Targeting eIF4A using rocaglate CR-1-31B sensitizes gallbladder cancer cells to TRAIL-mediated apoptosis through the translational downregulation of c-FLIP

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Abstract. Induction of the apoptosis of tumor cells is a promising therapeutic approach for the treatment of cancer. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a novel type of anticancer drug. However, gallbladder cancer cells (GBC) exhibit strong resistance to TRAIL. The aim of the present study was to assess the effect of rocaglate CR-1-31B (CR-31), an inhibitor of eukaryotic translation initiation factor 4A (eIF4A), on the sensitization of cells to TRAIL-induced apoptosis in TRAIL-resistant GBC. eIF4A was highly abundant in GBC tissues and cell lines (GBC-SD and SGC-996). GBC cells were treated using TRAIL and/or CR-31 and then apoptosis and TRAIL signaling were detected *in vitro*. CR-31 enhanced the sensitivity of TRAIL-resistant GBC cells, due to the CR-31-mediated eIF4A translational downregulation of c-FLIP and the subsequent activation of the caspase cascade. Furthermore, GBC-SD tumor xenografts models were established and the effects of CR-31 *in vivo* were assessed. CR-31 significantly reduced the growth and initiated the apoptosis of tumor cells, suggesting that CR-31 also increased sensitivity *in vivo*. Taken together, the results of the present study show that CR-31 treatment countered the resistance to TRAIL in GBC cells *in vitro* and *in vivo*. Therefore, eIF4A may serve as a novel therapeutic target and its combination with TRAIL-CR-31 as a therapy may serve as a novel strategy for GBC treatment.

Introduction

Gallbladder cancer (GBC) is among the most common and lethal types of human malignancy (1). However, classical chemotherapy is rarely effective and drug resistance represents a significant challenge in treating GBC, particularly advanced GBC cells, which exhibit a strong resistance to apoptosis-inducing chemotherapeutic drugs owing to the reprograming of apoptosis (2). Therefore, novel therapeutic targets are required to increase the pro-apoptotic effects of therapeutic drugs in GBC.

TNF-related apoptosis induced ligand (TRAIL) mediates the extrinsic apoptotic pathway by binding to Death Receptor 4 or 5 on the cell surface. Upon binding of ligands to these receptors, they assemble into homomeric and heteromeric complexes and recruit FADD and caspase-8, which trigger a downstream caspase cascade (3). TRAIL has shown notable efficacy as a therapeutic drug owing to its high selectivity of inducing apoptosis (4). However, an increasing number of studies have shown that certain GBC cells respond poorly to TRAIL alone, and cancer cell death is not induced (5,6). There are numerous causes underlying the development of resistance to TRAIL in GBC cells. As caspase-8 mediates TRAIL-induced apoptosis, the TRAIL resistance of GBC cells is related to downregulation of caspase-8, which results in the reduced activity of the apoptotic pathway (6). Of note, caspase-8 activity is reduced through c-FLIP in TRAIL-resistant GBC cells. In addition, c-FLIP is reported to be abundant in TRAIL-resistant GBC cells compared with TRAIL-sensitive cells. Furthermore, a decrease of c-FLIP can overcome resistance in GBC cells to TRAIL (7). Thus, reducing the abundance of c-FLIP or inhibiting its function may counter TRAIL resistance in GBC cells.

Targeting the initiation of translation is a rapidly emerging anti-tumor strategy in cancer treatment (8). Rocaglates are a class of natural compounds extracted from *Aglaia genus* that possess potent anti-neoplastic properties by targeting

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eukaryotic translation initiation factor 4A (eIF4A) (9,10). In particular, CR-1-31B (CR-31), is a synthetic rocaglate, which has been shown to exhibit powerful inhibitory effects over eIF4A by perturbing the interaction between eIF4A and RNA, sequentially impeding initiation during protein synthesis (11). However, the exact anticancer effects of rocaglate CR-31 in GBC remain to be determined.

In the present study, it was demonstrated that eIF4A was abundant in GBC tissues and cell lines, and its inhibitor CR-31 significantly sensitized GBC cells to TRAIL-induced apoptosis via the eIF4A-mediated translational downregulation of c-FLIP, in addition to mediating the caspase cascade *in vitro*. Furthermore, CR-31 strongly repressed the growth and enhanced the apoptosis of a GBC xenograft mouse model. Thus, it was shown that eIF4A may be a novel therapeutic target and that CR-31 may serve as an adjuvant to TRAIL treatment, which may be valuable for the management of GBC.

Materials and methods

CBC cell lines, chemicals, human GBC samples and ethics. GBC-SD and SGC-996 cells were obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Wuhan, China) and were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 μg/ml strepto-mycin and 100 U/ml penicillin (all from Gibco; Thermo Fisher Scientific, Inc.). Normal human intrahepatic biliary epithelial cells (HIBECs) were obtained from ScienCell Research Laboratories, Inc. and were incubated using epithelial cell medium (ScienCell Research Laboratories, Inc.). Cells were incubated at 37˚C with 5% CO_2. TRAIL was obtained from PeproTech, Inc. (cat. no. 310-04). Rocaglate CR-1-31B was synthesized as previously described (12) and other reagents were obtained from Sigma-Aldrich; Merck KGaA, unless otherwise stated.

Human GBC and paired normal gallbladder tissues were collected from 42 patients during gallbladder resection for GBC (22 males and 20 females) between July 2015 and June 2018 at the Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. The patients did not receive chemotherapy prior to surgery. The use of human GBC tissues was approved by Tongji Hospital Research Ethical Committee, and written informed consent was obtained from all the patients.

