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Raddeanin A induces human gastric cancer cells apoptosis and inhibits their invasion *in vitro*

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ABSTRACT

Raddeanin A is one of the triterpenoid saponins in herbal medicine *Anemone raddeana Regel* which was reported to suppress the growth of liver and lung cancer cells. However, little was known about its effect on gastric cancer (GC) cells. This study aimed to investigate its inhibitory effect on three kinds of different differentiation stage GC cells (BGC-823, SGC-7901 and MKN-28) *in vitro* and the possible mechanisms. Proliferation assay and flow cytometry demonstrated Raddeanin A's dose-dependent inhibitory effect and determined its induction of cells apoptosis, respectively. Transwell assay, wounding heal assay and cell matrix adhesion assay showed that Raddeanin A significantly inhibited the abilities of the invasion, migration and adhesion of the BGC-823 cells. Moreover, quantitative real time PCR and Western blot analysis found that Raddeanin A increased Bax expression while reduced Bcl-2, Bcl-xL and Survivin expressions and significantly activated caspase-3, caspase-8, caspase-9 and poly-ADP ribose polymerase (PARP). Besides, Raddeanin A could also up-regulate the expression of reversion inducing cysteine rich protein with Kazal motifs (RECK), E-cadherin (E-cad) and down-regulate the expression of matrix metalloproteinases-2 (MMP-2), MMP-9, MMP-14 and Rhoc. In conclusion, Raddeanin A inhibits proliferation of human GC cells, induces their apoptosis and inhibits the abilities of invasion, migration and adhesion, exhibiting potential to become antitumor drug.

1. Introduction

Gastric cancer (GC) is one of the leading causes of cancer-related death around the world [1]. It is the second most common cancer and the third leading cause of death among cancer patients in China [2]. Currently, surgery, with better clinical therapeutic effect when combined with chemotherapy, is the dominated therapy for GC. Increasing new antitumor agents are under investigation in both academic and industrial research laboratories all over the world. Nowadays, researchers have found that natural products are important sources of chemical anticancer medicine and many chemotherapeutic agents, directly or indirectly, derived from natural products such as paclitaxel and vincristine. It has been a hotspot in cancer treatment research to find antitumor drugs from natural products.

Anemone Raddeana Regel belonging Ranunculaceae family is used to treat cancer, rheumatism, paralysis, and pain around waist and legs [3]. During the past 20 years, various components were isolated and identified from this plant, among which the primary ones are triterpenoid saponins [4]. Recent studies have suggested that one of the triterpenoid saponins, Raddeanin A, exhibited cytotoxicity *in vitro* [5–7]. It had significant inhibitory effect on the growth of the tumor cells such as liver cancer and lung cancer cells [8,9]. However, its inhibitory effect on GC cells and the related mechanism has not yet been reported. Therefore, the purpose of our study was to investigate its inhibitory effect on GC cells and the related molecular mechanism.

2. Materials and methods

2.1. Cell lines and cell culture

Human GC cell lines BGC-823 (low differentiated), SGC-7901 (moderately differentiated) and MKN-28 (high differentiated) were obtained from Type Culture Collection, Chinese Academy of

Abbreviations: GC, gastric cancer; PARP, poly-ADP ribose polymerase; RECK, reversion inducing cysteine rich protein with Kazal motifs; E-cad, E-cadherin; MMP, matrix metalloproteinase; OD, optical density.

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Sciences (Shanghai, China), cultured in RPMI-1640 medium containing 10% bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in 5% CO_2 atmosphere.

