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Original Article

Targeting the eIF4E/β-catenin axis sensitizes cervical carcinoma squamous cells to chemotherapy

Hai Xu[1], Zhiyin Wang[1], Lang Xu[2], Guoyan Mo[3], Gangfeng Duan[4], Yali Wang[5], Zhengang Sun[6], Hao Chen[6]

[1]Department of Obstetrics and Gynaecology, Huangjiahu Hospital of Hubei University of Chinese Medicine, Wuhan, China; [2]Department of Pathology, School of Medicine, Wuhan University of Science and Technology, Wuhan, China; [3]China Key Laboratory of TCM Resource and Prescription, Hubei University of Chinese Medicine, Ministry of Education, Wuhan, China; [4]Department of Internal Medicine, Wuhan Integrated Traditional Chinese Medicine & Western Medicine Hospital, Wuhan, China; [5]Department of Obstetrics and Gynaecology, Wuhan General Hospital of Guangzhou Military, Wuhan, China; [6]Department of Gastrointestinal Surgery, Jingzhou Central Hospital, Yangtze University, Jingzhou, China

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Abstract: Chemotherapy has improved the clinical outcomes of cervical cancer patients. However, patients develop chemoresistance, whose underlying mechanisms are not well understood. In this study, we investigated the phosphorylation levels of eukaryotic translation initiation factor 4E (eIF4E) in cervical cancer cells subjected to chemotherapy. Results showed that chemotherapeutic drugs significantly increased eIF4E phosphorylation at S209 in HeLa and SiHa cells. Upregulation of phosphorylated eIF4E (p-eIF4E) levels has also been shown in cisplatin-resistant HeLa cells and has been observed to be a common response of cervical cancer patients undergoing chemotherapy. We further showed that chemotherapeutic drugs increase β-catenin activity and mRNA levels of Wnt/β-catenin target genes in cervical cancer cells but not in eIF4E-depleted cells, suggesting that chemotherapeutic drugs activate Wnt/β-catenin signaling in an eIF4E-dependent manner. Inhibiting eIF4E via siRNA knockdown or Wnt/β-catenin using the Wnt inhibitor pyrvinium effectively enhanced the anti-proliferative and pro-apoptotic effects of cisplatin in cervical cancer cells both *in vitro* and *in vivo*. Our findings demonstrate that eIF4E/β-catenin signaling plays a positive regulatory role in the resistance of cervical cancer cell to chemotherapy and thus highlight the therapeutic value of eIF4E or β-catenin inhibition in overcoming chemoresistance.

Keywords: eIF4E, β-catenin, pyrvinium, cervical cancer, chemoresistance

Introduction

Cervical cancer is one of the leading causes of cancer-related deaths in women worldwide [1]. Advances in screening and treatment methods have significantly improved clinical outcomes. However, treatment of cervical cancer in advanced stages remains a challenge due to the development of resistance to the standard treatment, chemotherapy [2]. Moreover, the molecular mechanisms underlying chemoresistance are complex and are not well understood. Overexpression of anti-apoptotic proteins, increased DNA repair, and enhanced epithelial-mesenchymal transition appear to contribute to the resistance of cervical cancer cells to chemotherapy [2-4]. Therefore, elucidating the molecular mechanisms underlying chemoresistance is essential to the development of targeted therapy against cervical cancer.

The gene encoding eukaryotic translation initiation factor 4E (eIF4E) is an oncogene, and its overexpression is associated with poor prognosis in various cancers, such as lung cancer and prostate cancer [5, 6]. eIF4E is a regulator promoting the synthesis of proteins that favor tumor growth and survival [7, 8]. Previous studies have reported that β-catenin is an eIF4E target that promotes tumor progression. Furthermore, the eIF4E/β-catenin axis has been shown to maintain leukemia stem cell function [9]. Aberrant activation of β-catenin plays important roles in tumor progression and metastasis of cancer cells, including cervical

cancer cells [10, 11]. Inhibition of eIF4E or β-catenin has been demonstrated to be effective in inhibiting proliferation, migration, and survival of cervical cancer cells [12-14]. However, the exact role of eIF4E in cervical cancer chemoresistance remains to be elucidated.

In this study, we first investigated eIF4E phosphorylation levels in response to chemotherapeutic drugs in cervical cancer using *in vitro* cultured cells and *in vivo* samples from patients. We then determined the role of eIF4E in the chemoresistance of cervical cancer and its downstream targets. We next investigated the effects of inhibiting eIF4E or its downstream targets in cervical cancer cells treated with chemotherapeutic drugs. Our study demonstrates eIF4E/β-catenin signaling as a critical positive regulator in cervical cancer response to chemotherapy. Results also showed that inhibition of eIF4E or β-catenin represents an alternative strategy to sensitize cervical cancer cells to chemotherapy.

