



## Genistein synergizes with RNA interference inhibiting survivin for inducing DU-145 of prostate cancer cells to apoptosis

Fan Yuan-jing<sup>a,\*</sup>, Huang Nan-shan<sup>a</sup>, Xia Lian<sup>b</sup>

<sup>a</sup>School of Biotechnology and Food Engineering, Hefei University of Technology, Hefei 230009, China

<sup>b</sup>ANYA Science and Technology Invests Co., Ltd., Hefei 230009, China

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### ABSTRACT

To further investigate the effect of a combination of genistein with survivin of RNA interference on the proliferation and apoptosis of DU-145 cells, the effect of genistein on the proliferation of DU-145 cells was detected by the MTT method and cytometry, and the apoptosis of cells was observed with fluorescence microscopy. In order to test combined genistein with transfection of small interfering RNA (siRNA) against survivin, a survivin siRNA plasmid was constructed and transfected into DU-145 cells. Genistein inhibited proliferation and induced apoptosis of cancerous DU-145 and Hela cells, whereas genistein had minimal effects for normal L-O2 cells. The stable transfected cell lines of DU-145, knockdown survivin by siRNA, displayed stronger apoptotic than untransfected DU-145, the transfected cell of DU-145 treated with genistein demonstrated the inhibition of proliferation and induction of apoptosis significantly; it showed genistein synergistic effect with RNAi in survivin for inhibition of prostate cancer cells.

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### 1. Introduction

The most intensively studied phytoestrogen group is that of the isoflavones, of which the effects of genistein (4,5,7-trihydroxyisoflavone) are the best-documented [1]. The present studies determined the effects of genistein on human prostate cancer cell lines *in vitro*. Genistein arrests cell cycle progression at the G2/M or G1/S phases. Genistein contributes to apoptosis and inhibits proliferation via promotion of antioxidant enzyme activities in prostate cancer cells of LNCaP and PC-3 [2], as well as DU-145 [3]. Genistein may also modulate correlative gene expression to promote cancer cell apoptosis through the

influence of growth arrest and DNA damage-inducible gene 45 (Gad45) in DU-145 [4]. The mechanism of action of genistein is complex and includes several cellular pathways. In addition to its estrogenic and/or anti-estrogenic activities, genistein has been reported to inhibit steroidogenesis and block several protein tyrosine kinases, including epidermal growth factor receptor and src tyrosine kinases [5].

Survivin is an anti-apoptotic protein within the inhibitor of apoptosis proteins (IAPs) family and one of the most prominent cancer-associated genes that have been analyzed [6]. Survivin has been reported to inhibit cell proliferation and changes in cell cycle distribution of stable transfected cells of human breast cancer cell lines (MCF7, HEK293, HT29, and Hela) with specific small interfering RNA (siRNA) of survivin gene RNA interference (RNAi) [7]. The cells were blocked in the G0/G1 phase and sharply reduced in the G2/M phase so as to increase apoptosis of cells [8]. Survivin inhibits activation of caspases, and its overexpression can lead to resistance to apoptotic stimuli [9]. Caspases are a family of cysteine proteases and play

**Abbreviations:** RNAi, RNA interference; siRNA, small interfering RNA; shRNA, small hairpin RNA; EGF, epidermal growth factor; PTK, protein-tyrosine kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; IAP, inhibitor of apoptosis protein; EDTA, ethylene diamine tetraacetic acid; EGTA, ethyleneglycol-bis(aminoethyl ether)-N,N-tetraacetic acid; MAPK, mitogen-activated protein kinases.

\* Corresponding author. Tel.: +86 551 2901505 8418.

E-mail address: [swf89105@mail.hf.ah.cn](mailto:swf89105@mail.hf.ah.cn) (F. Yuan-jing).

essential roles in apoptosis. There are two types of apoptotic caspases: initiator caspases (caspases-2, -8, -9, and -10) and effector caspases (caspases-3, -6, and -7). The proteolytic activity of caspase-3 is a critical determinant of whether a cell commits to death, and as a result, multicellular organisms have evolved strategies to tightly regulate caspase-3 activity within the cell [10].

In this study, we tested the influence genistein extracted from soybeans on cell proliferation and/or apoptosis in human cancer cell lines (DU-145 and Hela) and a human hepatic normal cell line (L-O2). Furthermore, we carried out experiments on the role of genistein with survivin RNAi inhibiting survivin for inducing DU-145 of prostate cancer cell apoptosis to verify the protective effect of survivin expression. We adopted a plasmid that carried short hairpin RNA reversed to the survivin gene by RNAi to cause cell cycle arrest or apoptosis in DU-145, and combined genistein and survivin RNAi to observe the synergistic effect. The purpose of this study was to explain survivin expression in prostate cancer and to provide new direction for chemopreventive and/or chemotherapy against prostate cancer.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Chemical materials

Genistein extracted from soybean (Gen, 4,5,7-trihydroxyisoflavone, 96% of purity, Shijiazhuang Biological Products Co., Ltd., Shijiazhuang, China) was dissolved in dimethylsulfoxide (DMSO; Sigma Co., Ltd. Steinheim, Germany) and diluted to final concentrations in each culture medium used (*vide infra*).

