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Original Paper

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 $p21^{Cip1}$ and $p27^{Kip1}$, resulting in G1 cell cycle arrest. YXQ-EQ induced apoptosis in HT-29 cells in association with decreased expression of antiapoptotic proteins Bcl-xL, 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.

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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% CO_2. 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.

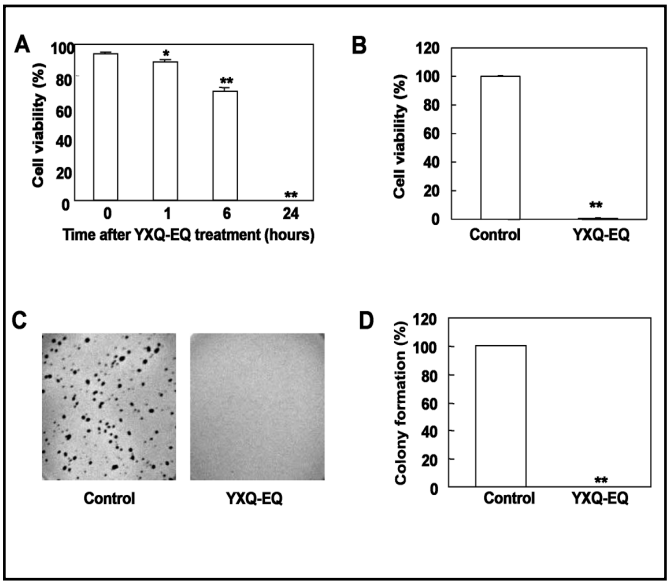

Fig. 1. YXQ-EQ exerts cytotoxic effect on HT-29 cells. (A) YXQ-EQ reduced HT-29 cell viability. HT-29 cells were exposed to YXQ-EQ for 5 min. Cell viability was determined using trypan blue exclusion assay at time points indicated. (B) HT-29 cells were subjected to prolonged YXQ-EQ treatment as described in Materials and Methods. Cell viability was evaluated by MTS assay. C and D. YXQ-EQ inhibited colony formation of HT-29 cells. HT-29 cells were treated for 5 min by YXQ-EQ and allowed to grow for 2-3 weeks. Colonies were fixed, stained, photographed (C) and counted (D). Data are presented as mean ± SD of % viability or colony formation (n=3). * $P < 0.05$; ** $P < 0.01$.

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 capsases-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, $p21^{Cip1}$ and 27^{Kip1} 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 $P \leq 0.05$.

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 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

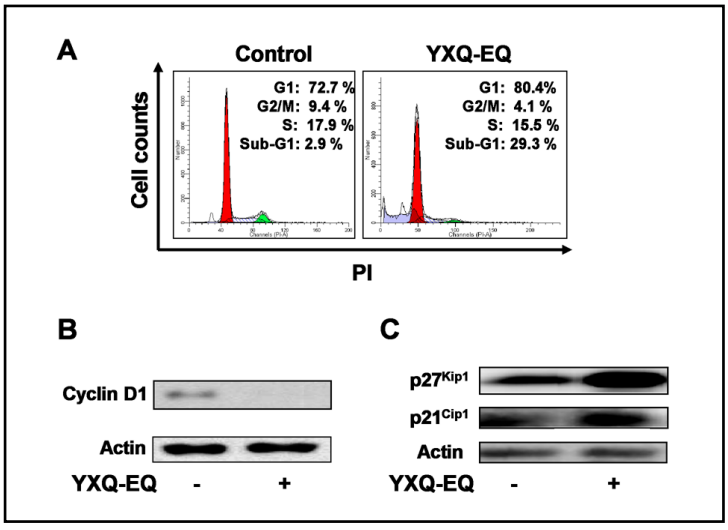

Fig. 2. YXQ-EQ induces cell cycle arrest in HT-29 cells. HT-29 cells were treated by YXQ-EQ for 5 min, incubated for 12 h and then subjected to cell cycle (A) and Western blot analysis (B and C). A. YXQ-EQ increased G1 and sub-G1 populations. B. YXQ-EQ downregulated cyclin D1 expression. C. YXQ-EQ increased $p21^{Cip1}$ and $p27^{Kip1}$ protein levels.