Materials and methods

Cell culture and treatment with chemotherapeutic drugs

The human cervical cancer cell lines, HeLa and SiHa (ATCC), were maintained in minimal essential media supplemented with 10% fetal bovine serum (Hyclone, UK), 2 mM L-glutamine, and penicillin/streptomycin (Invitrogen, US). HeLa-cis-r were established by culturing HeLa cells in media containing gradually increasing concentrations (0.1, 0.2, 0.4, 1, 2, 4, and 8 μM) of cisplatin for 4 months. HeLa-cis-r were then maintained in media containing 8 μM cisplatin. 5-fluorouracil, cisplatin, and pyrvinium (Sigma, US) were dissolved in DMSO and stored as aliquots at -20°C.

Patient tissue specimens and immunohistochemistry

Tissue samples were collected from patients at Huangjiahu Hospital of Hubei University of Chinese Medicine. Written informed consents were obtained from all patients following institutional review board-approved protocols. Tissue sections were deparaffinized and hydrated by incubating in xylene and subsequently in decreasing ethanol concentrations. Next, antigen retrieval was performed using citrate buffer. Sections were stained with primary antibody against phosphorylated eIF4E (p-eIF4E) at 4°C overnight and with secondary HRP conjugated-antibody for 1 h at room temperature. Sections were counterstained with hematoxylin (Sigma, US).

MTS proliferation assay

Cells were plated on 96-well plates at 10,000 cells per well, after which chemotherapeutic drugs were added to the culture media after 1 day. After 3 days of treatment, cells were added with MTS [3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyltetrazolium bromide] from the CellTiter 96 AQueous One Solution Cell Proliferation assay kit (Promega, US) and incubated for 2 h. The absorbance was measured at 595 nm.

Annexin V labeling and flow cytometry

Cells were plated onto 12-well plates at a density of 500,000 cells per well and treated with chemotherapeutic drugs for 3 days. Cells were detached using trypsin and labeled with Annexin V-FITC and PI (BD Pharmingen, US). Stained cells were analyzed using flow cytometry on a Beckman Coulter FC500. The percentage of Annexin V-positive cells was determined using CXP software.

siRNA transfection

eIF4E-specific knockdown was achieved by transfecting cells with siRNA against custom human eIF4E (GGUGGGCACUCUGGUUUUU) [15] from Ambion. Control siRNA was purchased from Ambion. Transfection was performed using DharmaFECT1 reagent according to the manufacturer's protocol. Cells were harvested for analysis 48 h after transfection.

Western blot analyses

For total protein extraction, cells were homogenized in RIPA lysis buffer (50 mM Tris·HCl, 150 mM NaCl, and 1 mM EDTA) containing protease inhibitor cocktail (Roche, US). Proteins were separated by SDS-PAGE and then processed for western blotting using antibodies directed against eIF4E, phosphor-eIF4E (S209), and β-actin (Cell Signaling Technology, US). Protein levels were quantified using Image J software.

