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Original Research Article

Withaferin A Suppresses Anti-apoptotic BCL2, Bcl-xL, XIAP and Survivin Genes in Cervical Carcinoma Cells

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Abstract

Purpose: *To investigate the effect of withaferin A on the suppression of the anti-apoptotic genes, BCL2, Bcl-xL, XIAP and Survivin), in cervical carcinoma cells.*
Methods: *Annexin V-FITC/propidium iodide (PI) staining was used for the investigation of cell apoptosis. RNA RNeasy Kits was used to isolate RNA and Omniscript RT to reverse and transcribe the mRNA. Quantitative real-time polymerase chain reaction (qPCR) was performed using Taq PCR Master Mix Kit.*
Results: *Withaferin A (WFA) treatment reduced mRNA and protein levels of antiapoptotic genes in MCF-7 and HeLa cervical carcinoma cells. Suppression of BCL2, Bcl-xL, XIAP and Survivin induced a significant anti-proliferative effect. Treatment with WFA at a concentration of 20 µM, decreased cell viability and induced apoptosis. In MCF-7 cells, knockdown of BCL2, Bcl-xL, XIAP and Survivin caused 4-fold enhancement in apoptosis rate and 53 % decrease in cell viability.*
Conclusion: *WFA significantly leads to knockdown of antiapoptotic genes and is, therefore, a promising treatment strategy for cervical cancer.*

Keywords: *Anti-apoptotic genes, Cervical cancer, Apoptosis, Cell viability, BCL2, Bcl-xL, XIAP and Survivin*

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INTRODUCTION

Cervical cancer is the most frequently observed cancer in women throughout the world. It is estimated that every year 500,000 new cervical carcinoma cases are detected globally and 80 % of them are from developing countries [1,2]. Currently, surgery, radiation and chemotherapy are used for the treatment of cervical cancer. Intracavitary brachytherapy is a technique used to deliver high radiation doses to tumor site without exposing normal tissues to radiation [3,]. Presently, the combination of external beam radiotherapy and intracavitary brachytherapy is considered to be the standard treatment strategy for cervical cancer. Although high cure rates are reported at the early stage of the disease using definitive radiotherapy, in locally advanced cervical cancer cases the cure rates are poor. The 5-year overall survival of only 66 % are reported at the advanced stages [5]. Thus, the discovery of molecules with roles in the treatment of locally advanced cervical cancer is needed.

Maintenance of homeostasis in normal tissues and selective removal of damaged and infected cells is achieved by the process of apoptosis [6].Tumor cells are bestowed with the ability to escape apoptosis [7]. It is reported that tumor cells express higher concentration of antiapoptotic genes including BCL2, Bcl-xL, XIAP and Survivin which enables them to evade

apoptosis. Among the antiapoptotic genes, BCL2 and Bcl-xL help to escape apoptosis by inhibiting cytochrome release from the mitochondria followed by failure to activate caspases [8]. Caspases on activation induce apoptosis in which proteins essential for cell function and stability are cleaved [9].

Extracts of withaferin A isolate from *Witha niasomnifera*, are used in traditional East Indian medicine [10]. Withaferin A is the major compound present in the extract of *Witha niasomnifera* [11,12]. Withaferin A has anti-angiogenic effects [10,13] from micromolar doses and inhibits soft tissue sarcoma growth and local recurrence in xenograft experiments [14]. It exhibits proapoptotic and anti-tumor activitities in breast and prostate cancers [15–17]. It is reported that withaferin A usually exerts its effect through NF-Kb, BCL-2, FOXO3A, Hsp90, phosphorylated STAT3 and annexin II [16,18–22]. In the present study, effect of WFA on knockdown of antiapoptotic genes including BCL2, Bcl-xL, XIAP and Survivin human cervical carcinoma cell lines was studied.

EXPERIMENTAL

Cell lines and cell culture

The human cervical carcinoma cell lines MCF-7, HeLa and ME-180 were purchased from American Type of Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured under standard conditions at 37 ^{o}C in humidified atmosphere containing 5 % CO_2.

