Original Article

Upregulation of sulfatase-1 decreases metastatic potential of SKOV3 human ovarian cancer cells *in vitro* and *in vivo*

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ABSTRACT

Aim: Sulfatase-1 (SULF-1) is one of the genes associated with the inhibition of several signaling pathways by desulfating HSPG in cancer cells. The aim of this study is to investigate the effect of SULF-1 upregulation on SKOV3 ovarian cancer cell line and its influence on cell proliferation, migration, invasion *in vitro*, and lymph node metastasis in 615 inbred mice *in vivo*.

Materials and Methods: In *in vitro* study, we upregulated SULF-1 in SKOV3 cells using SULF-1 expression plasmid. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) and western blotting were used to measure SULF-1 expression levels after stable upregulation. CCK-8, flow cytometry, Boyden Transwell-chamber, and scratch-wound healing assay were performed to explore the effect of SULF-1 on the proliferation, migration, and invasion. In *in vivo* study, immunohistochemistry and eosin stain (H and E) were used to evaluate the expression level of SULF-1 gene and to measure the lymph node metastatic rate of mice inoculated with SULF-1-SKOV3-expressed plasmid, SKOV3, and Nc-SKOV3 cells.

Results: qRT-PCR and western blot assay confirmed that SULF-1 was upregulated both in mRNA and protein levels. Following SULF-1 stable upregulation, the cell proliferation, migration, and invasion were significantly reduced in the SULF-1 upregulated cells (SULF-1-SKOV3) compared with the nontransfected (SKOV3) and the nonspecific sequence transfected cells (Nc-SKOV3). IHC results showed that SULF-1 was highly expressed after stably upregulation in SKOV3 cells, and H and E stain confirmed that the mice inoculated with SULF-1-SKOV3 cells decreased lymph node metastatic rate compared to the two control groups.

Conclusions: Our findings showed that overexpression of SULF-1 in SKOV3 results in a decrease in ovarian cancer cell proliferation, migration, and invasion *in vitro* and decreased lymph node metastasis *in vivo*. This finding could have a potential therapeutic window in the management of ovarian cancer.

KEY WORDS: Migration and invasion, lymph node metastasis, ovarian cancer, proliferation, sulfatase-1

INTRODUCTION

Ovarian cancer is one of the cancers topping the list of malignancies that are of major global health concern because of its high incidence and case fatality rate. Worldwide, about 239,000 women were diagnosed with ovarian cancer and among them 152,000 died in 2012.[1] It is the 7th most common cancer and the 8th most common cause of death worldwide.[1] Metastasis is the most important process in cancer progression and it accounts for about 90% of cancer-associated mortalities. To date, the pathogenesis and the genes associated with the cancer metastasis remain poorly understood.[2]

Sulfatase-1 (SULF-1) is a heparin degrading endosulfatase which is known to inhibit tumorigenesis by desulfating cell surface heparin sulfate proteoglycans (HSPGs) and inhibits signaling pathways such as Ras/Raf/MAPK, PI3K/Akt/mTOR, Wnt/β-catenin, Hedgehog and hepatocyte growth factor (HGF)/c-Met, and vascular endothelial growth factor and epidermal growth factor (EGF) pathway.[3-10] It has been reported that, in early stage of ovarian cancer, SULF-1 is shown to be downregulated, and interestingly, highly metastatic ovarian cancer cells such as TOVG21G and SKOV3 have been shown to lack the expression of SULF-1.[11] Upregulation of SULF-1 is shown to speed up the desulfation process on HSPG which leads to

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the inhibition of tumor progression in ovarian cancer.[12-14] In hypoxia, it has been documented that the expression and signaling of HS binding growth factors, such as fibroblast growth factor receptor-2/basic fibroblast growth factor-2 (bFGF2), CXCR4/SDF, and c-mat/HGF, are increased and lead to increased invasion and migration of the ovarian cancer cells, while upregulation of SULF-1 is shown to interfere with hypoxia-induced signaling pathways and leads to decreased migration and invasion of the cells.[15,16] A recent study reported that expression of SULF-1 in SKOV3 facilitates Bim expression and inhibits tumor growth by blocking ERK pathway.[17]

In this study, we validated the potential tumor suppressor role of SULF-1 in ovarian cancer. Using SULF-1-expression plasmid, we successfully upregulated SULF-1 in SKOV3 cells to obtain stable SULF-1-SKOV3 cells. Our results showed that upregulation of SULF-1 expectedly inhibited cell proliferation, migration, and invasion *in vitro* and decreased lymph node metastasis *in vivo*.

