

Effect of allyl isothiocyanate on the viability and apoptosis of the human cervical cancer HeLa cell line *in vitro*

GUANGYI QIN, PING LI and ZHUOWEI XUE

Department of Obstetrics and Gynecology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai 200233, P.R. China

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Abstract. The present study aimed to investigate the effect of allyl isothiocyanate (AITC) on the viability and apoptosis of the human cervical cancer HeLa cell line *in vitro*, and to explore the potential underlying mechanisms of this. HeLa cells were treated with varying concentrations of AITC for different durations. The cell viability was then measured using a Cell Counting kit-8 assay and the apoptosis rate of the cells was detected using flow cytometry. Additionally, the B cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax) mRNA expression levels were determined by reverse transcription-quantitative polymerase chain reaction, while the Bax and Bcl-2 protein expression levels in cells were detected by western blot analysis. AITC was revealed to inhibit the viability of HeLa cells. AITC was revealed to induce the apoptosis of HeLa cells, as the apoptosis rate increased gradually with an increase in the dose. As the concentration of AITC increased, the Bax mRNA expression level increased, whilst the Bcl-2 mRNA expression level decreased. Furthermore, the Bax protein expression intensity increased whilst Bcl-2 protein expression intensity decreased, thereby resulting in a decrease in the ratio of Bcl-2/Bax proteins. AITC may inhibit cell viability by inducing the apoptosis of HeLa cells and this may be accounted for by the imbalance in the Bcl-2/Bax expression ratio.

Introduction

Globally, ~200,000 women succumb to mortality from cervical cancer every year and the age of onset of this disease has decreased over time (1), indicating that it presents a notable threat to the health of women. At present, the treatment methods for cervical cancer include surgical treatment, chemotherapy, radiotherapy, biological therapy and traditional

Chinese medicine. In clinical practice, surgical treatment with radiotherapy or chemotherapy is the gold-standard. However, this treatment option has a number of shortcomings, including damage to the body and toxicity. Therefore, it is of interest to identify a novel treatment method for cervical cancer.

Allyl isothiocyanate (AITC) is widely distributed in cruciferous plants and their by-products, including mustard and horseradish (2,3). A number of studies have considered AITC to be associated with tumors. For instance, AITC may arrest cancer cells in mitosis, which in turn leads to apoptosis via B-cell lymphoma 2 (Bcl-2) protein phosphorylation (4-6). AITC may affect the proliferation of various tumor cells by inducing apoptosis and cell cycle arrest, or by inhibiting their invasion and metastasis, including in breast (7), bladder (8-10), lung (11), colon (12,13), liver (14,15) and prostate cancer cells (16). On the basis of the inhibitory effect AITC has on the proliferation of bladder cancer cells, population-based survey results have suggested that the intake of cruciferous plants may improve the survival rate of patients with bladder cancer (17), which may provide further evidence for the anti-cancer effect of AITC. However, few studies have reported on whether AITC has anticancer effects on cervical cancer. Therefore, in the present study, cervical cancer HeLa cells were treated with varying concentrations of AITC in order to investigate the effect of AITC on the viability and apoptosis of HeLa cells. In addition, the gene expression of Bcl-2 and Bcl-2-associated X protein (Bax), which are associated with apoptosis, was detected and the potential mechanisms underlying the effects of AITC on the apoptosis of HeLa cells was preliminarily discussed.

Materials and methods

Culture of HeLa cells. The cervical cancer HeLa cell line was sourced from the American Type Culture Collection (Manassas, VA, USA) and was cultured in Dulbecco's modified Eagle medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), prior to being incubated with 5% CO₂ at 37°C. Cells were passaged every 2-3 days, during which the culture medium in the culture dish was removed and discarded, and the cells were rinsed with phosphate-buffered saline (PBS) twice, prior to being digested with trypsin for 1 min at 37°C. Subsequently, the adherent

Correspondence to: Dr Guangyi Qin, Department of Obstetrics and Gynecology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, 600 Yishan Road, Shanghai 200233, P.R. China
E-mail: qinguangyi160716@sina.com

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cells were pipetted into single cell suspensions in PRMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) and centrifuged at 1,000 x g for 5 min at 37°C. The supernatant was discarded. The cells were resuspended with 1 ml culture medium and then 200 μ l was removed for subculture (subculture ratio 1:5).