Reagents and chemicals

Withaferin A (WFA) was purchased from Sigma Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) to a concentration of 100 μM as a stock solution. Rabbit antihuman Caspase-3, mouse antihuman Bcl-2, and β-actin were purchased from Cell Signaling (China).

Cell viability assay

Cervical carcinoma cells were incubated with various concentrations of WFA at 37 ^{o}C for 72 h. MTT viability assay was performed according to manual protocol (Roche Diagnostics). The absorbance was measured at the wavelength of 595 nm.

Apoptosis analysis

Annexin V-FITC/propidium iodide (PI) staining (Annexin V-FITC Apoptosis Detection Kit I, BD Biosciences, Heidelberg, Germany) was used to examine the apoptosis in cervical carcinoma cells. After 48 h of WFA treatment flow, cytometry (FACScan, BD Biosciences) was used to examine the cells. The quadrant analysis of Annexin V-FITC/PI plots was performed to determine the percentage of early (Annexin V-FITC positive, PI negative) and late (Annexin V-FITC positive, PI positive) apoptotic cells. For this purpose, WinMDI2.8 software was employed.

Reverse transcriptase PCR

For the isolation of total cell RNA RNeasy Kits (Qiagen) was used. The RNA sample (500 ng) was used for reverse transcription to cDNA using Omniscript RT (Qiagen). The cDNA was then employed for quantitative real-time PCR (qPCR) using Taq PCR Master Mix Kit (Qiagen) according to the manual protocol.

Western blot analysis

The cells after WFA treatment were lysed in ice cold lysis buffer supplemented with protease inhibitors. The cell lysate was subjected to SDS-PAGE separation. Proteins were transferred to polyvinyl idenedi fluoride (PVDF) membrane (GE Healthcare, Freiburg, Germany) and then incubated with the primary antibodies. The antibodies were used against BCL2, Bcl-xL, XIAP and Survivin (Sigma-Aldrich, St. Louis, MO, USA) and anti-β-actin was used as the loading control (Sigma-Aldrich, St. Louis, MO, USA). The polyclonal anti-rabbit immunoglobulin HRP-linked antibody and polyclonal rabbit anti-mouse immunoglobulin HRP-linked antibody were used as the secondary antibodies. For visualization, we used the Enhanced chemiluminescence Kit (GE Healthcare).

RESULTS

Antiapoptotic gene expression in MCF-7, HeLa and ME-180 cervical carcinoma cell lines

The expression levels of the four antiapoptotic genes in MCF-7, HeLa and ME-180 cervical cancer cells was analyzed by quantitative PCR analysis. In all the tested cervical carcinoma cell lines, BCL2, Bcl-xL, XIAP and Survivin were expressed at higher level (Table 1). Among the three tested cell lines, MCF-7 and HeLa cells expressed all the four genes which are antiapoptotic at significantly higher levels thus, were selected for further studies.

Table 1: Expression levels of mRNA in MCF-7, HeLa and ME-180 cervical cancer cell lines

Cell line	BCL2	Bcl-xL	XIAP	Survivin
MCF-7	0.411	33.6	4.38	5.76
HeLa	0.134	31.3	3.12	3.87
ME-180	0.132	11.2	2.64	2.59

Effect of WFA on expression of genes involved in anti-apoptosis

Among the range of WFA concentrations from 5 to 50 µM used to analyse the effect on antiapoptotic gene inhibition, the effect was significant at 30 µM concentration. Although, 20 µM WFA concentrations markedly reduced the mRNA levels by 52-59 % after 24 h treatment, the inhibition rate was increased to 81– 86 % at 30 µM concentration of WFA (Figure 1). Thus, a marked antiapoptotic gene inhibition was observed after WFA treatment in MCF-7 and HeLa cell lines.

