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• *RAPID COMMUNICATION* •

Genistein inhibits invasive potential of human hepatocellular carcinoma by altering cell cycle, apoptosis, and angiogenesis

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Abstract

AIM: To study the *in vitro* and *in vivo* inhibitory effects of genistein on invasive potential of Bel 7402 hepatocellular carcinoma (HCC) cells and to explore the underlying mechanism.

METHODS: Bel 7402 HCC cells were exposed to genistein. The invasive activity of tumor cells was assayed in transwell cell culture chamber. $p125^{FAK}$ expression and cell cycle were evaluated by a functional assay. Cell apoptosis analysis was performed with TUNEL method. In addition, bilateral subrenal capsule xenograft transplantation of HCC was performed in 10 nude mice. Genistein was injected and the invasion of HCC into the renal parenchyma was observed. Microvessels with immunohistochemical staining were detected.

RESULTS: Genistein significantly inhibited the growth of Bel 7402 cells, the inhibitory rate of tumor cells was 26–42%. The invasive potential of Bel 7402 cells *in vitro* was significantly inhibited, the inhibitory rate was 11–28%. Genistein caused G2/M cell cycle arrest, S phase decreased significantly. The occurrence of apoptosis in genistein group increased significantly. The expression of $p125^{FAK}$ in 5 μg/mL genistein group (15.26±0.16%) and 10 μg/mL genistein group (12.89±0.36%) was significantly lower than that in the control group (19.75±1.12%, $P<0.05$). Tumor growth in genistein-treated nude mice was significantly retarded in comparison to control mice, the inhibitory rate of tumor growth was about 20%. Genistein also significantly inhibited the invasion of Bel 7402 cells into the renal parenchyma of nude mice with xenograft transplant. The positive unit value of microvessels in genistein-treated group (10.422 ± 0.807) was significantly lower than that in control group (22.330 ± 5.696, $P < 0.01$).

CONCLUSION: Genistein can effectively inhibit the invasive potential of Bel 7402 HCC cells by altering cell cycle, apoptosis and angiogenesis, inhibition of focal adhesion kinase may play a significant role in this process.

Key words: Genistein; Human hepatocellular carcinoma; Invasion; Cell cycle; Apoptosis; Angiogenesis

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INTRODUCTION

Genistein (5,7,4'-trihydroxyisoflavone), an isoflavinoid in soy beans, has been identified as a potential cause for the low incidence of certain types of tumor such as breast cancer, gastric cancer, colon cancer, prostate cancer, *etc.*[1-4]. As a natural tyrosine kinase inhibitor[5-7], genistein can suppress the formation and development of these tumors[8-11]. However, only limited data are available to demonstrate the effects of genistein on human HCC. The purpose of this study was to investigate the invasive potential and apoptotic effects of genistein *in vitro* and *in vivo* on HCC cells and to gain insights regarding the underlying mechanism mediating the effects of genistein.

MATERIALS AND METHODS

Cell culture and genistein

The human HCC cell line, Bel 7402, was obtained from Cancer Institute of Sun Yat-Sen University in Guangzhou. The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL) and cultured at 37 °C in a humidified atmosphere containing 50 mL/L CO_2 in air. Genistein purchased from Sigma

Chemical Co. was suspended in dimethylsulfoxide (DMSO) for the experiments.

In vitro assays of Bel 7402 cell growth and viability

The cells were seeded at the density of 1×10^4 cells with 1mL of medium/well onto 24 plates and incubated with or without genistein for 6 d. On the indicated day thereafter, cells were trypsinized and the number of cells was scored. An equivalent volume of DMSO was added to control cultures.

Cell viability was assayed using methyl thiazol tetrazolium (MTT) method. A 96-well plate was incubated with exponentially growing cells at the density of 1×10^4/well, following incubation of Bel 7402 cells with or without genistein in different columns of 96-well microtiter plates on d 1, 3, 5, and 7, MTT was added to each well and incubated at 37 °C for further 4 h before 595 nm absorbance (A_{595nm}) was detected. Each assay was performed in quadruplicate.