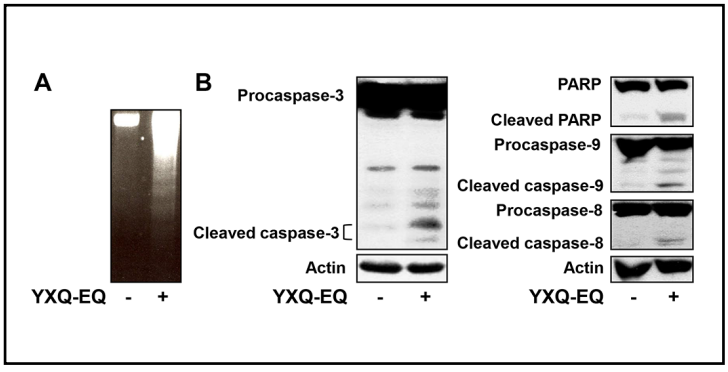

Fig. 3. YXQ-EQ induces apoptosis in HT-29 cells. HT-29 cells were treated by YXQ-EQ for 5 min and analyzed after incubation for 12 h. A. YXQ-Q induced DNA fragmentation. B. YXQ-EQ caused cleavage of procaspases-3, -8 and -9 and PARP.

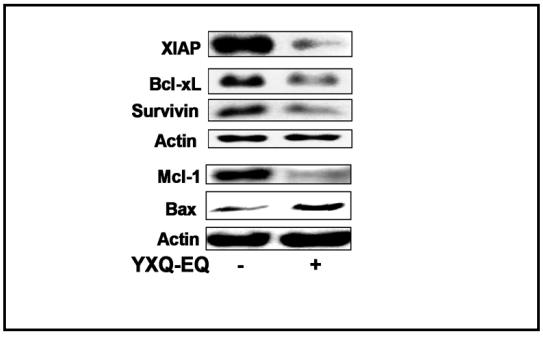

Fig. 4. YXQ-EQ downregulates anti-apoptotic proteins survivin, Bcl-xL, XIAP and Mcl-1 while increased proapoptotic Bax expression in HT-29 cells. HT-29 cells were treated by YXQ-EQ for 5 min, incubated for 12 h and then harvested for Western blot analysis.

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 penal 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).

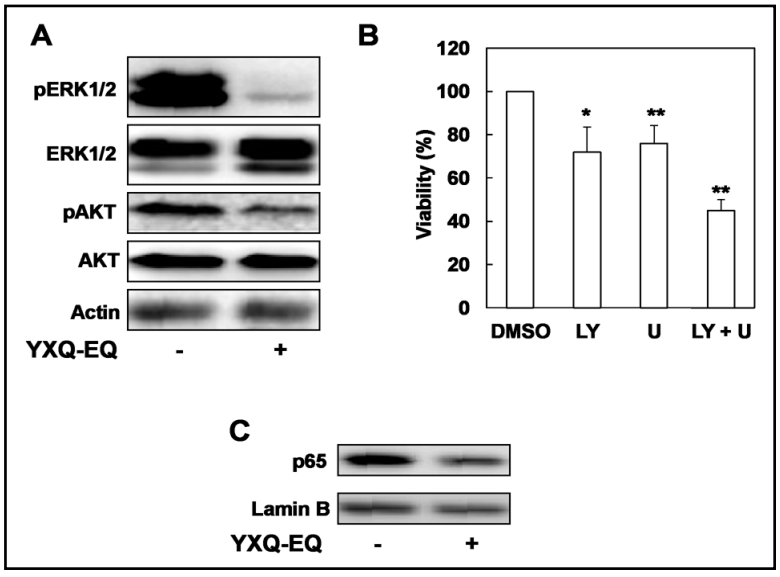

Fig. 5. YXQ-EQ inhibits Akt, Erk1/2 and NF-κB activation in HT-29 cells. A. Western blot analysis of phosphorylated and total Akt and Erk1/2. HT-29 cells were treated by YXQ-EQ for 5 min and analyzed after 1 h incubation. B. Inhibitors of PI3K/Akt and MEK/Erk1/2 reduced HT-29 cell viability. HT-29 cells were treated with 20 μM LY294002 (LY), U0126 (U), or LY294002 and U0126 (LY+U) for 3 days and subjected to MTS assay. Data are presented as mean ± SD (n=3). *P < 0.05; **P < 0.01. C. YXQ-EQ reduced nuclear p65 protein levels. Cells were treated by YXQ-EQ for 5 min, incubated for 1 h and then harvested for nuclear extract preparation. Nuclear p65 levels were evaluated by Western blot analysis of the nuclear extracts. Lamin B was used as loading control.

