Int J Clin Exp Med 2016;9(10):19381-19389
www.ijcem.com /ISSN:1940-5901/IJCEM0031974

Original Article

CK2 inhibitor CX4945 enhances the radiation sensitivity of human gastric cancer cells

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Received December 24, 2015; Accepted August 22, 2016; Epub October 15, 2016; Published October 30, 2016

Abstract: Chemotherapy and radiotherapy are often combined for treatment of cancers include gastric cancer. However, cancer cells are commonly developed cross-resistance to chemotherapy and radiotherapy and lead to the failure of treatment. Others and our previous results showed that XRCC1 plays important role in resistance to chemotherapy. CX4945, a first-in-class clinical stage inhibitor of CK2, was found to block the cisplatin induced DNA repair response by decreasing the phosphorylation of XRCC1. Here, the significantly cross-resistance to radiations of X-ray and UV-B were observed in two cisplatin resistant human gastric cancer cell lines. Our data showed that XRCC1 was a key regulator in GC cells responsible for cross-resistant to X-ray and UV-B radiation. Kncocking down XRCC1 expression by siRNA lead to more severe DNA damage. Moreover, Western bloting and immunofluorescence staining results showed that CX4945 enhanced DNA damage of GC cells induced by X-ray and UV-B radiation via inhibited phosphorylation of XRCC1. The colony formation and cytotoxic assay indicated that CX4945 sensitized cisplatin resistant GC cells to X-ray and UV-B radiation induced cytotoxicity. In summary, we reported for the first time that CX4945 is a valuable targeted drug for sensitizing radiotherapy in gastric cancer. These results revealed a precise mechanism of CX4945 in anti cisplatin resistant gastric cancer therapy and with potential clinical significance.

Keywords: CX4945, chemotherapy, radiotherapy, resistance, gastric cancer

Introduction

Gastric cancer (GC) is the fifth most common human malignant tumor worldwide and the third cause of cancer death. Current strategies for treatment of gastric cancer include surgery, chemotherapy and radiotherapy. Cisplatin, a widely used chemotherapeutic drug for treating gastric cancer [1], kills cancer cells by inducing DNA double strand breaks (DSB) through cross-linking of the DNA [2]. However, cancer cells often develop enhanced DNA repair capacity to overcome cisplatin-induced DNA damage, leading to drug resistance [3]. Radiotherapy is employed to kill cancer cells also by inducing DNA double strand breaks. Due to the cross-resistance to chemotherapy and radiotherapy, relapse and metastasis are common and result in high mortality of gastric cancer [4-6].

XRCC1 (X-ray repair cross complementing group1) is the first mammalian gene identified that facilitates protection of the cells from the effects of ionizing radiation [7]. XRCC1 is a genetic biomarker of therapeutic radiation sensitivity [8, 9]. However, XRCC1 abrogation increases the cisplatin cytotoxicity and cisplatin-induced DNA damage in HepG2 cells [10]. These evidences indicate a critical role of XRCC1 in the cross-resistance to cisplatin and radiotherapy.

CK2 (casein kinase 2) appears to play a central role in the regulation of gene expression and protein synthesis/degradation by acting at different levels on the complex apoptotic machinery [11]. Moreover, CK2 phosphorylates XRCC1 at 518S/519T/523T sites and is required for its stability and efficient DNA repair [12]. CX4945,

a first-in-class clinical stage inhibitor of CK2, was found to block the cisplatin-induced DNA repair response by decreasing the phosphorylation of XRCC1 at CK2-specific phosphorylation sites [13, 14].

Recently, we reported that overexpression of XRCC1 contributes to cisplatin resistance in gastric cancer cells and showed that XRCC1 protein is important for effective repair of cisplatin induced DSBs in gastric cancer cells [15]. Moreover, we found up-regulated levels of CK2 and P-XRCC1 (518S/519T/523T) in cisplatin resistant cells, and CX4945 selectively kills cisplatin resistant cells by inhibiting CK2 activated p-XRCC1 (518S/519T/523T) phosphorylation [16]. However, whether CX4945 sensitizes cisplatin resistant cells to radiotherapy was still unknown.