MATERIALS AND METHODS

Cell line and culture

Ovarian cancer cell line (SKOV3), purchased from cell research (Shanghai, China), was cultured routinely in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Gibco, USA) containing penicillin 100 IU/ml, streptomycin 100 μg/ml, and 10% fetal bovine serum (FBS) (PAA). The cells were maintained in an incubator at 37°C with 5% CO_2 atmospheric condition.

SKOV3 cells were divided into three groups: (1) SULF-1 expression plasmid in SKOV3 cells (SULF-1-SKOV3), (2) nonspecific sequence control plasmid in SKOV3 cells (Nc-SKOV3), and (3) unmanipulated SKOV3 cell line (SKOV3). A day before transfection, 5×10^5 cells per well was plated into a 6-well plate in a medium with 10% FBS and placed in an incubator with a temperature of 37°C with 5% CO_2. Then, the cells were stably transfected with SULF-1 overexpression plasmid (SULF-1-SKOV3) and nonspecific sequence control plasmid (Nc-SKOV3). The nontransfected group was labelled as unmanipulated SKOV3 cells (SKOV3). The transfection was done using lipofectamine 2000 Reagent (Invitrogen, USA) according to the manufacturer's protocol. Before the transfection, we empirically determined G418 selection concentration to be 400 μg/ml of effective drug concentration, and after 72 h posttransfection, the cells were cultured in a medium containing this predetermined G418 concentration until the entire cells in the nontransfected wells died off. We continued to culture the cells under the selection pressure of the G418 until they became completely resistant to the drug. The stably transfected cells were then cultured and the level of SULF-1 protein upregulation was determined by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) and Western blot procedure.

Detection of sulfatase-1 mRNA sequence by quantitative real-time reverse transcription-polymerase chain reaction analysis

Total RNA was extracted from SULF-1 to SKOV3, Nc-SKOV3, and SKOV3 cell lines using Trizol (Invitrogen, USA) according to the manufacture's instructions. Reverse transcription of purified RNA was performed using oligonucleotide dT primer. qRT-PCR was carried out using SYBR green I dye, and the quantification of gene transcripts was performed and normalized to Gapdh as the internal control. The sequences of primer pairs used in the present study are listed in Table 1. PCR was carried out under the following conditions: 45 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C, and extension for 30 s at 72°C, and the relative mRNA expression level was collected and measured using $^{\Delta}$Ct equation. The PCR was carried out with Mx 3005P qRT-PCR machine (Agilent Technologies, Germany).

Detection of sulfatase-1 by western blot analysis

Cells in the log phase of growth were harvested and washed twice with ice-cold phosphate-buffered saline (PBS). Total cell proteins were extracted and then quantified by the bicinchoninic acid method using a Nanodrop spectrophotometer (Thermofisher Scientific USA). Equal amounts of proteins prepared into equal volumes were loaded onto a gel (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and separated by electrophoresis. Guided by a prestained protein molecular weight ladder, portions of the gel corresponding to the molecular weights of SULF-1 and glyceraldehyde 3-phospate dehydrogenase (Gapdh) proteins were sectioned out and transblotted onto a PVDF membrane (Invitrogen, USA). The membrane was blocked in 5% nonfat dried milk for 1 h and then probed with monoclonal goat anti-SULF-1 antibody (Abcam, China, 1–3 μg/ml) and Gapdh (ZSGB-Bio, China, 1:7500) primary antibodies for 1 h. After washing the membrane 6 times, rabbit anti-goat secondary antibody was applied to SULF-1 and anti-mouse secondary antibody applied on Gapdh for 1 h and the bright bands were captured by Li-Cor Odyssey Infrared Imaging System (Version 3.0 software).

Table 1: Nucleotide sequences of the primers used in quantitative real-time reverse transcription-polymerase chain reaction

Genes	Primers	
	Forward	Reverse
SULF-1	GCCAAGCGCCATGATGAG	TTCCACGCTCTGGCTGACT
Gapdh	AAGGGTTTGGGACAGACGA	CATGAACAGCGCAAGGATTA

SULF-1=Sulfatase-1, Gapdh=Glyceraldehyde 3-phospate dehydrogenase

Cell proliferation analysis

The effects of SULF-1 upregulation on SKOV3 cell proliferation were measured using Dojindo's CCK-8 cell proliferation kit (Dojindo Molecular Technologies, Japan). Briefly, triplicates of 3×10^3 cells/well of SKOV3, SULF-1-SKOV3, and Nc-SKOV3 cells were plated in 96-well plates and cell proliferation was measured on days 1, 2, 3, and 4. The cell proliferation test was done by adding 10 μl of CCK-8/well and the absorbance was measured 30 min after adding the reagent at 450 nm using a Multiskan Go spectrometer (Thermofisher Scientific, USA).

