

## RESEARCH ARTICLE

## Sulforaphane Inhibits the Proliferation of the BIU87 Bladder Cancer Cell Line via IGFBP-3 Elevation

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### Abstract

**Aim:** To investigate effects of sulforaphane on the BIU87 cell line and underlying mechanisms involving IGFBP-3. **Methods:** Both BIU87 and IGFBP-3-silenced BIU87 cells were treated with sulforaphane. Cell proliferation was detected by MTT assay. Cell cycle and apoptosis were determined via flow cytometry. Quantitative polymerase chain reaction and Western blotting were applied to analyze the expression of IGFBP-3 and NF- $\kappa$ B at both mRNA and protein levels. **Results:** Sulforaphane (80  $\mu$ M) treatment could inhibit cell proliferation, inducing apoptosis and cell cycle arrest at G2/M phase. All these effects could be antagonized by IGFBP-3 silencing. Furthermore, sulforaphane (80  $\mu$ M) could down-regulate NF- $\kappa$ B expression while elevating that of IGFBP-3. **Conclusions:** Sulforaphane could suppress the proliferation of BIU87 cells via enhancing IGFBP-3 expression, which negatively regulating the NF- $\kappa$ B signaling pathway.

**Keywords:** Sulforaphane - bladder cancer - IGFBP-3 - NF- $\kappa$ B

*Asian Pac J Cancer Prev*, **15** (4), 1517-1520

### Introduction

Bladder cancer is a common urinary system cancer worldwide. In order to enhance long-time survival rate, patients with bladder cancer are subjected to an extended treatment modality. Thus, the expense of medication is a burden not only for patients themselves, but also for the economy and society (Montgomery et al., 2013). Despite the improvements made in surgical and chemotherapeutic modalities to combating bladder cancer, the 5-year survival rate still remains relative low due to highly invasiveness and metastasis of bladder cancer (Ferreira et al., 2007; Ge et al., 2012). Therefore, studies on novel medications and therapeutic modalities are warranted.

Sulforaphane, which belongs to isothiocyanate group, is ubiquitously presented in cruciferous plants. It has been reported that sulforaphane functions as an antioxidant in skin and neural tissue (Kleszczynski et al., 2013; Chen et al., 2013). Moreover, anticancer potential of sulforaphane by suppressing cancer cell proliferation has also been well documented in breast, prostate and colon cancer cells (Johnston et al., 2004; Frydoonfar et al., 2004; Herman-Antosiewicz et al., 2007). Sulforaphane mainly induces cell cycle arrest and apoptosis on cancer cells (Kim et al., 2011; Chang et al., 2013). However, the underlying mechanisms remain elusive.

Insulin-like growth factor-binding protein-3 (IGFBP-3) is a member of the insulin-like growth factor-binding protein family, which could specifically bind to and

thus modulate the effect of insulin-like growth factors (IGFs). Furthermore, recent study revealed that IGFBP-3 exhibited proapoptotic effect via novel IGFBP-3 receptor. Together with the decreased expression of IGFBP-3 in non-small cell lung cancer tissue (Wang et al., 2013), we therefore hypothesized that the down-regulation of IGFBP-3 might play a role in the resistance of tumor cell to apoptosis.

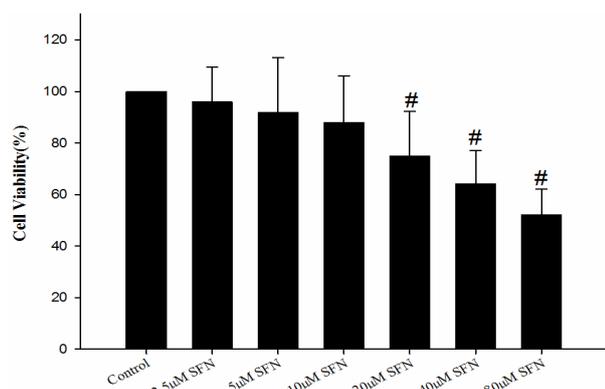
NF- $\kappa$ B is an inducible and ubiquitously expressed transcription factor which closely relates to cell growth, adhesion and survival. NF- $\kappa$ B also correlates with angiogenesis and apoptosis in cancer pathogenesis, as it is overexpressed in various cancer tissue and several tumor cell lines (Dolcet et al., 2005; Fang et al., 2011). Is there any possible connection between the decreased expression of IGFBP-3 and overexpression of NF- $\kappa$ B in tumor tissue? Therefore, the current study was designed to investigate the effect of sulforaphane on bladder cancer cell line BIU87 and to further unveil the underlying mechanism concerning IGFBP-3 and NF- $\kappa$ B signaling pathway regulation.