YXQ-EQ inhibits activation of Akt, Erk1/2 and NF-κB 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 critic 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 (10^6-10^8 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.

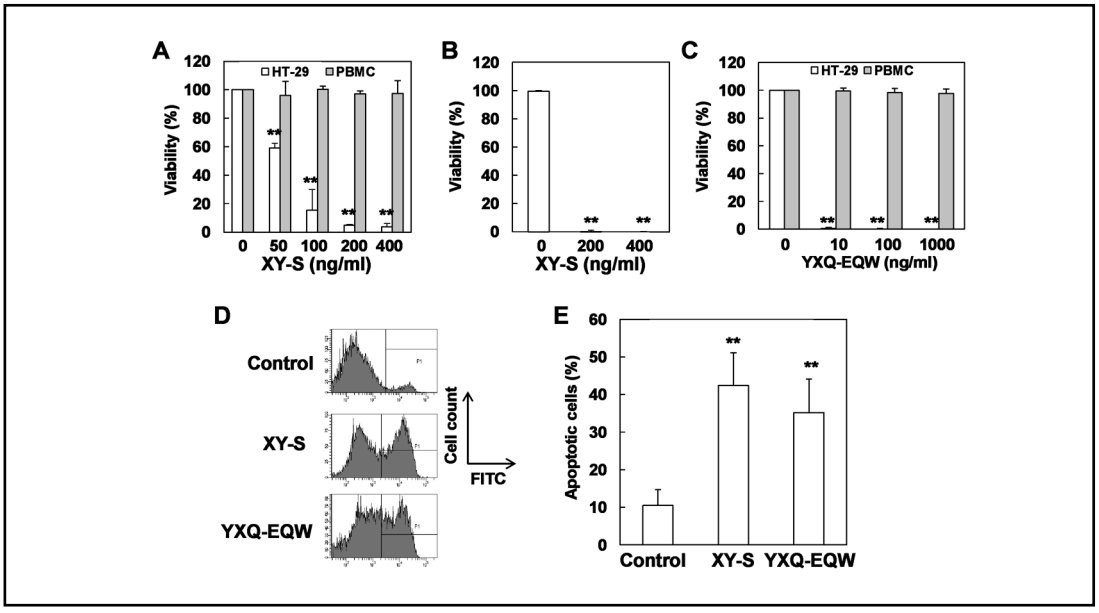

Fig. 6. XY-S and YXQ-EQW inhibit growth of HT-29 cells. A and B. XY-S reduced HT-29 cell viability. Cells were treated with indicated amounts of YX-S for 16 h and subjected to MTS (A) or trypan blue exclusion (B) assay. (C) YXQ-EQW reduced HT-29 cell viability. Cells were treated with indicated amounts of YXQ-EQW for 16 h and subjected to MTS assay. D and E. YXQ-EQW and XY-S induced apoptosis in HT-29 cells. Cells were treated with XY-S (200 ng/ml) or YXQ-EQW (100 ng/ml) for 12 h. Apoptosis was assessed by staining with annexin V-FITC and FACS analysis (D) and shown in bar graphs (E). Data are presented as mean ± SD (n=3). ** $P < 0.01$.

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 $p21^{Cip1}$ and $p27^{Kip1}$ 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 $p21^{Cip1}$ and $p27^{Kip1}$ 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 critic 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