#### 2.1.2. Plasmid and shRNA

Plasmid of U6 was constructed a recombinant encoding two survivin short hairpin RNA small hairpin RNA (shRNA) and named pU6 + 27-survivin, it was DNA template oligonucleotides corresponding to survivin gene which designed, fragment of 28nt, and synthesized as follows (Sangon Inc. Shanghai, China):

*Sall* sense: loop antisense

5'-TCGACGAAGGACCACCGCATCTCTACATTCAAGAATT-  
CAAGAGATTCTTGA ATGTAGAGATGCGGT

end *Xba*I

GGTCCTTTTTTTT-3'

3'-GCTTCTGTTGGCGTAGAGATGTAAGTTCTTAAGTTCT-  
CTAAGAACTTACATCTCTACGCCACCA GGAAAAAA AAA-  
GATC-5'

The above were subcloned into the *Sall*, *Xba*I and *Hind*III sites of pU6 + 27 plasmid (Promega, USA), in order to be helpful in confirming the inserted sequence, we added a *Sall* site to the terminal of siRNA. Two pairs of survivin-specific siRNAs were selected in this study and an unrelated siRNA was used as negative control [11]. The oligonucleotide templates were then incubated in annealing buffer, first at 94 °C for 5 min and at 37 °C for night's lodging. pU6 + 27-survivin was already accomplished (Xiao Weihua's Laboratory of University of Science and Technol-

ogy of China, USTC) and stored at -80 °C until they were used for transfections.

### 2.2. Cell culture

DU145 cells (androgen-independent human prostate cancer cell line, University of Science and Technology of China, USTC), Hela (human cervical cancer cell line, USTC) and L-O2 (normal human hepatic cell, Nanjing KeyGen Biotech. Co., Ltd., China) were, respectively, maintained in DMEM (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Hangzhou Sijiqing Biological Engineering Material Co., Ltd., China), each 100 U/ml of Penicillin/streptomycin (Invitrogen, USA), at 37 °C in the humidified atmosphere of a 5% CO<sub>2</sub> incubator. After about 80% of confluence, the cultured cells were digested using 0.25% trypsin (Amresco, USA) and subcultured.

### 2.3. Cell cycle analysis cell proliferation and viability assay

The effect of genistein on cell proliferation was measured by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Amresco USA) assay, based on the ability of live cells to cleave the tetrazolium ring in active mitochondria to a product molecule that absorbs at 570 nm of wavelength. Cells were plated in 96-well microtiter plates at an initial density of  $1 \times 10^4$  cells per well.

DU-145, Hela and L-O2 of Cells were treated with genistein (15, 30, 60, 120 μM in medium) each, respectively, the blank was uniform concentration of DMSO. All treated cell were incubated for 48 h, then washed in PBS and cleared away supernatant fluid, 180 μL of fresh DMEM and 20 μL of MTT solution (MTT was dissolved in PBS at 5 mg/ml. Briefly) were added to each well, followed by 4 h of incubation. After incubation, MTT-containing medium was removed and 150 μL of DMSO was added to each well to dissolve formazan crystals. The concentration of formazan was quantified spectrophotometrically (BIOTEK ELX800, Bio-Tek, Co., Ltd) at 570 nm. Cell proliferation was determined as above.

### 2.4. Cell calculation and cell modality observation

DU-145 and Hela were treated with 60 μM, 120 μM of genistein and incubated for 24 h, 48 h and 72 h, then cells were digested use 0.25% trypsin and living cell calculated by microscope; The same of DU-145 and Hela treated with 30 μM, 60 μM of genistein for 48 h, added 20 mg/mL of fluorescent dye (hoechst 33258, Sigma) and incubated at 37 °C for 20 min, observed by fluorescent microscope.

### 2.5. Western blotting analysis and activity assay toward caspase-3

DU-145 cells were harvested for 0, 1, 3, 6, 12, or 24 h times after genistein treatment (60 μM) and disrupted in lysis buffer (1% Triton X-100, 1 mM EGTA, 1 mM EDTA, and 10 mM Tris-HCl [pH 7.4]) containing protease inhibitors. Cell debris was removed by centrifugation at 10,000g for 10 min at 4 °C. The resulting supernatants were

resolved on a 12% SDS–polyacrylamide gel under denaturing reducing conditions and transferred to nitrocellulose membranes. The membranes were blocked with a 1% BSA solution for 3 h and washed twice with PBS containing 0.2% Tween-20, and incubated with a primary antibody against caspase-3, which contains 35 kDa and 17 kDa specificities for assaying pro- and cleaved-caspase-3 (product No. AC031; Beyotime Institute of Biotechnology, Co., Ltd., Nanjing, China). The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody (lamb anti-human, product No. A0201; Beyotime Institute of Biotechnology, Co., Ltd.). The immunoblot was analyzed using a BIORAD gel documentation system and quantified using BIORAD Quantity One software. The caspase-3 activity was measured using the colorimetric assay (Production No. BV-1008-1000, Bitab Biotech, Co., Ltd., Beijing, China) with Ac-DEVD-p-nitroanilide (pNA) as substrate, according to the manufacturer's protocols. The rate of caspase-3 enzymatic hydrolysis was measured by release of pNA from caspase substrate (405 nm).

## 2.6. Recombinant plasmid of pU6 + 27-survivin and transfection of DU-145

According to the sequencing of survivin, recombinant of plasmid pU6 + 27-survivin was gotten (Xiao Weihua's Laboratory of USTC) and was confirmable by *Sall*, *XbaI*, *HindIII* and sequence analysis (Sangon Inc. Shanghai, China), pU6 + 7-survivin is aimed at survivin (siRNA for survivin) and containing coding sequencing of shRNA of siRNA and loop.

