Genistein inhibits growth of human uveal melanoma cells and affects microRNA-27a and target gene expression

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Abstract. Genistein, an isoflavone isolated from soybean, has been found to be a potent antitumor agent. However, both the effect of genistein on human uveal melanoma cells and the precise mechanism by which genistein suppresses tumorigenesis remain unclear. In the present study, we explored the possible activity of genistein to inhibit human uveal melanoma cell growth and investigated the possible role of genistein on microRNA-27a (miR-27a) as well as its target gene zinc finger and BTB domain containing 10 (*ZBTB10*) expression levels. The results suggested a significant inhibition of uveal melanoma cell growth in a time- and dose-related manner. *In vivo* study also indicated treatment groups with genistein could significantly inhibit the growth of xenografts. Further functional assays revealed that the levels of miR-27a and its target gene *ZBTB10* were significantly different based on the dose of genistein. In conclusion, the present study demonstrates that genistein exerts growth inhibitory activities in human uveal melanoma cells. Moreover, we for the first time report a correlation between antitumor activity of genistein and miR-27a mediated regulatory mechanism.

Introduction

Uveal melanoma is the most common primary malignant tumor of the eye in adults (1). It is associated with a high mortality rate due to early hematogenous dissemination and preferentially affects the liver (2,3). In the past decades, despite the development of more diagnostic techniques and the introduction of new treatment modalities, the mortality of uveal melanoma still have not significantly changed. Moreover, it has been found to be highly resistant to various chemotherapeutic drugs in uveal melanoma cells (4). Therefore, it is critical to improve understanding of the molecular pathogenesis of cancers and eventually develop new therapeutic drugs that can be effected by interfere with a specific pathway in uveal melanoma development or progression.

MicroRNAs (miRNAs), a novel group of endogenous non-coding small RNAs, which negatively regulate gene expression in post-transcriptional manner by interfering with target mRNA (5). Abnormal expression of miRNAs has been shown to play crucial roles in a variety of cellular processes including differentiation, apoptosis and cell proliferation (6,7). A previous study showed that some special miRNAs can serve as both diagnostic markers and therapeutic targets for many different tumor types (8). Worley *et al* suggested that aberrant miRNA expression was considered as accurate biomarker for metastatic risk in uveal melanoma (9).

Genistein, an isoflavone isolated from soybean, has been reported to have the preventive and therapeutic effect on carcinogenesis and many other chronic diseases (10). It is widely considered as a classic tyrosine kinase inhibitor, which plays key roles in cell growth and apoptosis (11). In our previous study, we also found that genistein had an effect on the prevention and treatment of ocular neovascularization, by inhibiting vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) expression in cultured retinal pigment epithelium cells (12,13). Previous studies indicated that genistein inhibited melaonoma cell growth apoptosis *in vivo* and *in vitro* (14,15). Genistein has been revealed to inhibit the growth of various human cancers by affecting a wide variety of cellular processes and/or enzymes, such as cell cycle, apoptosis and angiogenesis, however, there is no related study between miRNA-mediated transcriptional modulation and the inhibitory effects of genistein on carcinogenesis. Moreover, both the effect of genistein on human uveal melanoma cells and the precise mechanism by which genistein suppresses tumorigenesis remain unclear. Therefore, we examined the effects of genistein on human uveal melanoma cells growth *in vivo* and *in vitro* and investigated the possible role of genistein on microRNA-27a and its target gene zinc finger and BTB domain containing 10 (*ZBTB10*) expression levels. MiR-27a is an oncogenic

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miRNA and has been reported to be widely expressed in various cancer cells (16,17). In the present study, we hypothesized that genistein could affect human uveal melanoma vitality and analyzed whether genistein inhibits tumor growth by influencing the expression of miR-27a and its target gene.

Materials and methods

Chemicals. Genistein was purchased from Sigma (St. Louis, MO, USA). For cell culture experiments, genistein was dissolved in dimethyl sulfoxide (DMSO) (Sigma) and the final concentration of 0.05% DMSO. *In vivo* studies, genistein was dissolved in 1% DMSO to a desired concentration and was sonicated into suspension in 0.9% saline prior to injection.

