

Plantamajoside represses the growth and metastasis of malignant melanoma

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Abstract. Plantamajoside (PMS) has been shown to have anti-cancer effects and is the main compound of *Plantago asiatica*. The aim of the present study was to investigate the effects of PMS on malignant melanoma and its molecular mechanisms. The malignant melanoma cell line A2058 was treated with different concentrations of PMS (0, 20, 80 and 160 $\mu\text{g/ml}$) for 24, 48 or 72 h, followed by cell viability detection using the Cell Counting Kit-8 assay. The present results suggested that PMS inhibited cell viability in a dose-dependent manner. In addition, flow cytometry was used to analyze cell apoptosis, and Transwell assays were used to investigate cell migration and invasion. The present results suggested that PMS induced A2058 cell apoptosis, and inhibited cell invasion and migration in a dose-dependent manner. In order to study the molecular mechanism by which PMS inhibited malignant melanoma growth and metastasis, reverse transcription-quantitative PCR and western blotting were used to determine the expression levels of apoptotic-related genes and PI3K/AKT signaling pathway-related proteins. The present results indicated that PMS inhibited the protein and mRNA expression of *Bcl-2*, and promoted the expression of *Bax* and caspase-3 in a dose-dependent manner. The protein expression level of phosphorylated-AKT was dose-dependently reduced by PMS treatment. Collectively, the present results suggested that PMS inhibited the invasion, migration and viability of malignant melanoma cells. In addition, PMS induced apoptosis by regulating the expression levels of apoptotic-related genes and the activation of the PI3K/AKT signaling pathway, thereby exerting anti-malignant melanoma effects.

Introduction

The prevalence of skin cancer is increasing at an alarming rate, with ~1 million new cases discovered annually worldwide (1). Malignant cutaneous melanoma occurs as a result of dysregulated proliferation of melanocytes in the epidermis and is one of the most aggressive forms of cutaneous neoplasms (2,3). Malignant melanoma has a high metastatic rate, thus it is considered to be the most severe skin cancer type (3-5). The mortality rate of melanoma accounts for 65% of skin cancer deaths, despite the incidence rate only representing approximately 3% of all skin cancer types (6). Skin cancer prognosis is poor and usually leads to mortality due to metastasis (1). The median survival time is 8 months, and the 5-year survival among patients with stage IV metastatic melanoma is only approximately 10% (7,8). Although there are many antimelanoma treatments, in many cases melanoma is treatment-resistant (1). Thus, malignant melanoma is difficult to successfully treat using conventional surgery and chemotherapeutics (1).

Plant compounds are being increasingly used to prevent and treat many diseases including tumors (9-11). A cross-sectional survey in the West Midlands in the UK found that a substantial number of patients with cancer are likely to be taking herbal medicine (12). Currently, over 60 herbal complexes are being studied as anticancer medicine (11). The clinically used plant-derived anticancer drugs can be divided into four important groups: Vinca, alkaloids, taxanes and podophyllotoxins (13). Therefore, it is feasible to find new Chinese herbal medicines that may inhibit melanoma metastasis (11).

Plantamajoside (PMS) is a major ingredient isolated from *Plantago asiatica* (14). PMS has long been applied in food and medicine (14). PMS exhibits anti-inflammatory and antioxidant properties (15,16). PMS has been applied to the treatment of many diseases due to its protective effects against cadmium-induced renal injury (17), and its anti-inflammatory (15) and antifibrotic effects (18). Currently, the antitumor effects of PMS in esophageal squamous cell carcinoma have been studied (19). However, to the best of our knowledge, the effect of PMS on malignant melanoma remains unknown.

Therefore, the aims of the present study were to investigate the effect of PMS on malignant melanoma *in vitro*, and to

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examine the underlying mechanism by which PMS may affect malignant melanoma.

Materials and methods

Cell culture and treatment. The malignant melanoma cell line A2058 was obtained from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. Human primary epidermal melanocytes were obtained from the American Type Culture Collection (cat. no. ATCC PCS-200-013). A2058 cells were cultured in RPMI-1640 media (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (HyClone; GE Healthcare Life Sciences) at 37°C with 5% CO₂ for 24 h. Normal melanocytes were grown in RPMI-1640 (HyClone; GE Healthcare Life Sciences) containing 10% FBS (HyClone; GE Healthcare Life Sciences), 100 U/ml penicillin and 100 µg/ml streptomycin (Beyotime Institute of Biotechnology) in a humidified incubator with 5% CO₂.

