Am J Transl Res 2020;12(4):1309-1318
www.ajtr.org /ISSN:1943-8141/AJTR0101957

Original Article

Upregulation of cell division cycle 20 in cisplatin resistance-induced epithelial-mesenchymal transition in osteosarcoma cells

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Received September 5, 2019; Accepted March 23, 2020; Epub April 15, 2020; Published April 30, 2020

Abstract: Cell division cycle 20 homologue (Cdc20) is characterized as an oncoprotein that is involved in carcinogenesis. Accumulated evidence reveals that Cdc20 plays an oncogenic role by governing cell growth, apoptosis, motility, and metastasis. The role of Cdc20 in drug resistance is elusive. In the present study, we exploited whether Cdc20 is involved in the cisplatin (DDP) resistance-induced epithelial-mesenchymal transition (EMT) of osteosarcoma cells. We found that DDP resistant U2OS and MG63 cells underwent EMT. Moreover, DDP-resistant cells exhibit the mesenchymal features such as enhanced attachment and detachment and increased invasion activity and migration. Mechanistically, Cdc20 was highly expressed in DDP-resistant osteosarcoma cells compared to parental cells. Consistently, downregulation of CdcC20 in DDP-resistant cells reversed the EMT phenotypes and changed the expression of EMT biomarkers. Our studies provide evidence for targeting Cdc20 as a promising approach to enhancing drug sensitivity for the treatment of osteosarcoma.

Keywords: Osteosarcoma, Cdc20, EMT, drug resistance, DDP, invasion

Introduction

Osteosarcoma is one of the most common bone tumors in the world. In the United States, 3500 new cases and 1600 deaths from bone and joint tumors are expected to occur in 2019 [1]. Bone tumors are the third most frequent cause of cancer-related death in patients less than 20 years old. Osteosarcoma is also observed in teenagers and young adults [2, 3]. The five-year survival rate of osteosarcoma patients is approximately 65%-70% [1]. However, osteosarcoma patients with metastasis often have a worse prognosis. The treatment strategies for osteosarcoma include surgical resection, radiation and chemotherapy [4]. To obtain a better therapeutic benefit, it is important to explore the mechanism of osteosarcoma development and progression.

Chemotherapeutic agents for osteosarcoma include cisplatin (DDP), doxorubicin, ifosfamide and methotrexate [5]. The cisplatin, methotrexate, and doxorubicin regimen is currently the preferred choice for osteosarcoma chemotherapy [6, 7]. Additionally, DDP, doxorubicin, oxazaphosphorine and methotrexate are often used in osteosarcoma chemotherapeutic treatment [8-10]. It is clear that patients with osteosarcoma acquire resistance to chemotherapeutic drugs during treatment progression [11]. Drug resistance is associated with epithelial-mesenchymal transition (EMT) in human cancers [12-14]. EMT is a phenotype in which epithelial cells are transformed into mesenchymal cells under various stresses [15]. During the EMT process, the expression of epithelial markers (such as E-cadherin) is downregulated, whereas the expression of mesenchymal markers (including N-cadherin, Snail, Slug, ZEB1, ZEB2, Vimentin and Twist) is upregulated [16]. DDP resistance-induced EMT has been observed in nasopharyngeal carcinoma cells and occurs due to overexpression of miR-205-5p [17]. Similarly, DDP resistant cells have mesenchymal characteristics because of downregulation of miR-574-3p in gastric carcinoma cells [18]. The miR-495-UBE2C-ABCG2/ERCC1 axis is involved in DDP

sensitivity in DDP-resistant nonsmall cell lung cancer cells [19]. The role of DDP resistance-mediated EMT in osteosarcoma cells has not been fully elucidated.

Cell division cycle 20 homolog (Cdc20) exhibits an oncogenic function in carcinogenesis. Higher expression of Cdc20 exists in a variety of human malignancies such as cancers of the lung, bladder, breast, prostate, colon, pancreas, and liver [20]. Moreover, high expression of Cdc20 is correlated with poor prognosis and poor survival in a number of cancers such as pancreatic [21], breast [22], lung [23], colon [24], and liver cancers [25]. Our previous study demonstrated that knockdown of Cdc20 inhibited cell proliferation, induced apoptosis and cell cycle arrest, and blocked cell invasion in osteosarcoma cells [26]. In agreement with this, overexpression of Cdc20 enhanced the cell proliferation, inhibited the apoptosis and promoted the invasion of osteosarcoma cells [26]. However, it is unclear whether Cdc20 is associated with DDP resistance-mediated EMT in osteosarcoma cells. Therefore, this study aimed to explore whether Cdc20 is critically involved in DDP resistance-induced EMT in osteosarcoma cells. The findings of this study could provide the molecular insight into DDP resistance-triggered EMT in osteosarcoma cells and provide evidence that inhibition of Cdc20 might be helpful in overcoming DDP resistance in osteosarcoma.

