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Apoptosis induction of colorectal cancer cells HTL-9 in vitro by the transformed products of soybean isoflavones by *Ganoderma lucidum*

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Abstract: Soybean isoflavones have been one of the potential preventive candidates for antitumor research in recent years. In this paper, we first studied the transformation of soybean isoflavones with the homogenized slurry of *Ganoderma lucidum*. The resultant transformed products (TSI) contained (703.21±4.35) mg/g of genistein, with transformed rates of 96.63% and 87.82% of daidzein and genistein, respectively, and TSI also could enrich the bio-active metabolites of *G. lucidum*. The antitumor effects of TSI on human colorectal cancer cell line HTL-9, human breast cancer cell line MCF-7, and human immortalized gastric epithelial cell line GES-1 were also studied. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay showed that TSI could dramatically reduce the viability rates of HTL-9 cells and MCF-7 cells without detectable cytotoxicity on GES-1 normal cells when the TSI concentration was lower than 100 μg/ml. With 100 μg/ml of TSI, HTL-9 cells were arrested in the G1 phase, and late-apoptosis was primarily induced, accompanied with partial early-apoptosis. TSI could induce primarily early-apoptosis by arresting cells in the G1 phase of MCF-7 cells. For HTL-9 cells, Western-blot and reverse-transcriptase polymerase chain reaction (RT-PCR) analysis showed that TSI (100 μg/ml) can up-regulate the expression of Bax, Caspase-3, Caspase-8, and cytochrome c (Cyto-c), indicating that TSI could induce cell apoptosis mainly through the mitochondrial pathway. In addition, the expression of p53 was up-regulated, while the expression of Survivin and nuclear factor κB (NF-κB) was down-regulated. All these results showed that TSI could induce apoptosis of HTL-9 cells by the regulation of multiple apoptosis-related genes.

Key words: Soybean isoflavones; *Ganoderma lucidum*; Transformation; Antiproliferative activity; Apoptosis
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1 Introduction

The GLOBOCAN 2012 shows that there are approximate 14.1 million new cancer cases and 8.2 million death cases each year worldwide. The most commonly diagnosed cancers are lung cancer, breast cancer, and colorectal cancer, and the colorectal cancer cases represent almost 1.4 million and account for 9.7% of the total cancer cases (Ferlay *et al*., 2015). Due to the rising incidence and mortality rate of cancers, more and more natural products are being used for cancer prevention and adjuvant treatment, such as polysaccharides, macrolide (Lim *et al*., 2012), phytoestrogen, peptides (Suarez-Jimenez *et al*., 2012), isoflavones (Szliszka *et al*., 2011), and anthranone (Gong *et al*., 2011).

Soybean isoflavones are the secondary metabolites formed during the period of soybean growth, and their main ingredients contain genistein, daidzein, and glycitein. Epidemiologic research has proved that soybean isoflavones have many pharmacological effects. They can enhance body immunity (Wei *et al*.,

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2012; Zhou *et al*., 2015) and improve learning ability and memory in menopausal women. Moreover, they can prevent and treat cardiovascular disease, osteoporosis (Srivastava *et al*., 2014; Baglia *et al*., 2015), breast cancer, prostate cancer (Li *et al*., 2012a), colon cancer (Tse and Eslick, 2016), and menopausal syndrome (Ollberding *et al*., 2012). The possible mechanisms of antitumor activity include an estrogen-like effect (Mense *et al*., 2008; Choi and Kim, 2013), the impact on the androgen receptor pathway by inhibiting the expression of prostate specific antigen (PSA) (Banerjee *et al*., 2012), the regulation of cell cycle and apoptosis induction (Prietsch *et al*., 2014; Tsuboy *et al*., 2014), and the regulation of mitogen activated protein kinase (MAPK) pathway (Cotrim *et al*., 2013; Chen *et al*., 2014).

In natural conditions, 97%–98% soybean isoflavones exist in the form of glycosides (Hati *et al*., 2015) which cannot be directly absorbed by the body. Isoflavones have to be hydrolyzed into aglycones for absorption and to exert their pharmacological activity (Andlauer *et al*., 2000; Handa *et al*., 2014). Therefore, more and more research focuses on the enzymatic hydrolysis of isoflavones by microorganisms (Yeo and Liong, 2010; Yeom *et al*., 2012; Maitan-Alfenas *et al*., 2014). Also there has been research on how to transform isoflavones by food microorganisms and then create transformed products as a health food of potential to be a new application of isoflavones (Ewe *et al*., 2012; Titiek *et al*., 2013; Yin *et al*., 2014).

Ganoderma lucidum (Leyss. ex. Fr.) Karst, a fungus in the group of Basidiomycetes, has increasingly been explored as a valuable traditional Chinese medicinal mushroom for over 2000 years. A lot of research has demonstrated that *G. lucidum* is extremely effective in prevention and treatment of various diseases, such as cancer (Zhao *et al*., 2011), hyperglycaemia (Guo *et al*., 2013), hepatitis (Kim *et al*., 2006), cardiovascular diseases (Lee and Rhee, 1990), human immunodeficiency virus 1 (HIV-1) (Kang *et al*., 2015). In addition, the polysaccharides and triterpenoids are the major bioactive metabolites and have some important medicinal properties (Keypour *et al*., 2010).

