Genistein inhibits the proliferation of human multiple myeloma cells through suppression of nuclear factor-κB and upregulation of microRNA-29b

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Abstract. Multiple myeloma (MM) is a malignant tumor and is the most common primary tumor of the bone marrow in the USA. Genistein is predominantly found in Leguminosae and various lines of evidence have indicated that it suppresses cell growth, induces programmed cell death and inhibits angiogenesis. As a result of these capabilities, genistein presents as a promising cancer chemopreventive agent. However, the effect of genistein on MM remains to be elucidated. The present study investigated the effect of genistein on the proliferation and apoptosis of MM cells through the regulation of nuclear factor-κB (NF-κB) and microRNA-29b (miR-29b). In the present study, cell proliferation was examined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. In addition, apoptosis was detected using an Annexin V-fluorescein isothiocyanate/propidium iodide apoptosis assay and caspase-3 activation assay. The expression of NF-κB and miR-29b was analyzed using western blotting and reverse transcription quantitative polymerase chain reaction, respectively. Finally, miR-29b and anti-miR-29b plasmids were transfected into U266 cells to determine the effect of genistein on MM. In the present study, the results demonstrated that genistein could significantly reduce cell proliferation, induce apoptosis and increase the activity of caspase-3 in U266 cells. Furthermore, it was found that genistein could suppress the protein level of NF-κB and promote the expression of miR-29b in U266 cells. The results also indicated that miR-29b could alter the expression of NF-κB in U266 cells. These findings suggest that genistein inhibits the proliferation of human MM cells by upregulating miR-29b resulting in suppression of NF-κB.

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Introduction

Multiple myeloma (MM) is a malignant tumor, which originates from terminally differentiated B lymphocytes and is characterized by clonal proliferation of a large number of plasma cells in the bone marrow of patients. MM has a complex multi-step and multi-stage pathogenesis, and a variety of factors and pathways are involved in its development and progression (1,2). MM is the most common primary tumor of the bone marrow in the USA (3).

Previous studies have focused on the interactions among cytogenetic abnormalities, the bone marrow microenvironment, myeloma cells, nuclear factor-κB (NF-κB) signaling pathways and resistance mechanisms (4,5). The active transcription factor NF-κB is a heterodimer consisting of two subunits p50 and p65. Non-activated NF-κB in the heterodimer also inhibits IκB. Following activation, NF-κB changes into an active heterodimer, which then enters the nucleus and combines with the promoter region of several target genes. This triggers transcription of the target genes, thus increasing the expression of various cytokines, chemical factors, adhesion molecules and cyclin D, which in turn promotes the growth and survival of cells (6,7). Suppression of NF-κB enhances the anti-MM effects of conventional chemotherapeutic agents (5). For example, celastrol, an inhibitor of NF-κB, has been demonstrated to induce cell cycle arrest and apoptosis of human MM cells via downregulation of NF-κB (8).

MicroRNAs (miRs) are a type of non-coding small RNA with a length of ~22 nt, which regulate gene expression following transcription of genes, involved in the regulation of cell differentiation, apoptosis and proliferation by inhibiting the mRNA of specific target genes (9). Previous studies have demonstrated that miRNAs are extensively involved in the occurrence, development and prognosis of a tumor. For example, the expression of miR-29b was increased 10-fold in MM cells compared with in the plasma cells of healthy individuals (10). Upregulation of miR-29b has been demonstrated to induce significant antitumor activity in human MM (11). For example, Zhang *et al* (12) demonstrated that

miRNA-29b-induced apoptosis was found to act antagonistically with IL-6 in human myeloma cell lines.

Genistein is predominantly found in Leguminosae, with the largest quantities identified in Fructus Sophorae and Subprostrate Sophora (13). Genistein is able to induce programmed cell death, increase the anti-cancer efficacy and inhibit angiogenesis, and thus is a promising cancer chemopreventive agent. The anticancer effect of genistein has broad application prospects (14,15) and thus requires further investigation. The aim of the present study was to examine the effects of genistein on the proliferation and apoptosis of human MM cells.