The objective of the present study was to investigate the role of CX4945 in radiotherapy sensitivity. Our results demonstrated that CX4945 sensitized cisplatin resistant cells to radiotherapy by inhibiting the function of XRCC1, and therefore leading to more DNA damage. CK2 and XRCC1 protein levels were negatively correlated with the efficacy of radiotherapy. These findings indicated a potential value of CX4945 for cancer comprehensive therapy.

Materials and methods

Cell lines and culture

Human gastric cancer cell lines BGC823 and SGC7901 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All the cells were cultured in RPMI 1640 medium supplemented with 10% of fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Life Technologies/Gibco, Grand Island, NY, USA). The cells were grown at 37°C in a humidified incubator with 5% CO_2. Cisplatin was obtained from Sigma-Aldrich (St. Louis, MO, USA). The cisplatin-resistant BGC823/DDP cells were developed from the parental BGC823 cells that were subjected to persistent gradient exposure to cisplatin for about 12 month, through increasing cisplatin concentration from 0.05 μg/ml until the cells acquired resistance to 1 μg/ml. The cisplatin-resistant SGC7901/DDP cells were obtained by the same procedure. Prior to each experiment, BGC823/DDP and SGC7901/DDP cells were cultured in drug-free RPMI 1640 medium for 2 weeks.

Plasmids and transfection

The GFP-XRCC1 plasmid was constructed from the RFP-XRCC1 plasmid that has been described previously [17]. Small interfering RNA (siRNA) specific for XRCC1 (5'-GGU CCU UCU AUA UCU GUA A dTdT-3') and nonspecific control siRNA were synthesized (Ribobio, Guangzhou, China). The plasmid DNA or siRNA was transfected into cells with Lipofectamine 2000 according to manufacturer's instructions.

Cytotoxicity assay

One day before treatment, BGC823, SGC7901, BGC823/DDP, SGC7901/DDP cells were plated at a density of 5000 cells per well in 96-well plates. The cells were treated with various concentrations of CX4945 (selleck, shanghai, China). After 24 h, the cell viability was determined using Cell Counting Kit-8 (CCK-8) according to the manufacturer's instructions (Dojindo, Kumamoto, Japan). The cell survival curves were plotted by Graph Pad Prism software (La Jolla, CA, USA).

Irradiation treatment of cells

For X-Ray radiation, the exponentially growing cells were seeded in the culture flasks or culture dishes. When the cellular confluence reached about 60%, the cells were treated with different single doses of 6-MV X-ray (0, 2, 4, 6, 8, and 10 Gy) at a dose rate of 200 cGy/min. Then the cells were used for other experiments. For UV-B radiation, before exposure, cell culture medium was removed; the cells were then rinsed once with phosphate-buffered saline (PBS 1X) and covered with a thin PBS layer. These prepared cells were irradiated for a period of time 100 J/m^2 for UVB.

Clonogenic assay

For colony formation assays, after 24 h of CX4945 treatment, cells were treated with different single doses of 6-MV X-ray, and then seeded into the six-well culture plates (different numbers of cells were used-600, 600, 800, 2000, and 6000 cells for irradiation doses 0, 2, 4, 6, and 8 Gy). Three replicates of each sample were performed. Two weeks later, all the culture plates were fixed with methanol and stained with crystal violet. Colonies containing >50 cells were counted under inverted microscope. Then the plating efficiency (PE) of non-irradiat-