Materials and methods

Cell culture and reagents

The osteosarcoma cell lines MG63 and U2OS cells were purchased and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. To obtain DDP-resistant osteosarcoma cells, U2OS and MG63 cells were cultured in DMEM with increased doses of DDP for more than 6 months. Calcein-AM and 3-4,5-dimethyl-2-thiazolyl-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-Cdc20, anti-ZO-1, anti-E-cadherin, anti-N-cadherin, anti-Snail, anti-Bim, anti-Securin, and anti-Vimentin antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). The anti-tubulin antibody was purchased from Sigma-Aldrich.

Cell viability assay

Parental and DDP resistant cells (5×10^3 cells/well) were cultured in 96-well plates overnight and exposed to various doses of DDP for 72 h. The DDP-resistant osteosarcoma cells were seeded into 96-well plates and infected with Cdc20 shRNA. After 72 h, the MTT assay was used to evaluate cell proliferation detected by a microplate reader as described previously [26].

Cell attachment and detachment

To detect the cell attachment activity, DDP-resistant cells (5×10^4/well) were maintained in a 24-well plate for 1 h. Then, both the unattached cells and the attached cells were counted. To measure cell detachment, DDP-resistant cells (5×10^4/well) were cultured in 24-well plates for 24 h. Then, the cells were digested with 0.05% trypsin for 3 min. Subsequently, detached and unattached cells were counted.

Cell invasion assay

Parental and DDP-resistant osteosarcoma cells were seeded in the upper chamber of Transwell inserts with Matrigel. FBS-free DMEM was added to the upper chamber, and complete medium was filled in the under chamber. After 20 h, the invasive cells on the bottom membrane surface were stained with Calcein AM and photographed by using a microscope [27].

Cell wound healing assays

Parental and DDP-resistant osteosarcoma cells were cultured in 6-well plates until cells grew to more than 90% confluence. A small pipette tip was used to generate a scratch wound. After the floating cell debris was removed, the cells were cultured for 20 h. Then, the scratched areas were photographed by a microscope.

Quantitative reverse transcription PCR (RT-qPCR)

Total RNA from parental and DDP-resistant osteosarcoma cells was extracted using TRIzol reagent, and RT-PCR was performed as described previously [26].

Western blotting analysis

The protein from parental and DDP-resistant osteosarcoma cells was harvested by protein

