The inhibitory effect of tumor suppressing component I in *Agkistrodon acutus* venom on oral squamous cell carcinoma

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Abstract: This study aimed to explore the effect of tumor suppressing component I from *Agkistrodon acutus* venom (AAVC-I) on the proliferation, migration, invasion, and apoptosis of oral squamous cell carcinoma (OSCC) and the effect on the tumors in vivo. Cal27 cells (a human OSCC cell line) were randomly divided into two groups: the experimental group (AAVC-I, 0.1 mg/mL) and the control group (AAVC-I, 0 μg/mL). AAVC-I was dissolved in an RPMI-1640 medium and added to the culture wells at different concentrations when the tumor cells reached the logarithmic growth phase. The cells were collected 24 hours later to observe the cell proliferation, migration, and invasion. Compared to the control group, the AAVC-I treatment not only inhibited Cal27 growth, but it also suppressed cell healing. Meanwhile, it was observed that Cal27 cell apoptosis was significantly increased. In the animal experiments, following AAVC-I intervention, the tumor growth in the experimental group was suppressed. Caspase-3 expression and apoptotic body cells were both increased. Both in vitro and in vivo studies demonstrated that AAVC-I inhibited cell migration and proliferation, and it also induced the apoptosis of OSCC Cal27 cells.

Keywords: Component I of *Agkistrodon acutus* venom, oral squamous cell carcinoma Cal27 cells, apoptosis

Introduction

Oral squamous cell carcinoma (OSCC) is a common malignancy [1]. It can be divided into tongue cancer, buccal mucosa cancer, lip cancer, and floor of the mouth cancer, etc., based on the location. In recent years, many researchers have been dedicated to exploring the pathogenesis and therapeutic targets of oral sarcoma cell carcinoma and conducted in-depth studies on the possible cause [3-9]. There are multiple ways to treat OSCC [10-12], and it is important to find an effective therapy.

Component I from *Agkistrodon acutus* venom (AAVC-I) is a tumor-inhibitory component extracted and purified from the crude venom of the snake *D. acutus*. Based on preliminary research [13, 14], in vitro research was conducted on the proliferation, migration, and apoptosis of OSCC Cal27 cells, and the inhibitory effect in vivo was also explored, in order to determine whether AAVC-I is able to inhibit tumor growth and reduce tumor size by affecting the proliferation, migration, and apoptosis of Cal27 cells.

This study provides a theoretical basis for the use of snake venom in the treatment of OSCC.

Materials and methods

Animals and cells

Twelve male nude mice, with an average weight of about 18 ± 3 g, were raised under SPF conditions for 20-35 days. The mice were provided by the Shanghai Silaike Experiment Animal Co., Ltd. The OSCC Cal27 cells were provided by ATCC (USA).

Equipment

A carbon dioxide thermostat incubator and a biosafety cabinet were purchased from ThermoFisher Scientific (USA). A high-speed centri...
fuge was purchased from Beckman, USA. An inverted fluorescent biological microscope was purchased from Leica (USA). A biological microscope was purchased from Olympus (Japan). A digital analytical balance was purchased from Ohaus (USA). A biological tissue dehydrator, a paraffin embedder, and a semi-automatic tissue microtome were purchased from Leica (USA).

Reagents

AACV-1 lyophilized powder was provided by the Research Institute of Snake Venom of the South Anhui Medical College. Cell culture media DMEM/F-12 was purchased from Thermo-Fisher Scientific (USA). Phosphate buffered saline (PBS) was purchased from Bioyuntian Bio (China). Mycoplasma free fetal bovine serum was purchased from Sijiqing (China). Trypsin was purchased from Hyclone (USA). Penicillin-streptomycin was purchased from Gibco (USA). Rabbit anti-Ki67 monoclonal antibody was purchased from Maixin Bio (Catalog MAB-0672, China). Rabbit anti-cleaved caspase 3 monoclonal antibody was purchased from Cell Signaling (Catalog #9664, China). DBA staining solution and a ready-to-use rapid immunohistochemistry MaxVision TM kit (rabbit/mouse) were purchased from Maixin Bio (China). An Annexin V-FITC/PI double staining apoptosis assay kit was purchased from Kaiji Bio (Jiangsu, China).

Cell culture

The OSCC Cal27 cells were thawed and cultured in an incubator which contained 5% carbon dioxide at 37°C following the standard procedure. The cells were digested with trypsin, followed by sub-culturation at a ratio of 1:2. DMEM/F-12 containing 10% fetal bovine serum was used as a culture medium. The medium was changed and the cells were passaged every 1-2 days. The cells were cultured until they reached the logarithmic phase.

Cell proliferation assay

The mature Cal27 cells were cultured and prepared in a suspension (2×10^5 cells/ml). Into each well in a 96-well plate 100 µl suspension was added. The cells were classified into four groups, with 5 wells in each group. The cells were cultured for 24 h in the incubator until all the cells adhered to the walls. The cells were washed and mixed with 0.1 ml medium containing the AAVC-1 (0.1 mg/mL). They were treated for 0 h, 24 h, 48 h, and 96 h respectively. 0.01 ml CCK8 solution was added to each well. After 2 h, we determined the absorbance at 450 nm using a microplate reader and recorded the value.