Cell lines. The human uveal melanoma cell line C918 was kindly provided by Professor Elisabeth A. Seftor (Children's Memorial Research Center, Chicago, IL). The origin and characteristics of C918 cell line has been described previously (18). The cells were maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum and 0.1% gentamycin sulfate in 37˚C incubator with 5% carbon dioxide.

Cell growth and viability. The effect of genistein on the *in vitro* growth of C918 cells was determined by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cells were plated at the density of $1x10^4$ cells/well onto a 96-well plate. After a 12 h preincubation, cells were exposed to different concentrations of genistein (0, 10, 25, 50, 100 and 200 μM) for 72 h. Subsequently, 20 μl of 0.5% MTT in phosphate-buffered saline (PBS) was added to each well and the incubation was continued for 4 h at 37˚C. At the end of the incubation period, the culture medium was replaced with 200 μl of DMSO and the optical density of each well was determined with a spectrophotometer at 492 nm. Eight duplicate wells were set up for each concentration sample.

Animal model. Six-week-old female BALB/C nu/nu mice were obtained from Vital River Laboratory Animal Technology (Beijing, China). They were kept under specific pathogen-free conditions in autoclaved cages and fed with sterilized food and water *ad libitum*. All experiments were performed in accordance with the official recommendations of the Chinese Community Guidelines. For *in vivo* injection, C918 cells were harvested when growing to 80-90% of confluence and washed twice with sterile PBS, then counted and resuspended in PBS at a density of $1x10^7$/ml. The suspension (0.1 ml/10 g body weight) was injected subcutaneously into the nude mice. When tumor nodules were palpable, the mice were randomized into five groups. Following our previous experiment, genistein was used at concentrations of 25, 50 and 100 mg/kg body weight/day intraperitoneally (i.p.), respectively. In brief, the groups were: group 1, untreated; group 2, control, injected with 1% DMSO; group 3, genistein, 25 mg/kg; group 4, genistein, 50 mg/kg; group 5, genistein, 100 mg/kg. The treatment was continued every day for 1 month. During this period, the xenograft volume was measured by caliper every other day and was calculated by the formula: length x width^2 x 0.5. At the study endpoint, the mice were sacrificed, the tumors were removed and weighed and livers were also collected. The inhibition rate (IR) of the tumor was determined using the formula:

IR (%) = (1- mean tumor weight of treatment group/mean tumor weight of control group).

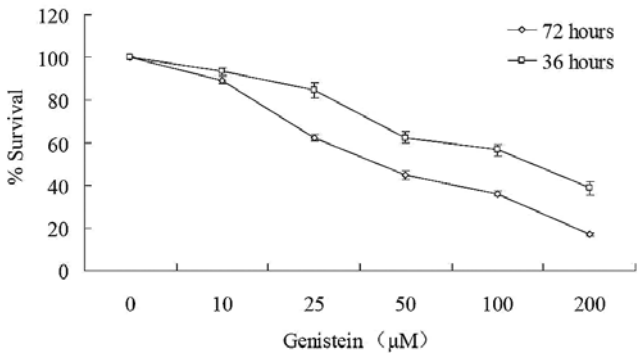

Figure 1. Effect of genistein on human uveal melanoma (C918) cell growth and viability. Cell viability of C918 cells was determined by MTT assay after 36 and 72 h stimulation with or without different concentrations of genistein. Each point represents the mean ± SEM. This figure is a representative of three independent experiments.

Quantitative RT-PCR. Total RNA from C918 cells was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. TaqMan microRNA assays (Applied Biosystems Inc.) were used to quantify miR-27a expression. Small nuclear RNA, U6B (Applied Biosystems Inc.), was treated as normalization control. *ZBTB10* and TATA binding protein (*TBP*) (control) mRNA levels were assessed by SYBR-Green quantitative PCR. Reactions were conducted according to manufacturer's instructions using Power SYBR-Green PCR Master Mix (Applied Biosystems Inc.). Forward (F) and reverse (R) primers were used as follows: *ZBTB10*, GCTGGATAGTAGTTATGTTGC and CTGAGTGGTTTG ATGGACAGA; *TBP*, TGCACAGGAGCCAAGAGTGAA and CACATCACAGCTCCCCACCA. All real-time amplifications were measured in triplicate and performed with the ABI Prism 7300 sequence detection system (Applied Biosystems Inc.). The fold-change of miR-27a and *ZBTB10* mRNA was calculated using the $2^{-\Delta\Delta CT}$ method.