Inhibitory rate of tumor cell growth = (average $A_{595\ nm}$ value of control group-average $A_{595\ nm}$ value of genistein group)/average $A_{595\ nm}$ value of control group[12].

In vitro assays of Bel 7402 cell adhesion and invasion

Ninety-six-well microtiter plates were precoated with 20 mg/L fibronectin and incubated at 4 °C overnight. Wells were blocked with 2% BSA for 45 min at 37 °C. The cells were cultured in 2% serum-containing medium for 24 h, harvested at about 70% confluency, resuspended in serum-free RPMI 1640 medium supplemented with 0.1% BSA and distributed to wells (8×10^4/well). The cells were incubated at 37 °C in a 50 mL/L CO_2 atmosphere for 20, 40, 60, and 90 min with or without genistein. The wells were washed thrice with PBS to remove unattached cells, then the attached cells were incubated with MTT and the absorbance was measured at 595 nm. Each assay was performed in triplicate.

Adhesion rate = average $A_{595\ nm}$ value of genistein group/average $A_{595\ nm}$ value of control group×100%.

Inhibitory rate of adhesion = (average $A_{595\ nm}$ value of control group-average A595 nm value of genistein group)/average A595 nm value of control group×100%.

The invasive activity of Bel 7402 cells was assayed in transwell cell chambers (Corning Inc., USA), according to the method reported by Kido *et al*[13]. The basement membrane Matrigel was obtained from the Department of Cell Biology, Peking University Health Science Center. Polyvinylpyrrolidone-free polycarbonate filters with an 8.0-μm pore size were precoated with 5 μg of fibronectin in a volume of 50 μL on the lower surface. The Matrigel was diluted to 100 μg/mL with cold PBS and applied to the upper surface of the filters (5 μg/filter), and dried overnight under a hood at room temperature. The coated filters were washed extensively in PBS, and then dried immediately before use. Log-phase cell cultures of Bel 7402 cells were harvested and washed thrice with serum-free RPMI 1640, and resuspended to a final concentration of 2×10^6/mL in RPMI 1640 with 0.1% BSA. Cell suspensions (100 μL) with or without genistein were added to the upper compartment and incubated for 10-20 h at 37°C in a 50 mL/L CO_2 atmosphere. The filters were fixed with methanol and stained with Giemsa. The cells on the upper surface of the filters were removed by wiping with cotton swabs. The cells invading the lower surface of the filter through Matrigel and filter were manually counted under a microscope at a magnification of ×400, and each assay was performed in triplicate.

Invasion rate = average cell numbers invading the lower surface of the filter in genistein group/average cell numbers invading the lower surface of the filter in control group×100%.

Inhibitory rate of invasion=(average cell numbers invading the lower surface of the filter in control group-average cell numbers invading the lower surface of the filter in genistein group)/average cell numbers invading the lower surface of the filter in control group×100%.

Cell cycle analysis

Bel 7402 cells were seeded at the density of 5×10^5/well in six-dishes. After 24 h, the cells were treated with or without genistein for 72 h and harvested by trypsinization. The cells were then centrifuged at 300 r/min for 10 min, washed in PBS, and resuspended in cold 70% ethanol. The cells were then subjected to flow cytometric analysis on a FACScan cytofluorimeter (Becton Dickinson) after propidium iodide labeling.

