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Cyramza induces apoptosis of HCC4006 cell by affecting the level of Bcl-w

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Abstract. – OBJECTIVE: Lung cancer seriously threats to patient's life and health. Cyramza is a therapeutic drug for inhibition of vessel formation and growth in clinical practice. The aim of this study was to investigate the effect of cyramza on growth and apoptosis of non-small lung cancer HCC4006 and explore the related mechanisms.

MATERIALS AND METHODS: Cell viability and apoptosis were examined by MTT assay and flow cytometry, respectively. Western blot was employed to examine the effect of cyramza on the apoptotic protein Bcl-w. After that Bcl-w knockdown and overexpression were respectively fulfilled by Bcl-w siRNA and plasmid transfection, effects of cyramza on cell apoptosis were determined by Western-blot.

RESULTS: Cyramza inhibited the cell growth and induced the cell apoptosis in HCC4006 cells, which was mediated by downregulation of Bcl-w level. Bcl-w knockdown and overexpression could increase and decrease the effect of cyramza on cell apoptosis, respectively.

CONCLUSIONS: Cyramza induced the apoptosis of non-small lung cancer cell line HCC4006 via the downregulation of Bcl-w.

Key Words:
Cyramza, Bcl-w, HCC4006 cells, Cell apoptosis.

Introduction

Non-small lung cancer (NSLC) is a type of malignant cancer in human respiratory system that occurs all over the world[1]. NSLC, which could be classified into squamous cell carcinoma and adenocarcinoma carcinoma, is composed of 90% of all types of lung cancers[2,3]. Due to the delayed diagnosis of NSLC, the surviving rate of the NSLC patients was very low[4-6]. Therefore, it is of great importance for in-time diagnosis for the treatment of NSLC. Currently, therapeutic choices for NSLC include chemotherapy, surgery and radiotherapy. Of note, combined therapy was usually employed in clinical practice[7-9], the advantage of which included reduction of the toxic and side effects caused by chemotherapy and radiotherapy and induction of the synergistic effect of different treatment. The most recent studies have shown that the combined use of chemotherapy and target therapy could effectively decrease the toxic and side effects caused by chemotherapy itself[10-12]. However, one of the most difficulties in target therapy is the choice of molecule target. Recent studies[13-15] demonstrated that the molecule target of chemotherapy agent cyramza could provide the theory evidence for employment of the target therapy in NSCLC. Currently, several chemotherapy agents have been introduced into the treatment of NSLC, including gemcitabine, vinorelbine, paclitaxel, and gefinitib[16-18]. Cyramza, approved by Food and Drug Administration of United States in 2014, is an important therapeutic agent for NSLC treatment. Cyramza, also named ramucirumab, could be applied in the treatment of gastroesophageal cancer, gastric cancer and adenocarcinoma by targeting vascular endothelial growth factor (VEGF)[19,20]. From the view of current research, cyramza exerted inhibitory effects on vessel formation and growth by cutting off the blood supply. The most advantage of cyramza was that it took effects in patients who were not allowed for surgery and inefficacy for cisplatin or fluorine pyrimidine based chemotherapy[21,22]. However, the therapeutic mechanism of cyramza is not fully explored[23,24]. Cell apoptosis caused by chemotherapy agents is one of the mechanisms involved in the anti-cancer effects. Studies[25,26] have shown that several molecules were involved in the programmed cell death, including proapoptotic factors Bax, Bak, and anti-apoptotic molecules Bcl-w, Bcl-2 and Bcl-xL. Among them, Bcl-w was a representative molecule with increasing interest, and Bcl-w exerted

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its effects by directly or indirectly inhibition of activity of caspase via its classical BH-2 structure domain[27,28]. According to the screening results, we found that cyramza could decrease the expression of Bcl-w in cancer cell lines, indicating the possible role of Bcl-w in the effect of cyramza. In the present study, we used the NSLC HCC4006 as the cell model to explore the effect and mechanism of cyramza on the HCC4006, thereby providing basis for its use in clinical practice.