In this study, soybean isoflavones were transformed by the homogenized slurry of *G. lucidum* for the first time. The antitumor effects of the transformed products on HTL-9 cells and MCF-7 cells were tested, followed by the determination of cell cycle arrest and apoptosis induction. The apoptosis-related genes and proteins were analyzed to preliminarily clarify the antitumor mechanism.

2 Materials and methods

2.1 Liquid cultivation of *Ganoderma lucidum*

G. lucidum was grown in a 250-ml flask containing 100 ml seed culture at 28 °C for 8 d with shaking at 180 r/min, and was then inoculated at 10% (v/v) into the fermentation culture and cultivated at 28 °C for 7 d with shaking at 180 r/min.

The seed culture contained (g/L): potato extract 10, glucose 20, peptone 18, KH_2PO_4 3, $MgSO_4$ 1.5, and vitamin B_1 (VB_1) 0.05 (pH 5.5). The fermentation culture contained (g/L): wort 41, peptone 18.9, KH_2PO_4 3, $MgSO_4$ 1.5, and VB_1 0.05 (pH 5.4) (Cui *et al*., 2015).

Wort was made from barley by means of official analysis methods of the European Brewery Convention (EBC) (Munck *et al*., 1989), and was assessed by its total sugar contents.

2.2 Transformation of soybean isoflavones by *Ganoderma lucidum*

After fermentation, the broth and mycelia of *G. lucidum* were homogenized and the activity of β-glucosidase was determined. A volume of 100 ml slurry with β-glucosidase of 1.0 U/ml was made as a reaction solution, and 5 g soybean isoflavones were added and transformed for 48 h at 60 °C with pH 5.0. The transformed products were freeze-dried and collected. The ingredients of the transformed products were extracted with 50% (v/v) ethanol at 60 °C for 1 h, and then the supernatant was collected, concentrated, and made as a stock solution by passing through a 0.22-μm filter (Millipore, USA). This solution was called TSI and assessed by its total isoflavone content.

The analysis of isoflavone ingredients and the determination of transformed rates of daidzein (R_d) and genistein (R_g) were made using an Agilent series 1100 high-performance liquid chromatography (HPLC) instrument (Agilent, Germany) and R_d and R_g were calculated by

$$R_d=C_d/C_{dd}\times100\%,$$
$$R_g=C_g/C_{gg}\times100\%,$$

where C_d and C_g are the contents of daidzein and genistein after transformation, respectively; while C_{dd} and C_{gg} are the contents of "daidzin+daidzein" and "genistin+genistein" before transformation, respectively. The sample was separated on a ZORBAX SB-C18 column (5 μm, 4.6 mm×250 mm; Agilent). The mobile phase consisted of 0.2% (v/v) aqueous acetic acid (A) and acetonitrile (B) with a linear gradient program of B including: 10%–63% in 0–25 min. The flow rate was 1 ml/min with a column temperature of 30 °C. The diode array detector (DAD) detector was monitored at 254 nm ultra violet (UV) spectra and 3D-plots were recorded between 200 and 400 nm.

2.3 Cell lines and cell culture

Human colorectal cancer cell line HTL-9, human breast cancer cell line MCF-7, and human immortalized gastric epithelial cell line GES-1 were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Science (Shanghai, China). These three cell lines were routinely suspended in RPMI-1640 medium (HyClone, USA) containing 10% (v/v) fetal bovine serum (FBS) and 100 U/ml streptomycin-penicillin, and incubated in a humidified atmosphere at 37 °C with 5% CO_2.

2.4 MTT assay of cell viability

The effects of TSI on the viability of these three cell lines were determined using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. These cells were harvested and seeded at a density of 1×10^5–5×10^5 cells/ml in 96-well plates. After incubation for 24 h, the serial dilutions of TSI (0, 20, 40, 60, 80, 100, 120 μg/ml) were added to each well and incubated for 24 h. The viability was determined by adding 20 μl of MTT solution (5 mg/ml in phosphate buffered saline (PBS)). Living cells reduce the yellow MTT to a blue formazan product. After 3 h of incubation at 37 °C, the formazan product was dissolved in 150 μl dimethyl sulfoxide (DMSO) from each well, and the plates were read at 490 nm using the Multiskan GO. The percentage of cell viability was expressed as a ratio versus control (van Meerloo *et al*., 2011).

2.5 Assay of cell apoptosis and cell cycle by flow cytometry

All these cells were seeded in 6-well plates at a density of 1×10^6 cells/ml and incubated for 24 h, and then treated with the suitable concentration of TSI for 24 h (media alone as control). At the end of the incubation period, the cells were collected and washed twice by cold PBS (200*g*, 5 min). For apoptosis assay, the cells (1×10^6 cells/ml) were resuspended in 500 μl binding buffer, and were stained with Annexin V-fluorescein isothiocyanate (FITC) (5 μl) and propidium iodide (PI) (10 μl) in the dark for 5–15 min at 2–8 °C. The treated cells were analyzed by the flow cytometry method (FCM) within 1 h (Priyadarsini *et al*., 2010). For cell cycle assay, the cells (1×10^6 cells/ml) were fixed with 70% cold ethanol overnight at −20 °C, and then washed twice by cold PBS (200*g*, 5 min), followed by treating with RNaseA (100 μl) for 30 min at 37 °C and PI (400 μl) staining in the dark for 30 min at 4 °C. Finally, these treated cells were analyzed by FCM within 1 h.

