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*Original Article*

# Ubiquitin-like protein D enhances the sensitivity of MDA-MB-231 cells to cisplatin via the Bcl-2/Bax/caspase-3 pathway in triple-negative breast cancer

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**Abstract:** Objective: To study the effects of ubiquitin-like protein D (UBD) expression on the sensitivity of cisplatin to human triple negative breast cancer MDA-MB-231 cells. Methods: Thirty-five cases of triple negative breast cancer patients who were treated by chemotherapy with cisplatin and surgery in our hospital were divided into a CR group and PR group according to postoperative pathological diagnosis. Expression of UBD was compared before chemotherapy and UBD over expression and silenced expression were generated in MDA-MB-231 cells. The expression of UBD, Bax, Bcl-2, and caspase-3 were compared and the $IC_{50}$ to cisplatin was determined in different MDA-MB-231 cells. Results: The positive cell ratio of UBD and Bcl-2 was (20.8+7.9%) and (41.3+12.9%) respectively in tumor tissue of the CR group, which was significantly lower than the PR group (68.3+12.4%) and (77.8+20.4%) ($P<0.05$). The positive cell ratio of Bax and activated caspase-3 was (56.9+15.7%) and (67.3+21.4%) respectively in tumor tissue of the CR group, which was significantly higher than the PR group that was (30.6+11.5%) and (36.6+10.1%) ($P<0.05$). The $IC_{50}$ of cisplatin in 231-Silence, MDA-MB-231, and 231-Over was (19.82+1.08) μmol/L, (35.69+1.21) μmol/L and (67.79+1.54) μmol/L respectively. The relative expression of Bcl-2 in MDA-MB-231 cells was (0.66+0.17), which was significantly higher than 231-Silence (0.075+0.05) ($P<0.05$), and significantly lower than 231-Over (1.05+0.22) ($P<0.05$). The relative expression of Bax and activated caspase-3 in MDA-MB-231 cells was (0.44+0.10) and (0.70+0.19) respectively, which was significantly lower than 231-Silence [(1.08+0.24) and (1.28+0.31)] ($P<0.05$) and significantly higher than 231-Over [(0.17+0.09) and (0.36+0.12)] ($P<0.05$). Conclusion: The high expression of UBD enhanced the resistance of cisplatin to MDA-MB-231, and the mechanism may be related to the high expression of UBD reduced Bax and caspase-3 gene expression and increased Bcl-2 gene expression.

**Keywords:** UBD, triple negative breast cancer, cisplatin, MDA-MB-231

## Introduction

Breast cancer is a malignancy that occurs in the mammary epithelial tissue, with approximately 99% of patients being females. Cancer statistics for China in 2015 showed that [1] the new cases of breast cancer accounted for approximately 15% of total cases of cancer. In addition, the statistics also revealed that incidence and mortality of breast cancer in women aged below 45 ranked the first among malignancies. Triple negative breast cancer (TNBC) refers to a subtype of breast cancer with negative expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2) in the tumor tissue, accounting for about 15%-24% of all breast cancer patients [2]. Like other subtypes of breast cancer, chemotherapy in combination with surgery still represents the best treatment for patients with TNBC. Diamminedichloroplatinum (DDP) is one of the most commonly used agents for preoperative chemotherapy in patients with TNBC. Although DDP confers good clinical effects in the early stage of chemotherapy for patients with TNBC, the resistance of tumor cells to DDP is enhanced with prolongation of the treatment cycle [3]. Therefore, studies on the regulation of molecular mechanisms underlying the sensitivity of TNBC to chemotherapeutic drugs, such as DPP, have become a hotspot in recent years.

Ubiquitin-like protein D (UBD) is one of the important members of the ubiquitin-like protein family. UBD not only regulates LPS- or TNF-α-

