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Original Article

High-mobility group box 1-induced epithelial-mesenchymal transition of ovarian cancer through Smad3/Snail/NF-κB pathways

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Abstract: Objective: Our aim was to explore the mechanism of HMGB1 induced epithelial-mesenchymal transition (EMT) in ovarian cancer. Method: Expression of HMGB1 in ovarian cancer and adjacent normal tissues were measured by immunohistochemistry. Over-all survival (OS) and disease-free survival (DFS) was analyzed by the Kaplan-Meier method. Ovarian cancer cell lines OVCAR3 and SKOV3 were transfected with HMGB1 overexpression or HMGB1 knockdown lentiviral vectors, respectively. Epithelial and mesenchymal markers were visualized by immunofluorescence staining and cell invasion and migration ability was assayed by transwell chamber and wound healing assay. Expression of mRNA or proteins were also measured. Results: HMGB1 was significantly upregulated in ovarian cancer tissues. In addition, HMGB1 expression was significantly associated with FIGO stage, histologic grade, and lymph node metastasis. Kaplan-Meier survival analysis showed that high expression of HMGB1 had poorer OS and DFS in ovarian cancer patients. We further found that HMGB1 induced EMT and promoted cell migration and invasion of ovarian cancer cells *in vitro* and these effects were attenuated by inhibiting expression of HMGB1. Moreover, Smad3/Snail/NF-κB signaling pathways may contribute to regulation of HMGB1-mediated EMT of ovarian cancer cells. Conclusion: HMGB1 acts as a promoter of ovarian cancer EMT via activation of Smad3/Snail/NF-κB pathways.

Keywords: High-mobility group box 1, ovarian cancer, epithelial-mesenchymal transition, signaling pathway

Introduction

Ovarian cancer (OC) is the second most common female reproductive tract cancer with more than 200,000 new cases reported every year, worldwide [1]. OC, with poor prognosis and high lethality, has attracted the attention of many researchers. The most obvious characteristics of OC are its high metastasis rate and strong invasive ability [2]. Despite many efforts to identify modifiable factors involved in ovarian carcinogenesis, little is known about the molecular mechanism related to metastasis and recurrence of ovarian cancer.

Epithelial-mesenchymal transition (EMT) plays a critical role in the metastasis and invasion of many tumors such as breast cancer, colorectal cancer, and lung cancer [3-5]. During EMT, epithelial cells lose apical-basal polarity and acquire mesenchymal phenotype through cell-cell contacts. Consistent with EMT, expression of epithelial junction proteins, including E-cadherin and Vitamin D3 receptor (VDR), is downregulated and expression of mesenchymal adhesion proteins such as N-cadherin and vimentin is upregulated. Numerous studies have reported that EMT confers resistance to chemotherapy of cancer cells, escape from immune surveillance, and resistance to cell death [6, 7]. Therefore, identifying the signaling pathway that involves cancer EMT offers drugs targets to inhibit cell metastases.

High-mobility group box 1 (HMGB1) is a nuclear protein that functions similary to a cytokine [8]. HMGB1 induces inflammatory reactions through multiple receptors in the cell surface and is recognized as an *in vivo* sensor of tissue damage. Mounting evidence has suggested

that cancer cells secrete HMGB1 into their microenviroment during the necrotic process and production of many inflammatory cytokines that promote invasiveness and metastases. Furthermore, cancer cell release of HMGB1 results in inflammatory responses that, in the setting of cancer, may lead to tumor metastases and survival [9]. In fact, there have been many studies that have demonstrated that HMGB1 promotes metastasis in a variety of cancers such as colon, gastric, liver, and prostate cancers. Recent studies have also indicated that OC cells undergo EMT through exposure to the extracellular milieu and cytokines that promote invasion of cells [10]. However, the mechanisms of EMT on ovarian cancer metastasis remain largely unknown.

Based on these previous findings, we hypothesized that HMGB1 can induce the EMT process in OC cells, possibly offering a novel therapeutic target for OC patients. There is an accumulating amount of evidence that has demonstrated that HMGB1 modulates ventricular remodelling via inhibition of the TGF-β/Smad pathway. We also explored whether HMGB1 inducing EMT depends on TGF-β signaling pathways.

Materials and methods

Specimen collection

OC samples and pair-matched adjacent normal tissues were obtained from eighty seven cases of OC patients that underwent surgical treatment at the First Affiliated Hospital of Soochow University, from June 2009 to September 2011. None of these patients received prior chemotherapy or radiotherapy. All patients provided informed consent prior surgical treatment and this study was approved by the Committees for Ethical Review of Research Involving Human Subjects at the First Affiliated Hospital of Soochow University. Tumor specimens and adjacent tissues were collected and stored in liquid nitrogen, immediately. Clinicopathologic features of OC patients were summarized and over-all survival (OS) was defined as the interval between surgery and death or the end of follow up. Disease-free survival (DFS) was defined as the interval between surgery and disease recurrence or the end of follow up.