PMS was purchased from Sigma-Aldrich (purity >99%; Merck KGaA), and DMSO (Sigma-Aldrich; Merck KGaA) was used to dissolve PMS. For PMS treatment, A2058 cells and normal melanocytes were treated with different concentrations of PMS (0, 20, 80 and 160 µg/ml) at 37°C for 0, 24, 48 and 72 h respectively following which the cells were subjected to further experimentation.

Cell Counting Kit-8 (CCK-8) assay. The CCK-8 method was used to detect cell viability. The cell concentration of normal melanocytes or A2058 cells was adjusted to 1x10⁴/ml, and 100 µl cell suspension was added to each well of the 96-well plates. Then, A2058 cells were treated with different concentrations of PMS (0, 20, 80 and 160 µg/ml) at 37°C for 0, 24, 48 and 72 h. The 96-well plates were cultured at 37°C with 5% CO₂ for 24 h. Subsequently, 10 µl CCK-8 reagent (Sigma-Aldrich; Merck KGaA) was added to each well according to the manufacturer's protocol. The absorbance at the wavelength of 450 nm was measured after 2 h of incubation at 37°C using an automatic enzyme-linked immune detector. The experiment was repeated three times, and data were normalized to the control group, which was cells without any PMS treatment.

Flow cytometry. After treatment with different concentrations of PMS (0, 20, 80 and 160 µg/ml) at 37°C for 48 h, 0.2% trypsin was used to digest the normal melanocytes or A2058 cells. Cells were then washed with PBS and fixed using the 70% ethanol overnight at 4°C. The apoptotic rate of the cells was detected using the Annexin V-FITC/PI kit (cat. no. 70-AP101-100; Hangzhou Multi Sciences Biotech Co., Ltd.) according to the manufacturer's instructions. The apoptotic rate of normal melanocytes was also measured using an AnnexinV-FITC/PI Apoptosis Detection kit (cat. no. A211-01; Vazyme Biotech Co., Ltd.) as per the manufacturer's instructions. BD FACSCalibur™ flow cytometer (Beckman Coulter, Inc.) coupled with the CellQuest™ software (version 5.1; BD Biosciences) were used to measure the apoptotic rate of cells. The experiments were performed in triplicate.

Transwell assay. Polycarbonate filters (pore size, 8 µm; Corning, Inc.) were used for the Transwell assay. Chamber

inserts pre-coated with 200 mg/ml of Matrigel at 37°C for 30 min were used for cell invasion detection, whilst chamber inserts without Matrigel were used for cell migration determination. The upper chamber was plated with 200 µl RPMI-1640 media (Gibco; Thermo Fisher Scientific, Inc.) containing 0.1% FBS and 1x10⁵ A2058 cells treated with different concentrations of PMS (0, 20, 80 and 160 µg/ml) at 37°C for 48 h. The lower chamber was plated with 600 µl RPMI-1640 media (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS. After 48 h incubation at 37°C, cells in the lower chamber were fixed with 4% paraformaldehyde (cat. no. P0099; Beyotime Institute of Biotechnology) at room temperature for 30 min and then stained with 0.5 ml 0.1% crystal violet (cat. no. C0121; Beyotime Institute of Biotechnology) at room temperature for 15 min. migrated or invaded cells were then counted using a light microscope (five fields per chamber; magnification x200). Each Transwell assay was repeated in five independent experiments.