Flow cytometry

The three groups of cells (SKOV3, SULF-1-SKOV3, and Nc-SKOV3 cells) were synchronized at G_0/G_1 phase by growth in 100% confluence with reduced serum for 3 days.[18] The cells were then passaged and cultured for 24 h after which they were harvested in the log phase of growth, washed twice with ice-cold PBS, and fixed in 75% cold ethanol overnight at 4°C. The following day, the cells were washed twice with ice-cold PBS after discarding the ethanol, following which 50 μg/ml of RNase (Sigma, USA) was added for 30 min and then stained with 20 μg/ml of propidium Iodide (Sigma, USA) overnight in darkness. The cells were analyzed by flow cytometry (Beckman Coulter, USA) and the data were analyzed by Multicycle Software (Phoenix Flow Systems, San Diego, USA) to get the cell cycle distributions.

Migration assay analysis

Transwell cell culture plates were used to determine the extent to which SULF-1 upregulation has inhibited the cells' migration potential. The upper chambers of the inserts were seeded with 2×10^4 cells in 200 μl serum-free DMEM while the lower chamber was filled with 750 μl of DMEM containing 20% FBS as a chemoattractant, except in control wells which contained serum-free DMEM in both upper and lower chambers. After 16 h of incubation in humidified incubator with 5% CO_2, the nonmigrated cells in the upper chamber were swabbed off and the plates were fixed, stained, and then observed under an inverted fluorescent microscope. Cells in five microscopic field views of representative areas in each of the groups were counted and averaged.

Cell invasion assay

The inner chambers of the transwell plates were coated with ECM gel (Sigma, USA) and incubated at 37°C for 1 h to produce an artificial basement membrane. The rest of the procedure was as described in migration assay above. Both the migration assay and the invasion assay were performed concurrently, and the former, aside being an assay on its own, was additionally used as control for the later.

Scratch-wound healing assay

We plated 1×10^5 cells per well in a 24-well plate and allowed the cells to form 100% confluence overnight. Thereafter, the cells were carefully wounded with 10 μl white pipette tip, washed twice with PBS, and then continued culturing in complete medium at 37°C with 5% CO_2. The rate of wound closer, which signifies cell migration, was monitored by imaging at the time interval from 0 h, 12 h, and 24 h using an inverted fluorescent microscope equipped with a digital camera.

In vivo tumor metastasis assay

The animals were provided by the Animal Facility of Dalian Medical University. All experimental procedures were approved by the Animal Ethics Committee of the Dalian Medical University, China. A total of 21 male inbred 615 mice (aged 6–8 weeks, weighing 18–22 g) were randomly divided into three groups. The left footpad of each mouse was inoculated with 0.1 ml cell suspension (approximately 2×10^6 cells) of SKOV3, SULF-1-SKOVE, and Nc-SKOV3 cells. At 28 days, post-inoculated, the implanted tumor lymph nodes were dissected. The dissected tumor tissues were cut into 5 μm sections and fixed in paraformaldehyde. IHC stain was employed to detect the expression of SULF-1. The tissues were fixed in 4% buffered formalin for 15 min at 4°C and rinsed in tris-buffered saline. Paraffin sections were dewaxed in xylene and rehydrated in a series of ethanol solutions after which antigen retrieval was done with Tris buffer at pH 9.0 in a microwave. Endogenous peroxidase activity was blocked by 20 min preincubation with 3% H_2O_2 and then incubated with the blocking solution (Horse serum) for 30 min at room temperature. The incubation with primary antibody (goat monoclonal anti-SULF-1 antibody, Abcam, China, 1–3 μg/ml) was carried out overnight at 4°C. It was then incubated with the secondary antibody for 1 hr and color was developed with diaminobenzidine (Zhongshan Biotechnology, China). The positive reaction manifested as a brown stain. The section was counterstained in Mayor's hematoxylin. Dehydration process started from 80%, 95%, and 100% of ethanol each for 2 min and then washed with xylene 2 times for 2 min each. Mounting and cover slipping were done and we proceeded to slide reading. Other slides were stained by routine hematoxylin and eosin staining method, and the rate of lymph node metastasis was determined.

Statistical analysis

Each assay was performed three times. SPSS 17 software was used for all statistical analysis. One-way ANOVA was used to determine the significant differences among the three groups (SULF-1-SKOV3, Nc-SKOV3 and SKOV3) at $P < 0.05$. The data were expressed as the mean ± standard deviation.

