

Cantharidin inhibits cell proliferation and promotes apoptosis in tongue squamous cell carcinoma through suppression of miR-214 and regulation of p53 and Bcl-2/Bax

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Abstract. Cantharidin, a type of terpenoid, is a chemical compound secreted by the blister beetle or *Mylabris phelara* of the Meloidae family. Cantharidin is known to have good antitumor activity. The present study aimed to investigate the anticancer effect of cantharidin and its possible underlying mechanism using tongue squamous cell carcinoma (TSCC) TCA8113 cells. TCA8113 cells were treated with various concentrations of cantharidin, and the cell viability and cytotoxicity were assessed using MTT and LDH assays, respectively. Flow cytometry was conducted to examine cell apoptosis and colorimetric protease assay was performed to analyze caspase-9/3 activities in TCA8113 cells. qPCR and western blot analysis were used to investigate microRNA-214 (miR-214) expression, as well as the expression of p53, Bcl-2 and Bax proteins in TCA8113 cells. miR-214 and anti-miR-214 were transfected with mimics to examine whether miR-214 expression regulated the anticancer effect of cantharidin on TCA8113 cells and p53, Bcl-2 and Bax protein expression. The anticancer effect of cantharidin significantly inhibited cell proliferation and increased cytotoxicity of TSCC Tca8113 cells in a dose- and time-dependent manner. In addition, cantharidin induced cell apoptosis and activated caspase-9/3 activities of TSCC Tca8113 cells. Cantharidin markedly weakened miR-214 expression level, activated p53 protein expression, and suppressed the Bcl-2/Bax signaling pathway in Tca8113 cells. Downregulation of miR-214 increased p53 protein expression and decreased the Bcl-2/Bax signaling

pathway of TSCC Tca8113 cells. However, the overexpression of miR-214 reduced the anticancer effect of cantharidin on the proliferation and apoptosis of TSCC Tca8113 cells, inhibited p53 protein expression, and increased the Bcl-2/Bax signaling pathway. The results suggested that cantharidin is a potential anticancer drug that can be used to regulate the proliferation and apoptosis of human TSCC Tca8113 cells. Additionally, its mechanism may partially be associated with the downregulation of miR-214, upregulation of p53 protein expression and suppression of the Bcl-2/Bax signaling pathway.

Introduction

The incidence of oral cancer accounts for ~5% of all the malignant tumors of body, and the 5-year survival rate is ~64% (1). The incidence of oral cancer in China is 5-6/100,000 individuals. Squamous epithelial cancer is most common in oral and maxillofacial malignancies, accounting for ~80% of oral and maxillofacial tumors (2). Tongue squamous cell carcinoma (TSCC) has the highest incidence of oral cancers with cervical lymph-node metastasis occurring in early stage, rendering it an early event for the development of TSCC. Thus, it is particularly important to identify clinical treatment as well as prediction of recurrence and prognosis (3).

Recent findings have shown that there is an abnormality of microRNA (miR) expression in a variety of tumors. This influences the biological behavior, such as proliferation, apoptosis, differentiation, movement, invasion, metastasis and angiogenesis, of tumor cells through the regulation of downstream target genes and plays a role in oncogenes or tumor-suppressor genes, which are involved in tumorigenesis and the development of tumors, a multi-step process (4,5). Recent studies have found miR-214 is associated with a variety of malignancies, which plays a tumor suppressor or tumor promotion effect through multiple signal transduction pathways. The knockdown of miR-214 which promotes apoptosis and inhibits proliferation in TSCC Tca8113 cells (6), and in nasopharyngeal (7) and hepatocellular carcinoma (8), has been previously reported.

The p53 gene is considered to be associated with cancer in humans. Proliferating cell nuclear antigen, also known as

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cyclin, is an auxiliary protein of DNA polymerase δ , involved in the regulation of DNA synthesis and closely associated with cell cycle proliferation (9). Qi and Zhang showed that alisertib induced apoptosis of human TSCC by activation of the p53 pathway (10). Yasumoto *et al* reported that apoptosis-related gene expression after hyperthermia harbors wild- or mutated-type p53 in human TSCC (11). Nagler *et al* identified that the possible biological significance of markers is an independent role for p53 in TSCC (12). Tumor occurrence is associated with hindered apoptosis. The *Bcl-2/Bax* gene is important for the inhibition of apoptosis, and upregulation of the *Bcl-2/Bax* signal may play an important role in the occurrence and development of TSCC Tca8113 (13,14). Xie *et al* reported that the *Bcl-2/Bax* expression ratio has a prognostic value in TSCC (15).

Cantharidin is an active pharmaceutical ingredient for cantharide, which has a good effect on malignant tumors such as liver (16), esophageal (17), lung (18) and gastric (19) cancer, and has been used in clinical treatment of cancer over a long period of time. As reported, cantharidin induces tumor cell apoptosis. However, the estimated anticancer effect of cantharidin on cell proliferation and apoptosis of human TSCC remains to be determined. Thus, we investigated the anticancer effect of cantharidin on the behavior of human TSCC TCA8113 cells and investigated the molecular mechanisms related involved in this activity.