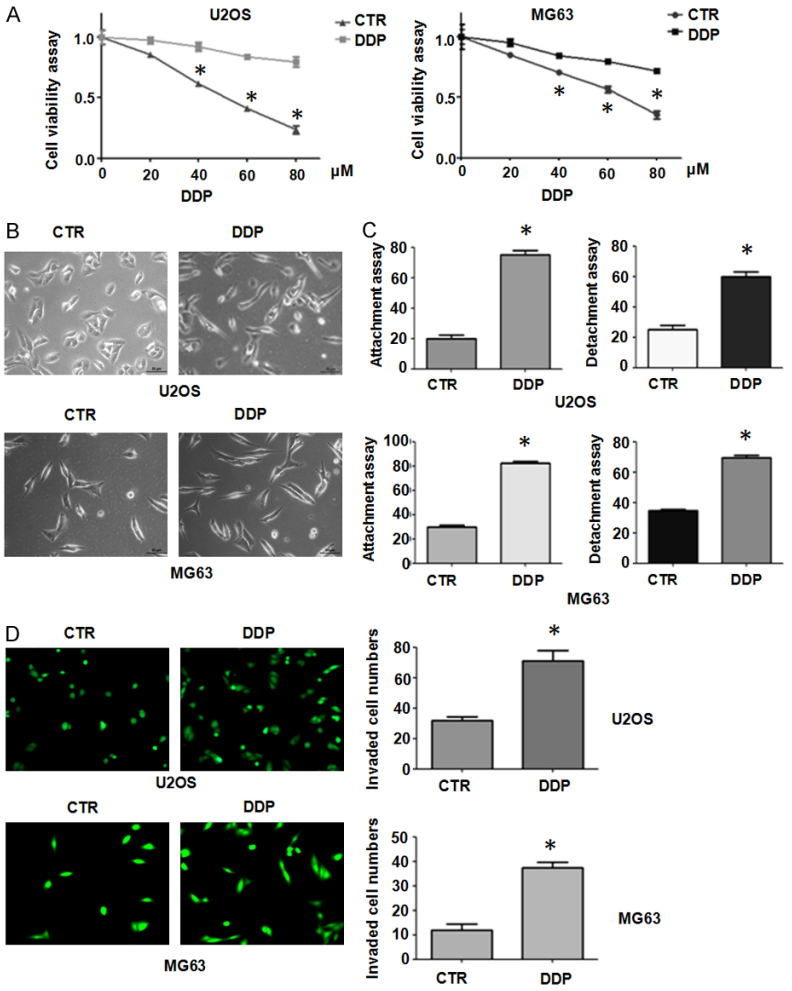

Figure 1. DDP-resistant cells acquire an EMT phenotype. A. MTT assay was used to measure the cell viability in DDP-resistant osteosarcoma cells after DDP treatment for 72 h. *P<0.05 vs control. B. Cell morohology of DDP-resistant U2OS and MG63 cells and their parental cells was photographed by using a microscope. C. Cell attachment and detachment assays were performed in DDP-resistant cells and their parental cells. *P<0.05 vs control. D. Left panel: Cell invasion assays were performed to determine the invasive ability of DDP-resistant cells and their parental cells. Right panel: Quantitative results are shown for the left panel. *P<0.05 vs control. CTR: control cells; DDP: cisplatin-resistant cells.

lysis buffer. A BCA protein assay kit was used to measure the concentrations of proteins. Protein samples were loaded and separated by SDS-PAGE and transferred onto PVDF membranes. Then protein expression was measured using an electrochemiluminescence assay as described previously [26].

Viral infection

DDP-resistant osteosarcoma cells were infected with Cdc20 short hairpin (shRNA, Cdc20-RNAi) or scrambled shRNA lentiviral particles for 48 h. Then, the cells were cultured with puromycin for approximately two weeks. The cells were passaged for experiments that were described in the Results section.

Statistical analysis

Statistical analysis was performed using the Student's t-test. $P<0.05$ was considered a significant difference.

Results

DDP-resistant osteosarcoma cells exhibit a mesenchymal phenotype

DDP-resistant cells were obtained through continuous treatment of parental osteosarcoma cells with increasing doses of DDP for more than 6 months. An MTT assay was used to evaluate the efficacy of DDP in the DDP-resistant osteosarcoma cells. The MTT data showed that 40 μM DDP significantly inhibited cell proliferation in both U2OS and MG63 parental cells (**Figure 1A**). However, this concentration of DDP did not inhibit the proliferation of DDP-resistant U2OS and MG63 cells (**Figure 1A**). Therefore, DDP-resistant U2OS and MG63 cells were maintained with 40 μM and 60 μM DDP, respectively. Cell morphology was changed in DDP-resistant cells compared with parental cells (**Figure 1B**). DDP-resistant cells exhibited elongated, spindle-like shapes, which are the properties of the mesenchymal phenotype, indicating that DDP resistance induced EMT in osteosarcoma cells.

DDP-resistant osteosarcoma cells exhibit EMT related functions

To determine whether DDP-resistant osteosarcoma cells change their biological functions, cell attachment and detachment assays were