2.6 RT-PCR analysis of apoptosis-related genes

The expression of apoptosis-related genes was measured by reverse transcription-polymerase chain reaction (RT-PCR), with the expression of human 18S rRNA as internal control. HTL-9 cells were seeded in 6-well plates with a density of 1×10^6 cells/ml and incubated for 24 h, followed by treating with a suitable concentration of TSI for 24 h (media alone as control). After treatment, the total RNA of cells was isolated using TRIzol reagent (Sangon Biotech, China) according to the manufacturer's protocol, and RNA quality was determined by NanoDrop-1000 (Thermo, USA). Reverse transcription was performed using the Roche Transcriptor complementary DNA (cDNA) Synth. Kit (Roche, Switzerland) and the primers of target genes are shown in Table 1. PCR reaction was performed using a Roche FastStart Universal SYBR Green Master (ROX) kit (Roche, Switzerland).

2.7 Western-blot analysis

HTL-9 cells were seeded into 6-well plates at a density of 1×10^6 cells/ml and incubated for 24 h, followed by treating with a suitable concentration of TSI for 24 h (media alone as control). After treatment, the cells were collected and washed twice by PBS. Lysis buffer (0.2–0.5 ml) mixed with phosphatase inhibitors (10 μl), protease inhibitors (1 μl), and 100 mmol/L phenylmethanesulfonyl fluoride (PMSF) (5 μl) was added to extract cell protein. After quantitative detection, the cell protein was separated by 5% (0.05 g/ml) sodium dodecyl sulfate-polyacrylamide

Table 1 RT-PCR primers of target genes

Gene sequence number	Gene name	Primer sequence
NR_003286	HOMO-18S-F	CAGCCACCCGAGATTGAGCA
	HOMO-18S-R	TAGTAGCGACGGGCGGTGTG
NM_002046	HOMO-P53/TP53-F	GAAGATGGTGATGGGATTTC
	HOMO-P53/TP53-R	GAAGGTGAAGGTCGGAGTC
NM_000546.4	HOMO-AMPKa2/PRKAA2-F	AGGCCTTGGAACTCAAGGAT
	HOMO-AMPKa2/PRKAA2-R	CCCTTTTTGGACTTCAGGTG
NM_006252.3	HOMO-Bax-F	CTGAGAAGCAGAAGCACGAC
	HOMO-Bax-R	ACAACATCTAAACTGCGAAT
NM_001291428.1	HOMO-Bcl2-F	CTCAGGATGCGTCCACCAA
	HOMO-Bcl2-R	CCTCTGCAGCTCCATGTTACTGT
NM_138578.1	HOMO-Caspase-3/CASP3-F	CCTGACGGGCATGACTGTGG
	HOMO-Caspase-3/CASP3-R	TGGACGGAGGATGTGGTGGA
NM_032991.2	HOMO-Caspase-8/CASP8-F	TGGTTCATCCAGTCGCTTTG
	HOMO-Caspase-8/CASP8-R	AATTCTGTTGCCACCTTTCG
NM_001228.4	HOMO-Caspase-9/CASP9-F	AGGAGCTGCTCTTCCGAATT
	HOMO-Caspase-9/CASP9-R	GTGTCTGGCACTGGCTGTTT
NM_032996.2	HOMO-Survivin/BIRC5-F	AAGCCCAAGCTCTTTTTCATC
	HOMO-Survivin/BIRC5-R	ACTCGTCTTCAGGGGAAGTG
NM_001168.2	HOMO-GAPDH-F	TGGGAAGGGTTGTGAATGAG
	HOMO-GAPDH-R	CAGTTTGGCTTGCTGGTCTC
NM_019887.5	HOMO-Smac/DIABLO-F	GCAGCGTAACTTCATTCTTC
	HOMO-Smac/DIABLO-R	CAAAGCCAATCGTCACAG

gel (SDS-PAGE). The protein expression of cytochrome c (Cyto-c), nuclear factor κB (NF-κB), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (internal control) was detected by Western-blot using protein-selective antibodies (KeyGen Biotech, China). Antibodies were incubated overnight at 4 °C and then incubated with goat anti-Rb IgG-HRP with shaking for 1–2 h at room temperature. The immunolabeled proteins were detected by G:BOX ChemiXR5 (Syngene, UK) using an enhanced chemiluminescence (ECL) reagent kit.

2.8 Statistical analysis

All experiments were performed in triplicate and the results were presented as the mean±standard deviation (SD) using SPSS 17.0. Comparisons among all groups were performed with the one-way analysis of variance (ANOVA) test. $P<0.05$ was considered statistically significant.

3 Results

3.1 Transformation of soybean isoflavones by *Ganoderma lucidum*

The contents of daidzein and genistein in soybean isoflavones extract were (85.45±1.72) and (31.48±1.04) mg/g, respectively. After transformation, the genistein content increased to (703.21±4.35) mg/g, and the transformed rates of daidzein and genistein could reach 96.63% and 87.82%, respectively (Fig. 1). More importantly, this is the first time soybean isoflavones have been transformed with the homogenate of *G. lucidum*, including (1.87±0.02) g of mycelia, (93.21±0.79) mg of intracellular triterpenoid, and (45.63±0.36) mg of intracellular polysaccharides. These could eventually be collected in the transformed products. In brief, the transformed products contained not only glycosides, but also bioactive metabolites of *G. lucidum*, and these could provide a new direction for the development of health products based on soybean isoflavones.

