Original Article
Bortezomib enhances the radiosensitivity of hypoxic cervical cancer cells by inhibiting HIF-1α expression

Heqing Cui[1*], Qin Qin[2*], Meilin Yang[2*], Hao Zhang[2], Zheming Liu[2], Yan Yang[2], Xiaochen Chen[1], Hongcheng Zhu[1], Di Wang[1], Cuicui Meng[1], Hongmei Song[1], Jianxin Ma[1], Guanhong Huang[1], Jing Cai[3], Xinchen Sun[2], Zhongming Wang[1]

[1]Department of Radiotherapy, The Second People's Hospital of Lianyungang, Lianyungang Hospital Affiliated to Bengbu Medical College, China; [2]Department of Radiation Oncology, The First Affiliated Hospital of Nanjing Medical University, China; [3]Department of Radiotherapy, Nantong Tumor Hospital Affiliated to Nantong University, China. *Equal contributors.

Abstract: Objective: This study aimed to investigate the radiosensitivity of bortezomib to cervical cancer and the possible underlying mechanism. Methods: HeLa and SiHa cell lines with or without hypoxia treatment were divided into control, radiation alone, bortezomib alone, and radiotherapy plus bortezomib groups. CCK8 assay, clone formation assay, flow cytometry, and immunofluorescence test were used to measure cell proliferation, colony formation, apoptosis, and DNA double-strand break (DSB). Western blot analysis was performed to detect the expression of HIF-1α, PARP-1, and caspase-3, -8, and -9. Result: Statistical analysis of data revealed that bortezomib at nanomolar level exerted a radiosensitization effect on both cervical cancer cell lines in normoxia or hypoxia. Western blot analysis showed that the drug could inhibit hypoxia-related HIF-1α expression to increase apoptosis-related caspase-3, -8, and -9 activation and DNA DSB-related PARP-1 cleavage. Conclusions: Radiotherapy sensitization of bortezomib on cervical cancer cell lines had a drug-dose relation, and sensitization in hypoxia was more remarkable than in normoxia. Bortezomib may be a potential radiotherapy sensitization drug for cervical cancer.

Keywords: Bortezomib, cervical cancer, radiosensitivity, HIF-1α, hypoxia

Introduction

Cervical cancer is the third most commonly diagnosed cancer and the fourth cancer death cause in women worldwide, with at least 85% occurring in developing countries in 2008. Mortality rate is as high as 52% [1, 2]. Radiation is the main therapy or adjuvant therapy for cervical cancer at different stages. Cervical cancer radio-resistance leads to a decline in the five-year survival rate, and to a certain extent, this situation indicates the importance of enhancing the radiation sensitivity of cervical cancer.

Solid tumor hypoxia is considered to be one reason of radio-resistance, leading to unsatisfactory survive rate [3, 4]. As the major component that causes the cellular response to hypoxia, HIF-1 can promote angiogenesis, increase glucose metabolism, and modulate apoptosis. It is a heterodimer composed of HIF-1α and HIF-1β [5-7]. HIF-1α is the key subunit that determines the presence of HIF-1, and it is finally degraded by 26S proteasomes through ubiquitin-proteasome pathway [8-12].

Bortezomib (BTZ) is an important 26-proteasome inhibitor used for the clinical treatment of human multiple myeloma [13]. The effect of BTZ on various solid tumors has been confirmed in previous studies. The proteasome inhibitor could inhibit HIF-1α protein expression to antitumor, and one of its mechanisms is the regulation of the apoptosis of tumor cells [14, 15].

Poly (ADP-ribose) polymerase (PARP-1) has an important function in cell death by participating in DNA double-strand break (DSB) repair [16] and apoptosis [17]. Moreover, HIF-1α knockdown induces PARP-1 cleavage [18]. Caspase-3, -8, and -9 are all very important in the apoptosis

of tumor cells. Caspase-3 could regulate PARP-1 expression, and its activity is regulated by HIF-1α [19]. Therefore, given this premise, drugs that suppress HIF-1α can be speculated to promote radiation sensitivity by regulating PARP-1 and the caspase family.

