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Original Research Article

Irigenin exhibits anticancer activity against human colon cancer cells via autophagy, inhibition of cell migration and invasion, and targeting of ERK/MAPK signal pathway

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Abstract

Purpose: *To study the anticancer effect of naturally-occurring irigenin isoflavonoid on colon cancer, and to determine the mechanism involved.*
Methods: *The effect of irigenin on viability of normal and cancerous colon cells was assessed by MTT assay, while clonogenic assay was used to measure colony generation. Autophagy was examined by transmission electron microscopy (TEM) and western blotting. Transwell chamber assay was used to determine the influence of irigenin isoflavonoid on cell migration and invasion. The expression levels of ERK/MAPK signal pathway-associated proteins were assayed using Western blotting.*
Results: *Irigenin significantly decreased the viability of Caco-2 colon cancer cells, in contrast to normal CCD841 colon cells, and produced concentration-dependent anti-proliferative effects ($p < 0.05$). The number of cell colonies in control group decreased significantly ($p < 0.05$) upon exposure to irigenin. Results from TEM revealed that irigenin caused dose-dependent formation of autophagosomes, and dose-based up-regulation of the expressions of Beclin-1, LC3-I and LC3-II ($p < 0.05$). Moreover, irigenin markedly suppressed the migration and invasion of Coca-2 cells. Furthermore, irigenin exposure dose-dependently blocked the expressions of proteins associated with ERK/MAPK signal pathway in Coca-2 cells.*
Conclusion: *These results indicate that irigenin exerts potent inhibitory effect on the growth and migration of colon cancer cells. Furthermore, irigenin induces autophagy, inhibits cell migration and invasion, and targets ERK/MAPK survival signal pathway. Therefore, irigenin may be a lead candidate drug for colon cancer treatment. However, there is need for further in vivo and clinical studies to validate these findings.*

Keywords: *Colon cancer, Isoflavonoids, Irigenin, Autophagy, Cell migration, Cell invasion*

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INTRODUCTION

Historically, medicinal plants have been used to overcome numerous human malignancies throughout human civilization [1]. The importance of medicinal plants to early *homo sapiens* is evident from records of Traditional Medicine Systems and scripts. The emergence of synthetic and modern medicine resulted in reduced interest in medicinal plants [2,3]. However, recent

years have seen tremendous awareness of the pharmacological properties of bioactive phytochemicals from medicinal plants. Indeed, medicinal plants provided drugs and lead molecules that led to breakthroughs in natural products chemistry. Traditional Chinese Medicine (TCM), a complex ancient therapy which is considered as comprehensive complementary system of medicine today, comprises mostly medicinal plants and plant-based products [4]. *Belamcanda chinensis* has been prescribed in TCM for the treatment of ailments affecting the liver, lungs, and stomach, as well as inflammation [5]. The chemical composition of the plant is diverse, and comprises flavonoids, isoflavonoids, triterpenoids and glycosides. The major isoflavonoids extracted from the rhizome of the plant are tectoridin, and iridin, as well as their aglycones tectorigenin and irigenin. Some traces of iristectorigenin and irisflorentin have been found in its rhizomes [6,7]. Isoflavonoids are of tremendous medicinal importance as indicated by their anti-inflammatory, anti-carcinogenic, anti-mutagenic, anti-oxidant and anti-proliferative properties [8,9].

The isoflavonoid irigenin (Figure 1) from rhizomes of the medicinal plant *Belamcanda chinensis* has been extensively studied. Irigenin exerts *in vitro* antitumor, anti-inflammatory and anti-oxidative activities. Moreover, in association with TRAIL therapy, it induced sensitizing effects on gastric cancer [10]. Colon cancer is a hazardous human malignancy associated with high morbidity and mortality [11]. Globally, colon cancer ranks third in prevalence and second in cancer-related mortality. Over 90 % of colorectal cancers originate from within the glandular epithelial cells of the rectum or colon, and they are termed adenocarcinomas [12,13]. It has been observed that over 60-65 % of the colon cancer cases occur sporadically. Colon cancer patients show no family history or inherited mutations, although acquired epigenetic and somatic aberrations increase the risk of colon cancer [14]. The extant management strategies for colon cancer are surgical resection, chemotherapy and radiation therapy. Although remarkable advances have been made in the treatment of colon cancer, the overall survival remains low, while relapse rates are high. Therefore, to overcome the shortcomings of currently adopted therapeutic approaches, there is need for novel treatment approaches and potential therapeutic agents. The current study was designed to unveil the anti-colon cancer potency of naturally occurring irigenin molecule. Its effects on autophagy induction, cell migration and invasion inhibition, and its influence on the ERK/MAPK signal pathway were also investigated.

