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ORIGINAL ARTICLE

β-ELEMENE INHIBITS THE PROLIFERATION AND MIGRATION OF HUMAN GLIOBLASTOMA CELL LINES *VIA* SUPPRESSING RING FINGER PROTEIN 135

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ABSTRACT

β-Elemene is commonly used as an anti-cancer agent in different types of cancers and its effects on glioblastoma have been studied through different pathways. However, its effect through ring finger protein 135 (RNF135, OMIM 611358) (RNF135), which is upregulated in glioblastomas, has not yet been explored. The current study is focused on the effects of β-elemene on human glioblastoma cell lines U251, U118, A172 and U87 through RNF135. A cell counting kit-8 assay and wound healing assay have been utilized to test the proliferation and migration of the cells. Western blot and quantitative real-time-polymerase chain reaction (qRT-PCR) were used to evaluate the level of expression of RNF135. A model of nude mice was used to explore progression of the tumor *in vivo*. It was observed that increasing treatment time or dose of β-elemene remarkably decreased viability of the cells. The cells that were treated with β-elemene had a much lower speed of moving toward the gap in comparison to untreated cell lines. β-Elemene-treated cells showed a much lower level of expression of RNF135 mRNA than control groups ($p < 0.05$) and the levels of RNF135 protein were lower in the cells treated with β-elemene than in control groups ($p < 0.05$). Moreover, tumor progression in subcutaneous xenograft nude mice

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was delayed with the injection of β-elemene. Altogether, our findings suggest that β-elemene inhibits proliferation, migration and tumorigenicity of human glioblastoma cells through suppressing RNF135.

Keywords: β-Elemene; Glioblastoma; Migration; Proliferation; Ring finger protein 135 (RNF135); Tumorigenicity.

INTRODUCTION

β-Elemene, which has very limited toxicity, has been proven to have a large spectrum of anti-cancer effects in various types of cancers [1]. It is proven that β-elemene inhibits the formation of DNA of various cancer cell lines triggering the suppression of cancer formation [2]. The basic oil extracted from Curcuma Wenyujin is composed of β-elemene, γ-elemene and δ-elemene, of which β-elemene is the major active component [3].

Glioblastoma, which is the most common glioma in adults and categorized as WHO grade IV tumor, has disappointing prognosis [4,5]. Even though its treatment regimen has been markedly meliorated but still most of the patients relapse soon after treatment with an average survival rate of 8 to 9 months after the progression of the tumor [6].

Ring finger protein 135 (RNF135, OMIM 611358) (RNF135) is up-regulated in glioblastomas [7] and it has a ring finger domain in its N-terminal, two more domains, which are coiled, and a C-terminal that is about 200 amino acid in size [8]. Therefore, as RNF135 regulates degradation of protein, it has significant effect in various biological processes [9].

As glioblastoma is the most common glioma of adults and has got the worst prognosis therefore many studies and researches have been focused on the treatment of this serious disease. In this study, we found that β-elemene, which is obtained from Curcuma Wenyujin, inhibits the proliferation and migration of human glioblastoma cell

lines U251, U118, A172 and U87 by suppressing RNF135. We also found that β-elemene inhibits the tumorigenicity of human glioblastoma cell line U251 in vivo.

MATERIALS AND METHODS

Cell Culture. Cell lines U251, U118, A172 and U87, which have been derived from human glioblastomas, were purchased from the Shanghai Cell Biology Institute, Shanghai, People's Republic of China (PRC), were cultured in high glucose Dulbecco's modified eagle medium (Gibco, El Paso, TX, USA), fetal bovine serum 10.0% (Ausbian, St. Lucia, Queensland, Australia), penicillin and streptomycin (100 IU/mL, 100 mg/mL). The cells were stored in the incubator that was providing 5% CO_2 at a temperature of 37 °C.

Animals. National Institutes of Health (NIH) guidelines were followed for the experimental procedures and animal care. The Ethics Committee of the First Affiliated Hospital of Dalian Medical University, Dalian, Liaoning Province, PRC approved the guidelines for the use of mice. The 5-6 week old female nude mice, born at the Animals Experimental Center of Dalian Medical University, Dalian, Liaoning Province, PRC.