Reverse transcription-quantitative PCR. TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract the total RNA from A2058 cells. Total RNA was reverse transcribed into cDNAs using the temperature protocol of 70°C for 5 min, 37°C for 5 min and 42°C for 1 h. PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd.) was used according to the manufacturer's instructions. qPCR was performed to analyze cDNAs using a TaqMan Universal PCR Master Mix kit (Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Primer sequences used for PCR were as follows: *GAPDH* forward, 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse, 5'-TGGTGAAGACGCCAGTGGA-3'; *Bcl-2* forward, 5'-TTCTTTGAGTTCGGTGGG GTC-3' and reverse, 5'-TGCATATTTGTTTGGGGCAGG-3'; *Bax* forward, 5'-TCCACCAAGAAGCTGAGCGAG-3' and reverse, 5'-GTCCAGCCCATGATGGTTCT-3'; and *caspase-3* forward, 5'-TGTGGCATTGAGACAGAC-3' and reverse, 5'-CACTTGCCATACAACTA-3'. The thermocycling conditions used for the qPCR were as following: Initial denaturation at 95°C for 10 min; followed by 35 cycles of 95°C for 15 sec and 55°C for 40 sec. *GAPDH* was used as the standardized control. $\Delta\Delta C_q = C_{q\text{gene}} - C_{q\text{reference}}$ is the relative level of gene expression, and the fold change in gene expression was calculated by the $2^{-\Delta\Delta C_q}$ method (20). The experiments were repeated in triplicate.

Western blotting. Total proteins were collected using RIPA lysis buffer (cat. no. P0013E; Beyotime Institute of Biotechnology) following the manufacturer's instructions. A bicinchoninic acid assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used to quantify the protein samples. Protein samples (40 µg per lane) were separated on 10% SDS-PAGE and electrophoretically transferred onto a PVDF membrane (EMD Millipore). After blocking with 5% skim milk for 2 h at room temperature, the membrane was incubated with primary antibodies: Anti-AKT (1:1,000; cat. no. 4691; Cell Signaling Technology, Inc.), anti-phosphorylated (p)-AKT (1:1,000; cat. no. 4060; Cell Signaling Technology, Inc.), anti-Bcl-2 (1:1,000; cat. no. 4223; Cell Signaling Technology, Inc.), anti-Bax (1:1,000; cat. no. 5023; Cell Signaling Technology, Inc.), anti-total caspase-3