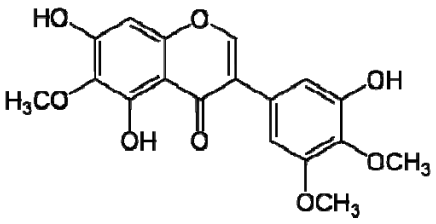

Figure 1: Chemical structure of irigenin molecule

EXPERIMENTAL

Cell culture and conditions

Human normal colon CCD841 cells and cancerous colon Caco-2 cells were bought from American Type Culture Collection (ATCC). The cancerous and normal cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing antibiotics (100 µg/mL of streptomycin and 100 units/mL of penicillin); 10 % fetal bovine serum (FBS) and 2 mM glutamine. The cells were incubated in a humid environment at 37 ºC in a 5 % CO_2 incubator (Thermo Scientific, Waltham, United States).

Cell viability assay

The effect of irigenin on the viability of normal colon CCD841 cells and cancerous Coca-2 colon cells was evaluated via MTT assay. The two types of cell lines were seeded in 96-well plates, each at a density of 2.5×10^3 cells/well, and were pre-cultured for 24 h prior to drug exposure. The CCD841 and Coca-2 cells were exposed to varying doses of irigenin drug viz 5, 25, 50 and 100 µM for 24 h. Untreated cells served as controls. After the irigenin treatment, 20 µL of MTT solution was added to each well, followed by incubation. Thereafter, the resultant formazan crystals were dissolved in dimethyl sulfoxide (DMSO), and the absorbance of each well was read at 540 nm using an ELISA plate reader.

Colony generation assay

Clonogenic assay was used to determine the effect of irigenin on colony formation of Coca-2 cells. The Coca-2 cells were seeded in 6-mm culture plate dishes at a level of 500 colonies per dish. Each dish was incubated for 48 h at 37 ºC to let the cells to adhere. Thereafter, the cells were exposed to varying doses of irigenin drug viz 5, 25 and 100 µM, and further subjected to incubation for 6 days. Post-incubation, the cells

were washed using PBS, and fixed in methanol. Finally, the irigenin-treated Coca-2 cells were stained with crystal violet for 15 min, followed by counting under a light microscope (OLYMPUS, Japan).

Autophagy assessment

Irigenin-treated and control Coca-2 cells were evaluated for autophagic cell death using transmission electron microscopy (TEM). The control and irigenin-treated cells were subjected to fixation with PBS containing 2.5% glutaraldehyde for 40 min, and fixed using PBS containing osmium tetroxide 1% for additional 40 min. Then, the irigenin-treated Coca-2 cells were dehydrated in ethanol, washed with propylene oxide, and loaded onto Epon. Then, 90-nm thick sections of the cells were cut using Reichert-Jung ultra-microtome. The thin cell sections were analyzed under transmission electron microscope (Hitachi H7100) at 75 kV after staining with 5 % lead citrate and 5 % uranyl acetate.

Transwell chamber assay

The invasion and migration potential of irigenin-treated Coca-2 cells were determined with Transwell chamber assay. Cells were harvested at exponential growth phase, and 200 mL of cell suspension was placed in culture medium containing 10 % FBS in the upper Transwell chambers, while the lower chambers contained culture medium only. The cells in the upper chambers were treated with irigenin at doses of 5, 25 and 100 µM, while control cells were untreated. After incubation with irigenin for 24 h, non-migrated cells were cleaned off with a cotton swab, while migrated cells were washed twice using PBS. The washed Coca-2 cells were then fixed with alcohol, stained with crystal violet, and examined under an inverted microscope. A similar procedure was followed for invasion analysis except that the Transwell chambers were coated with Matrigel.

