HMGB1 enhances the migratory and invasive abilities of A2780/DDP cells by facilitating epithelial to mesenchymal transition via GSK-3β

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Abstract. The aim of the present study was to investigate the impact and mechanism of high mobility group box 1 (HMGB1) on the regulation of cell migration and invasion in A2780/DDP cisplatin-resistant ovarian cancer cells. After transfecting small interfering (si)RNA-HMGB1 into A2780/DDP cells, Transwell migration and invasion assays were conducted to assess alterations in the cell migratory and invasive abilities. Additionally, western blotting analyses were performed to examine changes in HMGB1, phosphorylated (p)-GSK-3β, GSK-3β, E-cadherin and vimentin expression levels. The results of the present study demonstrated that the migratory and invasive abilities of A2780/DDP cells were significantly higher compared with those of A2780 cells. Additionally, the expression levels of HMGB1, p-GSK-3β and the mesenchymal phenotype marker, vimentin, in A2780/DDP cells were significantly elevated relative to the levels in A2780 cells. Conversely, the expression level of the epithelial phenotype marker, E-cadherin, was markedly decreased compared with that in A2780 cells. Following transfection of A2780/DDP cells with siRNA-HMGB1, there was a significant reduction in the rate of cell migration and invasion. Simultaneously, the expression levels of HMGB1, p-GSK-3β and vimentin were downregulated while the level of E-cadherin was upregulated. It was therefore concluded that the high expression of HMGB1 in A2780/DDP cells enhanced the cell migration and invasion abilities by facilitating epithelial to mesenchymal transition via GSK-3β.

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Introduction

Ovarian cancer is one of the most common gynecological malignancies and exhibits the highest mortality rate worldwide among all malignant tumors affecting the reproductive organs (1). Epithelial ovarian cancer (EOC) constitutes ~95% of ovarian malignancies worldwide (2) and typically lacks specific clinical symptoms during its early stages, leading to the majority of patients being diagnosed at an advanced disease stage. Currently, the first-line treatment for ovarian cancer involves surgical intervention followed by platinum-based chemotherapy (3). However, ~70% of patients with advanced stages of EOC worldwide will experience relapse within 2 years (4,5). Furthermore, the majority of patients with recurrent cancer exhibit resistance to platinum-based chemotherapy, resulting in a low 5-year survival rate (6). Therefore, comprehending the resistance mechanisms of platinum-based chemotherapy is advantageous in enhancing the prognosis of patients with EOC.

Cisplatin, the first platinum chemotherapy analogue approved for use in the treatment of ovarian cancer, induces DNA damage and subsequent cell death by crosslinking with DNA chains to inhibit the DNA replication process (7). Cisplatin and paclitaxel combination chemotherapy significantly enhances the outcome of patients with EOC (8,9). However, the majority of patients experience relapse due to the aforementioned development of platinum resistance (10). Growing evidence has suggested that the process of epithelial to mesenchymal transition (EMT) can contribute to the development of chemotherapy resistance (11,12). For instance, the presence of EMT has been associated with cisplatin resistance in EOC (13,14). Additionally, evidence suggests that the interplay between STAT3 and p53/RAS signaling pathways regulates both metastasis and cisplatin resistance in ovarian cancer cells through EMT (15).

High mobility group box 1 (HMGB1) is a highly conserved nuclear protein that binds to DNA and influences chromatin regulation and transcription (16), and is upregulated in various types of solid tumors, including breast cancer, sarcomas, pancreatic cancer, head and neck/oral squamous cell carcinomas, melanoma, hepatoblastoma and ovarian and non-small

cell lung cancer, in humans (17). HMGB1 plays a crucial role in the chemoresistance of tumor cells, including those from osteosarcoma and lung cancer (18,19). A previous study has shown that expression of HMGB1 is associated with chemotherapy sensitivity in patients with ovarian cancer (20). The upregulation of HMGB1 in carboplatin-resistant SKOV3 cells suggests its potential involvement in the development of resistance to carboplatin in this cell line (21). Furthermore, tumor cells can secrete HMGB1, which plays a crucial role in tumor growth, migration and invasion (22-24). As previously reported, elevated expression of HMGB1 promotes EMT in liver cancer and glioblastoma cells through the GSK-3β/Snail and STAT3/microRNA-34a/NF-κB signaling pathways (25,26). Furthermore, HMGB1 has been found to be upregulated in ovarian cancer cells and is associated with the growth and metastasis of ovarian cancer (27). However, the precise mechanism by which HMGB1 induces metastasis in ovarian cancer cells remains unclear.