DU-145 incubated as the same as above, divided into two groups, survivin-specific siRNA transfected cells and the empty vector transfected cells, untransfected cells were taken as controls. DU-145 was transfected with admixture of Lipofectamine2000 (Lipo2000, Gibco USA) plus pU6 + 27-survivin, stable transfected cell lines of DU-145 was named DU-3; DU-145 was transfected with empty vector, named DU-2, in admixture of Lipo2000 plus pU6 + 27, respectively. All of them were digested, and were plated in 96-well microtiter plates at an initial density of  $5 \times 10^4$  cells per well, once more incubated up to about 70% of confluence and then incubated at 37 °C and 5% CO<sub>2</sub> in a humid environment for 48 h. Double transfected cell lines were transferred to medium comprised G418 (1000 µg/mL) to incubate for 3 days, then replaced medium comprised G418 concentration of 500 µg/mL, and replaced instead the medium every 3 days. After 14 days, the cells returned to DMEM for incubation normally.

## 2.7. Test of survivin expression mRNA

RT-PCR analysis of survivin expression in the transfected DU-145 cells for reverse transcription-polymerase chain reaction (RT-PCR) analysis, total RNA was isolated by TRIZOL Reagent (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., China). Total RNA was reverse transcribed into cDNA. The synthesized cDNA was used for PCR (Eppendorf, Germany) amplification. The primer sequences were 5'-AGCCCTTCTCAAGGACCAC-3' and 5'-TGGCTGTTCTCAGTGGGGCAGT-3' for survivin and 5'-GAC-

CTGACTGACTACCTCA TGAAGAT-3' and 5'-GTCACATTCATGATGGAGTTGAAGG-3' for β-actin used as internal control. The PCR products separated by electrophoresis on a 2% agarose gel, Gels were stained with ethidium bromide, destained, and photographed by the image analysis system; levels of survivin mRNA were displayed in arbitrary units as the proportion of survivin PCR product intensity to β-actin PCR product intensity from the same RNA sample.

## 2.8. Flow cytometric analysis of apoptosis

Quantification of apoptotic cells was performed using an Annexin-V-FITC Apoptosis Detection FACSCalibur flow-cytometer (Becton Dickinson Medical Devices Co., Ltd.) according to the Annexin V-FITC of manufacturer instructions. Briefly, cells plated in a 60-mm disk and treated with drugs for 48 h. Then cells collected and resuspended in 500 µL of binding buffer, and 2 µL of Annexin-V-fluorescein isothiocyanate (FITC) and 5 µL of propidium iodide (PI) added. Analyses were performed with a FACS called flow cytometer (Becton Dickinson, Sunnyvale, CA, USA) and with Ex = 488 nm, Em = 530 nm. The cells in the FITC-positive and PI-negative fraction regarded as apoptotic cells. The sample incubated for 5 min in the dark before analysis by flow cytometer.

## 3. Results

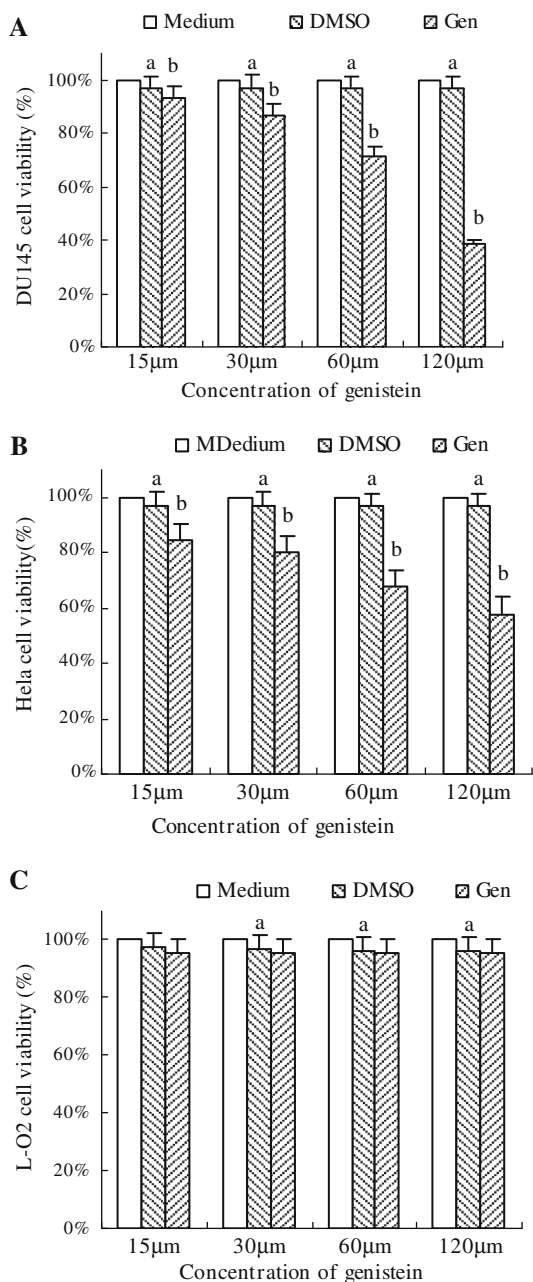
### 3.1. Effect of genistein treatment on cell viability

Determination of the damage with performed by measuring the MTT reduction ability of DU-145, HeLa and L-O2 cells on genistein. After three kinds of cells treated with genistein (15–120 µM) for 48 h, a concentration dependent decrease of cell viability found by MTT assay (Fig. 1). Compared with blank medium as control (Medium, Genistein (Gen) and its solvent (DMSO) inhibited cell viabilities in a dose-dependent, made allowance for DMSO results, Cell proliferation caused by genistein were respective as follows:  $0.93 \pm 0.044$ ,  $0.88 \pm 0.034$ ,  $0.72 \pm 0.027$  and  $0.44 \pm 0.029$  for DU-145 in Fig. 1A;  $0.88 \pm 0.079$ ,  $0.88 \pm 0.023$ ,  $0.83 \pm 0.051$  and  $0.78 \pm 0.038$  for HeLa in Fig. 1B;  $0.99 \pm 0.048$ ,  $0.97 \pm 0.018$ ,  $0.98 \pm 0.019$  and  $0.98 \pm 0.011$  for L-O2 in Fig. 1C, corresponding in sequence of concentration of 15 µM, 30 µM, 60 µM, 120 µM, respectively. The damage of L-O2, as normal cell, impacted growth of cells was basically DMSO but not genistein.