Cell Viability Assay. The cell counting kit-8 (CCK-8) (DHDB4000X; Dojindo Molecular Technologies, Tokyo, Japan) was utilized for measuring the viability of cells. At the beginning, 5000 cells were administered in a 96-well plate in high glucose DMEM with 10% FBS and 1% antibiotics (penicillin +streptomycin) and stored in incubator at 37 Celsius temperature and 5% CO_2 for a time interval of 24 hours. β-Elemene was used to treat the cells for 24, 48, 72, 96, 120 hours, respectively. Then 1:10 diluted CCK-8 solution was administered to the cells and stored for about 1-3 hours in an incubator at 37 °C and 5.0% CO_2. The absorbance was detected at 450 nm in a micro plate reader. Graph Pad Prism 7.04 (www.graphpad.com) was used to analyze the results.

Wound Healing Assay. Five thousand cells per well were administered into a 6-well plate and incubated for 24 hours in fresh medium. In order to create a scratch we used the tip of a 200 mL pipette. The scratch was washed with phosphate buffered saline. The wound was immediately photographed and its area was measured at time zero. Then the cells were cultured in fresh medium alone and (fresh medium plus β-elemene) for 12, 24, 36, 48 hours, respectively; the wounds were photographed and their areas were measured in the time intervals mentioned. The areas of the wounds were measured with Image J software (http://rsb. info. nih.gov/ij/).

Quantitative Real-Time Polymerase Chain Reaction and Reverse Transcription. Based on the method explained [10], the cell lines were washed twice with PBS and then Trizol (Cat: KGA1203; KeyGEN, Dalian, Liaoning, PRC) was used to extract RNA according to the manufacturer's guidelines. The kit for reverse transcription was used (Cat: RR037A; Takara, Shiga, Japan) and cDNA was then augmented with utilizing SYBR®Premix Ex Taq™ Kit for 40 cycles with the 7500 real-time polymerase chain reaction (qRT-PCR) system. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the loading control and 2-37 ΔΔCT method for calculating fold expression was used to analyze the data. The primers for GAPDH were 5'-GCA CCG TCA AGG CTG AGA AC-3' (forward) and 5'-TGG TGA AGA CGC CAG TGG A-3' (reverse) and the primers for RNF135 were 5'-TAC TGG GAA GTG GAC ACT AGG AAT T-3' (forward) and 5'-CTT GAC CAT GTG CCA TGC A-3' (reverse).

Western Blot. The total protein of glioblastoma cells were collected using 500 μL radioimunoprecipitation assay (RIPA) cell lysis buffer (Cat: ab156034; Abcam, Nanjing, Jiangsu, PRC) and then a BCA detecting kit (Cat: ab102536; Abcam) was utilized to qualify the protein. Equal amounts of protein were subjected and separated by 10.0% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred onto polyvinylidene difluoride (PVDF) membrane (0.2 μm Millipore, Cat: ab133411; Abcam) and blocked with 5.0% milk free of fats. The membranes were immunoblotted with primary specific antibodies targeting RNF135 (Cat: ab229959; Abcam; dilution rates of 1:2000) and GAPDH (Cat: ab245355; Abcam; dilution rates of 1:500) at 4 °C for overnight. On the second morning, the membranes were immunoblotted with a secondary antibody (Cat: ab205718, Abcam; dilution rates of 1:2000) for 2 hours at room temperature, and finally, enhanced chemiluminescence reagent was used to detect the proteins on the membranes.

Immunohistochemistry. The immunohistochemistry procedure was carried out according to the protocols and explanations [11]. Formalin-fixed paraffinized sections were embedded for 15 min. in pure xylene to dewax the sections. The sections were rehydrated in a series of graded ethanol, blocked in serum, stored with RNF135-specific rabbit anti-human antibody at 4 °C overnight. The sections were washed twice and stored in biotin-labeled goat anti-rabbit antibody at room temperature for 20 min. Finally, chromogenic reactions were carried out with horseradish peroxidase (HRP), 3,3-diaminobenzidine and hematoxylin. A Leica Wetzlar (DM2500 LED; Leica Microsystems GmbH, Wetzlar, Germany) microscope was used to analyze and photograph the samples.