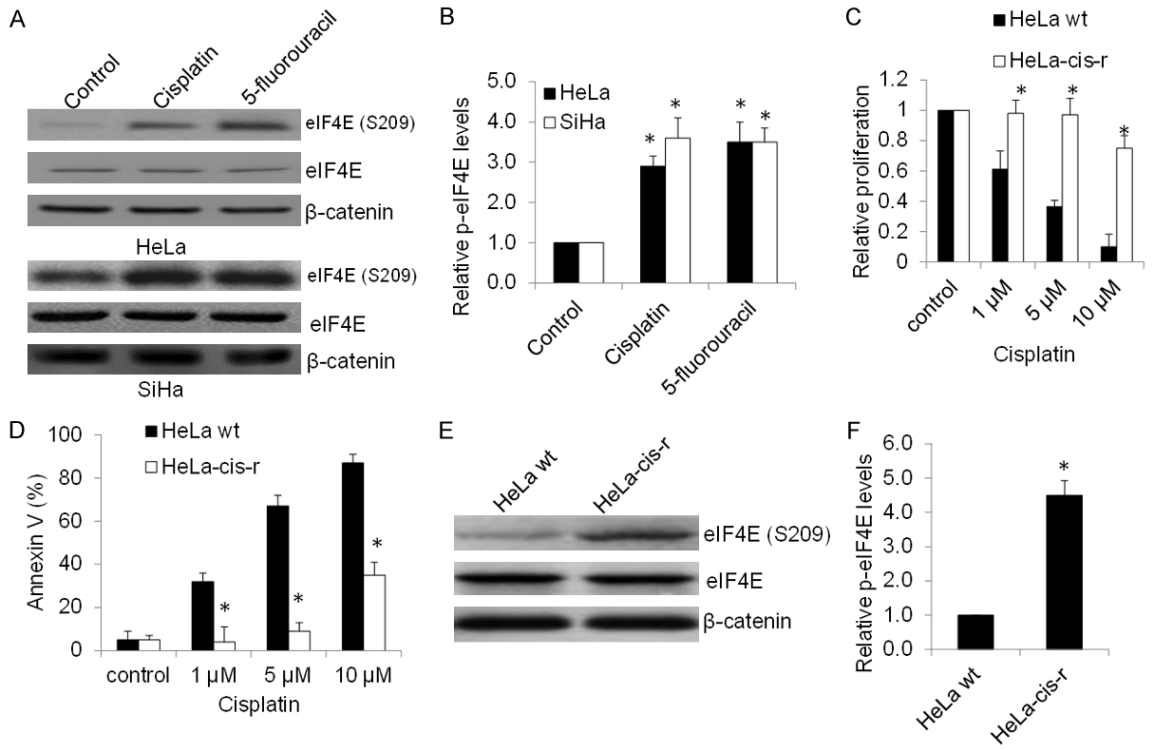

Figure 1. Chemotherapeutic drugs increase eIF4E phosphorylation in cervical cancer cells. Representative images of western blotting results (A) and quantification using Image J software (B) show increased levels of p-eIF4E at S209 in HeLa and SiHa cells treated with cisplatin or 5-fluorouracil compared to control. HeLa-cis-r cells showed reduced proliferation inhibition (C) and apoptosis induction (D) upon cisplatin treatment at 1, 5, and 10 μM compared to HeLa wild-type cells. Cells were treated with cisplatin or 5-fluorouracil for 3 days prior to performing MTS proliferation and Annexin V staining apoptosis assays. Representative images of western blotting results (E) and quantification using Image J software (F) showed increased levels of p-eIF4E at S209 in HeLa-cis-r compared to HeLa wild-type cells. *P<0.05, compared to control or wild-type HeLa cells.

Measurement of β-catenin activity

Cells were transfected with 5 μg of 16 × SuperTopFlash plasmid (Addgene, US) or β-gal using DharmaFECT1 reagent. Cells were harvested at 24 h post-transfection using the Luciferase Reporter Assay System (Promega, US). β-catenin activity was quantified by normalizing SuperTopFlash to β-gal.

RNA extraction and real-time PCR

Total RNA was isolated using TRIzol Reagent (Ambion, US) following the manufacturer's instructions and subsequently used for quantitative RT-PCR for mRNA expression analysis. First-strand cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-rad, CA). cDNA amplification was performed via PCR using a SsoFast EvaGreen Supermix (Bio-rad, CA). Primer sequences used were the same as previously reported [16].

Cervical cancer xenografts in SCID mice

Animal experiments were approved by the Institutional Animal Care and Use Committee of Hubei University of Chinese Medicine. Five million HeLa cells suspended in PBS were subcutaneously injected to the flanks of SCID/NOD mice (5-6 weeks old). Following development of palpable tumors, mice were intraperitoneally injected with vehicle (20%:80% DMSO: saline) alone, pyrvinium (0.5 mg/kg) alone, cisplatin (20 mg/kg) alone, or a combination of pyrvinium and cisplatin for 3 weeks. Tumor size was calculated using the following formula: $(\text{width})^2 \times \text{length}/2$.

Statistical analyses

Each experiment was performed at least thrice, and data were expressed as mean and standard deviation (SD). An unpaired Student t test was performed to compare against different

