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ORIGINAL ARTICLE

Genistein inhibits the proliferation, migration and invasion of the squamous cell carcinoma cells via inhibition of MEK/ERK and JNK signalling pathways

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Summary

Purpose: *The main purpose of the current research work was to investigate the anticancer activity of Genistein - a plant derived isoflavone - in squamous cell carcinoma SK-MEL-28 (SCC) cells along with studying its effects on cellular apoptosis, DNA damage, cell migration and invasion and MEK/ERK/JNK signalling pathway.*

Methods: *Cell proliferation was examined by CCK-8 (Cell Counting Kit-8) assay while the effects on apoptosis were evaluated by DAPI staining and Comet assay using fluorescence microscopy. Transwell assay was used for checking the effects on cell migration and invasion while western blot method was used to evaluate the effects on the expression of MEK/ERK/JNK proteins.*

Results: *The results showed that Genistein led to dose-dependent cytotoxic effects in these cells showing an IC_{50} value of 14.5 μM. It also led to dose-dependent apoptosis and induced DNA damage as shown by fluorescence microscopy. Genistein also inhibited cell migration and invasion dose-dependently, along with inhibiting matrix metalloproteinase (MMP)-9 expression. Genistein also led to inhibition of the expression of p-JNK with no apparent effects on the total JNK expression. It also showed significant and dose-dependent inhibition of the expression of p-MEK and p-ERK proteins.*

Conclusions: *Genistein has a significant anticancer activity in SK-MEL-28 human SCC cells, inducing apoptosis, DNA damage, cell migration and invasion and inhibiting MEK/ERK and JNK signalling pathway.*

Key words: *Genistein, squamous cell carcinoma, apoptosis, DNA damage, cell migration*

Introduction

Squamous cell carcinoma (SCC) ranks sixth in incidence and in cancer-related mortality. SCC develops in a ground of dysplasia and finally proceeds to tumor formation. SCC development involves initial accumulation of various genetic and epigenetic events leading to disruption of a series of biochemical signalling pathways. This signalling pathway disruption results in dysregulation of cell cycle phase distribution, which ultimately leads to imbalance between cell division and cell death [1,2]. The standard treatments for SCC are chemotherapy and radiotherapy. SCC poses various challenges concerning survival and prognosis. The 5-year survival rate during the past few decades has been at 50-60% despite the various advancements in chemotherapy and radiotherapy techniques. Moreover, this problem is further complicated by the fact that SCC cells have been reported to have acquired re-

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sistance to clinically used chemotherapy drugs [3,4]. These chemotherapeutic drugs are also associated with side effects affecting seriously the quality of life of the SCC patients. Therefore, there is an urgent need to identify new and novel anticancer agents with minimal side effects and are affordable. Plant-derived molecules have always played a pivotal role in drug discovery and have been used to alleviate human sufferings by treating different diseases including cancer [5,6]. Genistein is a potent anticancer molecule and has been reported to exhibit anticancer activity against a wide range of human cancer cells under *in vitro* conditions, including cervical, prostate, brain, colon and breast cancer cells [7-10]. It has also been reported that Genistein can sensitize cancer cells to radiotherapy making them more sensitive to radiation therapy [11]. In the current study, our main objective was to examine the anticancer effects of Genistein - a naturally occurring isoflavone mainly isolated from Genista tinctoria - against human SCC. We also examined its effects on apoptosis, cell migration and invasion and MEK/ERK/JNK signalling pathway.

Methods

CCK-8 cell proliferation assay

SK-MEL-28 human SCC line was purchased from Chinese Academy of Sciences, Shanghai, China (Department of Biochemistry and Biology). The cells were kept in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) at 37°C in incubator with 5% CO_2. The SK-MEL-28 cells were initially exposed to various doses of Genistein after which 20 µl CCK-8 (purchased from Dojindo Laboratories, Kumamoto, Japan) was added to the cell culture well plates. Subsequently, the cell culture plates were incubated for 12 h at 37°C. Finally, using a microplate reader (Bio-Rad, Hercules, USA) the absorbance was taken at 450 nm wavelength which was used for calculating the cell proliferation of human SCC cells.