Western blotting analysis

The expression levels of autophagy and ERK/MAPK signal pathway-allied proteins were determined with Western blotting assay. Coca-2 cells were harvested at exponential growth stage and treated with varying doses of irigenin (5, 25 and 100 µM). Then, the Coca-2 cells were lysed with lysis buffer, followed by incubation of the lysates at 99 °C for 10 min. The protein content of each lysate was quantified with BCA assay. About 40 µg protein from each sample was separated on SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with skimmed milk powder (5 %) prior to incubation overnight at 4 °C with primary antibodies against Beclin-1, LC3-I, LC3-II, P38 and ERK. Thereafter, the membranes were incubated with horseradish peroxidase-linked biotinylated secondary antibody (1: 1000 dilution) for 2 h at room temperature. Then, the membranes were washed with PBS, followed by visualization of the bands using ECL-PUS kit in accordance with manufacturer's protocol.

Statistical analysis

Data are presented as mean ± SD (n = 3). Statistical analysis was performed with the aid of GraphPad 6 using Student's *t*-test. Statistical significance of difference was assumed at $p < 0.05$.

RESULTS

Irigenin suppressed the viability of colon cancer cells

The MTT assay was used to determine the cytotoxic effects of irigenin molecule on normal CCD841 and Coca2 colon cancer cells. Repeated and uncontrolled proliferation are the primary features of cancer cells. Irigenin induced inhibitory effects on the proliferation of Coca-2 colon cancer cells, relative to normal CCD841 colon cells. The anti-proliferative effect of irigenin was concentration-dependent (Figure 2).

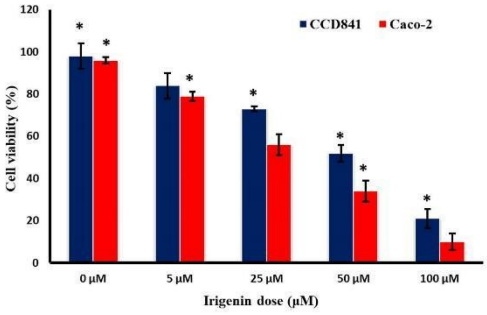

Figure 2: Effect of irigenin on proliferation of normal CCD841 and Coca-2 colon cancer cells. Irigenin exerted concentration-reliant inhibition of proliferation in Coca-2 cells, when compared to CCD841 cells. Data are presented as mean ± SD (n = 3)

Clonogenic assay was employed to evaluate the effects of irigenin on colony production propensity of Coca-2 cells. It was observed that the number of colonies was significantly decreased in the treatment groups, while control cells had negligible changes in colony generation. These results are presented in

Figure 3 A. The 500 cells seeded in each culture dish in control group were reduced to almost 50 by treatment with irigenin at a dose of 100 µM, as shown in Figure 3 B.

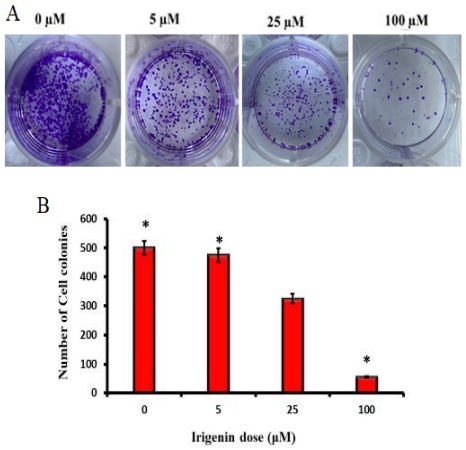

Figure 3: (A) Effect of irigenin on colony-forming potential of Caco-2 cells. (**B)** Number of Coca-2 colonies left after irigenin exposure

Irigenin induced autophagic cell death in Coca-2 cells

Autophagy was studied in Coca-2 cells post-irigenin treatment to investigate the mechanism of action associated with the antiproliferative effect the drug. Autophagy is one of key pathways of cell death either under natural circumstances or under stimulation by chemotherapeutic agents. Results from transmission electron microscopy (TEM) indicated the formation of autophagosomes or autophagic vesicles in irigenin-treated Coca-2 cells, when compared to control group (Figure 4 A). Thus, the antiproliferative effect of irigenin was mediated via autophagy which is usually hallmarked by formation of autophagosomes. Results from Western blotting showed increased protein expression levels of Beclin-1, LC3-I and LC3-II (Figure 4 B). These proteins are autophagy regulators. Therefore, these results confirm that irigenin induced autophagic cell death in Coca-2 cells.

