Resibufogenin inhibits the malignant characteristics of multiple myeloma cells by blocking the PI3K/Akt signaling pathway

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Received March 21, 2022; Accepted April 29, 2022

DOI: 10.3892/etm.2022.11368

Abstract. Resibufogenin (RBG) is an active ingredient of toad venom that also has antitumor potential. The present study aimed to investigate the role of RBG in multiple myeloma (MM) and the underlying action mechanism involving the PI3K/Akt signaling pathway. A human MM cell line, RPMI8226, was treated with RBG and/or insulin-like growth factor 1 (IGF-1; an activator of the PI3K/AKT signaling pathway). Cell viability and apoptosis were detected using Cell Counting Kit-8 and flow cytometry, respectively. Cell migration and invasion were detected using a Transwell assay. In addition, the epithelial-mesenchymal transition (EMT)-associated proteins (E-cadherin, N-cadherin and Vimentin) and the PI3K/AKT pathway-associated proteins [AKT, phosphorylated (p)-AKT, PI3K and p-PI3K] were measured using western blotting. RBG inhibited the viability, migration and invasion, and promoted the apoptosis of RPMI8226 cells in a dose-dependent manner. RBG at concentrations of 4 and 8 μM upregulated E-cadherin, and downregulated N-cadherin and Vimentin in RPMI8226 cells. RBG also decreased the protein expression of p-AKT and p-PI3K in a dose-dependent manner. In addition, the intervention of IGF-1 weakened the inhibitory effects of RBG on the malignant characteristics of MM cells. RBG-induced inhibition of EMT and the PI3K/AKT pathway were also weakened by IGF-1 treatment. In conclusion, RBG inhibited viability, migration, invasion and EMT, and promoted the apoptosis of MM cells by blocking the PI3K/AKT signaling pathway.

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Key words: resibufogenin, multiple myeloma, malignant characteristics, PI3K/AKT signaling pathway, antitumor

Introduction

Multiple myeloma (MM) is a malignant blood cancer characterized by the proliferation of clonal plasma cells in the bone marrow (1). As the second most common hematological malignancy worldwide, MM accounts for ~10% of all hematological malignancies, and 1% of all cancers (2,3). In the past few years, the development of novel agents, including immunomodulatory drugs, proteasome inhibitors, monoclonal antibodies and histone deacetylase inhibitors have achieved great advances on improving the response rate and survival time of patients with MM (4-6). However, Since MM is still unable to be cured (5), novel therapeutic agents against MM are still needed to be explored.

Toad venom, a dried product of toxic secretions of *Bufo bufo gargarizans* Cantor or *Bufo melanostictus* Schneider, is a traditional natural medicine widely used in China that has been revealed to have cardiotonic and analgesic activities (7). A recent study has confirmed that toad venom is also a source of antitumor drugs, and contains 96 types of bufadienolide monomers and 23 types of indole alkaloids (7). Resibufogenin (RBG) is an active ingredient of toad venom that exhibits potential in the treatment of diverse types of cancer, such as gastric carcinoma, colorectal cancer and osteosarcoma (8). It also has been reported that RBG is a detectable component of 'cinobufotalin injection', which is permitted for clinical administration in the treatment of liver and gastric cancer by the Chinese food and drug administration (9).

Han *et al* (2021) demonstrated that RBG inhibits the proliferative activity and induces the necrosis of colorectal cancer cells (10). Zhou *et al* (2019) revealed that RBG inhibits the proliferation, migration and invasion of ovarian clear cell carcinoma cells *in vitro*, as well as the growth of tumor xenografts *in vivo* (11). Guo *et al* (2020) revealed that RBG inhibits glycolysis and cell proliferation and promotes the apoptosis of breast cancer cells (12). Epithelial-mesenchymal transition (EMT) also is a notable factor contributing to the metastasis of MM (13,14). Han *et al* (2018) demonstrated that RBG inhibits the liver metastasis of colorectal cancer by repressing EMT (10). However, to the best of our knowledge, the specific role of RBG in the cell proliferation, invasion and EMT of MM is still unclear.