### 3.2. Microscopic counting and observation of DU-145 and HeLa cells treated with genistein and DMSO

Cells of DU-145 and HeLa were maintained in DMEM supplemented with 10% fetal bovine serum, all of cells ( $1 \times 10^5$ ) were seeded and 24 h later treated with 60 µM, 100 µM of concentrations of genistein, the blank was same quantities of DMSO to regard as contrast. Numbers of cells counted every 24 h. Fig. 2A shows the growth rate of DU-145 and HeLa cells, and (a) and (b) in Fig. 2A shows DU-145, and (c) and (d) were HeLa, cells number for 24, 48 and 75 h. After 72 h DU-145 cell growth was inhibited to 52.27%, 77.53%, and HeLa cell was 66.70%, 85.13% by genistein at 60 µM and 100 µM.

DU-145 were treated with genistein in concentration of 30 and 60 µM for 48 h then added 20 mg/mL of hoechst 33258. After DU-145 cells were incubated with or without genistein for 20 min in a 5% CO<sub>2</sub> incubator at 37 °C, cells were observed using an inverted fluorescence microscope (IX-70, Olympus, Japan). The addition of genistein to the cultures of DU-145 cells showed dose-dependent inhibition of cell growth. When the DU-145 cells were cultivated without genistein (DMSO), they proliferated normally and cells appeared enlarged with prominent nuclei (Fig. 2B). However, when genistein added at varying concentrations of genistein (30 µM, 60 µM), part of the cells suffered growth inhibition (Fig. 2B (b))



**Fig. 1.** Cells viability of DU-145, HeLa and L-O2 were detected by MTT. Effect of genistein (15, 30, 60, 120 μM) treatment on DU-145, HeLa of cancer cells and L-O2 of normal cell viability by MTT. Compared with blank medium as control (medium), Genistein (Gen) and its solvent (DMSO) inhibited cell viabilities in a dose-dependent, made allowance for DMSO results, genistein inhibitory cell proliferation was the strongest for DU-145 then HeLa and L-O2, and the weakest for L-O2 were treated with genistein (15–120 μM) for 48 h by MTT assay. (A) DU-145 cells; (B) HeLa cells; (C) L-O2 cells (a: Student's *t*-test,  $p < 0.05$  vs medium; b: Student's *t*-test,  $p < 0.05$  vs DMSO).

and (d) and appeared apoptotic cell. Genistein caused individual cells to shrink and separate from neighboring cells (Fig. 2B (d)). They appeared refringent and finally detached from the monolayer by 20 min after genistein administration.

### 3.3. Caspase-3 activity

Caspase-3 activity was assayed by Western blotting and a colorimetric method (Fig. 3), as the inactive 32 kDa proenzyme (procaspase-3) was decreasing, and after 12 h, reduced acutely. During apoptosis, procaspase-3 is activated by cleavage at specific Asp residues to generate active caspase-3, consisting of 17 and 12 kDa subunits. The quantitative results of caspase-3 activity in DU-145 cells treated with genistein (60 μM) were expressed using fold increase over control; the value at the beginning (time 0) was designated 1. After treatment with genistein (60 μM) for 0, 1, 3, 6, 12, or 24 h, the fold increases were  $1.01 \pm 0.022$ ,  $1.05 \pm 0.021$ ,  $1.23 \pm 0.14$ ,  $1.91 \pm 0.21$ , and  $2.78 \pm 0.24$ , respectively.

### 3.4. Genistein synergistic inhibition of cell growth with survivin gene RNAi

Compared with untransfected DU-145 cells (DU-1), transfected blank pU6 + 27 without shRNA (DU-2) and transfected pU6 + 27 with shRNA (DU-3). Total RNA, extracted from three kinds of DU-145 cells, were calculated concentration by measuring UV light absorbance at 260 nm, total RNA of DU-1 was 0.2025 μg/μL, DU-2 was 0.2075 μg/μL and DU-3 was 0.23 μg/μL.