**Table 1.** Design of PCR primers for the genes tested

| Gene | Primer sequence (5'-3') | Length (bp) |
|---|---|---|
| UBD | F: GCACAGCAAAGCTGGACAAA | 229 |
| | R: CCAGCCCCCGTTTCTAAGAT | |
| Bcl-2 | F: ACCCCAGGCTCTGTGTTAGT | 601 |
| | R: GTCCACGGCACAGAATCCA | |
| Bax | F: GTCCAGCTCTTTAATGCCCG | 205 |
| | R: TCCCGCCACAAAGATGGTC | |
| β-actin | F: AAGTACTCCGTGTGGATCGG | 615 |
| | R: TCAAGTTGGGGGACAAAAAG | |

induced intrinsic immune activation, but also is involved in the regulation of maturation of DC cells. Therefore, previous studies focused on the immune system [4]. However, a number of studies in recent years have shown that UBD can serve as an independent prognostic marker of chemotherapy for colon cancer [5], and that its expression may also be associated with the resistance of human ovarian cancer cells to DDP [6]. At the same time, the study on the regulation of UBD on breast stem cells showed that a high expression of UBD promoted the resistance of breast cancer cells MCF-7 to chemotherapeutic drugs [7]. However, UBD expression in the TNBC tumor tissue and the effects of its expression on resistance of TNBC MDA-MB-231 cells to DDP are rarely reported. Herein, we investigated the effects of UBD expression on sensitivity of TNBC patients to DDP by comparing the expression of UBD in the tumor tissue of TNBC patients with different chemotherapeutic outcomes and studying the effects of UBD expression on resistance of MDA-MB-231 cells to DDP.

## Materials and methods

### *Source of specimens*

A total of 35 specimens were collected from female breast cancer patients (age, 32 to 55 years, with a mean of 41.3 ± 6.2 years) receiving DDP-based chemotherapy in combination with surgery in our hospital from December 2014 to December 2015. All patients were diagnosed as TMBC via biopsy prior to treatment, with AJCC stage at II. Patients received DDP-based chemotherapy prior to surgery, and their tumor specimens were harvested for histopathological examination after the surgery. Of the 35 patients, 10 patients achieved complete remission (CR), and 25 achieved partial remission (PR).

### *Experimental reagents and cell strains*

MDA-MB-231 cell strains were purchased from ATCC. MDA-MB-231 cell strains with UBD overexpression and 231-Silence cell strains with silenced UBD expression were constructed by our laboratory. Total protein content was used by the BCA Protein Assay kit (Pierce). UBD antibody, Bcl-2 antibody, Bax antibody, caspase-3 antibody, β-actin antibody, and goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from the US. Santa Cruz Biotechnology Co., Ltd.

### *Experimental methods*

*Immunohistochemical assay of UBD protein in the tumor tissue:* Immunohistochemical staining was performed according to the instructions of the immunohistochemical kit and the primary antibody, with PBS selected as the primary antibody in the negative control group. For each section, 5 visual fields under 400× magnification were selected to calculate the proportion of cells with positive UBD. Yellowish-brown staining of the cell membrane or cytoplasm indicated positive UBD, brown staining of the cytoplasm or the nuclear membrane indicated positive Bcl-2, yellowish or yellowish-brown staining of the cytoplasm indicated positive Bax, and pale yellow or brown staining of the cytoplasm indicated positive caspase-3.

*Detection of mRNA expression of genes in the cells:* Total RNAs were extracted from the cells using the total RNA extraction kit. The cDNA templates were then established by reverse transcription of RNA, and semi-quantitative PCR was performed based on the templates. PCR products (10 ul) were subjected to 1.5% nucleic acid gel electrophoresis (120 V, 15 min), which were observed on a Bio-Rad gel imaging analyzer and photographed. The results were analyzed with a gel imaging system and Quantity One software and expressed as the relative content of the target gene and the β-actin band. The PCR primer for each gene was designed by the Primer function in the NCBI website. See **Table 1** for specific information.

*Detection of gene protein expression in the cells:* Total proteins were extracted by a whole

