Iran J Public Health, Vol. 52, No.7, Jul 2023, pp.1476-1486

Original Article

Two Enhances the Cisplatin Sensitivity of Cervical Cancer Cells via Suppression of c-MET Expression

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(Received 26 Feb 2022; accepted 19 May 2022)

Abstract

Background: The drug resistance of chemotherapeutic agents leads to unsatisfactory survival rates for cervical cancer (CC) patients. We aimed to explore the effect of FOXP2 on the sensitivity of CC cells to cisplatin (DDP) and its mechanism in Changde, China in 2018.

Methods: A Total of 6 cervical cancer tissue samples including 3 patients with cisplatin sensitivity and 3 patients with cisplatin resistance, who received DDP-based treatment, were obtained from Changde First People's Hospital, Changde City during 2021 , and FOXP2 level was detected by Western blot. The expression levels of FOXP2 and c-MET (hepatocyte growth factor receptor, c-MET) in cells were determined by q-PCR and Western blot analysis. The cell survival, apoptosis, and clone formation were analyzed by flow cytometry, MTT assay, or clone formation assay. Dual-luciferase reporter assays and Chromatin immunoprecipitation were applied to verify the regulation between FOXP2 and c-MET.

Results: FOXP2 was downregulated in cisplatin-resistant cervical cancer tissues and cells compared with control. FOXP2 overexpression in SiHa/DDP cells inhibited cell proliferation and promoted cell apoptosis, whereas down-regulation of FOXP2 in SiHa cells had the opposite result. FOXP2 enhanced chemosensitive to DDP in CC cells. FOXP2 is negatively correlated with c-MET expression level in SiHa and SiHa/DDP cells. Mechanistically, FOXP2 binds to the promoter region of c-MET to regulate its expression in CC cells negatively. Overexpression of c-MET can attenuate the enhancement of DDP-induced apoptosis caused by FOXP2 overexpression.

Conclusion: This is a novel study on the role of FOXP2 in promoting the DDP sensitivity of CC cells by inhibiting c-MET. The FOXP2/c-MET signaling axis uncovered in the present study may be a novel therapeutic target for the DDP therapy resistance of CC.

Keywords: Cervical cancer; Cisplatin (DDP); Chemoresistance

Introduction

Cervical cancer (CC) is still a worldwide pandemic cancer in women. Globally, approximately 600,000 new cases of cervical cancer and 340,000 deaths are recorded in 2020 (1). It represents ranks fourth in the incidence and mortality of female cancers. The burden of cervical cancer is expected to increase, and the HIV epidemic makes it even worse (1, 2). Platinum-based

chemotherapy is the standard first-line treatment for metastatic/recurrent cervical cancer. However, over time, numerous patients to its efficacy are ultimately restricted by the development of drug resistance (3). Therapeutic resistance remains a persistent challenge and we are not entirely investigating he molecular mechanism of cisplatin resistance in cervical cancer. Therefore, discovering the underlying mechanism of cisplatin resistance is of great significance and is one of the key strategies to improve the clinical efficacy of CC.

As a forkhead transcription factor, FOXP2 is one of the forkhead box family (4). It was implicated in language and speech development in humans and verified to be a tumor suppressor (5, 6). FOXP2 inhibits the transcriptional activity through its zinc finger domain, and involved in the occurrence and progression of numerous tumors (7). For instance, upregulating FOXP2 to accelerate the growth progression and metastasis of CC cells (8). Furthermore, FOXP2 is essential to inhibit the growth of osteosarcoma cells by regulating p21 (9). Moreover, FOXP2 protein level associated with Adriamycin-resistance in LoVo cells (10) decreased FOXP2 expression correlates with the chemotherapeutic agent resistance. FOXP2 plays a critical regulatory role in tumor occurrence, progression, and chemotherapy resistance. However, the specific mechanism of targeting FOXP2 in CC cell DDP-resistance remains unclear.

c-MET is the only known receptor for the hepatocyte growth factor (HGF), which closely participates in multiple cellular processes of cancer. Enhancing the expression and activation of c-MET promotes uncontrolled cellular proliferation, survival, angiogenesis, invasion, metastasis, and chemoresistance (11-13). Recently, activation and overexpression of the HGF/c-MET pathway can promote cervical cancer proliferation and invasion of cervical cancer (14, 15). More importantly, c-MET activates different downstream intracellular signaling pathways, such as Ras/MAPK/ERK and Ras/PI3K/AKT, pathways are intimate associations with resistance to various anti-tumor drugs (16). Furthermore, it was closely related to the DDP-resistance of cancer cells. Such as, delivered c-MET siRNA by exosome significantly reduces the expression of c-MET in gastric cancer cells and enhances the chemotherapy-sensitive to cisplatin (17).To sum up, c-MET may play an essential role in the resistance of CC cells to DDP chemotherapy and maybe a completely promising target for cancer therapy.

