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ORIGINAL ARTICLE

Cbl-b regulates the sensitivity of cetuximab through ubiquitin-proteasome system in human gastric cancer cells

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Summary

Purpose: *Cetuximab is a monoclonal antibody against epidermal growth factor receptor (EGFR) and is approved for clinical use in combination with chemotherapy in patients affected by colorectal cancer (CRC), non small cell lung cancer (NSCLC), and head and neck cancer. Compared with these cancers, gastric cancer is relatively resistant to cetuximab and its regulatory mechanism is still unclear.*

Methods: *In this study, we assessed whether the ubiquitin–proteasome pathway is involved in regulating cetuximab-induced cells apoptosis in MGC803 and BGC823 gastric cancer cell lines.*

Results: *The casitas B lineage lymphoma-b (Cbl-b), a kind of E3 ubiquitin ligase, was involved in this process. Specific silenced Cbl-b expression increased the expression of EGFR.*

Conclusions: *Our findings lead to a better understanding of the mechanism of cetuximab action, and suggests that Cbl-b increases the sensitivity of cetuximab in gastric cancer cells at least partially through affecting EGFR expression.*

Key words: *Cbl-b, cetuximab, EGFR, gastric cancer, ubiquitin-proteasome*

Introduction

Gastric cancer is one of the most common malignancies worldwide and a major cause of cancer deaths in Asian countries. Most patients present with advanced or metastatic disease at diagnosis and 5-year survival rates are only 10-15% [1]. Chemotherapy is the main therapeutic method for advanced gastric cancer. Although chemotherapy for advanced disease improves survival, the median overall survival is no more than 12 months [2]. Target therapy, one of the new methods based on the recognized genes and receptors, holds great promise for its potential to treat cancer selectively in recent years. It has attracted increasing attention and brought about good prospects for tumor treatments. The research on EGFR has recently been one of the focuses of cancer therapy.

EGFR is a member of transmembrane receptor with signal-transducing tyrosine kinase activity [3]. The function of EGFR includes migration, proliferation, differentiation and apoptosis [4]. Many studies showed that the EGFR gene is abnormally activated and overexpressed in many human tumors including breast cancer, lung cancer, head and neck cancer [5-7]. Cetuximab (C225) is a monoclonal antibody against EGFR and is approved for clinical use in combination with chemotherapy in patients affected by CRC, NSCLC, and head and neck cancer. Gastric cancer also overexpresses EGFR. However, this neoplasm shows a relative resistance to cetuximab. Therefore, a better understanding of the molecular mechanisms involved in cetuximab-induced gastric cancer cell proliferation and apoptosis is likely to provide theoretical support of tumor target therapy.

Previous studies showed that ubiquitin-proteasome pathway played a critical role in

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orderly proteolysis of intracellular proteins. To date, the effect of ubiquitin-proteasome system (UPS) in regulating the antitumor activity of cetuximab is controversial. Lu et al. have suggested EGFR ubiquitination and degradation as the mechanism for colon cancer cell resistance to cetuximab [8]. Conversely, Zage et al. have shown that the UPS enhanced the sensitivity to cetuximab in neuroblastoma [9]. Thus, the role of UPS in regulating the sensitivity of malignancies to cetuximab is not well understood.

The Cbl protein family is a member of a superfamily of RING finger E3 ubiquitin ligases and is known to be a major negative regulator of receptor tyrosine kinases (RTKs). A previous study has reported that c-Cbl ubiquitinated EGFR resulted in acquired resistance to cetuximab in colon cancer cells [8]. Our previous study has shown that Cbl-b, the second member of Cbl family, regulated EGFR redistribution in lipid rafts that antagonized TRAIL-induced apoptosis [10]. However, whether Cbl-b is involved in regulating the sensitivity of gastric cancer to cetuximab is presently unknown.

The purpose of the present study was to explore the mechanism of cetuximab action in gastric cancer cell lines. This study demonstrated that Cbl-b increased the sensitivity to cetuximab in gastric cancer cells, at least partially through affecting EGFR expression.