DAPI staining assay and Comet assay

The SK-MEL-28 human SCC (1×10^6 cells/well) were added to 6-well plates and grown for 12 h. The cells were exposed to treatment with several concentrations of Genistein (0, 12.5, 25 and 50 µM) and incubated for 24 h. Then, the cells were put on a glass slide and stained with 10 µl DAPI solution, following which the glass slides were cover-slipped and observed using a fluorescent microscope (Nikon Instruments Inc., NY, USA). Comet assay which measures the DNA damage was carried out by alkaline single cell gel electrophoresis as per the guidelines of the method previously published [12].

Transwell assay for cell invasion and migration evaluation

A Transwell chamber (8 mm pore size, Corning, New York, USA) with Matrigel was used to examine the anti-invasion potential of Genistein on SK-MEL-28 cells. The cell culture of SK-MEL-28 cells (300 ml) was placed onto the upper chamber and only Dulbecco's modified Eagle's medium (DMEM) was put into the lower chamber. The SK-MEL-28 cells were removed from the upper chamber after 24-h incubation. Afterwards, the cells that invaded through the chamber were methanol-fixed and then stained with 10 µl crystal violet. In order to count the number of SK-MEL-28 cells that had invaded, inverted microscope at 200X magnification was utilised. The migration was also measured in the same way except for the Martigel was not used in cell migration assay.

Western blot analysis

The SK-MEL-28 cells were initially washed with ice-cold phosphate buffered saline (PBS) and then lysed in RIPA lysis buffer containing the protease inhibitor. About 45 µg of proteins from each sample were separated through electrophoresis via SDS-PAGE gels. Separation was followed by transference to polyvinylidene difluoride (PVDF) membrane. Bradford assay was used for evaluating the protein content of each cell extract. Next, fat-free milk was used to block the membrane at room temperature for 1 h. Thereafter, the membranes were treated with primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with secondary antibodies. Finally, the signal was detected by Odyssey Infrared Imaging System. Actin was used as control for normalization.

Statistics

SPSS software package (version 16.0, IBM, Chicago, USA) was used for statistical analyses. All the results were expressed as mean ± standard deviation (SD) and $p<0.05$ was considered as statistically significant.

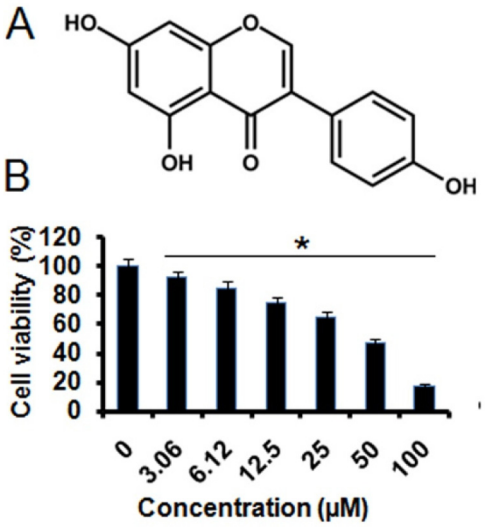

Figure 1. A: Chemical structure of Genistein isoflavone; **B:** CCK-8 assay showing the effects of genistein on the viability of the SK-MEL-28 human squamous cell carcinoma cells. The experiments were performed in triplicate and shown as mean ± SD (*$p<0.05$).

Results

Genistein triggered significant cytotoxicity in SK-MEL-28 human squamous cancer cells

Initially, we checked the cytotoxic effects of Genistein isoflavone on SK-MEL-28 cells for which CCK-8 (Cell Counting Kit-8) colorimetric assay was used. Genistein with increasing doses (0, 3.06, 6.12, 12.5, 25, 50, 100 μM) induced powerful cytotoxicity and it was seen that the Genistein-induced cytotoxicity was concentration-dependent. In order to evaluate its potency, IC_{50} value of Genistein was found to be 14.5 μM (Figure 1) indicating that this molecule has a high potency as cytotoxic agent.