Currently, there is no report about FOXP2 and c-MET regulating the resistance of cervical cancer cells to DDP. FOXP2 directly binds to the 5'regulatory region of c-MET, and FOXP2 leads to c-MET had a transcriptional inhibition (18). Nevertheless, so far, no studies have reported the role of FOXP2/c-MET in the resistance of cervical cancer cells to DDP. Collectively, we hypothesized that FOXP2 might regulate the DDP resistance of CC cells through c-MET.

To understand the specific mechanism of FOXP2/c-MET in CC DDP-resistance. We investigate whether FOXP2 can modulate CC cells' cisplatin resistance via c-MET to provide a new potential therapeutic target for alleviating the DDP therapy resistance of CC cells.

Materials and Methods

Human tissue samples

Cervical cancer tissue samples are from patients undergoing surgical resection after chemotherapy at Changde First People's Hospital (Changde, China) in 2018. Patients with no apparent clinical efficiency or with disease stabilization and progression were classified as chemo-resistant, while patients with complete or partial remission were defined as chemo-sensitive. Three patients with chemo-resistant and three cases of chemo-sensitive cervical cancer were collected.

The Ethics Committee approved this study of Changde First People's Hospital (2021-100-01), and all the patients signed informed consent.

RNA isolation and qRT-PCR

Extracted total RNA using the TRIzol™ Plus RNA Purification Kit (Invitrogen, USA), First-

strand cDNAs were obtained using the RevertAid First Strand cDNA Synthesis Kit (Invitrogen, USA), followed by qPCR using the SYBR™ Green PCR Master Mix (ThermoFisher, USA) and using in the Applied Biosystems (7300 Real-Time PCR System). The $2^{-\Delta\Delta Ct}$ method was used for data analysis with the internal control, β-actin.

Cell Culture and transfection

Cervical cancer cell lines SiHa were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The DDP-resistant cells (SiHa/DDP) were established through exposure to increasing concentrations of DDP referred to the previously published articles (19). The cells in the logarithmic growth phase were seeded into a 6-well plate at 2×10^5/mL. According to the manufacturer's instructions, when the cells reached 80% confluence, Lipofectamine 3000 (Invitrogen, USA) was used for cell transfection. All overexpression plasmid was purchased from SinoBuological Inc (Beijing, China. FOXP2 plasmid containing HA tag, Cat: HG12029-NY, c-MET, Cat: HG10692-NY). FOXP2 siRNAs and scramble were purchased from RiboBio Co. (Guangzhou, China). All the cells were cultured at 37 °C in a 5% CO_2 incubator.

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay

SiHa and SiHa/DDP cells were plated into a 96-well plate, with 5×10^3 cells in each well. After incubating overnight, the cells treated with DDP at various concentrations
(0, 2, 4, 8, 16, and 32 μg/mL). After 48 h of culture, MTT reagent was added (100 μL/well) for incubation at 37 °C for 4 h. The viability and inhibitory concentration 50 (IC_{50}) values were measured at 450 nm with a microplate reader (Microplate reader 3550-UV, Bio-Rad). All experiments were performed 3 times.

Clone formation assay

SiHa and SiHa/DDP cells in a logarithmic phase were inoculated into 35mm cell culture dishes, cells density of 500 or 800 cells/well, with 3 replicates for each group, and cultured in a 37 °C 5% CO_2 incubator overnight. Followed carry out CIS dosing treatment for 48 h, then change to standard complete medium, and continue culturing for 1-2 weeks, refreshed the medium every 3 days. When macroscopically visible clones are in the petri dish, stop the culture. Finally, the colonies were stained with crystal violet staining solution, and the clone formation rate was calculated.

Flow cytometry

Annexin V-FITC/propidium iodide (PI) staining assay was used to detect apoptosis of CC cells. 1×10^5 cells were collected and washed twice with PBS, then resuspended in 500 μL Annexin V-FITC binding buffer. Subsequently, the cell suspension was added with 5 μL Annexin V-FITC and 5 μL PI staining solution. After incubating for 15 min at room temperature in the dark, cell apoptosis was detected with a flow cytometer (BD Pharmingen, USA).