Cell viability using a Cell Counting kit-8 (CCK8) assay. The HeLa cells, which had been digested and counted during the logarithmic growth phase, were seeded onto 96-well plates at a density of 1×10^4 cells/well. After 12 h of incubation, the cells were treated with varying concentrations (0, 5, 15 and 45 μ M) of AITC (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The treatment duration was set as 24, 48 and 72 h at a temperature of 37°C. A total of 5 replicate wells were used for each concentration and a blank control group was treated with an equivalent volume of PBS (7). Following treatment, the cells were rinsed using PBS three times and 100 μ l CCK8 reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well (the ratio of CCK8 reagent to medium was 1:10). The cells were incubated at 37°C in the dark for 2 h. Subsequently, the absorbance values were determined using an enzyme microplate reader at a wavelength of 450 nm and the cell viability rate was calculated according to following expression: Cell viability rate = $(A_{\text{treatment group}} - A_{\text{blank control group}}) / (A_{\text{control group}} - A_{\text{blank control group}})$, where A denotes the absorbance value.

Apoptosis detection by flow cytometry. The HeLa cell suspension was added to a 6-well plate at a density of 1×10^5 cells/well. After 12 h of incubation, the cells were treated with varying concentrations of AITC (0, 5, 15 and 45 μ M) for 48 h. A total of 3 parallel samples were used for each concentration. Following completion of the treatment, the cells were digested with trypsin for 1 min at 37°C, washed twice with PBS, centrifuged at 1,000 x g for 5 min at 37°C and resuspended in PBS. Subsequently, 1×10^5 cells were counted and centrifuged again at 1,000 x g for 5 min at 37°C. The supernatant was discarded and 195 μ l Annexin V-fluorescein isothiocyanate (FITC), as part of a cell apoptosis detection kit (Beyotime Institute of Biotechnology, Haimen, China) was added for resuspension and agitated gently to mix following the addition of 5 μ l Annexin V-FITC. The mixture was gently agitated to mix again following the addition of 10 μ l propidium iodide staining solution (part of the aforementioned cell apoptosis detection kit). Next, the mixture was incubated at room temperature in the dark for 20 min, prior to being placed in an ice bath at 0°C for 20 min. The apoptosis rate was subsequently detected using a Gallios™ flow cytometer (Version no. A75199AA; BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using FCS Express 3.0 (DeNovo software, Glendale, CA, USA) and a cell apoptosis detection kit (Beyotime Institute of Biotechnology, Haimen, China).

Detection of Bax and Bcl-2 mRNA levels. The concentrations of AITC were set to 0, 5, 15 and 45 μ M. After 48 h of treatment at 4°C, total RNA was extracted from the HeLa cells using the TRIzol RNA extraction reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The purity and content of RNA were detected using a nucleic acid and protein analyzer. At the same time, the integrity of RNA was identified by 1% agarose gel electrophoresis (Abcam, Cambridge, MA, USA).

RNA (1 μ g) was used for cDNA synthesis through reverse transcription using a reverse transcription-quantitative polymerase chain reaction (RT-qPCR) kit (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocols. The primers of all genes were as follows: Bax forward, TCCTCATCGCCATGCTCAT and reverse, CCT TGGTCTGGAAGCAGAAGA; Bcl-2 forward, GATGAC CGAGTACCTGAACC and reverse, CAGGAGAAATCG AACAAAGGC; and β -actin forward, TGCTGTGTCCCC ATCTATCG and reverse, TTGGTGACAATACCGTGTTC. The $2^{-\Delta\Delta C_q}$ method was used for quantification (18), and an RT-qPCR reaction system was established according to the following conditions: 5 μ l 2 X SYBR-Green mixture (Qiagen GmbH, Hilden, Germany), 0.5 μ l cDNA, 0.5 μ l primer and 4 μ l purified H₂O. Reaction conditions were as follows: Following pre-denaturation at 95°C for 10 min (denaturation at 95°C for 15 sec, annealing and extension at 60°C for 60 sec), 40 PCR cycles were performed on a ViiA7 quantitative fluorescence PCR machine (ABI Corporation, Lee's Summit, MO, USA) at 95°C for 5 sec, 55°C for 30 sec and 72°C for 20 sec. A total of 3 parallel samples were set for each experiment, and β -actin was used as the internal control gene.