Statistical Analyses. One way analysis of variance (ANOVA) of repeated measures and a two-sample t-test was used to analyze the data. The data is presented as (mean ± SD) at the minimum of three independent experimental groups. The Statistical Package for the Social Sciences (SPSS) version 24.0 (IBM Inc., Armonk, NY,

USA), and GraphPad Prism 7.04 software were used to analyze and graph the data. A p value of <0.05 was judged to be statistically significant.

RESULTS

β-Elemene Suppresses RNF135 in Human Glioblastoma Cell Lines U251, U118, U87 and A172. The cells were cultured and treated with β-elemene in at least three independent experimental groups. The level of expression of RNF135 mRNA was detected with qRT-PCR. The cell lines that were treated with β-elemene showed a much lower level of expression of RNF135 mRNA than the control groups (p <0.05) [Figure 1(A)-1(D)]. As with PCR, the data from Western blot also confirmed that protein levels of RNF135 were lower in the cell lines which were treated with β-elemene than in the control groups [Figure 1(E)-1(H)].
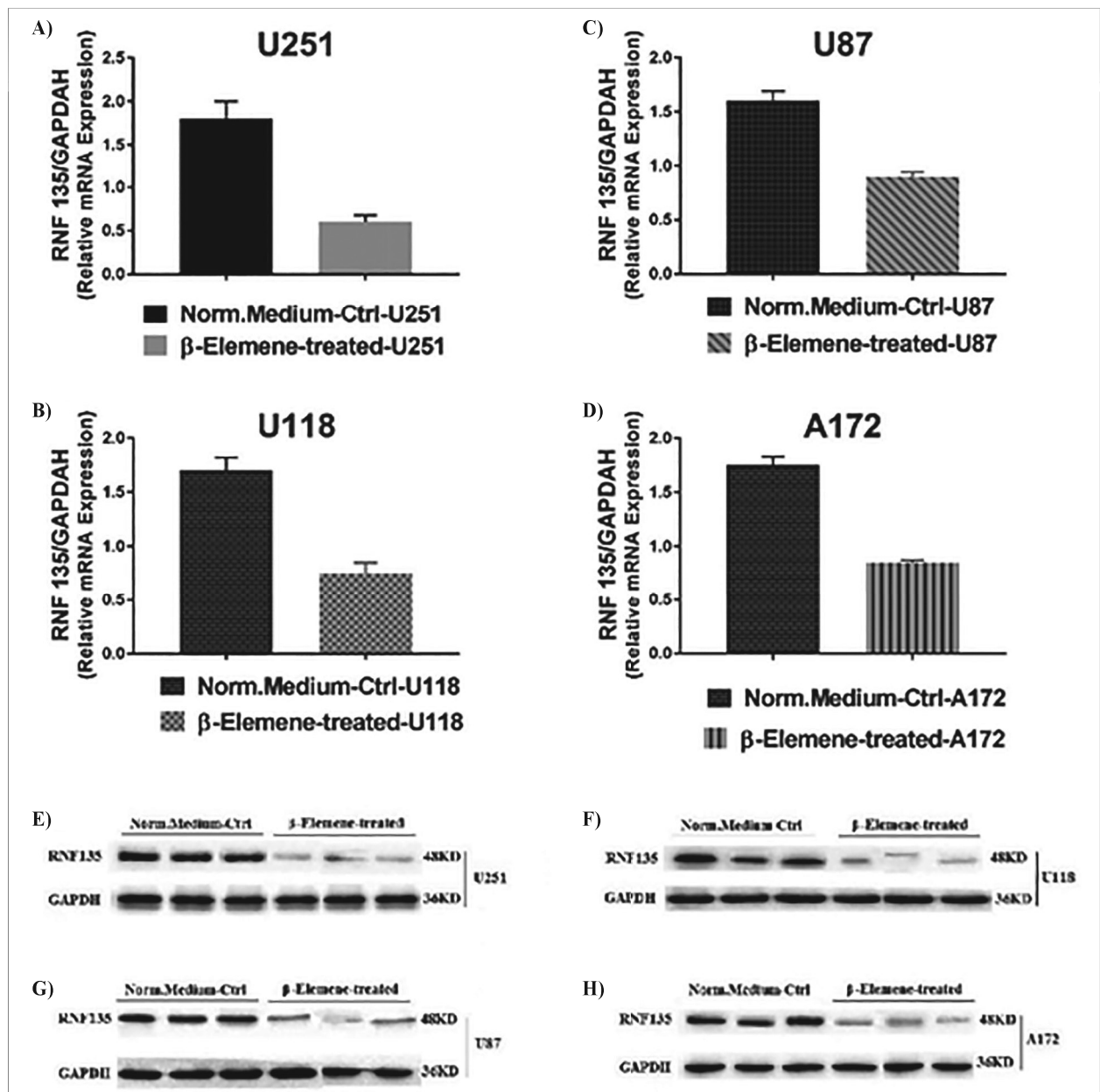