Cell viability analysis. GBC-SD and SGC-996 cells were cultured in 96-well plates in complete medium (Gibco; Thermo Fisher Scientific, Inc.) with CR-31 (0-200 nM for 48 h) and CR-31 (100 nM) and/or TRAIL (100 ng/ml) for the indicated time intervals (0-24 h). An MTT assay kit (cat. no. ab211091; Abcam) was used to assess cell viability. Briefly, following removal of the medium, MTT (150 μl; 0.5 mg/ml) was added to each well and then cultured for 2 h at 37˚C in an incubator. The medium was removed and DMSO (150 μl) was added to each well and the plates were shaken. Absorbance at 570 nm was analyzed using a microplate reader (BioTek Instruments, Inc.). Results were normalized to DMSO-treated cells.

Colony formation assay. For the colony formation assays, cells were plated in a 12-well plate and incubated for 12 h at 37˚C

with 5% CO_2, and then incubated with CR-31 (100 nM) and/or TRAIL (100 ng/ml) for another 12 h at 37˚C with 5% CO_2. Cells were washed with PBS, fixed using 4% paraformalde-hyde for 15 min, and subsequently stained using crystal violet for 15 min.

Flow cytometry analysis. Cells were treated with CR-31 (100 nM) or DMSO for 12 h, and incubated with TRAIL (100 ng/ml) for an additional 12 h at 37˚C with 5% CO_2. An Annexin V/propidium iodide (PI) kit (cat. no. v13242; Invitrogen; Thermo Fisher Scientific, Inc.) was used to assess cell apoptosis, according to the manufacturer's protocol. Flow cytometry was used to determine the proportion of apoptotic cells. Flow cytometry was performed on a FACSCalibur system (BD Biosciences).

Reverse transcription-quantitative (RT-q)PCR. RT-qPCR was performed as described previously (13). Briefly, total RNA was extracted from GBC-SD cells using TRIzol® reagent (cat. no. 15596018; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A total of 1 μg total RNA was used as the template for cDNA synthesis using a reverse transcription kit (cat. no. K1691; Thermo Fisher Scientific, Inc.). Equal quantities of cDNA were used for PCR analysis. The sequences of the primers were: c-FLIP forward, 5'-CGCTCAACAAGAACCAGTG-3'and reverse, 5'-AGG GAAGTGAAGGTGTCTC-3'; and β-actin forward, 5'-AGT GTGACGTCGACATCCGC-3' and reverse, 5'-GACTCG TCGTACTCCTGCTT-3'. PCR primers were purchased from Sangon Biotech Co., Ltd.

Western blot analysis. Western blot analysis was performed as described previously (13). Briefly, GBC-SD and SGC-996 cells were washed twice with ice-cold PBS and then lysed with ice-cold RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) supplemented using EDTA-free protease inhibitor cocktail (cat. no. 11836170001; Roche Diagnostics), and followed by protein concentration deter-mination with BCA assay. Lysates were kept on ice for 30 min and then centrifuged at 14,000 x g for 20 min at 4˚C. Equal amounts (20 μg) of proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were block with 5% bovine serum albumin (BSA) in Tris-buffered saline Tween-20 for 2 h and then incubated with primary antibodies at 4˚C overnight. Immunoreactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies. The primary antibodies used were: Caspase-8 (cat. no. 9746; Cell Signaling Technology, Inc.), caspase-3 (cat. no. 9662; Cell Signaling Technology, Inc.), cleaved-caspase-3 (cat. no. 9664; Cell Signaling Technology, Inc.), eIF4A (cat. no. ab31217; Abcam), c-FLIP (cat. no. ab8421; Abcam) and α-tubulin (cat. no. sc-5286; Santa Cruz Biotechnology, Inc.).

Immunofluorescence assay. The treated cells were fixed using 4% formaldehyde for 15 min at room temperature, washed and permeabilized using 0.5% Triton X-100 for 20 min at room temperature. The cells were treated for 1 h using Alexa Fluro 647-labeled IgG (H + L) (cat. no. ab150115; Abcam) and an

anti-c-FLIP antibody (cat. no. ab8421; Abcam). Nuclei were stained using DAPI. Cells were washed with PBS and visualized using confocal laser scanning microscopy (Olympus Corporation).

Knockdown of eIF4A. Highly pure small interfering (si)RNA non-targeting control (CTRL; 5'-aacuuacgcugaguacuucga-3') or siRNA eIF4A (Sangon Biotech Co., Ltd.) were transfected into GBC-SD cells using Lipofectamine® 3000 (cat. no. L3000008; Invitrogen, Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Briefly, 2.5 μl Lipofectamine 3000 and siRNA (50 nmol/l) were added to the medium (100 μl), and then the mixture was added to the cells, which had been washed. Cells were subsequently cultured for 5 h at 37°C, after which, the medium was removed and replaced with fresh medium for 48 h. Cells were treated using DMSO or TRAIL for 12 h following transfection. After that, the cells were used for subsequent experiments immediately.

In vivo BALB/c nude mice. GBC-SD cells (~2x10⁶ cells) were suspended in 100 μl 1:1 Matrigel/medium and then injected subcutaneously into the right flank of male BALB/c nude mice (n=20; age, 6-weeks old). The animals were housed and assayed under conditions of control temperature (22±2°C), humidity (45-65%), and artificial light (12-h light-dark cycle) with free access to rodent chow and water. The mice were randomly separated into four equal groups (n=5 per group): i) Control group [receiving vehicle (10% DMSO and 90% olive oil)]; ii) TRAIL group [intraperitoneal injection of TRAIL (80 μg/kg) in 60 μl olive oil]; iii) CR-31 group [intraperitoneal injection of CR-31 (2 mg/kg) in 60 μl olive oil]; and iv) TRAIL/CR-31 group [intraperitoneal injection of TRAIL (80 μg/kg) and CR-31 (2 mg/kg) in 60 μl olive oil]. Treatment was performed once every 2 days for 28 days. Body weight and tumor volume were monitored on a weekly basis. Tumor volumes (mm³) were calculated as follows: Volume=length x S²/2 (where S is the shortest diameter). After 28 days, mice were anesthetized by intraperitoneal injection using 10% chloral hydrate (350 mg/kg) and were sacrificed using cervical dislocation. A comprehensive evaluation of death by respiratory, heartbeat, pupil and nerve reflex of these mice was carried out and recorded. Finally, tumor specimens were collected for immunohistochemistry and TUNEL staining.