2.2. Chemicals and antibodies

Raddeanin A was purchased from China National Institute for the Control of Pharmaceutical and Biological Products (purity ⩾ 99%) and dissolved in DMSO. RPMI-1640 medium, bovine serum and penicillin–streptomycin were purchased from Gibco BRL (Gaithersburg, MD, USA). Pancreatin and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) from Biosharp (Hefei, China); Annexin-V/propidium iodide (PI) apoptosis detection kit from KeyGen Biotech Co., Ltd. (Nanjing, China); primescript reverse transcription reagent Kit with gDNA Eraser from TaKaRa (Dalian, China); TRIzol reagent and Power SYBR Green PCR Master Mix from Life Technologies (Grand Island, NY); monoclonal mouse β-actin antibody from Sigma Chemical Co. (St. Louis, MO). Antibody against Bcl-2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and antibodies against caspase-3, caspase-8, caspase-9, poly-ADP ribose polymerase (PARP), Bax, metalloproteinases-9 (MMP-9), MMP-14, reversion inducing cysteine rich protein with Kazal motifs (RECK), Rhoc and E-cadherin (E-cad) from Cell Signaling Technology (Beverly, MA).

2.3. MTT assay detects the inhibition of Raddeanin A on human GC cell lines growth (BGC-823, SGC-7901 and MKN-28)

Cells in the logarithmic growth phase were plated in 96-well culture plates at 8×10^3 cells/well. After the cells treated with Raddeanin A (2, 4, 8 or 16 μM) for 12 h, 20 μl of MTT (5 mg/ml) solution was added to each well, followed by further 4 h MTT incubation. Cells were lysed in 150 μl DMSO, and the optical densities (ODs) were measured by ELx800 microplate reader (BioTek, Winooski, VT) at 490 nm. The cell growth inhibition rate was calculated using the following formula: $1 - OD_{experiment}/OD_{control}$.

2.4. Flow cytometric analysis of apoptosis with Annexin-V/PI double staining

After treated with or without Raddeanin A (8, 16 μM), together with 50 μg/ml 5-fluorouracil (5-FU) for 12 h as a positive control, about 3×10^5 cells were harvested and washed twice with cold phosphate buffer saline (PBS), resuspended in 500 μl 1 × binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). About 5 μl Annexin-V and 5 μl PI were then added to the solution, and the cells were gently vortexed and incubated for 15 min at room temperature in the dark for flow cytometric analysis.

2.5. Transwell assay

BGC-823 cells were starved in serum-free medium for 24 h, resuspended in 200 μl serum-free medium with or without Raddeanin A (4, 8 or 16 μM) and then added to the upper chamber coated with Matrigel (5×10^5 cells per well), while the lower chamber was filled with 500 μl RPMI-1640 medium supplemented with 10% bovine serum were added. After 24 h incubation, cells attached to the upper side of the filter membrane washed twice with cold PBS while cells attached to the bottom were fixed with 75% alcohol and stained with crystal violet. The numbers of cells in five random fields of each group were counted by using inverted microscope.

2.6. Wound healing assay

BGC-823 cells which were cultured as confluent monolayers in a 24-well plate (1×10^6/well) were wounded by removing a 1 mm strip across the well with a standard 200 μl pipette tip when they adhered to the plate surface. The wounded monolayers were then washed twice with PBS to remove non-adherent cells before 1 ml RPMI-1640 medium with or without Raddeanin A (4, 8 or 16 μM) were added to the 24-well plate. After 24 h incubation, different concentration groups were photographed by inverted micrographs.

2.7. Cell matrix adhesion assay

After BGC-823 cells were treated with or without Raddeanin A (4, 8 or 16 μM) for 24 h, they were harvested and resuspended in RPMI-1640 medium. About 2×10^5 live cells were seeded into single well of a 96-well plate coated with fibronectin. Each concentration group contained 12 wells and every 3 wells were washed twice after 20, 40, 60 and 80 min to remove the non-adherent cells. At last, discarded the medium, 20 μl of MTT solution and 100 μl RPMI-1640 medium were added to each well for a further 4 h incubating. Similar to MTT assay, ODs were measured and the cell adhesion inhibition rates were calculated based on the means of three wells.

2.8. RNA purification and real time PCR (RT-PCR)

Total RNA was isolated by TRIzol reagent and reverse-transcribed into cDNA using the TaKaRa RT reagent kit and was quantified by ABI 7500 fast RT-PCR System. The primers for available gene were designed using Primer Express shown in Table 1. Cycle threshold (Ct) values were obtained graphically for the target genes and β-actin. $\Delta Ct = Ct_{target\ genes} - Ct_{endogenous\ reference\ gene}$. $\Delta\Delta Ct = \Delta Ct_{treated\ samples} - \Delta Ct_{control\ samples}$. The relative fold change in gene expression was calculated as $2^{-\Delta\Delta Ct}$.