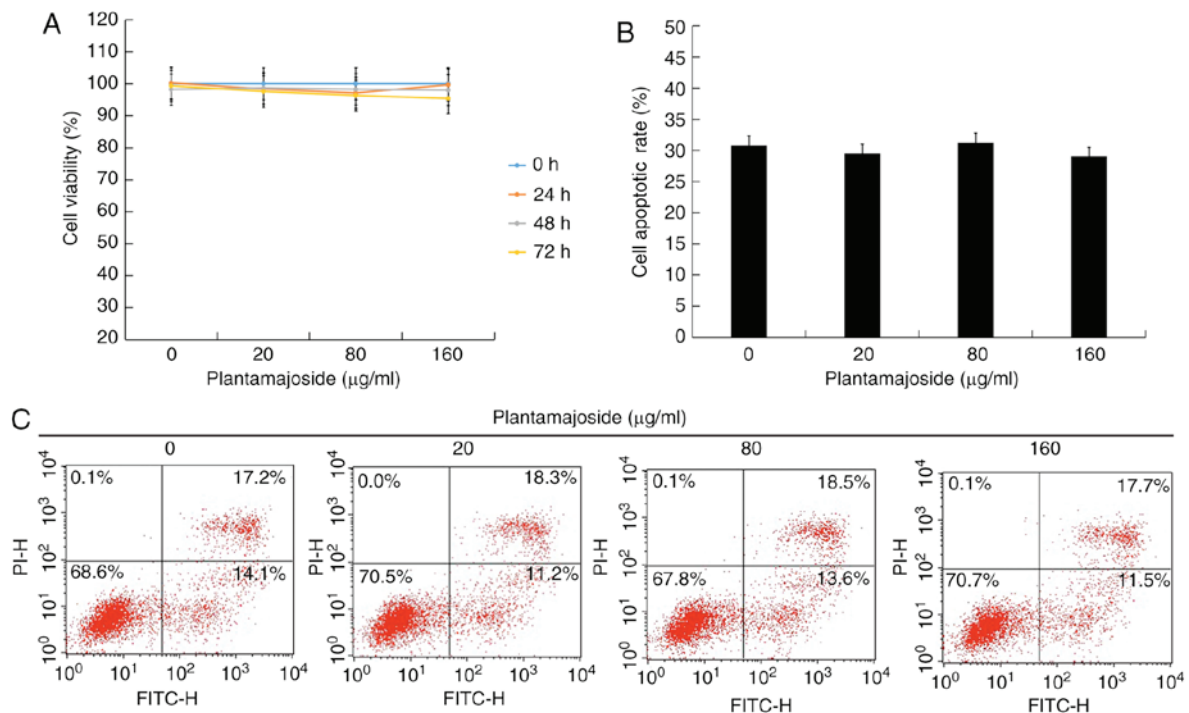


Figure 1. Effects of PMS on normal melanocytes. Normal melanocytes were treated with different concentrations of PMS (0, 20, 80 and 160 $\mu\text{g/ml}$) for 48 h. (A) Cell viability was assessed using a Cell Counting Kit-8 assay. (B) The apoptotic rate of A2058 cells was quantified from the (C) flow cytometry results. Data are presented as the mean \pm SD. PMS, plantamajoside.

(1:1,000; cat. no. 29629; Cell Signaling Technology, Inc.) and anti- β -actin (1:1,000; cat. no. 4970; Cell Signaling Technology, Inc.), overnight at 4°C. Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:1,000; cat. no. 7074; Cell Signaling Technology, Inc.) at room temperature for 2 h. Protein bands were visualized using chemiluminescent ECL reagent (EMD Millipore) and quantified by densitometry (QuantityOne 4.5.0 software; Bio-Rad Laboratories, Inc.).

Statistical analysis. Data are presented as the mean \pm SD of ≥ 3 independent experiments. One-way ANOVA followed by Tukey's post hoc test was used for comparison between groups. All data analyses were performed with SPSS 17.0 software (SPSS, Inc.). $P < 0.05$ was considered to indicate a statistically significant different.

Results

No significant effects of PMS on normal melanocytes. To investigate the effect of PMS on malignant melanoma cells, the present study examined the effect of PMS on normal melanocytes. After treatment with different concentrations of PMS (0, 20, 80 and 160 $\mu\text{g/ml}$) for 0, 24, 48 and 72 h, cell viability was determined. The present results suggested that there were no significant effects of PMS on the viability of normal melanocytes (Fig. 1A). In addition, after treatment with different concentrations of PMS (0, 20, 80 and 160 $\mu\text{g/ml}$) for 48 h, the apoptotic rate of normal melanocytes was also investigated. The present results suggested that PMS had no effect on normal melanocyte apoptosis (Fig. 1B and C).

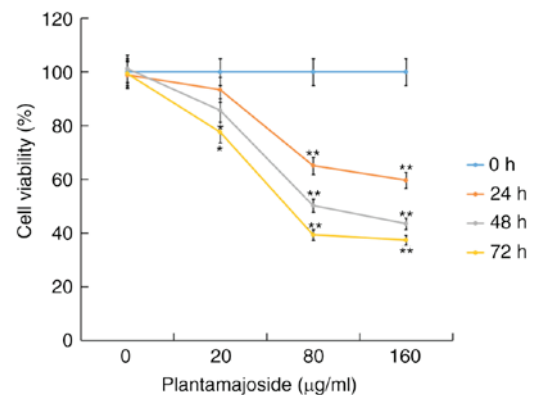


Figure 2. Effect of PMS on the viability of A2058 cells. Malignant melanoma cells were treated with different concentrations of PMS (0, 20, 80 and 160 $\mu\text{g/ml}$) for 0, 24, 48 and 72 h. Then, the viability of cells was assessed using a Cell Counting Kit-8 assay. Data are presented as the mean \pm SD. * $P < 0.05$, ** $P < 0.01$ vs. control group. PMS, plantamajoside.

PMS inhibits cell viability in a dose-dependent manner. The present study investigated the effect of PMS on the malignant melanoma cells. A2058 cells were treated with different concentrations of PMS (0, 20, 80 and 160 $\mu\text{g/ml}$) for 0, 24, 48 and 72 h, then cell viability was measured using a CCK-8 assay. The CCK-8 assay results suggested that PMS inhibited A2058 cell viability in a dose-dependent manner (Fig. 2).