Dual-luciferase reporter gene assay

To investigate whether c-MET is transcriptionally regulated by FOXP2, SiHa and SiHa/DDP cells were transfected with c-MET promoter (2000 bp fragment of upstream transcriptional start site) reporter constructs and further co-transfected with si-FOXP2 or FOXP2 overexpression plasmids, respectively. After 48 h transfection, harvested and lysed the cell using the luciferase reporter gene assay kit (Promega, USA), the Dual-Luciferase reporter assay system was used to assess promoter activity, with Renilla luciferase used for normalization.

ChIP-PCR assay

ChIP experiment was conducted by the Chromatin Immunoprecipitation (ChIP) Assay Kit (17-295, Millipore, USA) and carried out as previously described (20). Cells were fixed in 1% formaldehyde and quenched with glycine for 10 min. DNA was broken into 200 to 1000 bps through ultrasonication. The lysates were immunoprecipitated with anti-HA tag antibody (ab9110, Abcam,

USA) or rabbit IgG antibody (2729, CST, USA). PCR analyzed the ChIP product.

Western blotting

Extracted total protein and the concentration was measured using a BCA Protein Assay Kit (P0012, Beyotime, China). Electrophoresis resolved proteins by 10% SDS polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The unoccupied membrane sites were blocked in 5% bull serum albumin 1 h. Incubated with the primary antibodies (anti-FOXP2, 5337, CST; anti-Met, 8198, CST; anti-β-actin, 4970, CST) at 4 °C overnight. Later, washing thoroughly, hatched with horseradish peroxidase (HRP)-conjugated rabbit IgG secondary antibodies (7074, CST) at RT for 1 h, extensive rinsing, and then target proteins were visualized using Immobilon Western Chemiluminescent HRP Substrate Kit (Millipore, USA).

Statistical Analysis

Statistical analyses used Student t-tests or one-way ANOVA multiple comparisons test for this study data. Performed the statistical analysis by using the GraphPad Prism 8.0 software. $P<0.05$ was considered to be statistically significant.

Results

FOXP2 was downregulated in cisplatin-resistant SiHa cells and cervical cancer tissues.

Firstly, we analyzed the gene expression data from GEO DataSets (GSE168009) (|log FC|<1.0, $P<0.05$). Find FOXP2 mRNA was downer-expressed in the resistance group compared with the sensitivity group (Fig.1A).