Materials and methods

Reagents and instruments. The cantharidin chemical structure (Sigma-Aldrich, St. Louis, MO, USA), with a purity of $\geq 95\%$ is shown in Fig. 1. Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from HyClone, Invitrogen Co. (Australia). 3,3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich. Annexin V-fluorescein isothiocyanate (V-FITC)/propidium iodide (PI) double staining kits were purchased from Sigma-Aldrich.

Cell line and culture conditions. Human TSCC TCA8113 cells were obtained from the Shanghai Usea Biotech Company (Shanghai, China). TCA8113 cells were cultured in DMEM containing 10% FBS, 1% penicillin/streptomycin (Invitrogen Life Technologies), and maintained at 37°C in a humidified atmosphere of $5\% \text{CO}_2$.

Detection of cell proliferation by MTT assay. Proliferation of TCA8113 cells was screened by MTT colorimetric assay. Briefly, TCA8113 cells (2×10^3 cells/well) were seeded in 96-well plates and treated with cantharidin (0, 2.5, 5, 10, 20, 40 and $80 \mu\text{M}$) for 24, 48 or 72 h following treatment, respectively. TCA8113 cells were added with $20 \mu\text{l}$ MTT (5 mg/ml) and the cells were cultured for an additional 4 h. DMSO was added to each well and agitated for 20 min. The absorbance was measured with Multiskan Spectrum microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 490 nm.

Detection of cell cytotoxicity by lactate dehydrogenase (LDH) assay. Proliferation of TCA8113 cells was screened by LDH

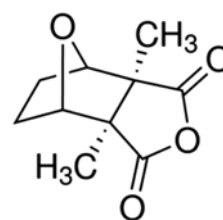


Figure 1. The chemical structure of cantharidin.

assay. Briefly, TCA8113 cells (2×10^3 cells/well) were seeded in 96-well plates and treated with cantharidin (10, 20 and $40 \mu\text{M}$) for 48 h following treatment. Each well was added with $100 \mu\text{l}$ LDH solution and incubated at room temperature for 30 min. The absorbance was read at 490 nm using a multiwell spectrophotometer (XL-818; Bio-Tek, Winooski, VT, USA).

Detection of cell apoptosis by flow cytometry. Apoptosis of TCA8113 cells was analyzed by flow cytometry. Briefly, TCA8113 cells (2×10^6 cells/well) were seeded in 6-well plates and treated with cantharidin (10, 20 and $40 \mu\text{M}$) for 48 h following treatment. TCA8113 cells were collected and washed twice with ice-cold phosphate-buffered saline (PBS). TCA8113 cells (1×10^6 cells/ml) were resuspended with binding buffer according to the manufacturer's instructions (Sigma-Aldrich). Annexin V-FITC ($10 \mu\text{l}$) was added into TCA8113 cells and stained for 30 min in the dark. The samples were immediately analyzed by flow cytometry.

Detection of caspase-9 and -3 activities by colorimetric protease assay. Apoptosis of TCA8113 cells was analyzed using flow cytometry. Briefly, TCA8113 cells (2×10^6 cells/well) were seeded in 6-well plates and treated with cantharidin (10, 20 and $40 \mu\text{M}$) for 48 h following treatment. TCA8113 cells were washed with twice PBS. The cells were lysed on ice in a buffer and cultured for 30 min. The total proteins were determined using a BSA kit (Pierce, Rockford, IL, USA). Equal protein was mixed with reaction buffer (Ac-DEVD-pNA for caspase-3, Ac-LEHD-pNA for caspase-9) and incubated at 37°C for 2 h in the dark. Caspase-3/9 activity was measured at an absorbance of 405 nm.

Detection of miR-214 expression by quantitative polymerase chain reaction (qPCR). miR-214 expression levels were measured with qPCR. Briefly, TCA8113 cells (2×10^6 cells/well) were seeded in 6-well plates and treated with cantharidin (10, 20 and $40 \mu\text{M}$) for 48 h following treatment. Total RNA was isolated from renal tissues using TRIzol, according to the manufacturer's instructions (Invitrogen, Chicago, IL, USA). cDNAs were produced and detected using the TaqMan 7900 (ABI) Real-Time PCR system, according to the manufacturer's protocol (Qiagen, Valencia, CA, USA). The expression of miR-214 was measured using a SYBR-Green kit, according to the manufacturer's instructions (Qiagen). The primers were compound and purchased from BeastBio Co., Ltd. (Shanghai, China). miR-214-F, 5'-AGCATAATACAGCAGGCACAGAC-3' and miR-214-R 5'-AAAGGTTGTTCTCCACTCTCTAC-3'; U6-F, 5'-CGCTTCGGCAGCACATATACTA-3' and U6-R, 5'-CGCTTCACGAATTTGCGTGTCA.