The mice were procured from HFK Bioscience Company. Animal experiments were performed according to the Guidelines of Laboratory Animals of Tongji Hospital, which is approved by the National of Health (NIH publication 85-23 revised 1996).

Immunohistochemistry and TUNEL assay. Immunohistochemistry was performed using anti-cleaved-caspase-3 (cat. no. 9664; Cell Signaling Technology, Inc.) and anti-eIF4A (cat. no. ab31217; Abcam) antibodies, as previously described (14). Two independent individuals examined the proportion of positive cells in ≥5 fields of view (magnification, x400), and tissues were scored as follows: 0, negative; 1, <25% positive cells; 2, 25-50; 3, 50-75; and 4, >75%. Images were visualized and calculated using a Nikon microscope (Nikon Corporation). TUNEL assays was performed

on paraffin-embedded tissue sections using a one-step TUNEL apoptosis assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Briefly, samples were incubated with TUNEL reaction mixture for 1 h at 37°C in the dark and then washed twice with PBS. Nuclei were counterstained with DAPI (300 nM; cat. no. D8417; Sigma-Aldrich) for 10 min at room temperature and mounting with antifade medium (cat. no. P0126; Beyotime Institute of Biotechnology) and then washed twice with PBS. The condensed or fragmented nuclei of apoptotic cells were observed using fluorescence microscopy (Olympus Corporation) (magnification, x200) in 20 fields of vision.

Statistical analysis. All data were analyzed using GraphPad Prism version 8.4.2 (GraphPad Software, Inc.). All data are presented as the mean ± standard deviation. Student's t-test was used to compare differences between two groups, and multiple groups were compared with one-way or two-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

CR-31 sensitizes GBC cells to TRAIL-mediated apoptosis. To assess whether CR-31 could sensitize GBC cells to TRAIL-induced apoptosis, GBC-SD and SGC-996 cells were used, as they exhibit strong chemoresistance to TRAIL (7). The treatment of GBC-SD and SGC-996 cells with CR-31 resulted in dose-dependent growth inhibition, with an IC$_{50}$ of ~100 nM (Fig. 1A). Furthermore, the efficacy of CR-31 on TRAIL-mediated cytotoxicity was assessed. CR-31 and TRAIL alone were weakly cytotoxic to GBC-SD and SGC-996 cells; however, the treatment of GBC-SD and SGC-996 cells using CR-31 increased the cytotoxicity to TRAIL (Fig. 1B). Moreover, although CR-31 or TRAIL did not reduce the colony numbers, the combined treatment with CR-31 and TRAIL significantly reduced the colony formation of GBC-SD and SGC-996 cells, indicating a synergistic effect (Fig. 1C and D). Similar results were observed based on the Annexin V/PI assay in GBC-SD and SGC-996 cells. TRAIL or CR-31 alone resulted in increased early apoptosis and necrosis in GBC-SD (22.28 and 9.77%, respectively) and SGC-996 cells (23.1 and 10.5%, respectively). However, combined apoptosis and necrosis were increased in both GBC-SD (41.48%) and SGC-996 cells (41.5%) (Fig. 1E and F). Collectively, the results showed that CR-31 may highly sensitize chemo-resistant GBC-SD and SGC-996 cells to TRAIL.