In the present study, the expression and function of HMGB1 in cisplatin-resistant A2780/DDP cells, its regulatory role in the migratory and invasive abilities of these cells and its associated mechanisms were investigated.

Materials and methods

Cell culture. The human ovarian cancer cell line, A2780, and the cisplatin-resistant cell line, A2780/DDP, were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. A2780 cells were cultured in RIPA 1640 medium containing 10% fetal bovine serum, both purchased from Gibco (Thermo Fisher Scientific, Inc.), and 1% penicillin and streptomycin. A2780/DDP cells were cultured in RIPA 1640 medium containing 1 μg/ml cisplatin, 10% fetal bovine serum and 1% penicillin and streptomycin. Cells were incubated at 37˚C in an atmosphere of 5% CO_2 and 95% air.

Cell Counting Kit-8 (CCK-8) assay. Cell viability and the half maximal inhibitory concentration (IC_{50}) of cisplatin were assessed using the CCK-8 assay. Briefly, A2780/DDP and A2780 cells ($2x10^4$ cells/well) were seeded into 96-well plates and incubated overnight. Then, concentration gradients of cisplatin-containing medium (0, 5, 10, 20, 40 and 80 μg/ml) were added to the plates with eight replicate wells/concentration. After 48 h, 10 μl CCK-8 solution (MedChemExpress), was added to each well and incubated for 1 h. Then, the absorbance was measured at 450 nm and the cell viability was calculated as follows: Cell viability (%)=(drug group A-blank group A)/(no cisplatin group A-blank group A) x100. The resistance index was calculated as follows: Resistance index=IC_{50} of A2780/DDP cells/IC_{50} of A2780 cells.

Western blotting analysis. Cells were lysed to extract total protein for western blotting as previously described (28). The RIPA Lysis buffer (MedChemExpress) was utilized for the extraction of total protein. The protein concentration was determined using Bicinchoninic acid. The protein samples (30 mg/lane) were evenly loaded onto 8-12% sodium dodecyl sulfate polyacrylamide gels for electrophoresis. Then, proteins were subsequently transferred onto a polyvinylidene difluoride membrane. After blocking with 5% skimmed milk powder (Wuhan Servicebio Technology Co., Ltd.) at room temperature for 1 h. The membranes were incubated with primary antibodies overnight at 4˚C and with secondary antibodies for 1 h at room temperature. The primary and secondary antibodies were diluted in a universally purchased antibody dilution buffer from Beyotime Institute of Biotechnology. The concentration of the primary antibody was 1:1,000, while that of the secondary antibody was 1:5,000. Primary antibodies used for immunodetection were anti-HMGB1, anti-phosphorylated (p)-GSK-3β, anti-GSK-3β, anti-E-cadherin, anti-vimentin and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and all primary antibodies were acquired from Cell Signaling Technology, Inc. Secondary antibodies were anti-rabbit and anti-mouse IgG peroxidase conjugate (OriGene Technologies, Inc.). The antigen-antibody immunocomplexes were detected using enhanced chemiluminescent substrate purchased from Thermo Fisher Scientific, Inc. The results were semi-quantified by densitometry, using Image J software (V1.8, NIH). Results were expressed as a ratio of the protein of interest and GAPDH. GAPDH was the loading control for each sample. The relative expression of p-GSK-3β was normalized to the level of (unphosphorylated) GSK-3β.

Transwell migration and invasion assays. Upon reaching 80-90% confluency, cells were digested and Transwell migration and invasion assays were performed as described in a previous study (28). Images (magnification, x200) of stained cells were captured by the Olympus IX51 inverted microscope (Olympus Corporation). Cells were counted manually in four random fields of each chamber.

Knockdown of the HMGB1 gene in A2780/DDP cells. The small interfering RNA sequences targeting human HMGB1 (siRNA-HMGB1; Table I) were designed by Shanghai GenePharma Co., Ltd. A2780/DDP cells were seeded in six-well plates without antibiotics for 12 h. When a 50-60% confluency was reached, cells were transfected with nothing (Con group), 20 nM of negative control (N.C.; scrambled sequence) or 20 nM of siRNA-HMGB1 (50 nmol/l) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 6 h. After 48 h of transfection, cells were digested for subsequent experiments.

Statistical analysis. GraphPad Prism version 5.01 (Dotmatics) was used for statistical analysis. All experiments were repeated three times and data are presented as the mean ± SEM. Paired Student's t-test was used to analyze the difference between two groups. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was conducted to analyze the difference among groups. Two-way ANOVA followed by Tukey's multiple comparisons test was used to analyze the difference in cell viability between the A2780 and A2780/DDP cell lines. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Determination of drug resistance in the A2780 and A2780/DDP ovarian cancer cell lines. Following treatment with a concentration gradient of cisplatin for 48 h, the CCK-8

Table I. siRNA-HMGB1 sequences.