Methods

Reagents and antibodies

Cetuximab was obtained from Merck (KgaA, Darmstadt, Germany). RPMI1640 medium was purchased from Gibco (New York, USA). Anti-actin (sc-1616-R,1:1000), anti-phospho-ERK1/2 (Thr202/Tyr204; sc-16982-R, 1:500), anti-ERK1/2 (sc-154, 1:2000), anti-Akt (sc-271984, 1:1000) and anti-phospho-Akt (sc-271984, 1:500), anti-Cbl-b (sc-376409,1:250) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Peroxidase conjugated secondary antibodies were goat anti-mouse IgG-HRP (sc-2005,1:200) and goat anti-rabbit IgG-HRP (sc-2004,1:2000) purchased by Santa Cruz Biotechnology Inc.

Cell cultures and transfection

The gastric cancer cells MGC803 and BGC823 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Both cell lines were cultured in RPMI 1640 medium (Gibco) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37 °C in an atmosphere of 95% air and 5% CO_2. For transfection, MGC803 cells were seeded into 6-well plates ($5x10^5$ cells/well) without antibiotics. After 24 hrs, cells were transfected with various plasmids using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The method of plasmid construction has been discussed in our previous study [11]. MGC803 gastric cancer cells were transfected with short hairpin RNA targeting Cbl-b using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the protocol. The method of selected stable transfected cell lines has been descripted in our previous study [12].

MTT assay

The cells were seeded at 5×10^4 cells/well in 96-well plates and then exposed to 1 or 10 μg/ml cetuximab for 24, 48 and 72 hrs. Then, 25 μL of MTT solution (5 mg/mL) were added to each well and the cells were incubated for another 4 hrs at 37 °C. Following this, the cells were lysed in 200 μL of dimethylsulfoxide (DMSO) and the optical density (OD) was measured at 570 nm with a microplate reader (Model 550, Bio-Rad Laboratories, USA).

Flow cytometry analysis

The cells were seeded at 3×10^5 cells/well in 6-well plates and then exposed to cetuximab for 24 hrs. The cells were collected and incubated with 5 μL Annexin V and 10 μL propidium iodide (PI) for 15 min in the dark. Mitochondrial depolarization was determined by means of the cationic lipophilic fluorochrome DiOC6. Cells were collected and incubated with 20 nm DiOC6 for 15 min in the dark. Finally, the samples were evaluated by flow cytometry.

Western blotting analysis

Total cellular proteins were extracted and quantified as described previously [11]. Aliquots (50 μg) of each lysate were electrophoresed on SDS–PAGE gels and then blotted onto nitrocellulose membranes. Membranes were blocked with 5% non-fat milk in TBST (10 mM Tris–HCl pH 7.4, 100 mM NaCl, 0.5% Tween-20) for 2 hrs at room temperature and were then incubated overnight at 4 °C in 5% non-fat milk in TBST containing either pAkt, Akt, Cbl-b, pERK, ERK or tubulin antibodies, followed by 1 hr incubation with the peroxidase-conjugated second antibody. After extensive washing (TBST) proteins were visualized using the enhanced chemiluminescence reagent (SuperSignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL). The final result was analyzed by NIH Image J software.

Statistics

All data are expressed as means ± SD. Differences between groups were compared using Student's *t*-test. SPSS13.0 computer software was used for statistical analysis and $p<0.05$ was considered statistically sig-