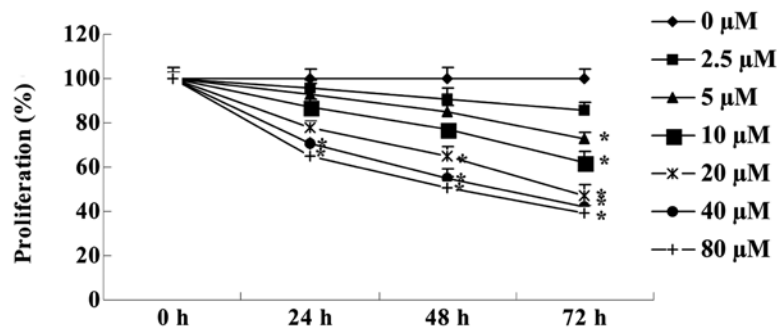


Figure 2. Cantharidin inhibits the proliferation of TSCC Tca8113 cell. * $P < 0.01$ compared with 0 μM cantharidin-treatment group. TSCC, tongue squamous cell carcinoma.

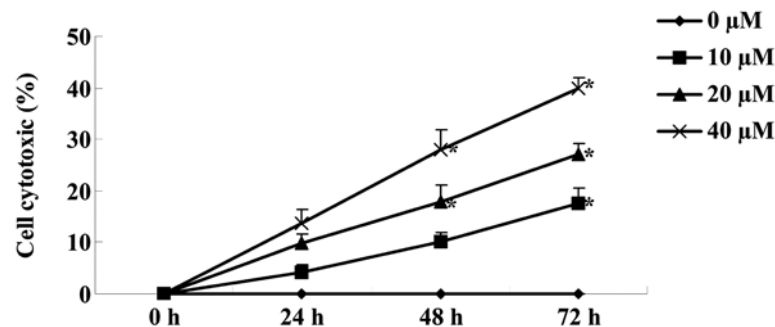


Figure 3. Cantharidin promotes the cytotoxicity of TSCC Tca8113 cells. * $P < 0.01$ compared with 0 μM cantharidin-treatment group. TSCC, tongue squamous cell carcinoma.

Detection of p53, Bcl-2 and Bax protein expression by western blotting. p53 and Bax protein expression levels were detected with western blotting. Briefly, TCA8113 cells (2×10^6 cells/well) were seeded in 6-well plates and treated with cantharidin (10, 20 and 40 μM) for 48 h following treatment. TCA8113 cells were washed with twice PBS. The cells were lysed on ice in a buffer and cultured for 30 min. The lysed solution was centrifuged at $12,000 \times g$ for 10 min at 4°C . The total proteins were determined using a BSA kit. Equivalent amounts of protein were separated by 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with Tris-buffered saline (TBS) containing 5% w/v non-fat milk to block non-specific binding sites. The membranes were blocked and incubated overnight with anti-p53 (1:1,000), anti-Bcl-2 (1:1,000), anti-Bax (1:1,500) (all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti- β -actin (1:500; Sangon Biotech, Shanghai, China) overnight at 4°C . The membranes were then incubated with IgG-conjugated goat anti-rabbit secondary antibodies at room temperature for 2 h using enhanced chemiluminescent reagents (Tiangen, Beijing, China) and exposed to X-ray film.

Transfection of miR-214 and anti-miR-214. The TCA8113 cells (2×10^6 cells/well) were seeded in 6-well plates 24 h prior to transfection. miR-214 inhibitors and mimics were established by BeastBio Co., Ltd. miR-214 inhibitors (10 nM duplex/well) and miR-214 mimics (10 nM duplex/well) were performed using Lipofectamine 2000 (Invitrogen Life Technologies).

Statistical analysis. Analysis of variance (ANOVA) or Student's t-test was performed with SPSS software. Data were presented as mean \pm SD. $P < 0.05$ was considered to indicate a statistically significant result.

Results

Cantharidin inhibits the proliferation of TSCC Tca8113 cells. To confirm the effect of cantharidin on the proliferation of TSCC Tca8113 cells, an MTT assay was used to measure the growth of Tca8113 cells. The results showed that cantharidin inhibited the proliferation of Tca8113 cells in a dose- and time-dependent manner (Fig. 2). After 72 h, the anticancer effect of cantharidin (5, 10, 20, 40 and 80 μM) markedly inhibited the proliferation of Tca8113 cells. After 48 h, treatment with cantharidin (20, 40 and 80 μM) significantly inhibited the proliferation of Tca8113 cells. Treatment with cantharidin (40 and 80 μM) significantly inhibited the proliferation of Tca8113 cells for 24 h.

Cantharidin promotes cytotoxicity of TSCC Tca8113 cells. An LDH assay was used to investigate the effect of cantharidin on the cytotoxicity of TSCC Tca8113 cells treated with 0, 10, 20 and 40 μM cantharidin for 48 h. In the Tca8113 cells treated with 0, 10, 20 and 40 μM cantharidin, cantharidin increased the cytotoxicity of Tca8113 cells in a dose- and time-dependent manner (Fig. 3). After 48 and 72 h, cantharidin (20 and 40 μM) notably increased the cytotoxicity of Tca8113 cells (Fig. 3).

