 weeks, body weight between 16 and 18 g.

Generation of Ovarian Cancer Transplant Model

Ovarian cancer xenograft mouse model was generated based on previous literatures[16]. In brief, Hey cells were passed until 90% confluence. Cells were digested in trypsin and centrifuged, and were re-suspended in PBS for adjusting cell density to 1 $\times 10^7$ per 200 µl. 0.2 ml cell suspension was injected subcutaneously at neck skin of nude mice. General conditions of nude mice were daily observed, including motility and food intake. 30 days later, nude mice were sacrificed by cervical dislocation and tumor tissues were removed. SIRT1 over-expression and inhibition lentiviral plasmids were designed and synthesized by Gimma (Shanghai, China). On the same day of tumor implantation, viral particles were infused into mouse by tail vein injection at 50 ng/ml concentration.

Statistical Analysis

Statistical software SPSS 15.0 was used for analyzing data (SPSS Inc., Chicago, IL, USA). All results were presented as mean ± standard deviation (SD). The Student's *t*-test was used to compare the differences between two groups. Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data between groups. $p<0.05$ was considered as statistical significance.

Results

SIRT1 and HMGB1 Expressions in Ovarian Cancer Tissues

We obtained ovarian cancer tissues and adjacent controlled tissues from our hospital and performed Western blot to analyze expressional profile of SIRT1 and HMGB1. As shown in Figure 1, SIRT1 expression level was significantly depressed in ovarian cancer tissues ($p<0.05$, Figure 1A), whilst HMGB1 showed opposite patterns and significantly elevated expression in cancer tissues (Figure 1B).

Effects of SIRT1 Knockdown or Over-Expression on HMGB1

We transfected SIRT1 knockdown or over-expression plasmid into cells, and measured SIRT1 and HMGB1 expression, plus HMGB1

acetylation level. As show in Figure 2, comparing to normal control group, SRIT1 knockdown group showed significantly lower SIRT1 expression, and over-expression group revealed elevated transcripts level (Figure 2A). The knockdown of SIRT1 further enhanced HMGB1 expression in cells and culture medium, and over-expression of SRIT1 inhibited intracellular expression and release of HMGB1 (Figure 2B, C). Moreover, we measured expression of acetylated HMGB1 in all groups of cells. We found that SIRT1 knockdown can enhance acetylated HMGB1 expression level inside cells, and SIRT1 over-expression suppressed expression of acetylated HMGB1 inside cells. All these results suggested that SIRT1 negatively regulated HMGB1 expression or acetylation level (Figure 2D, E).

Effects of SIRT1 Over-Expression on HMGB1 Translocation

Recent investigations showed that SRIT1, as an important histone deacetylase, could modulate deacetylation of HMGB1, thus suppressing its activation and release towards cytoplasm. Therefore, this study utilized immunofluorescence approach to measure the effect of SIRT1 expression on HMGB1 nuclear translocation. As shown in Figure 3, cytoplasmic HMGB1 expression was significantly elevated in SIRT1 inhibition group, and SIRT1 over-expression significantly suppressed the release of HMGB1 into cytoplasm. These results showed that SIRT1 could inhibit translocation of HMGB1 from nucleus towards cytoplasm.