Cell lines

Ovarian cancer cell lines, OVCAR3 and SKOV3, were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 medium (Gibco), supplemented with 10% fetal bovine serum (Invitrogen, USA) and 1% penicillin-streptomycin. Cells were kept at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Immunohistochemistry staining

Immunohistochemical staining was performed by Envision Plus System (Dako Ltd., Ely, UK), according to manufacturer protocol. Ovarian cancer sections were stained with anti-HMGB1 (BOSTER, Wuhan, China). Staining of HMGB1 in sections was examined in a blinded manner. Cell staining intensity and positive cells proportion of stained cells in 200 × magnification fields was calculated, according to immunoreactive score (IRS). IHC staining was evaluated by cell staining intensity and positive cells proportion. Cell staining intensity was scored as 0 (negative), 1 (pale yellow), 2 (yellow), and 3 (brown). Positive cells proportion was scored as 1 (0-26%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). Results of IRS were multiplication of intensity score and proportion score and ranged from 1 to 12 : IRS 4-12 scores were defined as high expression and IRS 0-3 scores were defined as low expression.

HMGB1 recombinant lentivirus and transfection

Procedures of synthesising HMGB1 overexpression or HMGB1 knockdown lentiviral vectors, corresponding negative controls, and protocols of lentivirus transfection were previously described [11]. The HMGB1 target sequence for interation as follows: 5'-AUUUCUCUUUCAUAACGGGTT-3'. For OVCAR3 cells, when selectively transfected with overexpressed HMGB1 lentiviral vectors or negative control, were named OVCAR3-HMGB1 or OVCAR3-NC. For SKOV3 cells, when selectively transfected with knockdown HMGB1 lentiviral vectors or negative control, were named SKOV3-siHMGB1 or SKOV3-NC. Expression of HMGB1, after transfection in cells, was confirmed by RT-PCR.

Immunofluorescence microscopy

Epithelial and mesenchymal markers were visualized by immunofluorescence staining. After transfection, the cells were fixed in formaldehyde (3.7%) for 30 minutes and then tested for permeability with Triton X-100. Cells were

added E-caderin, Vitamin D3 receptor (VDR), N-cadherin, or Vimentin (antibodies were purchased from Cell Signaling Technology) antibodies for 2 hours at room temperature, washed with phosphate-buffered saline (PBS), and incubated in fluorescein isothiocyanate-conjugated secondary antibody for 20 minutes. The nuclei was stained with DAPI for 15 minutes. Samples were measured by confocal laser scanning microscope (Carl Zeiss Jena, Germany).

Real time quantitative PCR (RT-PCR)

Total RNA from sections and cells were extracted by Trizol Reagent (Invitrogen, USA). cDNA was synthesized using PrimeScript TM RT reagent Kit (TaKaRa, Japan), according to manufacturer instructions. Relative expression of mRNA was amplified using SYBER Green (TaKaRa Bio, Tokyo, Japan). The sequences of primers were as follows: HMGB1 primers, F: 5'-ATGCCTGTAACAAAGAAGCCCC-3', R: 5'-CACACAGTTGCCCCCGTTTTTAC-3'; E-caderin primers, F: 5'-AAGGGGGCTATGGAAAGGCAA-3', R: 5'-AATCCACGATGAAGGGATGCT-3'; VDR primers, F: 5'-GAACCTCAGGGCAAGATGCT-3', R: 5'-CTGGTTTCCTCATTCCGGCT-3'; N-cadherin, F: 5'-GCTGAGCTGGTGGTTGAAAGGAATC-3', R: 5'-GCTTGGCCCACGATGACTTGG-3'; Vimentin, F: 5'-TGAGGTATCATAGGAGCAGCC-3', R: 5'-TGTTGGTGGAGAGTCAAGTGA-3'; GAPDH, F: 5'-CTCCCGCTTCGCTCTCTG-3', R: 5'-TCCGTTGACTCCGACCTTC-3'. Relative expression of the target gene was normalized by GAPDH, using $^{\Delta\Delta}$Ct comparative method.