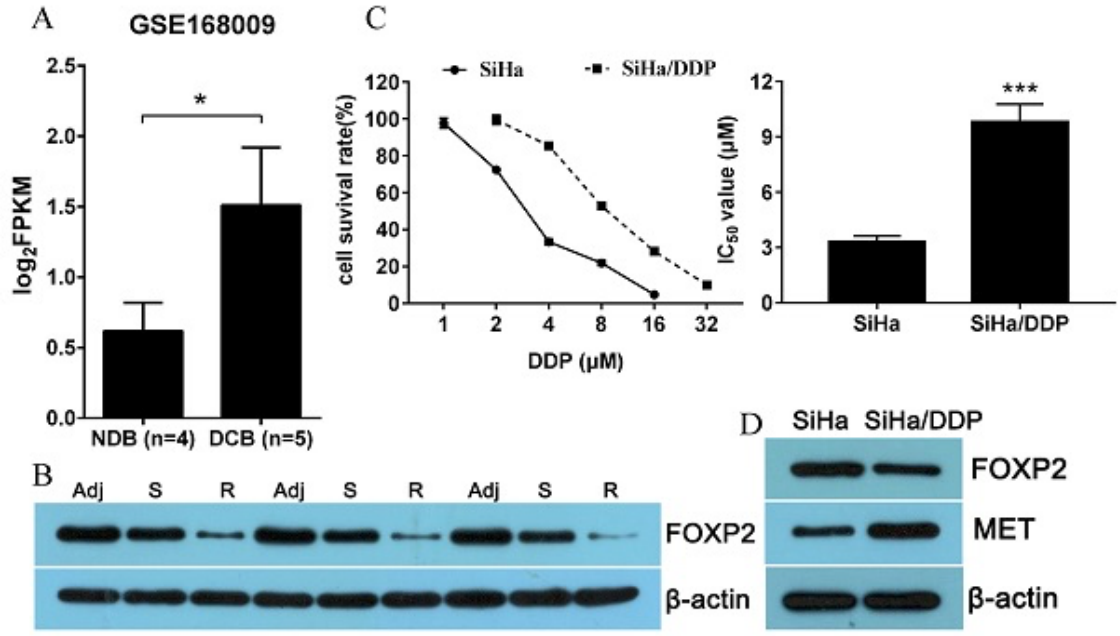

Fig. 1: FOXP2 was downregulated in cisplatin resistant SiHa cells and cervical cancer tissues
A: Relative mRNA level of FOXP2 was analyzed in DDP sensitivity and resistance cervical cancer tissues from the GEO DataSets (GSE168009). (NDB: effective sample, N = 4; DCB: non-effective sample, N = 5, * P < 0.05.); B: FOXP2 protein expression was detected in 3 cases of tissue samples using western blot. (Adj: adjacent tissues sample; S: DDP-sensitive cancer tissues sample; R: radioresistant radiosensitive sample); C: Viabilities (Left) and IC50 values (Right) of CC cells (SiHa) and the corresponding cisplatin-resistant cells (SiHA/DDP) treated with DDP at varying concentrations were assessed by MTT. D: Expression of FOXP2 and c-MET protein level in SiHa and SiHa/DDP cells assessed by western blot

In clinical tissues, FOXP2 was found downregulation in the cancer tissues, Most importantly, FOXP2 significantly decreased in DDP-resistant samples compared with DDP-sensitive cervical cancer patients (Fig. 1B). Furthermore, cisplatin-resistant SiHa cells (SiHa/DDP) were established through exposure to increasing concentrations of DDP. MTT assay showed that SiHa/DDP cells were more resistant to DDP compared with SiHa cells, and the half-maximal inhibitory concentration (IC50) of DDP was evidently higher in SiHa/DDP compared with the parental cells (Fig. 1C). Moreover, by western blot, FXOP2 expression in SiHa/DDP was much lower than that in SiHa. (Fig. 1D). These results suggested that FOXP2 may play an essential role in CC chemoresistance.

FOXP2 enhanced chemosensitive to DDP in CC cells.

Cellular models of functional overexpression and inhibition have been established (Fig. 2A). The MTT results showed that FOXP2 silencing enhanced DDP resistance in SiHa cells while restoring FOXP2 sensitized SiHa/DDP cells to DDP (Fig. 2B). Colony formation assay showed that FOXP2-knockdown obviously increased the colony formation in the absence or presence of DDP (2 μM) compared with that of the control group in SiHa cells (Fig. 2C and 2E). While FOXP2 overexpression reduced colony formation in the absence or presence of DDP (6 μM) in SiHa/DDP cells (Fig. 2D and 2F). Flow cytometry detection found that FOXP2-knockdown significantly reduced cell apoptosis after DDP treatment (2 μM) compared with that of the control group (Fig. 2G and 2I), While FOXP2 overexpression promoted apoptosis in SiHa/DDP cells following DDP treatment (6 μM) (Fig. 2H and 2J). The above data suggested that restoring FOXP2 might enhance the chemosensitive to DDP in CC cells.

FOXP2 acted as a transcription factor to inhibit the expression of c-MET in cervical cancer.

C-MET is a factor closely related to the occurrence and development of tumors and chemoresistance (11, 21). Notably, FOXP2 could bind to c-MET promoters and negatively regulate c-MET expression (18). Therefore, we hypothesized that FOXP2 mediates chemosensitive maybe via regulating c-MET. First, we detected the expression of c-MET in SiHa and SiHa/DDP cells. As shown in Fig. 1B, the expression of c-MET in SiHa/DDP cells was significantly upregulated compared to SiHa cells. Next, we tested the expression of c-MET protein in FOXP2 gain or loss of function cells. C-MET mRNA and protein were significantly increased in FOXP2-knockdown SiHa cells, while its expression was reduced in FOXP2 overexpression SiHa/DDP cells (Fig. 3A and Fig. 2A). FOXP2 might modulate c-MET expression in CC. Luciferase reporter assay showed that the si-FOXP2 enhanced luciferase intensity, and restoring FOXP2 reduced it (Fig. 3B). FOXP2 might negatively regulate the transcription of c-MET. Moreover, the ChIP-PCR assay showed that FOXP2 overexpression remarkably decreased the accumulation of FOXP2 on c-MET promoter, while FOXP2 knockdown, the result was opposite (Fig. 3C). The above results demonstrated that FOXP2 acted as a transcription factor to repress the expression of c-MET in cervical cancer.