Detection of Bax and Bcl-2 protein levels using western blot analysis. The concentrations of AITC were set to 0, 5, 15 and 45 μ M. Following 48 h of treatment at 37°C, the cells were added to cell lysis solution (155 mM ammonium chloride, 10 mM sodium bicarbonate and 0.5 mM EDTA; Miltenyi Biotec, Inc., Auburn, CA, USA) for 2 h at 37°C, and placed in a homogenizer for homogenization. Total cell protein was extracted using a total protein extraction kit (BestBio Co., Shanghai, China), according to the manufacturer's protocol, and was centrifuged at 1,000 x g for 5 min at 37°C, prior to the supernatant being obtained. The amount of protein was determined using the Coomassie brilliant blue protein assay kit (Shanghai Majorbio Pharmaceutical Technology Co., Ltd., Shanghai, China), according to the manufacturer's protocol. Next, the Laemmli sample buffer, containing 60 mM Tris-Cl (pH 6.8), 2% SDS, 10% glycerol, 5% β -mercaptoethanol and 0.01% bromophenol blue (Sigma-Aldrich; Merck KGaA), was added and boiled at 100°C. SDS-PAGE gel (6-12%; Sigma-Aldrich; Merck KGaA) was prepared as follows: The protein sample (50 μ g) was loaded for electrophoretic separation for 3 h and then placed into the electric transducers, in which the transfer buffer was added to the transmembrane for 1.5 h and the target protein was transferred onto nitrocellulose (NC) membranes. Non-specific binding was blocked using PBS with Tween (PBST; Sigma-Aldrich; Merck KGaA) containing 5% skimmed milk powder, and agitated at room temperature for 2 h. Bcl-2 (dilution, 1:100; cat. no. MS-123-A1; Biotium, Inc., Fremont, CA, USA), Bax (dilution, 1:100; cat. no. Rb-1486-R1; Biotium, Inc.) and GAPDH antibodies (dilution, 1:100; cat. no. MS-168-P1; Biotium, Inc.) were utilized. The membrane was washed 3 times with PBST and the horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (dilution, 1:1,000; cat. no. A-21422; Sigma-Aldrich; Merck KGaA) was added and incubated in a 4°C refrigerator overnight. The membrane was washed using PBST for 30 min, and the secondary horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (dilution, 1:1,000; cat. no. A-2418,

Table I. Effect of AITC on the viability of HeLa cells.

Treatment concentration, μM	Treatment duration, h		
	24	48	72
0	1.00 \pm 0.042	1.00 \pm 0.043	1.00 \pm 0.041
5	0.98 \pm 0.036	0.88 \pm 0.040 ^{a,b}	0.79 \pm 0.042 ^{a,b}
15	0.89 \pm 0.043 ^a	0.71 \pm 0.037 ^{a,b}	0.60 \pm 0.041 ^{a,b}
45	0.76 \pm 0.045 ^a	0.62 \pm 0.038 ^{a,b}	0.48 \pm 0.039 ^{a,b}

^aP<0.05 vs. 0 μM AITC; ^bP<0.05 vs. 24 h. AITC, allyl isothiocyanate.

