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Original Article

Eriocalyxin B induces apoptosis and inhibits migration through down-regulation of RTKN in human colon cancer

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Abstract: Like many epithelial-derived cancers, colon cancer results from a multistep tumorigenic process. However, the detailed mechanisms involved in colon cancer formations are poorly characterized. This study was aimed to elucidate the antitumor effect of Eriocalyxin B in colon cancer LOVO cells and the possible molecular mechanism involved. The results showed that Eriocalyxin B could inhibit the growth and migration, and induce apoptosis of LOVO cells significantly in a dose-dependent manner. The expression level of RTKN was significantly decreased after LOVO cells treated with Eriocalyxin B. However, these effects of Eriocalyxin B were reversed by overexpression of RTKN in LOVO cells. Furthermore, the expression level of NF-κBp65 was significantly increased in Eriocalyxin B-treated LOVO cells with RTKN overexpression. We therefore conclude that Eriocalyxin B exhibited significant growth and migration inhibition of LOVO cells and induced apoptosis of LOVO cells via the down-regulation of RTKN expression and inhibition of NF-κB activation.

Keywords: Colon cancer, Eriocalyxin B, RTKN, NF-κBp65

Introduction

Colon cancer is the third most commonly diagnosed cancer in the world and 1.2 million of new cases are yearly diagnosed [1]. As dietary habits have changed in recent years, the number of cases of colon cancer has been increasing faster in the Eastern world [2]. Colon cancer can be treated effectively with surgical resection, chemotherapy, radiation therapy and immunotherapy, among which surgical resection is considered as the first choice worldwide. However, 25% of patients with a five-year survival of 10% that present with metastatic disease [3]. Although a variety of therapeutic strategies for metastatic colon cancer have been evaluated over the last decade, the present knowledge of the cellular and molecular mechanisms of colon cancer can predict no biological parameter for the behavior of cancers. Therefore, an effective approach for the treatment of colon cancer patients is critical.

Natural products provide many promising sources of potential anticancer agents, which were originally isolated from plants, therefore, considered as one of the most vital sources for the development of novel anticancer drugs [4, 5]. Eriocalyxin B is a natural diterpenoid compound isolated from Isodon eriocalyx, a herb of the Labiatae family distributed in the southwest China. It has been reported to have wide spectrum of biological effects, including antibacterial and anti-inflammatory effects in traditional Chinese medicine [6, 7]. In murine xenograft lymphoma models, it significantly inhibited lymphoma cell proliferation and induced apoptosis associated with caspase activation [8]. Furthermore, Eriocalyxin B inhibited proliferation and induced apoptosis in cancer cells *in vitro*, including leukemia [6], ovarian cancer [9] and pancreatic cancer [10]. In some cases, the mechanism of Eriocalyxin B induced apoptosis of lymphoma and leukemia through increasing intracellular ROS levels and suppressing NF-κB pathway [11, 12]. However, the cytotoxic effects of Eriocalyxin B on colon cancer and its mechanism were still poorly understood.

RTKN is a Rho effector protein, initially isolated as a scaffold protein interacting with GTP-bound form of Rho [13]. Previous studies

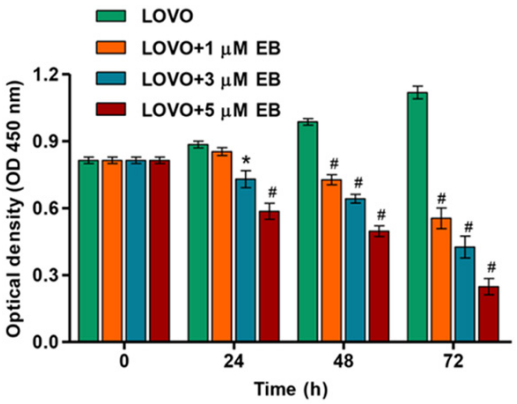

Figure 1. Eriocalyxin B inhibited the viability of LOVO cells. Cell viability was measured by the Cell Count Kit-8 (CCK-8). Eriocalyxin B (1, 3 and 5 μM) significantly inhibited LOVO cells viability in a time- and dose-dependent manner when compared with the control group. Date are presented as mean ± SD (standard deviation) (n = 3). EB, Eriocalyxin B. $^{*}P < 0.05$, $^{\#}P < 0.01$ compared with control groups.