3.2 Antiproliferative activity in vitro

Fig. 2 showed the viability of HTL9 cells and MCF-7 cells in the presence of different concentrations of TSI. The proliferation of cancer cells was dramatically reduced with TSI concentration affected. When the concentration of TSI was 80 μg/ml, the cell viability rates of HTL-9 cells and MCF-7 cells were decreased to (83.32±0.08)% ($P<0.05$) and (83.96±0.08)% ($P<0.05$), respectively, and when the concentration reached 100 μg/ml, the cell viability rate fell to (69.38±0.02)% ($P<0.01$) and (58.85±0.11)%

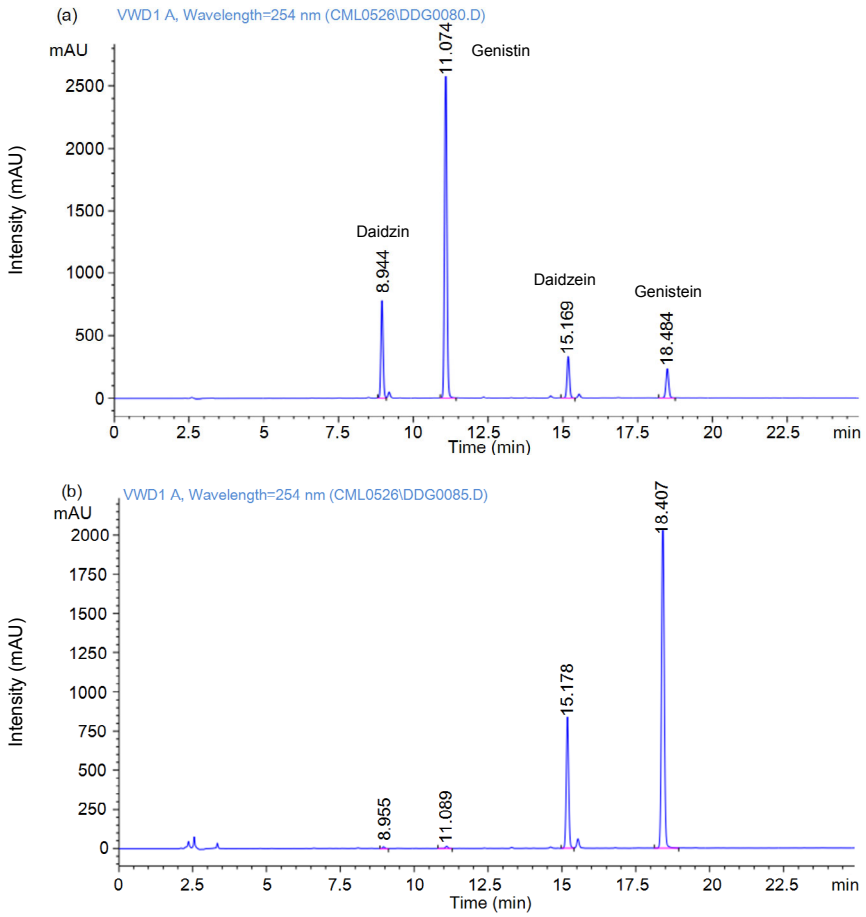

Fig. 1 HPLC analysis for soybean isoflavones

(a) Non-transformed soybean isoflavones; (b) Isoflavones of transformed products (transformation conditions: soybean isoflavones 5 g, 60 °C, 48 h). In non-transformed soybean isoflavones, the contents of daidzein and genistein were (85.45±1.72) and (31.48±1.04) mg/g, respectively. After transformation, the contents of genistein could reach (703.21±4.35) mg/g, and their transformed rates could reach 96.63% and 87.82%, respectively

(P<0.01), respectively. In addition, the viability of these two cancer cell lines was more seriously decreased as TSI concentration continuously increased. For GES-1 cells, the viability of cells was not significantly affected when the TSI concentration was lower than 100 μg/ml. However, the GES-1 cell viability rate decreased to (84.42±0.02)% (P<0.05) when the TSI concentration reached 120 μg/ml, which showed that this concentration was cytotoxic to GES-1. Therefore, TSI (≤100 μg/ml) had effectively antiproliferative activity on cancer cells in vitro, and TSI of 100 μg/ml was chosen for the following experiments.

3.3 Apoptosis-inducing and cell cycle arrest analyses

The apoptosis-inducing analysis of TSI on these cells was carried out by FCM with Annexin V-FITC and PI staining. As shown in Fig. 3, cells were gated into groups B1, B2, B3, and B4, which represented dead cells (Annexin V-FITC−/PI+), late-apoptotic cells (Annexin V-FITC+/PI+), viable cells (Annexin V-FITC−/PI−), and early-apoptotic cells (Annexin V-FITC+/PI−), respectively. After treatment with TSI (100 μg/ml), for HTL-9 cells, the late-apoptotic cells increased from 8.27% to 40.13%, and the early-apoptotic cells also increased from 0.95% to 9.05%, while the viable cells were decreased from 90.55% to 50.11%. This showed TSI could induce HTL-9 cells apoptosis primarily by means of late-apoptosis, accompanied with partial early-apoptosis. For MCF-7 cells, the late-apoptotic cells increased from 9.62% to 10.33%, and the early-apoptotic cells increased from 1.31% to 5.40%. This showed that TSI could induce MCF-7 cells apoptosis by early-apoptosis.