PI3K/AKT signaling pathway is well known as a notable cellular pathway that plays an important regulatory role in basic intracellular functions, such as cell proliferation, survival, autophagy, motility and differentiation (15,16). Because the PI3K/Akt pathway can be activated by diverse cytokines stimulated by the interaction of MM cells with bone marrow mesenchymal stem cells (multipotent adult stem cells), its blocking has become a promising therapeutic strategy for MM (17,18). For example, an Akt inhibitor, TAS-117, inhibits the growth in addition to inducing the apoptosis and autophagy of MM cells (19). Afuresertib, an ATP-competitive Akt inhibitor, exhibits a favorable safety profile and clinical activity against MM in a phase I clinical trial (20). A pan-PI3K inhibitor, BKM120, inhibits the survival of MM cells by inducing apoptosis and G_2/M arrest (21). In addition, the blocking of the PI3K/Akt pathway is also closely associated with the antitumor efficiency of numerous natural traditional Chinese medicines in MM, such as silybin (22), plumbagin (23), triptolide (24) and icaritin (25). Furthermore, RBG can exert antitumor effects by regulating the PI3K/Akt signaling pathway in multiple types of cancer, such as ovarian clear cell carcinoma and gastric carcinoma (11,26). Zhou *et al* (2019) revealed that RBG inhibits ovarian clear cell carcinoma growth and cell migration by downregulating the PI3K/AKT pathway (11). Lu *et al* (2018) suggested that the anticancer effect of RBG is achieved through the PI3K/AKT/GSK3β pathway (26). However, the action mechanism of RBG involving the PI3K/Akt pathway in MM has not been revealed.

In the present study, the antitumor potential of RBG was first evaluated on the malignant characteristics of MM cells. The action mechanism of RBG involving the PI3K/AKT signaling pathway was further studied. Overall, this study may reveal a promising therapeutic drug for MM.

Materials and methods

Cell treatments. A human MM cell line, RPMI8226, (American Type Culture Collection) was cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium (HyClone; Cytiva) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin/streptomycin at 37˚C with 5% CO_2. Different concentrations of RBG (2, 4 and 8 μM) were used to treat RPMI8226 cells for 12, 24 and 48 h at 37˚C. The doses for RBG treatment were selected according to previous reports (10,12,27). In addition, RPMI8226 cells also received the treatments of 8 μM RBG combined with 50 ng/ml insulin-like growth factor 1 (IGF-1; an activator of the PI3K/AKT signaling pathway) (14,28) for 12, 24 and 48 h at 37˚C in feedback verification assays. RPMI8226 cells without treatments were used as the control, and cells treated with RBG + PBS was used as a control for treatment with RBG + IGF-1.

Cell Counting Kit-8 (CCK-8) assay. CCK-8 (Beyotime Institute of Biotechnology) was used for the detection of cell viability. Simply, 100 μl cells ($2x10^4$ cells/ml) were seeded into 96-well plates and then treated with RBG and/or IGF-1 for 12, 24 and 48 h at 37˚C, respectively. CCK-8 solution (10 μl) was subsequently added into each well. After 2 h of incubation at 37˚C, the optical density at 450 nm was detected using a microplate reader (Wuxi Hiwell Diatek Instruments Co., Ltd.). In addition, the IC_{50} value of RBG was calculated at 48 h post treatment.

Flow cytometry. Flow cytometry was conducted to detect apoptosis using an Apoptosis Detection Kit (cat. no. C1062S; Beyotime Institute of Biotechnology). Briefly, cells ($1x10^5$ cells/ml) at 48 h post-treatment with RBG and/or IGF-1 were washed with PBS three times and then suspended in 300 μl binding buffer. After incubation with 5 μl Annexin V-Fluorescein isothiocyanate for 15 min at room temperature, cells were re-stained with 10 μl propidium iodide (PI) for 10 min at room temperature. The apoptotic ratio was measured on a flow cytometer (CytoFLEX S; Beckman Coulter, Inc.) using Cell Quest software (version 5.1; BD Biosciences).