Name	Sense (5'-3')	Antisense (5'-3')
HMGB1-siRNA-197	CCUAAGAAGCCGAGAGGCATT	UGCCUCUCGGCUUCUUAGGTT
HMGB1-siRNA-252	GGGAGGAGCAUAAGAAGAATT	UUCUUCUUAUGCUCCUCCCTT
HMGB1-siRNA-624	CUGCGAAGCUGAAGGAAAATT	UUUUCCUUCAGCUUCGCAGTT
N.C.	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

HMGB1, high mobility group box 1; N.C., negative control; siRNA, small interfering RNA.

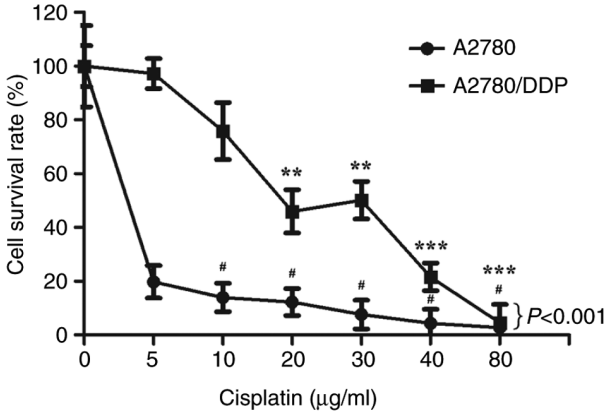

Figure 1. Cell viability and the IC_{50} of cisplatin in A2780/DDP and A2780 cells. Cisplatin repressed the cell viability of A2780/DDP and A2780 cells. #P<0.05 cell viability at different cisplatin doses in A2780 cell line compared with A2780 cell line at previous cisplatin concentration; **P<0.05, ***P<0.001 cell viability at different cisplatin doses in A2780/DDP cell line compared with A2780/DDP cell line at previous cisplatin concentration. Two-way ANOVA followed by Tukey's multiple comparisons test was used to analyze the difference in cell viability between the A2780 and A2780/DDP cell lines.

assay revealed that the cell viability of A2780/DDP cells was significantly higher than that of A2780 cells at lower cisplatin concentrations (Fig. 1). The IC_{50} values for A2780/DDP and A2780 were 17.66 and 2.24 μg/ml, respectively, meaning that the drug resistance index of A2780/DDP was 7.898.

Migratory and invasive abilities of A2780 cells and cisplatin-resistant A2780/DDP cells. The results of the Transwell migration assay demonstrated that the migratory ability of the cisplatin-resistant A2780/DDP cells was significantly higher than that of non-resistant A2780 cells (Fig. 2; P<0.001). Moreover, the invasive ability of A2780/DDP cells was significantly higher than that of A2780 cells, as shown in representative images from the Transwell invasion assay (Fig. 2; P<0.001).

Expression levels of HMGB1, p-GSK-3β and EMT-associated proteins in A2780 and A2780/DDP cells. As shown in Fig. 3, the expression levels of HMGB1 were significantly higher in the cisplatin-resistant A2780/DDP cells than in the A2780 cells (P=0.0003). Similarly, the expression levels of p-GSK-3β (P=0.0206) and the mesenchymal phenotype marker, vimentin, (P=0.001) were higher in A2780/DDP cells than in A2780 cells, while the levels of the epithelial phenotype marker, E-cadherin, were lower in A2780/DDP cells (P=0.005).

HMGB1 enhances the migratory and invasive abilities of A2780/DDP cells by facilitating EMT. To investigate whether HMGB1 increased the migratory and invasive abilities of A2780/DDP cells, the expression of HMGB1 in A2780/DDP cells was knocked down. The expression of HMGB1 was significantly decreased following the transfection of three siRNA-HMGB1 sequences in A2780/DDP cells (Fig. 4), particularly following the transfection of siRNA-HMGB1-197 (P=0.0082). Thus, siRNA-HMGB1-197 was used in all subsequent experiments. As shown in Fig. 5, the expression levels of HMGB1 (P=0.0028), p-GSK-3β (P=0.0242) and vimentin (P=0.0345) were significantly decreased in A2780/DDP cells following transfection with siRNA-HMGB1-197, whereas the expression of E-cadherin was significantly increased (P=0.0101). Moreover, Transwell migration assay demonstrated that the migratory ability of A2780/DDP cells was significantly decreased following transfection with siRNA-HMGB1-197 (Fig. 6A and B; P<0.0001). Similarly, the invasive ability of A2780/DDP cells was significantly decreased following transfection with siRNA-HMGB1-197 (Fig. 6A and C; P<0.0001). Therefore, the high expression levels of HMGB1 in cisplatin-resistant A2780/DDP cells may have enhanced cell migration and invasion by facilitating EMT via GSK-3β (Fig. 7).