PMS induces cell apoptosis in a dose-dependent manner. After treatment with different concentrations of PMS (0, 20, 80 and 160 $\mu\text{g/ml}$) for 48 h, the apoptotic rate of

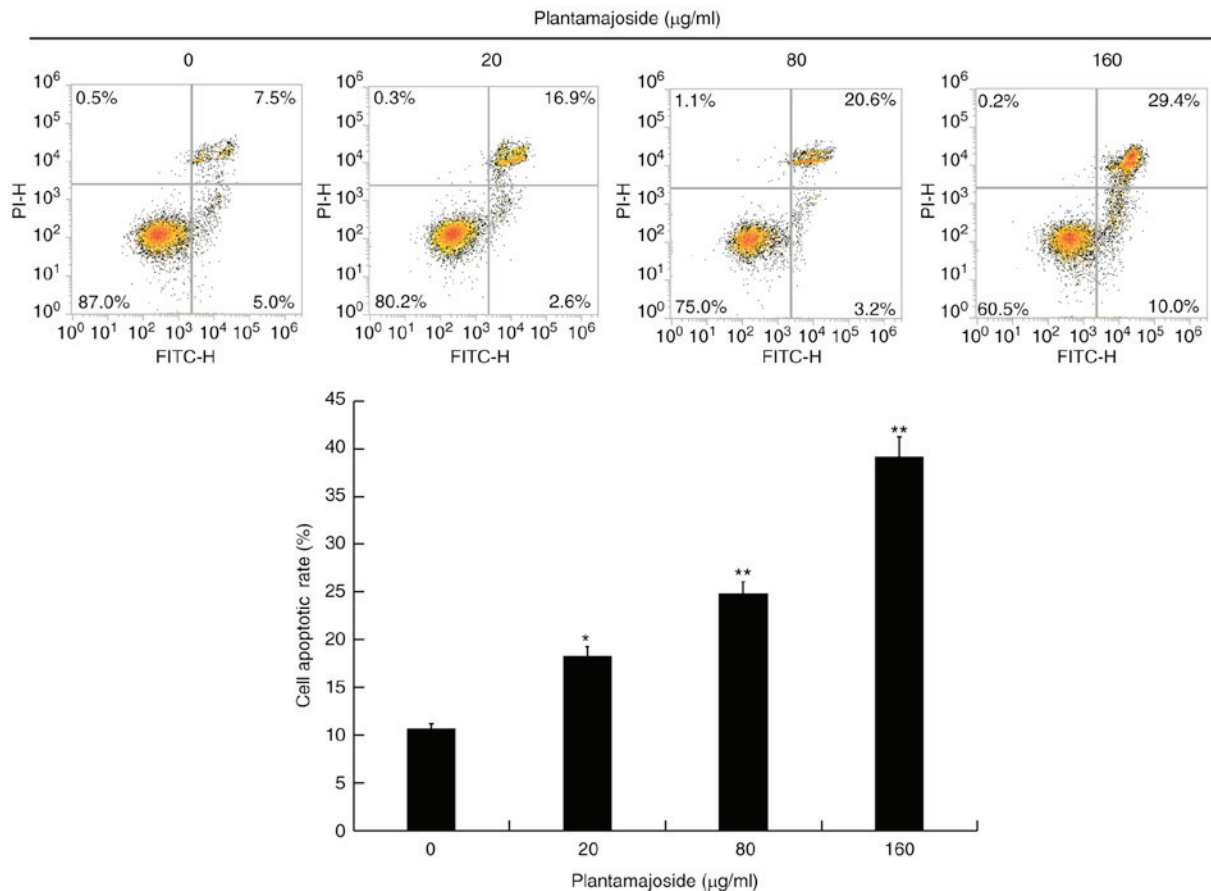


Figure 3. Effect of PMS on the apoptotic rate of A2058 cells. Malignant melanoma cells were treated with different concentrations of PMS (0, 20, 80 and 160 µg/ml) for 48 h. Then an Annexin V-FITC/PI apoptosis detection kit was used to detect the apoptotic rate of A2058 cells. Data are presented as the mean \pm SD. * $P < 0.05$, ** $P < 0.01$ vs. control group. PMS, plantamajoside.

malignant melanoma cells was measured using Annexin V-FITC/PI staining. The present results suggested that the apoptotic rate of A2058 cells significantly increased after 48 h of PMS treatment, and that PMS induced apoptosis in a dose-dependent manner (Fig. 3).