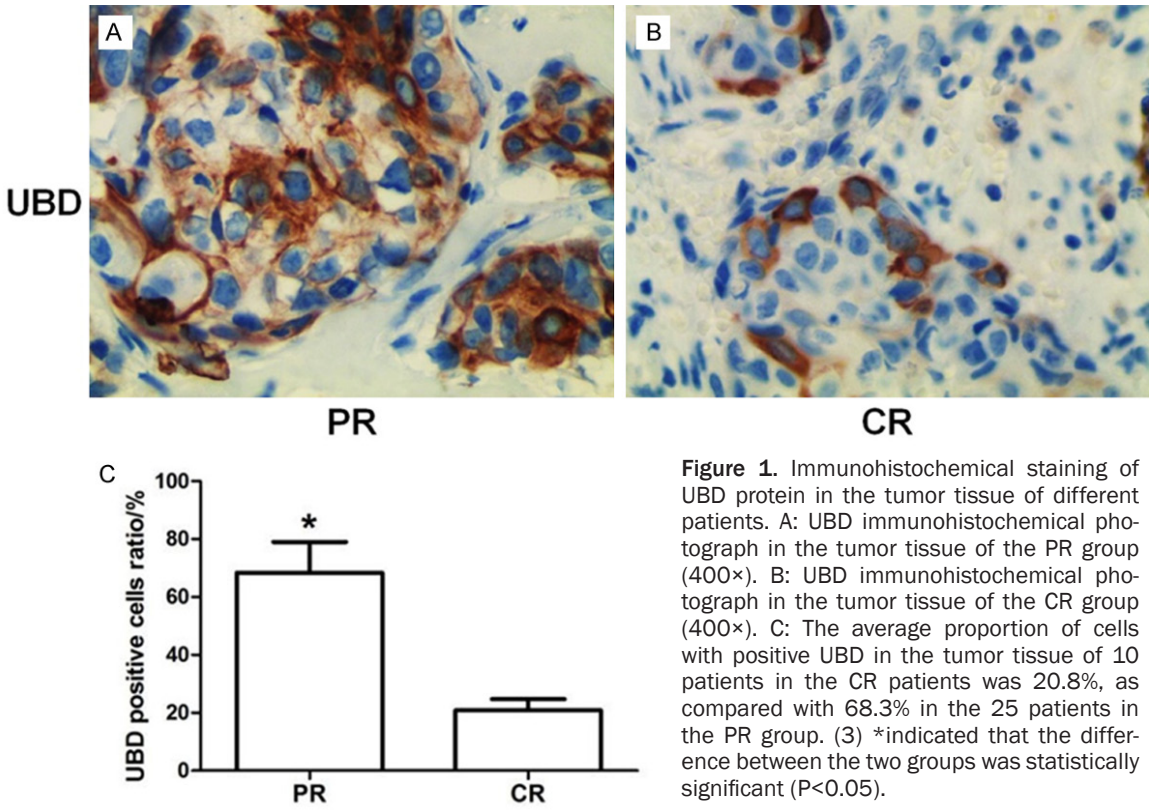


**Figure 1.** Immunohistochemical staining of UBD protein in the tumor tissue of different patients. A: UBD immunohistochemical photograph in the tumor tissue of the PR group (400×). B: UBD immunohistochemical photograph in the tumor tissue of the CR group (400×). C: The average proportion of cells with positive UBD in the tumor tissue of 10 patients in the CR patients was 20.8%, as compared with 68.3% in the 25 patients in the PR group. (3) *indicated that the difference between the two groups was statistically significant (P<0.05).

cell protein extraction kit (Amy Technology), and the concentrations of the total proteins were determined using the BCA kit. 50 mg of proteins was taken for SDS-PAGE. Afterwards, Western blot was performed, followed by mounting and addition of primary antibody and second antibody for visualization successively. Analysis of the brightness of the bands was performed using Image-J software, with the expression of genes represented as the relative contents of the target gene and the β-actin bands.

*Cell activity assay using MTT assay:* MDA-MB-231, 231-Over and 231-Silence single cell suspensions were prepared and seeded into 96-well plates, respectively, with 5000 cells (100 ml) in each well. The cells were cultured for 2 hours and 4 hours, and added with 0 μM, 10 μM, 20 μM, 40 μM, and 80 μM DDP (100 ml), respectively, followed by routine culture for 48 hours. Afterwards, each well was added with 5 mg/ml of MTT solution, followed by routine culture for 4 hours. Centrifugation was performed, with the culture medium discarded. The cells were washed twice with PBS and then 150 μl of DMSO was added to each well. The cells were shaken at a low speed at room temperature for 10 minutes, and the $OD_{450}$ value of each well was measured on a microplate reader.

*Statistical methods*

Statistical analysis of the data in this study was performed using the SPSS9.0 software, and the data were expressed as mean ± standard deviation or percentages. The difference between two groups was compared by t-test, and P<0.05 indicated significant difference.

## Results

*The effect of UBD expression on the efficacy of chemotherapy*

UBD protein is mainly expressed in the cell membrane or cytoplasm, and yellowish-brown staining of the cell membrane or cytoplasm via the immunohistochemical staining was deemed as positive expression. The results of immunohistochemical staining of UBD in the tumor tissue in different groups showed that the percentage of positive tumor cells in the PR group was (68.3+12.4%), significantly higher than that in the CR group [(20.8+7.9%), P<0.05]. See **Figure 1** for specific information.