Statistics. All data was represented as the mean ± SEM. The difference between the groups was determined by using the one-way ANOVA. A P-value of <0.05 was considered statistically significant. All the analyses were carried out with the SPSS 13.0 (SPSS Inc., Chicago, IL, USA) and were based on two-tailed probability.

Results

In vitro effect of genistein on C918 cell growth and viability. We investigated the effect of genistein on cell viability by MTT assay and the result suggested a significant inhibition of uveal melanoma cell growth in a time- and dose-related manner. As shown in Fig. 1, after a treatment period of 36 h, genistein decreased cell proliferation by ~60% in 200 μM

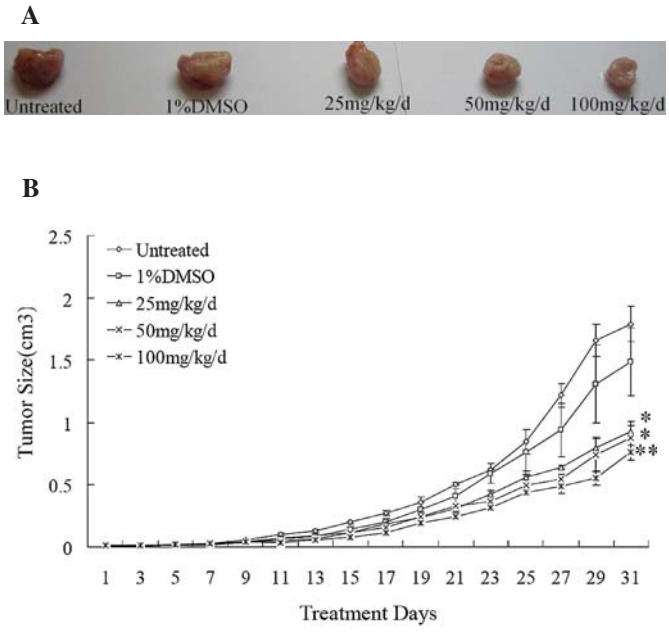

Figure 2. The inhibitory effect of genistein on the growth of uveal melanoma xenografts in nude mice. Animals were randomly assigned into five groups. The treatment groups were injected i.p. with 25, 50 and 100 mg/kg body weight/day of genistein, respectively. (A) Representative tumors in each experimental group are shown at the end of treatment. (B) Growth curves of xenografts revealed the mean tumor volume of the control and genistein treatment groups. Tumor size was determined as described in the Materials and methods. Data represent means ± SEM., n=4. *P<0.05; **P<0.01 vs. control (1% DMSO).

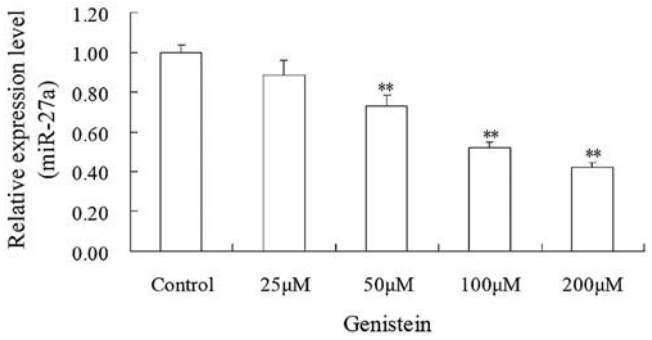

Figure 3. The effect of genistein on miR-27a expression in human uveal melanoma C918 cells. After being treated with 0, 25, 50, 100 and 200 μM of genistein for 48 h, miRNA was extracted and measured by TaqMan microRNA assays. The relative expression of miR-27a was normalized to that of the U6B small nuclear RNA gene (RNU6B) control. Data represent means ± SEM, n=4. **P<0.01 vs. control.