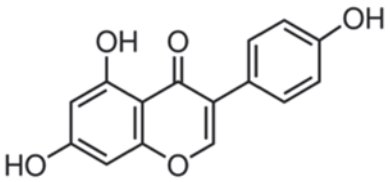

Figure 1. Chemical structure of genistein.

Materials and methods

Reagents and chemicals. The chemical structure of genistein is shown in Fig. 1. Genistein (Sigma-Aldrich, St. Louis, MO, USA; with a purity >98%) was dissolved in physiological saline according to the manufacturer's instructions (16,17). RPMI-1640 and fetal bovine serum (FBS) were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The Caspase-3 Activity Assay kit and BCA protein assay kit were purchased from Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection kit was purchased from BestBio (Shanghai, China). TRIzol reagent and quantitative polymerase chain reaction (qPCR) assays were purchased from Tiangen Biotech (Beijing) Co., Ltd. (Beijing, China).

Cell lines and cell culture. The human MM cell line U266 was purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in RPMI-1640 culture medium supplemented with 10% FBS, 100 units/ml penicillin and 100 mg/ml streptomycin in a humidified atmosphere in a 37˚C incubator with 5% CO_2.

Cell viability assay. Cells were seeded ($1x10^4$ cells) in 96-well plates and treated with the indicated dose of genistein (0, 10, 20 and 40 μM) for 24, 48 and 72 h. Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich). Absorbance was measured at λ=570 nm using a microplate reader (iMark™; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Annexin V-FITC/PI apoptosis assay. Cells were seeded ($1x10^6$ cells) in 6-well plates and treated with the indicated dose of genistein (0, 10, 20 and 40 μM) for 48 h. Briefly, apoptotic cells were measured using an Annexin V-FITC/PI Apoptosis Detection kit (BestBio). For flow cytometric analysis, a Cytomics FC500 flow cytometer with CXP software (Beckman Coulter, Fullerton, CA, USA) was used.

Caspase-3 activation assay. Cells were seeded ($1x10^4$ cells) in 96-well plates and treated with the indicated dose of genistein (0, 10, 20 and 40 μM) for 48 h. Briefly, caspase-3 activation was measured using the Caspase-3 Activity Assay kit (Shanghai Sangon Biotechnology Co., Ltd.). Protein cell lysate (10 μl per sample) was added to 80 μl reaction buffer with 10 μl substrate (Asp-Glu-Val-Asp-p-nitroanilide) and incubated at 37˚C for 4-6 h. Caspase-3 activation was measured using a microplate reader (Bio-Rad Laboratories, Inc.) at an absorbance of 405 nm.

Western blotting. Cells were seeded ($1x10^6$ cells) in 6-well plates and treated with the indicated dose of genistein (0, 10, 20 and 40 μM) for 48 h. Protein concentration was determined using the BCA protein assay kit (Shanghai Sangon Biotechnology Co., Ltd.). Protein samples were analyzed using 12% SDS-polyacrylamide gel electrophoresis followed by semi-dry transfer onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Inc.). The membrane was blocked with 5% non-fat milk in Tris-buffered saline and Tween-20 (TBST) buffer at 4˚C for 4 h. The membrane was incubated with rabbit anti-human anti-NF-κB p65 (cat. no. BA0610-2; 1:500 dilution; Wuhan Boster Biological Technology, Ltd., Wuhan, China) and rabbit anti-human anti-GAPDH (cat. no. PB0141; 1:2,000 dilution; Wuhan Boster Biological Technology, Ltd.) overnight at 4˚C. The membrane was washed with TBST and proteins were detected using a BCIP-NBT kit (Promega Corporation, Madison, WI, USA).