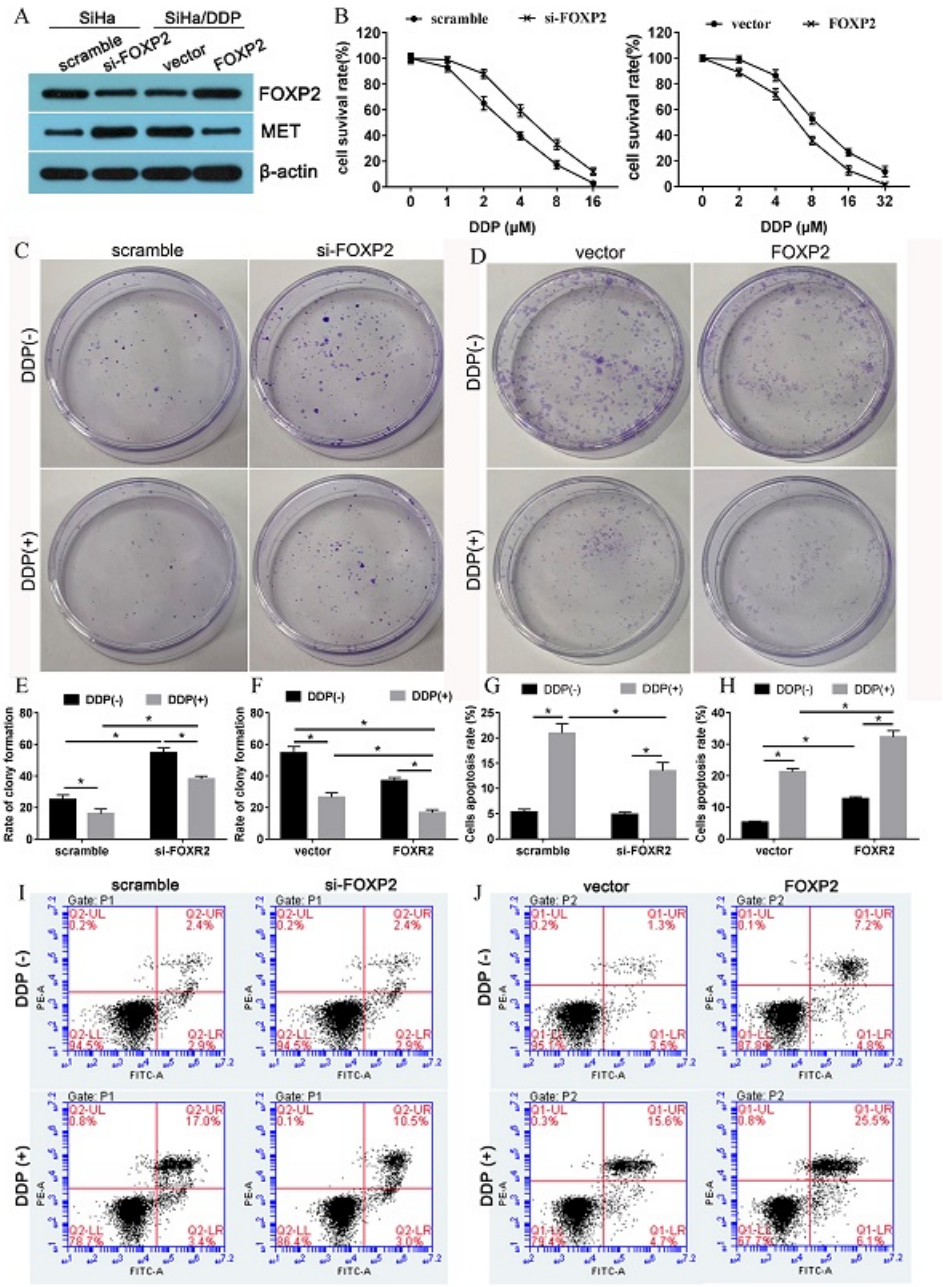

Fig. 2: FOXP2 enhanced chemosensitive to DDP in CC cells

A: Western blot verified the construction of loss or gain function cell models in SiHa and SiHa/DDP cells. B: SiHa (scramble, si-FOXP2) and SiHa/DDP (vector, FOXP2) model cells were exposed to different concentrations (0, 1, 2, 4, 8, 16, 32 μM) of DDP for 48 h, and cell proliferation was evaluated by MTT assay. C and D: Different model cells were exposed to DDP for 48 h (SiHa(C) and SiHa /DDP(D) cells were treated with 2 μM or 6 μM DDP, respectively.), survival cell colonies were assessed through clonogenic assays. E and F: Quantitative analysis of colony formation. * $P < 0.05$ VS. negative control. G and H: Treat transfected cells with or without DDP for 48 h, apoptotic cells were analyzed by flow cytometry, I and J: the apoptotic rates were calculated. * $P < 0.05$