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References

1 Siegel R, Naishadham D, Jemal A: Cancer statistics, 2012. CA Cancer J Clin 2012;62:10–29.
2 Yiu HY, Whittemore AS, Shibata A: Increasing colorectal cancer incidence rates in Japan. Int J Cancer 2004;109:777–781.
3 Haggar FA, Boushey RP: Colorectal Cancer Epidemiology: Incidence, Mortality, Survival, and Risk Factors. Clin Colon Rectal Surg 2009;22:191–197.
4 Yan X, Shen H, Jiang H, Zhang C, Hu D, Wang J, Wu X: External Qi of Yan Xin Qigong differentially regulates the Akt and extracellular signal-regulated kinase pathways and is cytotoxic to cancer cells but not to normal cells. Int J Biochem Cell Biol 2006;38:2102-2113.
5 Yan X, Shen H, Jiang H, Zhang C, Hu D, Wang J, Wu X: External Qi of Yan Xin Qigong induces G2/M arrest and apoptosis of androgen-independent prostate cancer cells by inhibiting Akt and NF-κB pathways. Mol Cell Biochem 2008;310:227-234.
6 Yan X, Shen H, Jiang H, Hu D, Zhang C, Wang J, Wu X: External Qi of Yan Xin Qigong Induces apoptosis and inhibits migration and invasion of estrogen-independent breast cancer cells through suppression of Akt/NF-κB signaling. Cell Physiol Biochem 2010;25:263–270.
7 Yan X, Li F, Dozmorov I, Frank MB, Dao M, Centola M, Cao W, Hu D: External Qi of Yan Xin Qigong induces cell death and gene expression alterations promoting apoptosis and inhibiting proliferation, migration and glucose metabolism in small-cell lung cancer cells. Mol Cell Biochem 2012;363:245-255.
8 Yan X, Shen H, Zaharia M, Wang J, Wolf D, Li F, Cao W: Involvement of phosphatidylinositol 3-kinase and insulin-like growth factor-I in YXLST-mediated neuroprotection. Brain Res 2004;1006:198-206.
9 Fong YH: Yan Xin Qigong informative product: Qi nutrition powder; in International Yan Xin Qigong Association (ed): Yan Xin Qigong Collectanea. Quebec, Les Editions LOTUS Publishers of Canada, 1997, vol 6, pp 299-318.
10 Lu Z: Scientific Qigong exploration: the wonders and mysteries of Qi. Malvern, Pennsylvania, Amber Leaf Press, 1997.
11 Ming Z: The new frontiers of modern sciences: an introduction to Yan Xin Qigong. Beijing, Xinhua Press, 1988.
12 Wang R, Zhu R: Yan Xin Qigong nutrition powder: report of nine cases; in International Yan Xin Qigong Association (ed): Yan Xin Qigong Collectanea. Quebec, Les Editions LOTUS Publishers of Canada, 1997, vol 1, pp 266-271.
13 Zhang ZW, Zhao JX, Zhang XR: Yan Xin Qigong nutrition powder: clinical report; in International Yan Xin Qigong Association (ed): Yan Xin Qigong Collectanea. Quebec, Les Editions LOTUS Publishers of Canada, 1997, vol 1, pp 319-329.
14 Li S, Sun M, Dai Z, Zhang P, Meng G, Liu Z, Ma Q, Zhu Q, Wang Q, Zhang L, Shan L, Sun Y, Hu Y, Chen Y, Pang Y, Wang G, Hao X, Lu A, Fan X, Yan X: Experimental studies on the feasibility of improving industrial strains with external Qi treatment. Nat J 1990;13:791-801.
15 Yan X, Zheng C, Zou G, Lu Z: Observations of the effects of external Qi on the ultraviolet absorption of nucleic acids. Nat J 1988;11:647-649.
16 Yan X, Zhao N, Yin C, Lu Z: The effect of external Qi on liposome phase behavior. Nat J 1988;11:572-576.
17 Yan X, Lin H, Li H, Traynor-Kaplan A, Xia Z, Lu F, Fang Y, Dao M: Structural and property changes in certain materials influenced by the external Qi of Qigong. Mater Res Innov 1999;2:349-359.
18 Yan X, Lu F, Jiang H, Wu X, Cao W, Xia Z, Dao M, Lin H: Certain manifestation and effects of external Qi of Yan Xin life science and technology. J Sci Explor 2002;16:381-411.
19 Yan X, Xia ZQ, Shen H, Traynor-Kaplan A: External Qi of Yan Xin life science and technology can revive or suppress enzyme activity of phosphatidylinositol 3-kinase. Bull Sci Tech Soc 2002;22:403-406.
20 Yan X, Fong YT, Wolf G, Wolf D, Cao W: Protective effect of XY99-5038 on hydrogen peroxide induced cell death in cultured retinal neurons. Life Sci 2001;69:289–299.