Transwell assay. Cell migration and invasion were detected using Transwell chambers. Cells at 48 h post-treatment with RBG and/or IGF-1 were adjusted to $1x10^5$/ml, and 200 μl cells were added into the upper chamber (pre-coated with Matrigel and air-dried naturally for invasion assay). The lower chamber was added with RPMI 1640 containing 10% FBS. After 24 h of incubation at 37˚C, cells in the lower chamber were washed with PBS, fixed with methanol for 30 min at room temperature, and stained with crystal violet for 20 min at room temperature. Cells were finally counted under a microscope (DMi3000 B; Leica Microsystems GmbH) in five randomly selected fields.

Western blotting. The protein expression of E-cadherin, N-cadherin, Vimentin, AKT, phosphorylated (p)-AKT, PI3K and p-PI3K were detected using western blotting. Total proteins were extracted by lysing cells in RIPA lysis buffer (Beyotime Institute of Biotechnology) and quantified using a BCA kit (Beyotime Institute of Biotechnology). After separation using 10% SDS polyacrylamide gel electrophoresis (50 μg protein per lane), the proteins were transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk for 1 h at room temperature and incubated with the following specific primary antibodies: Anti-E-cadherin (cat. no. ab133597; Abcam), -N-cadherin (cat. no. ab76057; Abcam), -Vimentin (cat. no. ab137321; Abcam), -GAPHD (cat. no. ab245355; Abcam), anti-AKT (cat. no. ab38449; Abcam), -PI3K (cat. no. 4292; Cell Signaling Technology, Inc.), -p-AKT (cat. no. 4060; Cell Signaling Technology, Inc.) and -p-PI3K (cat. no. AF3242; Affinity Biosciences Ltd.) (all 1:1,000) for 12 h at 4˚C. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:2,000; cat. no. ab205718; Abcam) for 1 h at 25˚C in the dark. After visualization using ECL kit (Pierce; Thermo Fisher Scientific, Inc.), images of the protein bands were captured using a Gel Imaging System (Tanon 3500; Tanon Science and Technology Co., Ltd.). Gray analysis for protein bands was performed using the ImageJ software (version 1.53r; National Institutes of Health) and the protein expression was normalized to GAPDH.

Statistical analysis. The software of GraphPad Prism 7.0 (GraphPad Software, Inc.) was used for statistical analysis. Each experiment was performed in triplicate. Data are presented as the mean ± standard deviation. Comparisons among different groups were determined using one-way analysis of variance