PMS inhibits cell migration and invasion in a dose-dependent manner. Subsequently, after treatment with different concentrations of PMS (0, 20, 80 and 160 µg/ml) for 48 h, a Transwell assay was used to examine the effects of PMS on the migratory and invasive abilities of malignant melanoma cells. The Transwell assay results indicated that PMS inhibited cell migration (Fig. 4A) and invasion (Fig. 4B) in a dose-dependent manner.

Effect of PMS on the expression levels of apoptotic related genes and the PI3K/AKT signaling pathway. In order to study the mechanism by which PMS may affect A2058 cells, the cells were treated with different concentrations of PMS for 48 h. Then, the expression levels of the apoptotic-related genes *Bcl-2*, *Bax* and *caspase-3*, and the relative proteins in the PI3K/AKT signaling pathway including AKT and p-AKT, were detected. The present results suggested that PMS inhibited the mRNA expression level of *Bcl-2*, and promoted the mRNA expression levels of *Bax* and *caspase-3* in a dose-dependent manner (Fig. 5A-C). Western blot analysis

revealed that PMS inhibited the protein expression level of *Bcl-2*, and promoted the protein expression levels of *Bax* and *caspase-3* in a dose-dependent manner (Fig. 5D-G). In addition, the protein expression level of p-AKT was inhibited by PMS treatment in a dose-dependent manner. (Fig. 5D and H). Collectively, the present results suggested that PMS may affect A2058 cells by impacting apoptotic related genes expression levels and the PI3K/AKT signaling pathway.

Discussion

Although the diagnosis and treatment of malignant melanoma is improving, the median survival time of patients diagnosed with malignant melanoma during metastasis is 6-9 months, the 5-year survival rate is $<15\%$ and the prognosis is still poor (21). Surgical removal of malignant melanoma is the preferred method of therapy, but treatment with radiation and/or chemotherapy is also used (6,21). However, in the case of metastasis the cure rate is almost zero and only palliative treatment is available (22). In addition, immunotherapy of cytokines, as well as antibodies and BRAF inhibitors, are used in the treatment of malignant melanoma (23,24). However, these therapies can cause serious side effects (25,26). Therefore, due to the aggressiveness of melanoma and the complications associated with treatment, it is important to find alternatives to improve melanoma treatment.

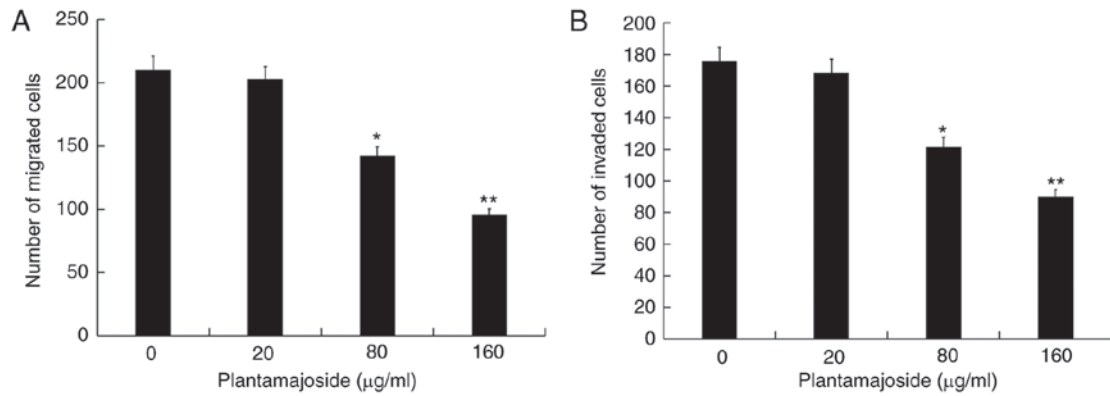


Figure 4. Effect of PMS on A2058 cell migration and invasion. Malignant melanoma cells were treated with different concentrations of PMS (0, 20, 80 and 160 μg/ml) for 48 h. Then the invasive and migratory abilities of cells were investigated using a Transwell assay. All experiments were performed in triplicate. (A) Effect of PMS on A2058 cell migration. (B) Effect of PMS on A2058 cell invasion. Data are presented as the mean ± SD. *P<0.05, **P<0.01 vs. control group. PMS, plantamajoside.