Western blotting

Total proteins of fresh tissues or cells were extracted and determined by BCA protein assay (Beyotime, Beijing, China), according to manufacturer instructions. Briefly, total protein was loaded onto SDS-polyacrylamide gel for electrophoresis and transferred onto PVDF membranes and blocked with non-fat milk for 2 hours. The membranes were incubated with specific primary antibody against HMGB1 (1:200; BOSTER, Wuhan, China), E-caderin (1:300; Cell Signaling Technology, Boston, USA), VDR (1:200; Cell Signaling Technology, Boston, USA), N-cadherin (1:500; Cell Signaling Technology, Boston, USA), Vimentin (1:300; Cell Signaling Technology, Boston, USA), Smad3 (1:750; Cell Signaling Technology, Boston, USA), phospho-Smad3 (1:500; Abcam, Cambridge, UK), NF-κB p65 (1:600; Abcam, Cambridge, UK), phospho-NF-κB p65 (1:300; Abcam, Cambridge, UK), Snail (1:500; Abcam, Cambridge, UK), and GAPDH (1:600; Abcam, Cambridge, UK) overnight at 4°C. After washing with PBST, membranes were incubated with H RP-labeled goat anti-rabbit immunoglobulin (1:5000; Abcam, Cambridge, UK) for 1 hour at room temperature. The band was scanned and digitized and intensities of band intensities were quantified by Image J software (Rawak Software, Inc. Germany). Target protein levels were expressed as respective ratios to GAPDH levels.

Transwell and wound healing assays

Cell invasion ability was assayed by a Transwell chamber assay (Costar, Pleasanta, CA, USA), according to manufacturer instructions. Serum-free DMEM was added to the upper chambers and DMEM containing 10% fetal bovine serum was added to lower chambers. After 48 hours of culturing, the cells that transferred to the lower chambers were fixed by 90% alcohol and stained by 0.08% crystal violet. Numbers of the lower chambers were counted. Migration of cells was measured by wound healing assay. Briefly, cunfluent cells were scraped with a pipette tip (100 μl). Cultures were rinsed with PBS twice and then serum-free medium was added. Cells that migrated into the denuded area were measured.

Statistical analysis

Related data of our experiments were analyzed by SPSS 19.0 (SPSS, Chicago, IL) and GraphPad Prism 7.0 (GraphPad Software Inc. La Jolla, CA, USA). Quantitative data are presented as mean ± standard deviation. Groups were compared with unpaired Student's t-tests and multiple groups were compared with one-way ANOVA. Categorical data were evaluated by χ^2 test. Kaplan-Meier method was used to analyze OS and DFS in patients with OC. A value of $P<0.05$ was considered statistically significant.

Results

HMGB1 was significantly upregulated in OC tissues

There have been several studies that have reported that HMGB1 is significantly related