Discussion

Platinum resistance can lead to the recurrence and metastasis of ovarian cancer (1). Understanding the underlying mechanisms behind recurrence is crucial in enhancing the prognosis of patients with ovarian cancer. The present study confirmed that the migratory and invasive abilities of cisplatin-resistant A2780/DDP cells were significantly higher than those of A2780 cells. In addition, the expression levels of HMGB1, p-GSK-3β and the mesenchymal phenotype marker, vimentin, were found to be significantly elevated in A2780/DDP cells, whereas the expression level of the epithelial phenotype marker, E-cadherin, was significantly reduced. After interference of HMGB1 expression in A2780/DDP cells, the migratory and invasive abilities of A2780/DDP cells were significantly reduced. Additionally, the expression levels of HMGB1, p-GSK-3β and vimentin were significantly downregulated, while the expression level of E-cadherin was significantly upregulated. Therefore, it was hypothesized that

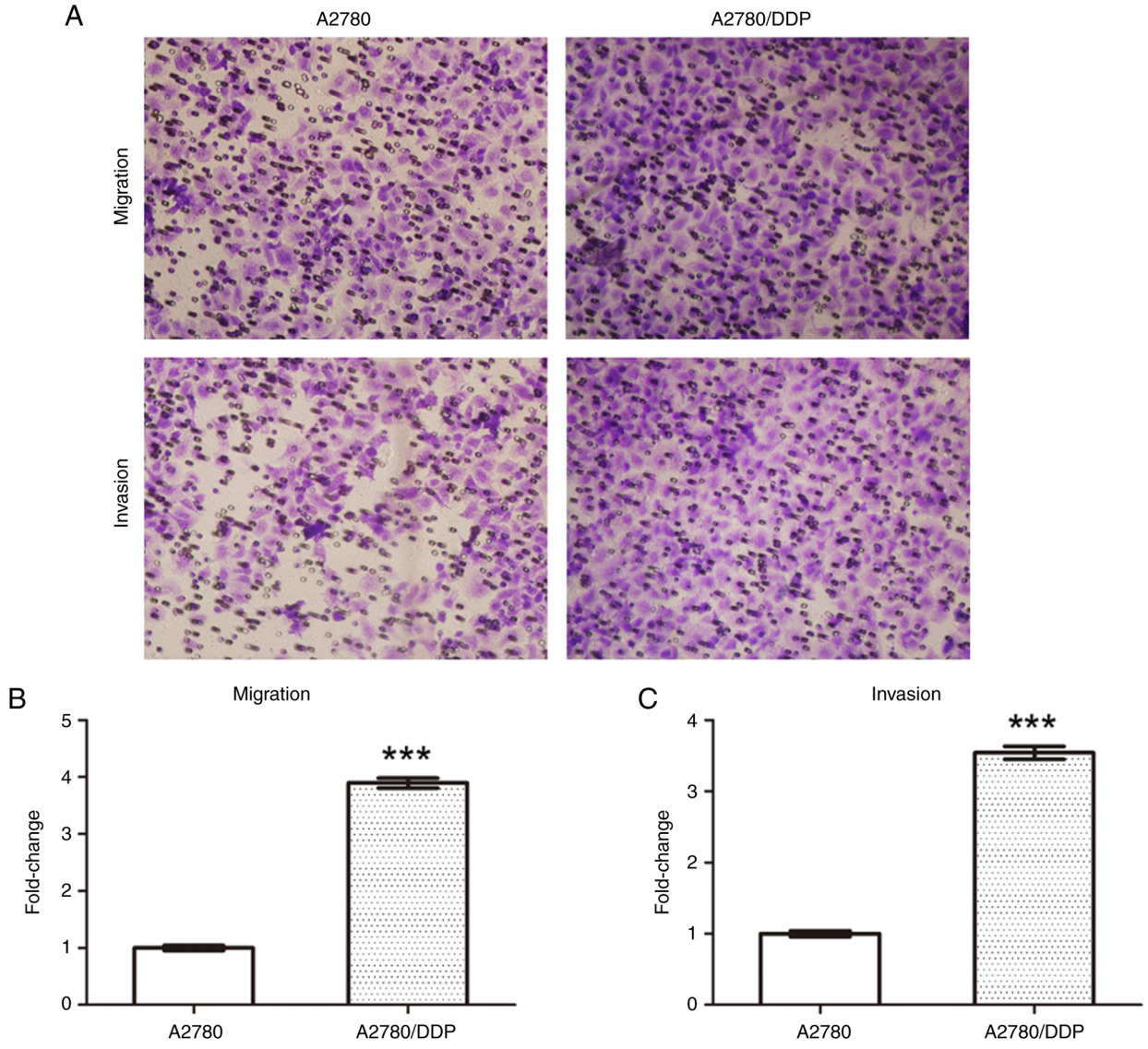