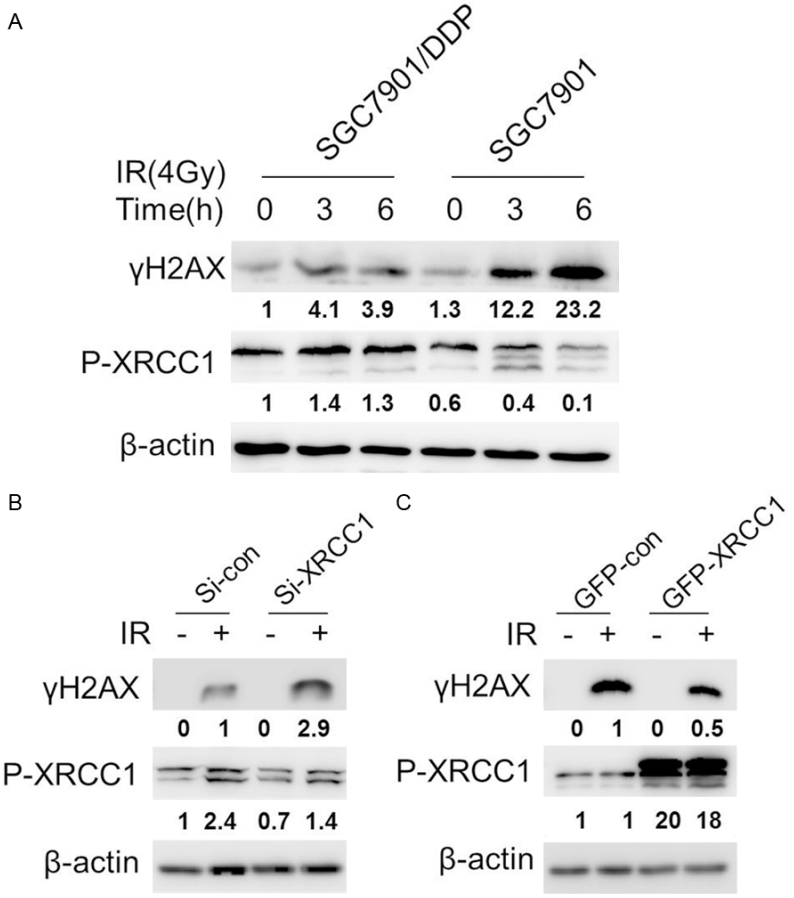

Figure 1. XRCC1 is required for DNA repairing of X-ray radiation induced DSBs. A: The SGC7901 and SGC7901/DDP cells were exposure to 4 Gy of X-ray radiation; after 3 h and 6 h, the Western blotting was used to determine the expression of γH2AX and P-XRCC1. B: The SGC7901/DDP cells were transfected with si-XRCC1 for 48 h and followed by exposure to 4 Gy of X-Ray radiation; after 6 h, the Western blotting was used to determine the expression of γH2AX and P-XRCC1. C: The SGC7901 cells were transfected with GFP-XRCC1 for 48 h and followed by exposure to 4 Gy of X-ray radiation; after 6 h, the Western blotting was used to determine the expression of γH2AX and P-XRCC1.

ed control cells and surviving fraction (SF) of each sample were calculated using the following equation:

PE (plating efficiency) = number of colonies counted/number of cells seeded ×100%

SF (surviving fraction) = number of colonies counted/number of cells seeded ×1/PE

Then the cell survival curve was obtained using the model of multi-target single-hit (SF=1-(1-e-D/D0)N) after analysis with GraphPad Prism 5 software. Radiobiological parameters were calculated: D0 (the dose required to reduce survival to 37% of its value), Dq (quasithreshold dose), N (extrapolation number), and SF2 (surviving fraction at 2 Gy). The assays were repeated at least three times.

Western blotting

Western blot analyses were performed as previously described [18]. The antibodies used were as follows: monoclonal anti-β-actin; monoclonal anti-γH2AX (1:2000, Epitomics, Burlingame, CA, USA); monoclonal anti-P-XRCC1 (518S/519T/523T) (Bethyl Laboratories, Montgomery, TX, USA).

Indirect immunofluorescence microscopy

Indirect immunofluorescence staining assays were performed as previously reported [19]. The cells were incubated with anti-γH2AX mouse monoclonal antibody at a 1:200 dilution. The confocal images of cells were sequentially acquired with Zeiss AIM software on a Zeiss LSM 700 confocal microscope system (Carl Zeiss Jena, Oberkochen, Germany).

Statistical analysis

Data were expressed as the mean ± SD. The statistical significance of the differences between the cell lines was determined by the parametric unpaired *Student's t test*. Differences were considered significant when $P<0.05$.