RESULTS

Quantitative real-time reverse transcription-polymerase chain reaction and western blot results for the expression of sulfatase-1

The levels of mRNA of SULF-1 were also measured by qRT-PCR and normalized to Gapdh, and Δct was used to calculate the values. In the SULF-1-SKOV3 cells, the result showed that the Δct values for SULF-1 was 0.32 ($^*P < 0.05$) compared to the two controls where the Δct values for SULF-1 were 1.46 and 1.55 for SKOV3 and Nc-SKOV3 cells, respectively. This indicates

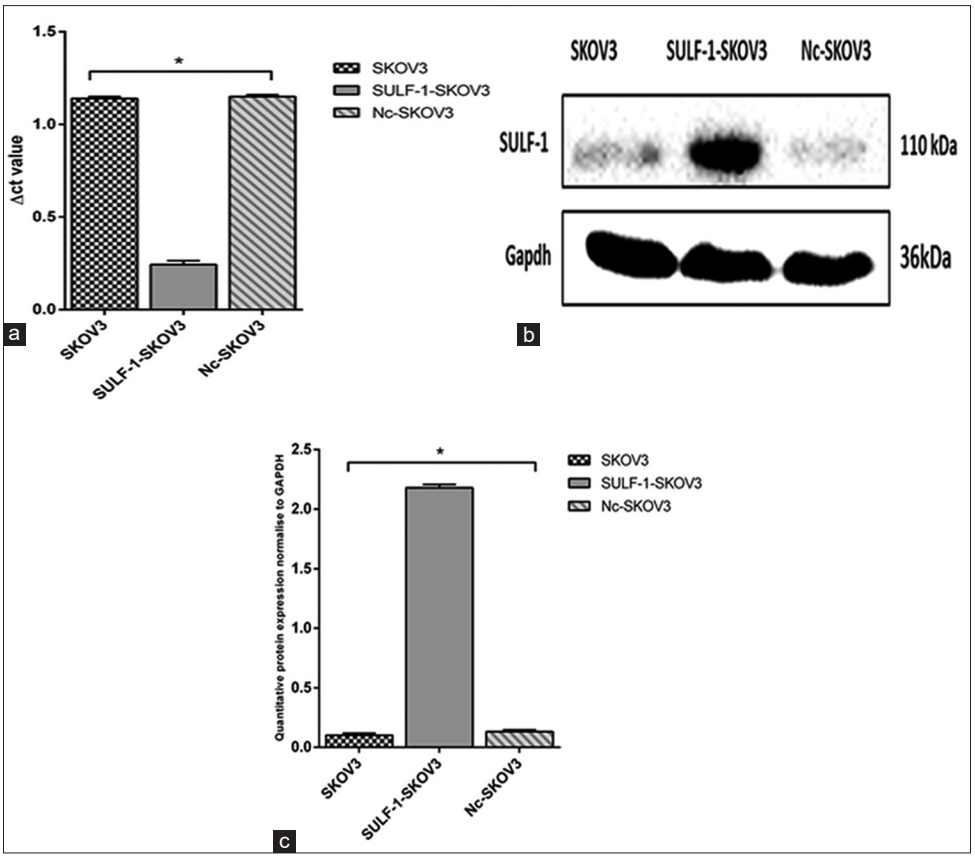

Figure 1: Quantitative real-time reverse transcription-polymerase chain reaction and Western blot analysis for sulfatase-1. (a) Δct values for mRNA expression levels of sulfatase-1. (b) Immunoblots for the expression of the sulfatase-1 proteins. (c) Graphical presentation of relative quantification of protein level. Data are presented in columns as mean ± standard deviation and the results were statistically significant at $P < 0.05$

that the mRNA expression level of SULF-1 in SULF-1-SKOV3 group was higher compared to the two controls [Figure 1a].

Western blot was performed to check the expression level of both SULF-1 in SKOV3, SULF-1-SKOV3, and Nc-SKOV3 cells. As shown in Figure 1b and c, the expression of SULF-1 in SKOV3 and Nc-SKOV3 cells was similar. However, and quite interestingly, the expression of SULF-1 following the upregulation significantly increased by 78% compared to the two controls.

Upregulation of sulfatase-1 decreased cell proliferation ability

CCK-8 cell proliferation assay was used to evaluate the role of SULF-1 on proliferation of SKOV3, SULF-1-SKOV3, and Nc-SKOV3 cells. The results showed that there was a significant inhibitory effect on cell proliferation by 65% ($^*P < 0.05$) in the SULF-1-SKOV3 cells compared to the SKOV3 and Nc-SKOV3 cells as shown in Figure 2. This indicates that SULF-1 played a tumor suppressor role in the ovarian cancer cells.