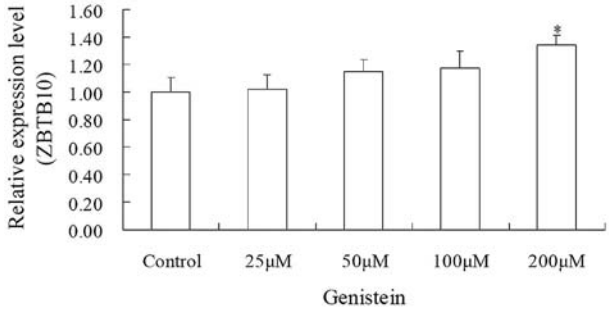

Figure 4. The effect of genistein on *ZBTB10* expression in human uveal melanoma C918 cells. After being treated with 0, 25, 50, 100 and 200 μM of genistein for 48 h, mRNA was extracted and assessed by SYBR-Green quantitative PCR. The relative expression of *ZBTB10* mRNA normalization was performed with TATA binding protein (*TBP*). Data represent means ± SEM, n=4. *P<0.05 vs. control.

group compared to control cells. When the treatment was extended to 72 h, the 50% inhibitory concentration (IC_{50}) value had decreased to 48.23 μM and at higher concentration (200 μM), the cell viability was only 16.7%. These data suggested that genistein could markedly inhibit human uveal melanoma cell growth and viability.

In vivo impact of genistein on uveal melanoma xenografts. To study the effect of genistein on human uveal melanoma, we established an ectopic model of human uveal melanoma in 28 athymic nude mice. There were no difference on body weight before the study (19.31±0.76 g, P=0.590). When tumors were observed, we administered genistein to tumor model mice daily for 30 days. We found that all treatment groups with genistein significantly inhibited the growth of xenograft *in vivo* (F=8.849; P=0.001) (Fig. 2). The tumors were harvested on the 37th day and the average size of the tumors in the 100 mg/kg treatment group was 0.76±0.60 cm^3, while for the control group, the average size was 1.49±0.27 cm^3. The average size of the tumors in the other treatment groups were also smaller than the control group (data was not shown). However, there was no difference between untreated group and control group. In the present study, we also tested the tumor masses and the results showed that the inhibition rate of tumor growth for 25, 50 and 100 mg/kg body weight/day genistein was 18.8%, 21.5% and 36.8% compared with the control group, respectively.

Effect of genistein on expression of miR-27a and its target gene ZBTB10. In order to investigate whether genistein inhibits human uveal melanoma cell growth through affecting miR-27a and its target gene expression, we measured their levels of expression by quantitative RT-PCR. The 'stem-loop' real-time PCR results indicated that genistein markedly inhibited miR-27a expression in a concentration-dependent manner. After the cells were treated with 25, 50, 100 and 200 μM of genistein for 48 h, compared with the control, the levels of miR-27a were decreased to 88.9±6.9, 72.9±5.4, 51.7±3.2 and 42±2.5%, respectively (Fig. 3).

To verify whether the decreased level of miR-27a could ultimately influence the target gene transcription activity, we examined the expression level of the known target gene *ZBTB10*. As shown in Fig. 4, after 48 h treatment with genistein, *ZBTB10* expression in 200 μM genistein group was significantly higher than that in control group (P=0.037). These data suggested that genistein inhibited human uveal melanoma cell proliferation at least in part through endogenous microRNA-mediated pathway.

Discussion

It is widely accepted that tumor development and progression is a multi-step and multifocal field process involving genetic and non-genetic factors, such as the environment and diet.