Figure 1. The expression level of RNF135 is suppressed in human glioblastoma cell lines U251, U118, U87 and A172 treated with β-elemene. The data from qRT-PCR (Author: see Abstract) of three independent experimental groups showed that the cell lines treated with β-elemene have a lower level of RNF135 mRNA than the control group (p <0.05) **(A-D).** The data from Western blot also confirmed that protein levels of RNF135 were lower in the cell lines that were treated with β-elemene than those of the control group **(E-H).** Glyceraldehyde 3-phosphate dehydrogenase was used for both qRT-PCR and Western blot as loading control.

The Viability of Different Human Glioblastoma Cells Decreases with Increasing Doses of β-Elemene. The CCK-8 was utilized, based on the report of the manufacturer's guidance, to test the effects of β-elemene on the proliferation of human glioblastoma cells. The U251, U118, A172 and U87 cells were treated with various doses of β-elemene such as 0.0, 20.0, 40.0, 60.0, 80.0, 100.0 μg/mL over a time interval of 24 hours to obtain noticeable effects on the proliferation of the cells. The viability of the cell was shown as the percentage of control [Figure 2(A)-2(D)], and it was observed that the half maximal inhibitory effect (IC50) for each cell line was at different doses of β-elemene, such as 47.44 μg/mL for U251, 49.68 μg/mL for U118, 57.36 μg/mL for A172 and 58.41 μg/mL for U87.

Increasing Time of Treatment with β-Elemene Results in Decreasing Viability of Different Cell Types of Human Glioblastoma. To determine the inhibitory effect of β-elemene on human glioblastoma cell lines in different time intervals we used CCK-8 based on the explanation mentioned in the Materials and Methods section. The U251, U118, A172 and U87 cells were utilized and 50.0 μg/mL of β-elemene was used for U251 and U118 cells and 60.0 μg/mL of β-elemene was used for U87 and A172 cells over a time interval of 0, 24, 48, 72, 96, 120,

144 hours. It was found that with increasing the time of treatment (we used IC50 dose for the cells), the viability of the cells decreases remarkably [Figure 3(A)-3(D)].

β-Elemene Inhibits the Migration of Human Glioblastoma Cell Lines U251, U118, A172 and U87. Wound healing assay was utilized to detect inhibitory effects of β-elemene on migration of cell lines U251, U118, A172 and U87. The results showed that the cell lines treated with β-elemene have a much lower speed of moving into the gap in comparison to the cell lines that were not treated ($p < 0.05$) [Figure 4(A)-4(D)].

β-Elemene Suppresses Tumorigenicity of Human Glioblastoma Cell Line U251 *In Vivo*. Human glioblastoma cells U251 were cultured *in vitro* and then (1×10^7) cells were suspended in 200 mL Dulbecco's modified eagle medium were injected subcutaneously into the 5-6 week old female nude mice. At 10 days post-injection, the mice developed tumors that were visible to the naked eye and were measured using Vernier Calipers. The tumors had an average volume of 3 mm^3. The equation (V = 1/2 5- to 6-week-old female nude mice × largest diameter × smallest diameter) was used for measuring the volumes of the tumor as reported previously [12] and the mice were divided into two groups (each group having five mice). One group was given daily intra-
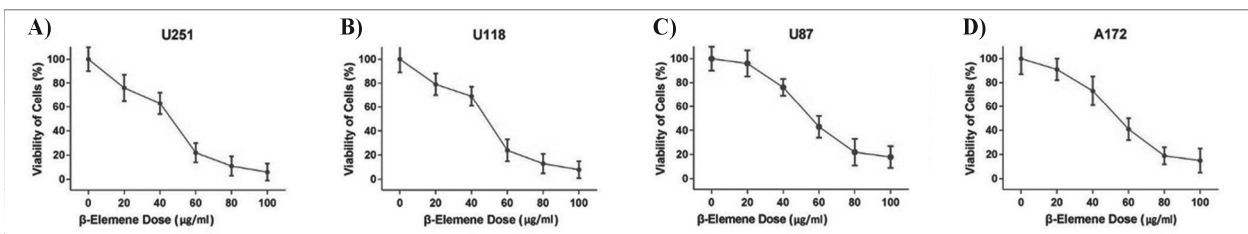