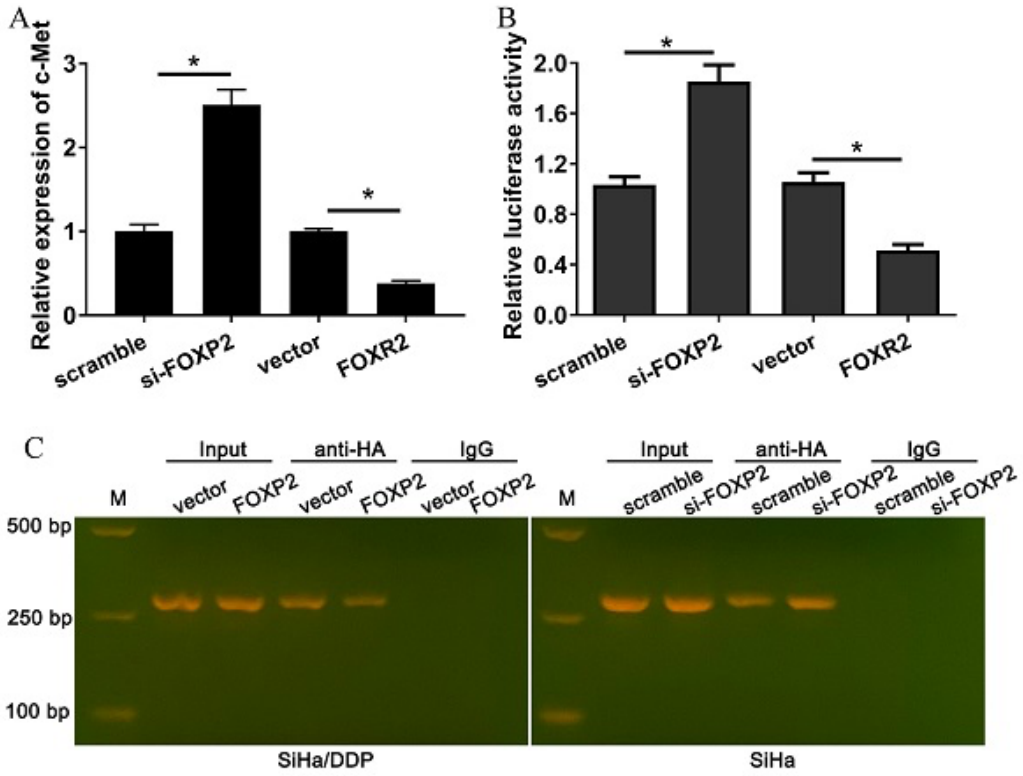

Fig. 3: FOXP2 acted as a transcription factor to inhibit the expression of c-MET in cervical cancer
A: The expression of c-MET mRNA level in different cell models (scramble and Si-FOXP2 transfer into SiHa cells, Vector and FOXP2 transfer into SiHa/DDP cells) was determined by q-PCR, * $P < 0.05$. B: c-MET promoter-luciferase reporter vector was constructed. The luciferase reporter vectors were co-transfected with si-FOXP2 or negative control into SiHa cells and FOXP2 or negative control into SiHa/DDP. Luciferase activities were then examined, * $P < 0.05$ VS. negative control. C: Gel image of ChIP-PCR indicating direct binding of FOXP2 to c-MET promoter

FOXP2 enhanced cervical cell sensitivity to cisplatin via c-MET.

Although our results indicate that FOXP2 can inhibit the expression of c-MET in cervical cancer cells, whether FOXP2 mediating the chemosensitivity via c-MET was unclear. The cell survival results showed that c-MET overexpression could antagonize chemosensitivity induced by FOXP2 in SiHa/DDP cells (Fig. 4A). Meanwhile, restoring c-MET relieved the suppressive effects of FOXP2 overexpression on cell colony formation (Fig. 4B and 4C). Besides, the proapoptotic effect induced by FOXP2 overexpression in SiHa/DDP was mitigated when restoring c-MET (Fig. 4D and 4E). FOXP2 may promote chemosensitivity in cervical cancer cells via c-MET.