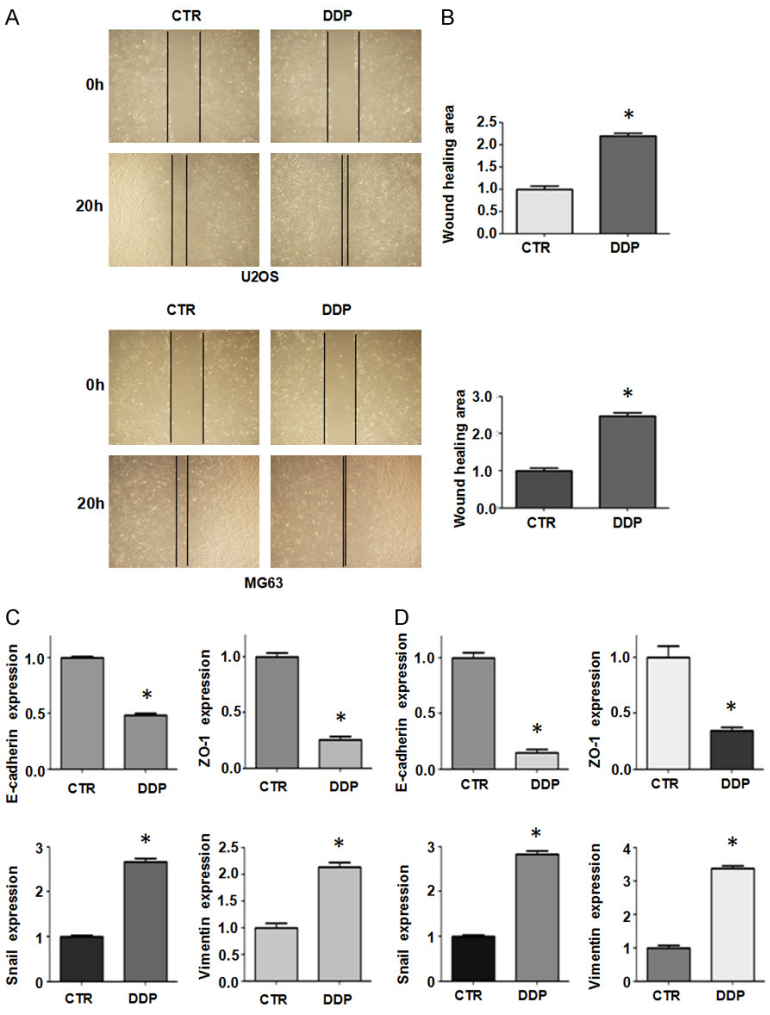

Figure 2. DDP-resistant cells acquire EMT characteristics. (A) Wound healing assays were used to evaluate cell migratory capacity in DDP-resistant cells and their parental cells. (B) Quantitative results are shown for (A). *P<0.05 vs control. (C, D) RT-qRCR was used to detect the mRNA expression levels of E-cadherin, ZO-1, Snail, and Vimentin in DDP-resistant U2OS (C) and MG63 (D) cells and their parental cells. *P<0.05 vs control.

examined in DDP-resistant cells. We found that the cell attachment ability was remarkably increased in DDP-resistant U2OS and MG63 cells compared to the parental cells (**Figure 1C**). In agreement with this, cell detachment activity was remarkably increased in DDP-resistant osteosarcoma cells (**Figure 1C**). Moreover, cell invasive capacity was evaluated using a Transwell invasion assay in DDP-resistant osteosarcoma cells. The data from the Transwell invasion assay demonstrated that DDP-resistant cells acquired an increase in invaded cell numbers compared with the control cells (**Figure 1D**). A wound healing assay was utilized to test whether DDP-resistant osteosarcoma cells have altered cell migration ability. Indeed, DDP-resistant cells obtained enhanced motility compared with that of their parental cells (**Figure 2A** and **2B**). Therefore, DDP-resistant cells acquired a phenotype and biological function related to EMT.

DDP-resistant osteosarcoma cells exhibits EMT marker changes

To further determine whether DDP-resistant cells have altered EMT marker expression, RT-qPCR was used to measure the mRNA expression levels of EMT markers including E-cadherin, ZO-1, Snail, and Vimentin, in DDP-resistant cells. RT-qPCR results demonstrated that the mRNA levels of epithelial markers, such as E-cadherin and ZO-1, were significantly decreased in DDP-resistant cells compared with their parental cells (**Figure 2C** and **2D**). In addition, the mRNA levels of mesenchymal markers including Snail and Vimentin were strikingly increased in DDP-resistant cells (**Figure 2C** and **2D**). Western blotting analysis was utilized to examine the protein expression levels of EMT markers in DDP-resistant cells. Western blotting revealed that the levels of E-cadherin and ZO-1 were downregulated in DDP-resistant cells (**Figure 3A-D**). In accordance with this, the protein expression levels of mesenchymal markers, including N-cadherin, Snail, and Vimentin, were remarkably upregulated in DDP-resistant cells compared with their parental cells (**Figure 3A-D**). These results suggested that DDP-resistant cells underwent EMT and acquired EMT molecular markers.

DDP-resistant osteosarcoma cells overexpress Cdc20

Cdc20 is involved in drug resistance in human malignancies. Therefore, the expression of