Figure 2. Increasing β-elemene treatment dose decreases viability of glioblastoma cells. Human glioblastoma cell lines U251, U118, U87 and A172 were sown at the concentration of 5000 cells per well in 96-well plates and treated with various doses (0, 20, 40, 60, 80 and 100 μg/mL) of β-elemene. The half maximal inhibitory effects (IC50) of the cells were obtained by doses of 47.44, 49.68, 58.41 and 57.36 μg/mL of β-elemene, respectively **(A-D)**. The CCK-8 was utilized to ascertain cell viability and inhibitory effect of β-elemene on the proliferation of the cells. The results are shown as mean ± SD of the three experiments ($p < 0.05$, the difference is statistically significant in the untreated control group).
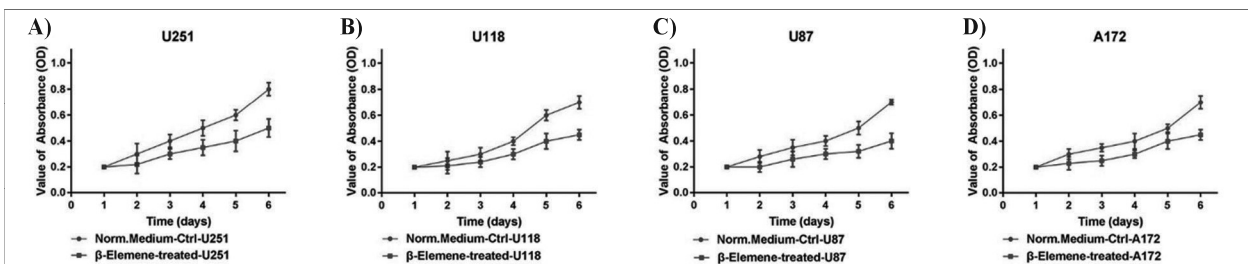

Figure 3. Increasing the time of treatment with β-elemene decreases glioblastoma cells viability. The 5000 cells per well were sown in 96-well plates. Cell lines U251 and U118 were treated with 50 μg/mL β-elemene and cell lines U87 and A172 were treated with 60 μg/mL β-elemene for 0, 24, 48, 72, 96, 120 and 144 hours **(A-D)**. The CCK-8 was utilized to determine inhibitory effect of β-elemene on the proliferation of the cells in different time intervals. The results are shown as mean ± SD of the three experiments ($p < 0.05$, the difference is statistically significant in the untreated control group).

peritoneal (50 mg/kg) β-elemene and the second group was kept as control and was not treated with β-elemene. At 28 days post-injection, the tumors were removed and weighed; on average, the tumors from the β-elemene-treated group weighed 1.315 g, and the tumors from the control group weighed 2.248 g ($p <0.05$) [Figure 5(A) and 5(B)]. The results from immunohistochemistry procedure showed that the levels of expression of RNF135 protein were down in the tumors of the group which was treated with β-elemene in comparison to the tumors of control group [Figure 5(C)].
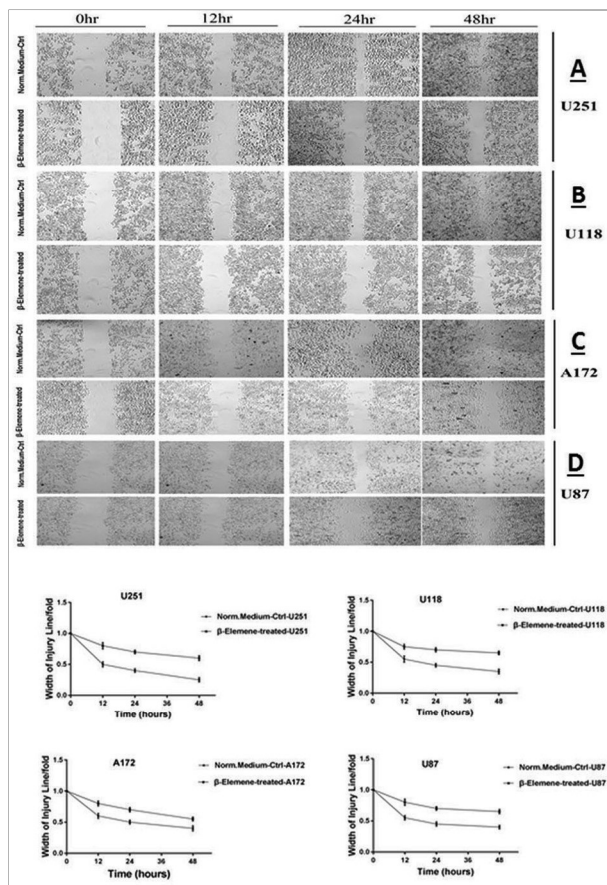