Materials and Methods

Reagents

Cell culture medium RPMI 1640, fetal bovine serum (FBS), penicillin-streptomycin (P/S) solution, Hank's buffer, polylysine, dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), EDTA and trypsin were purchased from Sigma-Aldrich (St. Louis, MO, USA). MTT was obtained from Sangon Biotech (Shanghai, China) whilst cell apoptosis reagents were purchased from Huamei Biotech (Beijing, China). Control siRNA and siRNA targeting Bcl-w were obtained from Sunbiotech (Beijing, China); Bcl-w overexpression plasmid was purchased from Sangon Biotech (Shanghai, China) whilst Bcl-w antibody and internal control actin antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell Culture

NSLC cell line HCC4006 was obtained from ATCC (Manassas, VA, USA) and maintained in 1640 supplemented with 10% FBS and P/S in a 37°C and 5% CO_2 incubator.

Cell Transfection

Cell transfection was performed using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacture's instructions. Briefly, cells were trypsinized, counted, seeded and allowed to adhesion for 12 h. Then these cells were grown to approximate 70% confluency. 1 μg Bcl-w siRNA or control siRNA was mixed with 5 μl Lipofectamine 2000, and incubated in RT for 5 min before adding to the cell culture plate. Medium change was performed after transfection for 24 h.

MTT Assay

The culture of NSLC HCC4006 cells was performed with above described method and the cell viability was performed by measuring absorbance value on a microplate reader as follows[24]: the cell was treated with cyramza and washed with PBS for 3 times, and 5 μl MTT solution (0.2 M, pH=7.4) was added. Cells were incubated in 37°C, 5% CO_2 condition for 4 h and DMSO was used to stop the reaction. The color of solution was determined by microplate reader at 560 nm wavelength.

Cell Apoptosis Assay By Flow Cytometry

The culture of NSLC HCC4006 cells was performed with above described method and the cell apoptosis was determined by flow cytometry as follows[24]: cells (2×10^5) were treated with cyramza followed by adding 50 μl Annexin V reagent and allowed to reaction in the dark for 20 min. The effect of cyramza on the cell apoptosis was determined by measuring the Annexin V fluorescence on the flow cytometry. The excitation and emission wavelength were 484 and 625 nm.

Measurement of Caspase-3 Activity in HCC Cells

The culture of NSLC HCC4006 cells was performed with above described method and the activity of caspase-3 was determined by a microplate reader as follows[24]: cells (2×10^5) were treated with cyramza followed by adding lysis buffer according to the description of manufacture to allow the release of the intracellular protein. Chromophoric substrate was then added and incubated in RT for 20 min. The OD value was determined on 492 nm wavelength on a microplate reader.

Western-Blot

The culture of NSLC HCC4006 cells was performed with above described method and the cell lyse was prepared by adding approximate volume of lysis buffer into the cell pellets. Then the protein extraction was performed using high-speed centrifugation and supernatant collection. Denature of protein was performed by adding SDS-loading buffer and metal bath at 100°C for 5-10 mins[24]. The above-prepared protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane. After membrane blocking, the primary antibody (anti-Bcl-w, 1:500; anti-β-actin, 1:1000) was added and allowed to reaction at RT for 1 h. After washing with PBS-T buffer for 3 times, appropriate horse radish peroxidase (HRP) labeled secondary

antibody (1:5000) was used for incubation (Abcam, Cambridge, MA, USA). Protein bands were detected with Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) on X-ray films (Kodak, Tokyo, Japan). Images were analyzed by ImageJ 6.0 software (Chicago, IL, USA).

Statistical Analysis

Statistical analysis was performed by using SPSS17.0 software (SPSS Inc., Chicago, IL, USA). Data was expressed as Mean±SD. Multiple group comparison was performed by One Way ANOVA assay, followed by LSD test. $p<0.05$ was considered as statistically significance.