Irigenin inhibited migration and invasion of Coca-2 cells

Irigenin suppressed the migration (Figure 5) and invasion (Figure 6) of Coca-2 cells, while cells in the control group showed almost no changes in migration and invasion pattern ($p < 0.05$).

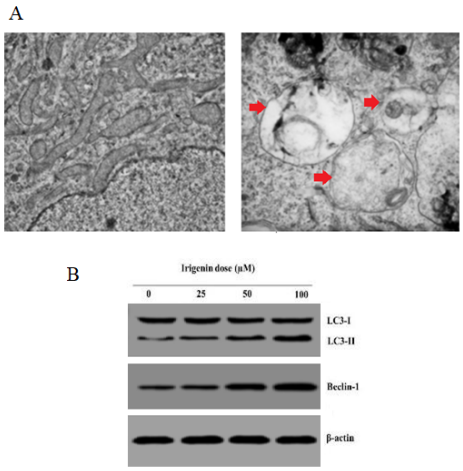

Figure 4: (A) Transmission electron microscopy showing formation of autophagosomes in irigenin-treated Coca-2 cells, relative to controls. Arrows point at autophagosomes. (B) Results of Western blotting assay showing marked up-regulations of the expressions of the autophagy-related proteins Beclin-1, LC3-I and LC3-II

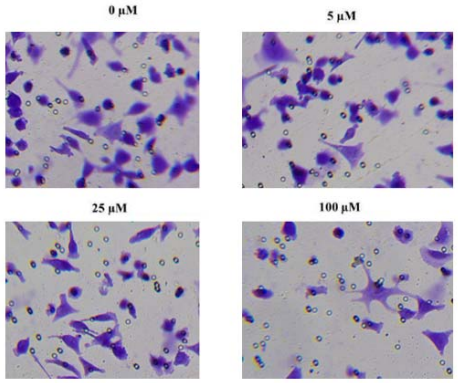

Figure 5: Cell migration potential of Coca-2 cells pre- and post-irigenin treatment at indicated doses

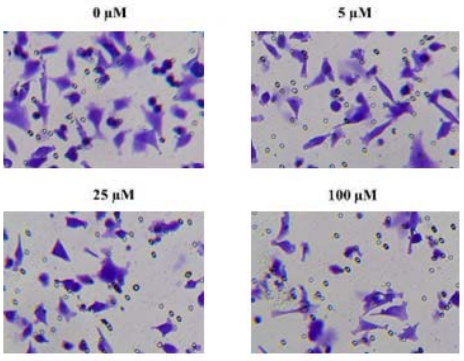

Figure 6: Cell invasion in Coca-2 cells pre and post irigenin treatment at indicated doses

Irigenin targeted ERK/MAPK signal pathway in Coca-2 cells

The ERK/MAPK signal pathway is one of the key pathways involved in growth, progression, proliferation and differentiation of cancer cells. Therefore, chemotherapeutic drugs act on cancer cells by targeting the ERK/MAPK signal pathway. Western blotting results revealed significant reductions in the protein expression levels of p-P38 and p-ERK in irigenin-treated Coca-2 cells, indicating downregulation of the ERK/MAPK pathway (Figure 7). However, there were no changes in the protein expressions of P38 and ERK in the irigenin-treated Coca-2 cells.