showed that RTKN is overexpressed in several human cancers, including gastric [14], breast [15], bladder [16] and colon [17], and inhibits apoptosis through activating Rho/RTKN/NF-κB pathway to induce the expressions of anti-apoptotic proteins [18]. In line with the pro-survival effect of Rho/RTKN/NF-κB signaling pathway, we also showed that overexpression of RTKN leads to resistance of Eriocalyxin B induced apoptosis and migration inhibition in LOVO cells, and activation of NF-κB greatly increases the resistance to Eriocalyxin B, suggesting that RTKN-dependent NF-κB pathway may represent a target for treating colon cancer.

Materials and methods

Cell culture

Human colon cancer cell line LOVO was obtained from Shanghai Institute of Cell Biology (Shanghai, China). LOVO cells were cultured to 80% confluence in low-glucose DMEM (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (Invitrogen Life Technologies), 100 IU/ml penicillin (Invitrogen Life Technologies) and 10 mg/ml streptomycin (Invitrogen Life Technologies). All cells were maintained at 37°C in 5% CO_2.

Cell viability assay

Cell viability was assessed by Cell Counting Kit (CCK)-8 kit (Tongren, Shanghai, China). Briefly, 4 × 103 LOVO cells were seeded in each 96-well plate and further treated with various concentrations of Eriocalyxin B (1, 3 and 5 μM), and incubated for 0, 24, 48 and 72 h, respectively. CCK-8 reagent was added to each well at 1 h before the endpoint of incubation. The optical density (OD) 450 nm values in each well were determined by a microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). Experiments were repeated at least three times each time in triplicate.

Cell apoptosis assay

Apoptosis was determined by flow cytometry analysis. Briefly, LOVO cells with different concentrations of Eriocalyxin B (1, 3 and 5 μM) treatment for 48 h and were seeded into 6-well plates. The cells were subsequently collected by trypsinization (JRDUN Biotechnology, Shanghai, China) and incubated with annexin V-fluoresce in isothiocyanate (FITC) and propidium Iodide, prior to analysis by a flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Cell migration assay

The migration of the LOVO cells were measured using a Transwell assay as previously described. Briefly, LOVO cells were treated with various concentrations of Eriocalyxin B (1, 3 and 5 μM) and plated into the top chamber. The chambers were subsequently placed in a 37°C incubator for 48 h. The filters were fixed with 4% methanol and stained with 0.5% methylrosanilinium chloride solution (JRDUN Biotechnology) for 30 min. Evaluation of the number of migratory cells was performed under a microscope (× 200; Olympus Corporation, Tokyo, Japan).

Quantitative real-time PCR

Total RNA was isolated from LOVO cells using the TRIzol reagent according to the manufacturer's instructions. The primers for RTKN and GAPDH were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The levels of RTKN and GAPDH were examined using the forward primer, 5'-GCCGCTGCTTACTATTGC-3' (RTKN) and 5'-CACCCACTCCTCCACCTTTG-3' (GAPDH), and reverse primer, 5'-GTGC-