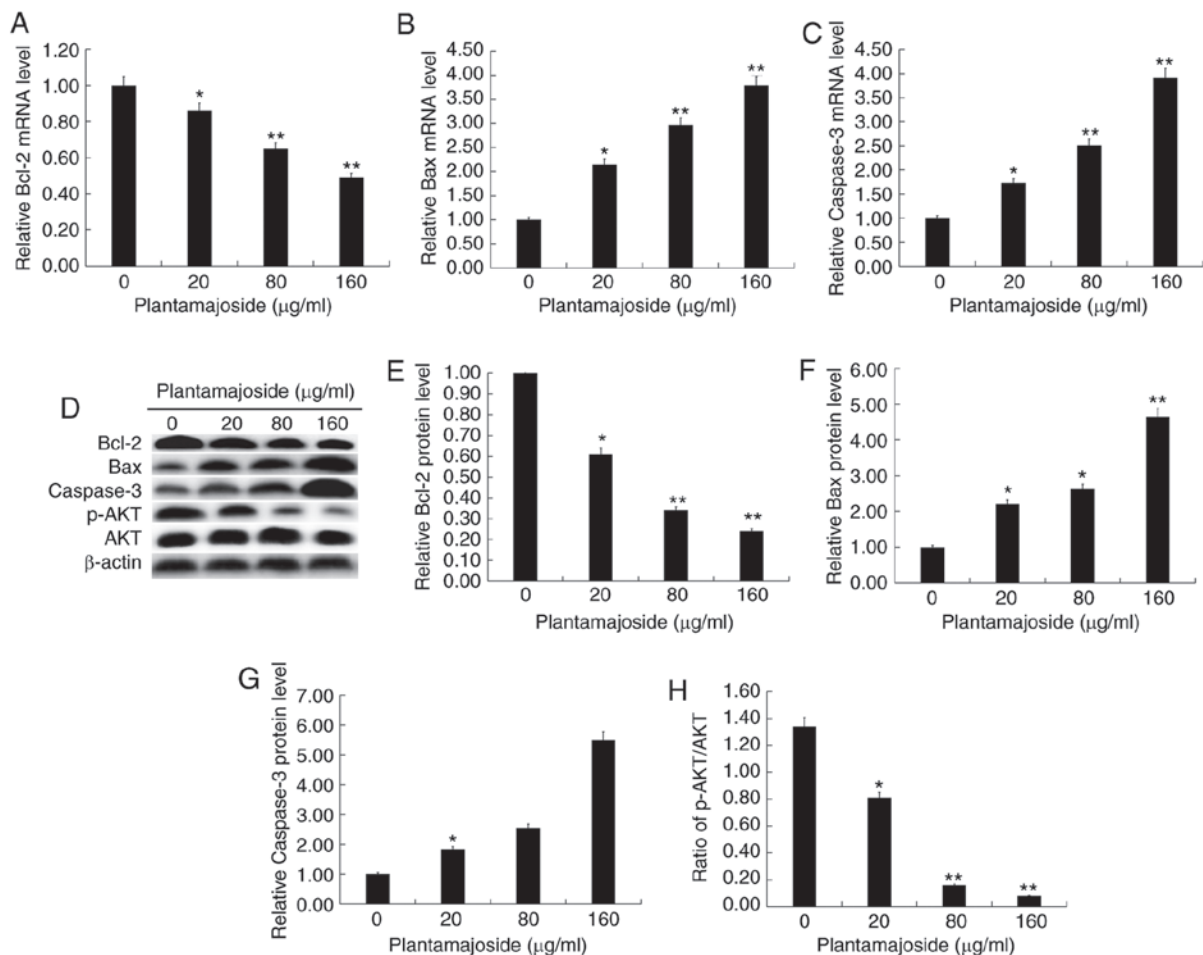


Figure 5. Effect of PMS on the PI3K/AKT signaling pathway related genes in A2058 cells. Malignant melanoma cells were treated with different concentrations of PMS (0, 20, 80 and 160 μg/ml) for 48 h. mRNA expression levels of (A) Bcl-2, (B) Bax and (C) caspase-3 were detected by reverse transcription-quantitative PCR. (D) Western blotting was used to investigate the protein expression levels of (E) Bcl-2, (F) Bax, (G) caspase-3 and (H) p-AKT/AKT. Data are presented as the mean ± SD. *P<0.05, **P<0.01 vs. control group. PMS, plantamajoside; p-, phosphorylated.

