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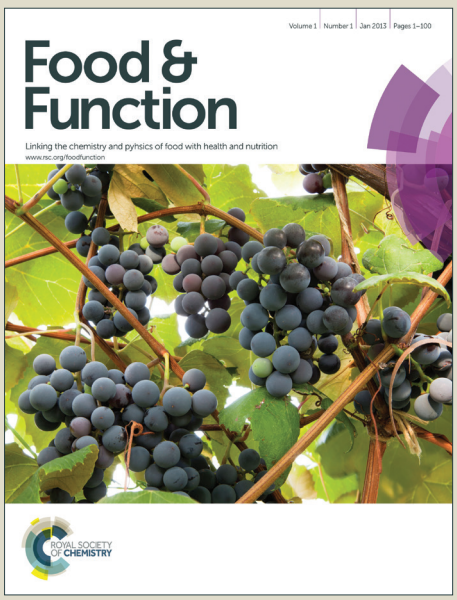

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Calycosin induces apoptosis by the regulation of ERβ/miR-17 signaling pathway in human colorectal cancer cells

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Abstract

Prior studies have suggested that a high intake of isoflavonoids was associated with a protective effect against hormone-related cancers, such as colorectal cancer (CRC). Calycosin, a main component of isofavones, has been shown to suppress the growth of hormone-dependent tumor through ERβ-mediated signaling pathway. However, the effects of calycosin on CRC remain unclear. In this study, we aimed to investigate the anti-tumor activities of calycosin on CRC and its potential mechanism. HCT-116 cells were treated with calycosin. Cell proliferation, apoptosis and invasiveness were measured by MTT assay, fow cytometry and transwell invasion assay, respectively. mRNA levels of ER beta (ERβ) and miR-17 were quantifed by real-time PCR. Protein expression of ERβ and phosphatase and tensin homolog deleted on chromosome ten (PTEN) were determined by Western blotting. We found that calycosin significantly induced apoptosis, and inhibited proliferation and invasiveness of HCT-116 cells in dose-dependent manner. In addition, ERβ expression was signifcantly increased in calycosin-treated HCT-116 cells, followed by decrease of miR-17, and up-regulation of PTEN. Our results indicate that calycosin has an inhibitory effect on CRC, which might be obtained by ERβ-mediated regulation of miR-17 and PTEN expression.

1. Introduction

Colorectal cancer (CRC) is one of the most common malignancies and a leading cause of cancer deaths worldwide. Increased incidence of CRC has been observed in recent years, likely due to associated changes in diet and environment. Despite improvements in surgical and chemotherapeutic treatments, five-year survival rate of CRC remains poor.[1,2] Thus, there is an urgent need to explore effective anti-tumor drug candidates with fewer side effects for the treatment of CRC.

Worldwide epidemiologic studies over several decades have showed that CRC has a higher incidence rate in men than in women, and hormone replacement therapy (HRT) in postmenopausal women could reduce the incidence of CRC, suggesting a therapeutic effect of sex hormones in CRC.[3,4] Preclinical data also supports that estrogen and its receptors play a potential protective role in the initiation and progression of CRC, and the inhibitory effects of estrogen are exerted through estrogen receptor beta (ERβ).[5] Moreover, it is widely known that there is a wide variation in cancer rates from country to country. Compared to Western countries, the incidence rate of CRC is historically much lower in Japan and China, which is thought to be attributed to higher amounts of phytoestrogen-rich soy diet in Asians.[6,7] And recent studies found that high intake of phytoestrogens, in particular isoflavonoids, could decrease the risk of CRC.[8] These findings demonstrate that the development of CRC is influenced by estrogen exposure, and phytoestrogens may possess anti-carcinogenic properties on CRC.