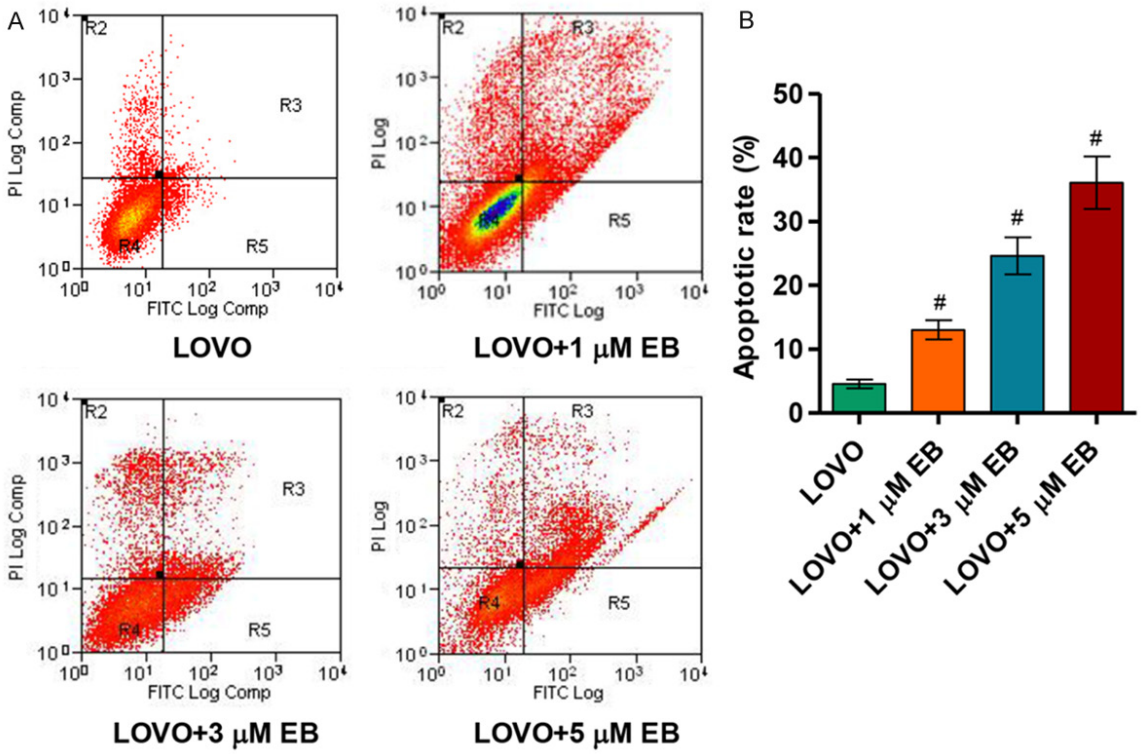

Figure 2. Effects of Eriocalyxin B on apoptosis of LOVO cells. A. Annexin-V/PI double stain assay and flow cytometry analysis were carried out to substantiate cell apoptosis. B. Treatment of Eriocalyxin B at doses of 1, 3 and 5 μM for 48 h dose-dependently increased the apoptotic population of LOVO cells. Date are presented as mean ± SD (standard deviation) (n = 3). EB, Eriocalyxin B. $^{\#}P < 0.01$ compared with control groups.

TTCCCGACTTTCTG-3' (RTKN) and 5'-CCACCACCCTGTTGCTGTAG-3' (GAPDH). RT-qPCR analyses were performed using SYBR Green (Takara Biotechnology Co., Ltd., Dalian, China), and data collection was conducted using an ABI 7500 (Applied Biosystems Life Technologies, Foster City, CA, USA). GAPDH was used an internal control for normalization. The gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

Cell lysates were prepared in RIPA buffer and total protein concentration was quantified by the BCA assay. Lysates were separated by 10% SDS-PAGE and transferred to PVDF membranes. Blots were blocked in 5% free-fat milk for 2 h and incubated with primary antibodies against RTKN (1:1000; Abcam, Cambridge, MA, USA), NF-κBp65 (1:1000; Cell Signaling Technology, Inc., Beverley, MA, USA), H3 (1:1000, Cell Signaling Technology) and GAPDH (1:1500, Cell Signaling Technology) overnight at 37°C, followed by horseradish peroxidase-conjugated secondary antibody IgG (1:1000, Beyotime Institute of Biotechnology, Haimen, China). The blots were visualized using enhanced chemiluminescence (EMD Millipore, Billerica, MA, USA), and the signals were quantified by densitometry using Quantity One software version 4.62 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Construction of stable cell lines