2.9. Western blot analysis

Proteins were extracted with RIPA buffer containing protease inhibitor cocktail and the concentration was determined using Bradford assay. Equal amounts of protein (20 μg) from each sample was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride

Table 1
Sequences of primers used in the real-time PCR amplifications.

Gene primer	Sequences (5′-3′)	Length of PCR product (bp)
Bax	F: TTTGCTTCAGGGTTTCATCC R: GCCACTCGGAAAAAGACCTC	213
Bcl-2	F: TCGCCCTGTGGATGACTGAG R: CAGAGTCTTCAGAGACAGCCAGGA	143
Bcl-xL	F: ATGAACTCTTCCGGGATGG R: TGGATCCAAGGCTCTAGGTG	166
Survivin	F: TTCTCAAGGACCACCGCATC R: GCCAAGTCTGGCTCGTTCTC	127
MMP-2	F: CTCCCGGAAAAGATTGATG R: GGTGCTGGCTGAGTAGAT	96
MMP-9	F: TCTATGGTCCTCGCCCTGAA R: CATCGTCCACCGGACTCAAA	219
MMP-14	F: ATCTGCCTCTGCCTCACCTA R: AAGCCCCATCCAAGGCTAAC	126
RECK	F: ATCTGTTCACCCTGGAGT R: TGTTGAAGTGTTTGCTTT	96
β-Actin	F: GGCCAACCGCGAGAAGAT R: CGTCACCGGAGTCCATCA	134