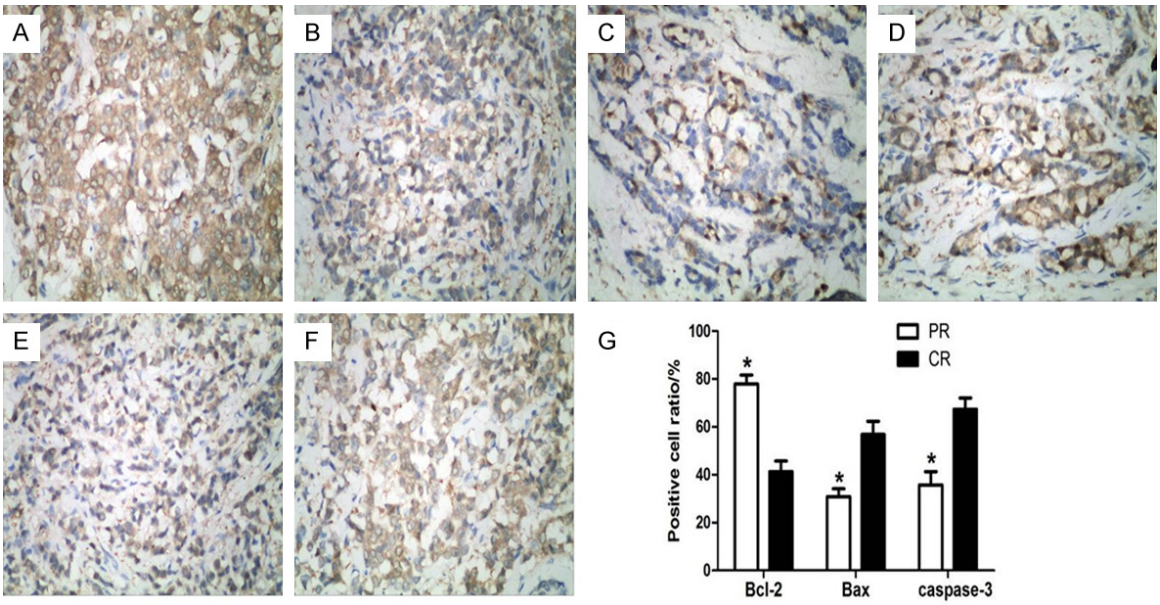


**Figure 2.** Immunohistochemical staining of Bcl-2, Bax, and activated caspase-3 in tumor tissues of different patients. A, C and E: Immunohistochemistry of Bcl-2, Bax and activated caspase-3 in the tumor tissue of the PR group; B, D and F: Immunohistochemistry of Bcl-2, Bax and Caspase-3 in the tumor tissue of the CR group (400×). G: The average proportion of cells with positive Bcl-2, Bax, and activated caspase-3 in tumor tissues of patients in the PR group was (77.8+20.4%), (30.6+11.5%) and (36.636.6 ± 10.1%), respectively, and in the CR group was (41.3+12.9%), (56.9+15.7%) and (67.3+21.4%), respectively. *indicated significant difference compared with the CR group.