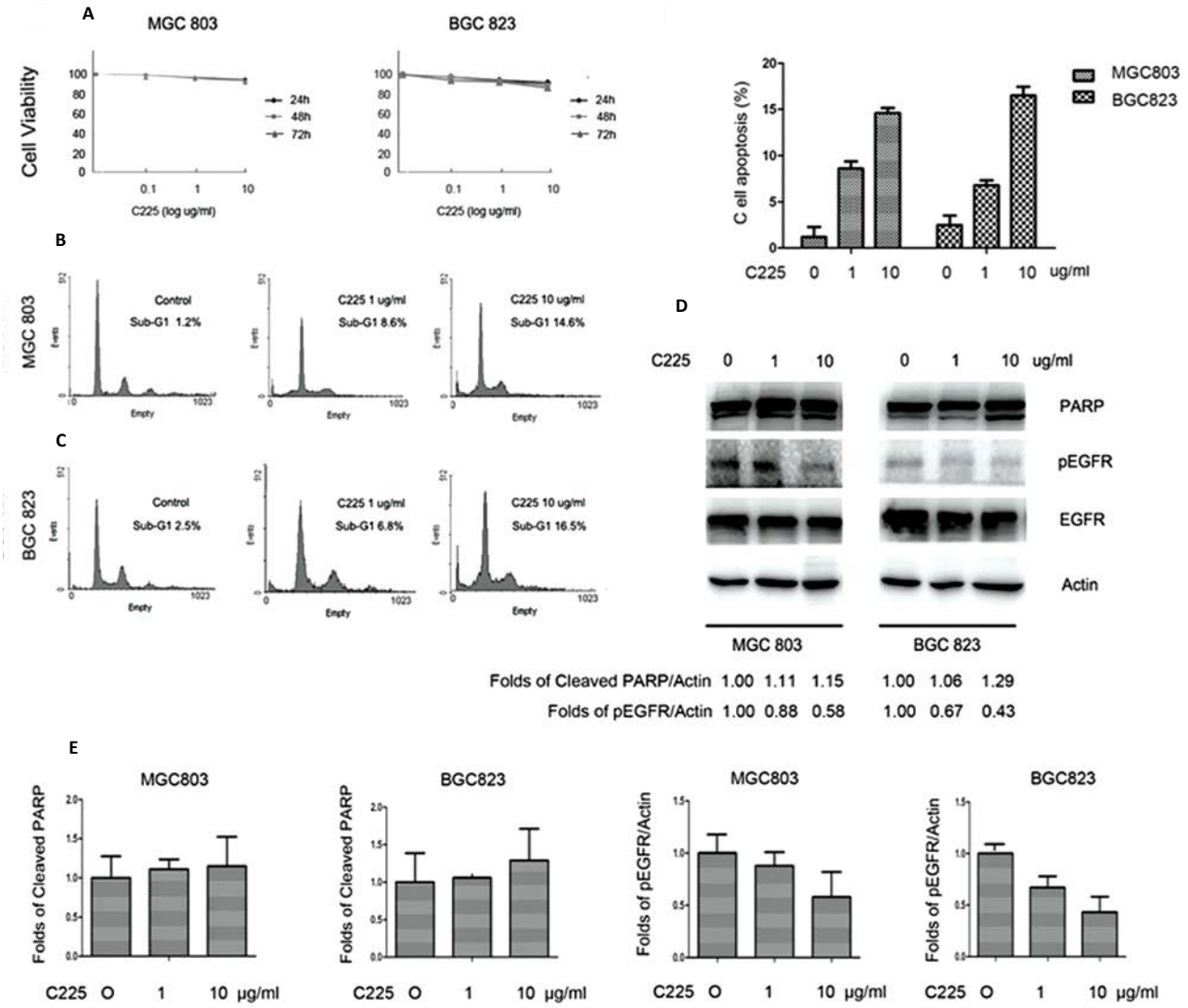

Figure 1. Effects of cetuximab on gastric cancer cell viability and apoptosis. MGC803 and BGC823 cells were treated with cetuximab for 24, 48 and 72 hrs. **(A)**: The cell viability was quantified by MTT assay (p=0.318 and p=0.111). **(B,C)**: MGC803 and BGC823 were treated with 0.1, 1, or 10 µg/mL cetuximab for 24 hrs, and apoptosis status was observed by flow cytometry. Treatment with 10µg/ml cetuximab for 24 hrs induced 14.6 and 16.5% cell apoptosis in MGC803 and BGC823, respectively ($p<0.05$, compared with control group). **(D)**: MGC803 and BGC823 were treated with 0.1, 1, or 10 µg/mL cetuximab for 24 hrs. Expression of PARP, pEGFR and EGFR was detected by Western blot. **(E)**: The cleavage of PARP was increased in a dose-independent manner in both MGC803 and BGC823, and significant increased in cells treated 10 µg/ml cetuximab group ($p<0.05$, compared with control group). The phosphorylation of EGFR was decreased in dose-independent manner in both MGC803 and BGC823 and significant decreased in cells treated 10µg/ml cetuximab group ($p<0.05$, compared with control group).

nificant. All means were calculated from at least three independent experiments.