Phytoestrogens, contained in plant foods, have nonsteroidal estrogen-like activities and are proposed as the natural alternatives to estrogen. They are subdivided into four main classes: isoflavones, stilbened, lignans, and coumestans. Isoflavones (e.g.,daidzein, genistein, formononetin, and calycosin) have dominated phytoestrogen research, because they are most active components from soybeans sources and exhibit significantly inhibit effects on a variety of malignancies, including CRC.[9-12] Calycosin is the major isoflavonoid in Huangqi (Radix Astragali Mongolici), synonyms: Astragalus membranaceus (Fisch.) Bunge and Astragalus membranaceus (Fisch.) Bunge var. mongholicus, a traditional Chinese herbal medicine,[13] and is proven to

possess anti-carcinogenic activities in breast cancer,[14-16] osteosarcoma,[17] and hepatocellular carcinoma.[18] Previous studies have shown that calycosin could induce tumor cell apoptosis, and inhibited cell proliferation, invasion, and angiogenesis by affecting the expression of ERβ and some tumor-related genes such as Akt, IGF-1R, MAPK, Bcl-2, Caspase-3, and RAS dexamethasone-induced 1 (RASD1). However, the role of calycosin in CRC remains unclear. Thus, we conducted the current study designed to determine whether calycosin affected human CRC growth and invasion.

As we know, estrogen exerts diverse effects through estrogen receptors, ERα and ERβ. It is found that the expression level of ERα is very low either in normal or pathologic colonic mucosa (adenoma and carcinoma), whereas the expression of ERβ is high in healthy colonic mucosa, and declines with the progression of CRC. This has led to the proposal that ERβ may function as a tumor suppressor, and protect against the tumorigenesis and development of CRC, suggesting ERβ as a potential diagnostic and therapeutic target of CRC.[5,19,20]

On the other hand, microRNAs (miRNAs) are endogenous, non-coding small RNAs, approximately 18–25 nucleotide molecules that negatively regulate the expression of a wide variety of genes by messenger RNA (mRNA) degradation or translation repression. Increasing evidences have shown that the deregulation of miRNAs is involved in a wide range of diseases, including human cancers. miRNAs might play important roles in biological processes that affect tumor progression including differentiation, proliferation, apoptosis, migration, and invasion by acting as tumor suppressor or oncogene.[21] miR-17 was reported to be frequently over-expressed in several cancers, including CRC. It could promote tumor cell growth and metastasis by negatively regulating its target gene PTEN, which function as a tumor-suppressor of CRC by restraining the PI3K/Akt pathway.[22-24] Furthermore, one recent study has found that miR-17 could be affected by ERβ, and involved in the anti-proliferative and apoptotic effects induced by ERβ in CRC cells.[25] In addition, considering that ERβ has been identified a protective role in the progression of CRC and responsible for calycosin–mediated anti-tumor effects, we thus hypothesized that calycosin may inhibit the promotion of CRC through ERβ-miR-17 pathway. Here we focused on

ERβ and miR-17 expression changes in HCT-116 cells after the treatment of calycosin.

Therefore in this study we sought to investigate the influence of calycosin on proliferation, apoptosis and invasion in human CRC cells HCT-116, and to explore the underlying mechanisms involved in the biological actions of calycosin.

2. Materials and Methods

2.1 Cell culture and calycosin treatment

The human CRC cell line HCT-116 were obtained from Shanghai Institute of Cell Biology (the Chinese Academy of Sciences, Shanghai, China). Cells were cultured in RPMI-1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone Co. Ltd, USA), 100 U/mL penicillin, 100 μg/mL streptomycin, and kept at 37˚C in a humidified 5% CO_2 incubator. The cells (60%–70% confluent) were treated with calycosin (Phytomarker Ltd, China) dissolved in dimethylsulfoxide (DMSO) and cells treated with vehicle (DMSO) served as control. All cells were starved with low-serum medium (contain 0.5% CS-FBS) for 24 h before the experiments.

2.2 Cell Proliferation assay

The effect of calycosin on cell proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. In brief, HCT-116 cells were plated into 96-well plates (4×10^3 cells/well). After overnight incubation, cells were treated with calycosin in concentrations ranging from 0 to 100 μM for 48 h. At the end of treatment, 20 μl MTT (5 mg/ml, Sigma-Aldrich, USA) was added into each well and incubated for additional 4 h. Then the medium was removed, and 150 μl DMSO was added to each well to dissolve the sediment. The Optical density (OD) for each well was determined at 570 nm on a microplate reader (Bio-Rad, USA) after shaken for 10 min at room temperature. To evaluate the anti-proliferation activity of calycosin, inhibitory rates were calculated as following formula: Inhibitory rate (%) = 1-(OD value of treated group/ OD value of control group) ×100%.

