Dichloroacetate enhances the cytotoxic effect of Cisplatin via decreasing the level of FOXM1 in prostate cancer

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Abstract: Improving the effectiveness of chemotherapy is a promising strategy for tumor patients. Recent studies have proved that Dichloroacetate (DCA) could dramatically enhance the anti-tumor effect of cisplatin in several tumor cells including prostate cancer, but the detailed mechanisms still need to be further investigated. In the present study, we use CCK-8 assay and Trypan blue exclusion assay to evaluate the anti-tumor effect, while we utilized western bolt to detect the change of molecular protein level. Interestingly, we at the first time found that DCA enhances the cytotoxic effects of cisplatin via decreasing the level of FOXM1 in prostate cancer. The down-regulation of FOXM1 leads to increased ROS in prostate cancer cells, which may be an important mechanism for the synergistic effect of Dichloroacetate and Cisplatin. In conclusion, our study demonstrated that targeting FOXM1 by DCA could efficaciously enhance the cytotoxic effects of Cisplatin in prostate cancer.

Keywords: Dichloroacetate, cytotoxic effect, cisplatin, FOXM1

Introduction

Prostate cancer is a common malignant neoplasm in male urinary system with poor prognosis. At the present time, chemotherapy is one of the primary treatment strategies for Prostate cancer. Among the chemotherapeutic drugs, Cisplatin is one that commonly used in clinic. However, parts of patients were not sensitive to Cisplatin treatment, which really need to explore new methods so as to improve the anti-tumor effect of Cisplatin.

Dichloroacetate (DCA) is a classical drug used in clinic for the treatment of lactic acidosis. Recent studies have demonstrated that DCA possessed potential anti-tumor capacity in kinds of tumors [1]. DCA alone treatment can induce cell apoptosis and inhibit cell proliferation in breast cancer cells, endometrial cancer cells, glioblastoma cancer cells and etc [2-4]. Moreover, DCA presented excellent synergistic effect with radiotherapy and chemotherapy drugs including Cisplatin [5-9]. Until now, the related anti-tumor mechanism of DCA is still not clear, which need further studies.

Forkhead transcription factor 1 (FOXM1), a member of the Forkhead family, plays an important role in cell division process via regulating cell cycle-dependent gene expression. Accumulating evidences have confirmed that is dramatically increased in various carcinomas, which is tightly associated with tumorigenesis, tumor progression and tumor therapy. Targeting FOXM1 may be a promising therapeutic strategy for neoplasia [10]. Previous studied have found that reducing FOXM1 expression could significantly enhance the cytotoxicity of Cisplatin, which may be an available method to overcome cisplatin resistance in clinic [11-14].

In this study, we demonstrated that DCA dramatically strengthened the cytotoxicity of Cisplatin in prostate cancer cells. The combination of DCA and cisplatin induced a reduction of FOXM1 level, which leads to an increased ROS. Our search provides a new insight for the anti-tumor effect of cisplatin plus DCA, which may be beneficial for its further clinical application.

Materials and methods

Cell culture

Human prostate cancer cell line PC3 and DU145 were purchased from American Type Culture Collection. The cells were cultured in
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DMEM medium (SH30022.01B, HyClone) containing 10% FBS (16000044, Gibco) and streptomycin (100 μg/mL) and penicillin (100 U/mL).

**Reagents**

Cell counting kit-8 (CCK-8) was obtained from Dojindo Laboratories. Dichloroacetate, Cisplatin and N-acetyl-L-cysteine were purchased from Sigma-Aldrich (Louis, MO, USA). Lipofectamin 2000 was from Invitrogen (Carlsbad, CA, USA). The primary antibodies of FOXM1, cleaved-caspase3 and cleaved-caspase9 were purchased from Abcam (Cambridge, UK); the antibodies against tublin were from Cell Signaling Technology (Boston, MA, USA). HRP-conjugated goat anti-rabbit and anti-mouse IgG were from KangChen Bio-tech (Shanghai, China).

**Cell viability detection**

The cell viability was assessed using CCK-8 assay according to the manufacturer's protocol. Briefly, PC3 and DU145 were plated in a 96-well plate at the density of 1×10^3 cells/well. After growing overnight, the cells were treated with indicated drugs and then the CCK-8 reagent was added to each well at the dose of 10 μL/well. Subsequently, the plate was incubated in the darkness for 2 h at 37°C. At last, the OD 450 value of each well was measured by Molecular Devices SpectraMax.

**Trypan blue exclusion assay**

PC3 and DU145 were seeded in a 6-well plate and cultured overnight. Then cells were treated with different drugs. Subsequently, the treated cells including adhering and suspended cells were all collected in PBS. Then the cells were incubated with trypan blue dye at room temperature for 3 min. Finally, the cells were observed under the optical microscope and the dyeing cells (blue cells) were considered as dead cells. The ratio of cell death was calculated as the percentage of dead cells in total cells.