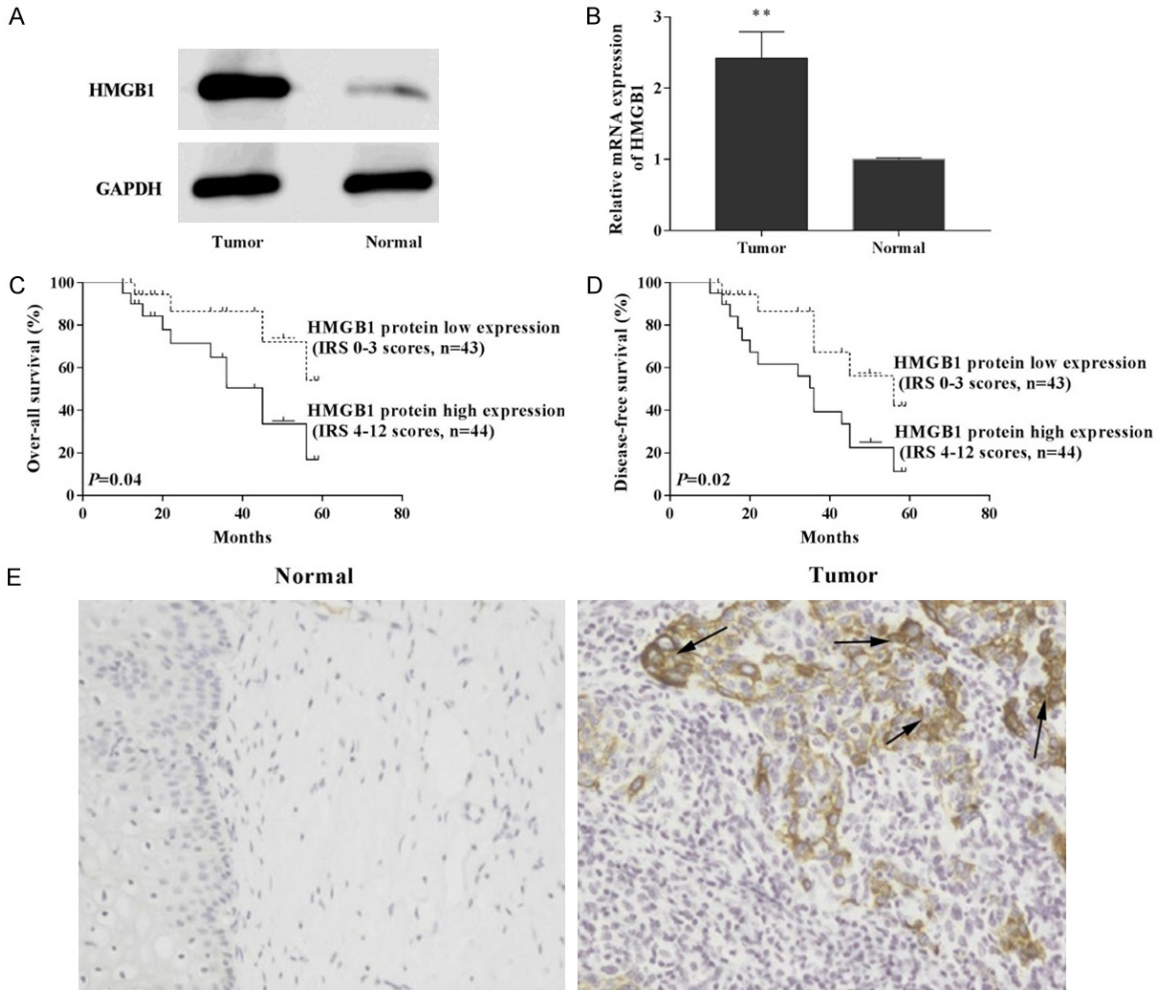

Figure 1. Upregulation of HMGB1 in ovarian cancer clinical specimens. A: Expression of HMGB1 protein in 87 pairs of ovarian cancertumor samples and adjacent normal tissues determined by Western Blotting. B: Expression of HMGB1 mRNA in 87 pairs of ovarian cancer tumor samples and adjacent normal tissues determined by RT-PCR. C and D: Overall survival (OS) and disease-free survival (DFS) of ovarian cancer patients determined by Kaplan-Meier method. E: Expression of HMGB1 protein in 87 pairs of ovarian cancer tumor samples and adjacent normal tissues determined by immunohistochemistry staining (indicated by arrows). $^{**}P<0.01$ compare to adjacent normal tissues. Data are expressed as mean ± standard deviation, all experiments were performed at least three times.

with advanced clinical stage and metastasis in breast cancer patients [12]. Additionally, expression of HMGB1 has been significantly related with poor outcome of cervical carcinoma patients [13]. To explore the clinical relevance of HMGB1 in OC patients, we first measured expression of HMGB1 in tumor samples and adjacent normal tissues by RT-PCR and Western blotting. As shown in **Figure 1**, expression of HMGB1 was significantly upregulated in tumor samples compared with adjacent tissues (**Figure 1A**, **1B**). In agreement with RT-PCR and Western blotting results, immunohistochemistry assay showed that IRS of HMGB1 was dramatically elevated in tumor tissues (**Figure 1E**).

To explore the clinical relevance of HMGB1 expression of OC patients, correlation between HMGB1 expression and clinicopathological parameters was analyzed. As indicated in **Table 1**, HMGB1 expression was significantly associated with FIGO stage ($P<0.001$), histologic grade ($P=0.01$), and lymph node metastasis ($P=0.035$) while there was no significant difference in patient age, tumor diameter, and histologic subtype. Moreover, patients with high HMGB1 expression had poorer OS ($P=0.04$) and DFS ($P=0.02$, **Figure 1C**, **1D**). Collectively, these results suggest that HMGB1 is overexpressed in OS and HMGB1 may be an oncogene accelerator in OC.

Table 1. Correlation between HMGB1 expression and clinicopathologic features in ovarian cancer patients

Clinicopathologic features	n	HMGB1		*P*
		High expression (IRS 0-3 scores)	Low expression (IRS 4-12 scores)	
Total	87	44 (50.6%)	43 (49.4%)	
Age (years old)				0.74
≤50	38	20 (52.6%)	18 (47.4%)	
>50	49	24 (49.0%)	25 (51.0%)	
Tumor diameter (cm)				0.68
≤3	63	31 (49.2%)	32 (50.8%)	
>3	24	13 (54.2%)	11 (45.8%)	
FIGO stage				<0.001***
I/II	37	8 (21.6%)	29 (78.4%)	
III/IV	50	36 (72.0%)	14 (28.0%)	
Histologic grade				0.01*
G1-G2	39	14 (35.9%)	25 (64.1%)	
G3	48	30 (62.5%)	18 (37.5%)	
Histologic subtype				0.44
Serous	60	32 (53.3%)	28 (46.7%)	
Other	27	12 (44.4%)	15 (55.6%)	
Lymph node metastasis				0.04*
Yes	34	22 (64.7%)	12 (35.3%)	
No	53	22 (41.5%)	31 (58.5%)	

FIGO, international federation of gynecology and obstetrics; *P<0.05; ***P<0.001.