RTKN coding sequence was purchased from Sangon Biotech (Shanghai, China). The sequence was cloned into the lentiviral vector (PLKO.1-EGFP, Sangon Biotech). The production, purification, and titration of lentivirus were performed as previously described [19]. The viruses were collected at 48 h following transfection and were used to infect the LOVO cells. Cell apoptosis, migration, qRT-PCR and Western blot were performed 48 h following infection, as previously described.

Statistical analysis

Experimental data were presented as mean ± SD of at least three independent replicates

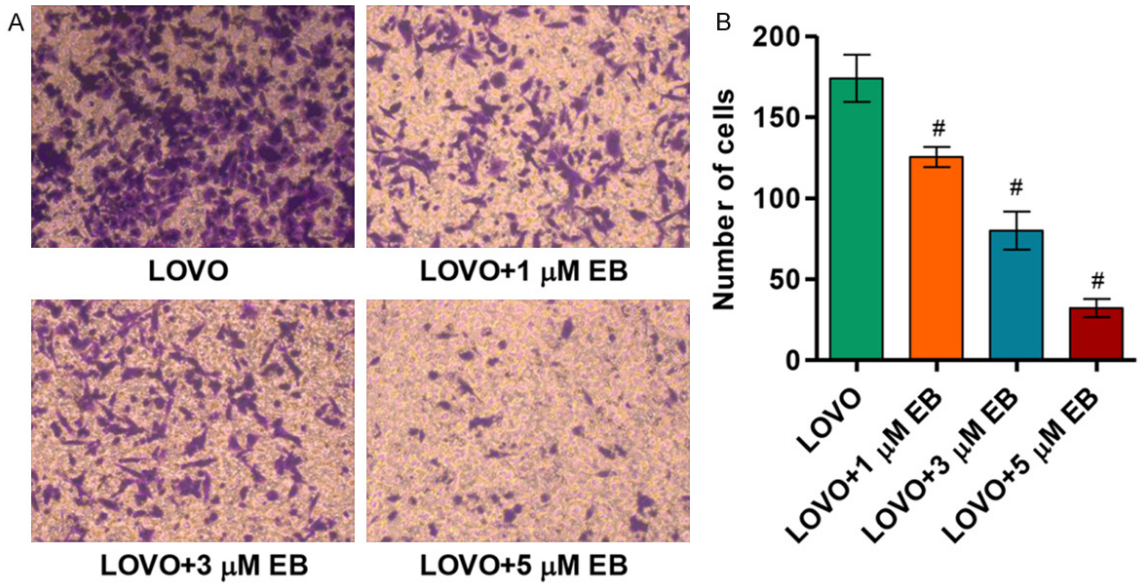

Figure 3. Effects of Eriocalyxin B on migration of LOVO cells. A. Transwell analysis was carried out to substantiate cell migration. B. Treatment of Eriocalyxin B at doses of 1, 3 and 5 μM for 48 h dose-dependently decreased the number of migratory LOVO cells. Date are presented as mean ± SD (standard deviation) (n = 3). EB, Eriocalyxin B. $^{\#}P < 0.01$ compared with control groups.

through analyzing with GraphPad Prism 5 (GraphPad Software, La Jolla, CA) and assessing comparisons between different groups by the Student's t test. Differences were considered significant at values of $P < 0.05$.

Results

Eriocalyxin B inhibits proliferation of LOVO cells

To investigate the growth inhibition effects of Eriocalyxin B on LOVO cells, cell viability was evaluated by CCK-8 after treatment with various concentrations of Eriocalyxin B for 0, 24, 48 and 72 h. As shown in **Figure 1**, Eriocalyxin B had significant growth inhibition effects on the LOVO cells in a dose- and time- dependent manner. Cell viability was decreased remarkably after the LOVO cells were treated with Eriocalyxin B at 1 μM for 48 h.