### Materials and Methods

#### Cell culture

BIU87 cell, purchased from Shanghai Institute for Biological Sciences (Shanghai, China), was maintained in RPMI1640 medium supplemented with 10% FBS (Thermo Scientific, Beijing, China) and was incubated

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**Figure 1. Effects of Sulforaphane on the Proliferation of BIU87 Cells.** <sup>#</sup> $p < 0.05$  vs. Control

in 5% CO<sub>2</sub> at 37 °C. when reaching 90% confluent, cells were passaged by using 0.25% trypsin.

#### Gene-silencing by small interfering RNA (siRNA)

Anti-sense siRNA against human IGFBP-3 was designed and synthesized by GenePharma Co., Ltd. (Shanghai, China). The transfection of siRNA was performed using Entranster™-R (Engreen Biosystem, Beijing, China) at a final concentration of 100 nM according to the manufacturer's instruction. Forty-eight hours after transfection, the gene-silencing effect was determined in both mRNA and protein level.

#### Cell proliferation assay

The proliferation of BIU87 cells was detected by MTT assay. Normal and IGFBP-3-silenced BIU87 cells ( $1.5 \times 10^5$  cells/well) seeded in 96-well plates were incubated with indicated concentrations of sulforaphane (0 µM, 2.5 µM, 5 µM, 10 µM, 20 µM, 40 µM, 80 µM) for 24 h. Four hours before the end of the incubation, aliquots (15 µL) of MTT were added into each well. The medium were aspirated and 100 µL DMSO were added to dissolve the formazan crystals. The absorbance at 490 nm presents cell proliferation.

#### Cell cycle analysis

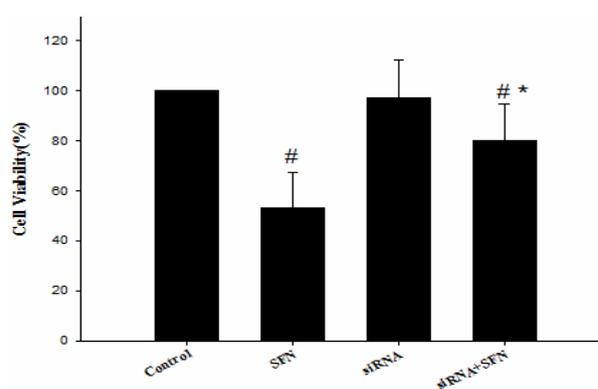
After indicated treatment for 24 h, cells were harvested and washed by ice cold PBS. The cells were further fixed in pre-cold 70% ethanol for 12 h. After that, 500 µL propidium iodide (PI, 50 µg/mL) was added and incubated away from light for 30 min. The cell cycle was determined by flow cytometry immediately.

#### Cell apoptosis analysis

Cell apoptosis was analyzed by using Annexin V-FITC/PI method. Cells were treated as indicated for 24 h and were further stained with Annexin V-FITC and PI according to the manufacturer's protocol (Sigma, St. Louis, MO, USA).