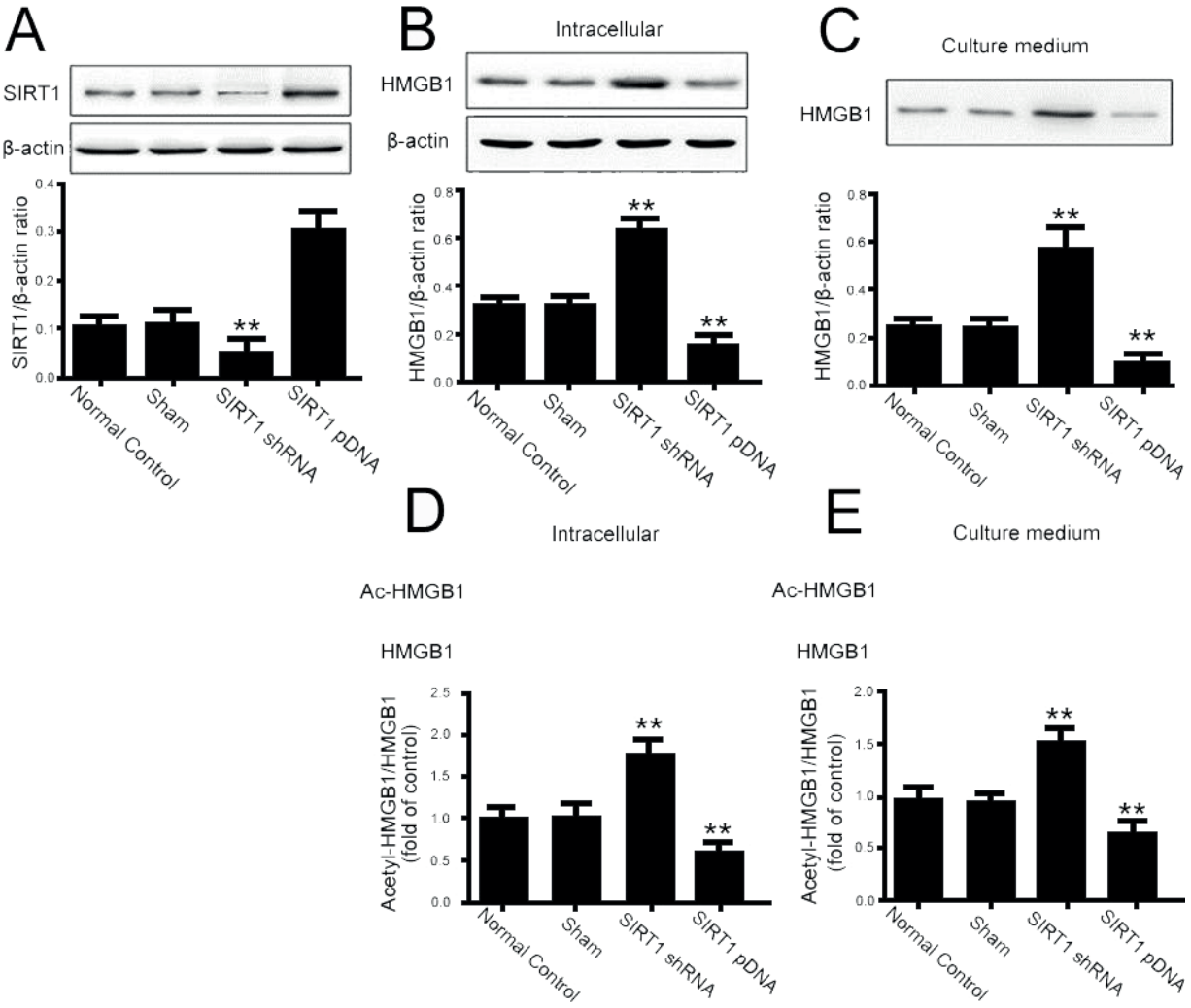

Figure 2. SIRT1 knockdown or over-expression and HMGB1 expression or acetylation. (A) SIRT1 expression. (B) HMGB1 expression inside cells. (C) HMGB1 levels in culture medium. (D) Intracellular expression of acetylated HMGB1. (E) Acetylated HMGB1 in culture medium. $^{**}p<0.05$ compared to normal control group.