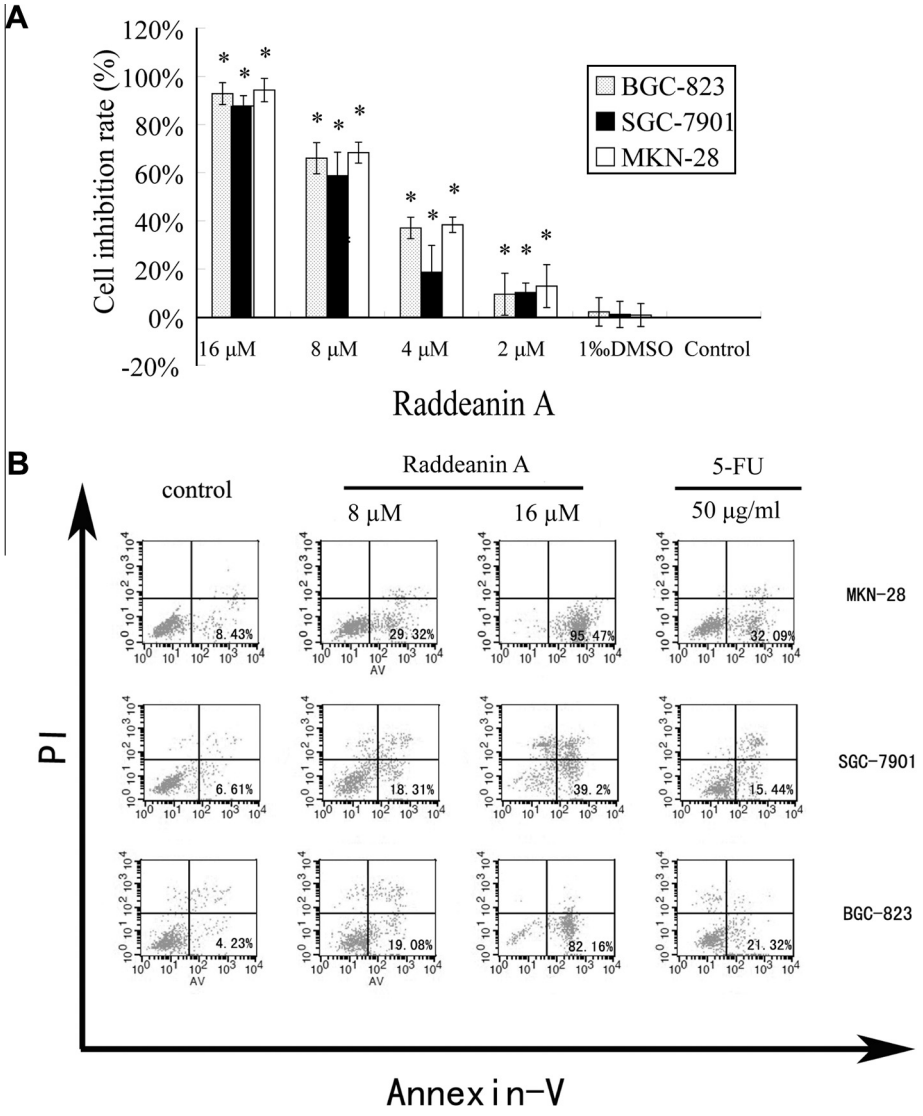

Fig. 1. (A) Anti-proliferation of Raddeanin A on human GC cells. MTT assay showed that after three human GC cells were treated by different concentrations (2–16 μM) of Raddeanin A or 0.1% DMSO for 12 h, the inhibition rates of three cells showed dose-dependent manner. *$P < 0.05$. (B) Flow cytometric analysis of human GC cells apoptosis induced by Raddeanin A using Annexin-V/PI staining. The left lower region of each quadrant shows the viable cells (Annexin-V−/PI−). The right lower region represents the early stage apoptotic (Annexin-V+/PI−). The top right region contains the nonviable necrotic cells (Annexin-V+/PI+).

(PVDF) membrane (Millipore, Billerica, MA), which was then incubated for 1 h at room temperature with 5% skim milk. All primary antibodies were diluted in phosphate buffer saline with Tween (PBST, 135 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM K_2HPO_4 and 0.05% Tween-20) containing 5% skim milk. Afterwards, the membranes were incubated with appropriate primary antibodies overnight at 4 °C. After washing three times with PBST and incubated with HRP-linked secondary antibodies (anti-rabbit or anti-mouse IgG) for1 h at room temperature, the signal was visualized using an ECL detection kit (Millipore, Billerica, MA). The β-actin was taken as a loading control. Western blot analysis was repeated independently for 3 times.

2.10. Statistical analysis

SPSS13.0 software was utilized to analyze the datas which were expressed as the mean ± SD. One-way ANOVA test was used to examine the statistical difference of experimental data between the groups. The significance was defined as $P < 0.05$.

3. Results

3.1. The inhibition of Raddeanin A on human GC cells proliferation in vitro

MTT assay results showed that the inhibition rates of three human GC cells presented dose-dependent manner after treated by different concentrations of Raddeanin A for 12 h. When the concentration of Raddeanin A reached to 16 μM, the inhibition rates for BGC-823, SGC-7901 and MKN-28 cells rose to 92.84% ± 4.5%, 87.74% ± 4.3% and 94.31% ± 4.9% (Fig. 1A) and the IC_{50} value of these cells were 5.34, 6.61 and 4.98 μM, respectively.

3.2. Raddeanin A induced GC cells apoptosis

To further confirm Raddeanin A's induction of cell apoptosis, Annexin-V/PI double staining was applied for the flow cytometric analysis after the cells were treated for 12 h. From 8 to 16 μM, early apoptosis rates of BGC-823, SGC-7901 and MKN-28 cell increased from 19.08% to 82.16%, from 18.31% to 34.01% and from