Results

XRCC1 is required for cross-resistance to cisplatin and radiotherapy in human gastric cancer cells

Recently, we have established two cisplatin resistant gastric cancer cell lines BGC823/DDP cells and SGC7901/DDP. The cisplatin resistant characteristic of these cells has been reported [15, 16, 20]. Here, we investigated

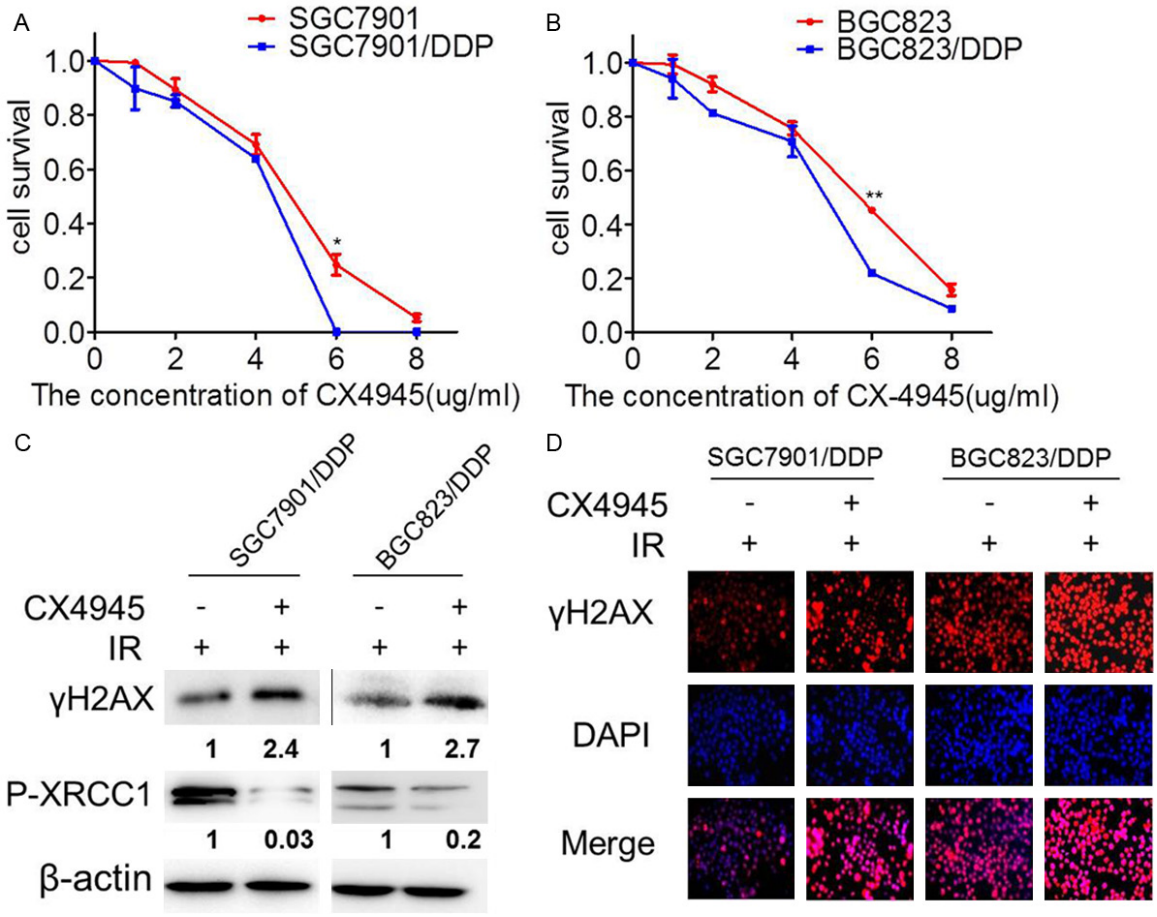

Figure 2. CX-4945 enhances DNA damage induced by X-ray radiation via inhibits phosphorylation of XRCC1. A and B: The cell viability was determined by exposure of SGC7901, SGC7901/DDP, BGC823 and BGC823/DDP cells to CX-4945 for 24 h (*P<0.05, **P<0.01). C: The SGC7901/DDP and BGC823/DDP cells were treated with 2 μg/ml CX4945 for 24 h and followed by exposure to 4 Gy of X-ray radiation; after 6 h, the Western blotting was used to determine the expression of γH2AX and P-XRCC1. D: The SGC7901/DDP and BGC823/DDP cells were treated with 2 μg/ml CX4945 for 24 h and followed by exposure to 4 Gy of X-ray radiation; after 6 h to give γH2AX foci staining.