eIF4A may be a therapeutic target and CR-31 can downregulate the expression of c-FLIP at the translational level in GBC. To investigate the clinical significance of eIF4A in human samples of GBC, the abundance of eIF4A was detected by immunohistochemistry in GBC and paired normal gallbladder tissues. Immunohistochemistry analysis suggested that eIF4A abundance was significantly higher in the 42 GBC samples compared with the paired normal control tissues (Fig. 2A). Furthermore, the eIF4A protein was strongly expressed in GBC-SD and SGC-996 cells compared with the HIBECs (Fig. 2B). Increased expression of c-FLIP in GBC cells has been shown to be associated
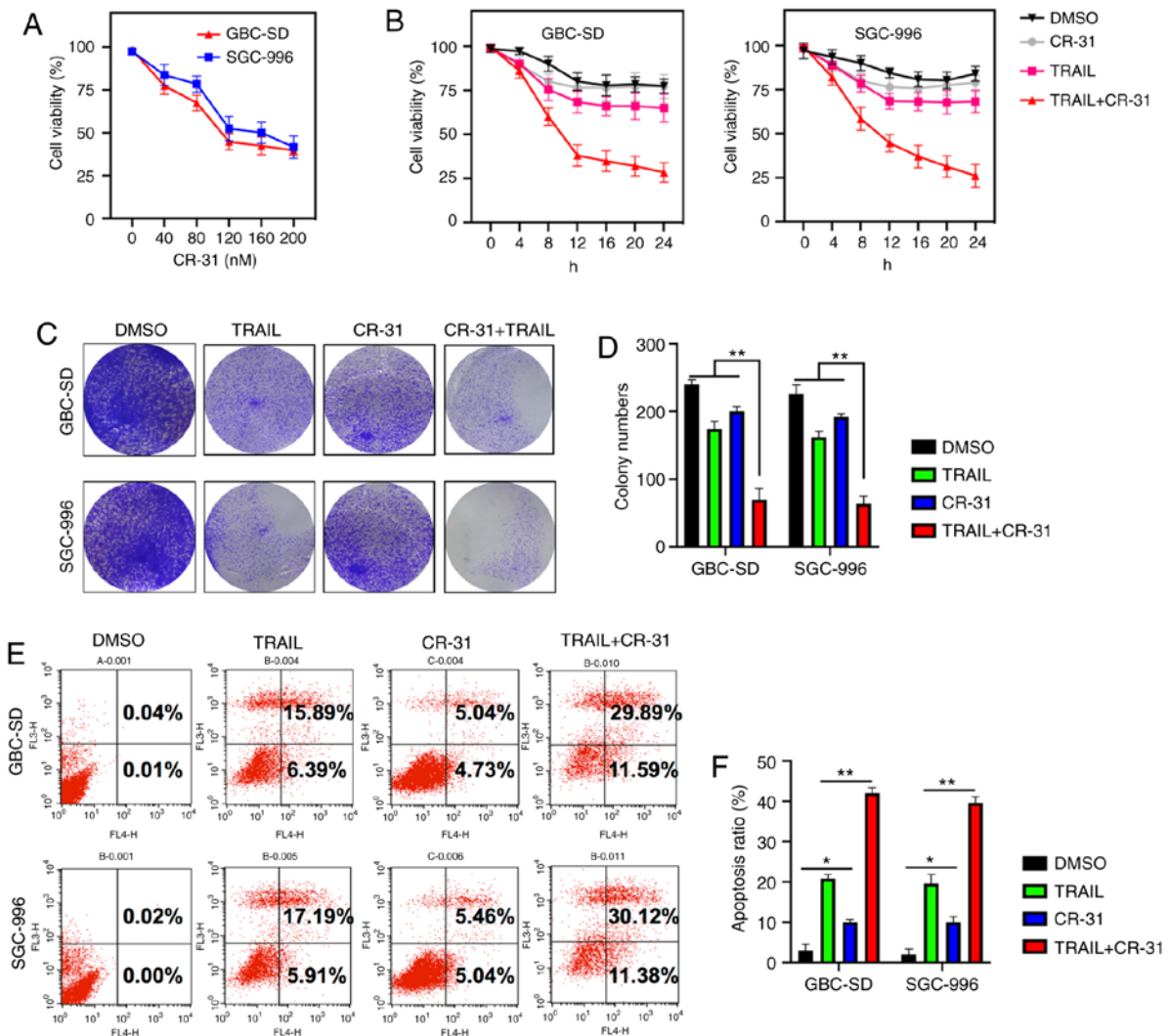
Figure 1. CR-31 sensitizes GBC cells to TRAIL-mediated apoptosis. (A) Cell viability of GBC-SD and SGC-996 cells treated with 0-200 nM CR-31 for 48 h. (B) Cell viability of GBC-SD and SGC-996 cells treated with 100 nM CR-31 and/or 100 ng/ml TRAIL for the indicated time-points. (C and D) GBC-SD and SGC-996 cells were treated with 100 nM CR-31 for 12 h and 100 ng/ml TRAIL for 12 h. Cells were washed with PBS, fixed using 4% paraformaldehyde and stained with crystal violet. The number of colonies was calculated and statistically analyzed. (E and F) Apoptosis was detected using Annexin V/PI staining and flow cytometry. Data are presented as the mean ± standard deviation. Representative images of three repeats are shown. *P<0.05, **P<0.01. CR-31, CR-1-31B; GBC, gallbladder cancer; TRAIL, tumor necrosis factor related apoptosis-inducing ligand.

with TRAIL resistance (7), thus the effect of CR-31 on the abundance of c-FLIP was determined. The results showed that eIF4A and c-FLIP were abundantly expressed in GBC cells, and CR-31 treatment significantly decreased the levels of c-FLIP protein in GBC-SD cells (Fig. 2C). Notably, there were no significant changes in c-FLIP mRNA levels (Fig. 2D), suggesting a translational mechanism of regulation of expression. Furthermore, confocal microscopy showed that CR-31 resulted in a decrease in the plasma localization of c-FLIP (Fig. 2E). Collectively, the results showed that eIF4A may be a therapeutic target and CR-31 can downregulate the abundance of c-FLIP at the translational level in GBC.

Knockdown of eIF4A downregulates the abundance of c-FLIP protein, mimicking the effects of CR-31 on GBC-SD cells. To assess the effect of knockdown of eIF4A on c-FLIP expression and CR-31 treatment, an eIF4A-knockdown GBC-SD cell line was established. siRNA-eIF4A resulted

in a significant decrease in both eIF4A and c-FLIP expression (Fig. 3A). Crystal violet staining showed eIF4A knockout GBC-SD cells exhibited significantly reduced colony formation when treated with TRAIL compared with the control group (Fig. 3B and D). Furthermore, the results of Annexin V-PI apoptosis by flow cytometry showed the same effect; the proportion of apoptotic and necrotic cells following TRAIL treatment was 24.78 and 8.73%, respectively, in the siRNA-eIF4A-transfected cells, and 43.15% in siRNA-eIF4A-transfected cells treated with TRAIL (Fig. 3C and E), showing that knockdown of eIF4A in GBC-SD cells resulted in similar effects to treatment with CR-31.