21 Yan X, Fong YT, Wolf D, Shen H, Zaharia M, Wang J, Wolf G, Li F, Lee GD, Cao W: XY99-5038 promotes long-term survival of cultured retinal neurons. Int J Neurosci 2002;112:1209–1227.
22 Franken NAP, Rodermond HM, Stap J, Haveman J, van Bree C: Clonogenic assay of cells in vitro. Nature Protocols 2006;1:2315-2319.
23 Sherr CJ, Roberts JM: CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 1999;15:1501-512.
24 Weiss RH: p21Waf1/Cip1 as a therapeutic target in breast and other cancers. Cancer Cell 2003;4:425–429.
25 Fang JY, Richardson BC: The MAPK signaling pathways and colorectal cancer. Lancet Oncol 2005;6:322–327.
26 Meier F, Schittek B, Busch S, Garbe C, Smalley K, Satyamoorthy K, Li G, Herlyn M: The RAS/RAF/MEK/ERK and PI3K/AKT signaling pathways present molecular targets for the effective treatment of advanced melanoma. Front Biosci 2005;10:2986–3001.
27 Vivanco I, Sawyers CL: The phosphatidylinositol 3-kinase AKT pathway in human cancer. Nat Rev Cancer 2002;2:489–501.
28 Sakamoto K, Maeda S: Targeting NF-κB for colorectal cancer. Expert Opin Ther Targets 2010;6:593-601.
29 Yan X, Shen H, Igor Dozmorov, Dan Hu, Jun Wang, Mark Barton Frank, Ming Dao, Feng Li, Michael Centola, Wei Cao: Induction of apoptosis and regulation of gene expression in NCI-H82 cell line of small cell lung cancer by YXLST-external-Qi-engineered culture medium [abstract]. 97th American Association for Cancer Research Annual Meeting. Washington, DC, April 1-5, 2006.
30 Yan X, Shen H, Wang L, Jiang H, Wu X, Wang J, Hu D, Wolf D, Yang Z, Dao M, Ni P, Zhang C: External Qi and Qi Water of Yan Xin Life Science Technology (YXLST) Potently Inhibit HIV-1 Replication. Antiviral Research 2005;65:A47–A48.
31 Morgan DO: Principles of CDK regulation. Nature 1995;374:131-133.
32 Budihardjo I, Oliver H, Lutter M, Luo X, Wang X: Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol 1999;15:269-290.
33 Reinacher-Schick A, Schoeneck A, Graeven U, Schwarte-Waldhoff I, Schmiegel W: Mesalazine causes a mitotic arrest and induces caspase-dependent apoptosis in colon carcinoma cells. Carcinogenesis 2003;24:443–451.
34 Gamet-Payrastre L, Li P, Lumeau S, Cassar G, Dupont MA, Chevolleau S, Gasc N, Tulliez J, Tercé F: Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. Cancer Res 2000;60:1426–1433
35 Reed JC: Mechanisms of apoptosis avoidance in cancer. Curr Opin Oncol 1999;11: 68–75.
36 Huang F, Hsu S, Yan Z, Winawer S, Friedman E: The capacity for growth stimulation by TGF beta 1 seen only in advanced colon cancers cannot be ascribed to mutations in APC, DCC, p53 or ras. Oncogene 1994;9:3701–3706.
37 Violette S, Poulain L, Dussaulx E, Pepin D, Faussat AM, Chambaz J, Lacorte JM, Staedel C, Lesuffleur T: Resistance of colon cancer cells to long-term 5-fluorouracil exposure is correlated to the relative level of Bcl-2 and Bcl-X(L) in addition to Bax and p53 status. Int J Cancer 2002;98:498-504.
38 Levine AJ: p53, the cellular gatekeeper for growth and division. Cell 1997;88:323–331.
39 Repasky GA, Chenette EJ, Der CJ: Renewing the conspiracy theory debate: does Raf function alone to mediate Ras oncogenesis? Trends Cell Biol 2004;14:639–647.
40 Yuen ST, Davies H, Chan TL, Ho JW, Bignell GR, Cox C, Stephens P, Edkins S, Tsui WW, Chan AS, Futreal PA, Stratton MR, Wooster R, Leung SY: Similarity of the phenotypic patterns associated with BRAF and KRAS mutations in colorectal neoplasia. Cancer Res 2002;62:6451–6455.
41 Fransén K, Klintenäs M, Osterström A, Dimberg J, Monstein HJ, Söderkvist P: Mutation analysis of the BRAF, ARAF and RAF-1 genes in human colorectal adenocarcinomas. Carcinogenesis 2004;25:527–533.
42 Parsons DW, Wang TL, Samuels Y, Bardelli A, Cummins JM, DeLong L, Silliman N, Ptak J, Szabo S, Willson JK, Markowitz S, Kinzler KW, Vogelstein B, Lengauer C, Velculescu VE: Colorectal cancer: mutations in a signalling pathway. Nature 2005;436:792-796.
43 Velho S, Oliveira C, Ferreira A, Ferreira AC, Suriano G, Schwartz S Jr, Duval A, Carneiro F, Machado JC, Hamelin R, Seruca R: The prevalence of PIK3CA mutations in gastric and colon cancer. Eur J Cancer 2005;41:1649–1654.