Results

Cetuximab affects cell viability and induces apoptosis in gastric cancer cell lines

MGC803 and BGC823 cell lines were treated with different concentrations of cetuximab for 24, 48 or 72 hrs, and viability was evaluated. Following treatment, the two lines exhibited growth inhibition (Figure 1A). Additionally, treatment with 10 µg/ml cetuximab for 24 hrs induced 14.6 and 16.5% cell apoptosis in MGC803 and BGC823, respectively (Figure 1B,C). Moreover, treatment with cetuximab significantly induced a dose-dependent decrease of EGFR in MGC803 and BGC823 cells accompanied by PARP cleavages (Figure 1D). These data indicate that cetuximab affects cell viability and induces apoptosis in gastric cancer cell lines.

Ubiquitin–proteasome pathway is involved in cetuximab-induced gastric cancer cells apoptosis

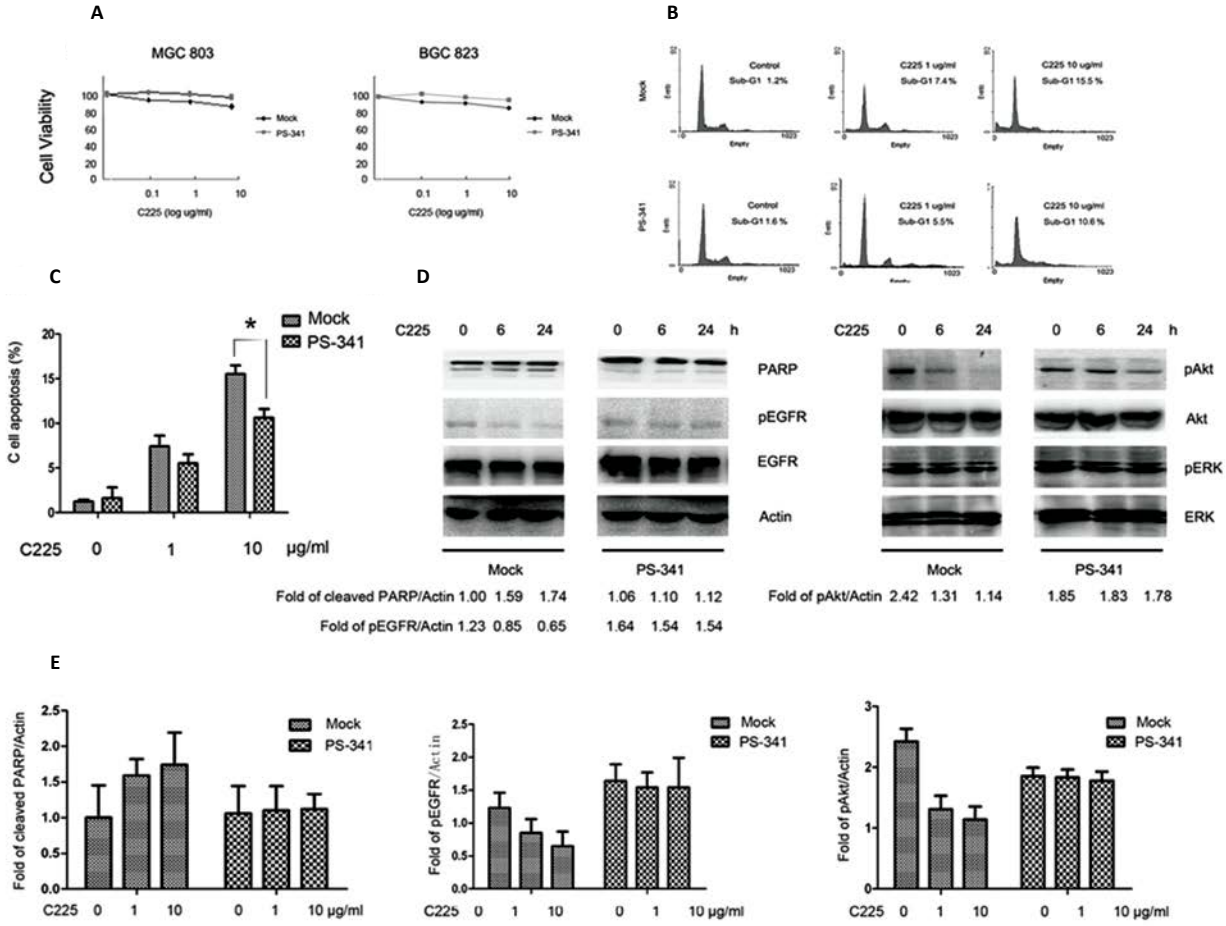

Figure 2. PS-341 decreased cetuximab-induced cell apoptosis by upregulating EGFR and phospho-Akt. MGC803 and BGC823 were treated with cetuximab (0.1, 1, or 10 µg/mL), with or without pre-treatment with PS-341 (10nM) for 4 hrs. **(A):** The cell viability was quantified by MTT assay (p=0.0396 and p=0.049683, respectively). **(B,C):** Cell apoptosis status was observed by flow cytometry. Treatment with 10 µg/ml cetuximab or pretreatment with PS-341 for 4 hrs followed by exposure to cetuximab for 24 hrs induced 15.5 and 10.6 % cell apoptosis in MGC803 cells (p=0.000302). **(D):** Treatment with 10 µg/ml cetuximab or pretreatment with PS-341 for 4 hrs followed by exposure to cetuximab for 24 hrs. Expression of PARP, EGFR, Akt and ERK were detected by Western blot. **(E):** The cleavage of PARP was decreased in cells pretreated with PS-341 in both cells treated 1 and 10 µg/ml cetuximab (p=0.001159 and p=0.000837, respectively). The phosphorylation of EGFR was increased in