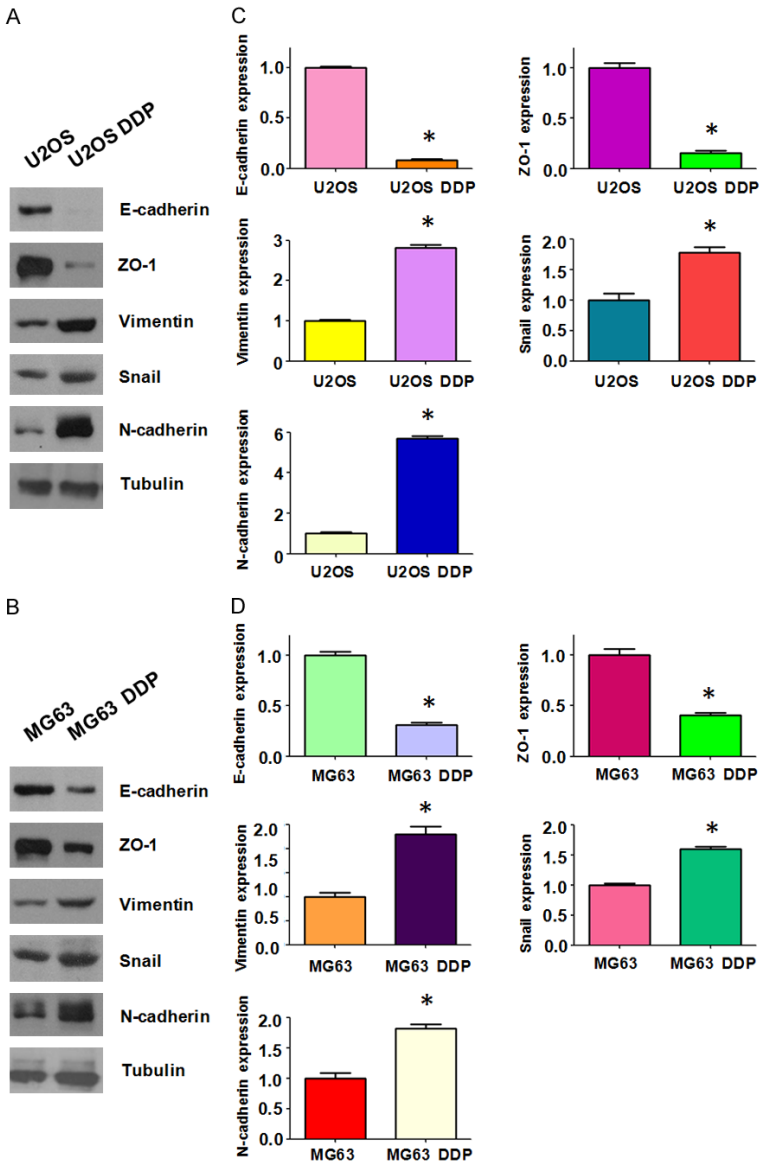

Figure 3. DDP-resistant cells modulate EMT marker expression. (A, B) Western blotting was utilized to evaluate the protein levels of EMT markers in DDP-resistant U2OS (A) and MG63 (B) cells. (C, D) Quantitative results are shown for (A, C) and (B, D). *P<0.05 vs control. U2OS DDP: DDP-resistant U2OS cells; MG63 DDP: DDP-resistant MG63 cells.

Cdc20 was determined in DDP-resistant cells using RT-qPCR and Western blotting analysis. The results of RT-qPCR demonstrated that Cdc20 mRNA levels were significantly increased in DDP-resistant U2OS and MG63 cells (**Figure 4A**). In line with this result, the Western blotting data showed that DDP-resistant U2OS and MG63 cells had higher expression of Cdc20 compared with their parental cells (**Figure 4B**). These findings indicated that Cdc20 could play a pivotal role in DDP-resistance-induced EMT in osteosarcoma cells.

Knockdown of Cdc20 reverses EMT in DDP-resistant cells

To investigate the critical role of Cdc20 in DDP-resistance-induced EMT, DDP-resistant cells were infected with Cdc20 shRNA lentiviral particles to knockdown the expression of Cdc20. The efficacy of Cdc20 shRNA infection in DDP-resistant U2OS and MG63 cells was detected by Western blotting. The results showed that Cdc20 shRNA infection led to significant inhibition of Cdc20 expression in DDP-resistant cells (**Figure 4C**). The Cdc20 shRNA1 was selected to knockdown Cdc20 expression in the following experiments. The morphological observations demonstrated that knockdown of Cdc20 in DDP-resistant U2OS and MG63 cells reversed EMT to mesenchymal-epithelial transition (MET) (**Figure 4D**). The elongated, spindle-like shapes of cells became rounded in DDP-resistant cells after Cdc20 knockdown (**Figure 4D**). This indicated that knockdown of Cdc20 may reverse EMT to MET in DDP-resistant cells.

Knockdown of Cdc20 inhibits the motility of DDP-resistant cells

The invasive ability was also determined in DDP-resistant cells after Cdc20 shRNA lentiviral particles infection using a Transwell chamber invasion assay. We found that knockdown of Cdc20 significantly inhibited the numbers of invaded DDP-resistant U2OS and MG63 cells (**Figure 4E**). Additionally, wound healing assay results demonstrated that knockdown of Cdc20 suppressed cell migratory ability in DDP-resistant U2OS and MG63 cells compared to that of the control cells (**Figure 5A** and **5B**). The attachment and detachment experiments revealed that knockdown of Cdc20 repressed the cell attachment and detachment activities