44 Balmanno K, Chell SD, Gillings AS, Hayat S, Cook SJ: Intrinsic resistance to the MEK1/2 inhibitor AZD6244 (ARRY-142886) is associated with weak ERK1/2 signalling and/or strong PI3K signalling in colorectal cancer cell lines. Int J Cancer 2009;125:2332–2341.
45 Wee S, Jagani Z, Xiang KX, Loo A, Dorsch M, Yao YM, Sellers WR, Lengauer C, Stegmeier F: PI3K pathway activation mediates resistance to MEK inhibitors in KRAS mutant cancers. Cancer Res 2009;69:4286–4293.
46 Aggarwal BB: Nuclear factor-κB: The enemy within. Cancer Cell 2004;6:203–208.
47 Karin M: Nuclear factor-kappaB in cancer development and progression. Nature 2006;441:431–436.
48 Voboril R, Weberova-Voborilova J: Constitutive NF-κB activityin colorectal cancer cells: Impact on radiation-induced NFκB activity, radiosensitivity, and apoptosis. Neoplasma 2006;53:518–523.
49 Kojima M, Morisaki T, Sasaki N, Nakano K, Mibu R, Tanaka M, Katano M: Nuclear factor-κB p65 (RelA) transcription factor is constitutively activated in human colorectal carcinoma tissue. World J Gastroenterol 2004;10:3255–3260.
50 Kojima M, Morisaki T, Sasaki N, Nakano K, Mibu R, Tanaka M, Katano M: Increased nuclear factor-κB activation in human colorectal carcinoma and its correlation with tumor progression. Anticancer Res 2004;24:675–681.
51 Yu HG, Yu LL, Yang Y, Luo HS, Yu JP, Meier JJ, Schrader H, Bastian A, Schmidt WE, Schmitz F: Increased expression of RelA/nuclear factor-κB protein correlates with colorectal tumorigenesis. Oncology 2003;65:37–45.
52 Lind DS, Hochwald SN, Malaty J, Rekkas S, Hebig P, Mishra G, Moldawer LL, Copeland EM 3rd, Mackay S: Nuclear factor-κB is upregulated in colorectal cancer. Surgery 2001;130:363-369.
53 Mukogawa T, Koyama F, Tachibana M, Takayanagi A, Shimizu N, Fujii H, Ueno M, Matsumoto H, Takeuchi T, Nakajima Y: Adenovirusmediated gene transduction of truncated IκBα enhances radiosensitivity in human colon cancer cells. Cancer Sci 2003;94:745–750.
54 Russo SM, Tepper JE, Baldwin AS Jr, Liu R, Adams J, Elliott P, Cusack JC Jr: Enhancement of radiosensitivity by proteasome inhibition:implications for a role of NF-κB. Int J Radiat Oncol Biol Phys 2001;50:183–193.
55 Wang CY, Mayo MW, Baldwin AS Jr: TNF- and cancer therapy- induced apoptosis: Potentiation by inhibition of NF-κB. Science 1996;274:784-787.
56 Aggarwal BB, Shishodia S, Sandur SK, Pandey MK, Sethi G: Inflammation and cancer: How hot is the link? Biochem Pharmacol 2006;72:1605–1621.