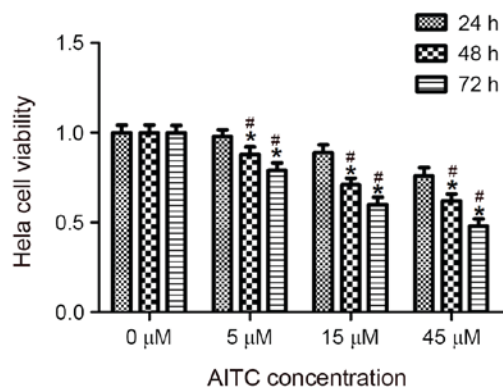
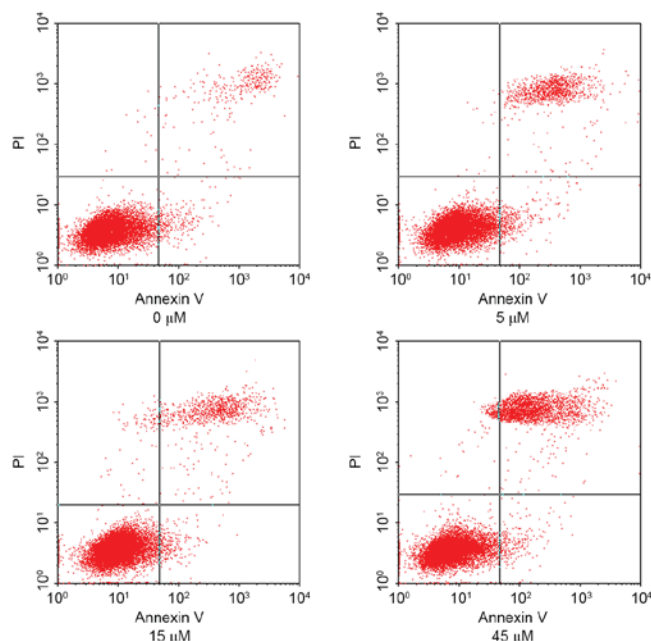
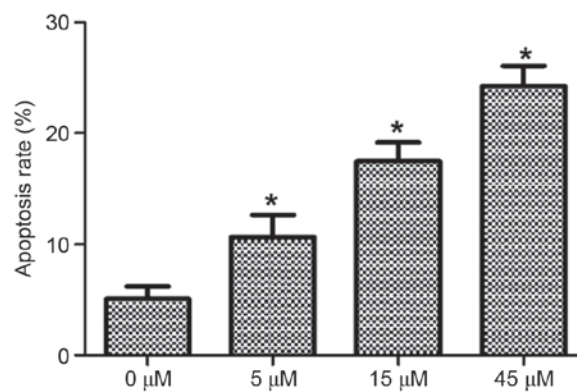
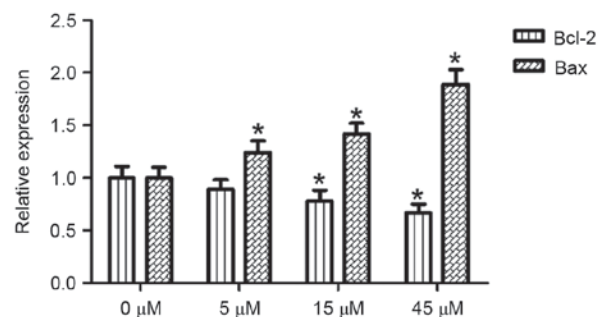
Figure 1. Effect of AITC on the viability of HeLa cells. *P<0.05, vs. 0 μM AITC; #P<0.05 vs. 24 h treatment group. AITC, allyl isothiocyanate.

Figure 2. Effect of differing concentrations of allyl isothiocyanate on the apoptosis rate of cells. PI, propidium iodide.

Sigma-Aldrich; Merck KGaA), prepared with PBST (containing 2.5% skimmed milk powder), was added and was incubated in a shaker at 58°C for 60 min. The NC membranes were washed with PBST 3 times and was then evenly coated

Figure 3. Comparison of the apoptosis rate with differing treatment concentrations. *P<0.05 vs. 0 μM . Allyl isothiocyanate.Figure 4. Effects of AITC treatment on the Bax and Bcl-2 mRNA expression levels *P<0.05 vs. 0 μM AITC. AITC, allyl isothiocyanate; Bcl-2, B cell lymphoma-2; Bax, Bcl-2-associated X protein.

