External Qi of Yan Xin Qigong Inhibits Activation of Akt, Erk1/2 and NF-κB and Induces Cell Cycle Arrest and Apoptosis in Colorectal Cancer Cells

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Key Words
Colorectal cancer • Akt • Erk1/2 • NF-κB • External qi of Yan Xin qigong • Apoptosis

Abstract
Background/Aims: Colorectal cancer (CRC) is the second leading cause of cancer death in the Western countries. Novel approaches of treatment are needed for CRC. The purpose of the present study was to investigate cytotoxic effect of external Qi of Yan Xin Qigong (YXQ-EQ) on human colorectal cancer cells. Methods: The effect of YXQ-EQ on viability, cell cycle progression and apoptosis in colorectal cancer HT-29 cells was investigated. Phosphorylation of Akt and Erk1/2, activation of NF-κB and the expression of proteins involved in regulation of cell cycle and apoptosis were examined by Western blot analysis. Results: YXQ-EQ markedly decreased viability and blocked colony formation of HT-29 cells. YXQ-EQ downregulated cyclin D1 expression and increased accumulation of cyclin-dependent kinase inhibitors p21\textsuperscript{Cip1} and p27\textsuperscript{Kip1}, resulting in G1 cell cycle arrest. YXQ-EQ induced apoptosis in HT-29 cells in association with decreased expression of antiapoptotic proteins Bcl-x\textsubscript{L}, XIAP, survivin and Mcl-1 and elevated expression of proapoptotic protein Bax. YXQ-EQ significantly repressed phosphorylation of Akt and Erk1/2 and NF-κB activation in HT-29 cells, suggesting that YXQ-EQ may exert cytotoxic effect through regulating signaling pathways critical for cell proliferation and survival. Furthermore, YXQ-EQ treated PBS and an YXQ-EQ treated plant extract induced apoptosis in HT-29 cells. Conclusion: These findings show that YXQ-EQ has potent cytotoxic effect on HT-29 cells and suggest that YXQ-EQ could be potentially used for colorectal cancer treatment either directly or indirectly via carriers.
Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of cancer death in the United States [1]. The incidence of CRC is increasing globally [2, 3]. Approximately 50% of CRC patients eventually develop metastatic disease. Despite significant progress in CRC treatment, this disease still causes considerable morbidity and mortality and for patients with metastatic CRC treatment is usually palliative. Novel approaches of treatment are needed for CRC, especially for metastatic disease.

The concept "External Qi of Qigong" refers to the technology and ability of “Qi deployment” therapy and health preservation of traditional Chinese medicine [4-7]. External Qi therapy has been used as one of the medical practices in China for thousands of years and is under management of the Chinese health authorities [8]. In the past three decades, positive effects of external Qi of Yan Xin Qigong (YXQ-EQ) on patients including those with cancers have been observed in many long-term and ongoing clinical studies and described in numerous case reports [9-13]. A great amount of YXQ-EQ experimental studies have shown significant effects of YXQ-EQ on structure and properties of DNA, RNA, protein and lipid molecules [10, 14-19]. YXQ-EQ has been shown to regulate signaling pathways and gene expression in normal and malignant cells [4-7, 18]. YXQ-EQ exerts potent cytotoxic effect on a variety of cancer cells and the underlying molecular mechanisms have recently begun to be addressed [4-7]. No cytotoxicity to normal cells was observed in these studies with YXQ-EQ. In fact, in vitro studies have found that YXQ-EQ was capable of protecting neurons from oxidative stress induced apoptosis and significantly prolonged survival of neurons [20, 21].

In the present study, we examined cytotoxic effect of YXQ-EQ on human colon cancer cells. We show that YXQ-EQ induced cell cycle arrest and apoptosis in HT-29 cells via inhibiting signaling pathways critical for proliferation and survival.

Materials and Methods

Cell culture

HT-29 colorectal cancer cells (American Type Culture Collection, Rockville, MD, USA) were grown in RPMI 1640 medium containing 10% FBS, 100 µg/ml penicillin G and 0.25% streptomycin at 37 °C and 5% CO₂. RPMI 1640 medium was supplemented with 2 mM glutamine.