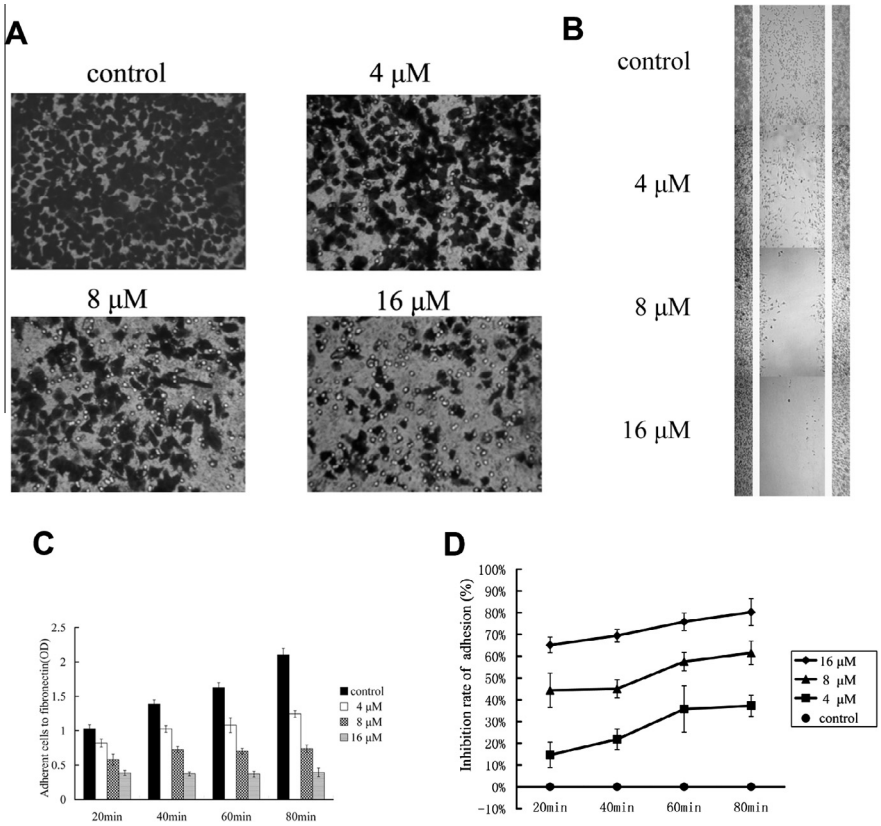

Fig. 2. (A) Transwell assay detected the effects of Raddeanin A's invasion on GC cells. After the treatment cells migrated across 8 μm diameter pores to the lower chamber for 24 h, light microscopic images of different concentrations (400×) were taken. The white dot was the 8 μm diameter pores of transwell chamber. (B) Wound assay detected migration of Raddeanin A for human GC cells, which were photographed 24 h after wounding (100×). White lines indicate wound edges. Cells between the two white lines were the ones which migrated to the wound area. (C) Cell matrix adhesion assay was used to detect Raddeanin A's adhesion for GC cells. (D) Each point in curve was the inhibition rate of cell adhesion of different concentrations (4, 8 or 16 μM) at different periods (20, 40, 60 and 80 min).

29.32% to 95.47%, respectively. Meanwhile, the cells were also treated with 50 μg/ml 5-Fu for 12 h. The early apoptosis rate of the each cell line was 21.32% (BGC-823), 15.44% (SGC-7901) and 32.09% (MKN-28), respectively (Fig. 1B). In summary, the above results suggested that Raddeanin A could effectively induce apoptosis, and the apoptosis rates of three cells all presented a dose-dependent manner.

3.3. Raddeanin A inhibited the invasion and migration abilities of the GC cells

In order to measure the effect of invasion and migration abilities of Raddeanin A on human GC cell, low differentiated BGC-823 cells were chosen as the target cells. As shown in Fig. 2A, the result of transwell assay showed that after BGC-823 cells were treated with or without Raddeanin A (4, 8 or 16 μM) for 24 h, the number of the cells invaded across 8 μm diameter pores to the lower chamber markedly decreased compared with control group, indicating that Raddeanin A could suppress the invasion of GC. Meanwhile, the treated cells in the 24-well plate migrated to the whole wound area markedly decreased compared with control group (Fig. 2B) which suggested that Raddeanin A could also suppress the migration of GC cells. In addition, the inhibition rates of both invasion and migration of Raddeanin A on GC cells presented a dose-dependent manner.