PMS has been previous found to exhibit antitumor properties (19). Therefore, the aims of the present study were to investigate the effect of PMS on malignant melanoma and its underlying mechanisms *in vitro*.

The present study examined the effect of PMS on normal melanocytes. After treatment with different concentrations of PMS (0, 20, 80 and 160 μg/ml) for 48 h, the present results suggested that there were no significant effects of PMS on

viability and apoptosis of normal melanocytes. The present study also investigated the effect of PMS on malignant melanoma cells. A2058 cells were treated with different concentrations of PMS (0, 20, 80 and 160 $\mu\text{g/ml}$) for 0, 24, 48 and 72 h, then cell viability was measured using a CCK-8 assay. In line with a previous study (19), the present results suggested that the viability of A2058 cells was significantly inhibited by PMS treatment in a dose-dependent manner. Furthermore, the present results indicated that PMS induced cell apoptosis in a dose-dependent manner. Moreover, Pei *et al* (11) reported that PMS inhibited the growth and metastasis of breast cancer by inhibiting the activity of matrix metalloproteinase (MMP)-9 and MMP-2. The present study investigated the effect of PMS on the migration and invasion of malignant melanoma cells, and in line with results from a previous study (11), the present results indicated that PMS inhibited the migration and invasion of A2058 cells in a dose-dependent manner. Collectively, the present results suggested that PMS could effectively inhibit malignant melanoma cell growth and metastasis. Therefore, alternative treatments using PMS alone or in combination with other methods may be beneficial in the treatment of malignant melanoma.

A number of specific inhibitors targeting different signaling pathways have been introduced into preclinical trials such as the RAF inhibitors (RAF/MEK/ERK pathway) (27). A major research direction in the therapy of advanced melanoma is individualized molecular-targeted therapy, which specifically targets the key enzymes involved in the cell cancerous signaling pathway for targeted inhibition and different types of genetic mutations in patients (28). Among them, two survival signaling pathways RAS/mitogen-activated protein kinase and PI3K/AKT have been investigated (29-31). The PI3K/AKT signaling pathway is continuously activated in cancer cells and is closely related to cancer cell survival, proliferation, angiogenesis and invasion (32). Inhibition of the PI3K/AKT signaling pathway is expected to be an effective treatment for cancer (33). However, to the best of our knowledge, there is no research on the inhibition of the PI3K/AKT signaling pathway by PMS in melanoma cells. Therefore, the present study investigated the effect of PMS on the PI3K/AKT signaling pathway by examining the protein expression level of p-AKT by western blotting. The present results indicated that PMS may inhibit malignant melanoma cell viability, invasion and migration. In addition, PMS may be able to induce apoptosis by regulating the expression levels of apoptotic-related genes and the activation of the PI3K/AKT signaling pathway, thereby exerting antimalignant effects on melanoma.

In conclusion, the present results suggested that PMS could effectively inhibit cell viability, migration and invasion, and induce apoptosis in malignant melanoma cells. The present results suggested that these effects were mediated by the regulation of apoptotic-related gene expression levels and activation of the PI3K/AKT signaling pathway. Therefore, PMS may be a promising new type of small molecule chemotherapy for treatment of malignant melanoma. However, the present study is only a preliminary study of the effect of PMS on melanoma and the effect of PMS on melanoma *in vivo* will need to be investigated in future studies. Follow-up experiments will need to continue to examine the effects of PMS *in vivo* in different species, as the effect of PMS on human malignant

melanoma may be different to *in vitro* results. Therefore, the role of PMS in human malignant melanoma requires further experimental research.

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Availability of data and materials

All data sets used and/or generated during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YW contributed to study design, data collection, statistical analysis, data interpretation and manuscript preparation. MZL and SGC contributed to data collection and statistical analysis. QW contributed to data collection, statistical analysis and manuscript preparation. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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