2.3 Flow Cytometry

The effect of calycosin on cell apoptosis was evaluated by flow cytometry analysis. After treatment with calycosin (0, 25, 50 and 100 μM) for 48 h, cells were dual stained with Annexin V-FITC and propidium iodide (PI) for 30 min at room temperature. Stained cells were immediately analysed by the flow cytometry (Becton Dickinson, USA). The early apoptotic cells is defined by Annexin V-FITC positive and PI negative.

2.4 Cell invasion assay

Cell invasion abilities were examined using 24-well matrigel-coated transwell chambers (Becton Dickinson, USA). HCT-116 cells were treated with calycosin (0, 25, 50 and 100 μM) for 48 h, washed with PBS, and resuspended at 1×10^5 cells/ml in serum-free medium. Then 0.2 ml cell suspension was added to the upper chamber, and 0.5 ml medium containing 10% FBS was added to the bottom chamber. After 24 h incubation, all non-invaded cells were removed from upper face of the filters and the invaded cells were fixed and stained by crystal violet solution. The experiments were repeated in triplicate wells, and the invaded cells were counted microscopically (400×) in five different fields per filter.

2.5 RNA isolation and real-time PCR

HCT-116 cells were treated with calycosin for 48 h, and then the totol RNA was isolated from cells using Trizol reagent (Invitrogen, USA) according to manufacturer's instruction. Then, cDNA was prepared using 10 ng of RNA and the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, USA). Next, quantification of ERβ and miR-17 was measured by qPCR using SYBR Premix Ex Taq™ (Takara, Japan) according to the manufacturer's protocol. Here GADPH and U6 small nuclear RNA (U6) were used as internal control gene for normalization. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.[26]

2.6 Western blot analysis

After treated with different concentrations of calycosin for 48 h, HCT-116 cells were harvested with ice-cold PBS and lysed on ice in lysis buffer for 30 min. The lysates were centrifuged 12,000 rpm for 10 min, and protein concentrations were

measured by protein assay kit (Bio-Rad, USA). Equal amounts of proteins were electrophosed by SDS-PAGE gels and subsequently transferred to PVDF membranes (Bio-Rad laboratories, USA). Membranes were blocked with 5% nonfat dried milk in TBST (Tris-buffered solution, pH 7.6, 0.05% Tween 20) and then incubated with mouse anti-ERβ (1:400, Santa Cruz Biotechnology, USA), mouse anti-PTEN (1:400, Santa Cruz Biotechnology, USA), and mouse anti–β-actin (1:1000, Santa Cruz Biotechnology, USA) at room temperature for 2 h. After three washes, the membranes were subsequently incubated with appropriate secondary antibodies (1:2000, Santa Cruz Biotechnology, USA) coupled to horseradish peroxidase at room temperature for 1 h, and then developed using electrochemiluminescence (ECL) western blot detection reagents (Beyotime, China). Expression levels of the proteins were compared to the control based on the relative intensities of the bands.

3.7 Statistical analysis

Data were analyzed using analysis of variance (ANOVA) as appropriate. All statistics and data analysis were performed using SPSS 13.0 software (SPSS Inc., USA). Data were presented as means ± standard deviation (SD). $P < 0.05$ was considered statistically significant.

3. Results

3.1 Calycosin inhibits proliferation of HCT-116 cell

In order to evaluate the anti-proliferative effects of calycosin, the HCT-116 cells were treated with increasing concentrations of calycosin for 48 h, and the cell viability was determined by MTT assay. The results are shown in Fig. 1. Compared with the negative control, calycosin significantly inhibited the proliferation of HCT-116 cell in a dose-dependent manner.

3.2 Calycosin induces apoptosis in HCT-116 cells

Flow cytometric analysis was performed to determine whether calycosin could induce apoptosis in HCT-116 cell. As shown in Fig. 2, the cells in negative control group showed 14.23% apoptosis rate. However, after treatment with calycosin (25, 50 and 100 μM) for 48 h, the apoptosis percentage increased to 23.37%, 34.07%, and 55.16% ($p < 0.01$), respectively. The results showed that calycosin could promote

apoptosis of HCT-116 cells in a dose-dependent manner, consistent with the results from MTT assay.