cells pretreated with PS-341 in both cells treated with 1 and 10µg/ml cetuximab (p=0.000012 and p=0.0013695, respectively). The phosphorylation of Akt was increased in cells pretreated with PS-341 in both cells treated with 1 and 10µg/ml cetuximab (p=0.010131 and p=0.000126, respectively).

To explore whether ubiquitin–proteasome pathway is involved in cetuximab-induced cell apoptosis, proteasome inhibitor PS-341 was used to inhibit the function of the proteasomal pathway. Pretreatment with 10nM PS-341 for 4 hrs decreased the sensitivity of cetuximab in both MGC803 and BGC823 cells (Figure 2A). In addition, treatment with 10 µg/ml cetuximab or pretreatment with PS-341 for 4 hrs followed by exposure to cetuximab for 24 hrs induced 15.5 and 10.6 % cell apoptosis in MGC803 cells ($p<0.05$; Figure 2B,C). Moreover, Western blot results showed that pre-treatment with PS-341 for 4 hrs, followed by exposure to cetuximab, increased the expression of phospho-EGFR, downstream phospho-Akt (Figure 2D). These results indicate that PS-341 decreases cetuximab-induced apoptosis in gastric cancer cell lines.

Cbl-b is involved in cetuximab-induced apoptosis in gastric cancer cell line

To determine whether the E3 ubiquitin ligase Cbl-b was involved in cetuximab -induced apoptosis in gastric cancer, previously established shRNA plasmids targeting MGC803 cells were transfected with Cbl-b and were used for further experiments. Non-silenced control and Cbl-b knockdown cells were treated with different concentrations of cetuximab. MTT assay showed that knockdown of Cbl-b obviously decreased the sensitivity of MGC803 to cetuximab cells. Cell proliferation was not inhibited in Cbl-b knockdown cells (Figure 3A). In addition, treatment with 10 µg/ml cetuximab for 24 hrs induced 18.4 and 10.6% cell apoptosis in non-silenced control and Cbl-b