with reagents from an enhanced chemiluminescence detection kit (Sigma-Aldrich; Merck KGaA) in a darkroom for 5 min. Following washing, the film was placed in a fixing solution (40% methanol and 7% acetic acid) for 5 min at 37°C, and hung to dry following flushing. The gel imaging system was sourced from ABI Corporation. The optical densities of the protein bands of Bax, Bcl-2 and GAPDH were analyzed using the GIS-2020D gel image analysis system (Sigma-Aldrich; Merck KGaA), while the expression intensities of Bax and Bcl-2 proteins were expressed as the ratio of the optical density of the Bax protein band to the optical density of the β -actin protein band and the ratio of the optical density of the Bcl-2 protein band to the optical density of the β -actin protein band by using SPSS 19.0 software (IBM Corp., Armonk, NY, USA).

Statistical methods. Data were statistically analyzed using SPSS 19.0 software (IBM Corp.). Normally distributed data are presented as the mean \pm standard deviation. The normally distributed data in different groups were compared using one-way analysis of variance and the data of two groups were compared using Fisher's least significant difference test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of AITC on the viability of HeLa cells. HeLa cells were treated with varying concentrations of AITC for different

Table II. Effects of AITC treatment on Bax and Bcl-2 protein expression levels.

Protein	Concentration, μM				P-value
	0	5	15	45	
Bax	0.58 \pm 0.07	0.71 \pm 0.06 ^a	0.83 \pm 0.04 ^a	1.02 \pm 0.08 ^a	<0.001
Bcl-2	0.98 \pm 0.09	0.89 \pm 0.08	0.65 \pm 0.07 ^a	0.50 \pm 0.05 ^a	<0.001
Bcl-2/Bax	1.69 \pm 0.14	1.25 \pm 0.13 ^a	0.78 \pm 0.11 ^a	0.49 \pm 0.12 ^a	<0.001

^aP<0.05 vs. 0 μM AITC. AITC, allyl isothiocyanate; Bcl-2, B cell lymphoma-2; Bax, Bcl-2-associated X apoptosis regulator.

durations, and a CCK8 assay was conducted to determine the effect of AITC on the viability of HeLa cells, the results of which are presented in Table I and Fig. 1. The results revealed that AITC inhibited the viability of HeLa cells, an effect that was most significant when the cells were treated with 45 μM AITC for 72 h ($P=0.024$).

Effect of AITC on the apoptosis of HeLa cells. HeLa cells were treated with 0, 5, 15 and 45 μM AITC for varying durations and the apoptosis rates of HeLa cells in different groups were detected using flow cytometry, the results of which are presented in Fig. 2 (in which the right upper quadrant presents the results for late apoptotic cells and the right lower quadrant presents the results for early apoptotic cells) and Fig. 3. As determined by flow cytometry, the apoptosis rate in the control group was 5.08 \pm 1.12%, which was significantly lower compared with 10.65 \pm 1.98, 17.49 \pm 1.68 and 24.26 \pm 1.83% in the 5, 15 and 45 μM AITC treatment groups, respectively. The apoptosis rates in the treatment groups were significantly higher than the apoptosis rate in the control group at all drug concentrations ($P<0.05$).

Effects of AITC on Bax and Bcl-2 mRNA expression in HeLa cells. With the 0 μM AITC treatment group serving as a control and β -actin acting as an internal reference gene, Bax and Bcl-2 mRNA expression levels in HeLa cells were detected using a semi quantitative method following 48 h of AITC treatment, the results of which are presented in Fig. 4. Compared with the control group, the Bcl-2 mRNA expression level was significantly decreased in the 15 and 45 μM treatment groups ($P<0.05$). The differences between the treatment groups (at all concentrations) and the control group were statistically significant ($P<0.05$).

Effects of AITC on Bax and Bcl-2 protein expression in HeLa cells. Following treatment with 0, 5, 15 and 45 μM AITC for 48 h, Bax and Bcl-2 protein expression levels in HeLa cells were detected. As illustrated in Fig. 5 and Table II, compared with the control group, the expression level of Bax protein was significantly upregulated, whereas the expression level of Bcl-2 protein was significantly downregulated following AITC treatment (all $P<0.001$).