Eriocalyxin B induces apoptosis of LOVO cells

An annexin-V fluorescein isothiocyanate (FITC)/propidium iodide (PI) double stain assay and flow cytometry analysis were carried out to substantiate cell apoptosis induced by Eriocalyxin B treatment under various concentrations. The number of apoptotic cells counted as late apoptotic cells shown in the upper right quadrant and the early apoptotic cells as shown in lower right quadrant of the histograms. As shown in **Figure 2A**, **2B**, treatment of Eriocalyxin B at the dose of 1, 3 and 5 μM for 48 h significantly increased the number of early apoptotic cells, respectively, from 13.02% ± 1.86% to 36.04% ± 5.03% in a dose-dependent manner compared with control cells with that of 4.57% ± 0.84%. The significant induction of apoptosis indicated the anticancer effect of Eriocalyxin B against LOVO cells.

Eriocalyxin B inhibits migration of LOVO cells

LOVO cells were treated with different concentrations of Eriocalyxin B for 48 h, and then cell migration analyzed by Transwell assay. As shown in **Figure 3A**, **3B**, treatment of Eriocalyxin B at the dose of 1, 3 and 5 μM for 48 h significantly decreased the number of migratory cells in a dose-dependent manner, from 125.4 ± 6.23 to 32.2 ± 5.63 compared with control cells at 174 ± 14.61.

Eriocalyxin B down-regulates RTKN expression in LOVO cells

To investigate the mechanism underlying Eriocalyxin B affects LOVO cell viability, apoptosis and migration, we also tested the expression of RTKN, which has been reported up-regulated

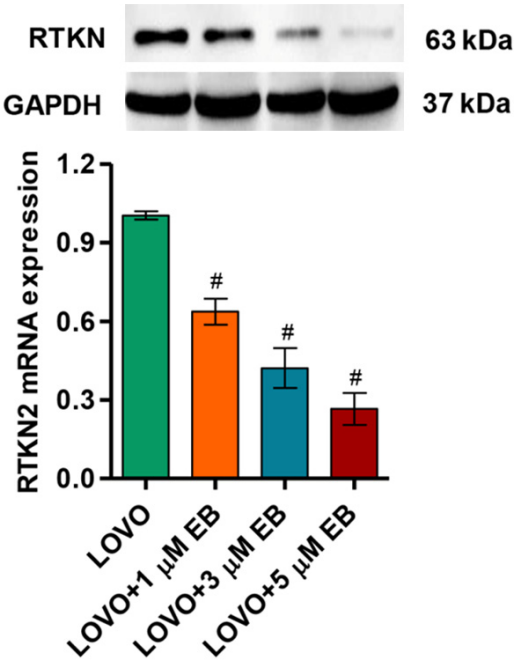

Figure 4. Effects of Eriocalyxin B on RTKN expression. The expression of RTKN was analyzed by qRT-PCR and Western blot assays. Eriocalyxin B significantly decreased RTKN levels in a dose-dependent manner. Date are presented as mean ± SD (standard deviation) (n = 3). EB, Eriocalyxin B. $^{\#}P < 0.01$ compared with control groups.

in colon cancer cells [17]. As shown in **Figure 4**, the expression of RTKN was significantly decreased in LOVO cells, at both mRNA and protein levels, treated with various concentrations of Eriocalyxin B, and the protein levels normalized by GAPDH decreased from 0.4005 ± 0.0601 to 0.1234 ± 0.0259 in a dose-dependent manner compared with control cells with that of 0.5946 ± 0.0657. These results suggest that Eriocalyxin B induced apoptosis may through down-regulation of RTKN.