#### Quantitative polymerase chain reaction (qPCR) assay

Total RNA was extracted by using RNAiso Plus kit (Takara, Dalian, China) according to the manufacturer's protocol. And the purity and concentration of extracted RNA was determined by a spectrophotometer and further



**Figure 2. IGFBP-3 Silencing Ameliorates the Inhibitory Effect of Sulforaphane on BIU87 Cell Proliferation.** <sup>#</sup> $p < 0.05$  vs. control; <sup>\*</sup> $p < 0.05$  vs. SFN group

reverse-transcribed to cDNA by using ImProm-II® kit (Promega, Beijing, China). qPCR with GO Taq® qPCR Master Mix (Promega, Beijing, China) was performed with primers for targeted genes as follows: IGFBP-3 (forward: 5'-CAG CCA GCG CTA CAA AGT TGA CTA-3'; reverse: 5'-CTG GGA CTC AGC ACA TTG AGG A-3'); NF- $\kappa$ B (forward: 5'-CAAGCGAGGAGGGGACGTG-3'; reverse: 5'-CCCCCAGAGCCTCCACCC'-3'). The expression of target genes were analyzed by  $\Delta\Delta$  cycle threshold ( $2^{-\Delta\Delta Ct}$ ) method with  $\beta$ -actin as reference gene by using Rotor-Gene 6 (forward: 5'-TGG CAC CCA GCA CAA TGA A-3'; reverse: 5'-CTA AGT CAT AGT CCG CCT AGA AGC A-3').

#### Western blot analysis

Proteins were extracted with RIPA lysis buffer (Applygen, Beijing, China) mixed with 1% proteinase inhibitors cocktail and the concentration were determined by BCA commercial kit (Applygen, Beijing, China). Samples (30 µg) were loaded on each well and further separated on 12% SDS-PAGE. Protein bands were then transferred to PVDF membranes and were blocked by 5% non-fat milk for 1 h at room temperature. After binding with specific primary antibodies (mouse anti-human IGFBP-3, 1:1000; mouse anti-human NF- $\kappa$ B, 1:1000; mouse anti-human  $\beta$ -actin, 1:1000), the protein bands were incubated with DyLight 800 conjugated secondary antibody for 2 h and further visualized using Odyssey infrared imaging system.

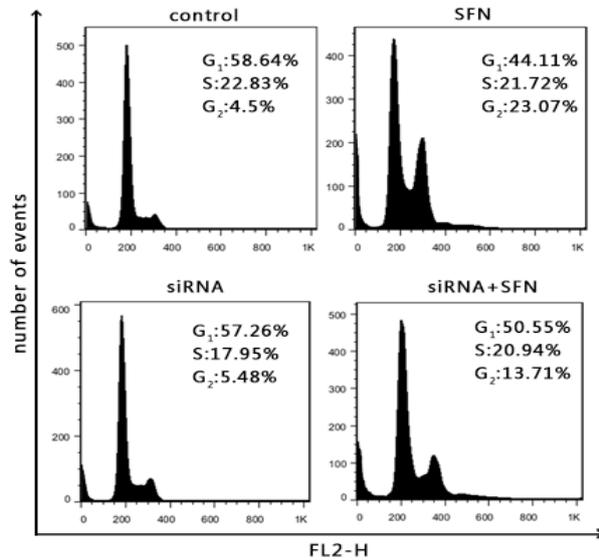
#### Statistical analysis

All data were expressed as mean  $\pm$  standard deviation (SD). Differences between control and experimental groups were analyzed by one-way analysis of variance, and  $p < 0.05$  was considered as statistically significant. All calculations were performed using SPSS 18.0 statistical software (SPSS, Chicago, IL, USA).

