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Original Paper

YXQ-EQ Induces Apoptosis and Inhibits Signaling Pathways Important for Metastasis in Non-Small Cell Lung Carcinoma Cells

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Key Words
NSCLC • Apoptosis • Migration • Invasion • EMT • EGF • TGF-β • YXQ-EQ

Abstract
Background/Aims: Lung cancer is one of the most prevalent malignancies in the world. The 5-year survival rate for non-small cell lung cancer (NSCLC) patients is only approximately 15%, with metastasis as the primary cause of death. This study was aimed to investigate cytotoxic effect of external qi of Yan Xin Qigong (YXQ-EQ) toward human lung adenocarcinoma A549 cells as well as its effect on signaling pathways promoting migration, invasion and epithelial-to-mesenchymal transition (EMT) in A549 cells. ***Methods:*** Cytotoxic effect of YXQ-EQ was evaluated using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] and cologenic assays. Apoptosis of treated cells was determined by Annexin V/propidium iodide staining and flow cytometry analysis, while cell migration and invasion were determined using transwell assays and EMT was assessed by morphological changes in cells. Protein expression and phosphorylation were examined by immunoblot analyses. ***Results:*** YXQ-EQ induced apoptosis in A549 cells, resulting in a pronounced reduction in viability and clonogenic formation. This was associated with inhibition of phosphorylation of AKT and ERK1/2 and reduced expression of anti-apoptotic proteins BCL-xL, XIAP and survivin. Furthermore, YXQ-EQ inhibited EGF/EGFR signaling and EGF mediated migration and invasion of A549 cells. While TGF-β1 induced phosphorylation of SMAD2/3 and EMT in A549 cells, YXQ-EQ suppressed TGF-β/SMAD signaling and induced cell death in these cells in the presence of TGF-β1. ***Conclusion:*** Our findings suggest that YXQ-EQ could exert anti-lung cancer effects via inhibiting signaling pathways that are important for NSCLC cell survival and NSCLC metastasis.

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Introduction

Lung cancer is the most common malignant disease and the leading cause of cancer related mortality worldwide [1]. Non-small cell lung cancer (NSCLC) comprises the majority of lung cancer cases, with most NSCLC patients having advanced stage disease at diagnosis [2]. The 5-year survival rate for NSCLC patients is only approximately 15%, with metastasis as the primary cause of death [3]. Conventional standard of care therapies for metastatic NSCLC include chemotherapy, targeted therapy and immunotherapy [4]. However, clinical efficacy of these therapies is limited by the development of intrinsic or acquired drug resistance [5]. Novel approaches are therefore needed for effective treatment of NSCLC.

External Qi therapy of traditional Chinese medicine (TCM) has long been one of the medical practices in China and managed by the Chinese health authorities [6]. The concept of External Qi (of Qigong) refers to the technology and ability of "Qi deployment" therapy and health preservation of TCM [6-11]. Clinical observations over the past 30 years and ongoing studies have documented significant beneficial effects of external Qi of Yan Xin Qigong (YXQ-EQ) on patients with cancer and other diseases [12-16]. Studies over the past three decades have demonstrated influence of YXQ-EQ on both the structural and functional properties of biomolecules [17-22]. Furthermore, YXQ-EQ has been shown to induce apoptosis in cancer cells while protect neurons from oxidative stress induced cell death via modulating signaling pathways and gene expression [6-11, 23]. These studies provide some molecular and cellular insights into the observed clinical benefits of YXQ-EQ.

We have previously shown that YXQ-EQ induces apoptosis and alters the expression of multiple genes involved in cell migration and energy metabolism in small cell lung cancer (SCLC) cells [10]. In the present study, we examined the effect of YXQ-EQ on NSCLC A549 cells. We report that YXQ-EQ exerted potent cytotoxic effect on A549 cells and inhibited EGF/EGFR and TGF-β/SMAD signaling that are important for NSCLC metastasis.

Materials and Methods

Cell culture

Human lung adenocarcinoma A549 cells (ATCC, Manassas, VA) were cultured in DMEM containing 10% FBS, 50 μg/ml penicillin and 100 μg/ml streptomycin. For EGF and TGF-β signaling studies, A549 cells were serum starved overnight and treated with EGF (10 ng/ml) or TGF-β1 (10 ng/ml) for 15 min and then harvested for immunoblot analyses.