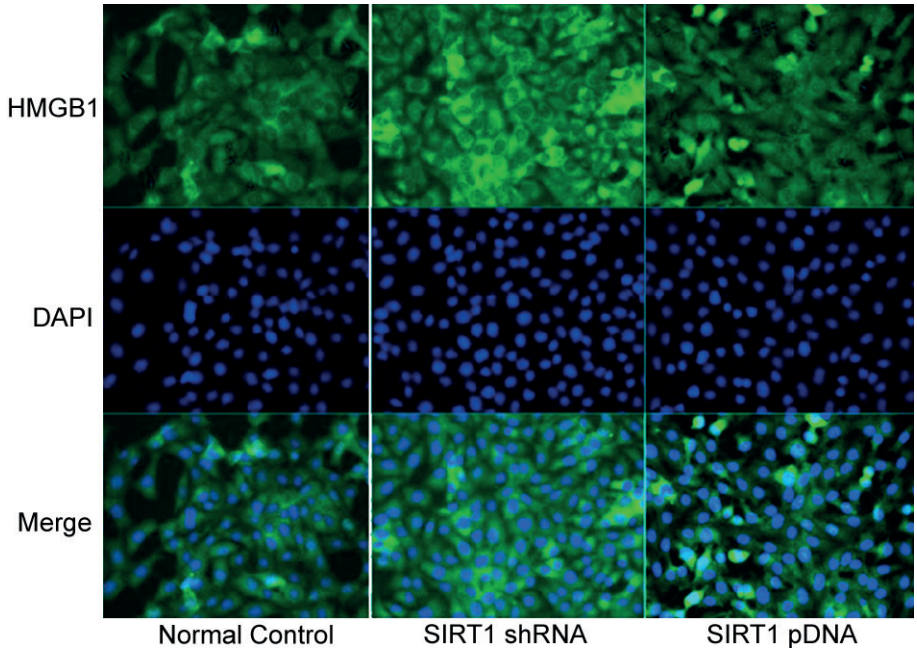

Figure 3. Effects of SIRT1 over-expression on HMGB1 translocation (200

Effects of SIRT1 Over-Expression on Cell Invasion

To measure cell migration status, we used scratch assay as previously described[17]. The width of scratch was measured at the same location from 0 h to 24 h. As shown in Figure 4, the cleft between cell scratch was significantly lower in SIRT1 inhibition group, and such cleft was significantly wide in SIRT1 over-expression group. These results showed that SIRT1 over-expression significantly inhibited cell migration.

Effects of SIRT1 Over-Expression on Cell Invasion

We used transwell chamber to measure the change of cell invasion potency. By analyzing the number of cells penetrating basal membrane within 24 h, we found significantly increased number of invading cells in SIRT1 inhibitor group comparing to blank control group, whilst SIRT1 over-expression remarkably decreased invading cell number. These results showed that SIRT1 over-expression could prevent cell invasion (Figure 5).

Effects of SIRT1 Over-Expression on Angiogenesis of Ovarian Cancer

iNOs is the early marker for angiogenesis[18]. In transplanted tumor tissues on nude mice, we measured iNOs expression. As shown in Figure 6A, compared to blank control group, SIRT1 knockdown mice displayed significantly lower SIRT1 expression, whilst SIRT1 over-expression mice had higher transcript levels. Meanwhile, we measured iNOs expression level in all group and found significant elevation in SIRT1 knockdown group (Figure 6B). In SIRT1 over-expression group, iNOs expression was significantly higher compared to cancer model group, but without significant change with blank control group. These results clearly suggested that up-regulation of SRIT1 expression could facilitate angiogenesis of ovarian cancer tissues. We also used immunohistochemistry staining to measure newly formed vessels using CD105 as the marker. SIRT1 knockdown lentivirus treated mice showed potentiated CD105 staining, and SIRT1 over-expression decreased staining rate of CD105, indicating that SIRT1 over-expression could inhibit angiogenesis (Figure 6C).

Discussion

Ovarian cancer is a commonly occurred malignant tumor in female reproductive organs, and has relatively higher incidence only lower than cervical carcinoma and uterus cancer[19]. Ovarian cancer frequently has early stage metastasis, making it unlikely to completely remove lesions

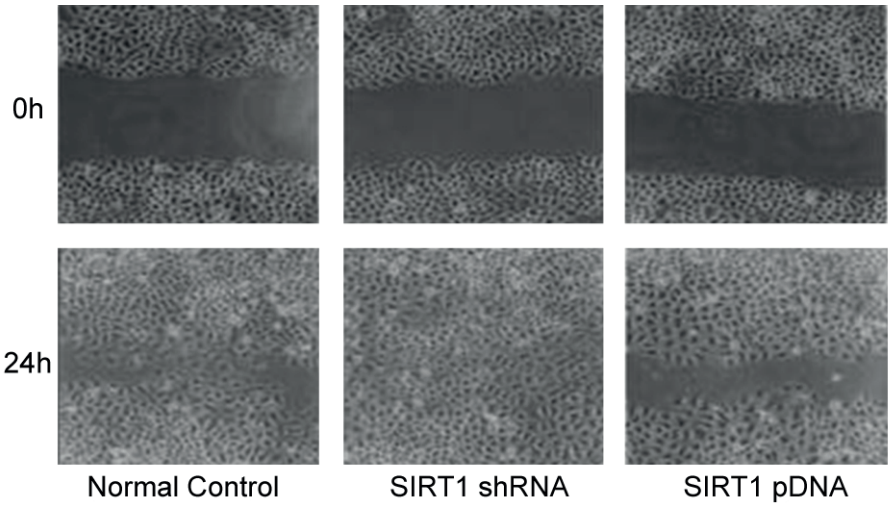

Figure 4. Effects of SIRT1 over-expression on cell migration (200 ×).