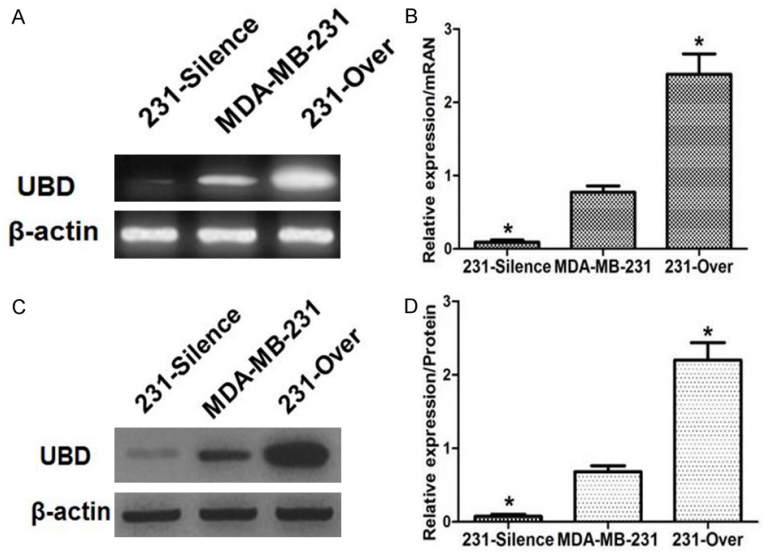


**Figure 3.** UBD gene expression in three groups. A: UBD and β-actin mRNA expression in different cells. B: The relative UBD gene mRNA expression in different cells. C: UBD and β-actin protein expression in different cells. D: The UBD protein expression in different cells. *denoted a significant difference compared with MDA-MB-231 cells (P<0.05).

*Bcl-2, Bax and caspase-3 protein expression in the tissue of different patients*

Bcl-2, Bax, and activated caspase-3 proteins are mainly located in the cytoplasm or nuclei. In immunohistochemical staining, the cell cytoplasm or nuclear membrane of positive Bcl-2 cells was stained yellow, the cytoplasm of positive Bax cells was stained yellow or yellowish-brown, and the cytoplasm of positive activated caspase-3 cells was stained pale yellow or brown. The results of immunohistochemical staining of Bcl-2, Bax, and activated caspase-3 proteins in tumor tissues of different groups showed that the proportion of positive Bcl-2 cells in the PR group was (77.8+20.4%), significantly higher than that in the CR group [(41.3 ± 12.9%), P<0.05)], that the proportion of positive Bax cells in the PR group was (30.6+11.5%), significantly lower than that in the CR group [(56.9+15.7%), P<0.05], and that the proportion of positive caspase-3 cells in the PR group was (36.6+10.1%), significantly lower than that in the CR group [(67.3+21.4%), P<0.05]. See **Figure 2** for specific information.

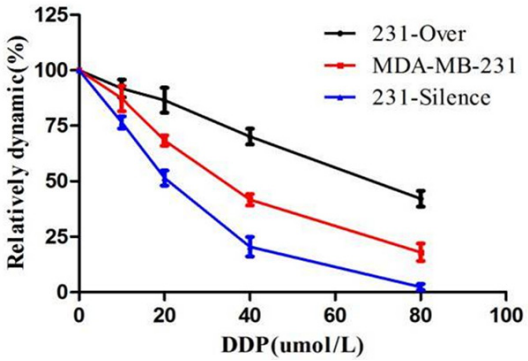


**Figure 4.** Sensitivity of different cells to DDP. The results showed that the $IC_{50}$ values of DDP in significantly inhibiting 231-Silence, MDA-MB-231, and 231-Over were (19.62+1.08) μM, (35.69+1.21) μM and (67.79+1.54) μM, respectively.

*UBD positively regulates sensitivity of MDA-MB-231 cells to DDP*

The MDA-MB-231 cell line 231-Over with UBD overexpression and the MDA-MB-231 cell line 231-Silence with silenced UBD expression were constructed using genetic engineering, and the relative expressions in different cell lines were determined. The results showed that the relative UBD gene mRNA and protein expressions in MDA-MB-231 cells were (0.77 ± 0.08) and (0.68 ± 0.12), respectively, significantly lower than those in 231-Over [(2.38 ± 0.32) and (2.2 ± 0.29), P<0.05] while significantly higher than those in 231-Silence [(0.091 ± 0.01) and (0.076 ± 0.02), P<0.05]. See **Figure 3** for specific information.

The $IC_{50}$ values of different cells to DDP were detected using MTT assay, and the results showed that the $IC_{50}$ values of DDP in significantly inhibiting 231-Silence, MDA-MB-231, and 231-Over were (19.62+1.08) μM, (35.69+1.21) μM and (67.79+1.54) μM, respectively. See **Figure 4** for specific information.