CR-31 enhances TRAIL-mediated apoptosis in a caspase-dependent manner. As TRAIL-mediated apoptosis is mediated through the activation of the caspase cascade (15), whether caspase-8 and caspase-3 expression was increased in GBC-SD cells treated with TRAIL or CR-31 alone was next determined. The results showed that the levels of pro-caspase-8
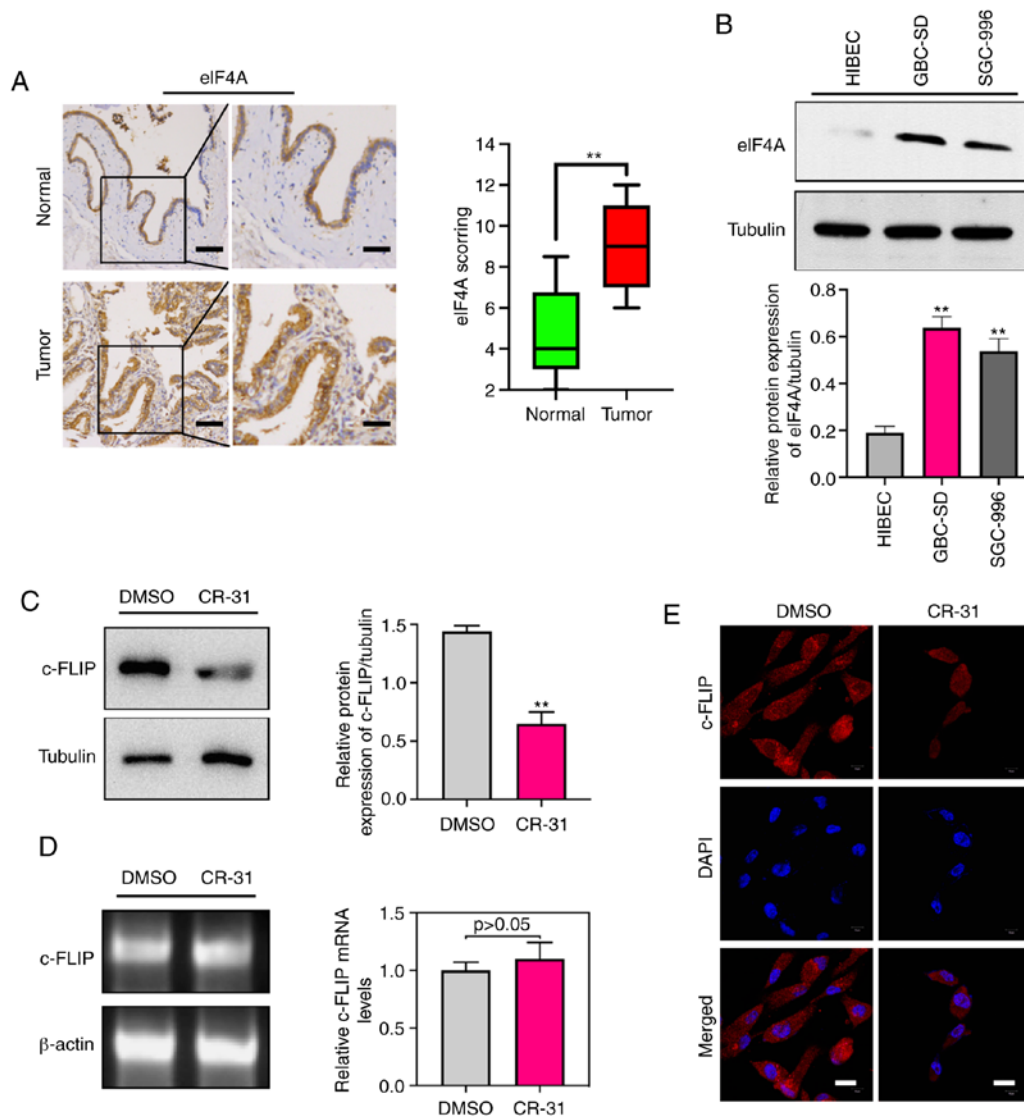
Figure 2. eIF4A expression is abundant in GBC, and CR-31 treatment reduces c-FLIP expression at the translational level in GBC cells. (A) Immunohistochemical staining of eIF4A expression in 42 human GBC tissues and paired-matched normal gallbladder tissues (left panel). Scale bar, 100 μm (left panels) and 50 μm (right panels). Statistical analysis of eIF4A expression in GBC tissues (right panel). [**]P<0.01. (B) Western blotting of eIF4A expression in GBC-SD, SGC-996 and HIBEC cells. α-tubulin was used as a control. Statistical analysis of eIF4A protein expression in GBC cells (panel below). [**]P<0.01. (C) Western blotting was performed on GBC-SD cells treated with 10 nM CR-31 for 12 h. DMSO was used as the control. Analysis of relative c-FLIP protein levels (left panel). [**]P<0.01. (D) c-FLIP mRNA levels in GBC-SD cells were determined by reverse transcription-quantitative PCR (left panel). Analysis of relative c-FLIP mRNA levels (right panel). β-actin was used as the loading control. (E) Immunofluorescence analysis of eIF4A expression (red) in GBC-SD cells treated with DMSO or 100 nM CR-31 for 12 h. Nuclei were stained with DAPI (blue). Scale bar, 10 μm. eIF4A, eukaryotic translation initiation factor 4A; CR-31, CR-1-31B; GBC, gallbladder cancer; HIBEC, human intrahepatic biliary epithelial cells.

were weakly decreased when treated with CR-31 or TRAIL alone, along with an increase in cleaved-caspase-8 expression (Fig. 4). Notably, when combined, CR-31 and TRAIL significantly increased the levels of cleaved-caspase-8 (Fig. 4). Notably, TRAIL or CR-31 alone resulted in slightly increased levels of cleaved-caspase-3 in GBC-SD cells (Fig. 4). However, CR-31 and TRAIL combined significantly increased the levels of cleaved-caspase-3 (Fig. 4). Therefore, the results suggest that CR-31 enhanced TRAIL-mediated apoptosis in a caspase-dependent manner.

CR-31 administration reduces the growth and initiates tumor apoptosis in a BALB/c nude mice model of GBC. To evaluate the effects of CR-31 on TRAIL *in vivo*, a BALB/c nude mice model of GBC was established using GBC-SD cells.