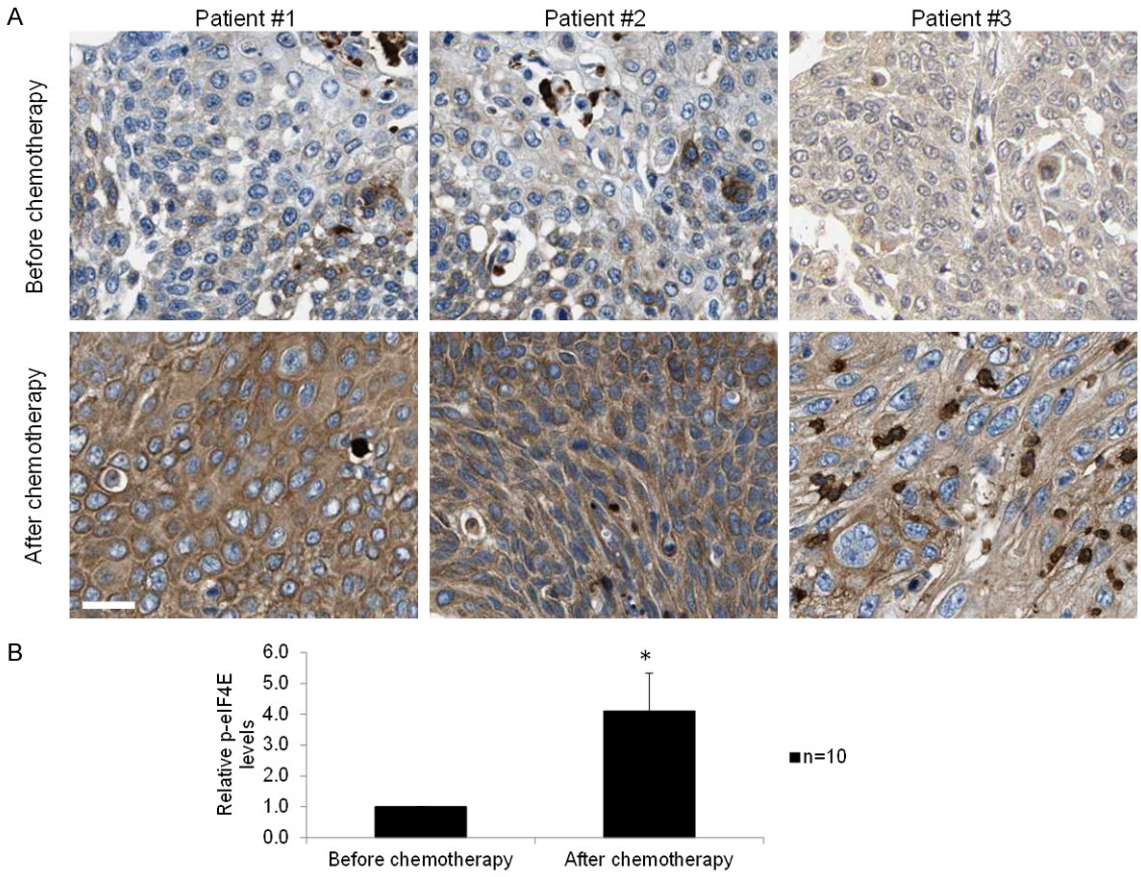

Figure 2. Chemotherapy significantly increases levels of phosphorylated eIF4E in cervical cancer cells. A. Representative results from immunohistochemistry analysis showing p-eIF4E levels in cervical cancer tissues collected from patients before and after chemotherapy. Patients were subjected to at least 1 month of chemotherapy prior to biopsy for analysis of p-eIF4E levels. Cisplatin and bleomycin were used for chemotherapy. Scale bar represents 50 μm. B. Cervical cancer tissues collected from patients showed higher p-eIF4E levels after chemotherapy than before chemotherapy. Results are expressed relative to control. *P<0.05, compared to before chemotherapy.

variables and P<0.05 was considered statistically significant.

Results

Chemotherapy significantly increases eIF4E phosphorylation in cervical cancer cells in vitro and in vivo

Aberrant eIF4E phosphorylation is involved in cell survival mechanisms that are triggered in response to various stress conditions, such as oxidative stress and DNA damage [17]. To investigate whether eIF4E confers resistance to chemotherapy, we examined p-eIF4E levels in cervical cancer cells subjected to treatment with standard chemotherapeutic drugs. Results showed that HeLa and SiHa cells exposed to cisplatin or 5-fluorouracil had significantly higher p-eIF4E levels (**Figure 1A** and **1B**). We next generated a HeLa-cis-r cell line that is resistant to cisplatin, a drug that inhibits proliferation and induces apoptosis (**Figure 1C** and **1D**). HeLa-cis-r cells consistently showed significantly higher p-eIF4E levels compared to parental HeLa cells (**Figure 1E** and **1F**).

To confirm the above results, we performed immunohistochemical analysis on cervical cancer tissues obtained from patients before and after chemotherapy. Results showed that most cervical cancer cells before chemotherapy had low phosphorylated eIF4E (S209) levels (**Figure 2A**). However, eIF4E phosphorylation levels were observed to significantly increase after subjecting the patients to chemotherapy (**Figure 2B**). Notably, increased p-eIF4E levels were detected in ten out of ten cervical cancer