Hypoxia is considered a remarkable reason for solid tumor radio-resistance, and certain studies have researched on BTZ as a chemotherapy drug. However, proteasome inhibitors have not been researched in hypoxia solid tumor cell lines. In the present study, we investigate whether BTZ could sensitize normoxic and hypoxic cervical cancer cells to X-ray by regulating HIF-1α expression and related expressions of caspase-3, -8, and -9 and PARP-1.

Materials and methods

Cell culture

The human cervical cancer cell lines HeLa and SiHa were purchased from Shanghai Institute of Cell Biology (Shanghai, China). The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, NY, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) in a humidified atmosphere of 5% CO_2 and 21% O_2 at 37°C. When subjected to hypoxia, HeLa and SiHa cells were incubated at 37°C in a sealed incubator chamber filled with air of 1% O_2, 5% CO_2, and 94% N_2.

Reagents and antibodies

BTZ (Novartis Pharmaceuticals, Basel, Switzerland) was dissolved in dimethyl sulfoxide (DMSO) as a stock solution of 10 mmol/l. The HIF-1α antibody (NB100-105, Novus Biologicals; 1:500), rabbit polyclonal antibodies against PARP-1 and caspase-3, and mouse monoclonal antibody against caspase-8 were purchased from Cell Signaling Technology. The rabbit antibody against caspase-9 was obtained from Bioworld Biotechnology Pharmingen (Bioworld Technology, St. Louis Park, MN). The mouse monoclonal antibody against GAPDH was from KangChen Biotechnology (Shanghai, China). The mouse antibody against β-actin was from Millipore (Billerica, MA).

CCK8 assay

Cell counting Kit-8 (CCK8; Dojindo Laboratories, Kumamoto) assay was used to assess cell via-bilities. The HeLa and SiHa cells were both seeded in 96-well cell culture plates at a density of 5 × 10³ cells/well. The cells were treated with different concentrations of BTZ (0-100 nmol/l) or DMSO 24 h later. After 24 or 48 h of incubation, CCK8 reagent was added into each well, and the cells were incubated at 37°C for 2 h. Cell viabilities were measured through absorbance (optical density) by a spectrophotometer at 450 nm.

Clonogenic assay

The HeLa and SiHa cells were seeded into six-well plates and incubated at 37°C overnight. On the next day, BTZ or DMSO was administered, and the cells were incubated in normoxia or hypoxia for 24 h. After irradiating at a dose of 0, 2, 4, 6, or 8 Gy (6 MV X-rays, 4.5 Gy/min), the cells were incubated in 5% CO_2 incubator at 37°C for 10 days for HeLa cells and 14 days for SiHa cells. Finally, the colonies were fixed with methanol, stained with Giemsa, and counted (> 50 cells/colony).

Flow cytometry

The HeLa and SiHa cells were seeded in six-well plates and incubated at 37°C overnight. The cells were then treated with BTZ (5 or 10 nmol/l) or DMSO (control) under normoxia or hypoxia. After 24 h, the cells were either irradiated (8 Gy, 6 MV X-rays, 4.5 Gy/min) or not. All cells were incubated in 5% CO_2 incubator at 37°C for another 48 h and then harvested and stained with annexin V-fluorescein isothiocyanate and propidium iodide (BD Bioscience, Oxford, UK) according to the manufacturer's instructions. Finally, the rates of apoptosis were obtained by flow cytometry.