Viability assay

Cell viability assessments for YXQ-EQ treated cells were performed as previously described [4-6]. Briefly, cells were plated in 96-well plates and allowed to grow overnight. Cells were exposed to YXQ-EQ for 5 min and viability was determined by trypan blue exclusion at indicated time points. In the prolonged YXQ-EQ treatment procedure, HT-29 cells were treated sequentially for 3 times in a period of 65 min, 5 min each time followed by a 25 min interval. Cell viability was evaluated 10 min after the last treatment using MTS assay. For HT-29 cells treated with varying amounts of YXQ-EQ water (YXQ-EQW) or XY -S, cell viability was determined by trypan blue staining and/or MTS assay after 16 h of incubation.

Clonogenic assay

Clonogenic assays were performed as previously described [6, 22]. In brief, HT-29 cells suspended in the growth medium were treated with YXQ-EQ for 5 min and plated in 6-well plates (1 x 10^3 cells per well). The cells were allowed to grow for 2-3 weeks with medium changed every 3-4 days. Colonies were fixed with ethanol and stained with crystal violet.

Cell cycle analysis

Cell cycle distribution was analyzed as previously described [4-6]. Briefly, cells were harvested, fixed in 70% ethanol and incubated in PBS containing 0.1% Triton X-100, RNase A (20 µg/ml) and propidium iodide (20 µg/ml) for 30 min before FACS analysis.
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Apoptosis assessment
Apoptotic cells were detected using annexin V-FITC staining as described previously [4-6].

Western blot analysis
Cell lysates were prepared as previously described [4-6]. Proteins were detected with specific antibodies. Antibodies against caspases-3, -8, and -9, Bax, cyclin D1, survivin, Bcl-xL, Erk1/2, pErk1/2, Akt, and pAkt were acquired from Cell Signaling Technology (Andover, MA). Antibodies against lamin B, PARP, Mcl-1, p21Cip1, and p27Kip1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against β-actin was obtained from Sigma (St Louis, MO).

Statistic analysis
Results are expressed as means ± SD. Statistic analysis was performed using Student's t-test and mean values were considered significantly different when P ≤ 0.05.

Results

YXQ-EQ exerts potent cytotoxic effect on HT-29 cells
HT-29 colon cancer cells were exposed to YXQ-EQ for 5 min and cell viability was assessed at 1, 6, and 24 h after treatment using trypan blue exclusion assay. YXQ-EQ treatment resulted in a time-dependent decrease in cell viability (Fig. 1A). A significant reduction in viability was detected as early as 6 h and no viable cells were observed 24 h after treatment. The viability of HT-29 cells decreased more rapidly when cells were sequentially treated with YXQ-EQ for three times within 65 min using the prolonged treatment procedure (Fig. 1B). In order to examine long-term effect of YXQ-EQ, we performed clonogenic assays and found that colony formation of HT-29 cells was completely inhibited by YXQ-EQ (Figs. 1C and D). These results indicate that YXQ-EQ exerted a potent cytotoxic effect on HT-29 cells.

YXQ-EQ induces G1 arrest and apoptosis in HT-29 cells
YXQ-EQ treatment of HT-29 cells resulted in an increase in G1 phase population that was accompanied by a significant increase in sub-G1 population and a modest decrease in G2/M phase population (Fig. 2A). These results indicate that YXQ-EQ induced G1 arrest and
apoptosis in HT-29 cells. To understand the mechanisms by which YXQ-EQ induced G1 arrest, we examined the expression of proteins important for G1-S phase transition of the cell cycle. Western analysis revealed that YXQ-EQ decreased cyclin D1 expression while increased the amount of cyclin dependent kinase inhibitors p21\(^{Cip1}\) and p27\(^{Kip1}\) (Figs. 2B and C). Cyclin D1 is known to promote G1 to S phase transition while p21\(^{Cip1}\) and p27\(^{Kip1}\) are prominent inhibitors of this process [23, 24]. Apoptosis was further confirmed by DNA fragmentation and cleavage of PARP and procaspases-3, -8, and -9 in YXQ-EQ treated HT-29 cells (Figs. 3A and B). In order to understand the mechanisms by which YXQ-EQ triggered apoptosis in HT-29 cells, we analyzed the expression of a panel of proteins that are known to play a role in regulation of apoptosis. Compared to control cells, the expression of prosurvival proteins survivin, Bcl-xL, XIAP and Mcl-1 was all reduced while that of the proapoptotic protein Bax was elevated in the treated cells (Fig. 4).
YXQ-EQ inhibits Akt, Erk1/2 and NF-κB activation in HT-29 cells