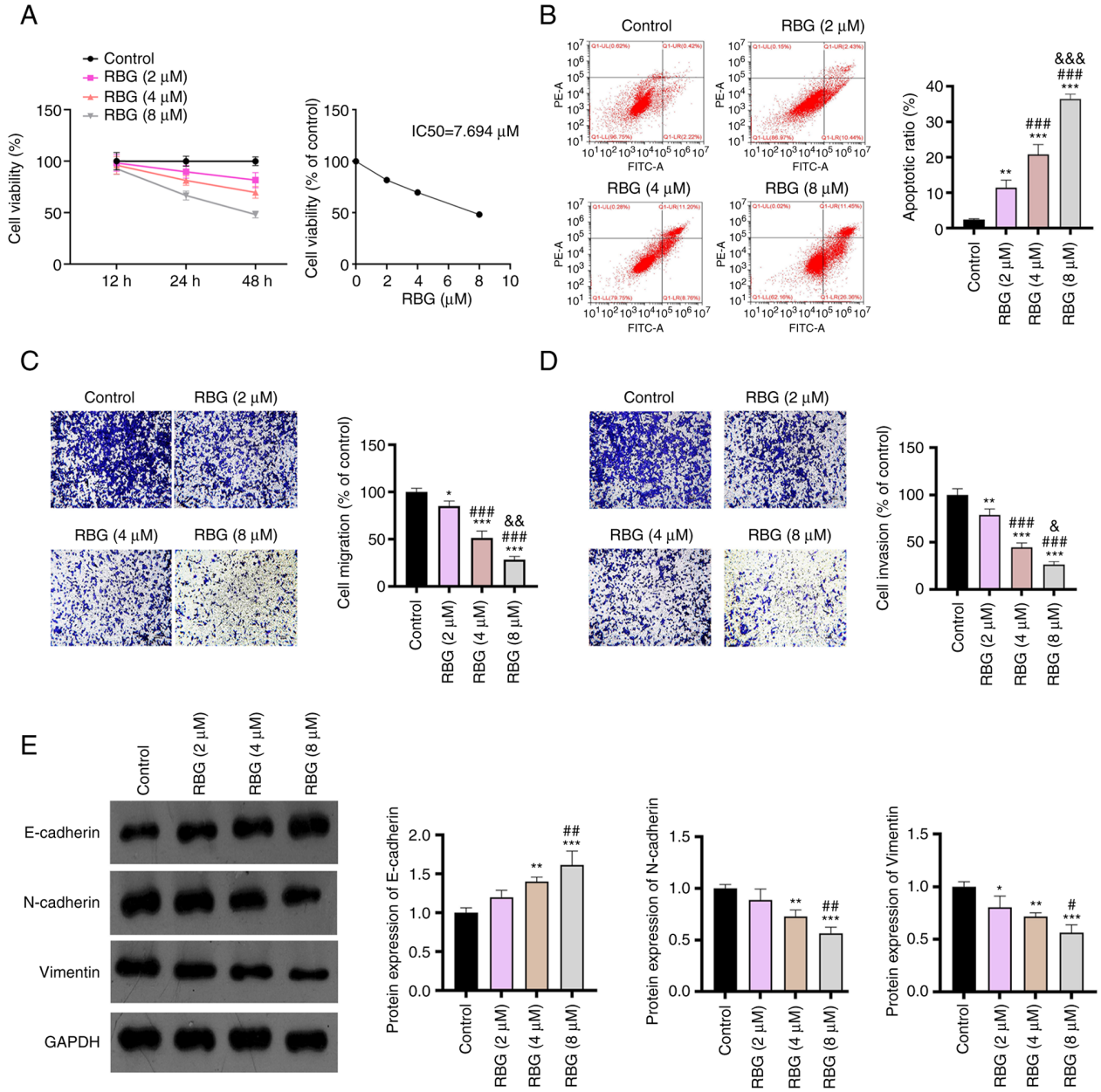

Figure 1. RBG inhibits the malignant characteristics of MM cells. (A) Cell viability and IC_{50} was measured using Cell Counting Kit-8 assay. (B) Apoptosis was measured using flow cytometry. (C) Cell migration was measured using a Transwell assay. (D) Cell invasion was measured using a Transwell assay (x400 magnification). (E) Protein expression of E-cadherin, N-cadherin and Vimentin was detected using western blotting. RPMI8226 cells were treated with different concentrations of RBG (2, 4 and 8 μM). *P<0.05, **P<0.01 and ***P<0.001 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. RBG (2 μM); $^{\&}$P<0.05, $^{\&\&}$P<0.01 and $^{\&\&\&}$P<0.001 vs. RBG (4 μM). RBG, resibufogenin.

followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

RBG inhibits the malignant characteristics of MM cells. The function of RBG in MM was first evaluated in RPMI8226 cells (a human MM cell line). As presented in Fig. 1A, the viability of RPMI8226 cells treated with 8 μM RBG for 12 h was markedly lower compared with that of the control. Both 24 and 48 h of RBG treatment could markedly decrease the viability of RPMI8226 cells in a dose-dependent manner. Meanwhile, the IC_{50} of RBG on RPMI8226 cells was determined as 7.694 μM at 48 h treatment (Fig. 1A). The time point of 48 h was then used for subsequent functional experiments. Flow cytometry demonstrated that RBG significantly promoted the apoptosis of RPMI8226 cells with increasing concentrations in comparison to the control group (P<0.01; Fig. 1B). In addition, the migration and invasion of RPMI8226 cells were both significantly inhibited compared with the control by the treatment of RBG in a dose-dependent manner (P<0.05; Fig. 1C and D). Western blotting further demonstrated that 4 and 8 μM RBG significantly upregulated E-cadherin and downregulated N-cadherin and Vimentin in RPMI8226 cells compared with the control (P<0.001, Fig. 1E). These results indicated that RBG inhibits the proliferation, migration and invasion of MM cells in a dose-dependent manner.