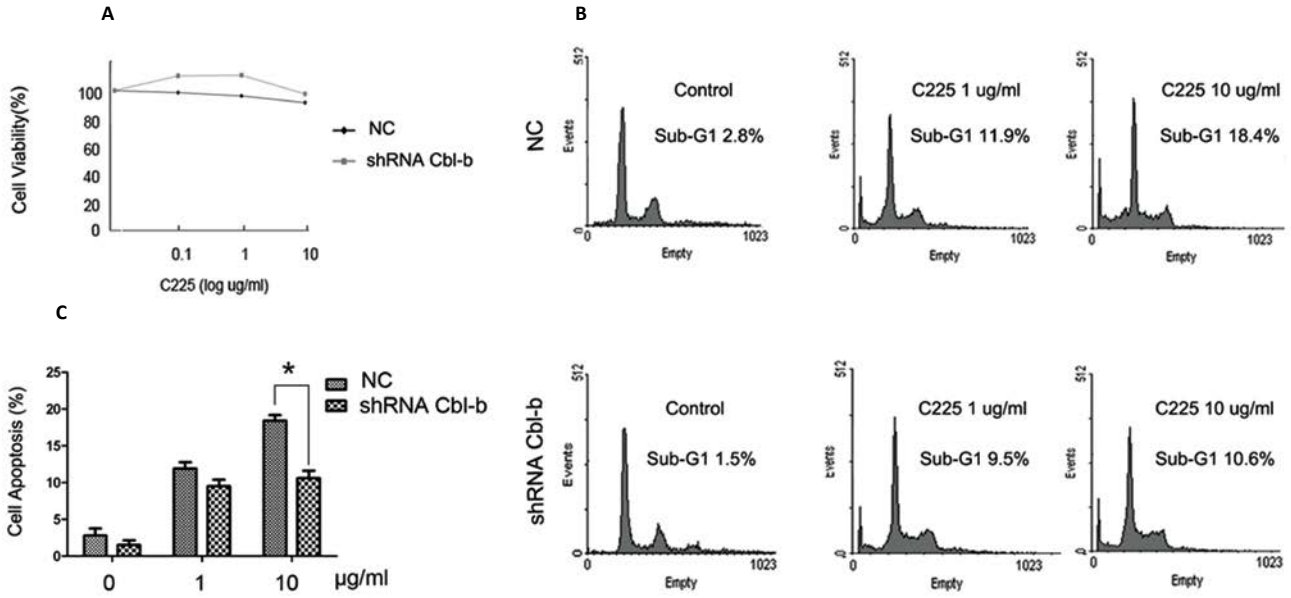

Figure 3. The inhibition of casitas B-lineage lymphoma-b (Cbl-b) expression decreased the sensitivity of cetuximab in gastric cancer cells. MGC803, non-silenced control and Cbl-b knockdown cells were treated with cetuximab (0.1, 1, or 10 μg/mL) for 24 hrs. **(A):** The cell viability was quantified by MTT assay. Cell proliferation was not inhibited in Cbl-b knockdown cells compared with non-silenced control group (p=0.044912). **(B,C):** MGC803, non-silenced control and Cbl-b knockdown cells were treated with cetuximab (0.1, 1, or 10 μg/mL) for 24 hrs. Cell apoptosis status was observed by flow cytometry. Treatment with 10μg/ml cetuximab for 24 hrs induced 18.4 and 10.6% cell apoptosis in non-silenced control and Cbl-b knockdown cells, respectively (p=0.000141).