Discussion

At present, the drugs used for the treatment of cervical cancer are often highly toxic and induce severe side effects, which

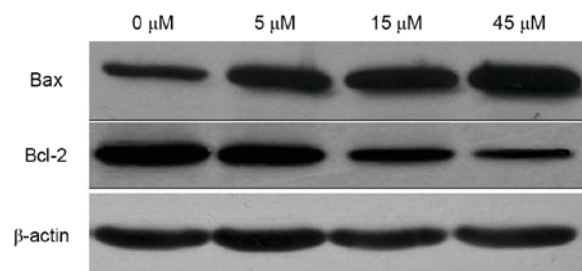


Figure 5. Effects of allyl isothiocyanate treatment on Bax and Bcl-2 protein expression levels. Bcl-2, B cell lymphoma-2; Bax, Bcl-2-associated X protein.

may cause damage to patient tissues during the treatment of cancer and may reduce the chance of survival (2,3). Therefore, the identification of a safe, effective and low toxicity anti-cancer drug has attracted interest from researchers, and the development of these drugs from natural sources has become an important strategy in the development of antitumor drugs. As a type of plant chemical that is prevalent in the natural diet of humans, AITC may affect the proliferation of a variety of tumor cells (19,20), and has attracted extensive attention from researchers. The anticancer effect of AITC has been verified by studies *in vivo* and *in vitro* (21,22), but the function of AITC in the treatment of cervical cancer has not been reported. Therefore, the present study confirmed the effect of AITC on cervical cancer cells through cell line experiments and preliminarily explored the underlying mechanisms of this.

The inhibitory effect on cell viability was most significant when the cells were treated with 45 μM AITC for 72 h ($P=0.024$). At present, the effect of AITC on HeLa cells has not previously been reported. However, a similar study was reported by Hasegawa *et al* (23), which revealed that isothiocyanate, including phenyl-ethyl isothiocyanate and benzyl isothiocyanate, may inhibit HeLa cell viability through cell cycle arrest. AITC is a type of isothiocyanate, which means that the conclusion of the present study is consistent with that of Hasegawa *et al* (23). In addition, when HeLa cells were treated with AITC for 48 h, the apoptosis rate of cells exhibited a dose-dependent increase, suggesting that AITC may inhibit HeLa cell viability by inducing cell apoptosis. However, the mechanism of AITC inhibiting cell viability is complex. For example, AITC may inhibit HeLa cell viability through cell cycle arrest and the induction of cell apoptosis.

Bax and Bcl-2 have attracted attention due to their regulation of cell apoptosis, and their function in promoting or inhibiting cell apoptosis may be realized through their protein expression (24). Bax and Bcl-2 are a pair of positive-negative apoptosis regulating genes. In addition to promoting cell apoptosis, Bax may also reduce the inhibitory effect of Bcl-2 on cell apoptosis by forming a dimer with Bcl-2 (25). Therefore, the occurrence of cell apoptosis is associated with an imbalance of Bcl-2/Bax expression. The present study revealed that AITC may induce the apoptosis of HeLa cells. By detecting Bcl-2 and Bax expression levels, it was demonstrated that, with an increase in the concentration of AITC, the expression levels of Bax mRNA and protein increased whilst Bcl-2 mRNA and protein expression levels decreased, resulting in a decrease in the ratio of Bcl-2/Bax proteins. The results of the present study suggested that the apoptosis of HeLa cells induced by AITC may be associated with an imbalance in Bcl-2/Bax expression. Sávio *et al* (8) also revealed that the apoptosis induced by AITC was associated with a Bcl-2/Bax expression imbalance.

The present study demonstrated that AITC may inhibit HeLa cell viability through the induction of cell apoptosis and that the imbalance of Bcl-2/Bax expression may be the mechanism accounting for AITC-induced cell apoptosis, which may provide evidence for further research for novel anticancer drugs for cervical cancer. However, the results of the present study remain far from being used for clinical applications and require further verification in future studies.

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