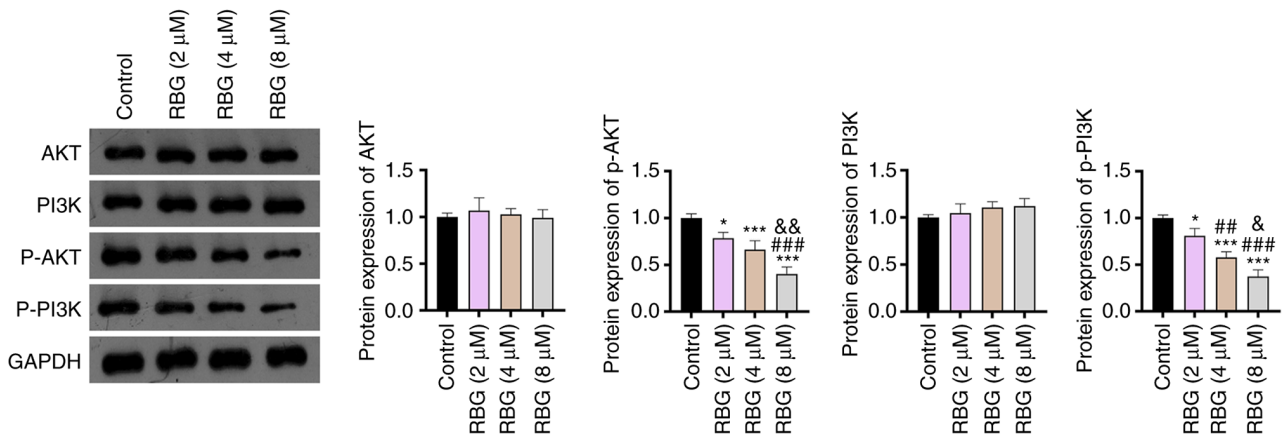

Figure 2. RBG blocks the PI3K/AKT signaling pathway in MM cells. RPMI8226 cells were treated with different concentrations of RBG (2, 4 and 8 μM). The protein expression of AKT, p-AKT, PI3K and p-PI3K were detected using western blotting. *P<0.05, ***P<0.001 vs. Control; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. RBG (2 μM); $^{\&}$P<0.05, $^{\&\&}$P<0.01 vs. RBG (4 μM). RBG, resibufogenin; p-phosphorylated.

RBG blocks the PI3K/AKT signaling pathway in MM cells. The action mechanism of RBG involving the PI3K/AKT signaling pathway was subsequently analyzed in RPMI8226 cells. Western blotting demonstrated that, compared with the control, RBG significantly reduced the protein expression of p-AKT and p-PI3K in RPMI8226 cells in a dose-dependent manner (P<0.05). The protein expression of AKT and PI3K was not significantly changed by the treatment of RBG in RPMI8226 cells (Fig. 2). These findings indicated that RBG blocked the PI3K/AKT signaling pathway in MM cells.