whether these cisplatin resistant cells cross-resistant to the radiotherapy. X-ray radiation was employed to treat cells. As a result, the DNA double strand breaks bio-marker γH2AX (phosphorylated histone H2AX) protein levels were elevated 12.2-fold or 23.2-fold after 3 or 6 h expsoure to 4 Gy of X-ray in cisplatin sensitive SGC7901 gastric cells, respectively. The P-XRCC1 had 1.5- or 6-flod inhibition after 3 or 6 h exposure to 4 Gy of X-ray in SGC7901 cells. In contrast, γH2AX protein levels were found a slight elevation in cisplatin resistant SGC7901/DDP gastric cells afterexposure to 4 Gy of X-ray 3 h (4.1-fold) or 6 h (3.9-fold). Similary, P-XRCC1 protein levels were up-regulated about 1.4- or 1.3-fold after 3 or 6 h exposure (**Figure 1A**). Knocking down of XRCC1 expression by transfection of XRCC1 siRNA resulted in decreased P-XRCC1 protein levels, but increased X-ray (4 Gy) generated γH2AX about 2.9-fold in SGC-7901/DDP cells (**Figure 1B**). In contrast, overexpression of XRCC1 increased P-XRCC1 protein levels, but decreased X-ray exposure (4 Gy) generatred γH2AX about 2-fold in SGC7901 cells (**Figure 1C**). These results confirmed that XRCC1 is required for repairing of X-ray radiation-induced DSBs and plays a critical role in human gastric cancer cells cross-resistance to cisplatin and radiotherapy.

CX4945 enhances X-ray radiation induced DNA damage via inhibits phosphorylation of XRCC1.

We have reported that CK2 is overexpressed in cisplatin resistant gastric cancer cells, and CK2