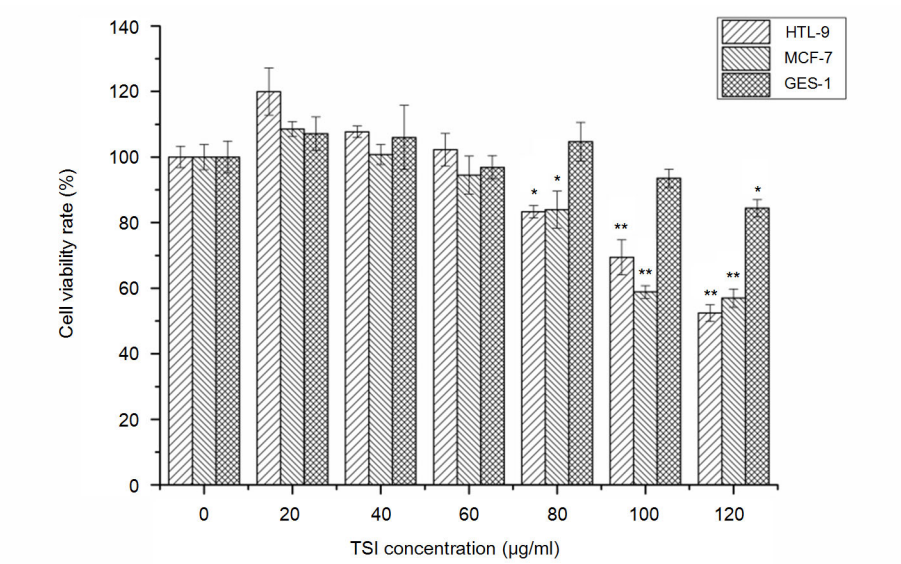

Fig. 2 Effect of TSI on viability of HTL-9 cells, MCF-7 cells, and GES-1 cells assayed by MTT

Values were expressed as the average of triple determination with ±SD. * $P<0.05$, ** $P<0.01$ (values of test groups compared with that of non-treatment). TSI could dramatically reduce viability of HTL-9 cells and MCF-7 cells as TSI concentration increased, whereas, the viability of normal GES-1 cells was not significantly affected when the TSI concentration was lower than 100 μg/ml

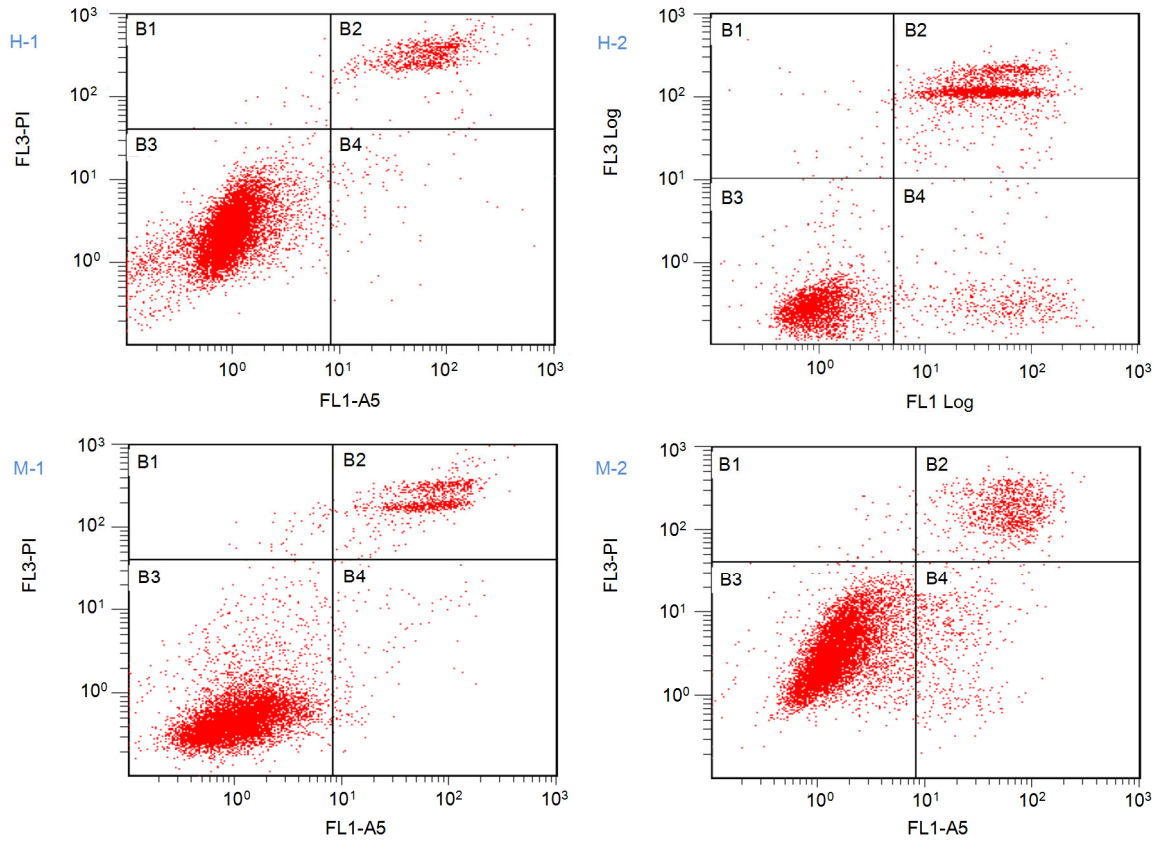

Fig. 3 Apoptosis induction by TSI on HTL9 cells and MCF-7 cells

(-1) Non-treatment; (-2) TSI (100 μg/ml). Proportions in different groups were as following: for HTL-9, (H-1) B2, 8.27%, B4, 0.95%, (H-2) B2, 40.13%, B4, 9.05%; for MCF-7, (M-1) B2, 9.62%, B4, 1.31%, (M-2) B2, 10.33%, B4, 5.40%, which showed that TSI (100 μg/ml) could effectively induce cell apoptosis of HTL-9 and MCF-7 cancer cells