Action of sulfatase-1 upregulation on cell cycle

The cell cycle analysis was carried out by PI staining followed by flow cytometry. The results showed that in the SULF-1-SKOV3 cells, there was 41% increase in cell

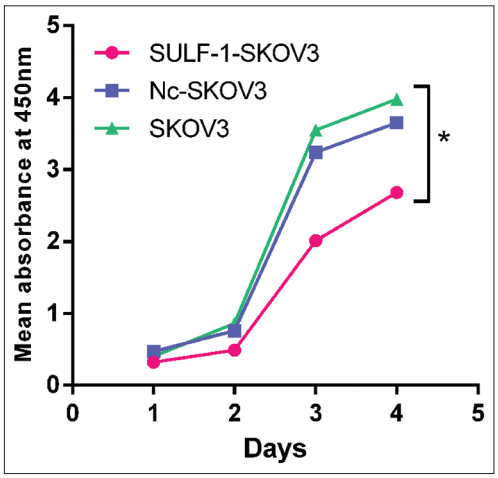

Figure 2: Cell proliferation assay (CCK 8). The curve for sulfatase-1-SKOV3 cells shows a lower proliferation rate in the CCK8 analysis. There was increased cell proliferation potential in both SKOV3 and Nc-SKOV3 with a statistical significant at $P < 0.05$

accumulation in G0-G1 phase with a concomitant reduction by 22% in the number of cells in S-phase compared to the two controls ($^*P < 0.05$) as shown in Table 2 and Figure 3. This result indicates that upregulation of SULF-1 inhibits ovarian cancer cell division.

Upregulation of sulfatase-1 inhibited cell migration and invasion

We performed Transwell assay to examine the mobility and invasion ability of the stably upregulated SULF-1-SKOV3 and the two controls (SKOV3 and Nc-SKOV3 cell lines). The migration and invasion ability of SULF-1-SKOV3 cells decreased by 3.25-fold and 2.8-fold with a statistical significance of $P < 0.05$. No significant differences were observed in migration and invasion abilities of the SKOV3 and Nc-SKOV3 groups as shown in Figure 4.

In the furtherance of ascertaining this inhibitory effect of SULF-1 upregulation on the SULF-1-SKOV3 cells motility potential, we also performed scratch-wound healing assay. The results, as illustrated in Figure 5, revealed near perfect congruity with the Transwell migration assays. There again, SULF-1-SKOV3 cells migrated at a slower rate than the two controls in terms of the wounded area covered over time as shown in Figure 5. The control groups were covering the wounded area at a rate nearly 2-fold (58.6 μm/h) that of the SULF-1-SKOV3 (30.4 μm/h). By 24 h time, the total wounded area remaining as a percentage of the original area was about 30% and 60% for the controls and SULF-1-SKOV3 cells, respectively.

Table 2: Cell cycle percentage distribution

Group	The percentage of cell cycle phase (%)		
	G0-G1	S	G2-M
SKOV3	42.67±0.67	49.31±1.4	8.03±0.85
SULF-1-SKOV3	81.59±0.07*	11.17±0.03*	8.10±0.02*
Nc-SKOV3	42.23±1.08	49.76±1.09	8.01±0.01

The difference for cell cycle analysis in SKOV3, SULF-1-SKOV3, and Nc-SKOV3 cells was statistical significant at *P<0.05. SULF-1=Sulfatase-1

Immunohistochemistry and histopathology analysis of lymph node *in vivo*

The lymph node was examined and the immunohistochemistry results showed that the intensity of SULF-1 expression is higher in SULF-1-SKOV3 group compared to the SKOV3 and Nc-SKOV3 [Figure 6a]. The H and E results revealed that the lymph nodes from SULF-1-SKOV3 bearing mice have less tumor cells and no necrotic tissues, while the lymph nodes from SKOV3 and Nc-SKOV3 have more tumor cells with mitotic figures in the nodal marginal sinus with necrotic tissues [Figure 6b]. This means that lymphatic metastatic level in SULF-1-SKOVE bearing mice was lower compared to the SKOV3 and Nc-SKOV3 bearing mice, and this confirmed that upregulation of SULF-1 in SKOV3 cells decreased LNM rates.

DISCUSSION

Ovarian cancer metastasis involves multistage processes with an extremely complex signal interaction. The involvement of SULF-1 in reducing the metastatic potential in human cancer has been reported; yet, little is known about the role of SULF-1 in ovarian cancer metastasis.[19,20] Many published studies reported that SULF-1 is downregulated in various cancers