3.4. Raddeanin A inhibited GC cells' adhesion

In order to evaluate the effect of Raddeanin A on GC cells' adhesion, the cell matrix adhesion assay was performed. Similar to the results of transwell invasion assay and wound assay, the adhesion rates of cells treated with Raddeanin A at all time points were lower than those of control group (Fig. 2C). Further data demonstrated that the inhibitory rate of adhesion increased as concentrations and time rose up (Fig. 2D). Consequently, Raddeanin A could dramatically inhibit the adhesion of GC cells to fibronectin and the inhibition rates presented a dose- and time-dependent manner.

3.5. Changes of the apoptosis-related mRNA and proteins

In order to investigate the potential mechanism involved in Raddeanin A inducing apoptosis, RT-PCR and Western blot were applied to analyze the mRNA and protein changes of the apoptosis-related molecules. BGC-823 (Fig. 3A), SGC-7901 (Fig. 3B) and MKN-28 (Fig. 3C) cells were treated for 12 h and all the three results showed that the Bax level significantly increased while Bcl-2, Bcl-xL and Survivin levels decreased in a dose-dependent manner. The ratios of Bax/Bcl-2 was further calculated, which increased in a dose-dependent manner (Fig. 3D). In addition, expressions of caspase family proteins and PARP protein were detected by Western blot, as shown in Fig. 3E, whose results demonstrated gradual increase of cleaved caspase-8 protein in a dose-dependent manner; while procaspase-3, procaspase-9 and pro-PARP decreased, indicating that Raddeanin A could significantly activated caspase-3, caspase-8, caspase-9 and PARP. To summarize, Raddeanin A induced the apoptosis of GC cells by up regulating Bax expression and down regulating the Bcl-2, Bcl-xL and Survivin expressions as well as activating the caspase-cascade response.