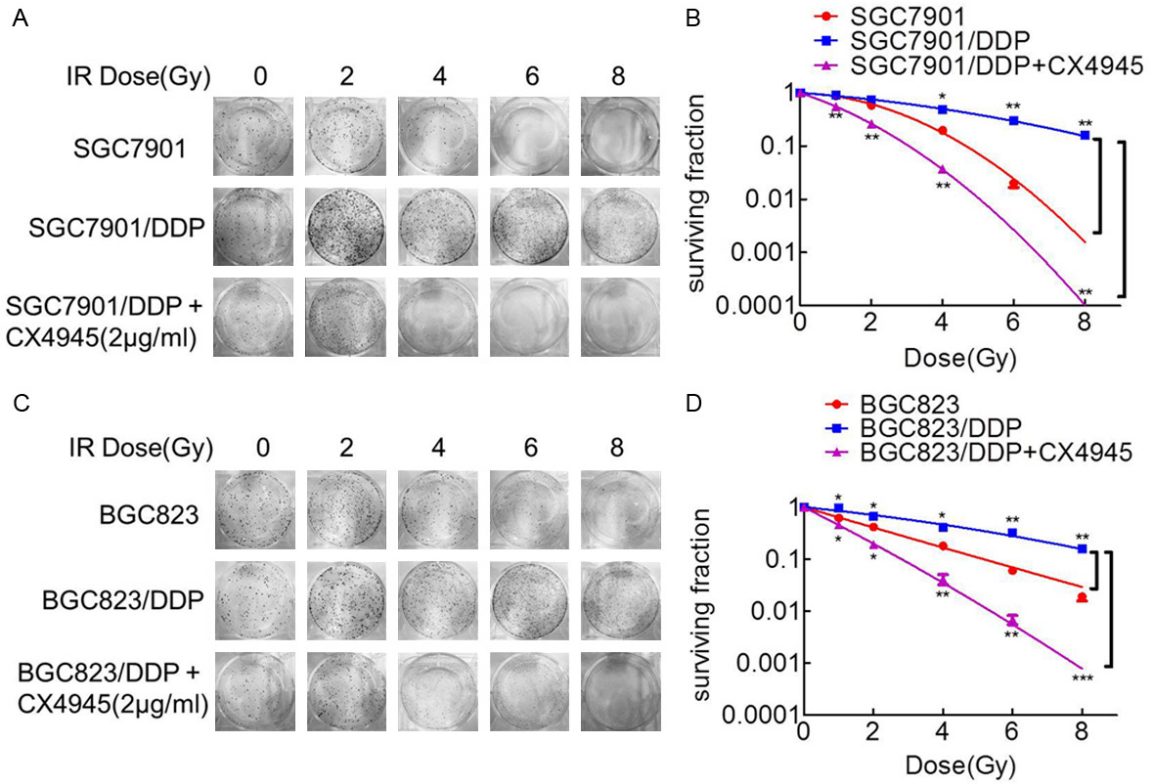

Figure 3. CX-4945 sensitizes cisplatin resistant cells to X-ray radiation induced cytotoxicity. A and C: Representative crystal violet staining of the colonies formed by SGC7901/DDP and BGC823/DDP cells. B and D: Survival curves of respective cell lines were established by the colony formation assays. The results were presented as means ± SD of values obtained in at least three independent experiments. (*P<0.05, **P<0.01, ***P<0.001).

inhibitor CX4945 sensitizes these cells to cisplatin [16]. However, whether CX4945 could also sensitize these cells to radiation was still unknown. Here, the cellular viability was determined by the CCK-8 assay in both cisplatin sensitive and resistant gastric cancer cells treated with CX4945. In accordance with our previous results, cisplatin resistant cells SGC7901/DDP and BGC823/DDP were more sensitive to CX4945 than their parental cisplatin sensitive cells SGC7901 and BGC823 (**Figure 2A**, **2B**). When the SGC7901/DDP cells and BGC823/DDP cells were treated with a combination of 2 μg/ml of CX4945 and 4 Gy of X-Ray, the levels of p-XRCC1 were 33-fold inhibited in SGC7901/DDP cells and 5-fold inhibited in BGC823/DDP cells compared to these cells treated with 4 Gy of X-Ray alone. At the same time, γH2AX protein levels were enhanced 2.4-fold or 2.7-fold in combined treatment in SGC7901/DDP cells or in BGC823/DDP cells compared to these cells treated with 4 Gy of X-Ray alone (**Figure 2C**). In addition, there were much more γH2AX positive cells in the combined treatment groups compared to the cells treated with 4 Gy of X-ray alone (**Figure 2D**). Indicating that CX4945 enhances X-ray radiation induced DNA damage via blocking the phosphorylation of XRCC1.

CX4945 sensitizes cisplatin resistant cells to radiation induced cytotoxicity

To further understand if CX4945 could enhance the toxcicity of X-ray to cisplatin resistant gastric cancer cells, we completed colony formation assays in both cisplatin sensitive and resistant cells. The results showed that the surviving fractions of cisplatin resistant cells SGC7901/DDP and BGC823/DDP after exposure to 2, 4, 6, or 8 Gy of X-Ray radiation were much higher than their parental cisplatin sensitive SGC7901 and BGC823 cells. In line with CX4945 enhances cisplatin induced DNA damage, the colony numbers were significantly decreased in SGC7901/DDP and BGC823/DDP cells by treatment with a combination of 2 μg/ml of CX4945 and different doses of X-ray radiation (**Figure 3A-D**). These results indicating that