Western blot analysis

The cells were lysed with RIPA lysis buffer (Beyotime Biotechnology) on ice for 20 min and centrifuged at 12,000 rpm for 20 min at 4°C. The protein concentrations of the supernatants were then quantified by BCA kit (Beyotime Biotechnology). Equal proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.05% Tween 20 at pH 7.5 for 30 min, incubated with primary antibodies at 4°C overnight, and then conjugated with secondary
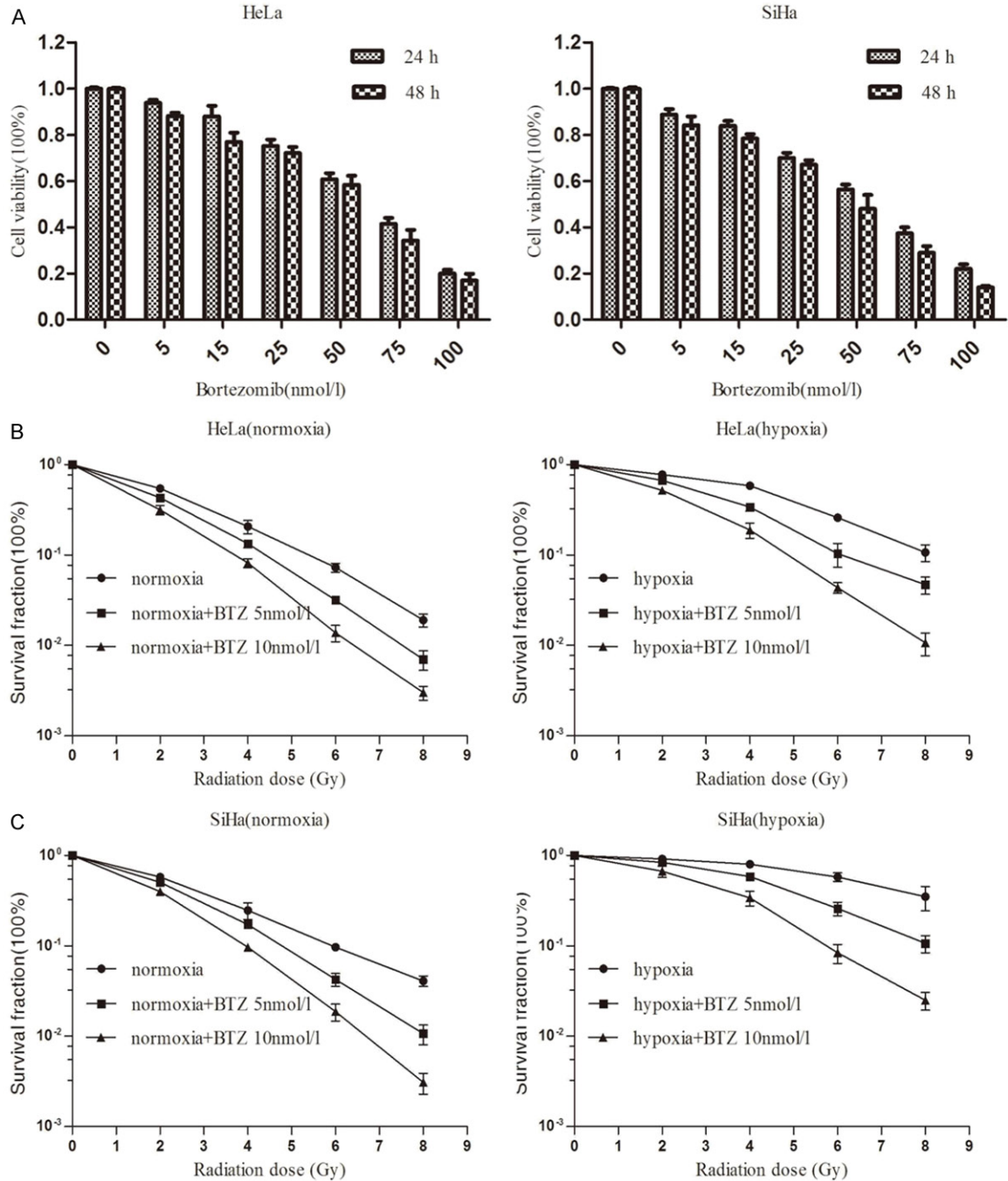
Figure 1. Bortezomib sensitizes normoxic and hypoxic cervical cancer cells to IR. A. Bortezomib inhibited growth of cervical cells in a time- and dose-dependent manner. B, C. Clonogenic assay showing that hypoxic cervical cancer cells became radio-resistant and was reversed by Bortezomib.

antibodies for 1 h at room temperature. The signals of immunoblotted proteins were visualized with an enhanced chemiluminescence detection kit by Chemidoc XRS imaging system (Quantity One Quantitation software, Bio-Rad Laboratories, Hercules, CA, USA).

Immunofluorescence test

Phospho-H2AX foci immunofluorescence was detected to monitor the DNA DSBs and DSB repair capacity of HeLa cells. The cells were seeded into laser-scanning confocal Petri dish-

Table 1. Radiosensitization activity of bortezomib in HeLa cells

	D_0	D_q	SER	SF_2
Control	1.833	1.203		0.545
BTZ 5 nmol/L	1.561	0.862	1.174	0.431
BTZ 10 nmol/L	1.395	0.477	1.314	0.318
Hypoxia	2.79	2.72		0.78
H + BTZ 5 nmol/L	2.10	1.78	1.33	0.67
H + BTZ 10 nmol/L	1.68	1.2	1.67	0.52

H, hypoxia; BTZ, bortezomib.