Detection of focal adhesion kinase expression by flow cytometry assay

On d 3 of cell culture, control and genistein-treated Bel 7402 cells were centrifuged at 300 r/min for 10 min, washed and fixed in 1 mL 70% ethanol at 4 °C, treated with 0.1% Triton X-100 and 1% FBS, and anti-focal adhesion kinase (FAK C-20 sc-558, Santa Cruz Biotechnology, Inc., USA) and IgG1 (1 ∶ 100) were added. Cells were then treated with RNAse (1 mg/mL, Sigma, USA) and propidium iodide (10 μg/mL) for 30 min at room temperature. FAK expression was measured using a FACScan cytofluorimeter (Becton Dickinson).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay

Control cells and genistein-treated cells were harvested at 72 h by trypsinization and collected by centrifugation. Cells were washed twice in PBS-0.1% bovine serum albumin and prepared for TUNEL assay. Cells were fixed for 30 min in 4% paraformaldehyde, washed twice in PBS, and then permeabilized in 0.1% Triton-X 100 and 0.1% sodium citrate. Cells were labeled with terminal deoxynucleotidyl transferase for 60 min at 37 °C in a dark humidified incubator. The samples were washed twice, resuspended in 500 μL of PBS, and then analyzed on a FACScan cytofluorimeter (Becton Dickinson).

In vivo experiments

Six-week-old male BALB/C nu/nu mice were obtained from the Medical Laboratory Animal Center, Sun Yat-Sen University, China. They were kept under sterile conditions

in autoclaved cages with filter bonnets in laminar flow units and fed with sterilized MF pellets and distilled water. The mice were maintained in accordance with institutional accredited guidelines.

Bel 7402 cells were grown to 80-90% of confluence and detached with 0.25% trypsin. The cells were washed twice, counted, and resuspended in PBS at 1×10^7/mL (viability over 95%). Cells (0.3 mL) were injected into the male nude mice. Each animal received two injections, one on each side of the neck. Animals were killed 3 wk after tumor inoculation, when the largest tumors reached about 10 mm in diameter. Surgical excision of primary tumor was carried out, and tumor tissues were cut into 1-mm^3 pieces for bilateral subrenal capsule xenograft transplantation in next 10 nude mice anesthetized with chloral hydrate. The xenograft volume was assessed and calculated by the formula: length×width×depth×0.5236.

Mice bearing subrenal capsule xenograft transplant were randomly selected for the treatment with genistein ($n = 5$) and those without genistein treatment served as control ($n = 5$). Genistein (50 mg/kg) was administered ip daily to each mouse in genistein group for 15 d, while control animals were given the same vehicle. The animals were killed by cervical dislocation 16 d after transplantation of the tissues. Autopsies were performed and the kidney was excised, fixed, and embedded in paraffin, and examined histologically. The inhibitory rate of transplant growth was calculated by comparing the changes in xenograft transplant volume[15,16]. The criteria for invasive capacity of tumor cells in subrenal capsule xenograft transplant were as previously described[14,16,17].

Immunohistochemical determination of angiogenesis

After deparaffinization, dehydration, and washing, sections from xenograft transplant were incubated with trypsin at 37 °C for 30 min, quenched with 0.3% H_2O_2-methanol for 30 min, and blocked with 10% normal goat serum in a buffer containing 100 mL of PBS, 1.0 g of BSA, and 0.1 mL of Tween-20. The sections were treated with a rabbit polyclonal antibody against human factor VIII-related antigen at a 1∶100 dilution with the PBS-BSA-Tween-20 buffer, followed by a biotinylated universal antibody at a 1∶100 dilution. The sections were then treated with avidin-biotin complex followed by 3,3-diaminobenzidine as a substrate for staining. Positive unit (PU) of the microvessels with staining was tested and calculated according to the method described by Shen[17].

Statistical analysis

The statistical significance of difference between the groups was determined by applying the one-way ANOVA and χ^2 using the Stata 6.0 program.

RESULTS

In vitro effects of genistein on Bel 7402 cell growth

Genistein significantly inhibited Bel 7402 cell growth over the 7 d of experiment. The inhibitory rate of tumor cell growth in 5 and 10 μg/mL genistein groups was 26.71%±10.2% and 42.64±16.1%, respectively. The inhibitory rate of tumor cell growth in 10 μg/mL genistein group was significantly higher than that in 5 μg/mL genistein group ($P<0.01$). The dose-dependent effects of genistein on Bel 7402 cell growth are presented in Figure 1.