It is well known that the PI3K/Akt, MAPK/Erk1/2 and NF-κB pathways regulate cell survival, proliferation and apoptosis and play critical roles in tumorigenesis, progression and resistance to chemotherapy of CRC cells [25-28]. Therefore, we assessed the effect of YXQ-EQ on these pathways. Phosphorylation of Akt and Erk1/2 was significantly reduced in YXQ-EQ treated HT-29 cells while the total protein levels of Akt and Erk1/2 were little affected (Fig. 5A). This suggests that inhibition of the PI3K/Akt and MAPK/Erk1/2 pathways may be part of the mechanisms underlying cytotoxic effect of YXQ-EQ. To address this notion HT-29 cells were treated with the PI3K/Akt inhibitor LY294002, the MEK/Erk1/2 inhibitor U0126 or their combination. Both inhibitors reduced HT-29 cell viability and the combination led to significantly further decrease in cell viability (Fig. 5B). We also examined the effect of YXQ-EQ on transcription factor NF-κB activation in HT-29 cells. It is well documented that activation of NF-κB leads to nuclear translocalization of its p65 subunit and inhibition of NF-κB activation impairs this process. Western analysis revealed substantially reduced p65 levels in the nuclear extract of YXQ-EQ treated HT-29 cells compared to that of control cells (Fig. 5C). These findings indicate that NF-κB activation was indeed repressed by YXQ-EQ and are in agreement with aforementioned downregulation of NF-κB target genes survivin, Bcl-xL and XIAP in the treated HT-29 cells (Fig. 4).

YXQ-EQW and XY-S induce apoptosis in HT-29 cells

Previous studies have shown that YXQ-EQ treated PBS (named as YXQ-EQ water or YXQ-EQW), YXQ-EQ treated cell culture medium, and YXQ-EQ treated natural nontoxic plant extracts (XY99-5038 and XY-S) possess some biological effects of YXQ-EQ, including anticancer, antiviral and antioxidative activity [9, 11, 12, 20, 29, 30]. Therefore, we examined whether YXQ-EQW and XY-S could inhibit the growth of HT-29 cells. Indeed, XY-S reduced the viability of HT-29 cells in a dose dependent manner (Figs. 6A and B). Highly (10⁶-10⁸ fold) diluted YXQ-EQW also markedly reduced viable HT-29 cells (Fig. 6C). In order to test if XY-S and YXQ-EQW had cytotoxic effect on normal cells, peripheral blood mononuclear cells (PBMC) were treated with the same amounts of XY-S and YXQ-EQW. In contrast to HT-29 cells, the viability of PBMC was not significantly altered by XY-S (Fig. 6A) or YXQ-EQW (Fig.
6C), indicating that XY-S and YXQ-EQW had no cytotoxic effect on PBMC. Both YXQ-EQW and XY-S increased annexin V positive cell population (Figs. 6D and E), indicating that YXQ-EQW and XY-S induced apoptosis in HT-29 cells.

Discussion

In the present study we showed that YXQ-EQ, YXQ-EQW and XY-S exerted potent cytotoxic effect on HT-29 colorectal cancer cells. YXQ-EQ induced G1 arrest and apoptosis was associated with suppressed activation of Akt, Erk1/2 and NF-κB in these cells. These findings suggest that YXQ-EQ may inhibit growth of CRC cells via targeting signaling pathways critical for cell survival and proliferation.

The progression of the cell cycle is controlled by interactions between positive and negative regulators including cyclins, cyclin dependent kinases (CDKs) and CDK inhibitors [31]. Cyclin D1 is a well known key positive regulator of G1-S transition while p21Cip1 and p27Kip1 are prominent negative regulators of cell cycle progression [23, 24]. Consistent with these known facts, YXQ-EQ induced G1 arrest was associated with decreased expression of cyclin D1 in combination with elevated accumulation of p21Cip1 and p27Kip1 in HT-29 cells.