3.3 Calycosin inhibits invasion of HCT-116 cells

The transwell invasion assay was performed to examine the effects of calycosin on invasiveness of HCT-116 cell. The results showed that calycosin significantly inhibited the invasiveness of CRC cells in a dose-dependent manner, when compared with negative control ($p < 0.01$).(Fig.3)

3.4 Calycosin regulates the expression of miR-17 and ERβ mRNA in HCT-116 cells

To study the possible mechanism for calycosin-induced anti-proliferation and apoptosis in HCT-116 cells, the expressions of miR-17 and ERβ mRNA were detected by real-time PCR. The results showed that calycosin significantly up-regulated mRNA expression of ERβ but down-regulated expression levels of miR-17 in a dose-dependent manner in HCT-116 cell when compared with negative control ($p < 0.01$), as shown in Fig. 4.

3.5 Calycosin regulates the expression of ERβ and PTEN protein in HCT-116 cells

After detection of ERβ mRNA and miR-17 expression levels in calycosin-treated HCT-116 cells, the levels of ERβ and PTEN (the direct target of miR-17) protein were determined by Western blot. In accordance with mRNA results, calycosin dose-dependently increased ERβ protein expression, compared with negative control ($p < 0.01$). Meanwhile, the PTEN protein level in HCT-116 cells was also significantly up-regulated after incubation with calycosin, especially in the 50 μM and 100 μM calycosin-treated group as compared with untreated cells ($p < 0.01$). (Fig.5)

4. Discussion

Calycosin, the main component of isoflavones, has been attracting attention for its possible anti-tumor effects, in particularly in hormone-dependent cancers (e.g. breast cancer). Previously, we have reported that calycosin significantly inhibited proliferation and induced apoptosis in ER-positive human breast cancer cells through ERβ-mediated selective regulation of downstream signaling pathway, such as MAPK and PI3K/Akt pathway.[14,15] While, to provide more valuble information in evaluating the superiority of calycosin in clinical application, we further observed and compared

the anti-proliferation effects between calycosin and other isoflavone components, genistein and formononetin, which have also been shown to have inhibitory effects on breast cancer. The results showed that calycosin has an advantage on inhibiting breast cancer growth in comparison with genistein and formononetin, implying that calycosin may be superior to other isoflavones in treating hormone cancers.[15,16] Furthermore, Qiu et al., reported that calycosin could dose-dependently inhibit osteosarcoma cells proliferation and xenografted tumor growth, and the antineoplastic mechanism was associated with activation of apoptotic signaling expression, such as IκBα, NF-κB p65, Bcl-2 and Caspase-3, thus inducing apoptosis.[17] Similarly, in the hepatocellular carcinoma cells, calycosin also exhibited inhibitory effects on cell proliferation involved in the modulation of cell cycle regulating genes, which resulted in cell cycle block.[18] However, until now little is known about the role of calycosin in CRC. Thus, in the present study we detected the *in vitro* biological effects of calycosin in CRC cells for the first time. The results showed that calycosin markedly inhibited cell proliferation, induced apoptosis, and suppressed cell invasion in Human CRC cells HCT-116 in a dose-dependent manner.

ER belongs to the steroid hormone receptor family and contains two subtypes, ERα and ERβ. Until now, more and more studies have demonstrated that ERβ signaling is implicated in the tumorigenesis and progress of estrogen-dependent cancer including CRC.[5] It showed that loss of ERβ expression in tumor tissue of patients with CRC has been associated with more advanced cancer stage and poorer prognosis.[27] Furthermore, transfection of CRC cell lines with ERβ resulted in inhibition of proliferation and cell cycle arrest. And CRC xenographts with ERβ expression had significantly reduction in the tumor weight, implying that targeting ERβ may be a promising therapeutic strategy in CRC.[28]

Meanwhile, the isoflavonoid has been reported to possess inhibitory effects on the growth of CRC via up-regulating ERβ in recent studies. For example, Pampaloni et al found that high dose of genistein can completely blocked the proliferation of HCT8 cells transected ERβ, but not non-transfected cells. And the anti-proliferative effects of genistein on CRC cells were accompanied by activation of ERβ, as

observed by luciferase activation.[10] similarly, Bielecki found that use of isoflavons (composed of genistein, daidzein, and glycitein) in human CRC cell line DLD-1, resulted in cell proliferation suppression and cell cycle arrest. However, the isoflavone-mediated anti-tumor effects were not observed when ERβ gene was silenced.[11] These findings suggest that maintaining the expression of ERβ is crucial in mediating the growth-suppressive effects of isoflavones against colon tumors. In this study, we also observed that calycosin could stimulate ERβ expression in CRC cells, which might explain the mechanism for calycosin-induced anti-proliferation, apoptosis and anti-invasion.