HMGB1 induces EMT in OC cells

Epithelial-mesenchymal transition (EMT) is a well-orchestrated process that gains migratory phenotypes and enhances invasiveness in many cancers, including ovarian cancer [14]. Concomitantly with EMT, epithelial phenotype is lost and mesenchymal phenotype is gained. In our present study, we ectopic expression of HMGB1 in OVCAR3 and SKOV3 cells. As shown in **Figure 2**, downregulation of HMGB1 mRNA in SKOV3-siHMGB1 and upregulation of HMGB1 mRNA in OVCAR3-HMGB1 was confirmed by realtime PCR (**Figure 2A** and **2B**). Then, we measured epithelial markers and mesenchymal marker proteins in two OC cell lines. Expression of epithelial markers (E-cadherin and VDR) was dramatically decreased and expression of mesenchymal markers (N-cadherin and Vimentin) was prominently increased in OVCAR3-HMGB1 (**Figure 2D**, **2F** and **2H**), while transfection of HMGB1 siRNA in SKOV3 cells produced an opposite effect (**Figure 2C**, **2E** and **2G**), reminiscent of the process of EMT. These data suggest that HMGB1 leads to switching from epithelial phenotypes to mesenchymal phenotypes in OC cells.

HMGB1 promotes invasiveness and metastasis of OC cells

Increasing publications have reported that HMGB1 induces EMT transitions in colorectal, breast, and gastric cancers and plays a key role in the process of tumor invasioness and metastasis [15-17]. To further explore the role of HMGB1-mediated EMT on invasiveness and metastasis of ovarian cancer, Transwell and wound healing assay was performed in OVCAR3 and SKOV3 cells after transfection with HMGB1 or si-HMGB1. Our results showed that OVCAR3 cells simulated with HMGB1 exhibited a significantly increased cellular migratory capacity compared with untreated cells (**Figure 3C**, **3D**). Migratory capacity was reversed through siRNA targeting of HMGB1 in SKOV3 cells (**Figure 3A**, **3B**).

HMGB1-mediated EMT of OC cells via Smad3/Snail/NF-κB pathways

TGF-β is known as a reliable inducer of EMT. TGF-β signaling is activated by Smad and directly upregulates expression of EMT transcription mediators, such as Snail, and is ultimately involved in activation of NF-κB [18]. To verify whether Smad signaling pathways are also responsible for HMGB1-mediated EMT, Western blotting was performed to determine protein expression of Smad3, Snail, and NF-κB. Of note, compared to untreated cells, protein expression of phospho-Smad3 (p-Smad3), Snail, and phospho-NF-κB p65 (p-NF-κB p65) induced by HMGB1 was significantly increased (**Figure 4B**) whereas silencing of HMGB1 produced downregulation of these proteins (**Figure 4A**). Taken together, these results suggest that Smad3/Snail/NF-κB signaling pathways be may involved in HMGB1-mediated EMT.