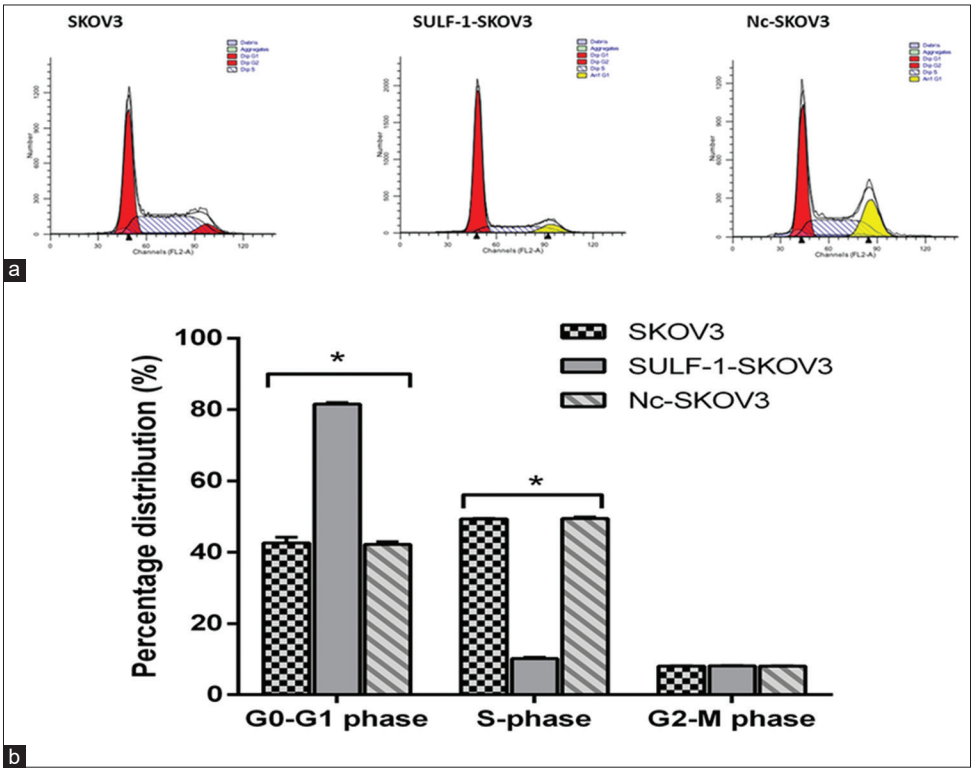

Figure 3: Cell cycle analysis. (a) Represent cell cycle analysis in sulfatase-1-SKOV3; SKOV3 and Nc-SKOV3 cells. (b) Cluster bar chart of the cell cycle showing percentage distributions of the cell cycle phases in the three cell groups. The values represent the number of cells in each phase of the cell cycle as a percentage of the total cells. The values were statistically significant at $P < 0.05$

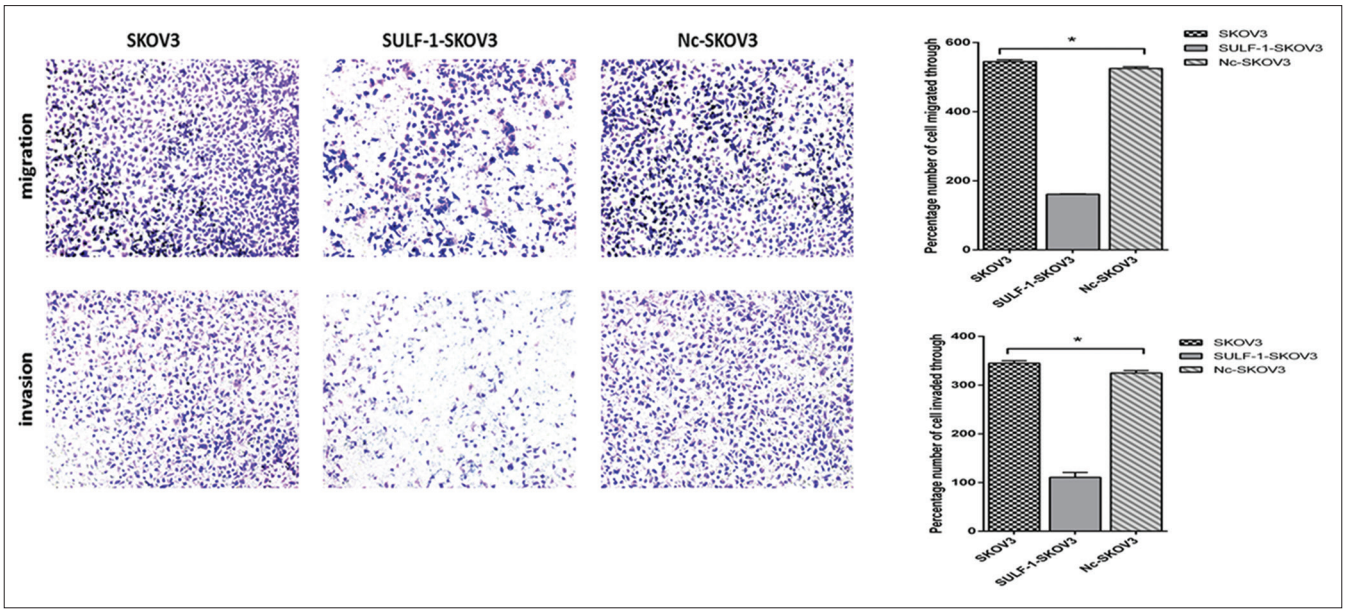

Figure 4: Migration and invasion assay. Transwell migration and invasion assays were performed in SKOV3, sulfatase-1-SKOV3, and Nc-SKOV3 cells. The results showed decrease in both migration and invasion in sulfatase-1-SKOV3 cells. Average number of migrating and invading cells presented as a bar chart with mean ± standard deviation. The results were statistically significant at $P < 0.05$