Viability assay

Cell viability assay was performed as previously described [7, 8]. Briefly, cells were plated in 96-well plates at 2 x 10^3 cells/well and incubated overnight prior to treatment with YXQ-EQ for 5 min. Cell viability was determined using an MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay with a CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI).

Clonogenic assay

Clonogenic assay was performed as previously described [9, 11]. Briefly, A549 cells were suspended in growth medium and plated in 6-well plates at 1 x 10^3 cells/well. Medium was changed every 3 - 4 days. Following two weeks after YXQ-EQ treatment, colonies were fixed with methanol, stained with crystal violet and counted.

Apoptosis analysis

Cell apoptosis was analyzed as previously described [9]. Briefly, cells were collected after trypsinization and incubated in Annexin V binding buffer containing FITC-Annexin V (BD Bioscience, San Jose, CA) and propidium iodide (PI) for 15 min. Stained cells were subjected to flow cytometric analysis and cell apoptosis was assessed using Flowjo.

Migration and invasion assay

Cell migration and invasion assay was performed in Transwell Boyden chambers and chambers coated with Matrigel as described previously [9]. Overnight serum starved A549 cells in DMEM containing 0.1% BSA were added to the inserts. The lower chambers were supplemented with 600 µl of DMEM containing 0.1% BSA with or without EGF (25 ng/ml). After incubation for 16 h, cells that did not migrate were carefully wiped off. Migrated cells were fixed with methanol, stained with crystal violet, and counted in 4 randomly selected fields then averaged.

Immunoblot analysis

Whole cell lysates were prepared and proteins of interest were detected by immunoblot analyses as described previously [9, 11]. Antibodies recognizing the following proteins were sourced from Cell Signaling Technology (Danvers, MA): AKT (catalog #9272), pAKT (Ser473, catalog #4060), ERK1/2 (catalog #4695), pERK1/2 (Thr202/Tyr204, catalog #4370), BCL-xL (catalog #2764), XIAP (catalog #2042), survivin (catalog #2808), PARP (catalog #9542), casapase-3 (catalog #9662), SMAD2/3 (catalog #8685), pSMAD2 (Ser465/467, catalog #3108), pSMAD3 (Ser423/425, catalog #9520). Other antibodies included EGFR (catalog #A300-388A, Bethyl Laboratories, Montgomery, TX), pEGFR (Tyr1068, catalog #44788G, Invitrogen, Grand Island, NY), and actin (catalog #A2228, Sigma-Aldrich, St. Louis, MI).

EMT induction

A549 cells were treated with TGF-β1 (10 ng/ml) for 48 h in growth medium to induce EMT. Morphological changes of cells were observed under an inverted microscope.

Statistical analysis

Data are presented as mean ± SD. Statistical significance was determined using two-tailed Student's *t*-test. $P < 0.05$ was considered significant.

Results

YXQ-EQ exerts potent cytotoxic effect on A549 cells

To examine any cytotoxic effect of YXQ-EQ, A549 cells were treated with YXQ-EQ for 5 min and cell viability was determined using an MTS assay 24 h later. YXQ-EQ treatment resulted in pronounced reduction (by approximately 93%) in cell viability (Fig. 1A). Furthermore, YXQ-EQ treated A549 cells failed to form colonies in contrast to the many colonies that developed from control untreated A549 cells (Fig. 1B). These findings demonstrated a potent cytotoxic effect of YXQ-EQ on A549 cells.

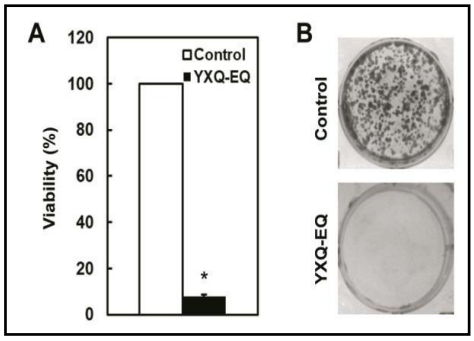

Fig. 1. YXQ-EQ reduced A549 cell viability and colony formation. A549 cells were treated or not with YXQ-EQ for 5 min. A. Cell viability was determined at 24 h after the treatment. Results are presented as mean ± SD of percent viability from 3 independent experiments. * $P < 0.01$, versus control cells. B. Colonies were stained and photographed 2 weeks after the treatment. Representative images from 3 independent experiments with similar results are shown.