Reverse transcription (RT)-qPCR analysis of miR-29b expression. Cells were seeded ($1x10^6$ cells) in 6-well plates and treated with the indicated dose of genistein (0, 10, 20 and 40 μM) for 48 h. Total RNA was extracted from cells using TRIzol reagent [Tiangen Biotech (Beijing) Co., Ltd.] and miRNAs were specifically amplified for individual miRNA TaqMan qPCR analysis [Tiangen Biotech (Beijing) Co., Ltd.]. Quantification of the miRNAs was performed using TaqMan miRNA qPCR assays [Tiangen Biotech (Beijing) Co., Ltd.]. The primers used were as follows: Forward, 5'-GGG GGTACCCTTCAGGAAGCTGGTTTC-3' and reverse, 5'-GGGGATATCTACATGTGAGGCAGGTTCTCAC-3' for miR-29b; forward, 5'-CGCTTCGGCAGCACATATACTA-3' and reverse 5'-CGCTTCACGAATTTGCGTGTCA-3' for U6.

Transfection of miR-29b and anti-miR-29b. miR-29b and anti-miR-29b plasmids were designed and purchased from Shanghai Sangon Biotechnology Co., Ltd. When the U266 cells reached 70-80% confluence, Lipofectamine 2000 (Invitrogen Life Technologies) was used to transfect miR-29b (100 nmol/l) or anti-miR-29b (100 nmol/l) into U266 cells according to the manufacturer's instructions. Cells were seeded ($1x10^6$ cells) in 6-well plates and treated with the indicated dose of genistein (20 μM) for 48 h.

Statistical analysis. All experiments were repeated at least three times and were analyzed using SPSS statistical software version 18 (SPSS, Inc., Chicago, IL. USA). Comparisons

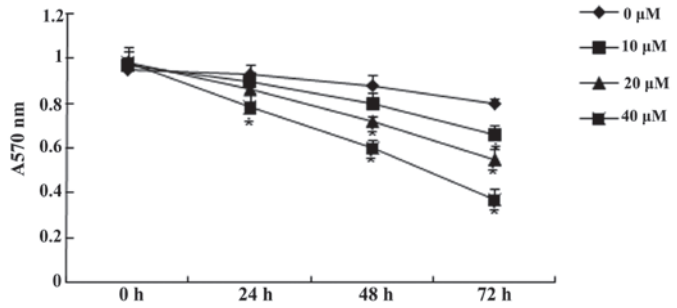

Figure 2. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide analysis of cell viability. *P<0.01, compared with the 0 μM genistein treatment group.

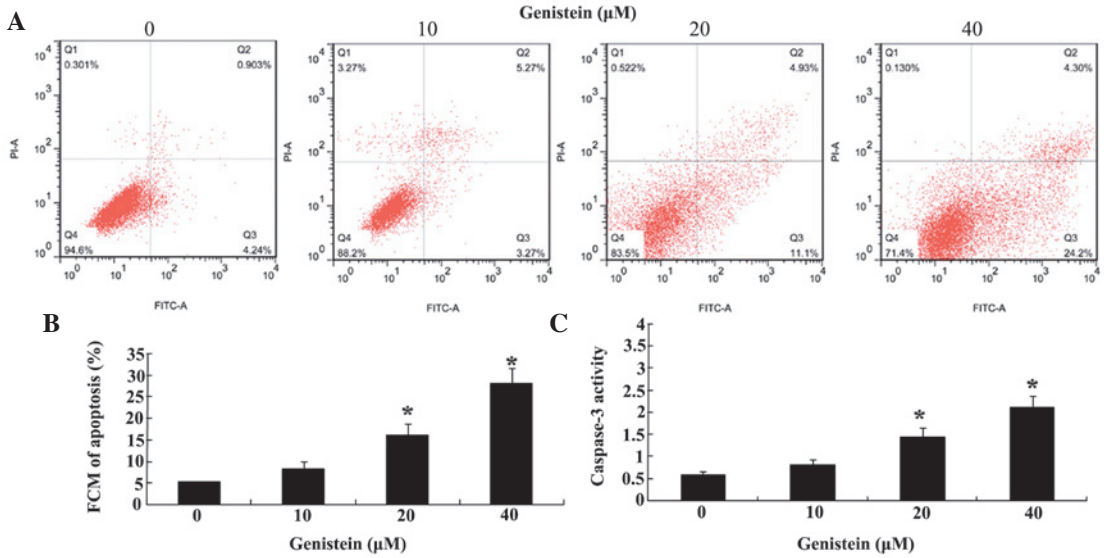

Figure 3. Flow cytometric analysis of apoptosis and measurement of caspase-3 activity. (A) Flow cytometric analysis of apoptosis, (B) statistical analysis of cellular apoptosis and (C) caspase-3 activity. *P<0.01, compared with the 0 μM genistein treatment group.