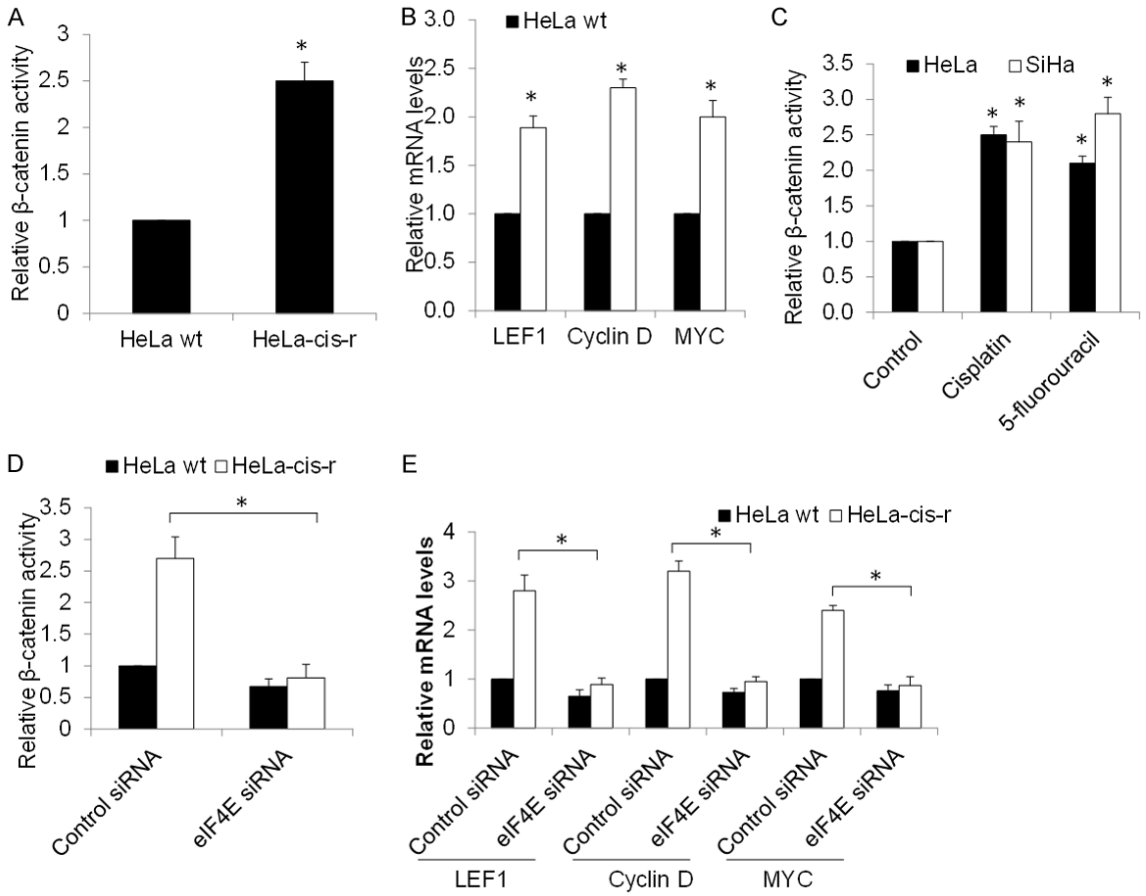

Figure 3. Chemotherapeutic drugs increase Wnt/β-catenin signaling in an eIF4E-dependent manner in cervical cancer cells. HeLa-cis-r cells showed increased β-catenin activity (A) and higher mRNA levels of LEF1, cyclin D, and MYC (B) compared to wild-type HeLa cells. (C) Treatment with cisplatin and 5-fluorouracil increased β-catenin activity in HeLa and SiHa cells. β-catenin activity (D) and mRNA levels of LEF1, cyclin D, and MYC (E) were decreased in eIF4E-depleted HeLa-cis-r cells. *P<0.05, compared to control.

patients, thereby demonstrating that increased eIF4E phosphorylation is a common feature in cervical cancer patients treated with chemotherapeutic drugs.

Chemotherapy activates Wnt/β-catenin signaling in an eIF4E-dependent manner

β-catenin has been reported as an eIF4E target that promotes tumor progression. Thus, to identify the downstream targets of phosphorylated eIF4E, we investigated Wnt/β-catenin signaling in cervical cancer cells treated with chemotherapeutic drugs [9]. Our results showed that HeLa-cis-r cells exhibit higher β-catenin activity and higher transcriptional upregulation of Wnt/β-catenin compared to parental HeLa cells after chemotherapy (**Figure 3A** and **3B**), demonstrating that Wnt/β-catenin signaling is activated in cervical cancer cells with prolonged exposure to cisplatin. Short exposure to cisplatin or 5-fluorouracil also consistently significantly increased eIF4E phosphorylation in HeLa and SiHa cells (**Figure 3C**). Importantly, eIF4E depletion abolished the increased Wnt/β-catenin activity and upregulation of Wnt/β-catenin target genes, including LEF1, MYC and cyclin D in HeLa-cis-r cells (**Figure 3D** and **3E**), suggesting that chemotherapy activates Wnt/β-catenin signaling in an eIF4E-dependent manner.

siRNA knockdown of eIF4E significantly sensitizes cervical cancer cell to chemotherapy

To elucidate the possible roles of p-eIF4E upregulation in cisplatin resistance, HeLa-cis-r cells were depleted of eIF4E via siRNA knock-