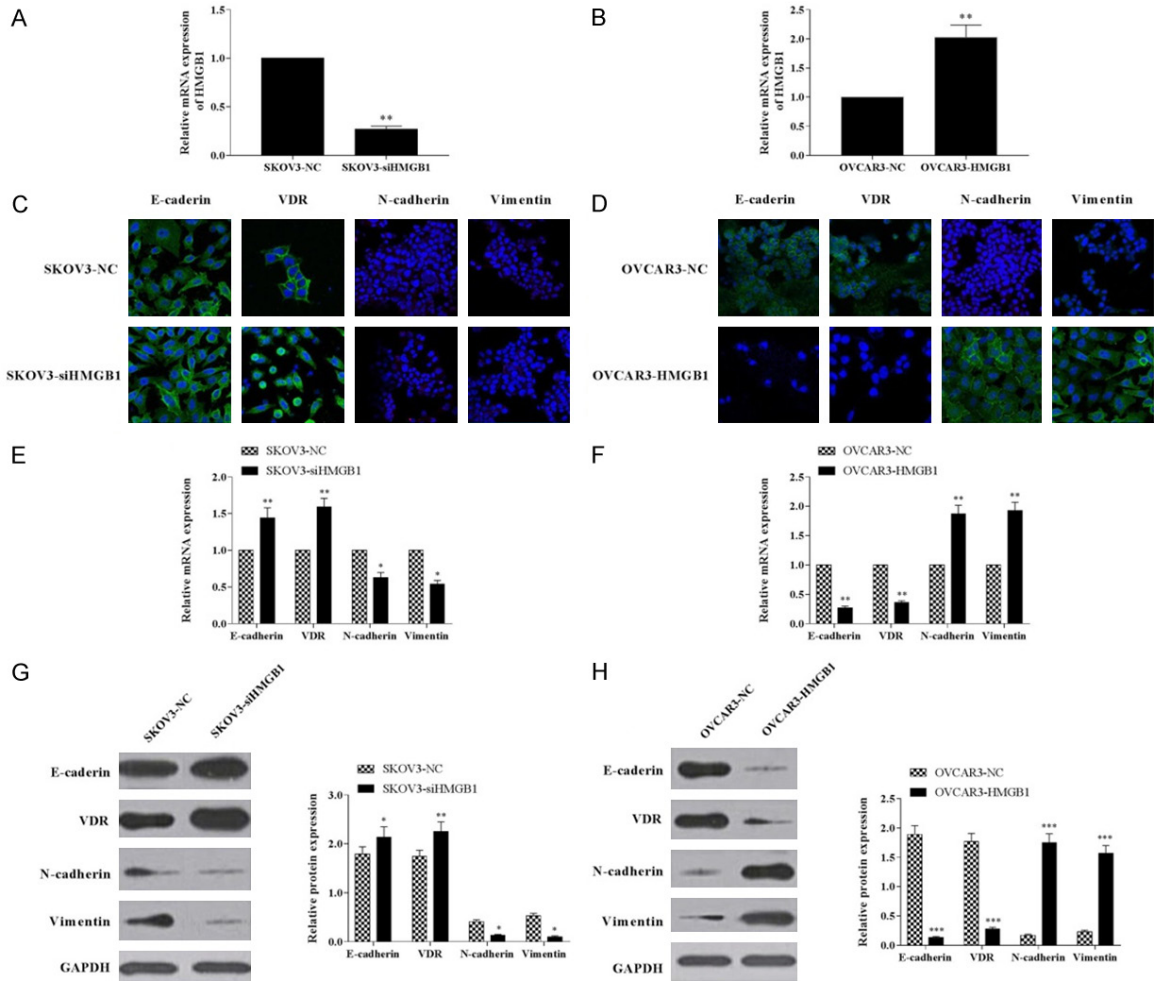

Figure 2. HMGB1 induces the EMT transition in ovarian cancer cell. A and B: OVCAR3 cells were transfected with overexpressed HMGB1 lentiviral vectors, the SKOV3 cells were transfected with knockdown HMGB1 lentiviral vectors. Expression of HMGB1 mRNA measured by RT-PCR in OVCAR3 and SKOV3 cells. C and D: Expression of E-cadherin, VDR, N-cadherin and Vimentin in OVCAR3 and SKOV3 cells analyzed by immunofluorescence staining. The green signal represents staining for E-cadherin, VDR, N-cadherin or Vimentin. Scale bar represents 20 μm. E and F: Expression of E-cadherin, VDR, N-cadherin, Vimentin mRNA in OVCAR3 and SKOV3 cell analyzed by RT-PCT. G and H: Expression of E-cadherin, VDR, N-cadherin, Vimentin protein in OVCAR3 and SKOV3 cell analyzed by Western bloting. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. All experiments were performed at least three times. Data are presented as mean ± standard deviation.

Discussion

Despite many efforts made in the early diagnosis of ovarian cancer, the 5-year survival rate of ovarian cancer is still less than 55.17% in China [19]. There is an accumulating amount of evidence demonstrating that the majority of OC patients die of metastasis rather than primary tumor lesions [20]. Metastasis is a multistep and complex biological process which may involve epithelial-mesenchymal transition (EMT), cell-cell contaction, and cytokines. Meng et al. [21] reported that the EMT process plays a vital role in cancer metastasis. During the EMT process, epithelial cells acquire mesenchymal phenotypes along with cancer cells losing their cell-cell adhesion and gaining fibroblast-like morphology. However, little is known about possible molecular mechanisms underlying EMT of ovarian cancer cells.