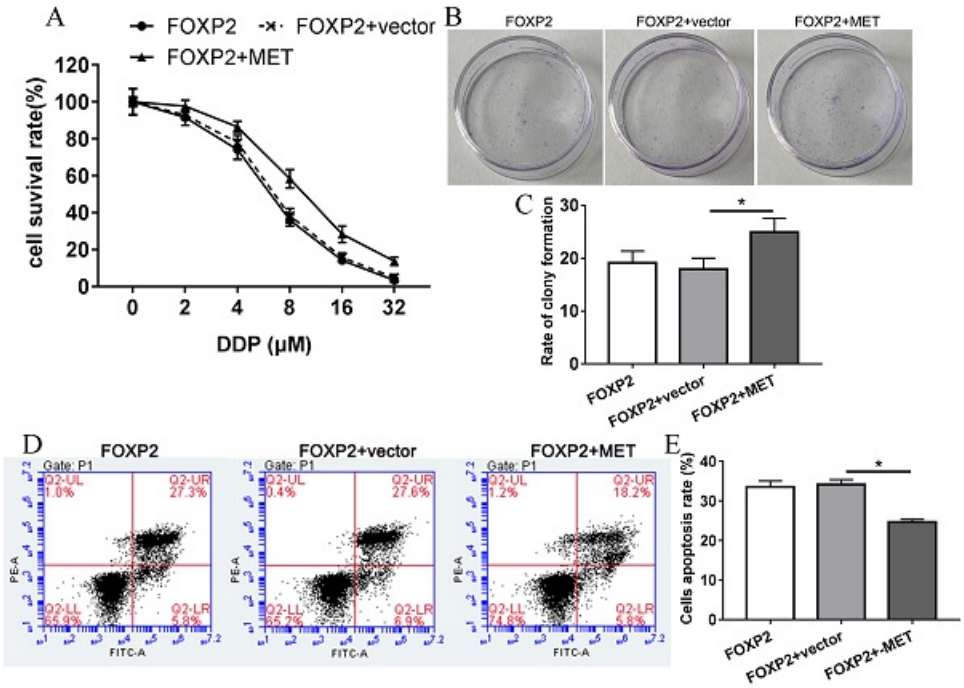

Fig. 4: FOXP2 enhanced cervical cell sensitivity to cisplatin via c-MET.
A: The results of an MTT assay showed the cell viability re-upregulated by overexpression of c-MET in FOXP2-SiHa/DDP cells. B: Colony formation assay was used to detect the effect of upregulating c-MET on the cloning ability of FOXP2-SiHa/DDP cells and quantitative analysis of colony formation (C). Formation assay in cells (*P<0.05). D and E: Cell apoptosis was determined by flow cytometric analysis in MET-transfected FOXP2-SiHa/DDP cells (* P < 0.05 VS. vector-transfected)

Discussion

In this study, FOXP2 was downregulated in cisplatin-resistant cervical cancer tissues and cells compared with control. FOXP2 overexpression in SiHa/DDP CC cells enhanced chemosensitive to DDP, whereas down-regulation of FOXP2 in SiHa cells has the opposite result. FOXP2 enhanced chemosensitive to DDP in CC cells. Moreover, FOXP2 acted as a transcription factor to inhibit the expression of c-MET in cervical cancer. Further exploration suggested that FOXP2 might enhance cervical cell sensitivity to cisplatin in part of c-MET.

Chemotherapy is one of the standard therapies for cervical cancer, and cisplatin is an effective chemotherapy therapeutic agent. It has become the mainstay in the treatment of locally advanced cancer or recurrent cervical cancers (22, 23). Although the initial clinical response was satisfactory, the drug resistance significantly impairs the effectiveness of DDP. Metastatic and recurrent cervical cancer has a poor prognosis, and the 5-year survival rate is less than one-fifth (24). At present, significant progress has been made in understanding the mechanisms of cisplatin drug resistance in CC; it is still necessary to understand the underlying mechanisms and molecular targets that lead to low response rates and drug resistance in the clinic.