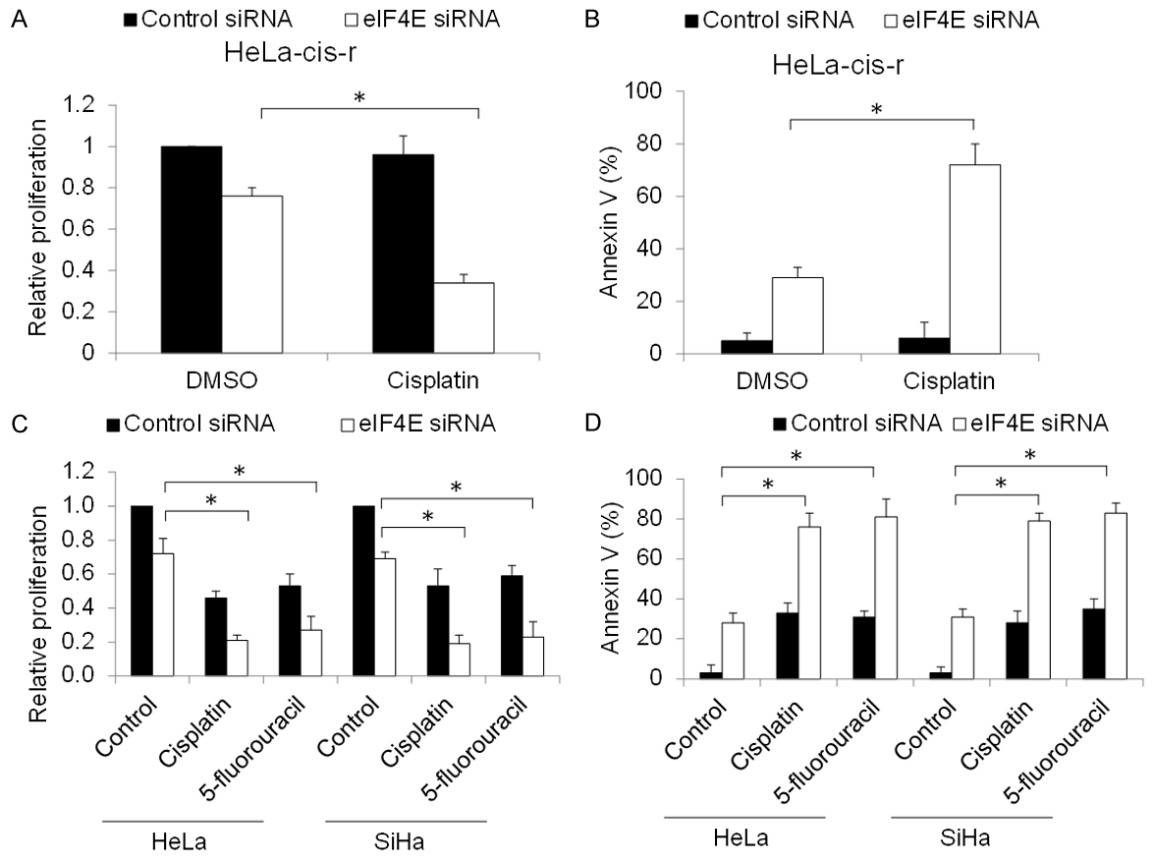

Figure 4. Inhibiting eIF4E enhances cervical cancer cell response to chemotherapeutic drugs. eIF4E depletion via siRNA knockdown significantly enhances the anti-proliferative (A) and pro-apoptotic (B) effects of cisplatin on HeLa-cis-r cells. Cisplatin was added to cells at 36 h post-transfection, and chemotherapeutic treatment was performed 3 days prior to MTS or Annexin V analysis. eIF4E depletion using siRNA significantly augments the anti-proliferative (C) and pro-apoptotic (D) effects of cisplatin and 5-fluorouracil on HeLa and SiHa cells. *P<0.05, compared to control.

down and subsequently treated with cisplatin. Results showed that eIF4E knockdown significantly attenuated cisplatin resistance in HeLa-cis-r cells in by reducing proliferation and apoptosis (**Figure 4A** and **4B**). In addition, the anti-proliferative and pro-apoptotic effects of cisplatin and 5-fluorouracil were further enhanced in eIF4E-depleted cervical cancer cells (**Figure 4C** and **4D**). These results clearly demonstrate that eIF4E inhibition sensitizes cervical cancer cells to chemotherapy.

Inhibition of Wnt/β-catenin signaling by pyrvinium sensitizes cervical cancer cells to chemotherapy in vitro and in vivo

Our data showed that eIF4E/β-catenin signaling contributes to chemoresistance in cervical cancer cells and eIF4E inhibition attenuates chemoresistance. Thus, we speculated that inhibition of Wnt/β-catenin signaling can be effective in enhancing the effectiveness of chemotherapeutic drugs against cervical cancer cells. Pyrvinium is an FDA-approved anthelmintic drug that has been identified as a potent Wnt inhibitor [18, 19]. To explore its potential for use in clinical applications, we evaluated the ability of pyrvinium to attenuate chemo resistance. Consistent with results of previous reports [18, 19], we first showed that pyrvinium is a Wnt inhibitor that decreases β-catenin activity in HeLa and SiHa cells (**Figure 5A**). Treatment with pyrvinium alone inhibited proliferation and induced apoptosis in cervical cancer cells in a dose-dependent manner (**Figure 5B** and **5C**). Importantly, pyrvinium treatment enhanced the anti-proliferative and pro-apoptotic effects of cisplatin and 5-fluorouracil on cervical cancer cells (**Figure 5D** and **5E**). In addition, pyrvinium enhanced the *in vivo* efficacy of cisplatin in a cervical cancer xenograft mouse model, as shown by greater decreases