between mean values were assessed using Student's unpaired t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

MTT analysis for cell viability. The effect of genistein (0, 10, 20 and 40 μM) on U266 cell viability was examined by the MTT assay. The results demonstrated that Genistein could inhibit the proliferation of U266 cells. Following treatment with 10 μM genistein for 72 h, 20 μM genistein for 48 and 72 h and 40 μM genistein for 24, 48 and 72 h, the proliferation of U266 cells was significantly reduced compared with that of the group treated with 0 μM genistein (Fig. 2). Thus, 20 μM genistein was selected as the standard treatment for further experiments.

Flow cytometric analysis of cell apoptosis and measurement of caspase-3 activity. In order to further assess the effect of genistein (0, 10, 20 and 40 μM) on the apoptosis of U266 cells, apoptosis and the activity of caspase-3 were analyzed. Following treatment with genistein (0, 10, 20 and 40 μM) for 48 h, apoptosis of U266 cells increased in a concentration-dependent manner (Fig. 3A and B). Apoptosis of U266 cells was significantly increased following treatment with genistein (20 and 40 μM) for 48 h (Fig. 3A and B). In addition, following treatment with genistein (20 and 40 μM) for 48 h, the activity of caspase-3 in U266 cells was significantly increased, compared with that of the group treated with 0 μM genistein (Fig. 3C).

Inhibition of NF-κB by genistein. Based on the abovementioned results, western blotting was used to analyze the protein level of NF-κB in U266 cells (Fig. 4A). Notably, treatment with genistein (0, 10, 20 and 40 μM) for 48 h had a pronounced inhibitory effect on the protein level of NF-κB in U266 cells (Fig. 4B).

Genistein activates miR-29b expression. To further investigate the effect of genistein on miR-29b expression, qPCR was used to examine the expression of miR-29b in U266 cells. Following treatment with genistein (20 and 40 μM) for 48 h, the expression of miR-29b in U266 cells was significantly promoted (Fig. 5).

Overexpression of miR-29b and NF-κB expression. To improve our understanding of the expression of miR-29b and NF-κB in U266 cells, miR-29b was transfected into U266 cells and the

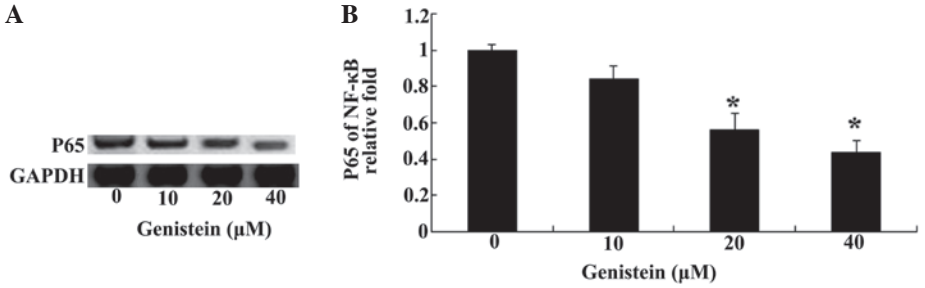

Figure 4. Inhibition of NF-κB following treatment with genistein. (A) Effects of genistein on NF-κB and (B) statistical analysis of the protein level of NF-κB. *P<0.01, compared with the 0 μM genistein treatment group. NF-κB, nuclear factor-κB.