HMGB1 is a nuclear protein, first identified in 1999. HMGB1 dyregulation is associated with a large range of cellular functions including proliferation, metastasis, and invasion resulting in progressive and uncontrolled tumor growth. Feng et al. [22] has reported that HMGB1 is upregulated in non-small cell lung cancer

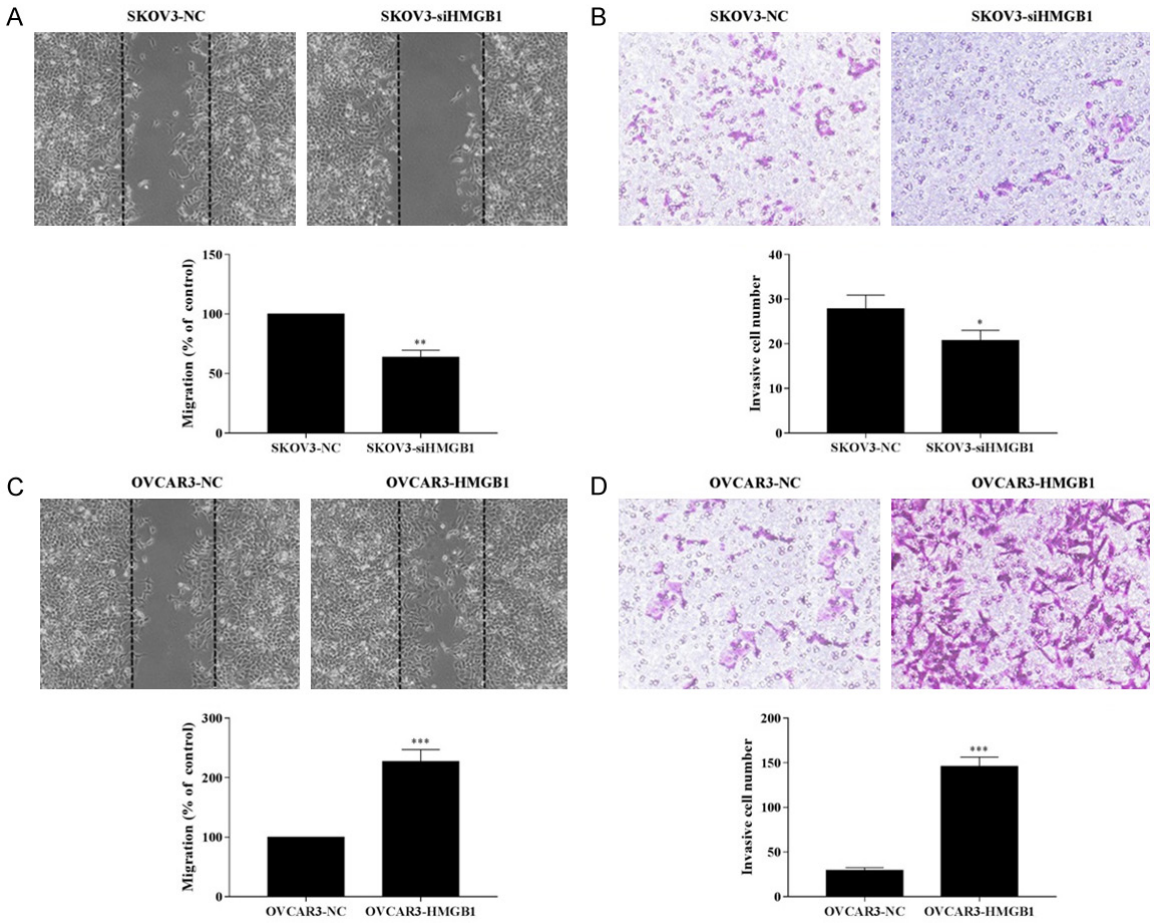

Figure 3. HMGB1 promotes the invasiveness and metastasis of ovarian cancercells. (A and B) The siRNA of HMGB1 inhibits the invasiveness (A) and migration (B) of SKOV3 cells. (C and D) HMGB1 exhibits a increased invasiveness (C) and migration (D) of OVCAR3 cells. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. All experiments were performed at least three times. Data are presented as mean ± standard deviation.