The tumor volume and weights of GBC-SD xenografts were measured. Tumor weights after 4 weeks of treatment showed a decreasing trend from control, to TRAIL to CR-31 to combination treatment (Fig. 5A and C). However, tumor weight did not exceed 10% of body weight of mice. Interestingly, there was a notable reduction in tumor volumes treated with CR-31 compared with those treated with TRAIL (Fig. 5A and C), and this may due to an increase in the production of TRAIL from natural killer cells *in vivo* (16). Notably, CR-31 neither resulted in the notable reduction of body weight nor showed evidence of toxicity (Fig. 5B) and the maximum percentage of weight loss did not exceed 8% of body weigh of mice, indicating that CR-31 was safe *in vivo*. The apoptotic effect of CR-31 and TRAIL were assessed *in vivo* using cleaved-caspase-3 and TUNEL staining. Upregulated expression of cleaved-caspase-3
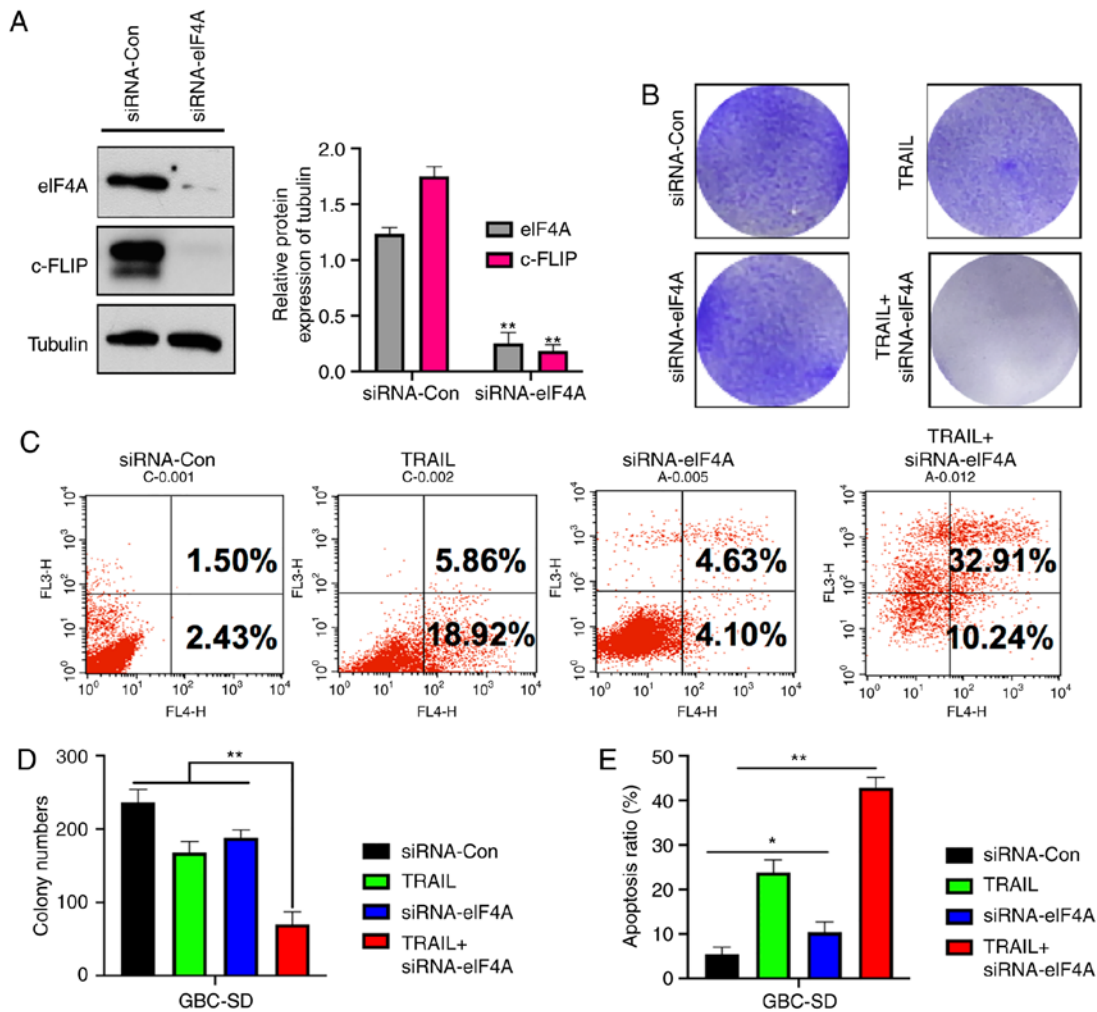
Figure 3. Knockdown of eIF4A mimics the effect of CR-31 on sensitization of GBC-SD cells to TRAIL. GBC-SD cells were transfected with siRNA-c-FLIP or control-siRNA for 48 h and treated with 100 ng/ml TRAIL for 12 h. (A) Western blotting showing the protein expression levels of eIF4A and c-FLIP in eIF4A-knockdown cells and control (left panel). Analysis of relative eIF4A and c-FLIP protein levels (right panel). **P<0.01. (B and D) Cells were washed with PBS, fixed using 4% paraformaldehyde and stained with crystal violet. The number of colonies was calculated and statistically analyzed. (C and E) Apoptosis was detected using Annexin-PI staining by flow cytometry, and statistically analyzed. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01. eIF4A, eukaryotic translation initiation factor 4A; CR-31, CR-1-31B; GBC, gallbladder cancer; TRAIL, Tumor necrosis factor related apoptosis-inducing ligand; siRNA, small interfering RNA.

were observed, and there was an increase in apoptosis when treated with the combined treatment compared with either TRAIL or CR-31 alone, which was in agreement with the *in vitro* results (Fig. 5D-G). Collectively, the results showed that CR-31 enhanced TRAIL-induced apoptosis of GBC xenograft tumors *in vivo*. Taking these findings together, we conclude that CR-31 can enhance TRAIL-triggered apoptosis by downregulating c-FLIP expression at the translational level and then activating the caspase cascade in TRAIL-resistant GBC cells (Fig. 6).

Discussion

GBC is one of the most malignant types of cancer and is associated with a poor prognosis that is largely attributed to late diagnosis and acquired drug resistance to traditional chemotherapy regimens (17). In particular, GBC cells exhibit significant resistance to TRAIL. Therefore, there is a need to develop novel strategies for overcoming TRAIL resistance in GBC cells. Recently, the effect of increasing apoptosis through

the use of natural compounds has been described in GBC cells (18,19). In the present study, it was shown that rocaglate CR-31, an inhibitor of eIF4A, enhanced the TRAIL-mediated apoptosis of GBC cells through the eIF4A-mediated translational downregulation of c-FLIP.