Table 2. Radiosensitization activity of bortezomib in SiHa cells

	D_0	D_q	SER	SF_2
control	2.078	1.226		0.5805
BTZ 5 nmol/L	1.619	1.175	1.283	0.507
BTZ 10 nmol/L	1.3065	0.967	1.59	0.4
Hypoxia	3.024	3.07		0.838
H + BTZ 5 nmol/L	1.977	1.84	1.529	0.67
H + BTZ 10 nmol/L	1.62	1.435	1.867	0.562

H, hypoxia; BTZ, bortezomib.

es and incubated in 5% CO_2 incubator at 37°C overnight. The cells were either treated or not with BTZ (5 nmol/l) under normoxia or hypoxia for 24 h. The cells were subjected to 4 Gy irradiation for 0.5, 2, 8, and 24 h and then fixed with immunol staining fix solution (Beyotime Institute of Biotechnology) at 37°C for 20 min. The cells were then treated with methyl alcohol of 4°C to rupture the membranes at -20°C for 30 min. The cells were blocked with a blocking buffer at room temperature for 1 h and then treated with phospho-H2AX S139 antibody (γH2AX, Millipore) overnight at 4°C before treating with Alexa 488 Fluor secondary antibody (Invitrogen, Carlsbad, CA) for 1.5 h at room temperature. The cell nuclei were then counterstained with DAPI for 15 min before mounting on Petri dishes. The images of the γH2AX foci were obtained by a laser scanning confocal microscope (Zeiss LSM510) to monitor the DNA DSBs in HeLa cells.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD) of triplicate independent assays, and the differences among groups were determined using student's *t* test. Statistical analysis was performed using SPSS statistical software system for Windows version 16.0 (SPSS Inc.), and *P* < 0.05 was considered statistically significant.

Results

*BTZ sensitizes cervical cancer cell lines to IR in hypoxia and normoxia*

To investigate the effect of BTZ on cervical cancer cell lines, we performed CCK8 assay of SiHa and HeLa cells at 24 and 48 h. The CCK8 assay showed that BTZ inhibited cell proliferation in a dose- and time-dependent manner (**Figure 1A**). At 48 h, the IC_{50} of BTZ for SiHa and HeLa cells were 33.94 and 40.06 nmol/l, respectively. Therefore, we chose the treatment dose of BTZ at a safe concentration (< IC_{50}) for the succeeding experiments.

We then performed clone formation assay of SiHa and HeLa cells to determine the influence of BTZ on the radiosensitivity of cervical cancer cells in hypoxia and normoxia. As illustrated by the survival curves (**Figure 1B** and **1C**) after IR, the clonogenic abilities of hypoxic cervical cancer cells were higher than that of normoxic cervical cancer cells. However, BTZ significantly reduced the clone-forming rates of the hypoxic cervical cancer cells when exposed to ionizing radiation. As shown in **Tables 1** and **2**, with BTZ treatment of 5 and 10 nmol/l, the SERs of hypoxic HeLa cells (1.33 and 1.67) increased more significantly than those of normoxic HeLa cells (1.17 and 1.31). A similar trend was observed for SiHa cells (SER = 1.28 and 1.59 for normoxia; SER = 1.53 and 1.87 for hypoxia). These data demonstrated that BTZ could enhance the radiosensitivity of hypoxic cervical cancer cells more remarkably than normoxic cervical cancer cells.

*BTZ promotes radiation-induced apoptosis of both normoxic and hypoxic cervical cancer cells*