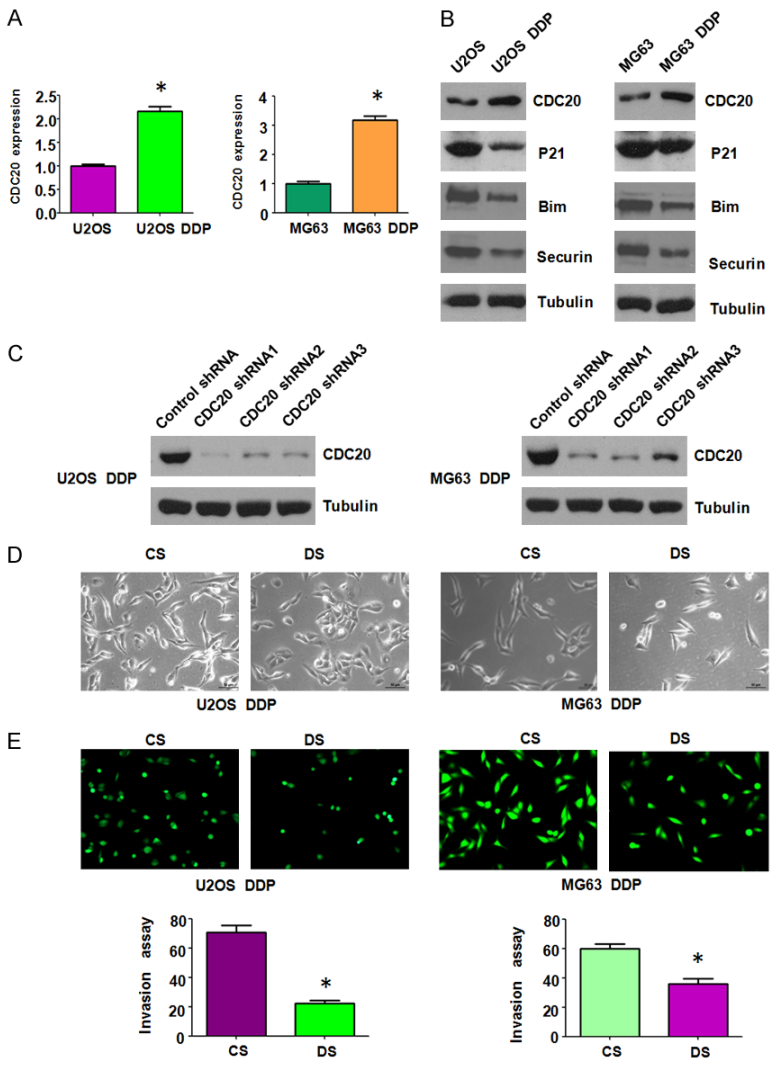

Figure 4. DDP-resistant cells have high expression of Cdc20. A. The mRNA levels of Cdc20 expression were evaluated by RT-qPCR in the DDP-resistant cells and their parental cells. *P<0.05 vs control. B. Western blotting analysis was used to evaluate the protein levels of Cdc20 and its target p21 in the DDP-resistant cells and their parental cells. C. Western blotting was used to test the efficacy of Cdc20 shRNA lentiviral particle infection in DDP-resistant cells. D. Cell morphology was photographed in DDP-resistant cells after Cdc20 shRNA lentiviral particle infection. E. Upper panel: Transwell chamber assay was used to detect the invasion ability in DDP-resistant cells after Cdc20 knockdown. Lower panel: Quantitative results are shown for the upper panel. *P<0.05 vs control. CS: control shRNA; DS: Cdc20 shRNA.

in DDP-resistant U2OS and MG63 cells (**Figure 5C**). Altogether, knockdown of Cdc20 impaired cell motility in DDP-resistant cells, suggesting that Cdc20 is involved in DDP-resistance-induced cell migration and invasion.

Knockdown of Cdc20 modulates the protein levels of EMT markers

Next, we measured the protein expression levels of EMT markers in DDP-resistant U2OS and MG63 cells following infection with Cdc20 shRNA lentiviral particles using Western blotting analysis. The data showed that knockdown of Cdc20 increased the expression of ZO-1 and E-cadherin in DDP-resistant cells (**Figure 6A-D**). In agreement with this, the protein expression levels of N-cadherin, Snail, and Vimentin were significantly downregulated in DDP-resistant cells compared to the control cells (**Figure 6A-D**). The data indicated that knockdown of Cdc20 modulated EMT marker expression in DDP-resistant cells, suggesting that Cdc20 may serve as a key molecule in the induction of EMT in DDP-resistant cells.

Knockdown of Cdc20 increases the sensitivityof osteosarcoma cells to DDP

The sensitivity of DDP-resistant cells to DDP was also measured using the MTT assay following Cdc20 shRNA lentiviral particle infection. We found that knockdown of Cdc20 significantly increased the sensitivity of resistant cells following DDP treatment (**Figure 6E**). This suggested that DDP resistance could be antagonized by knockdown of Cdc20. This also provided evidence that Cdc20 might be a new therapeutic target for combating DDP resistance in osteosarcoma.

Discussion

Emerging evidence has demonstrated that EMT is involved in DDP resistance in osteosarcoma cells [28]. One study showed that DDP treatment led to mesenchymal-like characteristics in osteosarcoma via upregulation of Snail [28]. Another study reported that DDP resistance-induced EMT is due to upregulation of visfatin and subsequent overexpression of Snail and Zeb1 in osteosarcoma [29]. Our current study