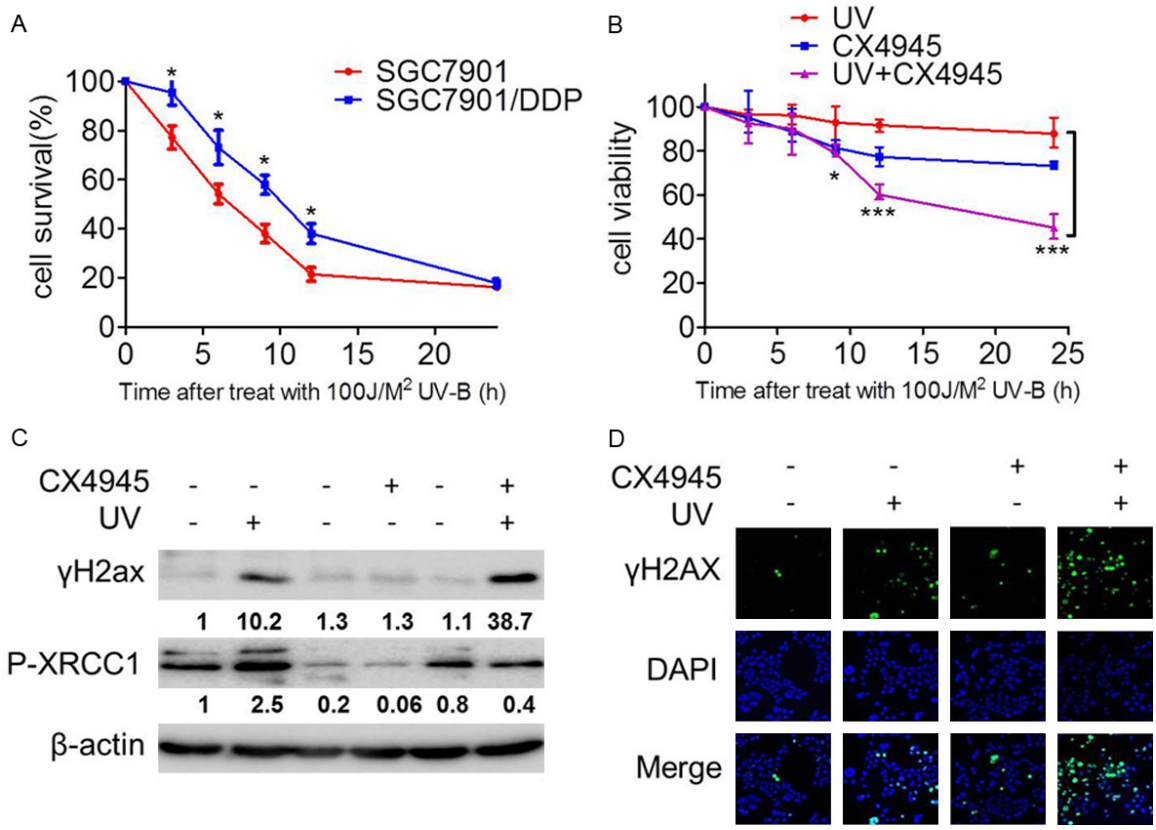

Figure 4. CX-4945 sensitized cisplatin resistant cells to UV-B radiation induced cytotoxicity. A: The cell viability was determined after exposure of SGC7901, SGC7901/DDP to 100 J/m^2 of UV-B radiation (*P<0.05). B: The SGC7901/DDP cells were treated with 2 μg/ml CX-4945 for 24 h and followed by exposure to 100 J/m^2 of UV-B radiation, then the cell viability was determined (*P<0.05, ***P<0.001). C: The SGC7901/DDP cells were treated with 2 μg/ml CX-4945 for 24 h and followed by exposure to 100 J/m^2 of UV-B radiation, after 6 h, the western blotting was used to determine the expression of γH2AX and P-XRCC1. D: The SGC7901/DDP cells were treated with 2 μg/ml CX-4945 for 24 h and followed by exposure to 100 J/m^2 of UV-B radiation, after 6 h to give γH2AX foci staining.

CX4945 increases cytotoxicity of cisplatin resistant cells to X-ray radiation.