Molecular effects of WFA on inhibition of genes involved in antiapoptosis

WFA at 30 µM concentration significantly inhibited the mRNA expression levels of antiapoptotic genes in the cervical carcinoma cell lines after 48 h treatment (Figure 2). BCL2, Bcl-xL, XIAP and Survivin were decreased respectively by 32 39 42 and 36 % in MCF-7 cells. The results from western blot analysis showed protein reduction after 48 h of WFA treatment in both MCF-7 and HeLa cervical cancer cell lines (Figure 3).

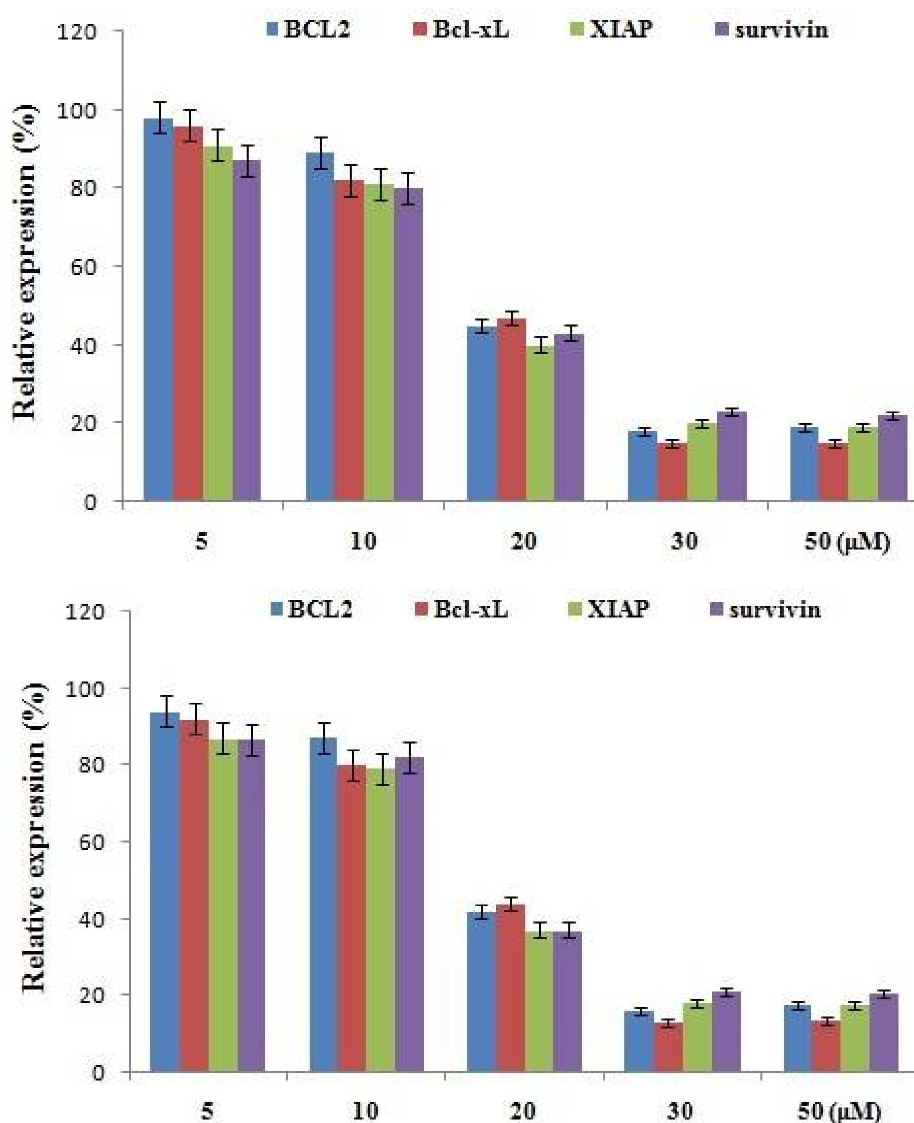

Figure 1: Decrease in the level of BCL2, Bcl-xL, XIAP and Survivin mRNA on treatment with WFA for 24 h in MCF-7 (upper figure) and HeLa (lower figure) cervical cell lines