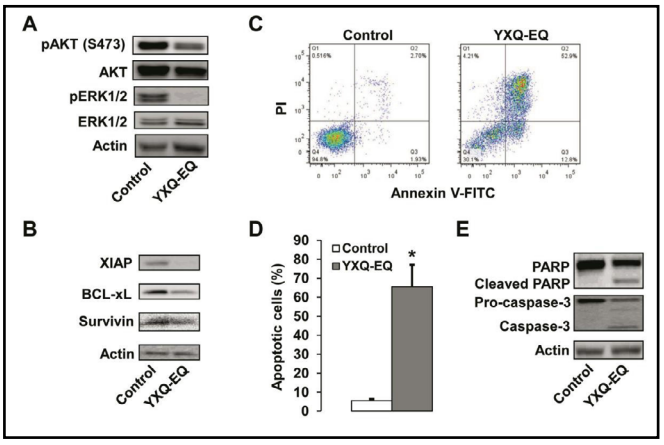

Fig. 2. YXQ-EQ induced apoptosis in A549 cells. A. YXQ-EQ inhibited phosphorylation of AKT (Ser473) and ERK1/2. B. YXQ-EQ reduced the expression of survivin, BCL-xL and XIAP. C and D. Cells were stained with Annexin V-FITC and PI and analyzed by flow cytometry. Representative results are shown in C and the mean ± SD of percent apoptotic cells from 3 independent experiments are presented in D. * $P < 0.01$, versus control cells. E. Immunoblot analysis of PARP and pro-caspase-3 cleavage. Cells were treated with YXQ-EQ for 5 min and collected 16 h and 20 h post treatment respectively for immunoblot analyses and Annexin V/PI staining.

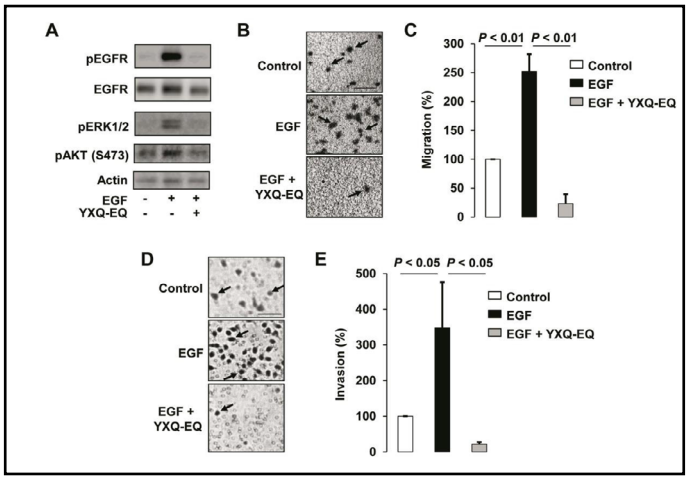

Fig. 3. YXQ-EQ inhibited EGF/EGFR signaling in A549 cells. Serum starved A549 cells were treated or not with YXQ-EQ for 5 min and then stimulated with EGF. A. YXQ-EQ inhibited EGF induced EGFR activation. Control and YXQ-EQ treated cells were stimulated with or without EGF for 15 min and subjected to immunoblot analysis of phosphorylation of EGFR, AKT (Ser473), and ERK1/2. B and C. YXQ-EQ inhibited EGF induced A549 cell migration. D and E. YXQ-EQ inhibited EGF induced A549 cell invasion. Migration and invasion of A549 cells were induced with EGF for 16 h. Migrated cells were fixed, stained, photographed and counted. Representative images (B and D) and the mean ± SD of percent migration (C) and invasion (E) from 3 independent experiments are shown. Arrows indicate migrated cells in panels B and D. Scale bar, 200 pixels.

YXQ-EQ induces apoptosis in A549 cells

To better understand the cytotoxic effect of YXQ-EQ on A549 cells, we next examined if YXQ-EQ influenced the PI3K/AKT and MAPK/ERK1/2 pathways that are known to be critical for A549 cell survival [24]. Immunoblot analyses revealed significant suppression of phosphorylation of AKT (Ser473) and ERK1/2 in YXQ-EQ treated A549 cells (Fig. 2A). Furthermore, the expression of anti-apoptotic proteins BCL-xL, survivin and XIAP was reduced in YXQ-EQ treated A549 cells (Fig. 2B). We next investigated if YXQ-EQ exerted its cytotoxic effect by inducing apoptosis in A549 cells. Apoptosis was measured using Annexin V-FITC/PI staining and flow cytometric analysis 20 h after YXQ-EQ treatment. Apoptotic cells consist of Annexin V positive/PI negative early apoptotic cells and Annexin V positive/PI positive late apoptotic cells. A small fraction (approximately 5%) of apoptotic cells were detected in control untreated A549 cells (Fig. 2C and D). However, approximately 65% of the YXQ-EQ treated A549 cells underwent apoptosis (Fig. 2C and D). Apoptosis in YXQ-EQ treated A549 cells was further confirmed by the cleavage of pro-caspase-3 and PARP as revealed by immunoblot analyses (Fig. 2E).