Genistein induced cellular apoptosis and DNA damage

In order to examine the mode of action of Genistein in SK-MEL-28 cells, fluorescence microscopy was used for Comet assay and DAPI staining assay. The results indicated that Genistein induced cellular changes in SK-MEL-28 cells reminiscent of apoptosis; these changes included nuclear fragmentation, chromatin condensation, and nuclei splitting. These apoptotic effects were seen to enhance with increasing Genistein dose (Figure 2). Further, DNA damage assessment was carried out by single-cell gel electrophoresis and the results, which are shown as photomicrographs in Figure 3, indicate that untreated control cells revealed intact DNA without any fragmentation, while at increasing concentrations of Genistein (12.5, 25 and 50 μM), the SK-MEL-28 cells showed considerable fragmented DNA that looked like a comet in this assay. The DNA fragmentation in these cells was found to be dose-dependent as can be easily seen from comet tails (Figure 3).

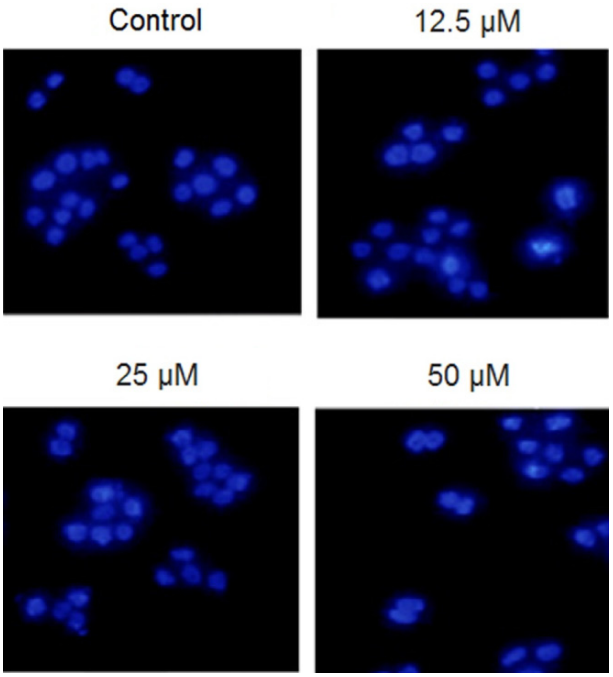

Figure 2. Fluorescence microscopy study using DAPI staining showing Genistein-induced apoptotic cell death as indicated by enhanced chromatin condensation and nuclear fragmentation with increasing Genistein dose. The experiments were performed in triplicate.

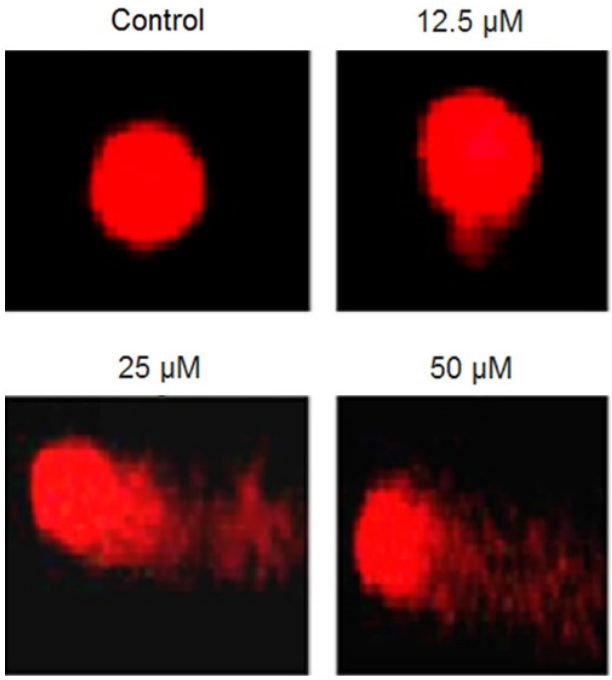

Figure 3. Fluorescence microscopy study with Comet assay to investigate the DNA-damaging effects of Genistein on SK-MEL-28 carcinoma cells. The results showed dose-dependent DNA fragmentation.