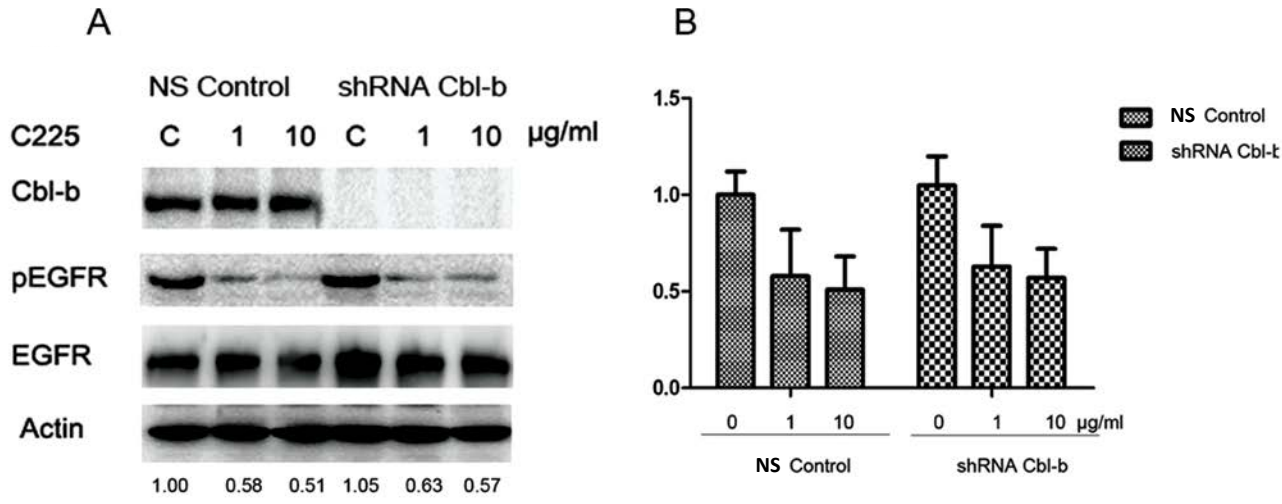

Figure 4. The inhibition of casitas B-lineage lymphoma-b (Cbl-b) expression increased the expression of EGFR. MGC803, non-silenced control and Cbl-b knockdown cells were treated with cetuximab (1,10 μg/ml) for 24 hrs. The expression of Cbl-b and EGFR were detected by Western blot. **A:** Specifically silenced Cbl-b increased the expression of EGFR (p=0.0674). The expression of phosphorylation of EGFR and EGFR was increased in cells with specifically silenced Cbl-b compared with non-silenced control. **B:** Cetuximab induced degradation of EGFR was decreased in cells specific silenced Cbl-b compared with non-silenced control (p=0.0674).

knockdown cells, respectively (p<0.05; Figure 3B,C). These results indicate that Cbl-b F 3B,C is required for cetuximab-induced apoptosis in MGC803 cells.

Cbl-b is involved in cetuximab-induced degradation of EGFR in gastric cancer cell line

Next, to characterize the effect of Cbl-b on cetuximab-induced apoptosis, non-silenced control and Cbl-b knockdown cells were treated with cetuximab for 24 hrs. Specific silenced Cbl-b increased the expression of EGFR (Figure 4). These observations collectively indicate that Cbl-b is involved in cetuximab-induced apoptosis in MGC803 cells.

Discussion

To clarify the mode of action of cetuximab in gastric cancer, we investigated its antitumor activity on gastric cancer cells. The results indicated that ubiquitin-proteasome pathway was involved in cetuximab-induced apoptosis in gastric cancer cell lines. Our data showed that Cbl-b was involved in this process. Knockdown Cbl-b increased the expression of EGFR, which indicated that Cbl-b mediated ubiquitin–proteasome pathway is involved in cetuximab-induced apoptosis in MGC803 cells.

EGFR is a member of the HER family of receptor tyrosine kinases and its activation leads to several signaling cascades, including MAPK and PI(3)K/Akt that influence cell proliferation, angiogenesis, and invasion [13]. Cetuximab, a monoclonal antibody targeting EGFR, prevents receptor activation and dimerization and ultimately induces receptor internalization and downregulation [14]. Cetuximab exhibits promising antitumor activity in head and neck cancer and metastatic colorectal cancer [15-18]. Recent studies have shown that UPS is involved in regulating the sensitivity of cetuximab [8,9]. However, the effect of UPS in the regulation of antitumor effect of cetuximab is controversial. In the present study, cells pretreated with PS-341 suppressed cetuximab-induced cell apoptosis accompanied with increased expression of EGFR in MGC803 and BGC823. These results indicate that ubiquitin–proteasome pathway is involved in cetuximab-induced apoptosis in MGC803 and BGC823 cells.

Results from previous studies have shown that UBE4B, an E3 ubiquitin ligase, mediates EGFR ubiquitination and enhances the sensitivity to cetuximab in neuroblastoma cells [9], and that c-Cbl promotes mono-ubiquitination of the activated EGFR at the cell membrane and thereby regulates receptor endocytosis [19-21]. Cbl-b is also a negative regulator of non-receptor tyrosine kinases, thereby resulting in their ubiquitination and downregulation. In the present study, knockdown of Cbl-b decreased the sensitivity of cetuximab in gastric cancer cells accompanied with increased expression of EGFR. These findings suggest that Cbl-b is involved in the regulation of sensitivity of cetuximab in gastric cancer cells.

Our observations indicate that ubiquitin-proteasome pathway was involved in the regulation of sensitivity of cetuximab in gastric cancer cells. Furthermore, the E3 ubiquitin ligase Cbl-b plays a key role in regulating the sensitivity of cetuximab in gastric cancer cells. These observations collectively indicate that the regulatory mechanism of cetuximab action and Cbl-b mediated ubiquitin–proteasome pathway are involved in cetuximab-induced apoptosis in MGC803 cells.

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Conflict of interests

The authors declare no confict of interests.

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