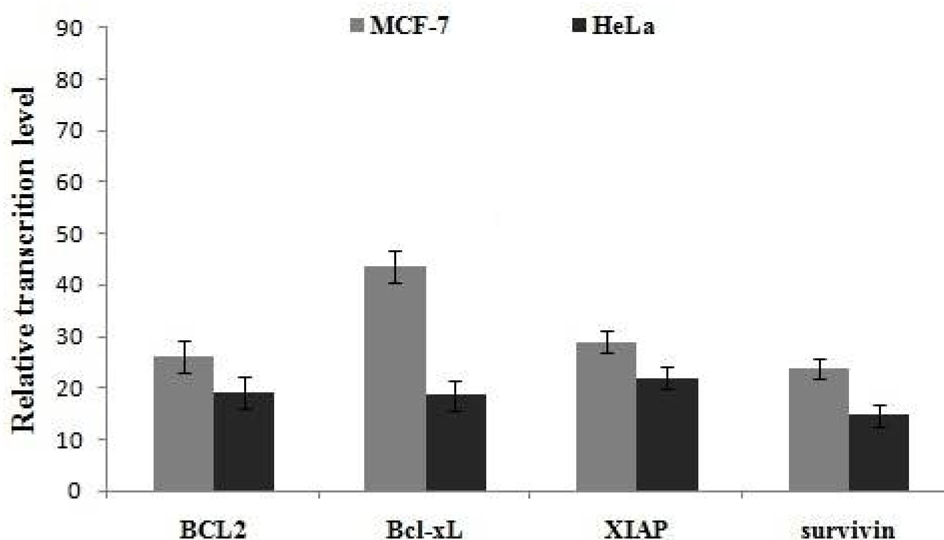

Figure 2: Relative mRNA expression levels of MCF-7 and HeLa cervical carcinoma cells 48 h after treatment with 30 µM WFA. The data presented is the average of three independent experiments

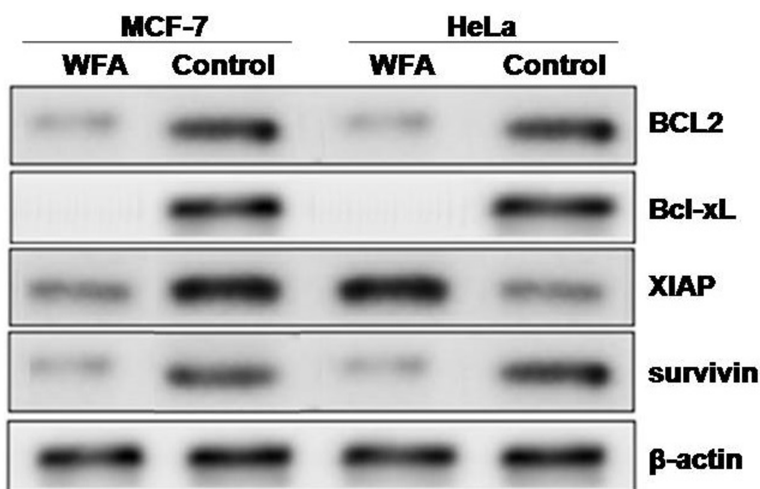

Figure 3: Western blot used to detect BCL2, Bcl-xL, XIAP and Survivin protein in MCF-7 and HeLa cervical carcinoma cells after 48 h of WFA treatment

Cellular effects of WFA treatment

The WFA-mediated inhibition of anti-apoptotic genes led to a significant decrease in cervical cancer cell viability (Figure4). The cervical carcinoma cell viability was decreased by 39 and 46 % in MCF-7 and HeLa cells respectively after treatment with WFA for 96 h. The rate of cell apoptosis was also significantly increased on treatment with WFA. In MCF-7cells, there was a 4-fold increase in the rate of apoptosis (Figure 5). In HeLa cells apoptosis was increased by 3.5 fold (Figure 5). However, no changes were observed in the cell cycle distribution in MCF-7 and HeLa cells on treatment with WFA.