The activation of PI3K/AKT signaling pathway weakens the antitumor effect of RBG in MM cells. In order to verify whether the antitumor effect of RBG was associated with the blocking of PI3K/AKT signaling pathway, IGF-1, an activator of the PI3K/AKT signaling pathway was used to treat RPMI8226 cells. As presented in Fig. 3A, the intervention of IGF-1 significantly weakened the inhibiting effects of RBG on the viability of RPMI8226 cells at 48 h post-treatment (P<0.001). By contrast, IGF-1 significantly inhibited the promoting effect of RBG on the apoptosis of RPMI8226 cells (P<0.01; Fig. 3B). The inhibiting effects of RBG on the migration and invasion of RPMI8226 cells were also significantly reversed by the intervention of IGF-1 (P<0.05; Fig. 3C and D). In addition, EMT of MM cells was evaluated by measuring the associated biomarkers (E-cadherin, N-cadherin and Vimentin). The upregulation of E-cadherin and downregulation of N-cadherin and Vimentin that were induced by RBG were partially reversed by IGF-1 (P<0.01; Fig. 4). Western blotting further verified that IGF-1 weakened RBG-induced blocking of the PI3K/AKT signaling pathway, as evidenced by the increased levels of p-AKT/AKT and p-PI3K/PI3K. (P<0.01; Fig. 4). These results indicated that RBG exerts an antitumor effect on MM by deactivating the PI3K/AKT signaling pathway.

Discussion

MM is a bone marrow-resident hematological malignancy involving plasma cells (29). With the development of immunomodulatory drugs, proteasome inhibitors and monoclonal antibodies, improvements have been achieved in the survival of patients with MM (5). However, MM remains incurable and its prognosis remains unsatisfactory, especially for elderly patients. Natural traditional Chinese medicine is a promising source of potential antitumor drugs with the advantages of having high efficiency and mild side effects (30). RBG is a bufadienolide isolated from toad venom that has been used to treat malignancies for several decades in China (11). In the present study, the antitumor ability of RBG against MM was preliminary revealed through the assessment of MM cell proliferation, migration, invasion and EMT. The underlying mechanism of RBG was revealed to be associated with the blocking of the PI3K/AKT signaling pathway.

Toad venom is a product of toxic secretions, containing a variety of active ingredients with antitumor activity, such as bufalin, cinobufagin, arenobufagin and RBG (7). Previous studies have demonstrated that bufalin and cinobufagin possess antitumor properties against MM (31,32). Hence, the present study hypothesized that RBG may also be used as a potential antitumor drug for MM. The current study revealed that RBG significantly inhibited the viability, migration and invasion, and promoted the apoptosis of a MM cell line (RPMI8226 cells) in a dose-dependent manner. These findings indicated that RBG was effective in inhibiting the malignant characteristics of MM cells.

The antitumor effect of RBG in MM cells was consistent with its role in a number of other types of cancer. For example, RBG inhibits the proliferation, migration and invasion, and induces the apoptosis of ovarian clear cell carcinoma cells (11). In addition, RBG inhibits the proliferation and promotes the apoptosis of gastric carcinoma cells (26) and breast cancer cells (12). Moreover, the present study also demonstrated that RBG increased E-cadherin expression, and decreased N-cadherin and Vimentin expression in RPMI8226 cells, which are markers of EMT. These results indicated that RBG could inhibit EMT in MM. As EMT confers enhanced tumor-initiating and metastatic potential in cancer cells (33), the RBG-induced inhibition of EMT may directly contribute to the treatment of MM. As aforementioned, this provided evidence that RBG may be an effective antitumor drug against MM.

The classic PI3K/Akt signaling pathway is an important participant in tumorigenesis, which acts a key regulator for