Next, we detected whether CX4945 sensitized radiation was X-ray specificity. UV-B radiation is known to induce DSBs, was used to treat these cisplatin resistant gastric cancer cells. The data showed that after treated with 100 J/m^2 of UV-B survival rates of SGC7901/DDP cells were higher than the rates of SGC7901 cells (**Figure 4A**). However, The UV-B induced cytotoxicity was increased in SGC7901/DDP cells exposure to a combination of 2 μg/ml of CX4945 and 100 J/m^2 of UV-B (**Figure 4B**). In line with these, γH2AX protein levels were increased about 10.2-fold after treatment with 100 J/m^2 alone. However, γH2AX were increased 38.7-fold in SGC7901/DDP cells after treated with a combination of 2 μg/ml of CX4945 and 100 J/m^2 of UV-B (**Figure 4C**). Similarly, there were much more γH2AX positive cells in the combined group compared to the cells treated with UV-B alone (**Figure 4D**). Indicating that CX4945 also sensitizes cisplatin resistant gastric cancer cells to UV exposure.

Discussion

Cisplatin is one of the most potent chemotherapeutics widely used in cancer chemotherapy. The anti-cancer mechanism of cisplatin is attributed to the platinum-DNA adducts induced DNA double strand breaks. Cisplatin combined with other modalities like radiotherapy is routinely used in clinic to treat advanced gastric cancer. However, cancer cells could develop several mechanisms including enhanced DNA repair to anti-cisplatin induced cell death, leading to cisplatin resistance. It seems that cross-resistance to radiotherapy is ines-

capability due to the radiation also induces DNA double strand breaks [21, 22]. Here, we treated cisplatin resistant gastric cancer cells with X-ray or UV-B radiation. Our results indicated that cisplatin resistant gastric cancer cells were cross-resistant to X-ray and UV-B radiation induced DNA damage and cell death. There is a challenge to develop new therapeutic strategy to reverse the cross-resistance.

XRCC1 is the key mediator response for radiation induced DNA damage [7]. Increasing evidence is mounting on the important role of XRCC1 in cisplatin resistance by others and our study [15, 23]. The phosphorylation of XRCC1 is required for its protein stability [24]. These evidences promote us to investigate the role of XRCC1 in cisplatin resistant cells cross-resistant to radiation induced DNA damage. Our data showed that knockdown of XRCC1 in the cisplatin-resistant cells resulted in increased γH2AX levels. On the contrary, overexpression of XRCC1 in cisplatin sensitive cells resulted in reduction of γH2AX levels when the cells were treated with X-ray radiation. These results indicate that XRCC1 plays a key role in cross-resistance to radiation and cisplatin in gastric cancer.

It is reported that phosphorylation of XRCC1 by CK2 is required for its efficacy on DNA repair where de-phoshorylated XRCC1 is ubiquitinylated by an E3 ubiquitin ligase and degraded by the proteasomal machinery [25]. The CX4945, a selective small molecule inhibitor of CK2 in clinical trial, blocks the cisplatin-induced DNA repair response by decreasing phosphorylation of XRCC1 at CK2-specific phosphorylation sites [26]. CX4945 has employed to treat various cancers include leukemia, non-small cell lung cancer and hematological malignancies [27-29]. Our previous data showed that CX4945 selectivity killed cisplatin resistant cells according to the levels of CK2α. Moreover, a synergistic effect of CX4945 combined with cisplatin triggered DNA damage in cisplatin resistant cells [16]. In the present srudy, we provided new evidences that treatment with CX4945 in the cisplatin-resistant cells resulted in higher sensitivity to X-ray and UV-B radiation. Mechanism study showed that P-XRCC1 was inhibited by treated with CX4945, lead to increase γH2AX levels.

Taken together, we report for the first time that CX4945 is a valuable targeted drug for sensitizing radiotherapy in gastric cancer. These finding suggest that CX4945 in gastric cancer may be of clinical significance.

Acknowledgements

This study was supported in part by the Natural Science Foundation of Jiangsu Higher Education Institutions (13KJA330001), the National Natural Science Foundation of China (8152-0108027), and the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions.

Disclosure of conflict of interest

None.

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