Figure 2. Migratory and invasive abilities of A2780 and A2780/DDP cells. (A) Representative Transwell migration and invasion assay images of A2780 cells and A2780/DDP cells (magnification, x200). (B) Quantification of A2780 and A2780/DDP cell migration. (C) Quantification of A2780 and A2780/DDP cell invasion. ***P<0.001. Paired Student's t-test was used to analyze the difference between the two groups.

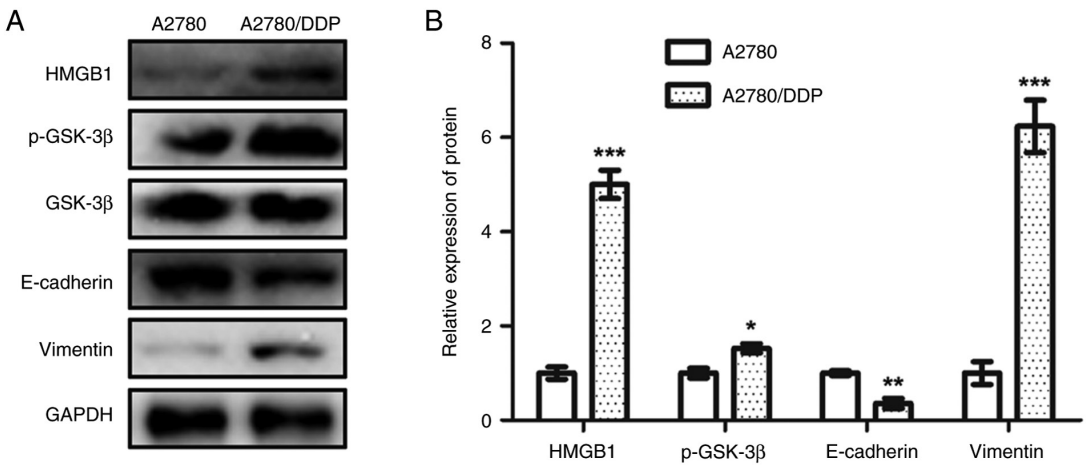

Figure 3. Expression levels of HMGB1, p-GSK-3β and EMT-associated proteins in A2780 and A2780/DDP cells. (A) Representative western blots showing the expression levels of HMGB1, p-GSK-3β, vimentin and E-cadherin in A2780 and A2780/DDP cells (the relative expression of p-GSK-3β was normalized to the level of GSK-3β). (B) Semi-quantitative analysis of the HMGB1, p-GSK-3β, vimentin and E-cadherin expression levels in A2780 and A2780/DDP cells. *P<0.05, **P<0.005, ***P<0.001. HMGB1, high mobility group box 1; EMT, epithelial to mesenchymal transition; p-, phosphorylated; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Paired Student's t-test was used to analyze the difference between the two groups.