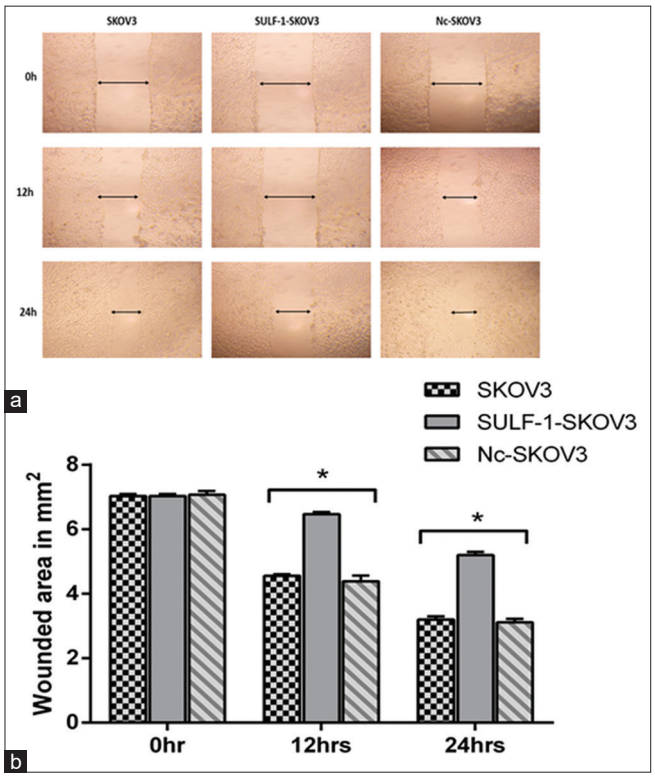

Figure 5: Scratch-wound healing assay. Cells were grown to 100% confluence and were then carefully wounded with a 10 ul pipette tip and the rate of closure of the wounded area was monitored and photographed at the same points at various time intervals. (a) SKOV3, sulfatase-1-SKOV3, and Nc-SKOV3. (b) Graphic representation of the wound closure showing changes in the wounded area remaining with time. Double arrow heads show the gap of the wounded area in micrometers (mm). Results represent mean of three values and were statistically significant ($P < 0.05$)

including ovarian cancer.[11,21-26] In this study, we observed that SULF-1 is minimally expressed in SKOV3 ovarian cancer cells, and forced expression of SULF-1 in the SKOV3 cells led to inhibition of cell proliferation, migration, and invasion *in vitro* and decreased lymph node metastasis *in vivo*. SULF-1 is a cell surface endosulfatase which has the ability to desulfate HSPG located on the cell membrane, and this action leads to the disruption of the structure of the HS growth factor complexes which are important in the facilitation of metastatic processes.[14] SULF-1 is reported to have increased expression in cancer cells sensitive to chemotherapeutic drugs; its overexpression is also observed to inhibit tumor progression.[27] The first study done by Dhoot *et al.* suggested that SULF-1 was localized on cell surface on quail and drosophila homologs. During their study, they found that SULF-1 is involved in desulfation of cell surface HSPG. HSPG is a major sulfated macromolecule located at the cell surface and consequently regulating tyrosine kinase signaling molecules.[28] Lai *et al.* performed a study which showed that SULF-1 expression causes desulfation of cell surface HSPG and led to inhibition of proliferation, migration, and invasion.[29-32]

Beneath the complexity of every cancer cells, a number of "mission critical" events happen that have propelled the tumor cells and its progeny into uncontrolled overproliferation. One of these is deregulated cell proliferation. One of the factors reported to facilitate overproliferation of cancer cells is the over sulfation of HSPG and this leads to the stimulation of the signaling pathway involved in the promotion of cell proliferation such as PI3K/AKT, Wnt/β and Hedgehog signaling.[28,33,34] Our results showed reduction in cell proliferation in the SULF-1 upregulated cells compared to the controls, and this confirms