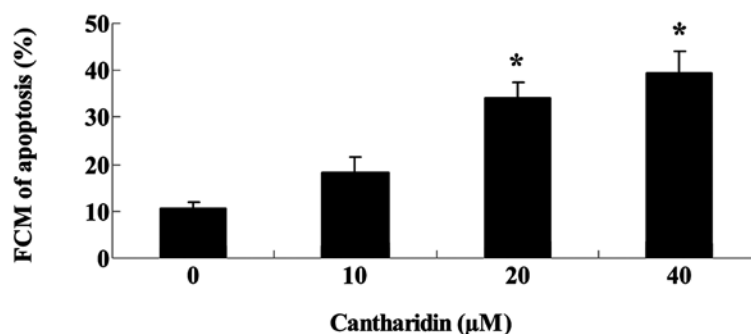


Figure 4. Cantharidin promotes the cytotoxicity of TSCC Tca8113 cells. * $P < 0.01$ compared with 0 μM cantharidin-treatment group. TSCC, tongue squamous cell carcinoma.

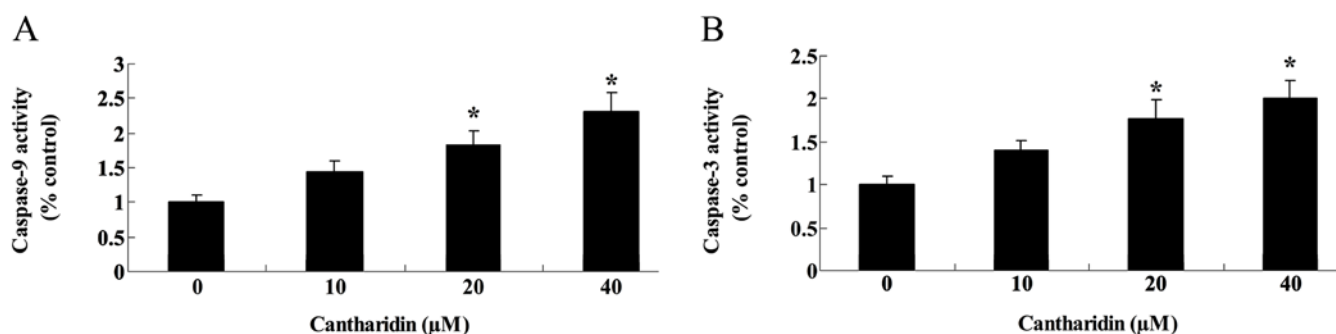


Figure 5. Cantharidin induced caspase-9 and -3 activities of TSCC Tca8113 cells. Cantharidin induced (A) caspase-9 and (B) caspase-3 activities of TSCC Tca8113 cells. * $P < 0.01$ compared with 0 μM cantharidin-treatment group. TSCC, tongue squamous cell carcinoma.

Cantharidin promotes apoptosis of TSCC Tca8113 cells. To investigate whether cantharidin induced inhibition of TSCC Tca8113 cells through cell apoptotic pathways, apoptosis of Tca8113 cells was measured by flow cytometry. After 48 h, treatment with cantharidin (20 and 40 μM) significantly promoted the apoptosis of Tca8113 cells (Fig. 4).

Cantharidin induces caspase-9 and -3 activities of TSCC Tca8113 cells. The possible signaling pathways through which cantharidin induced apoptosis in Tca8113 cells were investigated to detect caspase-9 and -3 activities of Tca8113 cells. Fig. 5 shows that caspase-9 and -3 activities of Tca8113 cells were markedly increased by treatment with cantharidin (20 and 40 μM).

Cantharidin suppresses miR-214 expression of TSCC Tca8113 cell. To investigate the functional role of miR-214 expression in TSCC Tca8113 cell, we carried out miR-214 expression of Tca8113 cells using qPCR. miR-214 expression level was markedly downregulated by treatment with cantharidin (20 and 40 μM) in Tca8113 cells (Fig. 6).

Cantharidin increases p53 protein expression of TSCC Tca8113 cells. The underlying signaling pathways through which cantharidin regulated the proliferation and apoptosis of TSCC Tca8113 cells were examined, and p53 protein expression of Tca8113 cells was examined using western blotting. After 48 h, treatment with cantharidin (20 and 40 μM)

repaired and markedly increased p53 protein expression of Tca8113 cells (Fig. 7A and B).

Cantharidin adjusts Bcl-2/Bax protein expression of TSCC Tca8113 cells. To investigate the potential connection between Bcl-2/Bax protein expression and the potential anticancer effect of cantharidin on TSCC Tca8113 cells, western blotting was used to analyze Bcl-2/Bax protein expression in Tca8113 cells. After 48 h, Bcl-2 protein expression was suppressed by treatment with cantharidin (20 and 40 μM) in Tca8113 cells (Fig. 8A and B). By contrast, the Bax protein expression of Tca8113 cells was activated by treatment with cantharidin (20 and 40 μM) for 48 h (Fig. 8C and D).

Anti-miR-214 and expression of p53 and Bcl-2/Bax proteins in TSCC Tca8113 cells. To investigate the potential connection between miR-214 silencing and the potential anticancer effect of cantharidin on TSCC Tca8113 cells, miR-21 inhibitors were transfected into Tca8113 cells to detect the p53 and Bcl-2/Bax protein expression in Tca8113 cells. Firstly, miR-21 expression of Tca8113 cells were significantly inhibited by miR-21 inhibitors (Fig. 9A). p53 protein expression of Tca8113 cells was subsequently increased by miR-21 inhibitors (Fig. 9B and C). Bcl-2 protein expression was attenuated and Bax protein expression was enhanced following miR-21 transfection (Fig. 9D and G). These results suggest that miR-214 inhibition regulated p53 and Bcl-2/Bax expression in Tca8113 cells.