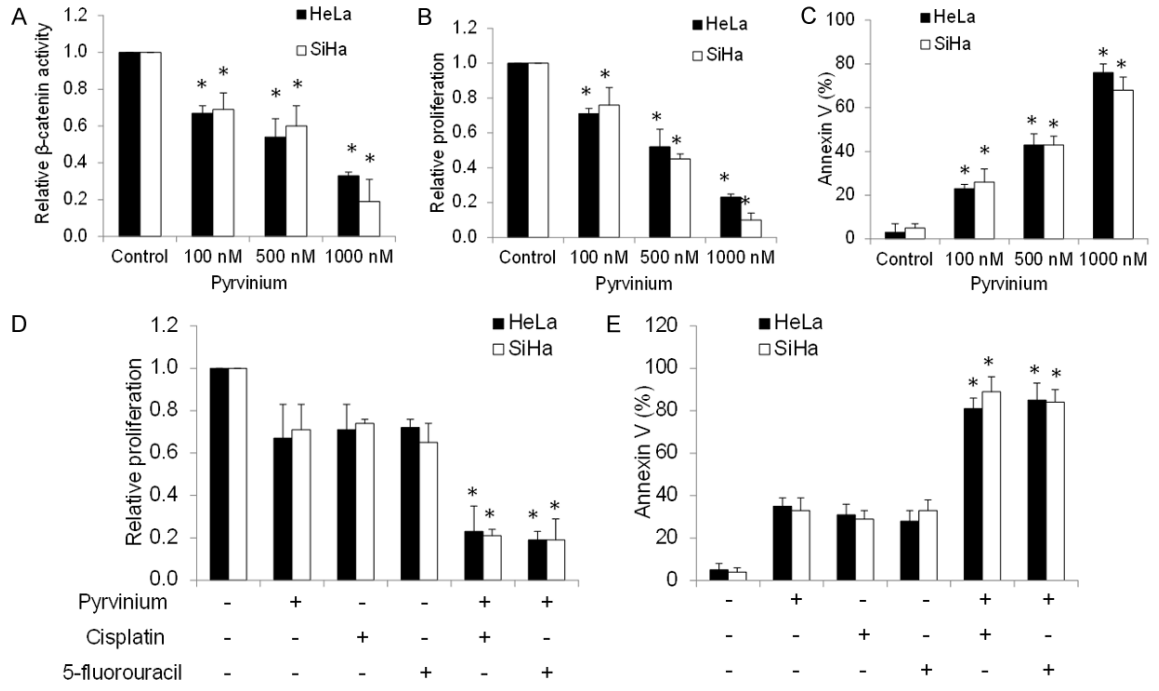

Figure 5. Pyrvinium treatment enhances cervical cancer cell response to chemotherapeutic drugs. (A) Pyrvinium treatment decreases β-catenin activity in a dose-dependent manner in HeLa and SiHa cells. Pyrvinium treatment inhibits proliferation (B) and induces apoptosis (C) in HeLa and SiHa cells. Combined treatment with pyrvinium and cisplatin or 5-fluorouracil is more effective in inhibiting proliferation (D) and inducing apoptosis (E) in cervical cancer cells compared to treatment with a single drug. *P<0.05, compared to control or single arm treatment.

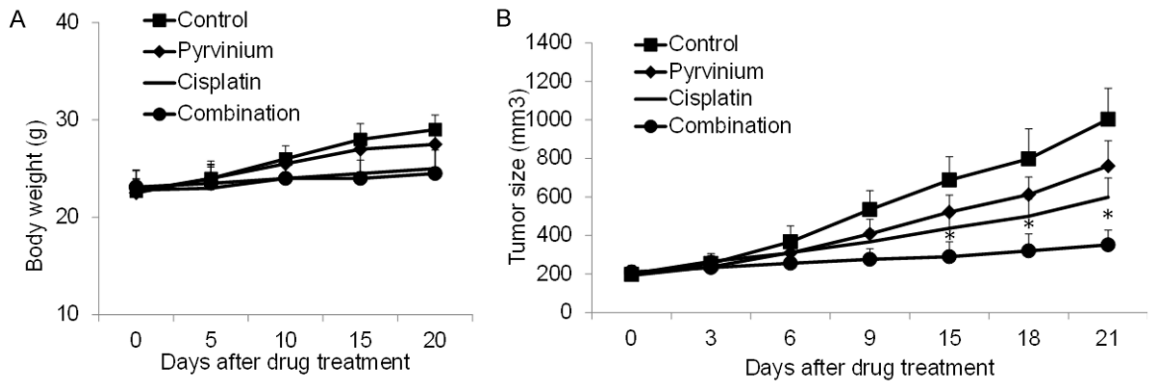

Figure 6. Pyrvinium sensitizes cervical cancer cell response in cisplatin *in vivo*. A. No significant differences in mouse body weights were detected between mice treated with a drug alone and those treated with combinations of chemotherapeutic drugs. B. Combination of pyrvinium and cisplatin more effectively arrests tumor growth compared to treatment with pyrvinium or cisplatin alone. *P<0.05, compared to single arm treatment.

in tumor weights and sizes in samples co-treated with pyrvinium and cisplatin than those treated with a single drug (**Figure 6**). Our *in vivo* data correlates well with *in vitro* data and confirm that inhibition of Wnt/β-catenin signaling through pyrvinium treatment sensitizes cervical cancer cell to chemotherapy.