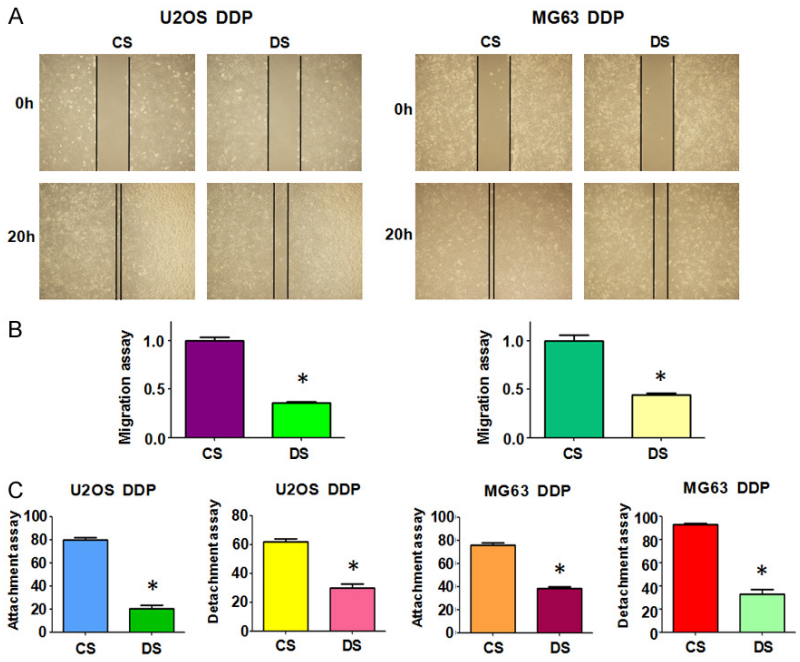

Figure 5. Knockdown of Cdc20 suppresses the motility of DDP-resistant cells. (A) Wound healing assay was utilized to measure cell migration in DDP-resistant cells following Cdc20 knockdown. (B) Quantitative results are shown for (A). *P<0.05 vs control. (C) Cell attachment and detachment abilities were evaluated in DDP-resistant cells following Cdc20 knockdown *P<0.05 vs control. CS: control shRNA; DS: Cdc20 shRNA.

also identified that DDP exposure triggered EMT in osteosarcoma cells. Several studies have dissected the mechanisms of DDP resistance in osteosarcoma cells. It has been reported that P-glycoprotein (P-gp) overexpression is involved in DDP resistance in osteosarcoma cells, indicating that inhibition of P-gp could be a strategy for overcoming DDP resistance [30]. In the present study, we identified that Cdc20 is critically involved in DDP resistance in osteosarcoma cells.

To overcome DDP resistance in osteosarcoma cells, many studies have elucidated various mechanisms and approaches to enhance DDP cytotoxicity. A study showed that knockdown of lncRNA taurine up-regulated gene 1 (TUG1) suppressed DDP resistance and enhanced DDP cytotoxicity via regulation of the MET/Akt signaling pathway in osteosarcoma cells [31]. Knockdown of LncRNA ANRIL caused cell sensitivity to DDP via inhibition of STAT3 in osteosarcoma cells [32]. LncRNA DNAJC3-AS1 promoted cell growth, migration and invasion and reduced DDP sensitivity through regulation of its sense-cognate gene DNAJC3 in osteosarcoma cells [33]. LncRNA OIP5-AS1 enhanced DDP resistance via activation of the LPAATb/PI3K/mTOR signaling pathway by sponging miR-340-5p in osteosarcoma [34]. LncRNA SPRY4-IT1 increased cell proliferation and motility via upregulation of Cdc20 in pancreatic cancer [35]. Therefore, it is important to explore whether lncRNAs are involved in DDP resistance-induced EMT via upregulation of Cdc20 in osteosarcoma.

Accumulating evidence has indicated that miRNAs play a vital role in DDP resistance in osteosarcoma. One study revealed that miR-221 overexpression led to DDP resistance through inhibition of PP2A subunit B PPP2R2A in osteosarcoma cells, suggesting that inhibition of miR-221 could be an approach for combating chemoresistance in osteosarcoma [36]. Similarly, miR-22 sensitized cells to DDP treatment via modulating autophagy-related genes in osteosarcoma cells [37]. Chen et al. uncovered that miR-504 regulated cell chemoresistance to DDP via modulating p53 in osteosarcoma cells [38]. In addition, downregulation of miR-19a-3p promoted cell sensitivity to DDP via elevation of PTEN expression in osteosarcoma cells [39]. Several miRNAs, including miR-494 and miR-451a, have been reported to regulate Cdc20 in cancer development and progression [40, 41]. Further investigation is needed to determine whether these miRNAs serve as upstream effectors to modulate the expression of Cdc20 in osteosarcoma.