**Real-time PCR**

Total RNA was extracted from the treated cells and quantified using Bioanalyzer. Then the cDNAs were obtained from the Total RNA using the method of reverse transcription. The primers for the FoxM1 gene were 5'-TGCAGCTAGGGATGTGAATCTTC-3' and 3'-GGAGCCAGTCC-ATCAGAICT-5'. Real-Time PCR with SYBR Green detection was carried out according to the standard protocol. The specificity of amplification was evaluated using the melting curve.

**Western blotting analysis**

The total proteins were extracted using whole-cell protein lysates and loaded for SDS-polyacrylamide gel electrophoreses. Then the protein was transferred to a PVDF membrane. Subsequently, the PVDF membrane were separately incubated with TBST containing 5% albumin, primary antibodies against FOXM1 (1:2000), Cleaved caspase9 (1:1000), Cleaved caspase3 (1:500) and GAPDH (1:5000) and goat anti-rabbit and anti-mouse IgG. Finally, Chemiluminescent Peroxidase Substrate was added on the membrane and the lanes were visualized.

**ROS detection**

The treated cells were washed by PBS and incubated with DCFH-DA fluorescent probes in darkness at 37°C for 20 min. Then the cells were washed by PBS and the fluorescence at excitation wavelength of 488 nm and emission wavelength of 525 nm were measured by Fluorescence microplate reader. The fold induction of ROS was calculated using the fluorescent value that deducting background value.

**Statistical analysis**

All the experiments in our study were performed more than 3 times. One-way ANOVA was used to analyze the statistical differences. All the data were expressed as the mean ± SD. The p-value of equal to or less than 0.05 was considered as statistically significant.

**Results**

*Dichloroacetate enhances the cytotoxic effects of Cisplatin in prostate cancer cells*

We firstly evaluate the anti-tumor effect of the combined Dichloroacetate and Cisplatin in prostate cancer cells. As shown in Figure 1A, the cell viability dramatically decreased in the group with the combined treatment compared to that with single drug treatment in both PC3 and DU145 cells. Similarly, Dichloroacetate significantly promoted cell death that induced by
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Cisplatin (Figure 1B). To further investigate the type of cell death, TUNEL apoptosis assay was used to detect the apoptotic cells in the study. As shown in Figure 1C, Dichloroacetate alone treatment didn’t increase the TUNEL-positive apoptotic cells in PC3 cells, while it obviously enhanced Cisplatin-induced apoptosis. Moreover, the apoptosis-related proteins cleaved caspase3 and caspase9 were detected using western blot. The results showed that the combination of Dichloroacetate and Cisplatin dramatically promoted the cleavage of caspase3 and caspase9, which indicated an enhanced mitochondrial apoptosis in this treatment. Taken together, Dichloroacetate indeed enhances the cytotoxic effect of Cisplatin in prostate cancer cells.

Targeting FOXM1 increases the sensitivity of prostate cancer cells to cisplatin

Previous studies have demonstrated that the level of FoxM1 is tightly associated with cisplatin resistance in several tumors and FOXM1 depletion could recover the sensitivity of tumor cells to cisplatin. In this study, we found that cisplatin could significantly up-regulated the protein level of FOXM1 in PC3 cells (Figure 2A). To further research the role of increased FOXM1, we made a screening for small interfering RNAs and choose the optimal sequence that targeting FOXM1 (Figure 2B). As shown in Figure 2C and 2D, knockdown FOXM1 by siRNA remarkably reduced the cell viability and promoted cell death in PC3 cells with cisplatin treatment. There data indicated that the increased expression of FOXM1 is tightly related to the sensitivity of prostate cancer cells to cisplatin and targeting FOXM1 could enhance the anti-tumor effect of cisplatin.

The synergetic effect of Dichloroacetate and cisplatin was partially dependent on the decrease of FOXM1

The down-regulation of FOXM1 leads to increased ROS in PC3 cells with the combined treatment of Dichloroacetate and cisplatin

Previous research has demonstrated that FOXM1 can regulate ROS level, and ROS could be an important factor to cell death. Therefore, we further investigated whether FOXM1 worked
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Figure 2. Targeting FOXM1 increases the sensitivity of prostate cancer cells to Cisplatin. A: PC3 cells were treated with different concentrations (μg/ml) of Cisplatin and then the protein level of FOXM1 was measured by western blot. B: PC3 cells were transfected with control siRNA sequence and different FOXM1 siRNA sequences. Subsequently, the mRNA level of FOXM1 was examined by real-time PCR. *, P<0.05; **, P<0.01. C: PC3 were transfected with control siRNA sequence and FOXM1 siRNA sequence, then the cells were treated with different concentrations (μg/mL) of Cisplatin. At last, the cell viability was detected by CCK-8 assay. *, P<0.05. D: PC3 were transfected with control siRNA sequence and FOXM1 siRNA sequence and then the cells were treated with 4 μg/mL of Cisplatin. Then the ratio of total cell death were detected by trypan blue exclusion assay. *, P<0.05.