Overexpression of RTKN inhibits apoptosis and induces migration of LOVO cells

The above results prompted us to examine whether the suppressive effect of Eriocalyxin B is mediated by repression of RTKN in LOVO cells. Therefore, a RTKN stably expressed LOVO cells was construction, and the expression of RTKN was also detected by qRT-PCR and Western blotting. The results showed that overexpression of RTKN significantly increased the expression of RTKN in LOVO cells with 1 μM of Eriocalyxin B treatment, as compared to the cells without RTKN overexpression (**Figure 5A**).

To further validate the hypothesis, we also detected the apoptotic rate and migratory ability of LOVO cells stably overexpressing RTKN. The results showed that RTKN overexpression significantly inhibited cell apoptosis in LOVO cells treated with 1 μM of Eriocalyxin B compared with the cells without RTKN overexpression. The apoptotic rate of Eriocalyxin B treated LOVO cells was decreased from 15.39% ± 1.75% to 10.03% ± 1.51% after RTKN overexpression, opposite to that induced by Eriocalyxin B (**Figure 5B**, **5C**). At the same time, the number of migratory cells was notably increased from 121.6 ± 13.13 to 158.0 ± 10.63 in Eriocalyxin B treated LOVO cells after RTKN overexpression (**Figure 5D** and **5E**). These data indicate that Eriocalyxin B induces apoptosis and inhibits migration through down-regulation of RTKN in LOVO cells.

Overexpression of RTKN activates NF-κBp65 in LOVO cells

Recent reports revealed that RTKN inhibits apoptosis through activation of NF-κB pathway, therefore we also detected the intranuclear activation of NF-κB in Eriocalyxin B-treated LOVO cells after RTKN overexpression. Overexpression of RTKN significantly increased the expression of NF-κBp65 in LOVO cells with 1 μM of Eriocalyxin B treatment, as compared to the cells without RTKN overexpression (**Figure 6**). These data indicate that the effect of Eriocalyxin B on LOVO cells may through inhibition of RTKN-dependent NF-κB activation.

Discussion

Human colon cancer is the fourth most common type of cancer in men and, the third most common in women worldwide, accounting for 8% of all cancer-associated mortality [20]. Accumulating evidence has shown that some natural products such as saffron [21] and curcumin [22] and many others have growth inhibitory and apoptosis inducing effects both *in vitro* and *in vivo*. To date, to the best of our knowledge, no study has explored the effects of Eriocalyxin B on the development and progression of human colon cancer. Therefore, our studies aimed to investigate the role of Eriocalyxin B in human colon cancer cell growth, apoptosis and migration.

Several studies have reported that Eriocalyxin B suppressed cancer cell proliferation and