Another group found that caffeine citrate could promote DDP antitumor effects in osteosarcoma, suggesting that the combination of DDP with caffeine citrate could be a promising way to improve the treatment outcome of osteosarcoma [42]. Oleandrin was reported to sensitize cells to DDP by preventing degradation of the copper transporter 1 in osteosarcoma cells [43]. Since Cdc20 is highly expressed in DDP-resistant osteosarcoma cells, suppression of

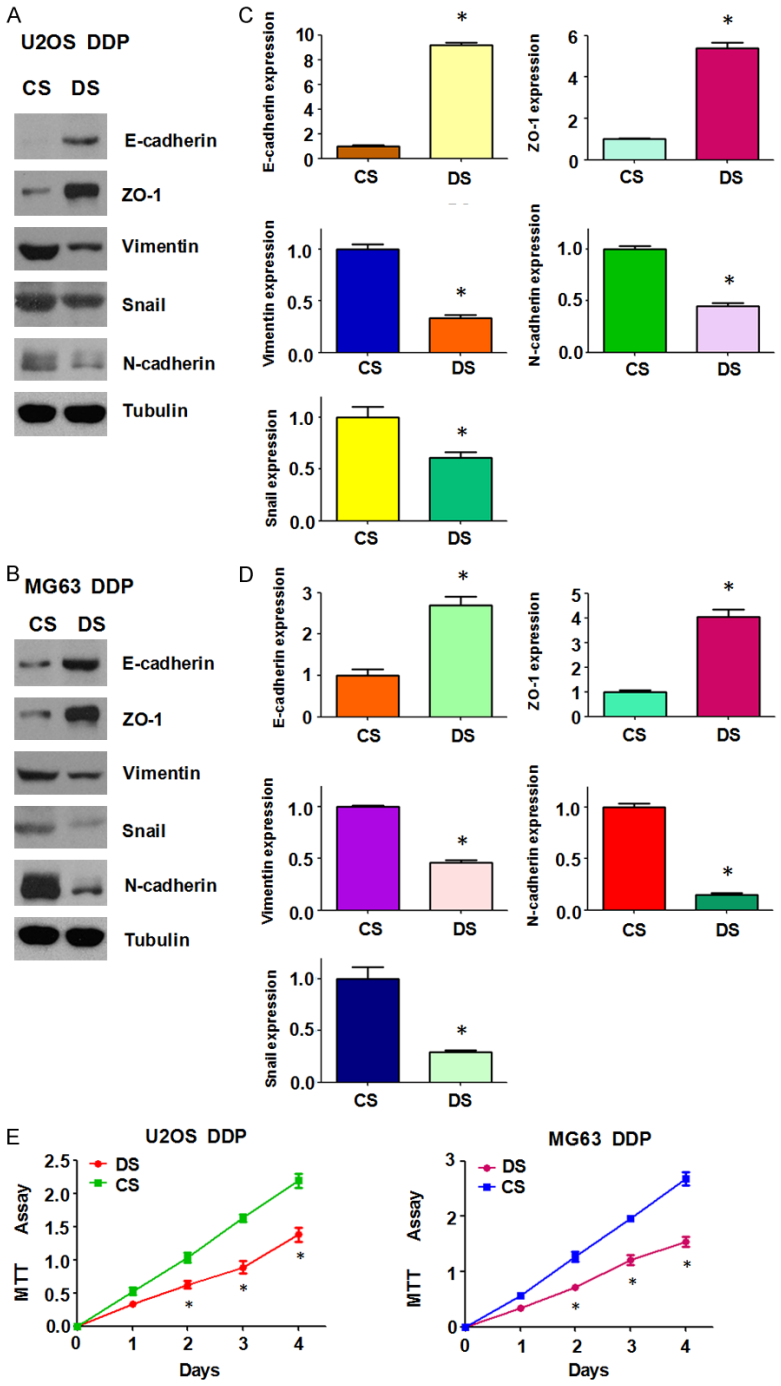

Figure 6. Knockdown of Cdc20 modulates the expression of EMT markers in DDP-resistant cells. (A, B) Western blotting analysis was used to evaluate the protein expression of EMT markers in DDP-resistant U2OS (A) and MG63 cells (B) following Cdc20 knockdown. (C, D) Quantitative results are shown for DDP-resistant U2OS (A) and MG63 cells (B). *P<0.05 vs control. CS: control shRNA; DS: Cdc20 shRNA. (E) MTT assay revealed that stable Skp2 knockdown restores DDP sensitivity in DDP-resistant cells. CS: control shRNA; DS: Cdc20 shRNA. *, P<0.05 compared with control shRNA.

Cdc20 expression by its inhibitors could be useful for overcoming DDP resistance in osteosarcoma. Apcin, a Cdc20 inhibitor, prevents its substrates from being recognized and degraded [44]. Our previous study demonstrated that apcin inhibited cell proliferation, induced apoptosis, and blocked motility in osteosarcoma cells [45]. Without a doubt, it is important to determine whether apcin could overcome DDP resistance in osteosarcoma in the near future. Altogether, this study provides evidence that Cdc20 may be a novel therapeutic target that could be used to reverse DDP resistance in osteosarcoma.

Acknowledgements

This work was supported by grant from Natural Science Foundation of Liaoning (No. 20180550547) and the program for high level talent for innovation research of Shenyang (No. RC180064).

Disclosure of conflict of interest

None.

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