*Effects of UBD expression on apoptosis-related gene expression in MDA-MB-231 cells*

The MDA-MB-231 cell line 231-Over with UBD overexpression and the MDA-MB-231 cell line 231-Silence with silenced UBD expression were constructed using genetic engineering, and the relative expression of Bcl-2, Bax, and activated caspase-3 in different cell lines was determined. The results showed that: (1) mRNA levels: the relative expression of Bcl-2 in MDA-MB-231 was (0.75+0.26), significantly lower than that in 231-Over (1.17+0.31, P<0.05) while significantly higher than that in 231-Silence (0.093+0.05, P<0.05); the amount of relative expression of Bax in MDA-MB-231 was (0.52+0.18), significantly higher than that in 231-Over (0.19 ± 0.08, P<0.05) while significantly lower than that in 231-Silence (1.07+0.28, P<0.05). (2) Protein levels: the relative expression of Bcl-2 in MDA-MB-231 was (0.66+0.17), significantly lower than that in 231-Silence (1.05+0.22, P<0.05) and significantly higher than that in 231-Silence (0.075+0.05, P<0.05); the relative expression of Bax in MDA-MB-231 was (0.44+0.10), significantly higher than that in 231-Over (0.17+0.09, P<0.05) and significantly lower than that in 231-Silence (1.08 ± 0.24, P<0.05); the relative expression of activated caspase-3 in MDA-MB-231 was (0.70+0.19), significantly higher than that in 231-Over (0.36+0.12, P<0.05) and significantly lower than that in 231-Silence (1.28+0.31, P<0.05). See **Figure 5** for specific information.

## Discussion

TNBC is a special type of breast cancer without expressions of ER, PR, and HER2 in the tumor tissue, thus the existing endocrine therapy and HER2 targeted therapy specific to breast cancer patients yield no effects on TNBC. Currently, chemotherapy or surgery is mainly used in clinical practice for the treatment of patients with TNBC. Although studies have confirmed that TNBC patients are more sensitive to chemotherapy compared to other subtypes of breast cancer, TNBC patients, when undergoing chemotherapy, still suffer from such side reactions as nausea, vomiting, and bone marrow hematopoietic dysfunction [8]. Therefore, improving the sensitivity of tumor cells to chemotherapeutic drugs is particularly important for reducing chemotherapy-induced toxic side effects. At present, there are no standard adjuvant chemotherapy regimen for TNBC clinically, and paclitaxel, anthracyclines, and DPPS are mainly used as chemotherapy for patients with TNBC.

DDP is a heavy metal complex in which platinum (2+) at its center binds with two Cl atoms and two NH3 molecules. Since it is effective in inhibiting the proliferation of cancer cells by prevented DNA replication and transcription, it is extensively used in the treatment of cancer

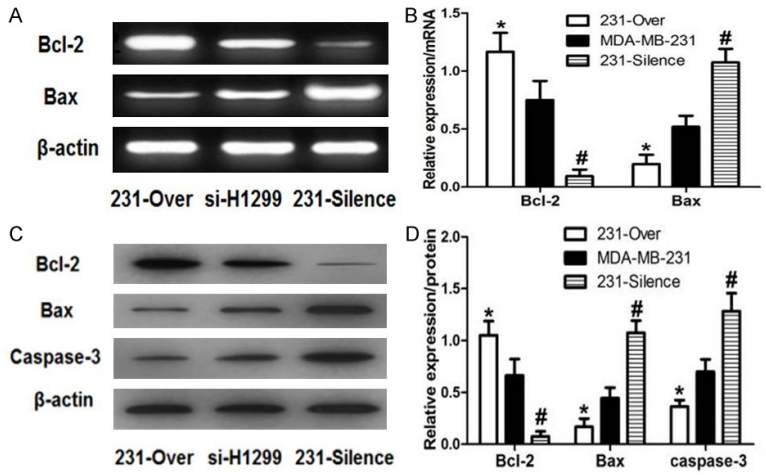


**Figure 5.** Effects of UBD expression on genes related to apoptosis of MDA-MB-231 cells. 231-Over, MDA-MB-231, and 231-Slience denote MDA-MB-231 cell lines with UBD overexpression, normal expression and silenced expression, respectively. A: Relative expression of Bcl-2, Bax, and β-actin mRNA in different cell lines. B: Relative expression of Bcl-2 and Bax gene mRNAs in different cell lines relative to β-actin gene mRNA. C: Relative expression of Bcl-2, Bax, activated caspase-3, and β-actin proteins in different cell lines. D: Relative expression of Bcl-2, Bax, activated caspase-3, and β-actin protein in different cell lines relative to β-actin protein. *indicates that there is significant difference between 231-Over and MDA-MB-231 ($P<0.05$); #indicates that there is significant difference between 231-Silence and MDA-MB-231 ($P<0.05$).