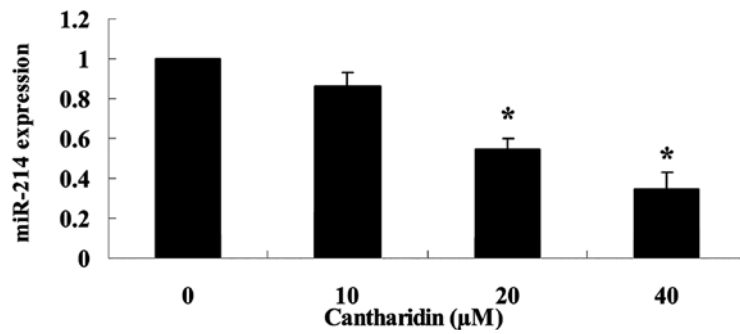


Figure 6. Cantharidin suppressed miR-214 expression of TSCC Tca8113 cells. *P<0.01 compared with 0 μM cantharidin treatment group. TSCC, tongue squamous cell carcinoma.

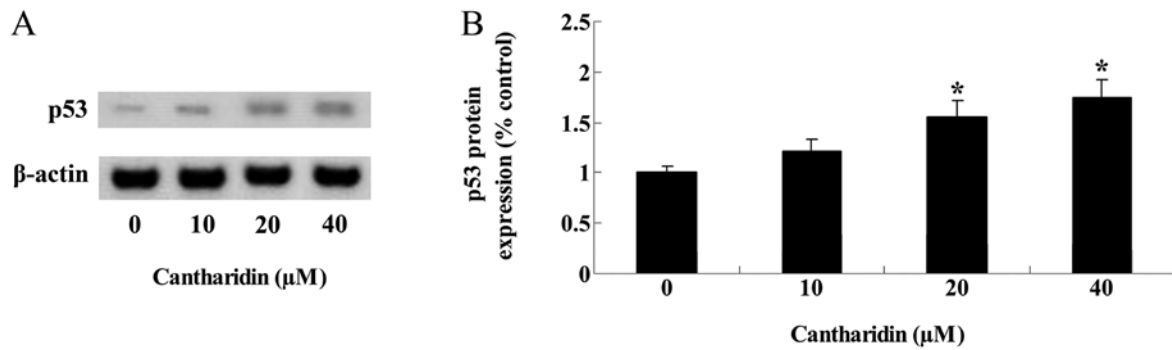


Figure 7. Cantharidin increases p53 protein expression of TSCC Tca8113 cells. (A) The effects of cantharidin on p53 protein expression by western blotting assays. (B) Statistical analysis of p53 protein expression levels of TSCC Tca8113 cells. *P<0.01 compared with 0 μM cantharidin treatment group. TSCC, tongue squamous cell carcinoma.