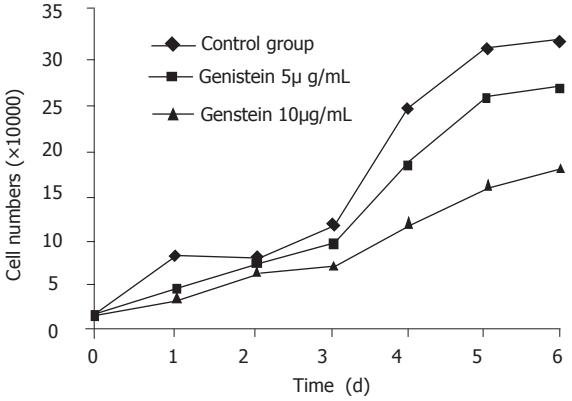

Figure 1 Effects of genistein on Bel 7402 cell proliferation *In vitro* effects of genistein on adhesion and invasion of Bel 7402 cells.

The adhesion rate of Bel 7402 cells for 20, 40, 60, and 90 min was 30.61%, 56.48%, 61.89% and 81.55% in 5 μg/mL genistein group, and 17.78%, 15.82%, 42.98% and 64.48% in 10 μg/mL genistein group. The inhibitory rate of Bel 7402 cells for 20, 40, 60, and 90 min was 69.39%, 43.52%, 38.11%, and 18.45% in 5 μg/mL genistein group, and 82.22%, 84.18%, 57.02%, and 35.52% in 10 μg/mL genistein group. Our results showed that genistein could inhibit tumor cell adhesion to fibronectin-coated substrates in a concentration-dependent fashion, and more potent inhibitory effect of genistein on adhesion occurred within 40 min.

We also investigated the capability of metastatic tumor cells through reconstituted basement membrane Matrigel. The cells invading the lower surface of the filter through Matrigel in control group, 5 μg/mL genistein group, and 10 μg/mL genistein group were 243.7±12.6/filed, 216.7±21.3/filed, and 174.5±9.6/filed, respectively. The invasion rate in 5 and 10 μg/mL genistein group was 88% and 71%, respectively, the inhibitory rate of invasion was 11% and 28%, respectively. Our results showed that genistein could inhibit the in vitro invasion of Bel 7402 cells, the inhibitory effect on invasion of Bel 7402 cells in 10 μg/mL genistein group was more significant than that in 5 μg/mL genistein group ($P<0.05$).

In vitro effects of genistein on cell cycle progression

Bel 7402 cells treated with genistein in the G0/G1 and G2/M phases increased significantly than cells in control group, the increase of cells in G0/G1 and G2/M phases was more remarkable in 10 μg/mL genistein group than in 5 μg/mL genistein group ($P<0.05$). S fractions decreased significantly in cells treated with genistein ($P<0.05$). Percentage of apoptotic cells in genistein-treated

group increased significantly compared to that in control group (P<0.05). The results of cell cycle analysis by flow cytometry in Bel 7402 cells are presented in Table 1.

Table 1 Effects of genistein on cell cycle progression in Bel 7402 cell line (mean±SD)

Treatment	Cell cycle phase			Apoptosis (%)
	G0/G1 (%)	G2/M (%)	S (%)	
Control	64.58±8.46	0.78±0.02	34.64±1.36	0.47±0.01
Genistein 5 μg/mL	71.74±4.46	2.75±0.03[b]	25.50±2.28[b]	1.02±0.06[a]
Genistein 10 μg/mL	75.27±6.12[ac]	6.37±0.08[d]	18.36±1.53[b]	2.12±0.12[b]

[a]P<0.05 *vs* control; [b]P<0.01 *vs* control; [c]P<0.05 *vs* 5 μg/mL genistein; [d]P<0.01 *vs* control and 5 μg/mL genistein.