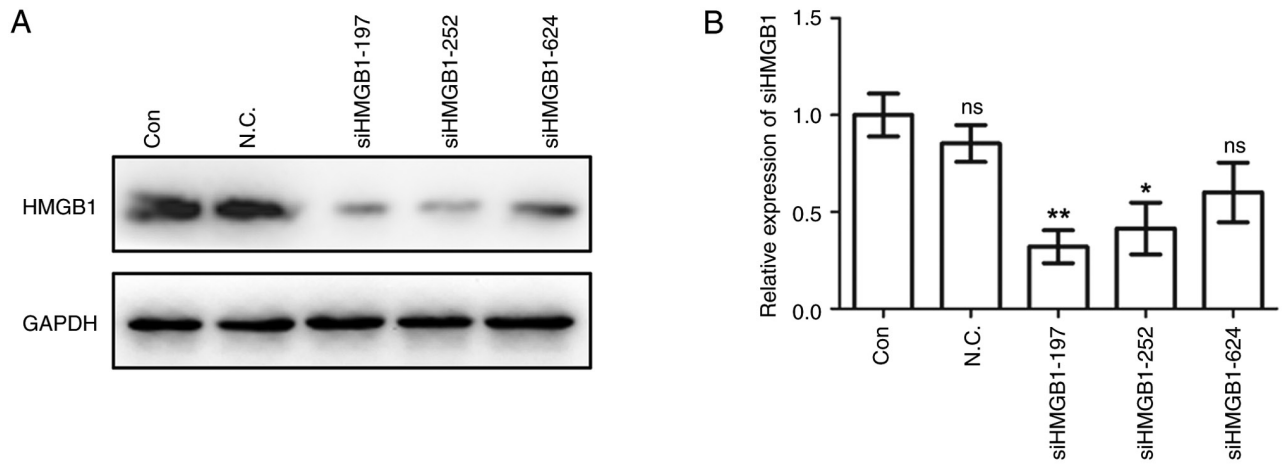

Figure 4. Knockdown effect of siRNA-HMGB1 on HMGB1 expression levels in A2780/DDP cells. (A) Representative western blots showing the expression level of HMGB1 in A2780/DDP cells following transfection with different types of siRNA-HMGB1. (B) Semi-quantitative analysis of HMGB1 in A2780/DDP cells following transfection with different types of siRNA-HMGB1. *P<0.05, **P<0.005. N.C., negative control; ns, not significant; HMGB1, high mobility group box 1; Con, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; si(RNA), small interfering RNA. One-way analysis of variance followed by Dunnett's multiple comparisons test were conducted to analyze the difference among groups.

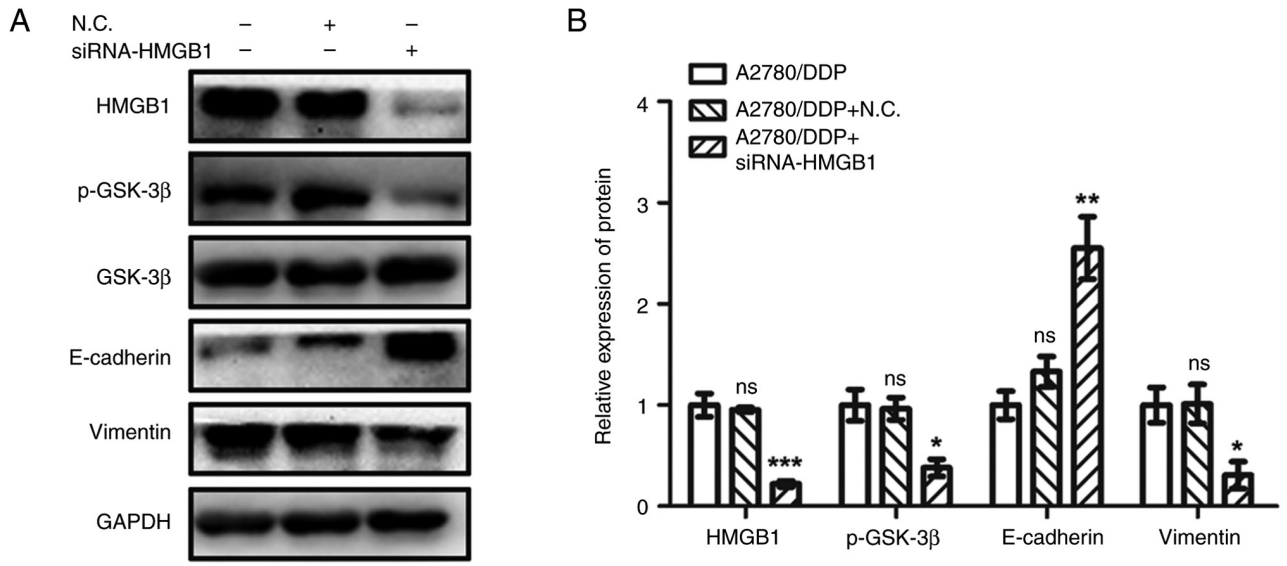

Figure 5. Expression levels of HMGB1, p-GSK-3β and EMT-associated proteins in A2780/DDP cells following transfection with siRNA-HMGB1. (A) Representative western blots showing the expression level of HMGB1, p-GSK-3β, vimentin and E-cadherin in A2780/DDP cells following transfection with siRNA-HMGB1 (the relative expression of p-GSK-3β was normalized to the level of GSK-3β). (B) Semi-quantitative analysis of HMGB1, p-GSK-3β, Vimentin and E-cadherin expression levels in A2780/DDP cells following transfection with siRNA-HMGB1 *P<0.05, **P<0.005, ***P<0.001. N.C., negative control; ns, not significant; HMGB1, high mobility group box 1; p-, phosphorylated; EMT, epithelial to mesenchymal transition; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; si(RNA), small interfering RNA. One-way analysis of variance followed by Dunnett's multiple comparisons test were conducted to analyze the difference among groups.

the high expression of HMGB1 in A2780/DDP cells promotes cell migration and invasion by facilitating EMT via GSK-3β.