As the cancer cell type may influence the response to TRAIL, the highly TRAIL-resistant GBC cell lines, GBC-SD and SGC-996 cells, were chosen (7). Subsequently, the effect of CR-31 on GBC-SD and SGC-996 cells and their sensitivity to TRAIL treatment was determined. Notably, GBC-SD and SGC-996 cells were less responsive to TRAIL treatment. However, TRAIL-resistant GBC-SD and SGC-996 cells treated with CR-31 showed notably reduced growth. Therefore, the results suggest that CR-31 has the potential to sensitize cells to TRAIL-mediated cell death.

Targeting translation initiation may serve as a promising anti-tumor strategy. In the present study, eIF4A was shown to be highly abundant in GBC tissues and cell lines. In addition, eIF4A inhibitor rocaglates CR-31 is currently the most potent translation initiation inhibitor that functions via eIF4A,
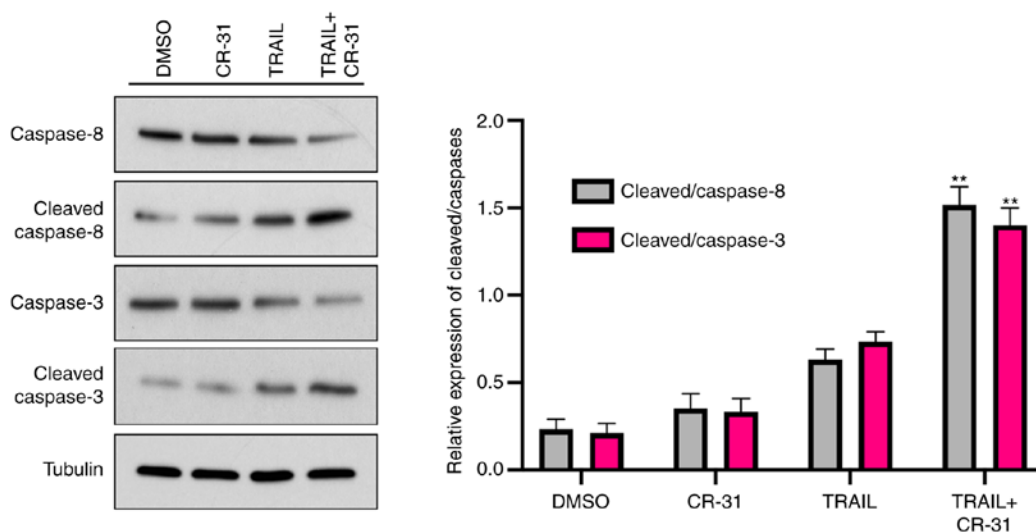
Figure 4. CR-31 augments TRAIL-mediated caspase activation. Western blotting from GBC-SD cells treated using 100 nM CR-31 for 12 h followed by treatment with 100 ng/ml TRAIL for 12 h. Treatment with DMSO was used as the control. Analysis of relative caspase-8, cleaved caspase-8, caspase-3 and cleaved caspase-3 protein levels (left panel). Data are presented as the mean ± standard deviation. $^{**}P<0.01$. CR-31, CR-1-31B; GBC, gallbladder cancer; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.
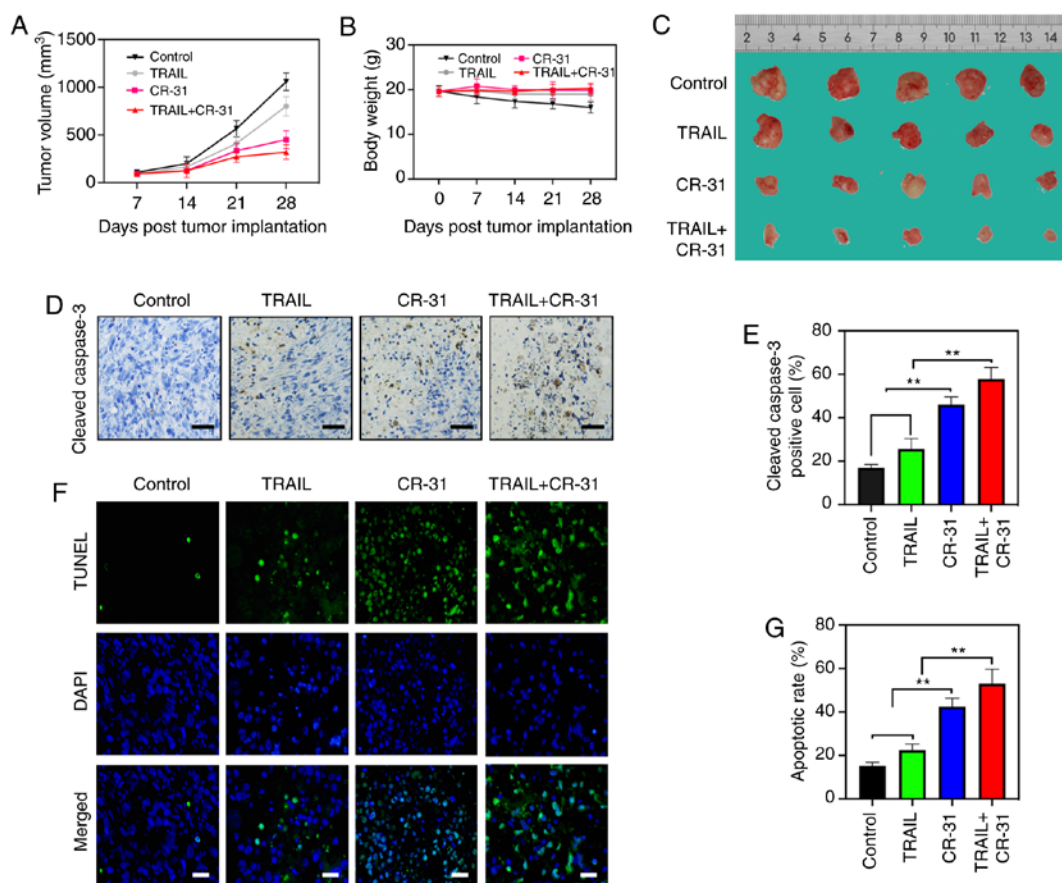