Effects of genistein on induction of apoptosis of Bel 7402 cells

TUNEL assay in our studies showed that the percentage of cells undergoing apoptosis was significantly higher in 10 μg/mL genistein group (1.05±0.09%) and 5 μg/mL genistein group (0.80±0.12%) than in control group (0.43±0.08%, P<0.01 and P<0.05, respectively) being consistent with genistein's ability to induce apoptosis observed in cell cycle analysis.

Evaluation of expression of p125^{FAK} protein

We investigated whether genistein could modulate protein expression of the signal transduction molecule-p125FAK. After 72-h treatment with genistein, p125FAK expression in 10 μg/mL genistein group (12.89±0.36)% was significantly lower than that in control group (19.75%±1.12%, P<0.05). p125FAK expression in 5 μg/mL genistein group (15.26±0.16)% also decreased, but there was no statistically significant difference between 5 μg/mL genistein group and control group (P>0.05).

Effects of genistein on growth of subrenal capsule xenograft transplant

The anti-tumor activity of genistein was evaluated in nude mice bearing subrenal capsule xenograft transplant. Treatment with genistein inhibited the local tumor growth significantly compared to the control group. At the end of the treatment (15 d post implantation), the increase of tumor volume in genistein group (63.32±8.96 mm^3) was significantly less than that in control group (79.25±6.85 mm^3, P<0.05). Tumors in mice treated with genistein reduced in volume by 20% compared to the control group.

Effects of genistein on in vivo tumor invasion

The criteria for invasive capacity of tumor cells in subrenal capsule xenograft transplant were as previously described[17]. The 0-IV invasion rank to renal parenchyma was recorded[14,16]. In the control group, invasion rank 0 was observed in 1 of 10 mice (1/10), invasion rank I in 8 of 10 mice (8/10), and invasion rank II in 1 of 10 mice (1/10). In genistein-treated group, invasion rank 0 was observed in 8/10, rank I in 2/10 and rank II in 0/10 mice. No rank III or rank IV invasion was observed in both groups. Our results showed that treatment with genistein could significantly inhibit the invasion of Bel 7402 cells to the renal parenchyma (P<0.05). Hematoxylin-eosin stained specimens are shown in Figure 2.

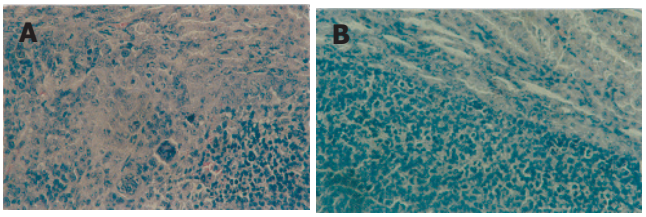

Figure 2 Tumor invasion of renal parenchyma in control (**A**) and genistein-treated nude mice (**B**). HE, magnification ×200.

Effects of genistein on tumor angiogenesis

In untreated tumor tissues, tumor cells were arranged in large nests with plenty of blood sinusoids. Whereas, in genistein-treated tumor tissue, tumor cells were characterized by small cancerous nests with scanty blood vessels. PU value of microvessels in the subrenal capsule xenograft transplant, as a marker of tumor angiogenesis, significantly decreased in genistein group (10.422±0.807) compared to that in control group (22.330±5.696, P<0.01). Immunohistochemical staining for the determination of angiogenesis in subrenal capsule xenograft transplant of the nude mice is shown in Figure 3.

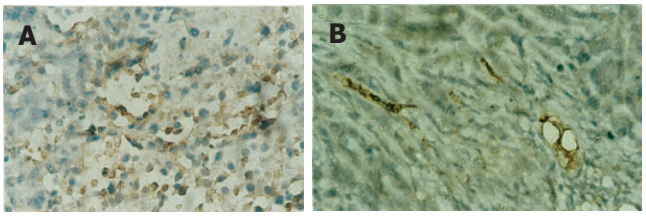

Figure 3 Tumor tissue with plenty of blood vessels (**A**) and scanty blood vessels (**B**) in control and genistein-treated nude mice. HE, magnification ×200.