EMT refers to the process in which epithelial phenotype-associated genes (for example E-cadherin and cytokeratin) are downregulated, while mesenchymal phenotype-associated genes (for example N-cadherin and vimentin) are upregulated under certain conditions, including wound healing, fibrosis and cancer metastasis, resulting in structural transformation of the cell into the mesenchymal form (29). This process can significantly upregulate the migratory and invasive abilities of cells (29). In addition, EMT also increases the proliferation and metastatic abilities and resistance to chemotherapy of cancer cells (30). A previous study by Li *et al* (31) reported that the EMT process contributed to cell proliferation, migration and invasion of nasopharyngeal carcinoma cells. Cui *et al* (32) similarly concluded that EMT promoted pancreatic cancer cell motility and metastasis. Furthermore, Liang *et al* (33) demonstrated that upregulation of ZEB1, an EMT transcription factor, promoted EMT, invasion and metastasis in serous ovarian cancer. Kim *et al* (34) demonstrated that long non-coding RNA steroid receptor RNA activator mediated cell migration, invasion and the progression of ovarian cancer via EMT. In the present study, Transwell migration and invasion assays indicated that the migratory and invasive abilities

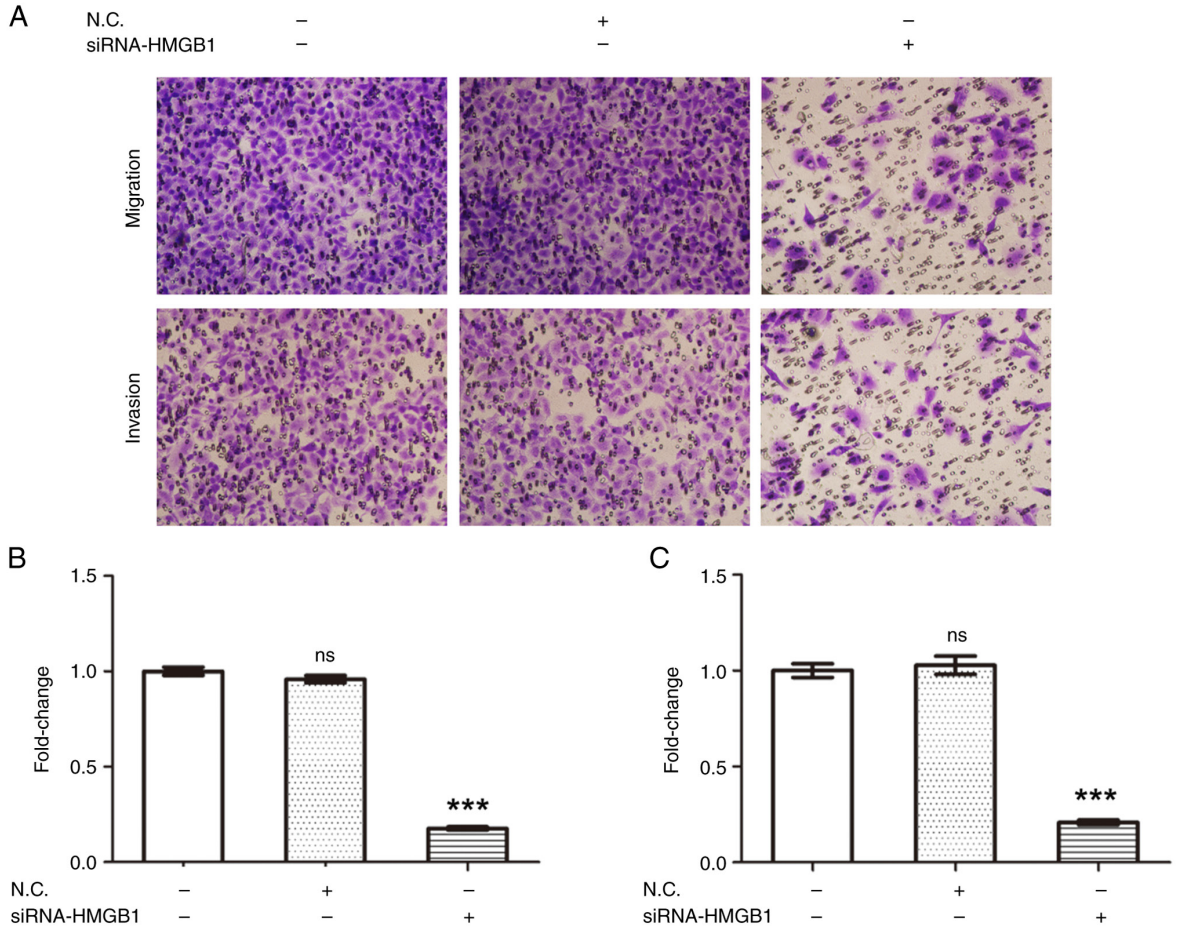

Figure 6. Migratory and invasive abilities of A2780/DDP cells following transfection with siRNA-HMGB1 (A) Representative Transwell migration and invasion assay images of A2780/DDP cells following transfection with siRNA-HMGB1 (magnification, x200). (B) Quantification of A2780/DDP cell migration following transfection with siRNA-HMGB1. (C) Quantification of A2780/DDP cell invasion following transfection with siRNA-HMGB1. ***P<0.001. ns, not significant; N.C., negative control; HMGB1, high mobility group box 1; si(RNA), small interfering RNA. One-way analysis of variance followed by Dunnett's multiple comparisons test were conducted to analyze the difference among groups.

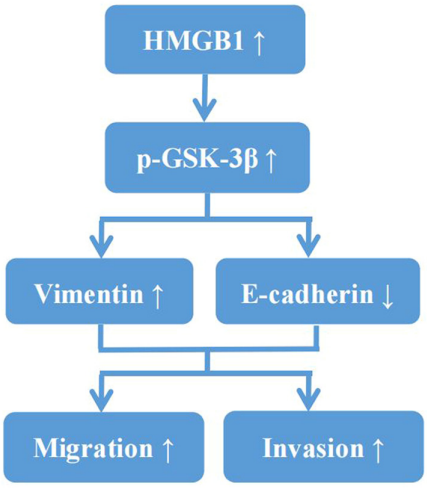

Figure 7. Signaling pathway schematic. High expression of HMGB1 in cisplatin-resistant A2780/DDP cells may enhance cell migration and invasion by facilitating the EMT via GSK-3β. HMGB1, high mobility group box 1; EMT, epithelial to mesenchymal transition.