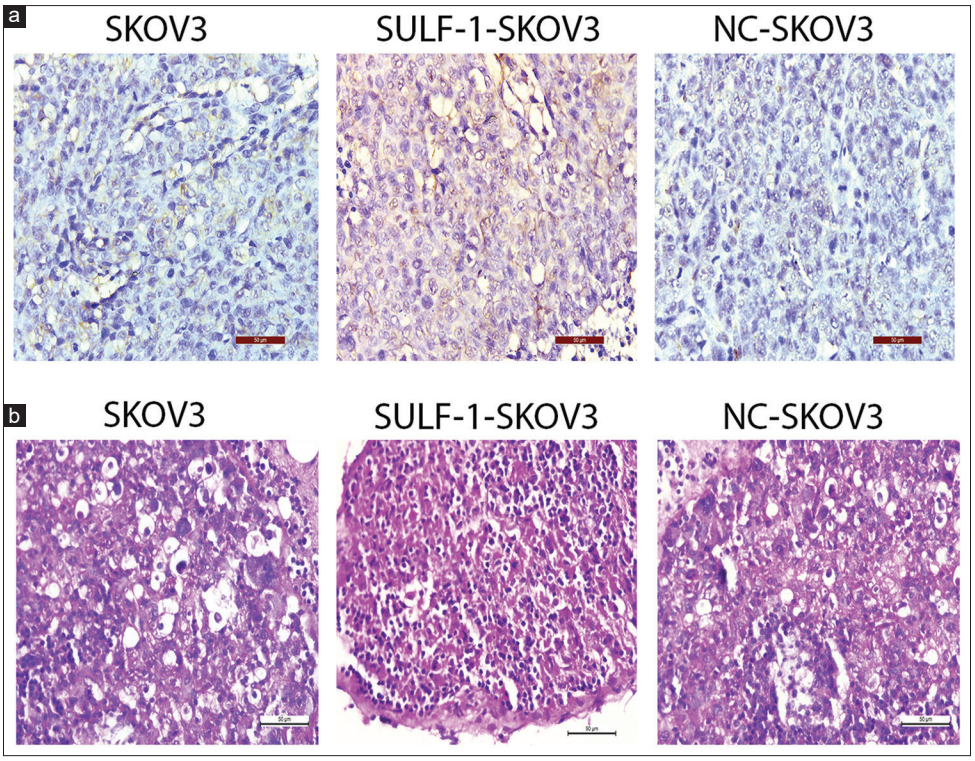

Figure 6: (a) Immunohistochemistry Analysis in the three different groups: SKOV3, sulfatase-1-SKOVE, and Nc-SKOV3 cell groups. The sulfatase-1 expression is higher in sulfatase-1-SKOV3 group compared to the SKOV3 and Nc-SKOV3 groups. (b) The H and E staining showed the lymph nodes from SKOV3 and Nc-SKOV3 groups had tumor giant cells and abnormal mitotic figures and were generally of high grade compared to that of the sulfatase-1-SKOVE group

the tumor suppressor role of SULF-1 as reported in other studies. Consistently, expression of SULF-1 is reported to suppress the growth and cell proliferation of ovarian cancer and gastric cancer cell line by desulfating HSPG.[10,24]

We further observed that there was a significant increase in the accumulation of cells in G0-G1 phase by 41% with an attendant 22% decrease in the S-phase cell number in the SULF-1 upregulated group compared to the controls. This finding is in agreement with the previous study from Xu *et al.*, who reported that SULF-1 causes cell cycle arrest in hepatocellular carcinoma by reducing the binding affinity of bFGF to its receptor through HSPGs desulfation.[6]

In cancer metastasis, tumor cells must acquire invasive and migratory phenotypes before they can metastasize. Downregulation of SULF-1 by hypoxia inducible factor 1 in hypoxic condition in breast cancer MCF10DCIS cells resulted in increased migration and invasion abilities, while on the other hand, its overexpression inhibited the bFGF signaling and led to reduction in migration and invasion of the breast cancer cells.[35] Similarly, our findings showed that SULF-1 upregulated cells had significantly reduced migration and invasion abilities compared to the two controls. Furthermore, our *in vivo* study showed that there was significantly reduction in lymph node metastasis in the mice inoculated with SULF-1-SKOV3 cells. This result implying that upregulation of SULF-1 has a restrictive interference effects on the metastatic ability of ovarian cancer cells. In other words, upregulation of SULF-1 might be of therapeutic benefits to the management of ovarian cancer.

CONCLUSION

In summary, our study highlights that upregulation of SULF-1 in SKOV3 cells inhibits cell proliferation, migration, and invasion *in vitro* and decreases lymph node metastasis *in vivo.* Therefore, in our strive to get a better understanding and a cure for ovarian cancer, more research is required to further explore the role of SULF-1 in cancer metastasis. This exploitation could be a key in unlocking a novel strategy toward finding a cure for ovarian cancer which is one of the diseases with a high case fatality rate.

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Conflicts of interest
There are no conflicts of interest.

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