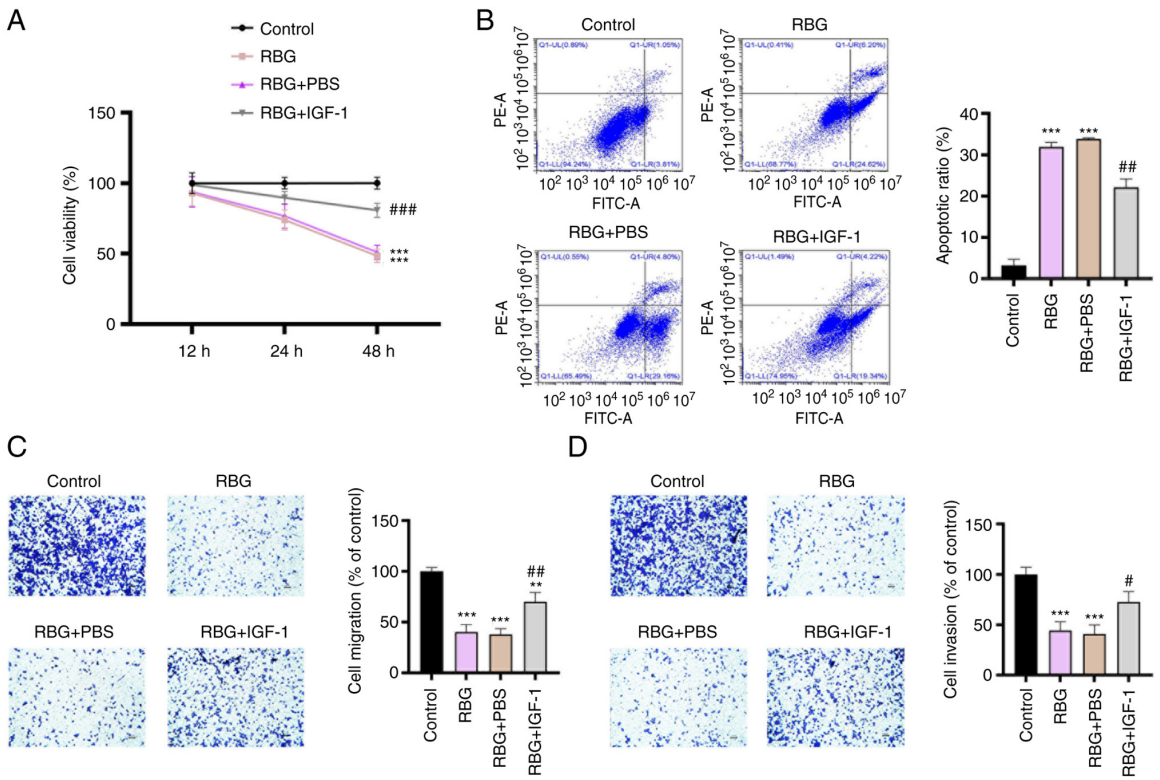

Figure 3. IGF-1 weakens the inhibiting effects of RBG on the malignant characteristics of MM cells. RPMI8226 cells were treated with 8 μM RBG and 50 ng/ml IGF-1 (an activator of the PI3K/AKT signaling pathway). (A) Cell viability was measured using a Cell Counting Kit-8 assay. (B) Apoptosis was measured using flow cytometry. (C) Cell migration was measured using a Transwell assay. (D) Cell invasion was measured using a Transwell assay (x400 magnification). **P<0.01, ***P<0.001 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. RBG. RBG, resibufogenin; IGF-1, insulin-like growth factor 1.