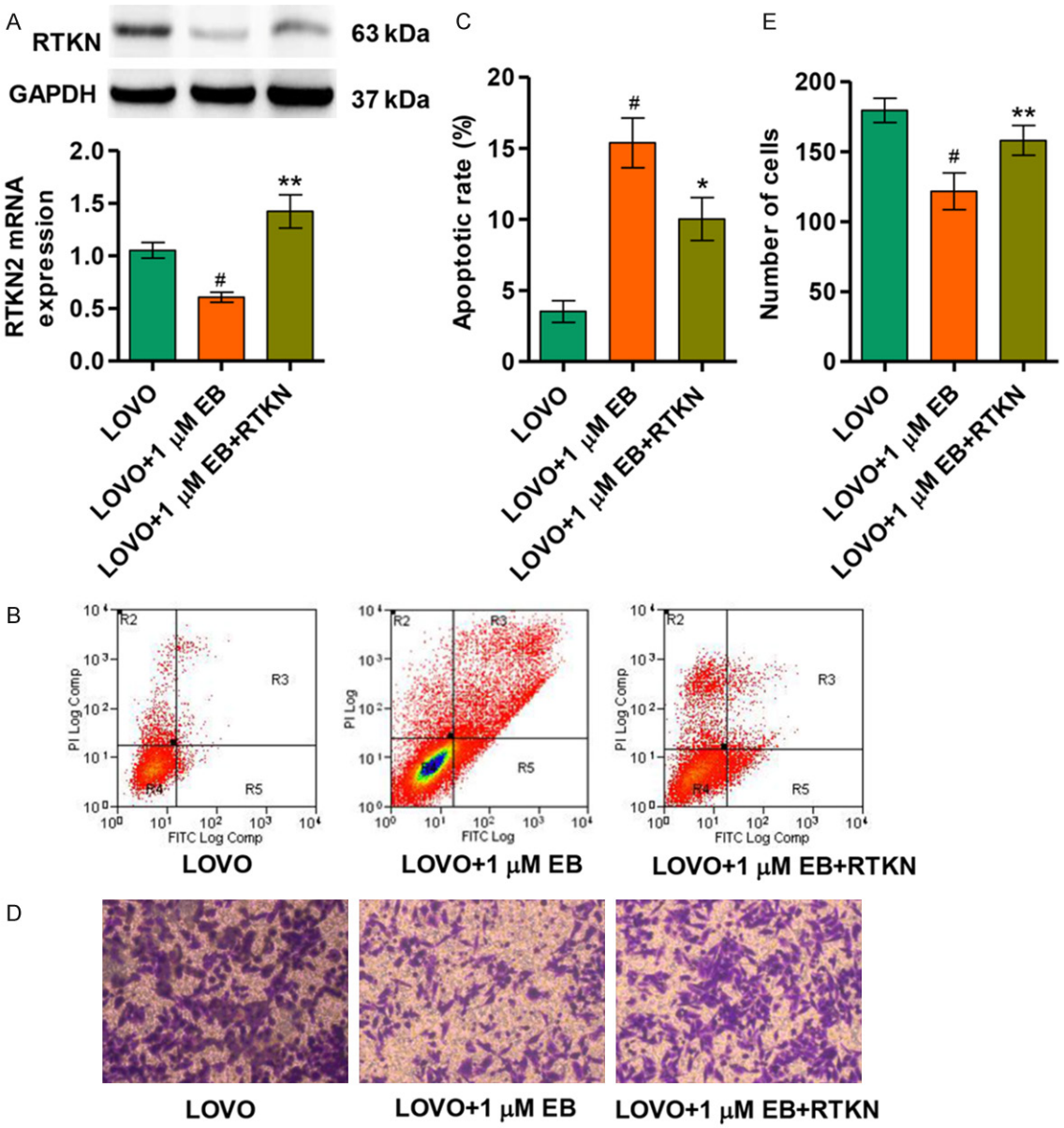

Figure 5. Effects of RTKN overexpression on apoptosis and migration of LOVO cells. A. The RTKN expression was analyzed by qRT-PCR and Western blot in LOVO cells treated with 1 μM of Eriocalyxin B and stably overexpressing RTKN. B. Annexin-V/PI double stain assay and flow cytometry analysis were carried out to substantiate cell apoptosis. C. Overexpression of RTKN significantly increased apoptotic rate in Eriocalyxin B-treated LOVO cells. D. Transwell analysis was carried out to substantiate cell migration. E. Overexpression of RTKN significantly decreased the number of migratory cells in Eriocalyxin B-treated LOVO cells. Date are presented as mean ± SD (standard deviation) (n = 3). EB, Eriocalyxin B. $^{\#}P < 0.01$ compared with control groups. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with LOVO cells treated with 1 μM of Eriocalyxin B.

induced apoptosis through a mechanism that differentially in different cancers [23]. For example, Eriocalyxin B induces apoptosis and cell cycle arrest in pancreatic adenocarcinoma cells through caspase- and p53-dependent pathways [10]. Additionally, Eriocalyxin B has been shown to induce apoptosis of leukemia cells through NF-κB and MAPK signaling pathways [6]. In this study, we demonstrated that Eriocalyxin B inhibited human colon cancer LOVO cells proliferation and migration, and induced apoptosis in a dose-dependent manner.