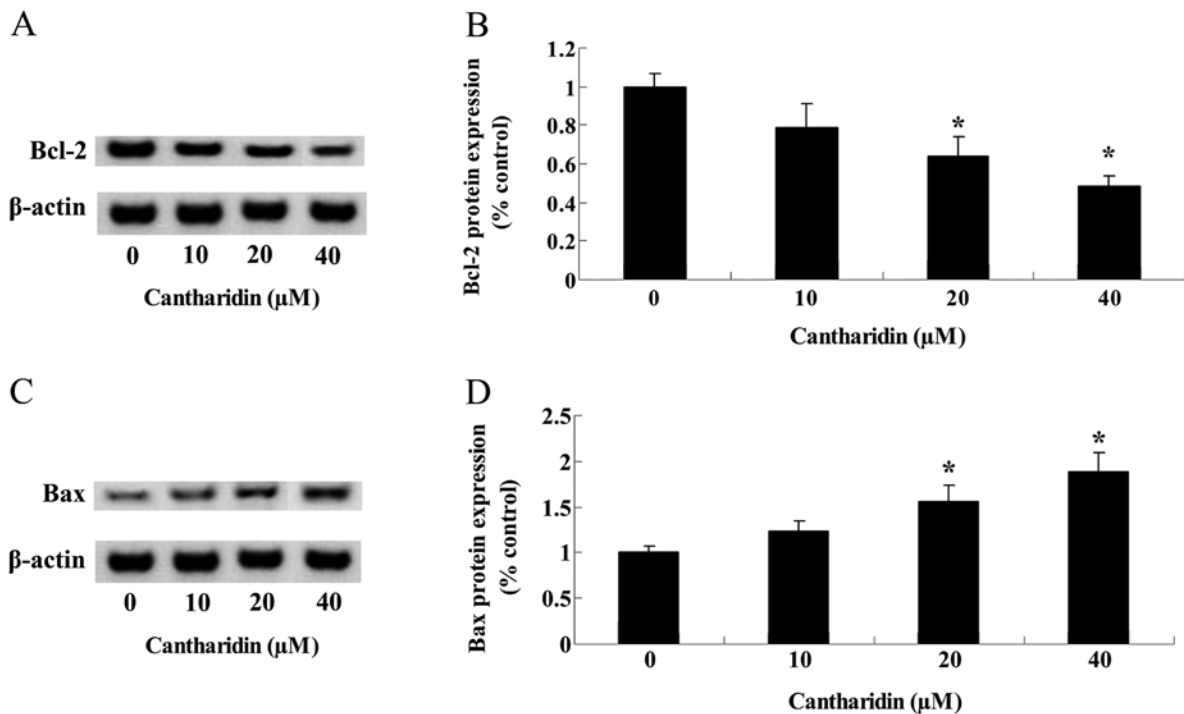


Figure 8. Cantharidin adjusts Bcl-2/Bax protein expressions of TSCC Tca8113 cell. (A and C) The effects of cantharidin on Bcl-2/Bax protein expression by western blot assays. (B and D) Statistical analysis of Bcl-2/Bax protein expression levels of TSCC Tca8113 cells. *P<0.01 compared with 0 μM cantharidin treatment group. TSCC, tongue squamous cell carcinoma.

Overexpression of miR-214 and the anticancer effect of cantharidin on TSCC Tca8113 cells. To assess the potential

connection between miR-214 overexpression and the anticancer effect of cantharidin on TSCC Tca8113 cells, miR-214

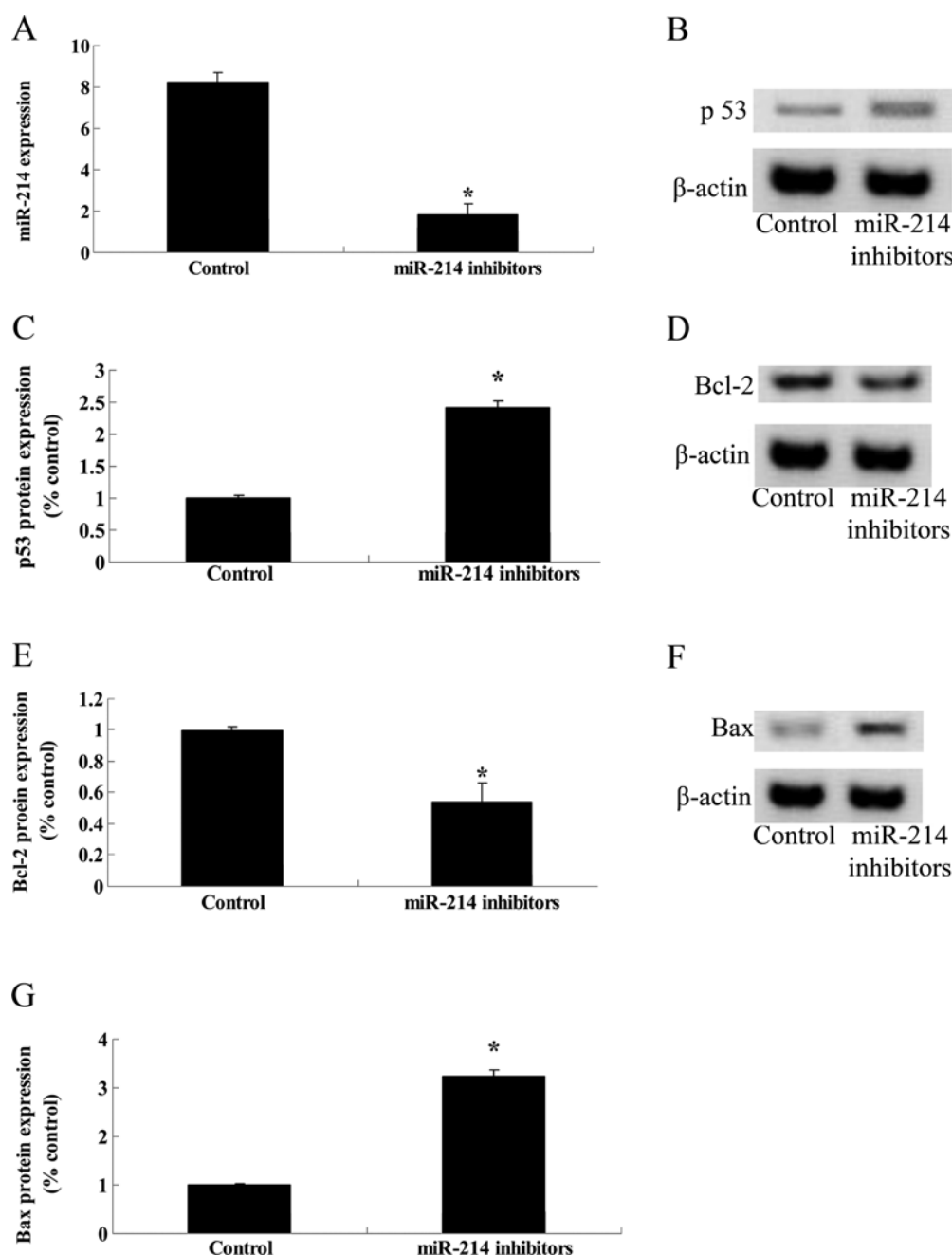


Figure 9. Anti-miR-214 and expression of p53 and Bcl-2/Bax protein expression of TSCC Tca8113 cells. miR-214 inhibitors inhibited (A) miR-214 expression. (B, D and F) p53, Bcl-2 and Bax protein expression by western blot analysis. (C, E and G) Statistical analysis of p53, Bcl-2 and Bax protein expression levels of TSCC Tca8113 cells. *P<0.01 compared with 0 μ M cantharidin treatment group. TSCC, tongue squamous cell carcinoma.