patients [9]. Because TNBC itself is incapable of DNA damage repair, it is more sensitive to DDP or other platinum-based drugs. A study has shown that [10] DDP monotherapy for four cycles results in a 22% CR in TNBC patients. Although TNBC is highly sensitive to DDP, one of the major causes of DDP chemotherapy failure is still resistance of cancer cells to DDP. Liang S et al. [11] found that the MDA-MB-231 cells, which high expressed CXCR4 in the human triple negative cell group, had increased resistance to DDP, and silencing the CXCR4 gene could significantly enhance the sensitivity of MDA-MB-231 cells to DDP and improve the effects of DDP chemotherapy. This study suggested that expression of certain proteins in TNBC tumor cells could serve as an indirect regulator to reduce the sensitivity of cancer cells to DDP.

In this study, it was revealed that the proportion of positive cells expressing UBD in the tumor tissue of 10 patients who had achieved complete remission after DDP monotherapy was significantly smaller than that in the 25 patients who had achieved partial remission ($P<0.05$). This finding indicates that UBD expression in TNBC tumor cells might be related to its sensitivity to DDP. UBD, a unique member of the ubiquitin-like protein family, is located at position 6p22.1 of the human chromosome and encodes 165 amino acids. It has a molecular weight of 18 kDa, and is expressed in lungs, brain, kidneys, ovaries, thymus, and other organs of human [12]. A number of studies in recent years have shown that the UBD expression can not only serve as an independent predictor of survival of colon cancer patients after chemotherapy [5], but is also associated with sensitivity of cancer cells to chemotherapeutic agents, such as breast cancer [7] and ovarian cancer [6]. This study found that, in human TNBC, with the increased expression of UBD, its resistance to DDP was gradually reduced.

Cha TL et al. [13] pointed out that although different chemotherapeutic regimens or antitumor drugs might vary in the targets of action, they eventually demonstrate antitumor effects by inducing tumor cell apoptosis or altering the tumor tissue's internal environment that leads to tumor necrosis. In the experiment in which the sensitivity of different TNBC cells to DPP was determined, it was also found that when DPPs had the same concentration and duration of action, the higher the expression of UBD in the cells, the lower the apoptosis rate, indicating that UBD expression may affect apoptosis of MDA-MB-231 cells.

Bcl-2 is expressed on the outer membrane of the mitochondria and can inhibit apoptosis caused by a variety of factors, and negatively regulate apoptosis of tumor cells. Its overexpression in tumor cells is one of the major causes of progression and drug resistance of malignancies [14], and inhibition of Bcl-2 expression can induce tumor cell apoptosis. Therefore, a batch of Bcl-2 targeting molecular inhibitors have been developed for the treatment of malignancies [15]. In addition, Pan R et al. [16] found that the Bcl-2 inhibitor BI97D6

could effectively reduce the resistance of acute myeloid leukemia patients to ABT-737. Bax protein is an important member of the Bcl protein family, whereas its effect is opposite to that of Bcl-2, which plays a role in promoting apoptosis [17]. In terms of the apoptotic effects, the transfected recombinant plasmids overexpressing Bax can enter ovarian cancer cells to promote their apoptosis and reduce its resistance to chemotherapeutic drugs [18]. Caspase protein can induce apoptosis of cells, and caspase-3 protein is an important member of the caspase protein family. Activated caspase-3 can activate a variety of apoptotic expression, and is the common signal pathway of the caspase pathway, a mitochondrial pathway [19, 20]. In the mitochondrial pathway of cell apoptosis, cell apoptosis-related signals stimulate the mitochondrial outer membrane to release cytochrome C and then enter the cytoplasmic cytochrome C, activating caspase-9 by binding with apaf-1. The activated caspase-9 further activates caspase-3, which can activate caspase-6/7/8, leading to cell apoptosis [19]. This study showed that highly expressed UBD could increase expression of Bcl-2 to inhibit cell apoptosis and decrease Bax and caspase-3 proteins in MDA-MB-231 cells. Therefore, UBD could affect the sensitivity of MDA-MB-231 cells to DDP by regulating the expression of proteins associated with cell apoptosis.

In conclusion, highly expressed UBD can enhance resistance of MDA-MB-231 cells to DDP, and the mechanism involved may be associated with decrease in the expression of Bax and caspase-3 and increase in the expression of Bcl-2.

## Disclosure of conflict of interest

None.

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