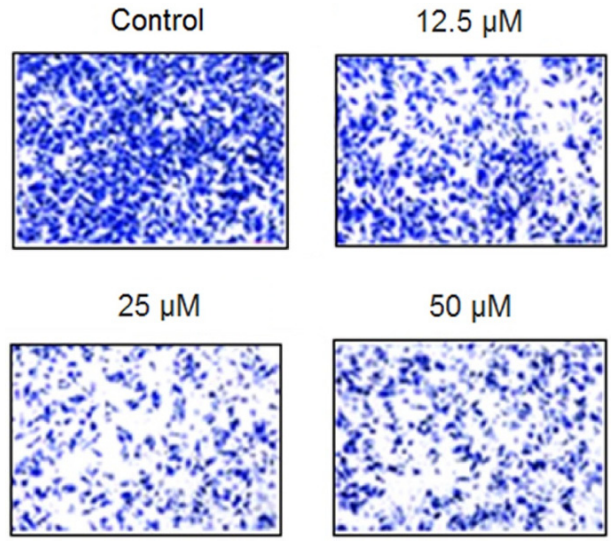

Figure 4. Transwell assay for evaluation of effects of Genistein on the SK-MEL-28 cell migration. The experiments were performed in triplicate. Genistein was shown to induce significant dose-dependent cell migration inhibitory effects. The experiments were performed in triplicate.

Genistein led to suppression of cell migration and cell invasion

In order to assess the effects of Genistein on cancer cell migration and invasion, transwell chamber assay was performed. The results, which are shown in Figure 4, indicate that the molecule inhibited the migration of SK-MEL-28 cells and this effect was found to be concentration-dependent. The effects of Genistein on the invasion of SK-MEL-28 cells are shown in Figure 5 and reveal that Genistein inhibits the cancer cell invasion in a dose-dependent manner. These results suggest that Genistein could be developed as an anti-metastatic agent which can possibly curb the spread of cancer to other parts of the body. Genistein-induced cell migration and invasion inhibition was also accompanied by inhibition of Matrix metalloproteinase-9 (MMP-9) expression. The MMP-9 expression has been shown to be a diagnostic marker in skin cancer tissues (Figure 6).

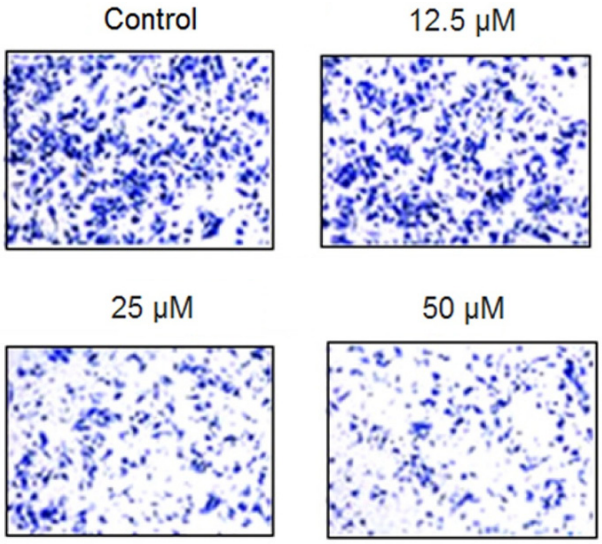

Figure 5. Transwell assay for evaluation of the effects of Genistein on the SK-MEL-28 cell invasion. The experiments were performed in triplicate. Genistein was shown to induce significant cell invasion inhibitory effects in a dose-dependent manner. The experiments were performed in triplicate.

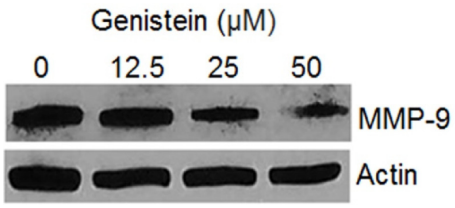

Figure 6. Effects of Genistein on the expression levels of MMP-9 (metalloproteinase-9) using western blot assay which is involved in cell migration and invasion. The experiments were performed in triplicate.

Genistein led to inhibition of MEK/ERK and JNK signalling pathways

The effects of this molecule were studied on the ERK/MAPK and JNK signalling pathways. The results showed that the molecule led to significant inhibition of the expression of p-MEK and p-ERK (Figure 7). Genistein treatment also led to inhibition of the expression of p-JNK with no apparent effects on the total JNK expression (Figure 8). Both MEK/ERK and JNK signalling pathways are involved in cell growth as well as in tumorigenesis. These signalling pathways also regulate the activity of several proteins associated with cellular apoptosis [13].