Apoptosis is activated by two major pathways of caspase activation: the cell surface death receptor pathway and the mitochondria-initiated pathway [32]. Activation of caspase-8 is the critical event that transmits the death signal in the death receptor pathway while caspase-9 is activated in the mitochondria-initiated pathway. We found that both caspases-8 and -9 was cleaved and activated in YXQ-EQ treated HT-29 cells. These findings suggest
that YXQ-EQ triggered both apoptosis pathways in HT-29 cells, similar to apoptosis induced by YXQ-EQ in pancreatic and prostate cancer cells [4, 5]. YXQ-EQ induced apoptosis in HT-29 cells was associated with downregulation of prosurvival proteins including Bcl-xL, survivin, XIAP and Mcl-1 and upregulation of proapoptotic Bax protein. Decreased expression of these prosurvival/antiapoptotic proteins has been reported in drug induced apoptosis in HT-29 cells [33, 34]. Bcl-xL has been reported to prevent mitochondrial permeability transition pore opening and the release of cytochrome c following DNA damage [35]. Decreased Bcl-xL expression in YXQ-EQ treated HT-29 cells suggests that mitochondrial apoptosis pathway may be initiated by the inhibition of Bcl-xL function. Further studies are needed to understand the molecular mechanisms by which YXQ-EQ triggers apoptosis. It is worthy to note that HT-29 cells carry a mutated and thus inactive p53 [36, 37]. Solid tumors harboring a non-functional p53 commonly deprive of p53-dependent apoptosis [38]. Our findings that YXQ-EQ induced apoptosis in HT-29 cells indicate that YXQ-EQ is capable of inducing p53 independent apoptosis. Similar to YXQ-EQ, XY-S and YX-EQW also induced apoptosis in HT-29 cells. While our results showed that apoptotic death was triggered by YXQ-EQ, YXQ-EQW and XY-S, induction of other cell death processes could not be excluded and remains to be studied.

The PI3K/Akt and MAPK/Erk1/2 pathways regulate cell survival, proliferation and apoptosis and play critical roles in CRC progression and are important targets for CRC treatment [25-27]. The PI3K/Akt pathway can be activated by KRAS and the MAPK/Erk1/2 pathway can be activated by KRAS and BRAF [39, 40]. KRAS and BRAF mutations have been frequently found in CRC [40, 41]. It has been reported that 22% of CRC harbor mutations in both KRAS and PI3K [42, 43]. HT-29 cells harbor the active BRAF mutant gene and constantly activated downstream MEK1/2 and Erk1/2 [44]. Activation of the PI3K/Akt pathway has been reported to contribute to the resistance to MAPK kinase (MEK) inhibitors in CRC cells [44, 45], suggesting that combined inhibition of the PI3K/Akt and MAPK/Erk1/2 pathways may be a more effective treatment for CRC. Indeed, the combination of PI3K/Akt and MEK/Erk1/2 inhibitors resulted in greater growth inhibition of HT-29 cells than either inhibitor alone. Interestingly, YXQ-EQ inhibited phosphorylation of Akt and Erk1/2 in HT-29 cells, similar to our findings in previous studies with pancreatic cancer cells [4]. These findings suggest that suppression of Akt and Erk1/2 signaling pathways may be part of the acting mechanisms of YXQ-EQ against cancer cells.

NF-κB is a transcription factor that regulates expression of a number of genes promoting cell proliferation, invasion, metastasis, angiogenesis, apoptosis suppression and drug resistance in a wide range of tumors [46, 47]. Constitutive NF-κB activation is frequently found in CRC but not in normal colorectal ductal epithelial cells [28, 48, 49]. It is well documented that NF-κB activation plays an important role in CRC progression, angiogenesis, and resistance to chemo- and radiotherapy [28, 47-55], suggesting that inhibition of NF-κB activation could be effective in CRC prevention and intervention [46, 56]. In the present study we showed that NF-κB activation was suppressed by YXQ-EQ in HT-29 cells, similar to our previous findings with breast, prostate and pancreatic cancer cells [4-6]. These findings combined suggest that suppression of NF-κB activity may be a common anticancer mechanism of YXQ-EQ.

One of the interesting findings of the present study is that YXQ-EQ treated PBS and an YXQ-EQ treated extract of natural plants retained cytotoxic activity of YXQ-EQ toward HT-29 cells while have no such effect on PBMC. Similar to our findings reported here, a cell culture medium treated by YXQ-EQ induced apoptosis in non-small cell lung cancer cells and an YXQ-EQ treated natural plant extract protected neurons against oxidative stress induced apoptosis [20, 29]. More importantly, long-term clinical observations have seen anticancer effects of XY-S [9, 11, 12]. These findings suggest that common buffers, cell growth media, or natural plant extracts can be treated by YXQ-EQ to gain anticancer activity without cytotoxicity to normal cells and thus could have remarkable potential of clinical application.
Acknowledgements

This work was supported in part by Yan Xin Foundation.

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