by surgery, and largely limited application and efficiency of radiotherapy. Therefore, systemic chemotherapy has become a critical auxiliary treatment approach[20], as it plays important roles in suppressing ovarian cancer cell migration, invasion and angiogenesis target for suppressing metastasis of ovarian cancer. High motility group box-1 protein (HHMB1) is one polypeptide strand with 215 amino acid residues with highly conserved sequence. On its N terminus, there is abundantly lysine distribution with large amounts of positive charges. Its C terminal region has amounts of glutamate with negative charges. HMGB1 obtained its name due to fast mobility velocity in PAGE gel electrophoresis[13, 21, 22]. Seidu et al[9] showed that up-regulation of HMGB1 might facilitate migration and invasion of ovarian cancer. By peptide micro-array assay, HMGB1 was found to be the candidate of SIRT1 substrate. HMGB1 participates in chromosome remodeling and transcriptional regulation[23]. Within cell secretion process, HMGB1 can work as an inflammatory affinitive-alerting marker. Under most scenarios, macrophage, NK cells and dendritic cells can actively secret HMGB1. However, increasing evidence showed the involvement of non-immune active cells in secretion of HMGB1, such as those in hepatocytes and most of tumor cells[23]. Lan et al[13] has shown that SIRT1 could suppress inflammation occurrence or progression in fatty liver disease or hepatic sepsis via mediating HMGB1. Other studies showed that microRNA could affect progression of gastric cancer, liver carcinoma and ovarian cancer targeting SIRT1[12,14]. Increasing evidence showed the central role of SIRT1 in tumor pathogenesis. However, whether HMGB1's role in ovarian cancer invasion, migration and angiogenesis was under the regulation of SIRT1 has not been reported.

SIRT1 down-regulation or loss of activity has been found in multiple tumors[9]. Moreover, some potent SIRT1 agonist has been confirmed to exert protective roles in tumors[24]. In this research, we used ovarian cancer tissue samples and adjacent tissues, on which expression of SIRT1 and HMGB1 were measured. We found significantly decreased SIRT1 expression in tumor tissues, and further substantiated such down-regulation in ovarian cancer cells. As consistent with predicted results, HMGB1 level was elevated in ovarian cancer cells. To further substantiate regulatory role of SIRT1 on HMGB1 in ovarian cancer, we established SIRT1

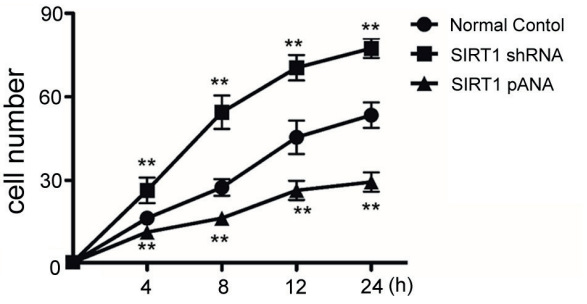

Figure 5. Effects of SIRT1 over-expression on cell invading potency. $^{**}p<0.05$ compared to normal control group.