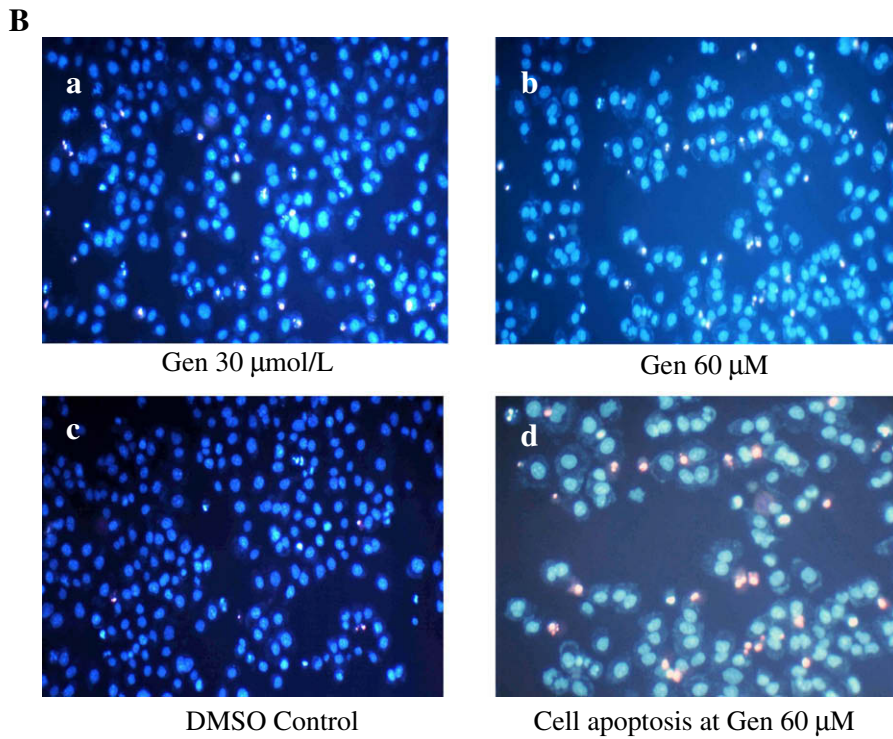
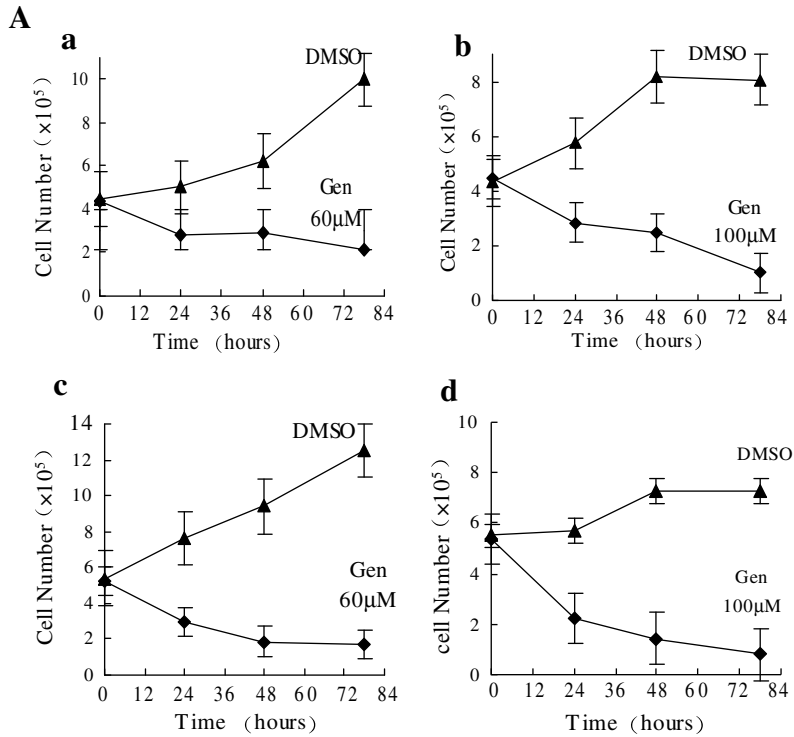
The expression levels of survivin mRNA in DU-3 showed further low compared with DU-1 and DU-2 obviously (Fig. 4A). The result confirmed that plasmid of shRNA was transfected into DU-145 cell (DU-3) successfully, and it performed in the role of RNA interference (RNAi) with survivin expression. Thus, synergistic effect of genistein with RNAi inhibiting survivin carried out with MTT method (Fig. 4B). Compared with control, the cell viability of DU-2 was  $0.97 \pm 0.04$ , DU-3 and genistein (60 μM) was  $0.65 \pm 0.056$ ,  $0.72 \pm 0.019$ , respectively, DU-3 plus genistein (60 μM, abbreviation DU-3 + Gen) was  $0.41 \pm 0.056$ , and inhibitory effect of DU-3 + Gen was the strongest among all of samples.

### 3.5. Genistein synergistic DU-145 apoptosis with RNAi to survivin

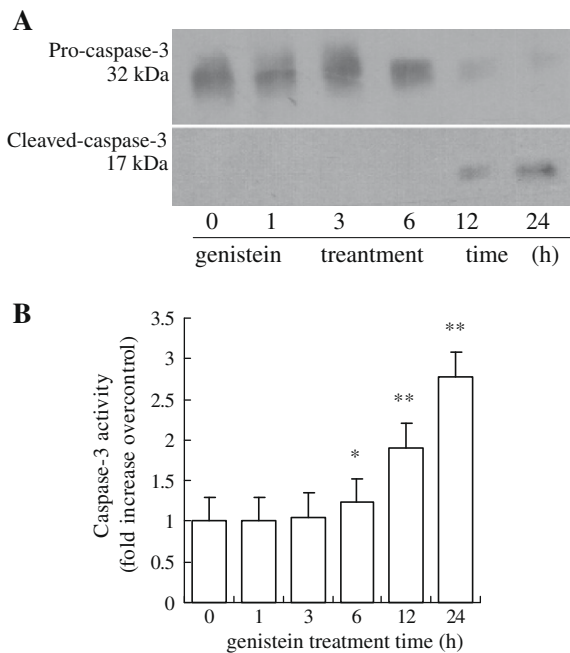
The synergistic effect of genistein with RNAi in survivin, arrested the growth and induced apoptosis of the DU-145, was quantitatively measured using flow cytometry. DU-145 cells of DU-1 (untransfected), DU-2 (transfected without shRNA) or DU-3 (transfected with shRNA) were cultured in DMEM up to about 80% confluence, and then were washed in PBS and centrifuged at 300 rpm for 5 min. Genistein was dissolved in DMSO. It was diluted to final concentrations in each culture medium to 30 μM, 60 μM and 120 μM, blank was equiareal DMSO, in medium for DU-3. Treated cells were incubated at 37 °C and 5% CO<sub>2</sub> in a humid environment for 48 h. To observe influencing factors from experimentation, DU-1 and DU-2 dealt with similarly. Fig. 5 displays the difference apoptosis of DU-1, DU-2 and DU-3 in natural state (Fig. 5 (a), (b) and (c)), the factor of DMSO was 0.91% (early) and 4.51% (later), deduct DMSO influence effect from Fig. 5 (e), genistein brought 6.87% (4.05% plus 2.82%) total ratio of apoptosis of DU-2 practically. In this way we counted ratio of apoptosis of DU-3 by genistein. In Fig. 6, the cell of DU-3, this was transfected shRNA for survivin and suffered from siRNA interference in survivin, showed apoptosis as result of synergetic RNAi and genistein. The synergetic effect genistein was demonstrated concentration dependent, make allowance for impacts of control or DMSO, We counted total rate of apoptosis were 10.56% at 30 μM of Gen, 19.61% at 60 μM of Gen and 30.00% at 120 μM of Gen, respectively.