Results

Cyramza Inhibits the Proliferation and Viability of HCC4006 Cells

As shown in Figure 1, cell viability and proliferation significantly decreased in HCC4006 cells treated with cyramza, compared to the control group treated with DMSO ($p = 0.011$).

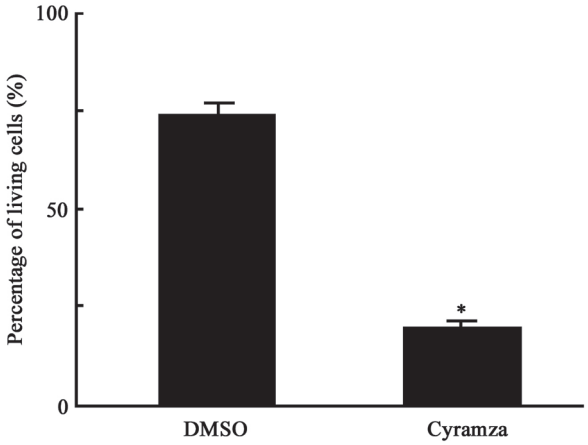

Figure 1. Cyramza incubation inhibited the growth of NSLC cell line HCC4006. *$p < 0.05$, compared with DMSO control group.

Cyramza Induces Cell Apoptosis in HCC4006 Cells

The cell apoptosis was detected by flow cytometry shown in Figure 2. According to the results, cyramza significantly increased the apoptosis of HCC4006 cells, compared to the control group ($p = 0.014$). We also determined the activity of

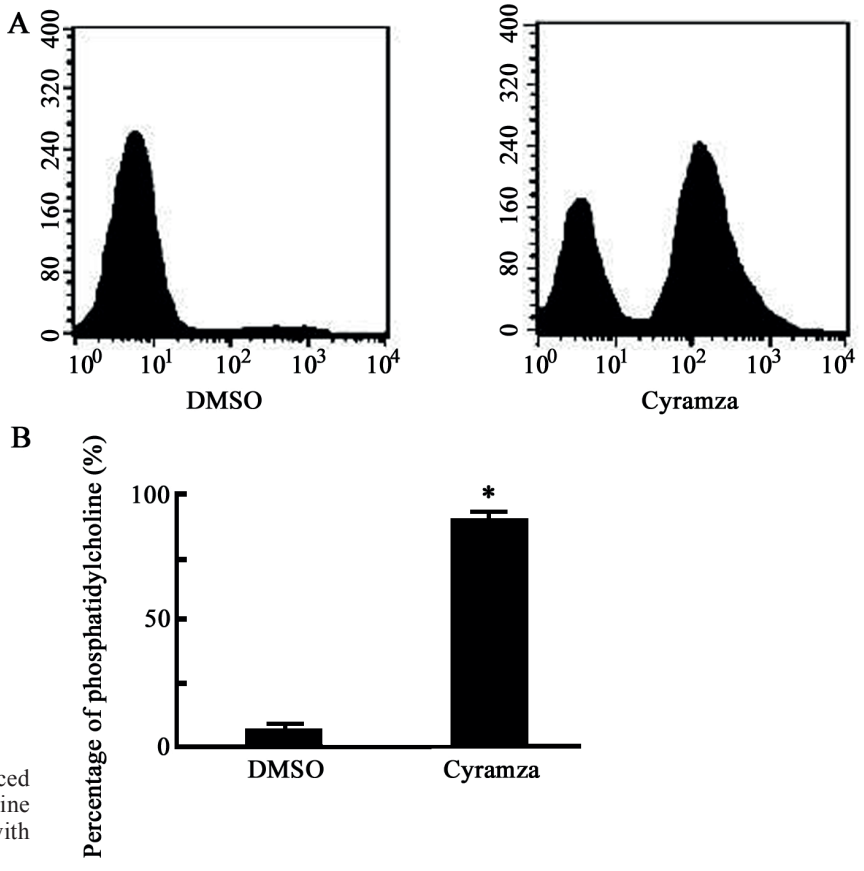

Figure 2. Cyramza incubation induced the cell apoptosis of NSLC cell line HCC4006. *$p < 0.05$, compared with DMSO control group.

caspase in cells affected by cyramza. As shown in Figure 3, cyramza significantly induced the caspase level in HCC4006 cells, compared to the control group ($p = 0.011$).