Improve cisplatin sensitivity effective strategies are still lacking. We analyzed GEO DataSets (GSE168009) in this work and found that FOXP2 is a low expression in DDP-resistant samples. FOXP2 may be related to tumor drug resistance. FOXP2 is involved in tumor drug resistance; FOXP2 is negatively correlated with the resistance of colorectal cancer cells to doxorubicin (10). Nevertheless, the mechanism of FOXP2

promoting DDP sensitivity of CC cells has not been reported yet.

To explore the mechanisms underlying drug resistance in CC by FOXP2. Firstly, we detected the FOXP2 level in vivo and vitro, and the result is consistent; FOXP2 is low expressed in DDP-resistant cells. We demonstrated that FOXP2 plays a specific role in CC cells' resistance to DDP. DDP-resistance was closely correlated with cells' decreased apoptosis and increased proliferative capability. Further investigation showed that the downregulation of FOXP2 could weaken the sensitivity of the SiHa cells to DDP, and upregulation of FOXP2 could enhance the sensitivity of the SiHa/DDP cells to DDP. Our in vitro experiments pointed out that FOXP2 enhanced chemo-sensitive to DDP in CC cells by improving cell apoptosis and reducing cell proliferation. This is the first time FOXP2 is closely related to DDP resistance in cervical cancer and upregulation of FOXP2 re-sensitizes DDP-resistant cervical cancer cells to DDP. However, the exact molecular mechanism of how FOXP2 regulates cisplatin chemosensitivity in cervical cancer cells remains explored. Gaining a better understanding of related molecular biological factors could assist in the development of better molecular treatment options. A comprehensive understanding of molecular mechanisms underlying cervical cancer chemo-resistance will promote effective treatment.

Cell resistance to DDP implicates multiple cellular pathways. c-MET is confirmed to be related to the occurrence, development, and chemotherapeutic agent resistance of various tumors (11, 13, 21). Previous research reports, c-MET is an oncogene in lung cancer, hepatocellular carcinoma, breast cancer, bladder cancer, ovarian cancer, cervical cancer, and so on (12, 25). Simultaneously, c-MET is thought to play an important role in chemotherapy resistance. c-MET-amplified and activation always promote cell resistance to chemotherapy drugs (12, 26, 27). Salvianolic acid A reverses cisplatin resistance by targeting the c-MET and attenuating Akt/mTOR pathway (28). Scutellarin overcomes cisplatin resistance through ERK/p53 and c-MET/AKT signaling pathways in lung cancer cells (29), c-MET signaling in normal and colorectal cancer stem cells overcomes EGFR inhibition drug resistance (30). In addition, in cervical cancer, PDGFα receptor-mediated signal pathway plays a crucial role in up-regulating and activating c-MET. More importantly, inhibition of PDGFα receptors in cervical cancer cell lines eliminated cisplatin-dependent c-MET expression (31). Collectively, the c-MET signaling axis may play an important role in the DDP resistance of cervical cancer cells.

As a transcriptional regulator, FOXP2 can regulate the expression of its downstream genes in various ways. We speculate FOXP2 may also be a transcriptional regulator of c-MET in CC cells. We tested the expression of c-MET after down-regulation of FOXP2 in SiHa cells and up-regulation of foxp2 in SiHa/DDP cells found that c-MET mRNA and protein level were both negatively correlated with FOXP2. Mechanistically, the luciferase reporter assay and CHIP-PCR assay also demonstrated that FOXP2 directly targeted the promoter region of c-MET. We confirmed that FOXP2, as a transcription factor, could negatively regulate the expression of c-MET by binding to its promoter region. It is consistent with the previous study (6). A potential interaction between FOXP2 and c-MET might contribute to DDP-resistance in CC cancer. Furthermore, our result illustrated overexpression of FOXP2 in SiHa/DDP cell lines suppressed the growth and promoted apoptosis, whereas these effects were reversed by transfection with c-MET, suggesting that FOXP2 promoted SiHa cells to DDP-resistance via regulating c-MET. In general, our study shows that FOXP2 mediated DDP-resistance in SiHa cell line. Further research has found that FOXP2 negatively regulates the expression of c-MET by binding to its promoter region, thereby participating in DDP resistance in cervical cancer cells.

Conclusion

The present study demonstrated a new mechanism, first report that FOXP2 decreases cisplatin

resistance in cancer cells via negative regulation of c-MET, implied that the FOXP2/c-MET signaling axis uncovered in this research may be a novel therapeutic target for the DDP therapy resistance of CC patients.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgements

This study was supported by the General project of Changde Science and Technology Bureau , 2018S019.

Conflict of interest

The authors declare that there is no conflict of interest.

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