miRNAs, which were dysregulated in nearly every types of human cancer, have a major impact on tumorigenesis and progression by regulating numerous important cancer-related gene expression at posttranscriptional level.[21] In CRC, miR-17 was shown to be up-regulated in tumor tissues with distant metastases and higher clinical stages. And Over-expression of miR-17 could promote CRC cell proliferation and invasiveness by repressing PTEN expression.[22] As we know, the PTEN tumor suppressor is a negative regulator of PI3K/Akt pathway, which has profound effects on multiple malignancy-related processes including cell uncontrolled proliferation, lack of apoptosis, invasion and metastasis.[29,30] Furthermore, ERβ has been implicated to have effects on the expression of miRNAs in CRC. It showed that the oncogenic miR-17 was strongly down-regulated by ERβ, and re-introduction of miR-17 could reverse the anti-proliferative and pro-apoptotic effects of ERβ in CRC cells.[24]

On the other hand, more and more studies have shown that natural agents, such as isoflavonoids, exert their anti-tumor efects through the regulation of one or more miRNAs.[31] For instance, xu at al., found that miR-27a was over-expression in human ovarian cancer, and genistein could block ovarian cancer cell growth and migration by suppressing oncogenic miR-27a.[32] In addition, our previous studies revealed that treatment with calycosin significantly down-regulated the relative expression level of miR-375, the tumor suppressor in breast cancer.[15] Hence, we also proposed to elucidate the effects of calycosin on miRNAs expression in CRC in this study. The results showed that along with increased ERβ levels, the miR-17 expression

dose-dependently decreased with calycosin treatment. Moreover, PTEN, the target of miR-17, was also up-regulated by calycosin. Based on these data, we hypothesize that the ERβ-mediated regulation of miR-17/PTEN/Akt pathway may be involved in the inhibitory effect induced by calycosin in HCT-116 cells. Nonetheless, further research was needed to confirm this hypothesis.

In conclusion, our results show that calycosin inhibits CRC cell proliferation and invasiveness through up-regulating ERβ and PTEN, and down-regulating miR-17. These findings improve our understanding in the anti-tumor activity and potential mechanisms of calycosin, and offer the valuable references for clinical application of calycosin in treating CRC. But further studies are required to evaluate the *in vivo* effects and molecular mechanisms of calycosin in CRC.

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Fig. 1 Anti-proliferative effects of calycosin on CRC cell HCT-116. Cells were treated with various concentrations of calycosin for 48 h, and then analyzed for viability by MTT. Data were shown as mean ± SD of three independent experiments.

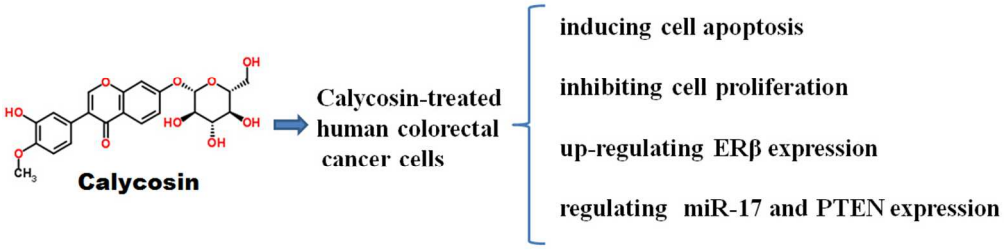
Fig. 2 Apoptosis-induced effects of calycosin on CRC cell HCT-116. Cells were treated with 0, 25, 50, and 100 μM calycosin for 48 h, and then the cell apoptosis was examined by flow cytometry. Representative scatter grams from flow cytometry profile represent Annexin V-FITC staining on the x axis and PI on the y axis. Percentages of apoptotic cells are shown in the histogram. Data were shown as mean ± SD of three independent experiments. **$p < 0.01$ *vs* negative control.

Fig. 3 Inhibitory effects of calycosin on invasiveness of HCT-116 cells. Cells were treated with 0 (A), 25 (B), 50 (C), and 100 (D) μM calycosin for 48 h, and then the invasive cells were examined by transwell invasion assay. Pictures of representative cell fields for each treatment were taken by a camera connected to the microscope (400×). Numerical representation of the data was obtained by counting average numbers of cells from five different fields for each treatment. The average number of invasive cells per field for each treatment is shown in the histogram. Data were shown as mean ± SD of three independent experiments. **$p < 0.01$ *vs* negative control.