HTL-9 cells and MCF-7 cells were treated with 100 μg/ml of TSI for 24 h and analyzed by FCM with PI staining for cell cycle arrest analysis. It is evident from the data shown in Fig. 4 that TSI (100 μg/ml) produced a significant arrest of growth of the cells in the G1 phase (47.73% to 75.24%) with a decrease in the S phase (37.11% to 19.01%) and the G2 phase (15.15% to 5.74%) of HTL-9 cancer cells compared with the untreated control. An increase in the G1 phase (68.98% to 73.54%) and the G2 phase (0.86% to 6.51%) with a decrease in S phase (30.15% to 19.94%) was observed in MCF-7 cells. It can be seen that treatment with TSI (100 μg/ml) can arrest cells in the G1 phase and prevent cell DNA synthesis and further division of HTL-9 cells and MCF-7 cells, and these eventually induce cell apoptosis.

From the cell apoptosis and cell cycle analyses, TSI (100 μg/ml) is more effective in inducing cell apoptosis in HTL-9 cancer cells than in MCF-7 cells, so HTL-9 cells were chosen for further study to explore the apoptosis mechanism with TSI treatment.

3.4 Apoptosis-related protein and gene expression of HTL-9 cells with TSI treatment

The protein expression of Cyto-c and NF-κB (P65) was detected using Western-blot analysis. As shown in Fig. 5, TSI (100 μg/ml) can induce a significant increase in the protein expression of Cyto-c, while an obvious decrease occurred in the expression of NF-κB. The relative protein expression level of Cyto-c was approximately 5.5 times higher than that in non-treated cells, while the NF-κB expression was approximately 0.53 times that of control.

RT-PCR was performed for the expression of apoptosis-related genes of HTL-9 cells with TSI (100 μg/ml) treatment. The method of $2^{-\Delta\Delta C_T}$ was used to calculate the variation of gene expression levels, and $2^{\Delta C_T}\times 10^6$ was used for gene relative expression levels. Compared with the untreated cells, the relative mRNA expression of p53, Bax, Caspase-3, Caspase-8, and Survivin showed significant changes in HTL-9 cells with TSI treatment. As shown in Table 2, the mRNA expression of p53, Bax, Caspase-3, Caspase-8