mimics were transfected into Tca8113 cells. The result in Fig. 10A shows that miR-214 mimics significantly increased the expression levels of miR-214 in Tca8113 cells. However, miR-214 mimics reversed the potential anticancer effect of cantharidin on the proliferation, thereby reducing caspase-3 activation in Tca8113 cells (Fig. 10B and C). Notably, p53 and Bax protein expression of Tca8113 cells were weakened by miR-214 overexpression (Fig. 10D and E, and H and I). miR-214 overexpression increased Bcl-2 protein expression of Tca8113 cells (Fig. 10F and G). The results suggested that overexpression of miR-214 reduces the anticancer effect of cantharidin on TSCC Tca8113 cells regulated by p53 and Bcl-2/Bax expression in Tca8113 cells.

Discussion

Oral cancer refers to oral and maxillofacial malignancies, of which the majority are squamous epithelial cancer, including tongue, buccal, gum, palate, lip and jaw bone cancer, oral floor carcinoma and oropharyngeal cancer (3). TSCC is a common oral cancer. The occurrence and development of cancer is a multi-step process with multi-gene mutations, while tumor growth and metabolism are associated with growth scores and self-programmed death (20). Autophagy, as a form of programmed death, is a highly conserved cellular behavior, closely associated with cell growth and proliferation, as well as the tumor development process (21). In the present study,