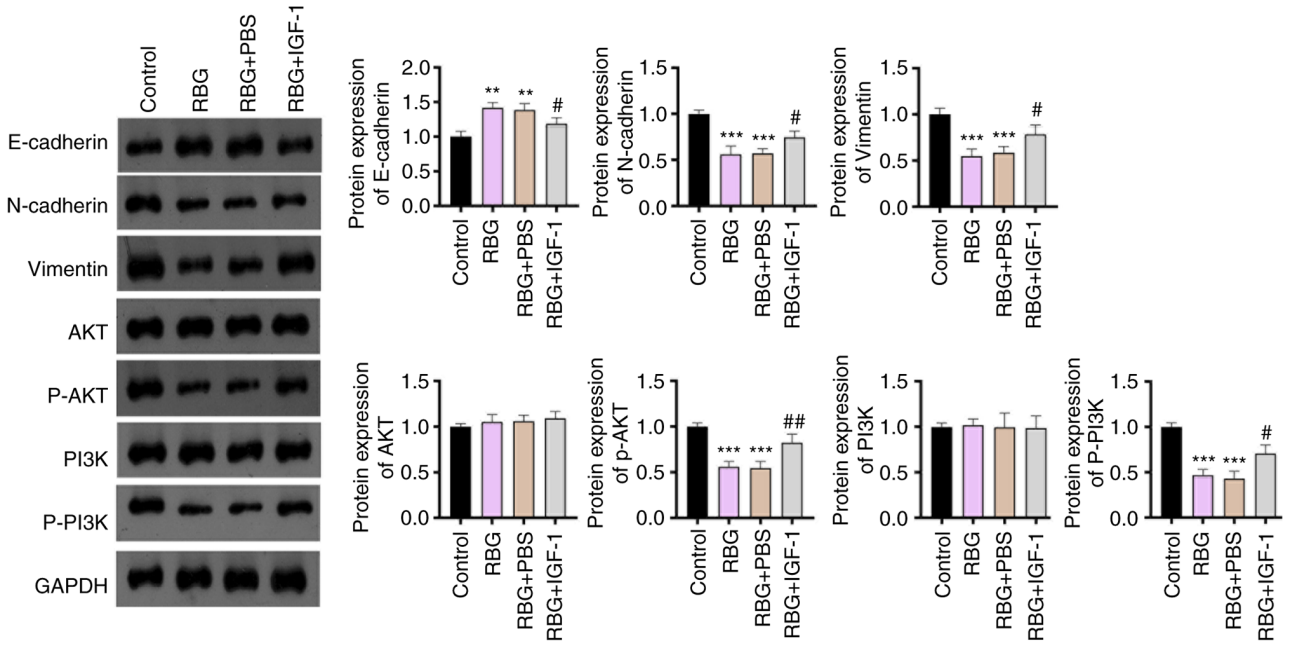

Figure 4. IGF-1 weakens the inhibiting effects of RBG on the EMT and PI3K/AKT signaling pathway in MM cells. RPMI8226 cells were treated with 8 μM RBG and 50 ng/ml IGF-1 (an activator of the PI3K/AKT signaling pathway). The protein expression of E-cadherin, N-cadherin, Vimentin, AKT, p-AKT, PI3K and p-PI3K was detected by Western blot. **P<0.01, ***P<0.001 vs. Control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. RBG. RBG, resibufogenin; IGF-1, insulin-like growth factor 1; EMT, epithelial-mesenchymal transition; MM, multiple myeloma; p, phosphorylated.

cell proliferation, migration, adhesion, angiogenesis and drug resistance (34). A previous study determined that the PI3K/AKT signaling pathway is a promising therapeutic target for MM (35). Various inhibitors targeting this pathway have been developed for the treatment of MM, such as TAS-117 (19), Afuresertib (20), BKM120 (21), BENC-511 (36) and

PIK-C98 (37). In the present study, the potential mechanism of RBG in MM involving the PI3K/Akt signaling pathway was analyzed. The results revealed that RBG blocked the PI3K/AKT signaling pathway in MM cells. It is hypothesized that RBG may inhibit the malignant characteristics of MM cells by blocking the PI3K/Akt signaling pathway. The present study's feedback assays further verified this speculation, as evidenced by the fact that the intervention of IGF-1 weakened the inhibiting effects of RBG on the malignant characteristics of MM cells.

In conclusion, RBG is a potential therapeutic drug against MM, which could inhibit cell viability, migration, invasion and EMT, and promoted apoptosis *in vitro*. The blocking of the PI3K/Akt signaling pathway is an underlying action mechanism of RBG against MM. However, the present study is still limited to the cellular level. The underlying mechanisms of RBG are not limited to the PI3K/Akt signaling pathway. Further research on these limitations is still needed.

Acknowledgements

Not applicable.

Funding

This work was supported by Chinese Medicine Research Fund Project of Zhejiang Province (grant no. 2021ZB092).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ, ZH and SD substantially contributed to the conception and the design of the study. YZ, ZH, KJ, CL, JX, HG, ZZ and JS were responsible for the acquisition, analysis and interpretation of the data. HG, ZZ, JS and SD confirm the authenticity of all the raw data. YZ, ZH and JS contributed to manuscript drafting and critical revisions of the intellectual content. SD approved the final manuscript to be published and obtained the funding. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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