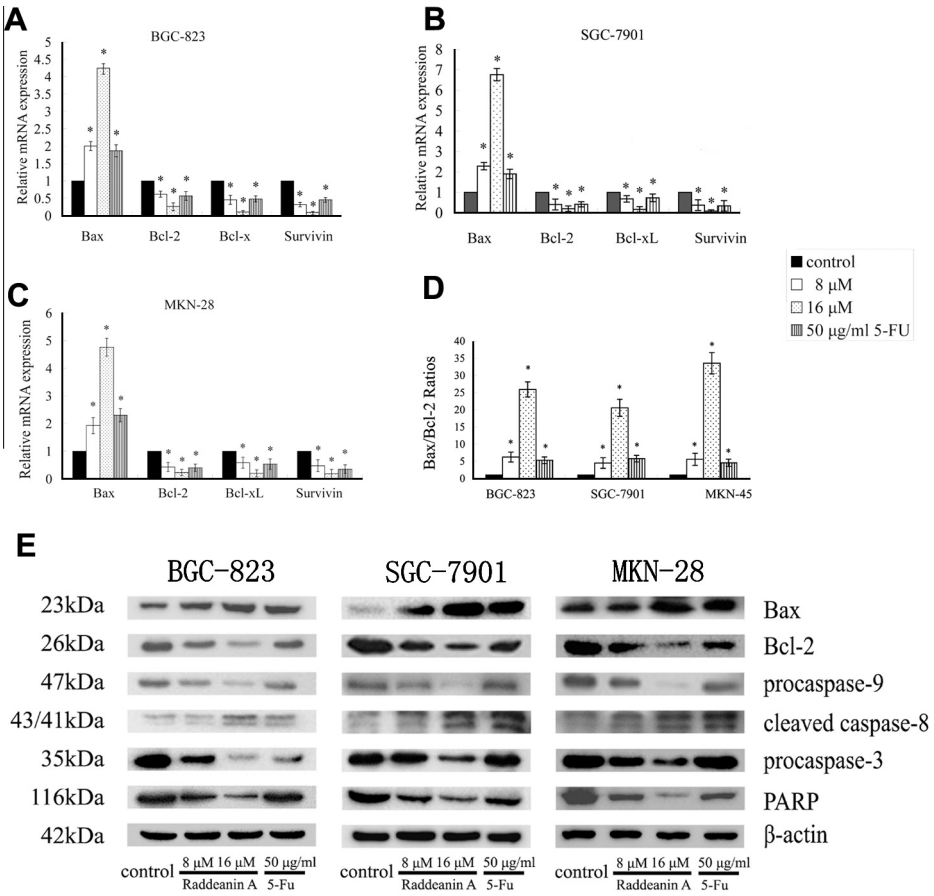

Fig. 3. Real time PCR and Western blot analysis of the apoptosis-associated molecules. After BGC-823 (A), SGC-7901 (B) and MKN-28 (C) cells were treated for 12 h with or without Raddeanin A (8, 16 μM), the mRNA levels of apoptosis-related genes (Bax, Bcl-2, Bcl-xL, and Survivin) were determined by RT-PCR. The β-actin was taken as a loading control and 5-FU (50 μg/ml) as a positive control. (D) The Bax/Bcl-2 ratios of mRNA levels in GC cells treated with 8 or 16 μM Raddeanin A. $^{*}P < 0.05$. The data represented from three independent experiments. (E) Representative images of Western blot.

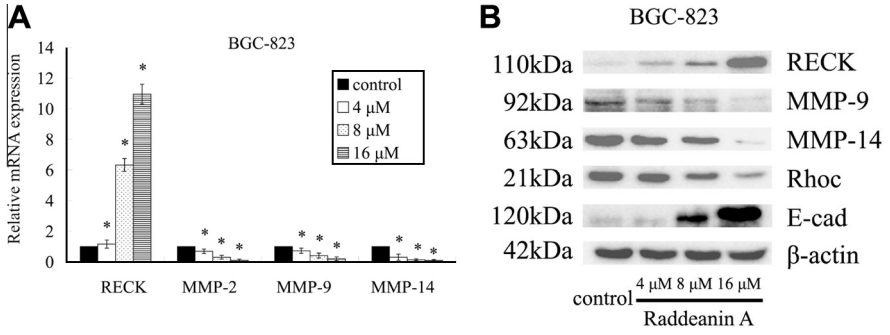

Fig. 4. (A) RT-PCR analysis of the invasion-related molecules. After BGC-823 cells were treated with or without Raddeanin A (4, 8 or 16 μM) for 24 h, the mRNA levels of invasion-related genes (MMP-2, MMP-9, MMP-14, RECK) were detected. The β-actin was as an internal control. $^{*}P < 0.05$. The data represent the means ± SD from three independent experiments. (B) Representative images of Western blot. After BGC-823 cells were treated with or without Raddeanin A (4, 8 or 16 μM) for 24 h, the expression changes of the proteins closely associated with invasion and adhesion (RECK, MMP-9, MMP-14, Rhoc, E-cad) were detected. The β-actin was as an internal control. The images were representative of three independent experiments.

3.6. Changes of the invasion-related mRNA and proteins

In order to investigate the potential mechanism of Raddeanin A's inhibition of cell invasion, RT-PCR and Western blot were utilized to detect the invasion-related molecules such as RECK, MMPs. The results of both RT-PCR (Fig. 4A) and Western blot (Fig. 4B) showed that RECK expression significantly increased while MMP-2, MMP-9, MMP-14 expressions were declined as dose decreased. Besides, changes of Rhoc expression were investigated. Similar to the MMPs family, it revealed a decreasing trend with the increase of drug concentrations. Raddeanin A was found in the experiment to markedly prevent GC cells from adhering to fibronectin. Therefore, the E-cad expression which was closely associated with adhesion was further detected, finding that the treated BGC-823 cells resulted in a dose-dependent increase of E-cad.