Previous studies indicated that diets containing a high intake of soy products and green tea had a beneficial effect on tumorigenesis (19,20). Genistein, the predominant isoflavone found in soy, has been demonstrated to exert potential antitumor activity in a range of human cancer cell lines *in vitro* and in xenograft models. For example, Shao *et al* revealed that genistein exerted multiple suppressive effects on human breast carcinoma cells (21). Lakshman *et al* indicated that dietary genistein could inhibit metastasis of human prostate cancer (22). It has received wide attention by its potential health-beneficial effects in the last few years (23). Therefore, to improve our understanding of genistein antitumor activity, we measured its effects on human refractory cancer-uveal melanoma. In the present study, we for the first time revealed that genistein concentration-dependently inhibited human highly aggressive uveal melanoma cells growth *in vivo* and *in vitro*. In the further functional assays, interestingly, we found that this effect was mediated, at least in part, through affecting miR-27a-associated regulatory mechanism.

To date, the mortality rate remains high because of the frequent occurrence of metastases and no effective chemotherapy has been developed for uveal melanoma patients. Therefore, it is necessary to develop an effective and non-toxic drug. It is well known that angiogenesis plays an important role in the development as well as the progression of solid tumors. Especially for malignant uveal melanoma, neovascularization is regarded as crucial step for metastasis. Our previous studies indicated that genistein 50, 100 and 200 μM dose-dependently inhibited ocular neovascularization by affecting VEGF, HIF1α and bFGF expression (12,13,24). In the current study, we observed that genistein had a dose-dependent growth inhibition on human uveal melanoma cells *in vitro* and a tumor suppressor effect *in vivo*, possibly associated with the inhibitor activity on cell proliferation and angiogenesis. Similarly, Russo *et al* suggested that genistein reduced significantly the vitality of human melanoma M14 cells and showed a protective effect on ultraviolet light-induced DNA damage (25). Record *et al* also indicated that genistein inhibited the growth of B16 melanoma cells in a dose- and time-related manner, the IC_{50} was only 35 μM after 48 h treatment (14). Together, these investigations suggest that genistein exhibits strong antitumor effect in the human malignant melanoma. Moreover, Tamura *et al* indicated that genistein could enhance the chemosensitivity of melanoma patients (15).

MiRNAs, a class of novel posttranscriptional regulators, had aroused increasing interest in biological and medical sciences. Recent studies indicated that exogenous miRNAs played crucial roles in the development of multidrug resistance (MDR) in cancer cells (26-28). MiR-27a is located at chromosome 19 and has been shown to be widely expressed in breast cancer, gastric adenocarcinoma and cervical cancer (16,17,29). Mertens-Talcott *et al* observed that transfection of antisense miR-27a (as-miR-27a) led to increased *ZBTB10* levels in MDA-MB-231 breast cancer cells (17). Consistently, Scott *et al* also suggested that the oncogenic miR-27a suppressed *ZBTB10* expression, which interfere with transcription factor *Sp1* activation (30). Sp protein expression and activation play important roles in human tumor development and progression, by regulating cancer cell survival, growth, and angiogenesis (31-33). More recently, Zhu *et al* reported that miR-27a and miR-451 were involved in activating the MDR1 gene P-glycoprotein expression, which was associated with cancer cell resistance to a series of chemotherapeutics (34). In present experiments, we found that genistein modulated the expression levels of miR-27a and its target gene *ZBTB10*, which might be responsible for the effect on human uveal melanoma. Therefore, miR-27a and its target gene could be considered as targeted therapeutic strategy in cancer with resistant to traditional chemotherapeutic drugs.

In conclusion, the present study for the first time demonstrates that genistein exerts growth inhibitory activities in highly aggressive uveal melanoma cells *in vivo* and *in vitro*. Moreover, we for the first time reported the role of genistein on miR-27a and its target gene *ZBTB10* expression in uveal melanoma. Combined with other observations, it is biologically plausible that the decrease of miR-27a expression resulting in post-transcriptional gene regulation in uveal melanoma cells might partly account for the inhibitive effect of genistein on human uveal melanoma. Further study is needed to clarify the mechanism underlying the interaction between the miR-27a and tumor chemoprevention of genistein. More importantly, we provide novel insights into the molecular mechanisms of genistein therapeutic actions. We expect our findings could eventually promote the development of chemo-interventions for uveal melanoma.

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