Flow cytometry was performed to investigate whether the radiosensitivity of BTZ to cervical cancer cells is related to apoptosis induction. As illustrated by **Figure 2**, the apoptosis rates of the cervical cancer cells exposed to single IR in the hypoxia group was less compared with that in the normoxia group. The apoptosis rates' statistical differences between the single IR group and IR-combined high-dose (10 nmol/l)
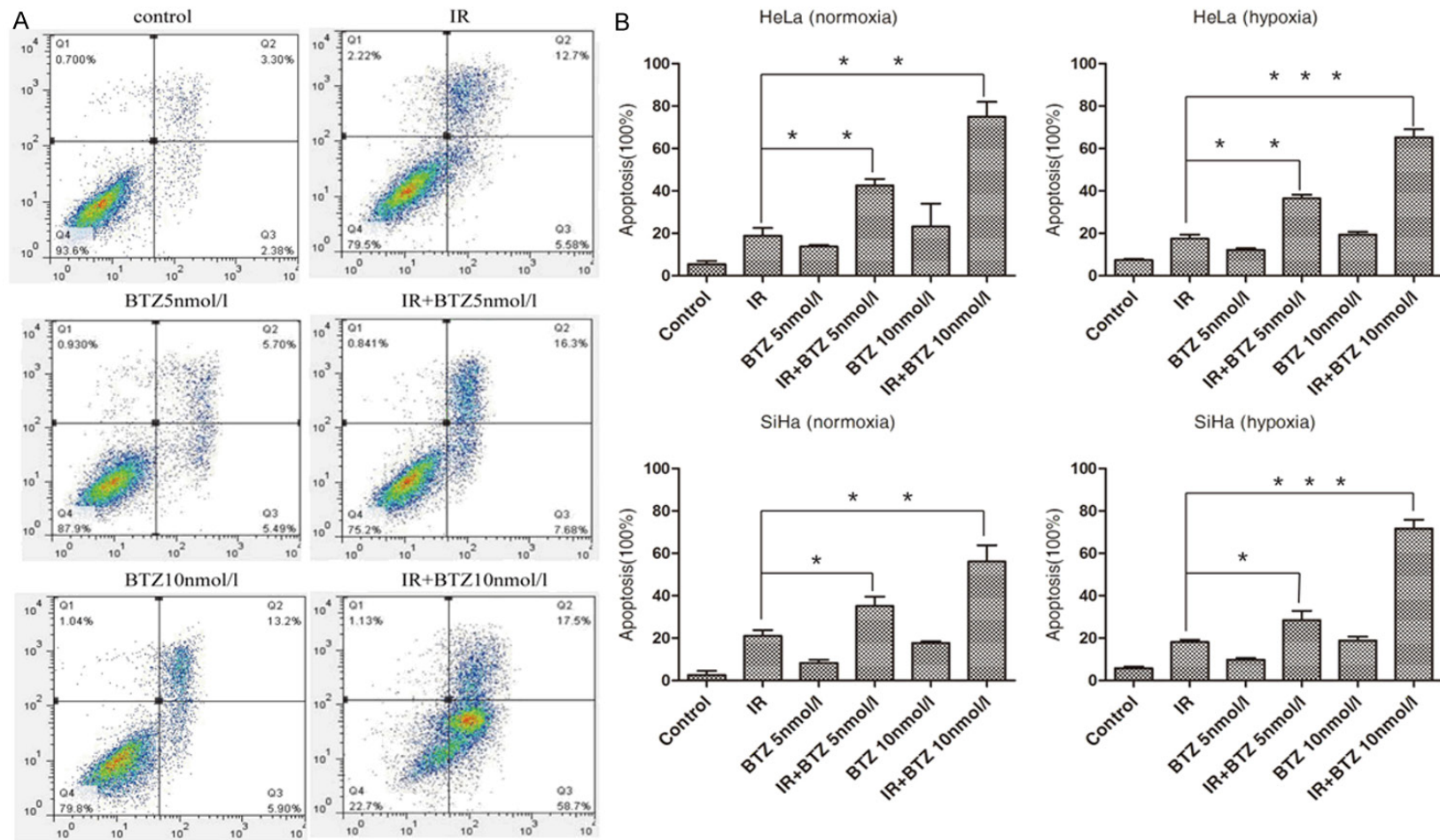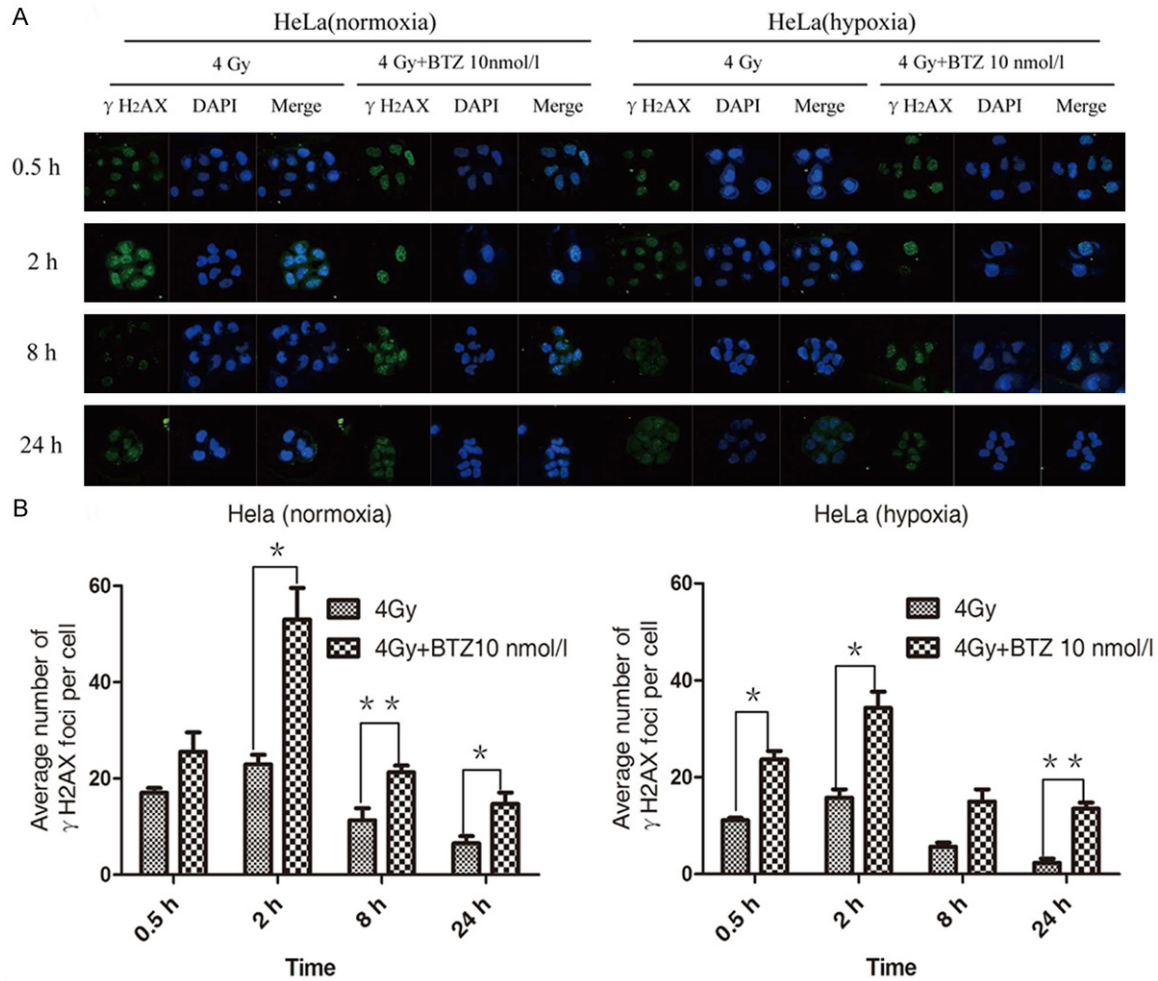