4. Discussion

Apoptosis, or programmed cell death, is a well-documented phenomenon in many cellular systems, which plays a crucial role

in maintaining cellular homeostasis between cell division and cell death. Caspase-cascade is a central part of cell apoptosis and regulated by various kinds of molecules, such as Bcl-2 family proteins. Generally, the caspase family proteases can be activated through two pathways: one is the death signal-induced, death receptor-mediated pathway; the other is the mitochondrion-dependent pathway. Once the specificity substrate such as PARP has been cut by the cleavage of caspases, apoptosis will be induced [10,11]. Therefore, the research of caspase is significant for exploring new targets of cancer treatment. The present research used flow cytometry to detect cell apoptosis rate, finding the apoptosis rates of three GC cells increased significantly when treated with Raddeanin A (8, 16 μM) for 12 h. Further Western blot data revealed that the caspase-3, 8, 9 and PARP were activated by Raddeanin A (8, 16 μM), which indicated the cell apoptosis. Therefore, we inferred that activating caspase cascade is the mechanism of Raddeanin A inducing cell apoptosis. However, more research is in need to investigate Raddeanin A inducing the apoptosis of GC cells through endogenous pathways or exogenous pathways or through both.

Although the caspases is a central point to apoptosis, their activation is regulated by many other factors, among which Bcl-2 family plays a pivotal role in either inhibiting (Bcl-2, Bcl-xL) or promoting (Bax, Bak) cell death [12,13]. Recently, it has been reported that Bax inactivating Bcl-2 proteins regulates the apoptosis mediated by mitochondria and the ratio of Bax to Bcl-2 proteins increases during apoptosis induction [14].Therefore, we continued to detect the expression change of Bcl-2 family. As shown in Fig. 3E, the protein level of Bax increased in a dose-dependent manner, while the Bcl-2 protein decreased. Further data revealed that the ratio of Bax/Bcl-2 proteins also increased in a dose-dependent manner (Fig. 3D). These results partly interpreted the molecular mechanism of Raddeanin A inducing human GC cells *in vitro*.

Invasion and metastasis, the main reasons for shortening long-term survival, are regarded as essential features of cancer. Therefore, in addition to apoptosis-inducing, inhibiting metastasis is also the key to cancer treatment. The degradation of the extracellular matrix plays a crucial role in tumor invasion and metastasis. MMPs, a family of zinc-dependent endopeptidases, could degrade the extracellular matrix, thus promoting the invasion and metastasis of cancer cells. MMP-2, MMP-9, MMP-14 are the important members of this family [15,16], which were reported to excessively express in breast cancer and colorectal cancer, closely associated with tumor invasion and metastasis [17–23]. The present study observed the changes of invasion and migration by transwell and wounding heal assays, finding that the inhibition rates of GC cells of invasion and migration exhibited dose-dependent after treated with Raddeanin A for 24 h. RT-PCR and Western blot analysis showed that the expression of MMP-2, MMP-9 and MMP-14 decreased in a dose-dependent manner. The expression of RECK was further detected, as it is a critical tumor suppressor that can potently inhibit angiogenesis and metastasis of tumor and closely related to the MMPs family [24,25]. The mechanism may be due to negative regulation of at least MMP-9, MMP-2 and MMP-14's activation to suppress the invasive and metastatic potentials [26–28]. The results showed that RECK expression significantly increased, contrary to that of the MMPs family. So, RECK gene negatively regulating the expression of MMPs family was supposed to be a key mechanism of Raddeanin A inhibiting invasion and metastasis of GC cells. However, tumor invasion and metastasis were multi-step processes with complex molecular mechanism, which requires further experiments to explore other possible molecular mechanisms.

According to the above results, it comes to the conclusion that Raddeanin A could inhibit proliferation of human GC cells and induce its apoptosis. Its induction of apoptosis may activate the caspase-cascades and up-regulate the expression of Bax, down-regulate the expression of Bcl-2, Bcl-xL and Survivin. Raddeanin A could inhibit invasion, migration and adhesion of BGC-823 cells. Its molecular mechanism may lie on up-regulating the expression of RECK, E-cad and down-regulating the expression of MMP-2, MMP-9, MMP-14 and Rhoc. These findings provide an experiment basis for Raddeanin A as chemotherapy drugs against GC cells, thereby facilitating the development of new anticancer agents.

Acknowledgments

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