DISCUSSION

Invasion and metastasis are the most devastating aspects of cancer. Advances in surgical techniques and adjuvant therapies have been proved to be useful in the treatment of primary tumors[18]. However, invasion and metastasis remain a major cause of poor prognosis and death in cancer patients. Reports from epidemiological and experimental studies indicate that genistein plays an important role in the prevention and inhibition of tumors such as breast cancer, prostate cancer, colon cancer, leukemia, melanoma, *etc*.[19]. However, there are still a few reports of studies on the correlation between genistein and the invasion and metastasis of human HCC. Our data provide evidence that genistein can also inhibit HCC cell proliferation and invasion.

In this report, we haveshould for the first time that genistein could significantly inhibit the growth and

viability of Bel 7402 cells. The inhibitory rate of tumor cell growth was about 26%-42%. We also found that genistein induced cell cycle arrest in the G0/G1 and G2/M phases. These findings are in agreement with other reports[19-24]. Although the exact mechanisms of genistein await further elucidation, induction of apoptosis may be partly responsible[25-27]. In our studies with TUNEL assay, the percentage of Bel 7402 cells undergoing apoptosis was significantly higher in genistein group than in control group, which is consistent with those found in other studies[28,29].

The adhesion and invasiveness of tumor cells represent some important properties necessary for the formation of metastases. We investigated the effect of genistein on the adhesive properties of Bel 7402 cells, and found that genistein could inhibit tumor cell adhesion to fibronectin-coated substrates, the most potent inhibitory effect of genistein on adhesion occurred within 40 min, demonstrating that reduction in cell adhesion after the treatment with genistein may account for the ability of Bel 7402 cells to transgress normal tissue boundaries and disperse to the adjacent sites. The invasion assay both *in vitro* and *in vivo* was further performed in our experiments. Bel 7402 cells invading the lower surface of the filter through Matrigel was significantly inhibited in genistein-treated groups compared to control group. Our experiments with the subrenal capsule xenograft transplant of nude mice showed that the treatment with genistein could significantly inhibit the invasion of Bel 7402 cells to the renal parenchyma, which was correlated with the biological behavior *in vitro*.

Inhibition of angiogenesis was observed in our studies. Angiogenesis is virtually absent in the healthy adult organism and is restricted to a few conditions including wound healing, placenta, endometrium, etc., representing the ordered and self-limited processes[30,31]. In certain pathological conditions, angiogenesis is dramatically enhanced and is no longer self-limited[30]. The most important manifestation of pathological angiogenesis is induced by solid tumors[32]. In our immunohistochemical studies, tumor cells were characterized by small cancerous nests with scanty blood vessels in genistein-treated mice. The PU value of microvessels was significantly decreased in genistein group compared to the control group. As angiogenesis is an important step in the invasion and metastasis process of tumors[33,34], the changes in angiogenesis caused by genistein may play a crucial role in inhibition of the invasiveness of Bel 7402 cells.

FAK is a cytoplasmic tyrosine kinase that plays an important role in integrin-mediated signal transduction pathways closely related to cell adhesion, motility, and growth[35-39]. Upregulation of FAK expression is associated with oncogenesis[40-43] and decrease in FAK is associated with the loss of ability to attach, decreased migration and induction of apoptosis[44-48]. We have reported that FAK is overexpressed in HCC, the expression of FAK in invasive or metastatic HCC is significantly higher than that in non-invasive or non-metastatic HCC[16]. Therefore, FAK seems to be an important pharmacologic target site[49]. In the present study, a significant downregulation of p125FAK after genistein treatment was observed, suggesting that genistein may serve as a potential important anticancer agent for HCC progression by blocking the FAK signaling process, which play a crucial role in angiogenesis and apoptosis.

In summary, our results provide the preliminary evidence that genistein is an effective chemopreventive agent for HCC. Further in-depth studies coupled with clinical trials are needed to establish the scientific basis for the use of genistein in the prevention and treatment of HCC.

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