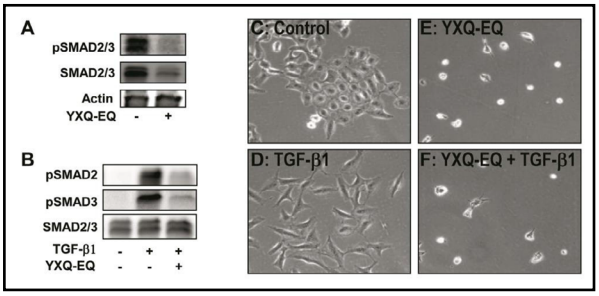

Fig. 4. YXQ-EQ inhibited TGF-β/SMAD signaling in A549 cells. A. YXQ-EQ reduced SMAD2/3 protein expression and phosphorylation. Cells were treated with YXQ-EQ for 5 min, collected and lysed for immunoblot analyses 16 h later. B. YXQ-EQ suppressed TGF-β1 stimulated SMAD2/3 phosphorylation. Serum starved A549 cells were treated or not with YXQ-EQ for 5 min then treated with TGF-β1 for 15 min. C and D. A549 cells alone (C) underwent EMT after 48 h of TGF-β1 treatment (D). E and F. YXQ-EQ treated A549 cells lost intact morphology and died either in the absence (E) or presence (F) of TGF-β1 48 h after YXQ-EQ treatment. For co-treatment, cells were incubated with TGF-β1 following 5 min of YXQ-EQ treatment.

YXQ-EQ inhibits EGF/EGFR mediated A549 cell migration and invasion

EGFR is known to be overactive in lung cancer cells due to overexpression or gain-of-function mutations [25-27]. The binding of EGFR with its ligands activates several signaling cascades important for lung cancer cells, principally the RAS/RAF/MEK/ERK pathway and the PI3K/AKT pathway [28, 29]. To investigate if YXQ-EQ influenced EGFR signaling in A549 cells, we examined the effect of YXQ-EQ on EGF/EGFR mediated AKT and ERK1/2 activation. As expected, treatment of A549 cells with EGF only increased phosphorylation of EGFR and downstream effectors AKT (Ser473) and ERK1/2 (Fig. 3A). Furthermore, EGF treatment alone promoted A549 cell migration and invasion (Fig. 3B-E). Treatment of A549 cells with YXQ-EQ for 5 min prior to EGF incubation abrogated EGF stimulated phosphorylation of EGFR (Tyr1068), AKT (Ser473) and ERK1/2 (Fig. 3A). YXQ-EQ treatment prior to EGF incubation not only completely inhibited EGF induced migration (Fig. 3B and C) and invasion (Fig. 3D and E) but also significantly reduced migration and invasion below the basal levels in A549 cells.

YXQ-EQ inhibits TGF-β signaling in A549 cells

Tumor metastasis is a major challenge in the management of NSCLC. EMT of cancer cells is associated with increased motility and a landmark of carcinoma progression during the metastatic phases. TGF-β is known to induce EMT in cancer cells [30, 31]. TGF-β transduces signals via binding to the heteromeric TGF-β receptor (TGF-βR) composed of type I and type II serine/threonine kinases, which in turn phosphorylate and activate downstream effectors SMAD2 and SMAD3 [31, 32]. Compared to untreated cells, SMAD2/3 expression and phosphorylation were markedly reduced in YXQ-EQ treated A549 cells (Fig. 4A), indicating that YXQ-EQ might negatively regulate TGF-β/TGF-βR signaling. To further address this notion, we examined the effect of YXQ-EQ on TGF-β1 induced SMAD2/3 phosphorylation in A549 cells. As expected, TGF-β1 treatment increased phosphorylation of SMAD2 and SMAD3 in these cells (Fig. 4B). TGF-β1 induced SMAD2/3 phosphorylation was abrogated by 5 min of YXQ-EQ pretreatment followed by the same TGF-β1 incubation (Fig. 4B). As reported previously [33], A549 cells underwent EMT after TGF-β1 treatment for 48 h (Fig. 4C and D). To examine the effect of YXQ-EQ on TGF-β1 induced EMT, A549 cells were treated with YXQ-EQ for 5 min prior to incubation with TGF-β1. It was observed that A549 cells had lost their intact morphology and died 48 h post YXQ-EQ treatment, regardless of treatment with TGF-β1 (Fig. 4E and F).