Discussion

SCC is the most frequent kind of cancer targeting skin and involving damage to skin cells due to building-up of genetic alterations in cancer cells. There are various challenges in the treatment of this cancer which is still the principal cause of both morbidity as well as mortality in skin cancer patients. These challenges include multidrug resistance and side effects due to chemotherapy and

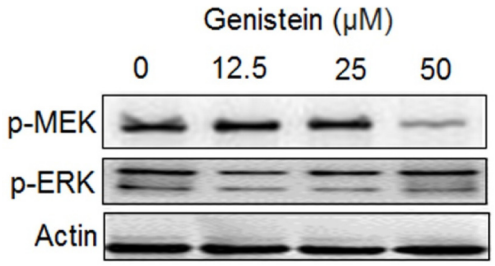

Figure 7. Effects of Genistein on the expression levels of MEK/ERK proteins. Genistein treatment led to significant inhibition of the expression of p-MEK and p-ERK. The experiments were performed in triplicate.

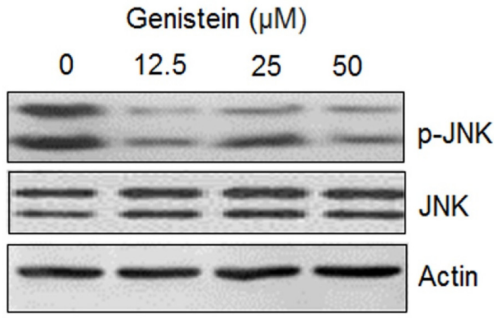

Figure 8. Effects of Genistein on the expression levels p-JNK/JNK pathway. Genistein treatment led to inhibition of the expression of p-JNK with no apparent effects on the total JNK expression. The experiments were performed in triplicate.

radiotherapy [14,15]. These factors create an urgent need to find alternative treatment modalities which are devoid of any side effects. In this direction, natural products have always played a major role in anticancer drug discovery. Molecules isolated from plants have been used as medicines for cancer treatment. It has been reported that about 25-30% of all latest medicinal agents are directly or indirectly obtained from various plant sources indicating the significant medicinal potential of the plant kingdom [16]. Chemoprevention using plant-derived natural products is suggested to be a cheap and effective alternative to prevent various life-threatening diseases including cancer. In the current study, the anticancer activity of Genistein - a plant isoflavone - was investigated along with its mode of action by studying its effects on apoptosis, DNA-damage, cell migration and invasion, and MEK/ERK/JNK signalling pathway. There is a huge number of published reports on the *in vitro* and *in vivo* anticancer action of Genistein including liver, gastric, lung, colorectal and breast cancers [17-23]. The mode of anticancer action of Genistein has been reported to involve induction of apoptosis, cell cycle arrest, inhibition of metastasis and angiogenesis, targeting mitogen-activated protein kinase (MAPK), Bax, Bcl-2, nuclear transcription factor κB (NF-κB), Wnt/β-catenin and PI3K/AKT signalling pathways. Genistein has also been shown to enhance the anticancer action of various antitumor drugs including tamoxifen, docetaxel, and adriamycin [24,25].

In the present study it was seen that Genistein showed anticancer activity in SK-MEL-28 human SCC cells. Genistein with increasing doses (0, 3.06, 6.12, 12.5, 25, 50, 100 μM) induced powerful cytotoxicity and it was also seen that the Genistein-induced cytotoxicity was concentration-dependent and showed an IC_{50} value of 14.5 μM. Furthermore, Comet and DAPI assays showed that this molecule could induce cellular apoptosis and DNA damage. In addition, transwell assay revealed that Genistein could inhibit both cell migration and invasion in SK-MEL-28 cells, along with inhibiting MMP-9 expression. Genistein treatment also led to inhibition of the expression of p-JNK with no apparent effects on the total JNK expression.

Conclusion

In conclusion, the present study indicates that Genistein has a significant anticancer activity in SK-MEL-28 cells by targeting apoptosis, DNA damage, cell migration and invasion and inhibiting MEK/ERK and JNK signalling pathway.

Conflict of interests

The authors declare no conflict of interests.

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