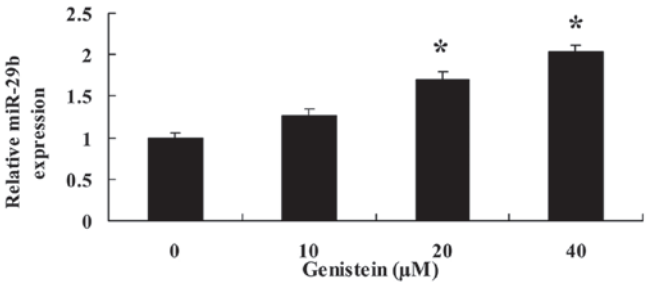

Figure 5. Genistein activates the expression of miR-29b. *P<0.01, compared with the control group. miR, microRNA.

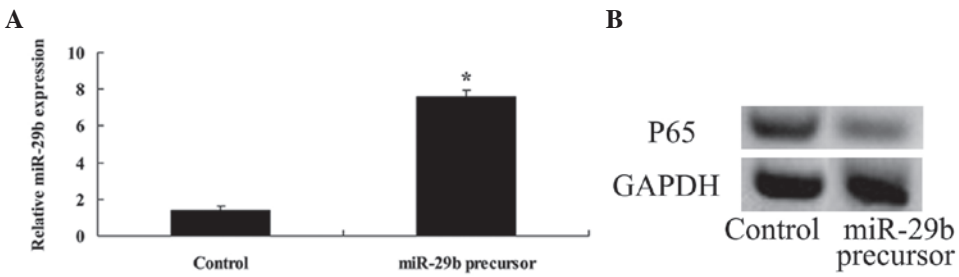

Figure 6. Overexpression of miR-29b can suppress the expression of NF-κB. (A) Overexpression of miR-29b increased the expression of miR-29b and (B) inhibited the protein expression of NF-κB. *P<0.01, compared with the 0 μM genistein treatment group. miR, microRNA; NF-κB, nuclear factor-κB.

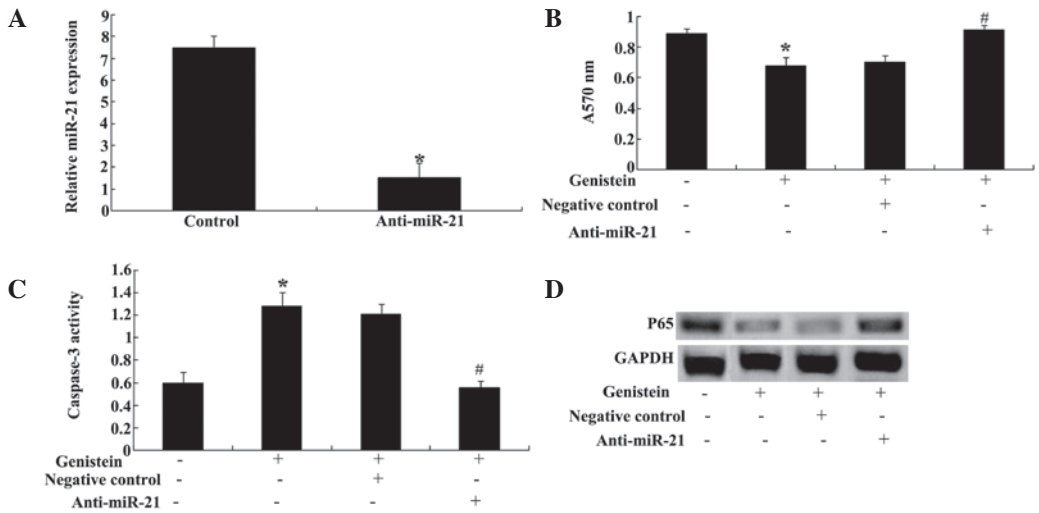

Figure 7. Anti-miR-29b reverses the effect of genistein. (A) Anti-miR-29b reversed the effect of genistein on the expression of miR-29b in U266 cells. (B) Following treatment with 20 μM genistein for 48 h, anti-miR-29b significantly promoted the cell proliferation of U266 cells. (C) Following treatment with 20 μM genistein for 48 h, anti-miR-29b evidently inhibited apoptosis of U266 cells. (D) Anti-miR-29b significantly increased NF-κB activity in U266 cells following genistein (20 μM) treatment for 48 h. *P<0.01, compared with the 0 μM genistein treatment group and #P<0.01, compared with the genistein-treated group transfected with the negative control. miR, microRNA; NF-κB, nuclear factor-κB.

expression of NF-κB in U266 cells was detected. These data indicated that transfection of miR-29b plasmids significantly increased the expression of miR-29b in U266 cells (Fig. 6A). Furthermore, overexpression of miR-29b significantly inhibited NF-κB expression in U266 cells (Fig. 6B).