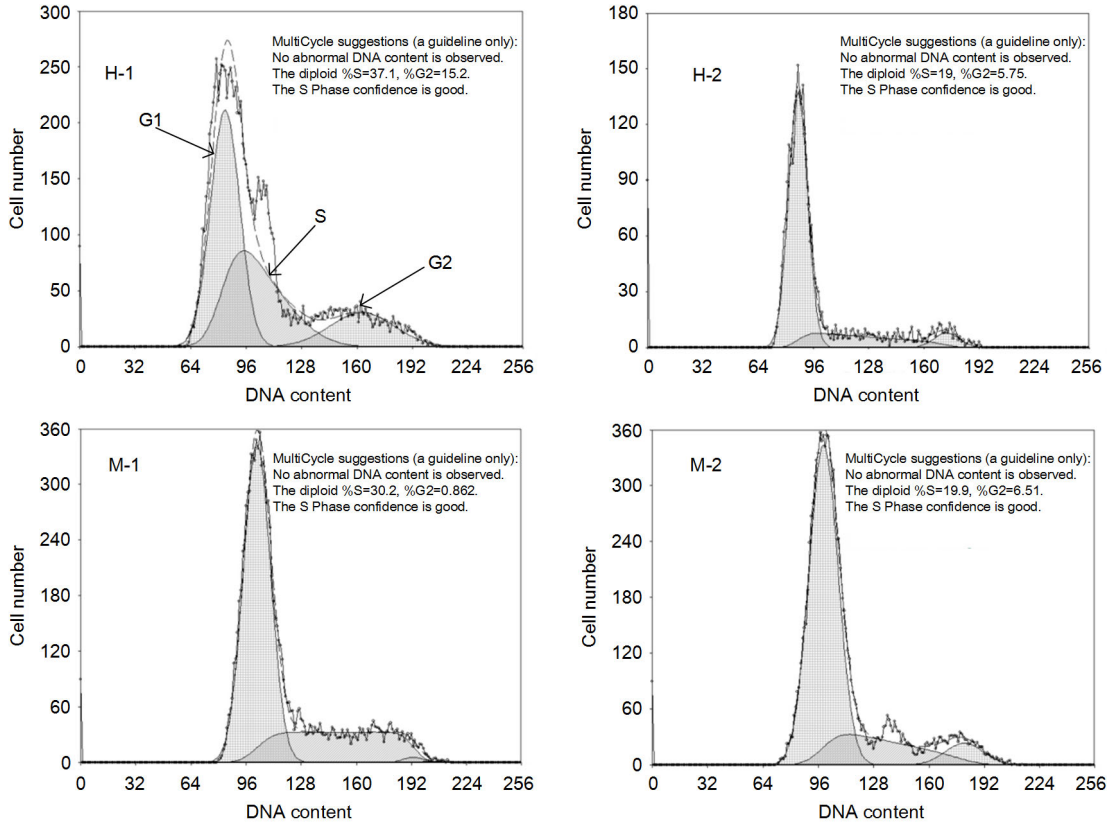

Fig. 4 Cell cycle analysis of HTL-9 cells and MCF-7 cells by FCM

(-1) Non-treatment; (-2) TSI (100 μg/ml). For HTL-9 cells: TSI (100 μg/ml) produced a significant arrest of growth of the cells in G1 phase (47.73% to 75.24%) with a decrease in S phase (37.11% to 19.01%) and G2 phase (15.15% to 5.74%). For MCF-7 cells: an increase in G1 phase (68.98% to 73.54%) and G2 phase (0.86% to 6.51%) with a decrease in S phase (30.15% to 19.94%)

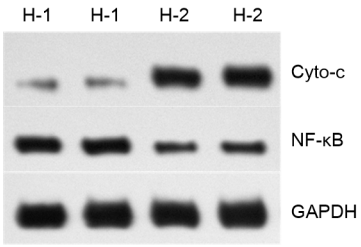

Fig. 5 Expression of Cyto-c and NF-κB of HTL-9 cells with TSI treatment

(-1) Non-treatment; (-2) Treatment with TSI (100 μg/ml). GAPDH was used to normalize protein loading. TSI could induce a significant increase (5.5 times higher) in the protein expression of Cyto-c, while an obvious decrease (0.53 times) occurred in expression of NF-κB

Table 2 Relative expression of the target genes in HTL-9 cells measured by RT-PCR

Gene	Expression level ($2^{\Delta C_T}\times10^6$)	
	Control	TSI (100 μg/ml)
p53	0.943±0.050	1.587±0.050**
Smac	1.093±0.090	1.160±0.140
AMPKa2	1.006±0.080	1.090±0.010
Bax	1.016±0.040	1.446±0.130**
Bcl-2	1.000±0.180	1.179±0.180
Caspase-3	1.000±0.020	1.330±0.020**
Caspase-8	1.083±0.070	1.438±0.130**
Caspase-9	0.923±0.090	0.917±0.040
Survivin	3.450±0.300	1.156±0.140**

The results were expressed as the average of triple determination with ±SD. * $P<0.05$, ** $P<0.01$ (values of test groups compared with that of non-treatment)

was significantly increased, while the mRNA expression level of the anti-apoptosis factor Survivin was significantly decreased to 1.156±0.140 while the expression in the control was 3.450±0.300.

From the comprehensive results of Western-blot and RT-PCR analyses with TSI treatment, it is shown that the relative mRNA expression of Bax significantly increased, the ratio of Bcl-2/Bax mRNA expression was significantly decreased, and the protein expression of Cyto-c was also significantly increased; simultaneously, Caspase-3 and Caspase-8 were activated and their expression was both significantly increased. All these indicated that TSI treatment could induce cell apoptosis mainly through the mitochondrial pathway. Moreover, the mRNA expression of pro-apoptosis factor p53 was significantly increased, and the expression of anti-apoptosis factor Survivin and NF-κB was significantly decreased. In conclusion, TSI could induce HTL-9 cells apoptosis by the regulation of multiple apoptosis-related genes.

4 Discussion

Increasing lines of evidences have showed that both *G. lucidum* (Tang *et al*., 2006; Loganathan *et al*., 2014; Hsin *et al*., 2015) and soybean isoflavones (Kurahashi *et al*., 2007; Li *et al*., 2012b) have antitumor activity. In this study, for the first time, the transformation of soybean isoflavones was performed using the homogenate of *G. lucidum*, and the genistein contents could reach (703.21±4.35) mg/g, with transformed rates of 96.63% and 87.82% of daidzein and genistein, respectively. The transformed products also contained the bioactive metabolites of *G. lucidum*, including (1.87±0.02) g of mycelia, (93.21±0.79) mg of intracellular triterpenoids, and (45.63±0.36) mg of intracellular polysaccharides.

We evaluated the viability and apoptotic effects of TSI on human colorectal cancer cells HTL-9. The results showed that TSI can dramatically reduce cell viability of HTL-9 cells as TSI concentration increased, and 100 μg/ml of TSI can induce HTL-9 cells apoptosis primarily by means of late-apoptosis by arresting cells in the G1 phase. These findings were consistent with previous reports that isoflavones inhibited the proliferation of human colorectal cancer cells (Yan *et al*., 2010; Budhathoki *et al*., 2011). Therefore, it is possible to make TSI as a competitive candidate for antitumor research.