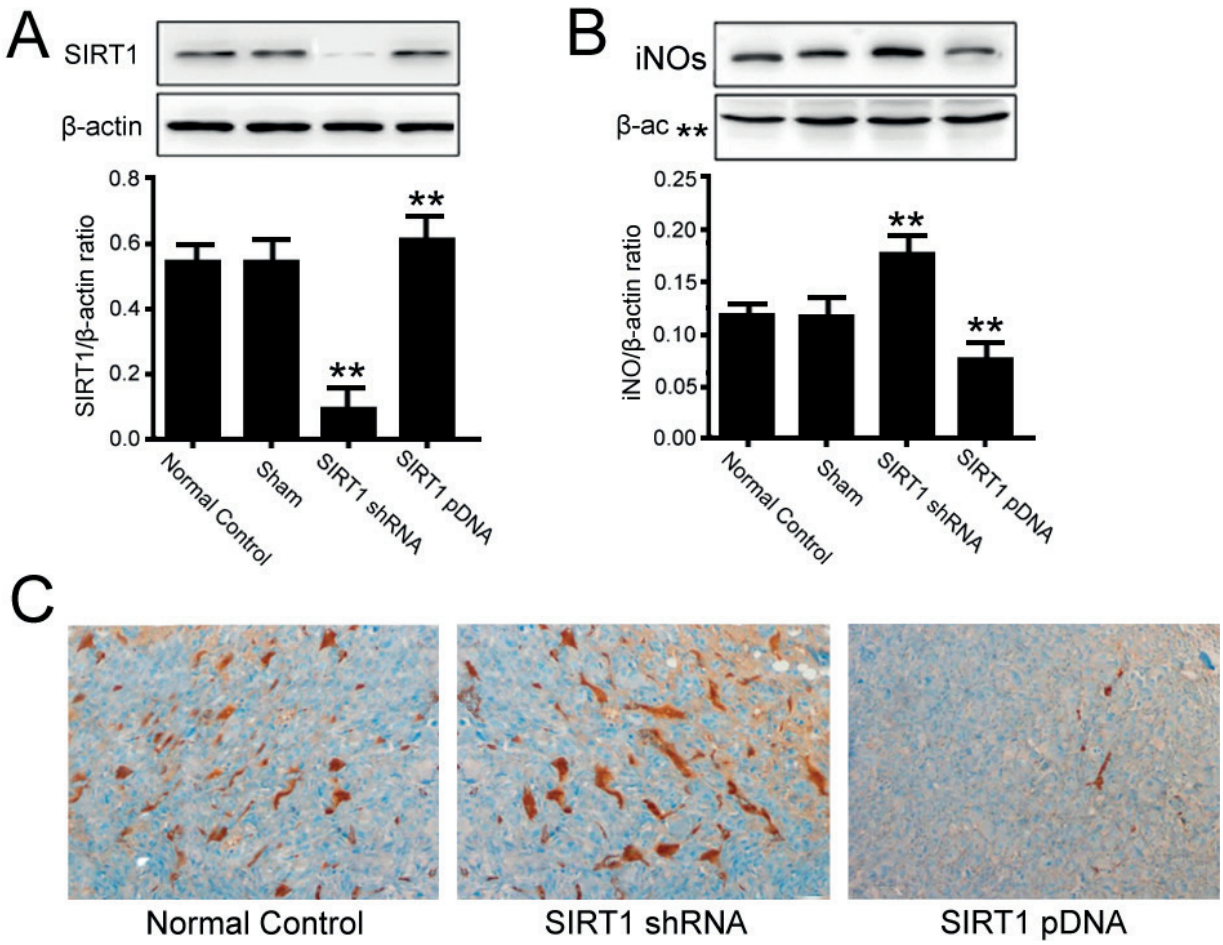

Figure 6. Effects of SIRT1 over-expression on ovarian cancer angiogenesis. (A) SIRT1 expression level. (B) iNOs expression level. (C) CD105 immunohistochemistry staining (200 ×). $^{**}p<0.05$ compared to normal control group.

over-expression and SIRT1 knockdown cell lines, and found decrease and increase HMGB1 expression or acetylation, respectively. These data demonstrated regulatory role of SIRT1 on HMGB1. Meanwhile, migration/invasion potency of cells, and angiogenesis ability were also weakened or enhanced with HMGB1 down- or up-regulation, respectively. These data collectively proved that the regulation of cell migration/invasion and angiogenesis by HMGB1 was under the direction of SIRT1. We investigated the role of SIRT1-HMGB1 axis on migration/invasion and angiogenesis of ovarian cancer cells, and demonstrated that the effect of HMGB1 on ovarian cancer cell behaviors was dependent on SIRT1. This work, however, did not illustrate whether SIRT1 had full protective role in mice carrying ovarian cancer cell xenograft, or any inhibitory effects on tumor growth and metastasis. Future studies can be performed to evaluate the regulatory role of SIRT1 within ovarian cancer, and possible mechanisms, in addition to its role in mice with ovarian cancer transplantation, all of which require comprehensive and detailed illustration.

Conclusions

We found that SIRT1 over-expression can inhibit HMGB1 expression or acetylation, thus suppressing migration, invasion or angiogenesis of ovarian cancer cells. Modulation on ovarian cancer cell behaviors by HMGB1 requires the involvement of SIRT1. Therefore, SIRT1 can work as therapeutic target for inhibiting ovarian cancer migration.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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