Cyramza Downregulates the Level of Bcl-w in HCC4006 Cells

As shown in Figure 4, cyramza remarkably decreased the expression of Bcl-w in HCC4006 cells, compared to the control group ($p = 0.011$).

Bcl-w Knocking-Down Enhances the Cyramza Induced Cell Apoptosis in NSLC HCC4006 Cells

The activity of caspase-3 after the inhibition of Bcl-w expression was shown in Figure 5. As the level of Bcl-w was knocked down, the caspase-3 activity was significantly increased in cyramza group, compared to that in control group ($p = 0.022$).

Bcl-w Overexpression Decreases the Cyramza Induced Cell Apoptosis in NSLC HCC4006 Cells

The activity of caspase-3 after Bcl-w over-expression was shown Figure 6. As Bcl-w over expressed in cyramza-treated HCC4006 cells, caspase-3 activity showed a significant decrease, compared to that in control group ($p = 0.0054$).

Discussion

Currently, the molecular mechanism on therapeutic effects of cyramza on the NSLC remains to be elusive. Therefore, we used the *in vitro* cell culture system to investigate the effects of cyramza on NSLC cell line HCC4006. Three main discoveries were presented in our study.

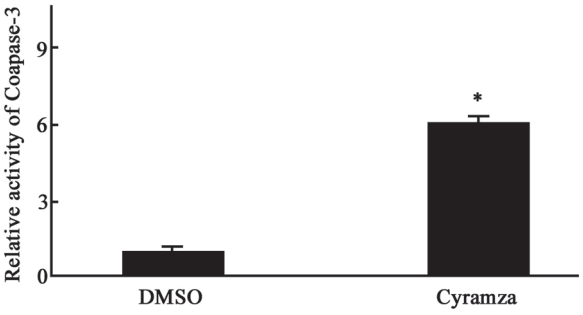

Figure 3. Cyramza incubation increased the activity of caspase-3 in NSLC cell line HCC4006. *$p < 0.05$, compared with DMSO control group.

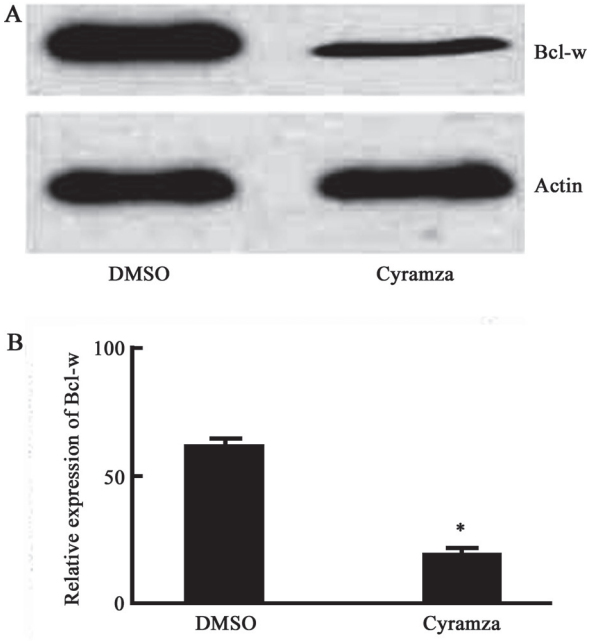

Figure 4. Cyramza decreased the level of Bcl-w in NSLC cell line HCC4006. *$p < 0.05$, compared with DMSO control group.