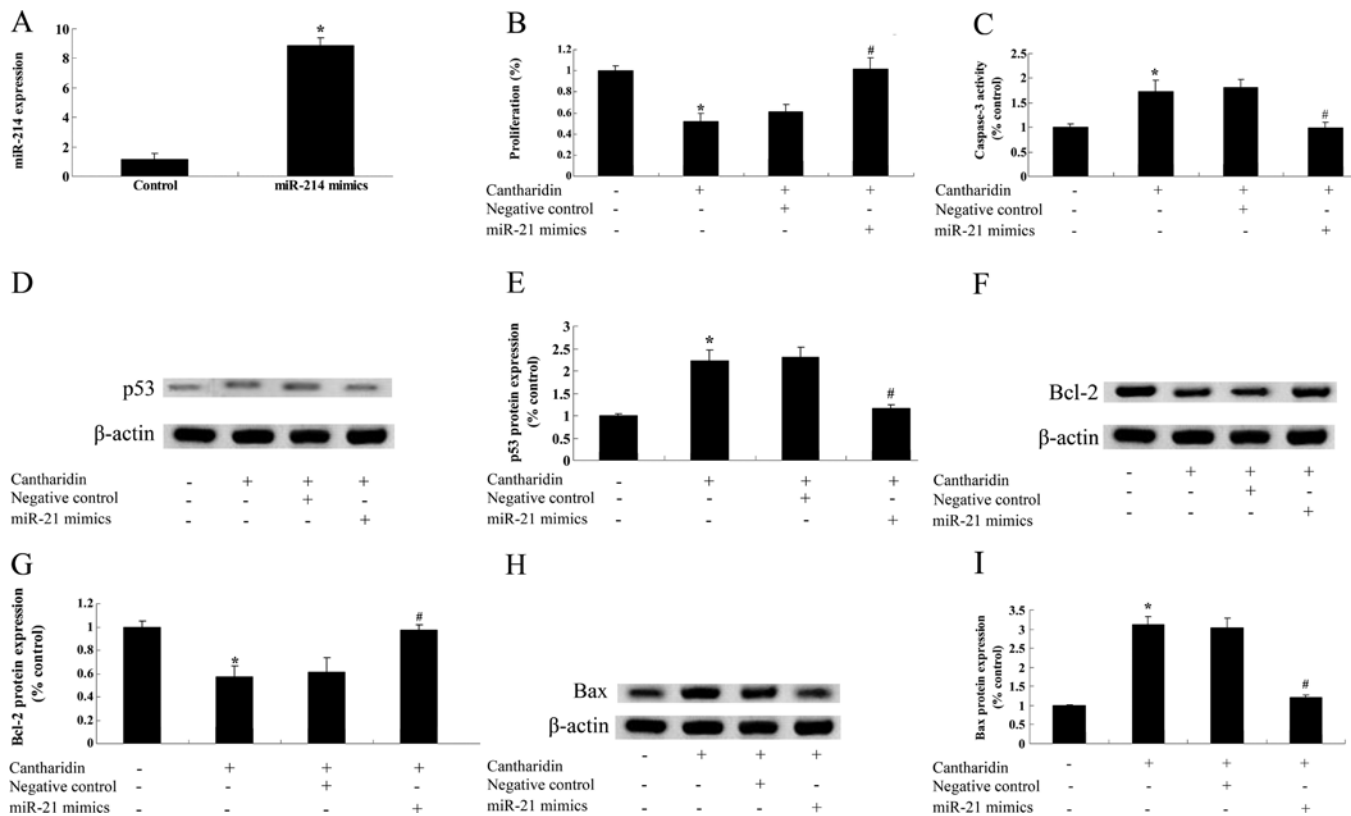


Figure 10. Overexpression of miR-214 and the anticancer effect of cantharidin on TSCC Tca8113 cells. Following treatment with cantharidin (20 μ M) for 48 h, miR-214 mimics induced (A) miR-214 expression, (B) promoted cell proliferation, and (C) inhibited caspase-3 activity. (D, F and G) p53, Bcl-2 and Bax protein expression by western blot analysis. (E, H and I) Statistical analysis of p53, Bcl-2 and Bax protein expression levels of TSCC Tca8113 cells. * $P < 0.01$ compared with 0 μ M cantharidin treatment group. # $P < 0.01$ compared with the cantharidin treatment group transfected with negative control. TSCC, tongue squamous cell carcinoma.

treatment with cantharidin significantly inhibited the proliferation and increased cytotoxicity of TSCC Tca8113 cells in a dose- and time-dependent manner. Cantharidin also stimulated cell apoptosis and enhanced caspase-9/3 activities of TSCC Tca8113 cells. The results were in concordance with those of other studies. For example, Zhang *et al* suggested that cantharidin induced apoptosis by activating caspase-3, -7, -8 and -9 in human gastric cancer (19). Hsia *et al* reported that cantharidin induces apoptosis by activating caspase-3 and -8 in H460 human lung cancer cells (22). Therefore, cantharidin is an optimal potential therapeutic agent for TSCC.

Results of miRNAs studies have identified that a large number of miRNAs with abnormal expressions regulate the development of various types of cancer including pancreatic cancer by regulating the expression of target genes. Thus, large-scale screening of the abnormal expression of miRNAs in the tumor development process may provide a comprehensive understanding of the biological characteristics of tumor in human (23). Findings of a recent study showed that miR-214 is abnormally expressed in a variety of malignancies. However, the expression in different tumors are not the same, with certain specificity (24). We found that cantharidin decreased the miR-214 expression levels of Tca8113 cells. This result was consistent with that of the study by Lu *et al* whereby cantharidin exerted an anticancer effect by miR-214 modulating in hepatocellular carcinoma (8). However, the detailed mechanisms on how cantharidin regulates miR-214 expression remain to be determined in future studies.

The p53 tumor-suppressor gene has been extensively studied. The p53 gene is a tumor-suppressor gene that is associated with human cancer and plays an important role in the regulation of the cell cycle (25). Its product, p53 protein, inhibits tumorigenesis, although following gene mutation the p53 protein loses its tumor-suppressor effect, with the promotion of the activity of malignancy (26). In the present study, we found that treatment with cantharidin effectively activated p53 protein expression of Tca8113 cells. Hsia *et al* showed that cantharidin inhibited DNA repair-associated protein levels (p53 and H2A.X) and induced DNA damage in NCI-H460 human lung cancer cells (18). Kuo *et al* reported that cantharidin induced the apoptosis of human bladder cancer by promoting the p53 level (27).

Apoptosis is a type of cell death with specific morphological change, which is a physiological process caused by a series of gene actions. Bcl-2 oncogene is a cancer gene found at t(14;18) chromosomal translocation breakpoint, of which the overexpression protects tumor cells from apoptosis (28). Cantharidin reduced the Bcl-2 protein expression and activated the Bax protein expression of Tca8113 cells in the present study. Li *et al* suggested that cantharidin induced oxidative stress-independent growth inhibition of pancreatic cancer cells through suppression of the expression of anti-apoptotic Bcl-2 (29). These findings suggested cantharidin induced apoptosis via Bcl-2/Bax in human bladder carcinoma (30), pulmonary carcinoma (31), and pancreatic cancer (29) cells.

The present study has limitations. To clarify the mechanism involved in the suppression of Tca8113 cells, the effect of cantharidin on miR-214, p53 and Bcl-2/Bax expression was examined. We found that the miR-214 silence expression stimulated the p53 protein expression and inhibited the Bcl-2/Bax signaling pathway in Tca8113 cells. Additionally, miR-451 overexpression reversed the anticancer effect of cantharidin on TSCC Tca8113 cells, attenuated p53 protein and stimulated the Bcl-2/Bax signaling pathway in TSCC Tca8113 cells. With respect to the anticancer effect of cantharidin on TSCC and its mechanism, further studies on knockdown of miR-214 may provide more information for an improved understanding of the effect of cantharidin affected by p53 and Bcl-2/Bax signal on the proliferation and apoptosis in TSCC.

In conclusion, the results show the anticancer effect of cantharidin inhibits cell proliferation and promotes apoptosis of TSCC. Knockdown of miR-214 activates the effect of cantharidin on cell proliferation and apoptosis *in vivo*. Our findings suggest that cantharidin is an important potential therapeutic agent in TSCC and a potential therapeutic target for TSCC patients.

Acknowledgements

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