Discussion

Patients with advanced cervical cancer develop resistance to standard therapy and continue to relapse. Substantial progress has been made in the understanding of the factors that contribute to the development of cervical can-

cer, such as human papilloma virus (HPV) infection. However, the mechanisms responsible for the persistence of cervical cancer cells after chemotherapy are not well understood. This study is the first to demonstrate that eIF4E/β-catenin signaling mediates chemoresistance in cervical cancer cells. We further demonstrate that inhibition of eIF4E or β-catenin is effective in sensitizing cervical cancers to chemotherapy agents.

The principal finding of the present study is that cervical cancer cells exposed to chemotherapy agents and cervical cancer patients subjected to chemotherapy consistently exhibit increased eIF4E phosphorylation (**Figures 1** and **2**). These results suggest that upregulation of phosphorylated eIF4E levels is a common response of cervical cancer patients subjected to chemotherapy. These findings are also consistent with the study by Alba et al. demonstrating that eIF4E phosphorylation confers resistance to cellular stress and DNA damage [17]. Aberrant protein synthesis as a result of eIF4E phosphorylation has been demonstrated to be essential for the development and progression of various tumors [20]. However, this study and that of Alba et al. show that eIF4E phosphorylation is also a critical player in cancer chemoresistance.

We found that Wnt/β-catenin is activated as a result of increased eIF4E phosphorylation in cervical cancer cells. Increased β-catenin activity and transcriptional upregulation of Wnt/β-catenin target genes were observed in cervical cancer cells treated with cisplatin or cisplatin-resistant HeLa cells (**Figure 3A-C**). In addition, eIF4E depletion abolishes the activation of Wnt/β-catenin signaling in cervical cancer cells treated with chemotherapeutic agents or cisplatin-resistant HeLa cells (**Figure 3D** and **3E**). Our findings demonstrate that cervical cancer cells acquire resistance to chemotherapy by promoting eIF4E-dependent Wnt/β-catenin activation. The primary roles of Wnt/β-catenin signaling in tumor development have been extensively investigated, particularly in tumor stem cells [18, 21-23]. Jesus et al. have shown that Wnt/β-catenin signaling participates in cervical carcinogenesis since it is regulated by the oncoproteins, HPV E6 and E7 [22]. In line with these efforts, our work highlights the roles of Wnt/β-catenin in the development of cervical cancer chemoresistance.

eIF4E inhibition has been shown to suppress growth and induce apoptosis in various tumors [12, 24]. Consistent with previous findings, our study also showed that eIF4E inhibition via siRNA knockdown significantly suppresses proliferation and induces apoptosis in HeLa and SiHa cells (**Figure 4C** and **4D**). Importantly, eIF4E inhibition significantly enhances the anti-proliferative and pro-apoptotic effects of chemotherapeutic agents (**Figure 4**), suggesting that eIF4E inhibition can overcome chemoresistance in cervical cancer. Besides cervical cancer, eIF4E inhibition has been reported to sensitize leukemia, glioblastoma, and breast cancer cells to chemotherapy [9, 17, 25]. Our study extends our previous work showing that targeting eIF4E presents a potential sensitizing therapy for cancer treatment.

We further show that pyrvinium, a potent Wnt inhibitor [18, 19], enhances the inhibitory effects of cisplatin in cervical cancer cells both *in vitro* and *in vivo* (**Figures 5** and **6**). This finding is consistent with those of previous studies showing that drugs targeting Wnt/β-catenin, such as tigecycline, act synergistically with chemotherapy agents in the treatment of cervical cancer [14]. The known pharmacokinetics and toxicity profiles of the anthelminthic drug, pyrvinium, facilitate the translation of our findings into the clinical setting.

Taken together, our findings show that cervical cancer cells acquire resistance to chemotherapy via enhanced eIF4E/β-catenin signaling. Inhibition of eIF4E or β-catenin represents an alternative therapeutic strategy to sensitize cervical cancer and possibly other eIF4E/β-catenin-driven cancers to chemotherapy.

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Disclosure of conflict of interest

None.

Address correspondence to: Hao Chen, Department of Gastrointestinal Surgery, Jingzhou Central

Hospital, Renmin Road 1, Jingzhou 434020, China. E-mail: chenhaochho000@outlook.com

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