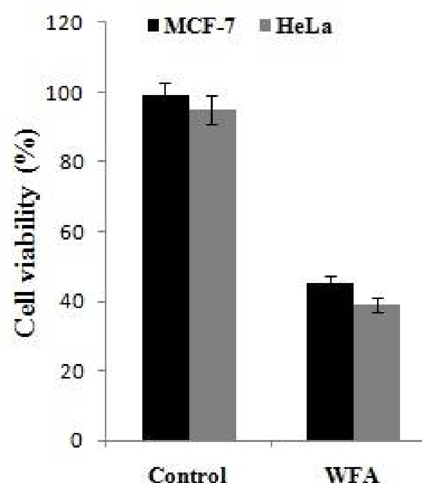

Figure 4: Viability of MCF-7 and HeLa cervical carcinoma cells 96 h after WFA treatment

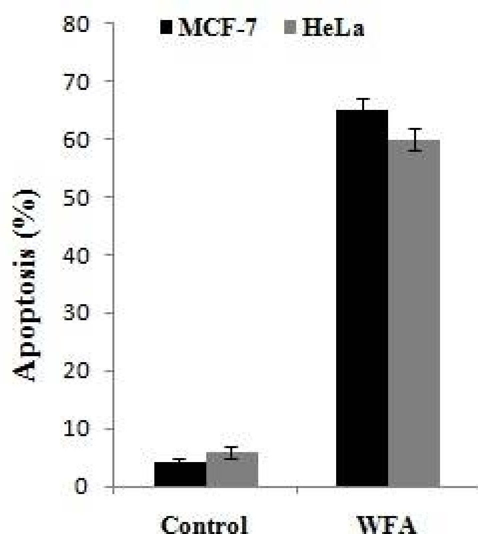

Figure 5: Percentage of early and late apoptotic cells 48 h after WFA treatment of MCF-7 and HeLa cells. Values represented are the mean of three independent experiments

DISCUSSION

In tumor tissues the expression of various antiapoptotic genes is found to be markedly higher and plays an important role in inhibiting the induction of apoptosis [23-25]. Thus, antiapoptotic gene inhibition can be a potent strategy for antitumor therapy. It is believed that the inhibition of one antiapoptotic gene can be compensated by the expression of other genes, therefore, inhibition of all the major antiapoptotic genes can have the greatest effect. In the present study, treatment of MCF-7 and HeLa cervical carcinoma cells with 30 μM of WFA resulted in the suppression of mRNA corresponding to antiapoptotic gene. WFA significantly inhibited the mRNA and protein levels of anti-apoptotic genes and exhibited strong anti-proliferative effects on MCF-7 and HeLa cervical carcinoma cells. In MCF-7 and HeLa cervical carcinoma cells the proliferation was reduced by 39 and 46 %, respectively following WFA treatment for 96 h. Tumor cells are bestowed with the ability to avoid the process of apoptosis [7]. It is reported that the tumor cells express higher level of BCL2, Bcl-xL, XIAP and Survivin antiapoptotic genes which enables them to evade apoptosis. The rate of cell apoptosis was also significantly increased on treatment with WFA. In MCF-7 cells, there was a 4-fold increase in the rate of apoptosis. In case of the HeLa cells the proportion of apoptotic cells was enhanced by 3.5 fold on treatment with WFA. However, in both MCF-7 and HeLa cells WFA treatment could not induce any alteration in the progression of cell cycle. Thus, the current study demonstrates that WFA induces suppression of antiapoptotic gene expression which can be a vital importance for the treatment of cervical cancer.

CONCLUSION

Thus withaferin A significantly inhibits cervical cancer via knockdown of antiapoptotic genes and is thus a potential therapeutic agent for cervical cancer.

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