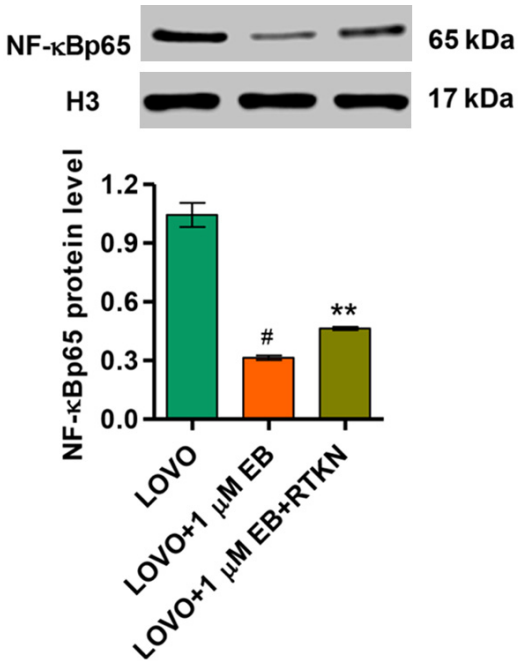

Figure 6. Effects of RTKN overexpression on NF-κB activation. The expression of intracellular NF-κBp65 was measured by Western blotting. Overexpression of RTKN significantly increased NF-κBp65 levels in Eriocalyxin B-treated LOVO cells. Date are presented as mean ± SD (standard deviation) (n = 3). EB, Eriocalyxin B. $^{\#}P < 0.01$ compared with control groups. $^{**}P < 0.01$ compared with LOVO cells treated with 1 μM of Eriocalyxin B.

Numerous genes regulated by signal transducers and transcription activators were involved in cancer processes, therefore it was hypothesized that Eriocalyxin B functions in human colon cancer by modulating genes that associated with cancer.

RTKN, the gene coding for the Rho effector, was shown to be expressed at a low level in normal cells and was overexpressed in many cancer cell line [18]. Importantly, RTKN has been reported overexpressed in human colon cancer cells and associated with growth, cycle, apoptosis and migration [17]. Therefore, we further detected the effect of Eriocalyxin B on expression of RTKN, which may clarify the mechanism involved. Our findings showed that Eriocalyxin B significantly decreased the expression of RTKN in a dose-dependent manner in LOVO cells at both mRNA and protein levels. These data suggest that the effects of Eriocalyxin B on LOVO cells proliferation, apoptosis and migration may through down-regulation of RTKN. To further validate the hypothesis, we introduced the LOVO cells stably overexpressed RTKN. Overexpression of RTKN in Eriocalyxin B-treated LOVO cells significantly inhibited the apoptosis and increased the number of migratory cells, suggesting that RTKN could resistant of Eriocalyxin B induced effects on LOVO cells.

Elevated NF-κB activity has been found in a number of malignant tumors [24-26]. In certain breast and pancreatic tumors and cell lines, the autocrine action of IL-1 and IL-1β has been described to induce NF-κB-dependent protection from apoptosis [27, 28]. Complementary to these findings, our data have clearly demonstrated that NF-κB activity was a results of activated Rho signaling pathway through overexpressed RTKN in LOVO cells. These data suggest that RTKN overexpression renders cells resistant to apoptotic insults and recovered the ability of migration, and may therefore represent an important molecular event in human colon cancer.

In summary, we found Eriocalyxin B inhibits proliferation and migration, and induces apoptosis and RTKN expression decreased in LOVO cells. However, overexpression of RTKN in LOVO cells resistant to apoptosis and promotes migration induced by Eriocalyxin B and also activation of NF-κB pathway. This may suggest that Eriocalyxin B inhibits colon cancer processes through down-regulation of RTKN-dependent NF-κB pathway.

Disclosure of conflict of interest

None.

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