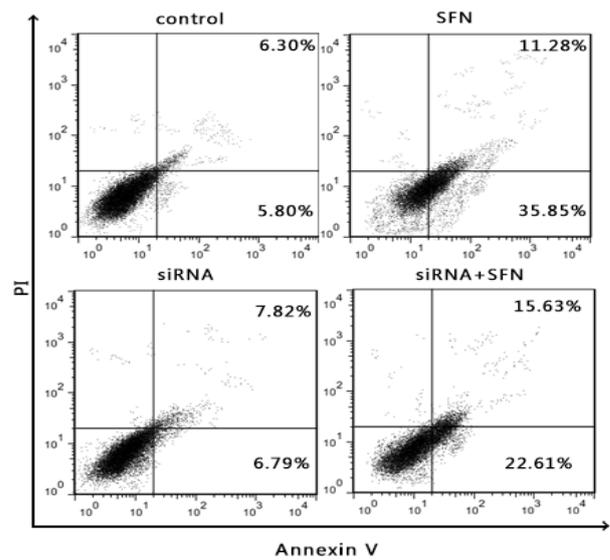
## Results

#### Effects of sulforaphane on the proliferation of BIU87 cells

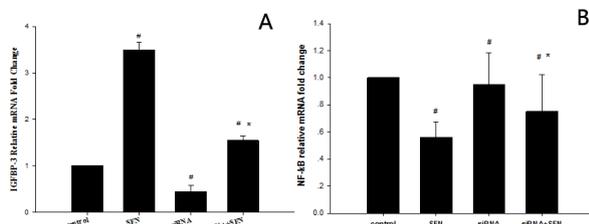
As shown in Figure 1, sulforaphane (10 µM and below) had no effect on cell proliferation, while sulforaphane (20, 40 and 80 µM) exhibited significant inhibition on the



**Figure 3. Effect of Sulforaphane on Cell Cycle of BIU87 Cells**



**Figure 4. Effect of Sulforaphane on Apoptosis of BIU87 Cells**



**Figure 5. Effect of Sulforaphane on IGFBP-3 and NF- $\kappa$ B mRNA Expression.** # $p < 0.05$  vs. control; \* $p < 0.05$  vs. SFN group

proliferation of BIU87 cells, compare to the control group ( $p < 0.05$ ).

#### *IGFBP-3 silencing ameliorates the inhibitory effect of sulforaphane on BIU87 cell proliferation*

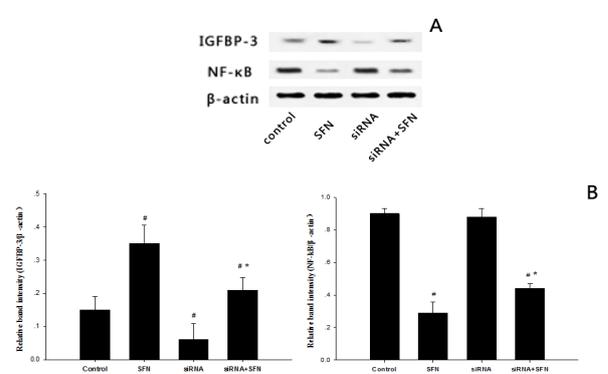
As shown in Figure 2, the cell viability of sulforaphane (80  $\mu$ M)-treated normal BIU87 group was significantly lower than untreated control group. Single IGFBP-3 silencing did not influence cell viability, while it could significantly ameliorate the inhibitory effect of sulforaphane on BIU87 cell proliferation ( $p < 0.05$ ).

#### *Effect of sulforaphane on cell cycle of BIU87 cells*

As shown in Figure 3, in the control group the percentage of cell population was (57.2 $\pm$ 3.8)% in G1 phase and (4.5 $\pm$ 0.9)% in G2 phase. In the group without siRNA treatment, due to the sulforaphane (80  $\mu$ M) treatment, the proportion of G1 phase fell to (43.2 $\pm$ 5.63)%, meanwhile the proportion of G2 phase rose to (22.3 $\pm$ 4.1)%, it indicated a significant cell cycle arrest at G2/M phase. IGFBP-3 silencing slightly elevated the percentage of cells in the G2/M phase, while it significantly attenuated the cell cycle arrest caused by sulforaphane treatment.

#### *Effect of sulforaphane on apoptosis of BIU87 cells*

As shown in Figure 4, the apoptotic cells ratio elevated from 12.31% $\pm$ 6.5% in the control group to 45.32% $\pm$ 16.11% after sulforaphane (80  $\mu$ M) treatment. IGFBP-3 silencing did not influence cell apoptosis.