## 4. Discussion

The results of our studies and others have shown that genistein arrests proliferation and induces apoptosis in cancer cells [12,13]; these findings are in agreement with other *in vitro* models in which DU-145 and normal human prostate epithelial cells are treated with genistein [1]. Our results demonstrated that genistein arrested growth of cancer cells (HeLa and DU-145) rather than normal cells (LO-2) at lower concentrations ( $\leq 30$  μM). The potential of targeting apoptotic pathways by genistein in cancer cells indicates that genistein is an ideal chemoprevention agent to

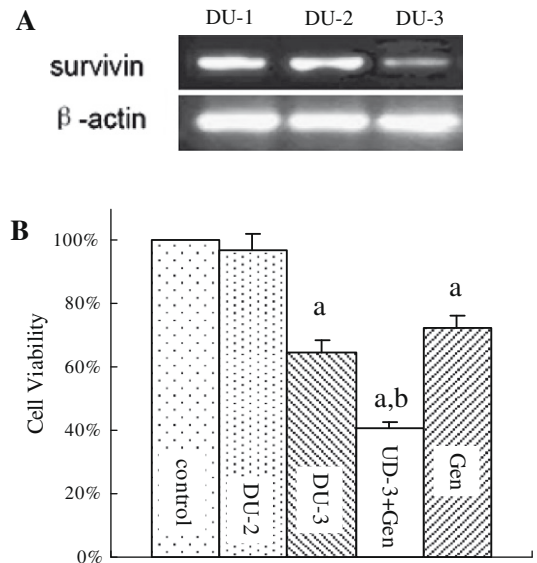


**Fig. 2.** Effect of genistein (Gen) on the growth and cell cycle of DU145 and Hela cells: (A) 24 h after inoculation of DU145 and Hela cells, Gen dissolved in DMSO was added for 24, 48 or 75 h and at concentrations of 60 or 100  $\mu$ M, and cells growth was compared with a control culture with equivalent DMSO alone. (a, b) of A was survival of DU-145 cells, and (c, d) of A was Hela cells. (B) Photographs of DU-145 cells cultivated without or with Gen for 48 h by hoechst staining. The rate of apoptotic cells increased with increasing concentration of Gen in DU-145 cells and the data not shown: (a) 30  $\mu$ M of Gen; (b) 60  $\mu$ M of Gen; (c) DMSO control and (d) apoptotic cell at 60  $\mu$ M of Gen.



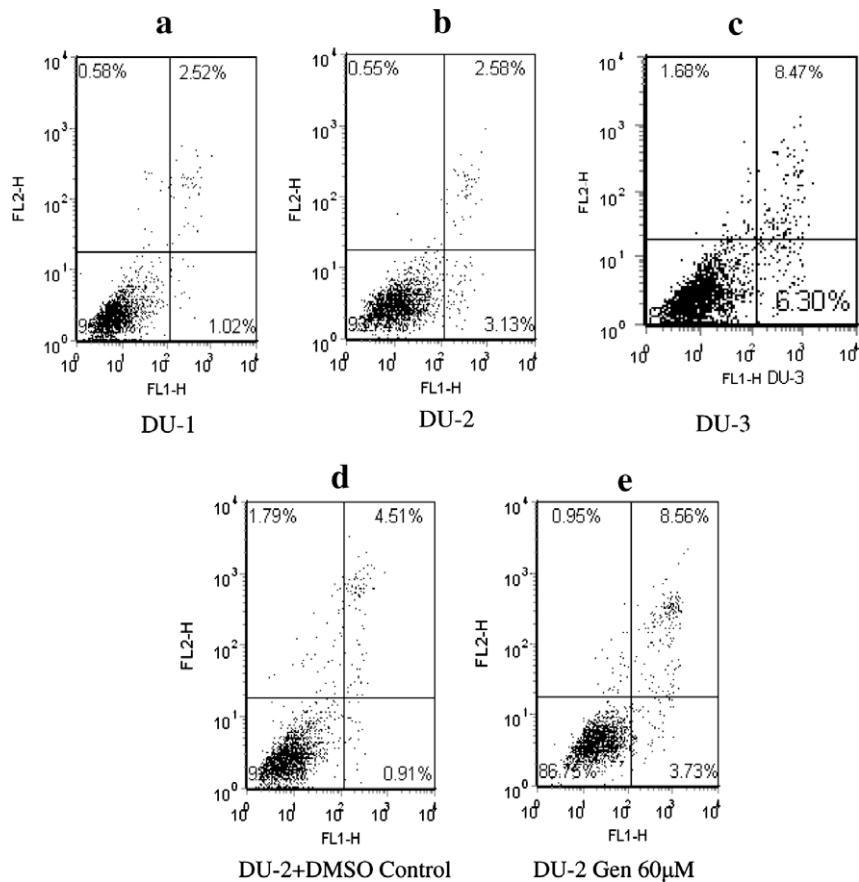
**Fig. 3.** Effect of genistein on caspase-3 activity in DU-145 cells. (A) Western blot assay caspase-3, procaspase-3 is inactive pro-enzyme with 32 kDa, during apoptosis, procaspase-3 is activated by cleavage at specific Asp residues to generate active caspase-3, consisting of 17 kDa subunits. Genistein-mediated apoptosis via caspase-3 cascade in DU-145 cells time-depend. (B) Effects of genistein on caspase-3 activity. Caspase-3 activity in lysates of DU-145 cells exposed to ethanol at 60  $\mu$ M of genistein for 0, 1, 3, 6, 12 and 24 h was determined. \*Significantly different from control,  $p < 0.05$ ; \*\* $p < 0.01$  ( $n = 3$ ).