Anti-miR-29b reverses the effect of genistein. To further investigate the correlation between the expression of miR-29b and the effect of genistein, the effect of genistein on MM was examined. The results demonstrated that transfection of anti-miR-29b plasmids into U266 cells significantly reduced the expression of miR-29b in U266 cells (Fig. 7A). Furthermore, anti-miR-29b plasmids could significantly reduce the effect of genistein on cell proliferation (Fig. 7B) and apoptosis of U266 cells (Fig. 7C). In addition, anti-miR-29b plasmids could promote the levels of NF-κB in U266 cells (Fig. 7D).

Discussion

MM is a malignant disease of plasma cells, of which the main clinical manifestations include hyperglobulinemia, renal dysfunction, bone damage and pancytopenia (18). At present there is no cure, however, there has been significant progress in treatment in previous years, including thalidomide, proteasome inhibitors and the application of bone marrow transplantation. However, the survival time of MM patients has not yet significantly improved. Therefore, possible treatments for MM are being continuously investigated. In the present study, genistein (5, 10 and 20 μM) inhibited the proliferation of U266 cells. Genistein possesses estrogen and anti-estrogen properties, as well as exerting antioxidant effects to inhibit the activity of protein tyrosine kinase, which inhibits the activity of topoisomerase II (19). In the present study, genistein significantly increased apoptosis and activity of caspase-3 in U266 cells. A previous study revealed that genistein combined with doxorubicin had a synergistic cytotoxic effect on breast cancer cells (20). Genistein has also been demonstrated to induce apoptosis of MCF-7 and 3T3-L1 cells via regulation of ERα expression (15).

Sustained activation of NF-κB can promote MM cell proliferation, mediate the secretion of IL-6 and the expression of adhesion molecules, upregulate anti-apoptotic proteins, inhibit death receptor pathways and promote angiogenesis, contributing to the proliferation of malignant myeloma cells and their resistance to apoptosis (21). Drugs targeting NF-κB can prevent NF-κB activation, promote apoptosis and improve prognosis, providing a broad prospect for the treatment of MM (22). In the present study, genistein was found to have a pronounced inhibitory effect on the protein level of NF-κB in U266 cells. In addition, Luo *et al* reported that genistein could induce apoptosis in human colon cancer through inhibiting the NF-κB pathway (23). Chung *et al* demonstrated that genistein inhibits phorbol ester-induced NF-κB transcriptional activity in human mammary epithelial cells (24).

miRNAs comprise ~1-2% of the known eukaryotic genome and are important in tumor biology, acting as tumor suppressor genes and proto-oncogenes. Overexpression of miRNA-29b reduces the level of Mcl-1 protein, thereby inhibiting the growth of MM cells, suggesting that miRNA-29b may be a tumor suppressor gene. Furthermore, miR-29b suppresses MM and endothelial cells by inducing the expression of SOCS-1 (25). Based on this, the present study found that the expression of miR-29b in U266 cells was also significantly promoted following treatment with genistein. It has been reported that genistein exerts its anti-tumor effect through downregulation of miR-1260b in prostate cancer cells (26). Xia *et al* reported that genistein can suppress pancreatic cancer cells through inhibition of miR-27a (27). The results of the present study demonstrated that overexpression of miR-29b significantly inhibited NF-κB expression in U266 cells. miR-29b was able to alter and control the expression of NF-κB in U266 cells. The decreased expression of miR-29b reduced the effect of genistein on U266 cells and increased the expression of NF-κB in U266 cells. In conclusion, genistein inhibits the proliferation and induces the apoptosis of human MM cells through suppressing NF-κB via upregulation of miR-29b.

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