Figure 5. Effect of CR-31 on the growth and apoptosis on an *in vivo* xenograft tumor model of GBC. Tumors were derived from GBC-SD cells implanted subcutaneously in BALB/c nude mice treated with control, TRAIL, CR-31 or TRAIL+CR-31: n=5 per group. (A and B) Tumor volume and body weight were monitored. (C) Representative images of xenograft tumors from mice treated with control, TRAIL, CR-31 and TRAIL+CR-31. (D and E) Histological analysis of cleaved-caspase-3 as a measure of apoptosis in xenograft GBC tumors. Scale bar, 50 μm. Quantification of cleaved-Caspase-3 staining. (F and G) TUNEL assay of apoptosis in xenografted GBC tumors. Scale bar, 50 μm. Quantification of apoptotic rates. Data are presented as the mean ± standard deviation. $^{**}P<0.01$. GBC, gallbladder cancer; TRAIL, Tumor necrosis factor related apoptosis-inducing ligand.

and is well tolerated *in vivo* (20,21). Moreover, inhibitors of eIF4A, such as hippuristanol, can induce apoptosis of adult T-cell leukemia (22). Downregulating c-FLIP by targeting the translation of c-FLIP may be a promising therapeutic strategy.
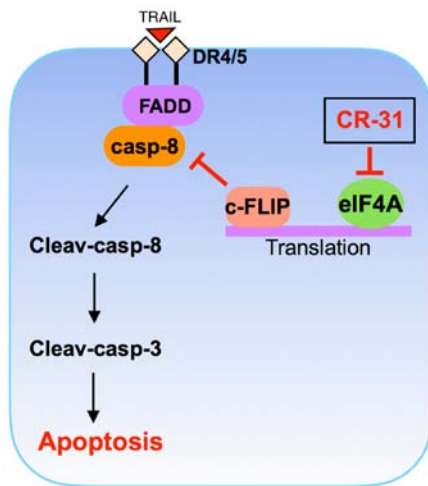
Figure 6. Schematic summarizing how the eIF4A inhibitor CR-31 down-regulates c-FLIP expression and enhances TRAIL-mediated apoptosis in GBC cells. eIF4A, eukaryotic translation initiation factor 4A; CR-31, CR-1-31B; GBC, gallbladder cancer; TRAIL, Tumor necrosis factor related apoptosis-inducing ligand.

Therefore, whether CR-31 treatment attenuated the translation of c-FLIP was determined. However, there was no significant change in c-FLIP at the mRNA expression level, suggesting that the effect of CR-31 on c-FLIP was at the translational level. The results showed that eIF4A may be a valuable therapeutic target and CR-31 can downregulate the translational abundance of c-FLIP and can sensitize GBC cells to TRAIL.

Due to its specificity against cancer cells and minimal toxicity on normal cells, TRAIL-based chemotherapy may serve as a favorable strategy in the treatment of cancer (23). However, GBC shows resistance to TRAIL-mediated apoptosis, suggesting that TRAIL alone is not suitable for the treatment of GBC. In the present study, GBC-SD cells were resistant to TRAIL; however, following the treatment with CR-31, the cells became sensitized to TRAIL. These data suggest that CR-31 may be used as an adjuvant in the TRAIL-based chemotherapy of GBC. Moreover, CR-31 sensitizes TRAIL-mediated apoptosis at nanomolar concentrations, suggesting the efficacy of CR-31 was potent, but also safe on normal cells. Although the mechanisms of TRAIL resistance in GBC cells remain to be determined, emerging evidence has demonstrated that the activity of death-inducing signaling complex (DISC)-recruited proteins caspase-8 and c-FLIP influenced the sensibility of TRAIL-induced cancer cell apoptosis (24). The present study showed that CR-31 markedly activated caspase-8 in GBC cells, following the upregulation of cleaved-caspase-3 *in vitro*.

The formation of DISC is a critical initiating process of the extrinsic signaling of apoptosis, activating the caspase cascade, which then induces apoptotic death. c-FLIP prevents the accumulation of caspase-8, resulting in the disruption of DISC (25). Moreover, the abundance of c-FLIP confers resistance in tumor cells to apoptotic stimuli (26). Additionally, a low abundance of c-FLIP increases the sensitivity of GBC cells to chemotherapy (27,28). Therefore, these reports indicate that c-FLIP may be a target of GBC. In the present study, it was shown that CR-31 significantly reduced c-FLIP levels in GBC-SD cells at nanomolar concentrations. In addition, CR-31 and TRAIL combined notably increased cell death, indicating

that CR-31 increased the sensitivity to TRAIL through downregulation of c-FLIP in GBC-SD cells. Using siRNA specifically to knock down c-FLIP in GBC-SD cells showed that the downregulation of c-FLIP in GBC-SD resulted in a similar effect to CR-31 treatment.

In summary, it was shown that eIF4A was highly abundant in GBC tissues and cell lines, and its inhibitor rocaglate CR-31 enhanced TRAIL-mediated apoptosis by downregulating c-FLIP expression at the translational level and then activating the caspase cascade in TRAIL-resistant GBC cells, both *in vitro* and *in vivo*. Therefore, the data indicate that eIF4A may be a therapeutic target, and the present study highlights a potentially valuable strategy, that is, the combination of rocaglate CR-31 with TRAIL, for the treatment of GBC.

Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

Authors' contributions

YC and YH performed the experimental work. LY analyzed the experimental data. ZL designed this study and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Medical Research, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. Written informed consent for publication was obtained from all participants (approval no. 20180302536). Also, this study was approved by the Ethics Committee of Laboratory Animals, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (approval no. 20180405482).

Patient consent for publication

Consent for publication was obtained from each patient.

Competing interests

The authors declare that they have no competing interests.

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