**Figure 6. Effect of Sulforaphane on IGFBP-3 and NF- $\kappa$ B Protein Expression.** (A) Western blot bands; (B): relative band intensity normalized by  $\beta$ -actin. # $p < 0.05$  vs. control; \* $p < 0.05$  vs. SFN group

However, it partially abrogated the effect of sulforaphane on BIU87 cell apoptosis.

#### *Effect of sulforaphane on IGFBP-3 and NF- $\kappa$ B mRNA and protein expression*

As shown in Figure 5, the IGFBP-3 mRNA expression was significantly enhanced by sulforaphane treatment, while the mRNA level of NF- $\kappa$ B decreased after sulforaphane incubation. IGFBP-3 silencing partially reversed the effect of sulforaphane on the mRNA expression of IGFBP-3 and NF- $\kappa$ B (Figure 6).

## Discussion

Sulforaphane could induce cell apoptosis and cell cycle arrest by regulating various targets. Studies showed that the apoptotic effect of sulforaphane is actually relying on cell cycle control (Wang et al., 2012). Interestingly, this cell cycle control is p53-independent (Shen et al., 2006). Our current results showed that sulforaphane could promote BIU87 cell apoptosis and arrest BIU87 cell at G2/M phase by elevating IGFBP-3 expression. Silencing IGFBP-3 by siRNA interfering technique could partially reverse the effect, and thus suggesting that sulforaphane

exerted an anti-tumor effect on BIU87 cells via IGFBP-3 regulation.

It has been reported that IGFBP-3 could regulate cell proliferation through either IGF-1-dependent or IGF-1-independent signaling pathway. As a binding protein, IGFBP-3 specifically binds to IGF-1, reducing free IGF-1, and therefore, inhibits the binding and activation of IGF-1 signaling pathway (Kalluri et al., 2011). On the other hand, IGFBP-3 could regulate cell cycle by acting directly with other cell cycle regulating proteins (Kim, et al., 2010). In addition, IGFBP-3 also elicits apoptotic effect by interfering with MAPK signaling pathway, caspase, Bax and Bad proteins (Butt et al., 2001). Moreover, it has been reported that IGFBP-3 could down-regulating various proinflammatory cytokines by inhibiting NF- $\kappa$ B activity (Williams et al., 2007), while NF- $\kappa$ B has an anti-apoptotic effect. Lee et al has reported that IGFBP-3 could activate caspase-3 and therefore negatively regulate the NF- $\kappa$ B signaling pathway. As shown in current study, sulforaphane elevated IGFBP-3 level while inhibiting NF- $\kappa$ B expression (Lee et al., 2011), which is consistent with previous study. Silencing IGFBP-3 in BIU87 cell suggested that sulforaphane might directly or indirectly modulate NF- $\kappa$ B activation by IGFBP-3.

The activation of NF- $\kappa$ B signaling pathway could lead to the anti-apoptotic effect of various cancer cells. The underlying mechanism is the imbalance between anti-apoptotic protein (Bcl-2 and Bcl-XL) and proapoptotic protein (Bax and Bad). The activation of NF- $\kappa$ B signaling pathway would lead to the translocation of NF- $\kappa$ B to the nucleus and binding to specific binding site in the promoter of downstream genes, including Bcl-2 and Bcl-XL. However, there are no binding sites for NF- $\kappa$ B in the promoters of Bax and Bad (Adams et al., 1998). In our study, NF- $\kappa$ B level also correlated with cell apoptosis after sulforaphane and IGFBP-3 silencing treatment, which was in agreement with previous studies.

During bladder and other cancer treatment, tumor cells often exhibit resistance to therapeutic modalities. Studies showed that NF- $\kappa$ B overexpression was frequently found in these cancer tissue or cells (Zhang, 2013). Therefore, elucidating underlying mechanism of certain drug would benefit the avoidance of adverse effects and drug resistance.

In conclusion, sulforaphane could suppress NF- $\kappa$ B signaling pathway by elevating IGFBP-3 level, and therefore inhibiting the proliferation of BIU87 cells.

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