Figure 2. Bortezomib promotes radiation-induced apoptosis of both normoxic and hypoxic cervical cancer cells. A. Hypoxic HeLa cells. B. The bars present mean ± SD of three separate experiments. BTZ, Bortezomib. ***P < 0.001; **P < 0.01; *P < 0.05.
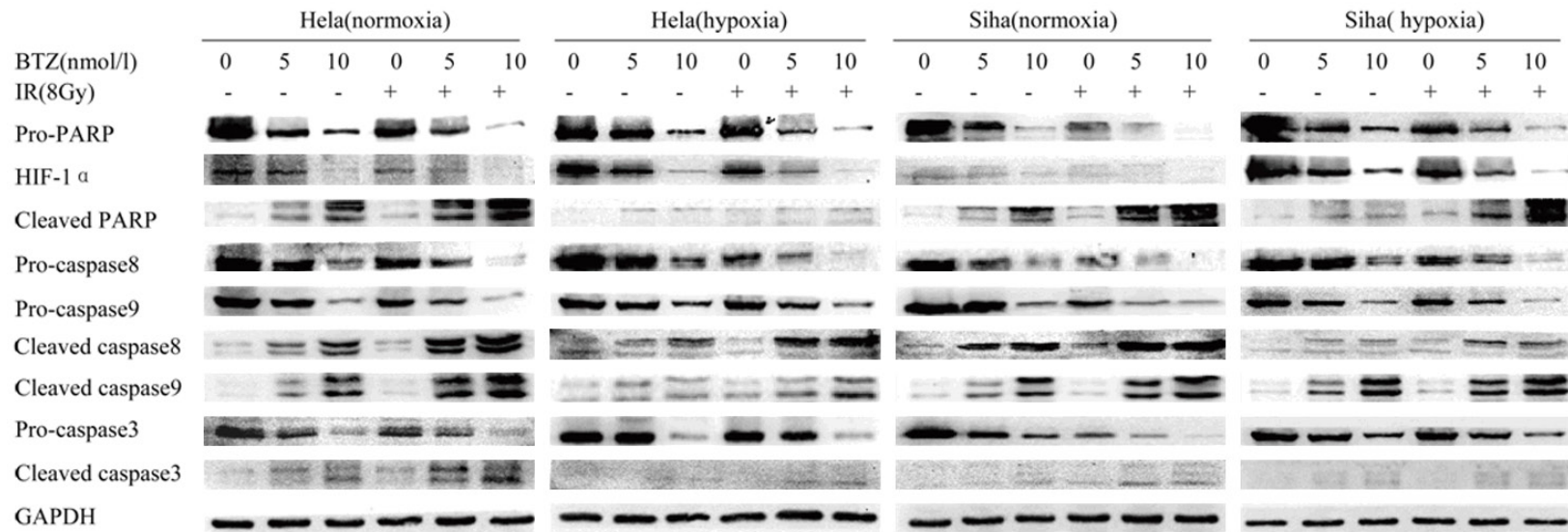
Figure 3. Bortezomib affects the expression of HIF-1α, caspase-3, -8, and -9, and PARP-1 in cervical cancer cells. The levels of HIF-1α, caspase-3, -8, and -9, and PARP-1 in normoxic and hypoxic cervical cancer cells were detected by Western blot analysis. Western blot showed that the drug could inhibit HIF-1α expression and increase caspase-3, -8, and -9 activation and PARP-1 cleavage.