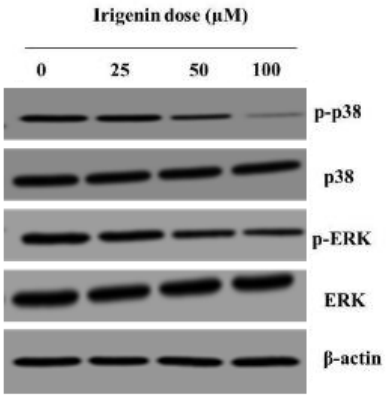

Figure 7: Expression levels of ERK/MAPK signal pathway-associated proteins

DISCUSSION

Colon cancer is a dangerous malignancy that is associated with high morbidity and mortality. Modern life styles constitute a big challenge for researchers and scientists because they present increased risk factors for colon cancer [15]. There is a higher incidence of colon cancer in developed nations than in developing and under-developed nations. Indeed, there has been an abrupt upsurge in incidence of colon cancer in developing and under-developed nations in the past one decade due to adoption of modern/western life styles [16]. Various therapeutic targets that can help in eliminating cancer have been identified by researchers.

Autophagy is one of the principal targets of chemopreventive drugs used in modern treatment techniques for cancer, and it is termed type II programmed cell death [17,18]. It is an evolutionary and intracellularly conserved catabolic process that helps in degradation of pathogens, damaged organelles, protein aggregates, and macromolecules. Autophagosomes engulf these entities and attack them with lysosomal hydrolases which transform them into adenosine triphosphate (ATP), sugars, fatty acids, amino acids and nucleotides. The autophagosomal mechanism of degradation plays a key role during starvation, stress and eliminations.

Another therapeutic strategy is to suppress cell migration and invasion which are primary causes of cancer metastasis [19]. There are different cellular-level signal pathways involved in the maintenance of normal growth, differentiation and hemostasis. One of such vital pathways is the ERK/MAPK signal pathway which is responsible for maintaining normal functioning of cancer cells. Therefore, this pathway often serves as a leading therapeutic target for various chemopreventive drugs [20]. The targeting of ERK/MAPK signal pathway in cancer cells halts their normal functioning and ultimately leads to cell death. Moreover, ERK/MAPK pathway is involved in the degradation process catalyzed by matrix metalloproteinases which hydrolyze the extracellular matrix (ECM) [21]. Furthermore, this pathway is involved in signal cascades and plays a vital role in proliferation, stress and differentiation.

The current research was undertaken to ascertain the anti-proliferative effects of irigenin on human colon cancer. Moreover, the effect of irigenin on autophagy induction, cell migration and invasion, and its effect on the ERK/MAPK signal pathway, were investigated. The results indicated that irigenin induced significant and dose-dependent anti-proliferative effect on Coca-2 colon cancer cells, when compared to normal CCD841 colon cells. Irigenin reduced the colony generation potential of Coca-2 cells, as was shown through clonogenic assay. Previous studies have demonstrated that isoflavonoids exert strong pro-autophagic potential. In the present study, TEM analysis showed formation of autophagosomes in the irigenin-treat group, when compared to controls. Autophagosomes are the hallmark of autophagy. Furthermore, the expressions of autophagy-allied proteins were enhanced in irigenin-treated Coca-2 cells. Therefore, these results are consistent with previous reports on isoflavonoid-induced autophagy-allied antiproliferative effects. It has already been established that irigenin inhibits the migration and invasion of cancer cells. However, this is the first study on its effect on Coca-2 cancer cells. The results revealed that cell migration and invasion potency of Coca-2 cancer cells were significantly suppressed by irigenin exposure. Moreover, this is the first study on the effect of irigenin on ERK/MAPK

signal pathway in Coca-2 cells. Results from Western blotting revealed that irigenin markedly down-regulated the expressions of ERK/MAPK signal pathway-allied proteins in Caco-2 cells.

CONCLUSION

The findings of this study reveal that naturally-occurring irigenin significantly inhibited human colon cancer growth and proliferation via induction of autophagy, inhibition of cell migration and cell invasion, and targeting of the important cell survival pathway, i.e., ERK/MAPK signal pathway. These findings are considered beneficial in colon cancer drug discovery and research. However, there is need to validate the therapeutic potential of irigenin on Caco cancer cells through more detailed investigations.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yan Zhan, Shuangxi Kong performed all the experiments. Liang Fan collected the data and did statistical analysis. The whole study was supervised by Jun Jiang.

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