Figure 3. The synergetic effect of Dichloroacetate and Cisplatin was partially dependent on the decrease of FOXM1. A: PC3 cells were treated with different concentrations (μg/ml) of DCA and then the protein level of FOXM1 was measured by western blot. B: PC3 cells were treated with or without 4 μg/ml Cisplatin in the presence or absence of 5 mM DCA, then the protein level of FOXM1 were measured by western blot. C and D) PC3 were transfected with pcMV-control plasmid and pcMV-FOXM1 plasmid. Subsequently, the cells were treated with Cisplatin and DCA. Ultimately, the cell viability and the ratio of total cell death were separately measured by CCK-8 assay (C) and trypan blue exclusion assay (D), *, P<0.05.

via modulating ROS in the cells with the combination treatment. As shown in Figure 4A, Dichloroacetate can enhance Cisplatin-mediated up-regulation of ROS, while antioxidant NAC attenuated the synergistic anti-tumor effect of Cisplatin plus Dichloroacetate (Figures 3C and 4B). These data suggested that elevatory ROS in the combination treatment could contribute to cell death. Moreover, Overexpression of FOXM1 can inhibit the increase of ROS in PC3 cells with Cisplatin and Dichloroacetate co-treatment. Collectively, Dichloroacetate increase ROS level in Cisplatin-treated prostate cancer cells via reducing FOXM1 expression, which lead to cell death.

Discussion

Cisplatin resistance is a common occurrence in clinic for cancer patients and the related mechanisms have been paid much attention nowadays. Recent studies have identified that increased expression of FOXM1 is an important mechanism of acquired cisplatin resistance in several tumors including breast cancer and non-small cell lung cancer [11, 14, 15]. Our studies demonstrated that inhibition of FOXM1 could significantly enhance the anti-tumor effect of Cisplatin in prostate cancer cells, which is consistent with the previous reports [11, 13-15]. FOXM1 expression in prostate cancer cells is dramatically upregulated that is tightly related to the carcinogenesis and progression of prostate cancer.
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As an anti-metabolic drug, DCA can repress aerobic glycolysis of tumors via regulating the activity of pyruvate dehydrogenase kinase (PDK) so as to block the primary energy supplement, which ultimately affected the survival of tumor cells. Apart from the function of blocking tumor’s glycolytic, DCA can also play an anti-cancer role via directly targeting oncoprotein such as Bcl-2. In this study, we firstly found that FOXM1 is a novel target of DCA in prostate cancer cells. However, the detailed mechanism of DCA-induced down-regulation of FOXM1 is not clear now and needs further studies. As targeting FOXM1 can also enhance the therapeutic effect of other drugs [17-19], we deduced that DCA could be a widely-used potent sensitizer in the cancer cells with high level of FOXM1.

Previous study have reported that the level of ROS was remarkably increased in FOXM1-knockout HCC cells, which indicated that FOXM1-mediated alternation of ROS played a critical part in the survival of the HCC cells [20]. High levels of FOXM1 may inhibit ROS production via increasing ROS scavenger genes, such as MnSOD, catalase and PRDX3 [21]. As the elevated Reactive Oxygen Species (ROS) can make tumor cells vulnerable to oxidative stress [21, 22], and Cisplatin treatment can also cause an obvious state of oxidative stress [23-25], we deduced that DCA-induced decrease of FOXM1 upregulated ROS level that sensitized prostate cancer cells to Cisplatin. However, the detailed mechanism of FOXM1-mediated regulation of ROS in prostate cancer cells with co-treatments of Cisplatin and DCA still need further investigation.

In conclusion, our research revealed that DCA could remarkably promote Cisplatin-induced cell death in prostate cancer through reducing FOXM1 expression. The decreased FOXM1 render the cells sensitive to chemotherapy via regulating ROS level. These data provided a novel insight for explaining the synergistic effect of DCA and Cisplatin, which may be beneficial for improving the therapeutic effect of Cisplatin in clinic. As Cisplatin and DCA are both drugs that widely used in clinic, we really hope this combination of drugs will be a promising strategy for prostate cancer patients in the future.

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Disclosure conflict of interest

None.

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