Fig. 4 Regulation of calycosin on miR-17 and ERβ mRNA expression in CRC cell HCT-116. Cells were incubated with 0, 25, 50, and 100 μM calycosin for 48 h, and then miR-17 and ERβ mRNA expression was detected by real-time PCR reaction array. The results were normalized to U6 or GAPDH expression and expressed as fold change relative to the negative control. Data were shown as mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$ *vs* negative control.

Fig. 5 Regulation of calycosin on ERβ and PTEN protein expression in CRC cell HCT-116. Cells were incubated with increasing concentrations of calycosin for 48 h. Then, the protein expression of ERβ and PTEN was detected by western blot analysis. Band density was quantified using the software program Bio-Rad Quantity One v4.62. The results were normalized to β-actin protein expression. Data were shown as mean

± SD of three independent experiments. *p < 0.05, **p < 0.01 *vs* untreated cells.



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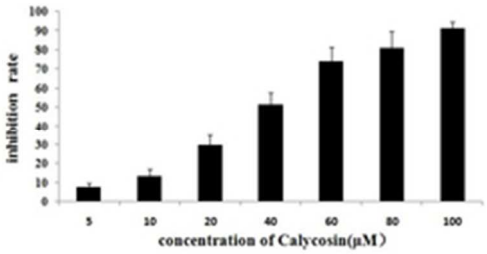

Fig. 1 Anti-proliferative effects of calycosin on CRC cell HCT-116. Cells were treated with various concentrations of calycosin for 48 h, and then analyzed for viability by MTT. Data were shown as mean ± SD of three independent experiments.
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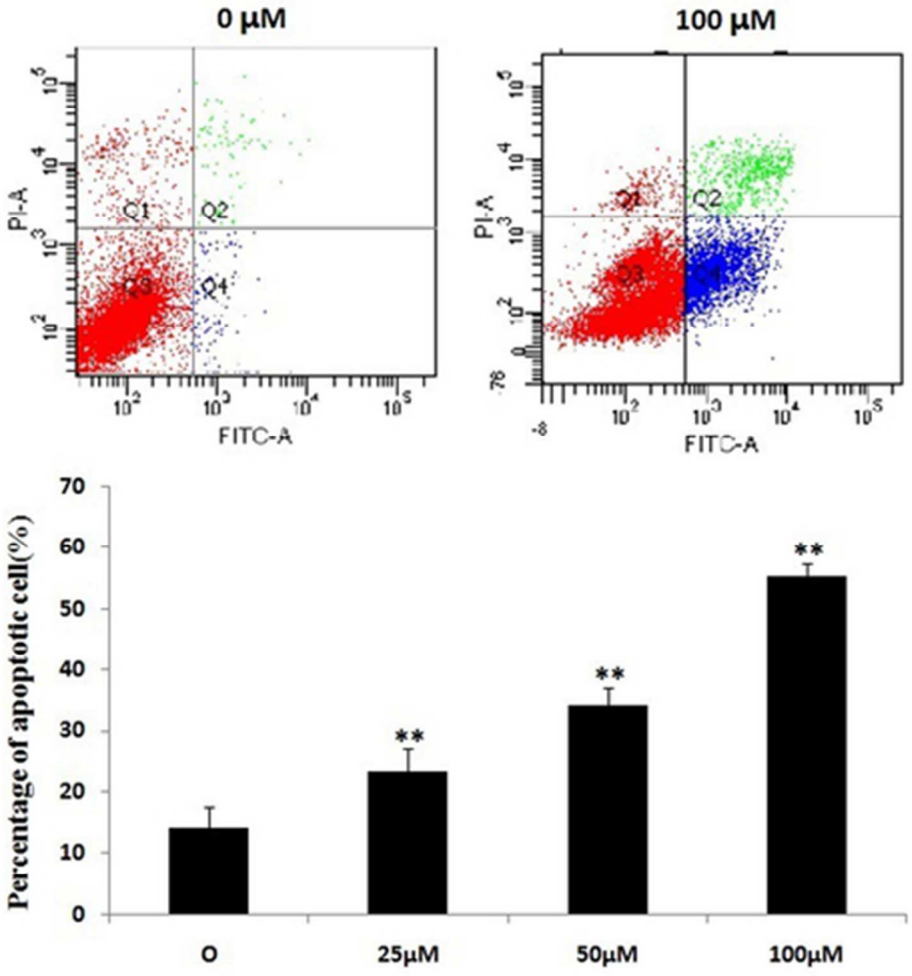