of cisplatin-resistant A2780/DDP cells were significantly elevated compared with A2780 cells. Additionally, expression of the mesenchymal phenotype marker, vimentin, was significantly upregulated in A2780/DDP cells, while expression of the epithelial phenotype marker, E-cadherin, was significantly downregulated. These results supported the hypothesis that EMT plays a crucial role in the migration and invasion of cisplatin-resistant A2780/DDP cells.

HMGB1 plays a prominent role in tumor development and metastasis through its ability to promote cell migration and angiogenesis (35,36). Overexpression of HMGB1 in gastric cancer cells promoted EMT activation, cell invasion and tumor growth (37). Moreover, a study by Liu *et al* (38) indicated that downregulation of HMGB1 inhibited EMT, invasion and migration of lung cancer cells. As demonstrated by Li *et al* (39), serum HMGB1 levels were significantly upregulated in patients with ovarian cancer. Paek *et al* (40) demonstrated that positive staining of HMGB1 in ovarian cancer tissue was detected in 80% of patients with EOC, and high HMGB1 expression was an independent predictor for progression-free survival of patients. Moreover, Jiang *et al* (41) found that downregulation of HMGB1 expression inhibited ovarian cancer migration, invasion and angiogenesis. In addition, another study demonstrated that HMGB1 promoted Snail-mediated EMT in glioblastoma cells via the degradation of GSK-3β (25). However, the present

study did not investigate whether forced overexpression of HMGB1 in A2780 cells could enhance their migratory and invasive capabilities. This limitation highlights the need for further research in this area.

In conclusion, the results of the present study demonstrated that high expression of HMGB1 in cisplatin-resistant A2780/DDP cells may enhance cell migration and invasion by facilitating EMT via GSK-3β. Consequently, targeting HMGB1 represents a promising therapeutic strategy for ovarian cancer treatment in the future. As such, the present study offers novel insights into the clinical management of platinum-resistant ovarian cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors contributed to the study conception and design. QZ also contributed to protocol development and manuscript writing; JW also contributed to project development and data collection; YZ also contributed to data collection and analysis; SC also contributed to data analysis; LC also contributed to data analysis and manuscript editing. QZ, JW and LC confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethical approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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