Figure 4. Migration of human glioblastoma cell lines U251, U118, A172 and U87 were inhibited by β-elemene. In this wound healing assay, 5000 cells/well were sown into 6-well plates, cultured and photographed at the time intervals of 12, 24, 36 and 48 hours in at least three independent groups (β-elemene treated *vs.* control groups). The data were analyzed as mean ± SD and $p <0.05$. The human glioblastoma cells treated with β-elemene was found to have much lower speed moving to the gaps compared to the untreated control group cells (**A-D**).

DISCUSSION

Glioblastoma, which is the most aggressive primary tumor of the brain and accounts for more than 50.0% of all brain tumors, arises from neuroglial progenitor cells with proportionately higher incidence rates in males. After treat-
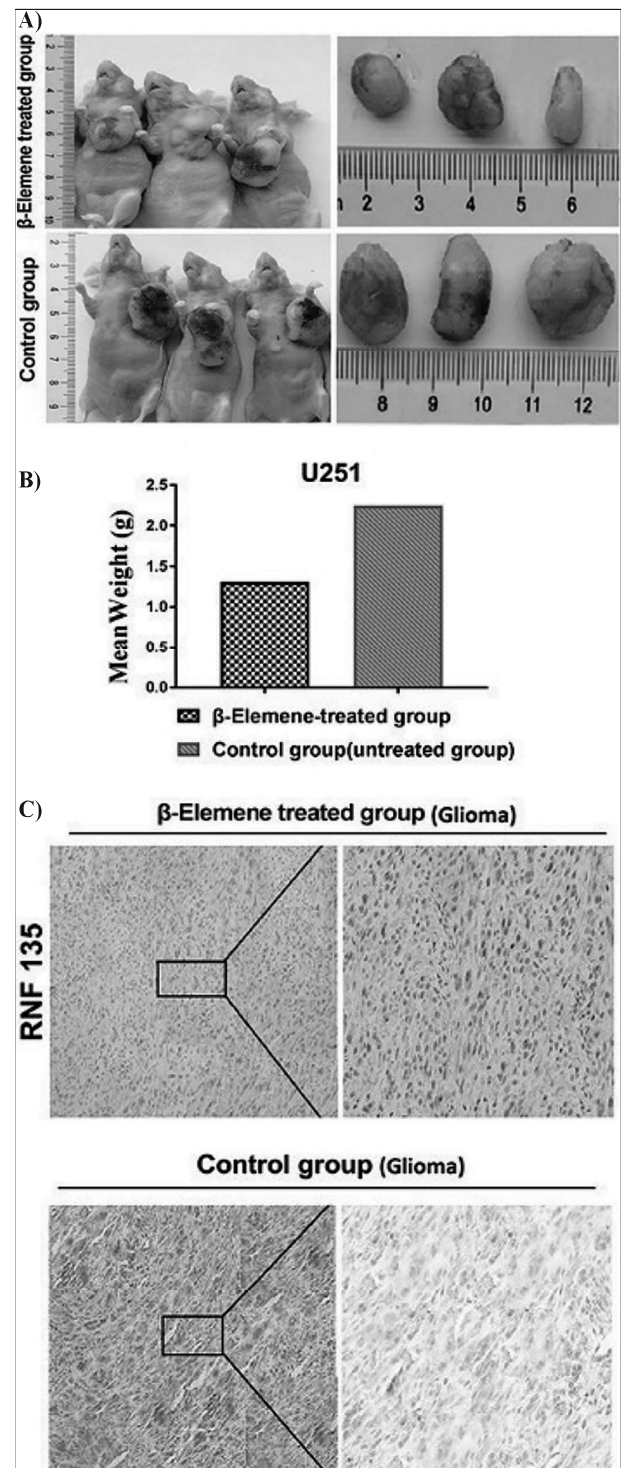