Figure 4. Bortezomib reduces DSB repair after X-rays in cervical cancer cells. A. HeLa cells under normoxic and hypoxic were treated with DMSO or 10 nmol/l bortezomib prior to 4 Gy irradiation, stained with Phospho-H2AX antibody, and observed under a confocal microscope. DSBs were stained green, and the nuclei were stained blue. B. DSB repair assay was performed by counting Phospho-H2AX foci. DSBs were quantified at each time point as the average number of foci in 50 nuclei. The bars present mean ± SD of three separate experiments. BTZ, Bortezomib. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

BTZ group in the hypoxic cervical cancer cells ($P < 0.001$) were higher than in the normoxic cervical cancer cells ($P < 0.01$). However, when the drug dose was 5 nmol/l, the abovementioned statistical differences were similar in the hypoxic and normoxic cervical cancer cells (HeLa, $P < 0.01$; SiHa, $P < 0.05$).

BTZ affects the expression of HIF-1α, caspase-3, -8, and -9, and PARP-1 in cervical cancer cells

To evaluate whether BTZ influence on the radiosensitivity of hypoxic and normoxic cervical cancer cells was related with HIF-1α, caspase-3, -8, and -9, and PARP-1, we detected

their expressions by Western blot analysis (**Figure 3**).

When treated with BTZ or IR alone, the expressions of HIF-1α of the hypoxic cervical cancer cells were lower than that of the control group. With combined treatment, the expressions of HIF-1α decreased more significantly. Under normoxia, the expression of HIF-1α was low, and the tendency of HIF-1α expression was similar. The result showed that BTZ could remarkably reduce the hypoxia-related HIF-1α expression in cervical cancer cells.