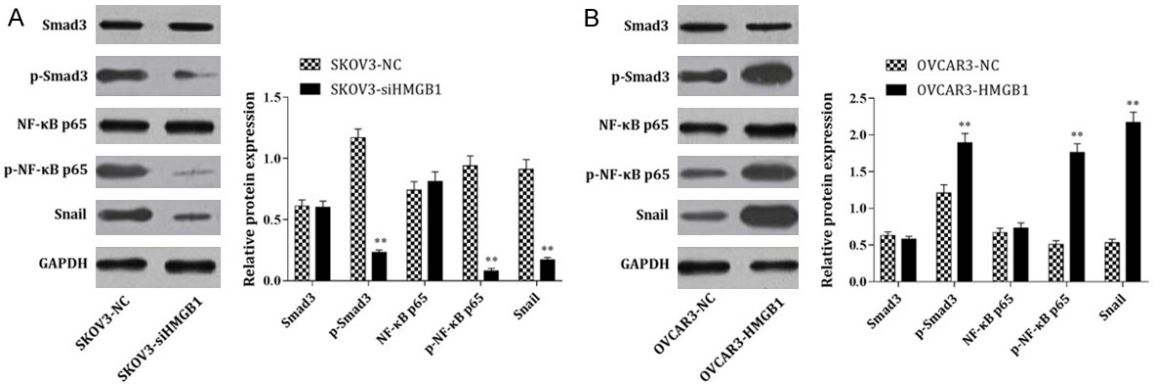

Figure 4. HMGB1-mediated EMT of ovarian cancer cells via Smad3/Snail/NF-κB pathway. A: HMGB1 stimulated the activation of phospho-Smad3, phospho-NF-κB p65 and Snail in SKOV3 cells. B: The siRNA of HMGB1 decreased the expression of phospho-Smad3, phospho-NF-κB p65 and Snail protein in OVCAR3 cells. $^{**}P<0.01$. All experiments were performed at least three times. Data are presented as mean ± standard deviation.

patients. They also reported that HMGB1 regulates non-small cell lung cancer cell invasion and proliferation and may be a potential prognostic predictor for NSCLC. Our present study

shows upregulation of HMGB1 in OC tissues. In addition, HMGB1 expression was significantly associated with FIGO stage, histologic grade, and lymph node metastasis. These data indicate that HMGB1 may play an oncogenic role in ovarian cancer. Li et al. [23] reported that abdominal surgery trauma releases a large amount of HMGB1 in the peritoneal cavity and promotes metastasis of colon cancer. We further investigated the prognostic value of HMGB1 by the Kaplan-Meier method. The results showed that ovarian cancer patients with high HMGB1 expression had poorer OS and DFS. This analysis indicates that HMGB1 may play a role in development and improvement of ovarian cancer.

Many studies have demonstrated that ovarian cancer cells exhibit the properties of EMT. Primed exposure to extracellular or cytokines promotes the EMT process [24]. Consistent with EMT process, the epithelial phenotype proteins are downregulated and mesenchymal phenotype proteins are upregulated. HMGB1 has been identified as an important mediator in EMT, associating with progression of tumor growth and metastasis. In this study, expression of E-cadherin and VDR was downregulated and expression of N-cadherin and Vimentin was upregulated, consistent with HMGB1 treated OC cells. Epithelial and mesenchymal markers, mentioned above, produced an opposite effect when treated with siHMGB1, clearly implying that HMGB1 acts an inductor of EMT in OC cells.

EMT is an important process which provides a therapeutic target for resistance and metastasis. Lou et al. [25] demonstrated that migration of pancreatic cancer cells was suppressed by naringenin and that the mechanism may be related to inhibition of the EMT process. Ono et al. [26] also reported prevention or reversal of EMT in colorectal cancer cells leading to inhibition of drug resistance and metastasis of tumors. In the present study, we found that HMGB1 induced metastasis and invasion of OC cells, *in vitro*. We also found that downregulating expression of HMGB1 significantly attenuated HMGB1-mediated metastasis of OC cells. Based on these results, our data suggests that HMGB1 may induce to undergo EMT and result in metastasis of OC cells.

It is increasingly evident that induction of EMT depends on activation of NF-κB and NF-κB induces upregulation of transcription factors participating in the control of EMT, such as Snail and Twist [27]. In fact, NF-κB promotes stabilization of Snail and is correlated with invasion and metastasis of cancer cells [28]. In addition, Smad pathways also mediate activation of TGF-β, leading to phosphorylation of Smad3 and stimulation of the EMT process. According to our current study, phospho-Smad3, phospho-NF-κB p65, and Snail were activated by HMGB1 and the silencing of HMGB1 reversed activation of Smad3/Snail/NF-κB signaling pathways. Liu et al. [29] reported that inhibition of HMGB1/Snail/NF-κB pathways could attenuate HMGB1-mediated EMT and prevent migration and invasion of colorectal cancer cells. Another study showed that HMGB1-induced upregulation of Snail led to enhaned metastaisi fomation *in vivo* and overexpression of Snail correlated with distant metastases of ovarian cancer [30]. Taken together, our findings imply that Smad3/Snail/NF-κB signaling pathways may play a role in regulation of HMGB1-mediated EMT of OC cells.

Collectively, we have demonstrated that expression of HMGB1 is associated with metastasis and survival of ovarian cancer patients. We also found that HMGB1 acts as a promotor of EMT via Smad3/Snail/NF-κB pathways. These findings suggest that HMGB1 represents a target for treatment of ovarian cancer metastasis.

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Disclosure of conflict of interest

None.

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