Figure 5. β-Elemene suppresses U251 cell tumorigenicity *in vivo*. Five- to six-week old female nude mice were injected subcutaneously with (1×10^7) U251 cells and the tumor weights from the β-elemene-treated group and control group (untreated group) were measured at 28 days post-injection (**A**).The tumorigenicity of the U251 cells was remarkably reduced in the group that was treated with β-elemene as compared to the group that was not treated with β-elemene ($p <0.05$) (**B**). Immunohistochemistry staining of RNF135 of the tumors treated with β-elemene and not treated with β-elemene (control group), original magnification ×400, ×100 (**C**).

ment with surgery, radiotherapy and chemotherapy, the survival rate still hardly reaches up to 15 months [13,14]. β-Elemene is the main constituent of elemene that is obtained from a herb known as Curcuma Wenyujin, and it has tumor-suppressing effects in various kinds of cancers with minimum side effects in the anti-cancer agents [15]. Ring finger protein 135 has a ring finger domain in its N-terminal, two more domains that are coiled and a C-terminal that is about 200 amino acids in size and is located on 17q11.2 [8,16]. Ring finger protein 135 that is up-regulated in glioblastomas, is a positive regulator for innate immunity mediated by the retinoic acid-inducible gene-I (RIG-I) signaling pathway [8,17].

In this study, we used human glioblastoma cell lines U251, U118, A172 and U87 to have a look at the effects of β-elemene on proliferation and migration of these cell lines. We observed that β-elemene inhibits the proliferation and migration of glioblastoma cells *via* suppressing RNF135.

We tried different doses in order to find an effective dose of β-elemene for treatment of the cells, and it was found that every cell line has different responses to the different doses of β-elemene and the responses were dose-dependent, meaning that elevated doses resulted in lower viability of the cells, as was also found in the previous study of Liu *et al.* [18]. Moreover, to study the duration of treatment and its outcome, we treated the cell lines for different time intervals such as 0, 1, 2, 3, 4, 5, 6 days, and it was found that the efficacy of the treatment increases with increasing the time of treatment, which was consistent with the results of Zhu *et al.* [19].

In the previous studies, it was confirmed that RNF135 is up-regulated in human glioblastomas and the level of expression of RNF135 is correlated with the grade of glioma [7]. It was also found that genetic disorganization of RNF135 causes learning disabilities in human beings and overgrowth syndrome [20]. In the present study, we used human glioblastoma cells to investigate the relationship between β-elemene and RNF135. The results from qRT-PCR showed that levels of RNF135 mRNA were lower in the cell lines treated with β-elemene in comparison to the untreated cell lines. The results from Western blot confirmed that the levels of protein expression in β-elemene-treated cells were lower than untreated cell lines, which means that β-elemene suppresses the expression of RNF135 in human glioblastoma cells.

An earlier study from Feng *et al.* [21] showed that U87 results in tumorigenicity in the nude mice xenografts, and Zhu *et al.* [22] reported that β-elemene regulates the stemness of U87 cells. Our findings also agree with the earlier results, and we observed that U251 cells results in tumorigenicity in female nude mice and β-elemene sup-

pressed the tumors formed in the mice by *via* suppressing the RNF135.

Previously, researchers reported that β-elemene prevents the migration of various cancer cells, such as cells of bladder cancer, cells of melanoma, cells of lung cancer, cells of stomach cancer, cells of cervical cancer and cells of breast cancer [15,23-27]. In our study, using a wound healing assay, we discovered that β-elemene inhibits the migration of human glioblastoma cells.

In conclusion, we found that β-elemene, which is obtained from an herb Curcuma Wenyujin, inhibits the proliferation and migration of human glioblastoma cell lines U251, U118, A172 and U87 *via* suppressing RNF135 and β-elemene also inhibits the tumorigenicity of human glio-blastoma cell line U251 *in vivo*.

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