block or delay the process of carcinogenesis. On the other hand, prostate cancer is an attractive target for chemoprevention because of its ubiquity, treatment-related morbidity, long latency between premalignant lesions and clinically evident cancer, and defined molecular pathogenesis. The major isoflavone components of soy, including genistein, daidzein, and their metabolites, inhibit benign and malignant prostatic epithelial cell growth, down-regulate androgen-regulated genes, and reduce tumor growth in some animal models [14,15]. The early mechanisms of action against prostate cancer was that the soy isoflavone genistein is a potent and specific inhibitor of tyrosine autophosphorylation of the epidermal growth factor (EGF) receptor and genistein attenuates growth factor and cytokine-stimulated proliferation of both normal and cancer cells, in addition to including the signaling pathways of transforming growth factor (TGF $\beta$ 1) receptor [16] and insulin-like growth factor (IGF) receptor [17]. Our studies also verified that genistein acts as an EGF receptor inhibitor via inhibition of phosphorylation activity derived from protein-tyrosine kinase (PTK) *in vitro* (unpublished). EGFR dimerization stimulates its intrinsic intracellular protein-tyrosine kinase activity, as a result, autophosphorylation of several tyrosine residues in the C-terminal domain of EGFR occurs [18] and elicits downstream signaling proteins initiate several signal transduction cascades, principally the MAPK, Akt and JNK pathways, leading to DNA synthesis and



**Fig. 4.** RNA interference inhibited survivin gene expression in DU-3 cells, and synergistic effect of Gen with RNAi inhibiting survivin expression on DU-145. (A) mRNA expression of survivin was detected by semi-quantitative RT-PCR. Total RNA from DU-1, DU-2 and DU-3 cell lines were reverse transcribed and amplified by the primers from survivin and  $\beta$ -actin. The products of PCR were separated on a 2% agarose gel. Densitometric analysis was performed using scion image software and the inhibitory rates of survivin mRNA expression in DU-145 was 14.3%. (B) Compared with control, DU-3, Gen and DU-3 plus Gen all inhibited the growth of prostate cancer cells, and DU-3 + Gen was the strongest among of them. (a: Student's *t*-test,  $p < 0.05$  vs. control; b: Student's *t*-test,  $p < 0.05$  vs. DU-3, Gen).

cell proliferation [19]. Genistein antagonised the activation of the PTK signal pathway by growth factors but did not suppress JNK protein phosphorylation [20]. The PTK-inhibitory effect is one of the most important biological functions of genistein. However, it is difficult to understand the multiple activities of genistein through interfering with signaling pathways of the EGF receptors in cells. Further studies are required to elucidate the precise process by which genistein acts on the signal transduction pathways in cancer cells and also the changes of signal molecules at various time phases. In brief, the efficacy of signaling targets, cell cycle regulators, cell survival, apoptotic targets, and angiogenic and metastatic target pathways are some common mechanisms of potential targets for prostate cancer chemoprevention agents [21,22], of which genistein, epigallocatechin gallate (EGCG) [23], quercetin [24], curcumin [25], resveratrol [26], and indole-3-carbinol [27] are now well-known. These agents have been shown to ultimately inhibit prostate cancer cell proliferation *in vitro*, as well as tumor growth *in vivo* [25]. Epidemiologic studies suggest that diet can act as a chemopreventive agent to reduce the incidence of prostate cancer as well as to reduce the mortality of the disease [28]. Prostate cancer has a long latency period, which means that it grows slowly and may exist for years before it is detected or causes symptoms. Herbs, nutritional supplements, and dietary changes can prevent the induction, growth, or progression of cancer. Researchers examined the effects of pure soy isoflavones