Cell apoptosis is regulated by complicated apoptosis-related genes. In the mitochondria apoptotic signaling pathway, when cells are at mitochondrial dysfunction, Cyto-c accesses into cytoplasm from mitochondria membrane (Mayola *et al*., 2011), binding with apoptotic protease activating factor-1 (APAF-1). In the presence of deoxyadenosine triphosphate (dATP), the caspase family is activated and triggers apoptotic cascade reactions (Indran *et al*., 2011; Miranda *et al*., 2014). Of these, the *Bcl-2* family genes play a vital role in the regulation of apoptosis, and contain anti-apoptotic genes (*Bcl-2*, *Bcl-xL*, etc.) and pro-apoptotic genes (*Bax*, *Bid*, etc.) (Thomas *et al*., 2013). Proliferation of cancer cells will increase with overexpression of Bcl-2, while the accumulation of apoptotic cells will occur with overexpression of

Bax, with the determination of cell's susceptibility for apoptosis by the ratio of Bcl-2/Bax expression (Xu *et al*., 2013; Al-Fatlawi *et al*., 2014). In this study, to further understand the apoptosis mechanism, apoptosis-related genes and proteins were detected by Western-blot and RT-PCR analyses. The results showed that TSI induced the expression of Bax, Caspase-3, Caspase-8, and Cyto-c while reducing the ratio of Bcl-2/Bax, indicating that TSI could induce cell apoptosis mainly through the mitochondrial pathway.

In cell apoptosis, NF-κB mainly acts as an anti-apoptosis factor and its expression can significantly increase in malignant tumors (Liu *et al*., 2012; Varfolomeev *et al*., 2015). Because of gene mutation in cancer cells, p53 is dysfunctional and cannot inhibit generation of glucose transporters (GLUTs), and this can result in significant accumulation of the level of glucose metabolism. Therefore, activation of p53 can induce cell apoptosis by down-regulating the glucose metabolism level (Liu *et al*., 2015). In addition, Survivin is a newly discovered member of inhibitors of apoptosis proteins (IAPs) family, and can inhibit cell apoptosis mainly through inhibition of the activity of the caspase family (Selent *et al*., 2013). In this study, with TSI treatment, the relative expression of NF-κB was approximately 0.53 times that of the control, the mRNA expression of p53 was significantly increased, while the expression of Survivin was significantly decreased. In summary, TSI can induce cell apoptosis mainly through the mitochondrial pathway, accompanied by the regulation of other apoptosis-related genes.

From the above experiment, we can obtain anti-tumor products with multiple-factors from the transformation, and these have a good effect on apoptosis induction as demonstrated in the cell experiment.

In addition, 5 g soybean isoflavones were added for the transformation, and then the ingredients of the transformed products were extracted with 50% (v/v) ethanol for 1 h. Because of the difference of polarity and extracted rates between bioactive compounds in *G. lucidum* and soybean isoflavones, we can see that the content and extracted rate of aglycones were the highest in the extracted supernatant. The extracted rates were not only affected by the solvent selection, but also by their own contents to a large extent. Therefore, it was speculated that the contribution of aglycones was much greater in the observed apoptosis induction.

5 Conclusions

Soybean isoflavones were transformed using the homogenized slurry of the mycelia and broth of *G. lucidum*. The transformed products (TSI) can significantly reduce viability of HTL-9 and MCF-7 cells in vitro, arrest HTL-9 cells in the G1 phase, and primarily induce cell late-apoptosis by means of the mitochondrial pathway, along with the regulation of other apoptosis-related genes. Our results suggested the potential use of TSI as functional food or nutraceutical ingredients for chemotherapy. Further studies should be carried out to detail the interreaction and optimal proportion between aglycones, polysaccharides, and triterpenoids for apoptosis induction, as well as the mechanism underlying TSI-induced apoptosis.

Compliance with ethics guidelines

Mei-lin CUI, Huan-yi YANG, and Guo-qing HE declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：灵芝菌生物转化大豆异黄酮及其产物对结直肠癌细胞 HTL-9 的体外凋亡诱导研究

目　的：通过灵芝菌生物转化大豆异黄酮，得到富含苷元及灵芝活性成分的多因子转化产物，并研究了转化产物对结直肠癌细胞 HTL-9 的体外凋亡诱导，初步探讨转化产物的抗癌活性及机理。

创新点：灵芝是一种珍贵的药用真菌，大豆异黄酮的苷元物质也具有重要的药理活性，本文首次利用灵芝菌液体发酵的匀浆液生物转化大豆异黄酮，所得到的产物中大豆苷元与染料木素转化率高，同时还富集了灵芝菌的活性成分，并对转化产物的抗癌活性及机理进行了初步探讨。

方　法：首先利用灵芝菌液体发酵的匀浆体系生物转化大豆异黄酮（图 1）。其次，对转化产物的抗癌活性进行研究，主要包括对癌细胞存活率（图 2）、细胞凋亡（图 3）及细胞周期分布（图 4）的影响。最后，利用蛋白质印迹（Western-blot）与逆转录聚合酶链反应（RT-PCR）技术对凋亡相关的基因和蛋白进行检测（图 5 和表 2），初步探讨转化产物的体外抗癌机理。

结　论：本实验结果显示，转化产物中大豆苷元及染料木素的转化率分别为 96.63%和 87.82%，其中染料木素的含量可达(703.21±4.35) mg/g，同时转化产物中还富含了灵芝菌的活性成分。其次，对转化产物抗癌活性研究发现，其能有效降低 HTL-9 细胞的存活率，可通过将细胞阻滞于 G1 期而诱导细胞晚期凋亡。此外，转化产物（100 μg/ml）还可明显上调 Bax、Caspase-3、Caspase-8、Cyto-c 和 p53 的表达量，而 Survivin 和 NF-κB 表达量发生明显下调。结果表明，转化产物主要通过线粒体途径诱导细胞凋亡，但同时还调控多个与凋亡相关的基因。

关键词：大豆异黄酮；灵芝；转化；抗增殖活性；凋亡