Fig. 2 Apoptosis-induced effects of calycosin on CRC cell HCT-116. Cells were treated with 0, 25, 50, and 100 μM calycosin for 48 h, and then the cell apoptosis was examined by flow cytometry. Representative scatter grams from flow cytometry profile represent Annexin V-FITC staining on the x axis and PI on the y axis. Percentages of apoptotic cells are shown in the histogram. Data were shown as mean ± SD of three independent experiments. **p < 0.01 vs negative control.
40x41mm (300 x 300 DPI)

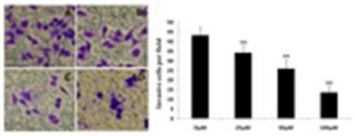

Fig. 3 Inhibitory effects of calycosin on invasiveness of HCT-116 cells. Cells were treated with 0 (A), 25 (B), 50 (C), and 100 (D) μM calycosin for 48 h, and then the invasive cells were examined by transwell invasion assay. Pictures of representative cell fields for each treatment were taken by a camera connected to the microscope (400×). Numerical representation of the data was obtained by counting average numbers of cells from five different fields for each treatment. The average number of invasive cells per field for each treatment is shown in the histogram. Data were shown as mean ± SD of three independent experiments. **p < 0.01 vs negative control.
14x5mm (300 x 300 DPI)

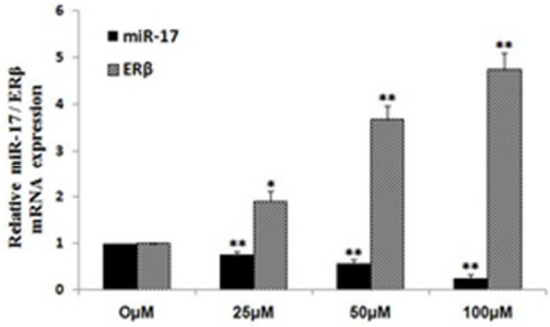

Fig. 4 Regulation of calycosin on miR-17 and ERβ mRNA expression in CRC cell HCT-116. Cells were incubated with 0, 25, 50, and 100 μM calycosin for 48 h, and then miR-17 and ERβ mRNA expression was detected by real-time PCR reaction array. The results were normalized to U6 or GAPDH expression and expressed as fold change relative to the negative control. Data were shown as mean ± SD of three independent experiments. *p < 0.05, **p < 0.01 vs negative control.
23x14mm (300 x 300 DPI)

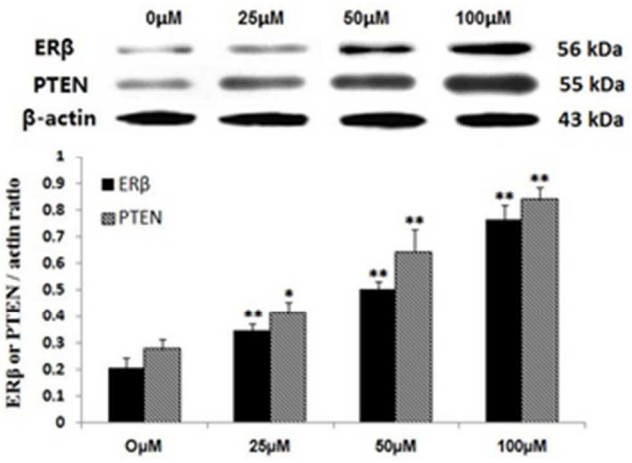

Fig. 5 Regulation of calycosin on ERβ and PTEN protein expression in CRC cell HCT-116. Cells were incubated with increasing concentrations of calycosin for 48 h. Then, the protein expression of ERβ and PTEN was detected by western blot analysis. Band density was quantified using the software program Bio-Rad Quantity One v4.62. The results were normalized to β-actin protein expression. Data were shown as mean ± SD of three independent experiments. *p < 0.05, **p < 0.01 vs untreated cells.
27x19mm (300 x 300 DPI)