Discussion

In the present study, we show that YXQ-EQ treatment exerted profound cytotoxic effect on lung cancer A549 cells and inhibited EGF/EGFR and TGF-β1/SMAD signaling pathways that play an important role in lung cancer metastasis. These findings are in agreement

with previously reported potent cytotoxicity of YXQ-EQ toward cancer cells and inhibition of multiple signaling pathways by YXQ-EQ in cancer cells [7-11]. These studies together provide some important molecular and cellular insights into the clinically observed anti-cancer activities of YXQ-EQ.

EGFR is expressed in 60% of NSCLC and mutated in approximately 10 - 35% of NSCLC [25-27]. The EGF/EGFR signaling is involved in a wide variety of cellular events, including proliferation, migration, and invasion [34, 35]. Growing evidence show that activation of AKT and ERK1/2 is critical for survival and proliferation driven by EGFR signals, either activated by EGFR ligands or activating EGFR mutations, in NSCLC [28, 29]. EGFR tyrosine kinase inhibitors (TKIs) are effective in treatment of lung cancer with activating EGFR mutations and currently the standard treatment option for advanced NSCLC; however, often resistance to TKIs develops within months after the start of treatment [34, 36]. TKIs alone have limited effect on NSCLC with wild type EGFR [35, 37] and dual inhibition of EGFR and AKT is required to induce apoptosis and inhibit growth of EGFR wild type NSCLC cells [38, 39]. A549 cells express wild type EGFR that is important for A549 cell growth [40]. Concomitant inhibition of AKT and ERK1/2 synergistically inhibits A549 cell growth and induces apoptosis *in vitro* and *in vivo* [24, 41, 42]. In agreement with these findings, YXQ-EQ induced apoptosis was associated with dual inhibition of AKT and ERK1/2 activation in A549 cells.

Lung cancer has a high potential for metastasis and this poses a challenge to lung cancer therapy. As reported previously [43, 44], EGFR activation with EGF enhanced A549 cell migration and invasion. AKT activation is required in EGF mediated lung cancer cell migration and invasion [43, 44]. Furthermore, ERK1/2 is also involved in A549 cell migration and invasion [45, 46]. These findings suggest that inhibition of AKT and ERK1/2 activation may be at least partly involved in the mechanism of inhibition of EGF mediated A549 cell migration and invasion by YXQ-EQ.

TGF-β is a pleiotropic cytokine known to drive cancer progression by multiple mechanisms including promotion of angiogenesis and metastasis, and suppression of anti-tumor immunity [47, 48]. TGF-β induced EMT increases the potential for tumor metastasis by enhancing motility and invasion of cancer cells [30, 49]. TGF-β induces EMT in cancer cells including A549 cells by activating SMAD2/3, AKT, and ERK1/2 [33, 50-52]. Inhibition of TGF-β/SMAD signaling by YXQ-EQ reported here suggests that YXQ-EQ may also exert anti-tumor effects by inhibiting the pro-tumor activities of TGF-β.

It remains to be investigated how YXQ-EQ inhibits EGF/EGFR and TGF-β/SMAD signaling. This may involve structural alterations of the receptors imposed by YXQ-EQ, since YXQ-EQ has been shown to influence the molecular structure of proteins and enzyme activity in previous studies [20-22]. The suppression of activation of AKT and ERK1/2 by YXQ-EQ may result from direct inhibition of the kinases themselves and/or indirectly from inhibition of upstream receptors or mediators. For example, YXQ-EQ may inhibit AKT activation via suppressing PI3K enzyme activity, shown in a previous study [22]. While more studies are needed to elucidate the mechanisms underlying YXQ-EQ's cytotoxic effect and inhibitory effect on EGF/EGFR and TGF-β/SMAD signaling in A549 cells, findings presented here suggest that YXQ-EQ may have anti-NSCLC activity via inhibiting multiple signaling pathways important for NSCLC cell survival and metastasis.

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Disclosure Statement

The authors declare to have no competing interests.

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