Cleaved caspase-3, -8, and -9 and cleaved PARP-1 of hypoxic cervical cancer cells ex-

pressed higher in the cells treated with BTZ or IR alone than in the control group. With combined BTZ and IR treatment, the decrease in expression was more remarkable. A similar trend was observed under normoxia. The results indicate that BTZ could sensitize hypoxic and normoxic cervical cancer cells to IR and were related to promoting apoptosis-related caspase-3, -8, and -9 activation and DSB repair-related PARP-1 cleavage.

BTZ reduces DSB repair after X-rays in cervical cancer cells

To investigate the influence of BTZ on the DSB repair of hypoxic cervical cancer cells exposed to IR, we detected the γH2AX foci of HeLa cell at 0.5, 2, 8, and 24 h (**Figure 4**). At 2 h after IR, a peak γH2AX foci number of HeLa cell was observed. From 0.5 h to 24 h, the number of γH2AX foci reduced gradually, indicating DSB repair. Treatment with BTZ significantly increased the foci at each time point, suggesting delayed DSB repair. Meanwhile, the most evident statistical difference between combined treatment group and single IR group appeared at different time points in hypoxia (24 h) and normoxia (8 h). The above results meant that BTZ could inhibit DSB repair after X-rays.

Discussion

Radio-resistance decreases the clinical effect of cervical cancer, and one main reason is the hypoxia tumor microenvironment. Studies have extensively reported that this situation is related to the influence of apoptosis and DSB repair, and HIF-1α performs an important function in these mechanisms [20, 21]. In this study, we demonstrated for the first time that BTZ could sensitize hypoxic HeLa and SiHa cells to X-ray by repressing the expression of HIF-1α and regulating the expression of PARP-1 and caspase-3, -8, and -9.

BTZ is the first proteasome inhibitor anticancer drug that targets the ubiquitin-proteasome pathway. It is already applied to the clinical therapy of hematologic neoplasms. Preclinical studies of BTZ in solid tumors showed that the drug could induce many cancer cell apoptosis and sensitize them to other anticancer agents [22-24]. Shin et al. demonstrated that BTZ could inhibit tumor adaptation to hypoxia by repressing HIF-1α [25]. We used Western blot

analysis and found that BTZ enhances SiHa and HeLa cells' radiation sensitivity to IR by inhibiting HIF-1α expression, which meant that BTZ could reverse hypoxia-induced radiation resistance.

HIF-1α could reduce cell apoptosis by inhibiting caspase-3 activation [19, 26]. The flow cytometry results demonstrated that the radiosensitization of BTZ was related to apoptosis increase. To further explore the apoptotic pathway, Western blot analysis was carried out. When the two cells were exposed to both BTZ and IR, the activation of caspase-3, -8, and -9 all increased more remarkably than the single treatment. The trend in normoxia was similar but less remarkable. Therefore, BTZ sensitizes cervical cancer cells to IR through both endogenous and exogenous apoptotic pathways.

The main way of radiation-induced anticancer effect is DNA DSB [26]. As a DNA repairase, PARP-1 induces DSB repair. Meanwhile, PARP-1 is sniped by caspase-3 in vivo and signals apoptosis. Furthermore, a previous study reported that HIF-1α knockdown significantly increased PARP-1 cleavage at a high level [18]. Western blot showed that the trend of cleaved PARP-1 expression was similar with cleaved caspase-3. Immunofluorescence test could display a more intuitive visual representation of the DSB trend. The results suggested that BTZ sensitizes cervical cancer cells to IR in hypoxia situation by inhibiting DSB repair.

In conclusion, we demonstrated that BTZ could sensitize hypoxic cervical cancer cells to IR, and this finding was related to up-regulating the expression of cleaved caspase-3, -8, and -9 and cleaved PARP-1 by down-regulating the expression of HIF-1α. The mechanism included increasing cell apoptosis and inhibiting DSB repair. Therefore, the 26-proteasome inhibitor could be a radiosensitization agent to solid tumor, with HIF-1α as the key target. This research did not involve detection in an animal experiment. Hence, more investigations should be performed to determine the radiosensitization effect of BTZ in vivo and in vitro.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zhongming Wang, Department of Radiotherapy, The Second People's Hospital of Lianyungang, Lianyungang Hospital Affiliated to Bengbu Medical College, 161 Xingfu Road, Lianyungang, Jiangsu Province, China. Tel: 86-25-6813-5700; Fax: 86-25-6813-5700; E-mail: W_zhongm@163.com

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