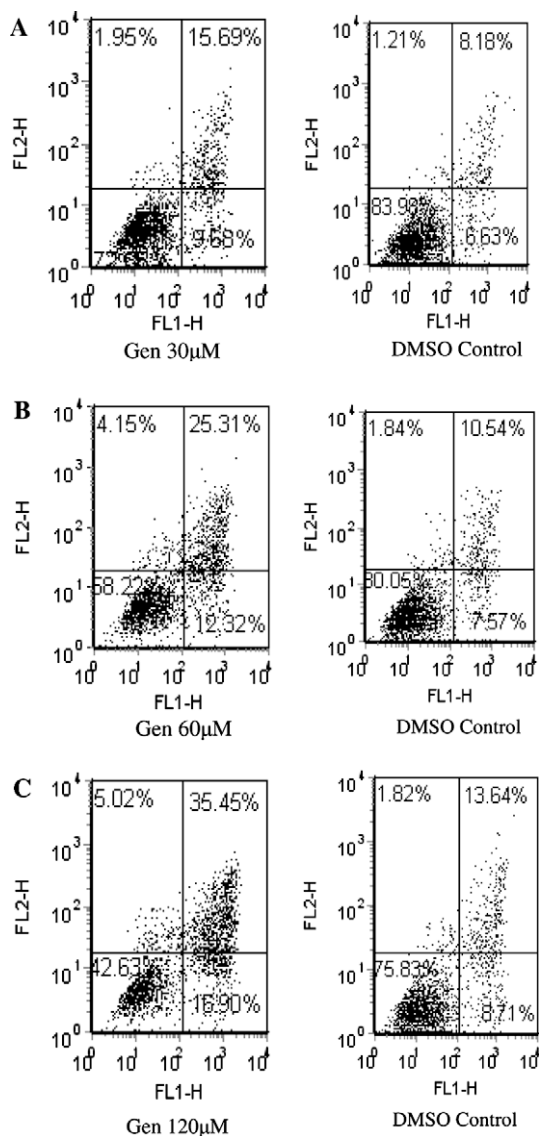
**Fig. 5.** To compare the effect of cell apoptosis by survivin of RNAi, untransfected DU-1 of DU-145, the empty vector transfected DU-2 of DU-145 and survivin-specific siRNA stable transfected DU-3 of DU-145 were analysed apoptotic level using flow cytometry. The apoptotic level of DU-3 (c) was higher than DU-1 and DU-2 (a, b) obviously, and the level of genistein causing apoptosis approached DU-3 (d, e).

(genistein, genistin, daidzein, and biochanin A) and soy phytochemical concentrate on the growth of mouse and human prostate cancer cell lines *in vitro* and *in vivo* [29].

Survivin is the inhibitor of the apoptosis protein which protects cancer cells [30]. To verify the protective effect of survivin expression against the chemotherapeutic substances of genistein, survivin expression was silenced using siRNA. Our results demonstrated that genistein arrests the growth and induces apoptosis or death of cancer cells both with and without suppressed survivin expression. Genistein is able to interfere with the protective effect of survivin to induce apoptosis of HeLa and DU-145 cancer cells untreated by RNAi, and genistein synergistically effects DU-145 apoptosis with silenced survivin expression that induces survivin down-regulation. Our data are in agreement with the induction of apoptosis by genistein in ovarian cancer cells of A2780, CaOV3, and ES2 [31]. Genistein can induce apoptotic the ovarian carcinoma cell lines involved in autophagic cell death and it has the potential to circumvent chemoresistance due to alterations in apoptotic signaling. Studies related to the combined survivin and chemopreventive compound of quercetin, butyrate, in cancer cells have appeared. Quercetin-enhanced tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)

cytotoxicity was not related to quercetin-induced cell cycle arrest in DU-145, but the expression of survivin was down-regulated by treatment with quercetin [32]. Butyrate can induce cell cycle arrest and apoptosis through interaction with the caspase signaling cascade. IAPs involved in survivin are over-expressed in several colon cancer cell lines leading to apoptotic resistance; the effect on the down-regulation of survivin is intensified by co-treatment with butyrate in Caco-2 cells [33]. Compared with the above, genistein not only arrests the cancer cell cycle, but also down-regulates IAPs expression. Genistein was shown to inhibit the growth of various cancer cells through the modulation of genes that are intimately related to the regulation of cell cycle and apoptosis. As an ideal chemopreventive and therapeutic agent, the inhibitory effects of genistein on cancer cells unveil the multi-targeted and pleiotropic molecular mechanisms [34].

The molecular mechanisms of survivin regulation by genistein are still not fully understood; however, the fact that survivin becomes an ideal target for cancer therapy as cancer cells are targeted while normal cells are spared was verified *in vitro* by the current study and another study [35]. In summary, the data presented in this study with the prostate cancer cell line, DU-145, provide a new basis to



**Fig. 6.** Induction of apoptosis, measured by Annexin V assay following treatment with genistein 30, 60, 120  $\mu\text{M}$ , and control cells treated with equal of DMSO, respectively. (A) apoptosis of DU-3 at 30  $\mu\text{M}$ , (B) at 60  $\mu\text{M}$ , and (C) at 120  $\mu\text{M}$  of genistein. Base on survivin of RNAi, it was demonstrated that genistein induced cells apoptosis with concentration dependent.

combine treatment cancer or tumor cells with survivin RNAi and genistein for therapy of previous *in vivo* studies. Genistein inhibited viability of DU-145 ranged from 28% to 56% and of HeLa ranged from 17% to 22%, which were dose-dependent. Caspase-3 is an enzyme that plays a key role in programmed cell death, or apoptosis. Caspase-3 has been likened to the henchman that goes around and executes the cell; it is a member of the family of aspartate-specific cysteinyl proteases. Genistein mediates apoptosis via the caspase-3 cascade in DU-145 cells. Combined treatment with survivin RNAi and varying concentrations of genistein showed a stronger inducible apoptotic effect (59%) on prostate cells (DU-145). It may be further explored for

devising chemopreventive and/or therapeutic strategies for prostate cancer. However, genistein induced apoptosis independently and had a synergistic effect on the inhibition of prostate cancer cells.

### Conflict of interest

None declared.

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