Firstly, the treatment of cyramza could inhibit the cell growth and apoptosis of NSLC cell line HCC4006 by decreasing the Bcl-w level. Secondly, the decrease of Bcl-w in HCC4006 cell line could enhance the cell apoptosis of HCC4006 induced by cyramza. Thirdly, the overexpression of

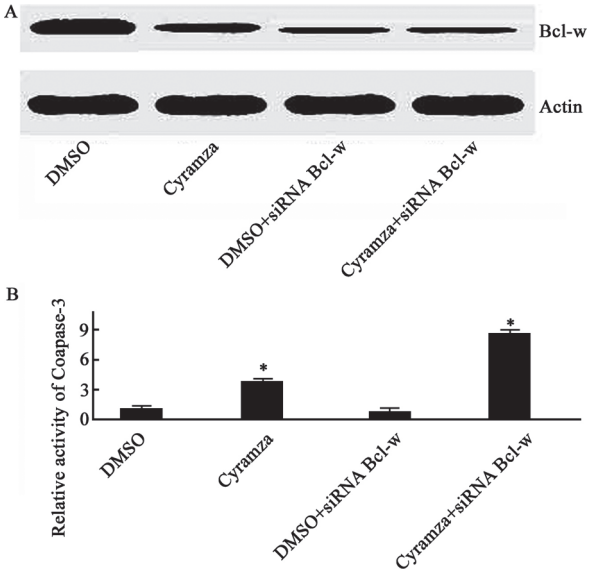

Figure 5. Bcl-w knocking down increased the cyramza induced cell apoptosis in NSLC cell line HCC4006. *$p < 0.05$, compared with DMSO control group.

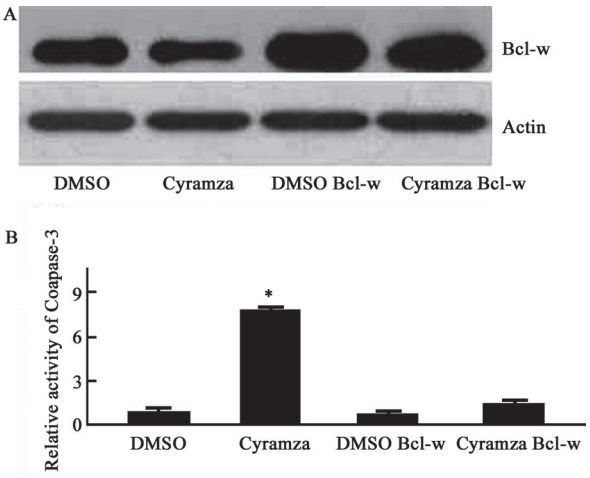

Figure 6. Bcl-w overexpression decreased the cyramza induced cell apoptosis in NSLC cell line HCC4006. * $p < 0.05$, compared with DMSO control group.

Bcl-w decreased the apoptosis of HCC4006 cells. These results indicated that cyramza could induce cell apoptosis via the modulation of Bcl-w, which was consistent with previous study[7]. Caspase-3, an intracellular molecule, which could mediate both cell receptor-mediated cell apoptosis and mitochondrial apoptosis, is the main factor in cell functions and processes[16]. The alternation of caspase-3 level also validated the promoting effect of cyramza on cell apoptosis. Previous studies showed that the target genes were screened for the apoptosis proteins[13, 16-18] by Western-blot and our results indicated no change of apoptosis inhibitory molecules (Bcl-2 and Bcl-xL), but significantly increased level of apoptosis promotion factors (Bax and Bak; data not shown). We found that the level of Bcl-w was significantly decreased and, therefore, we performed the overexpression and knocking-down of Bcl-w to identify the molecular mechanism of cell apoptosis effects induced by cyramza. There are some limitations in this study. The exact mechanism of cyramza on the regulation of the Bcl-w level still needs to be clarified. Also, the experiment with the animal model was required to validate the effect of cyramza.

Conclusions

Our data indicated that cyramza could induce the apoptosis of non-small lung cancer cell line HCC4006, and downregulation of Bcl-w was involved in the mechanisms.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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