Cell migration assay

The Cal27 cell suspension was inoculated in the 6-well plates at a concentration of 5×10^5 with three replicates for each group. 0.1 ml AAVC-1 at a concentration of 0.1 mg/mL was added into each well of the experimental group. The same volume of DMEM/F-12 without AAVC-1 was added in the control group. After 48 h, the cells were scratched with a micropipette tip. After they were washed with PBS three times, the scratched cells were removed and cultured in serum free DMEM/F-12. After 12 and 24 hours, we observed the cells migrating into the scratch using lighted microscopy, and we took pictures.

Cell invasion assay

The Matrigel gel and blank medium were mixed at a ratio of 1:8, and 20 µl of the matrix was added uniformly to coat the 8 µm wells. The gel solidified within 4-6 hours. 0.1 ml of cell suspension (1×10^6 cells/ml) was seeded around the chambers of the experimental group in the culture medium containing either serum or chemokine, and the DMEM/F-12 culture medium with or without AAVC-1 was added. The medium which contained FBS (10%) was in the bottom chamber. The cells were incubated at 37°C for twenty-four hours in a cell incubator containing 5% CO_2. The cells in the top chamber were removed. The cells in the bottom chamber were fixed with formaldehyde, stained with crystal violet for 15 minutes and washed. Five fields were randomly selected, and images were taken for cell counting.

Cell apoptosis assay

Annexin-V-PI double staining was used to observe the apoptosis of the Cal27 cells after the AAVC-I intervention. The Cal27 cells were seeded in six-well plates. When the cell confluence reached approximately 50-60%, AAVC-1 (0.1 mg/ml) was added. An equal amount of PBS
AAVC-I inhibits Cal27 cells migration, proliferation, and apoptosis

was added to the control group. After incubation for 48 hours, the cells were treated according to the instructions of the cell apoptosis detection kit.

Establishment of the tumor models in nude mice

The mature OSCC Cal27 cells were adjusted to a suspension of 1×10^7 cells/ml. 0.1 ml of cell suspension was subcutaneously injected into the backs of the nude mice. We continued to raise the animals in an SPF environment until the tumors reached a size of around 80-120 mm^3.

Animal grouping and drug administration

The animals with successful tumor formation were randomly divided into two groups: the experimental group and the control group, with 6 animals in each group, for a total of 12 animals. The dosage was calculated based on the body weight of each animal. The mice in the experimental group were injected with 1.0 mg/kg AAVC-1 (diluted with 0.01 mL/g saline), and the mice in the control group were injected with 0.01 mL/g saline. The dosing started from the third day of grouping, and the mice were dosed at 8:00 am every two days. The mice were dosed a total of five times and euthanized at 48 hours after the last dosing.

Tumor size recording and tumor growth curve plotting: Starting from the 3rd day, the long diameter (a) and the short diameter (b) were measured every two days. We calculated the tumor size based on the following formula, and the tumor growth curve was plotted.

Tumor size calculation formula: TV = 1/2×a×b^2, unit cm^3.

Measurement of the tumor weight and the tumor inhibition rate calculation: 48 hours after the last dosing, the mice were euthanized the next day. The tumors were completely dissected from the back of mice and the weight was measured immediately.

Inhibition rate = [(mean tumor weight of the control group - mean tumor weight of the experimental group)/mean tumor weight of the control group] ×100%.

HE staining: The completely dissected tumors were fixed, embedded, and sectioned onto 5 μm slides. The slides were stained with HE and observed under a lighted microscope. The results were recorded.

Determination of the Ki-67 protein expression: The tissue slides were dewaxed using the immunohistochemistry SP method, hydrated with an ethanol gradient, repaired with citric acid antigen repair solution, washed with a PBS buffer, and incubated with an anti-Ki-67 protein antibody at room temperature. The secondary antibody was added for the incubation. DBA was added for the color reaction and the slides were re-stained with hematoxylin. Positive cells were shown by the brownish yellow particles in their nuclei and cytoplasms. Images were taken and recorded.

Determination of cleaved caspase 3 (CC3) protein expression: After dewaxing the tissue slides, we added rabbit anti-rat cleaved caspase 3 monoclonal antibody (1:100), and a secondary antibody was added the following day. DBA was added for the color reaction, and the slides were re-stained with hematoxylin. The apoptosis was examined through the detection of the apoptosis associated protein CC3. The positive cells were characterized by a light yellow or brownish yellow color in their cytosols and their nuclei. Images were taken and recorded.

Statistical analysis

The data were analyzed using SPSS 17.0 statistics software and expressed as the mean ± standard deviation (X ± s). The quantitative data from all the groups were expressed as the mean ± standard deviation. The differences between the two groups were analyzed using t-tests. P<0.05 indicated a significant difference.

Results

The effect of AAVC-I on the migration, proliferation, invasion, and apoptosis of the OSCC Cal27 cells revealed by an in vitro assay

A CKK8 assay was used to measure the cell proliferation. The result showed that the number of proliferating cells in the experimental group was significantly reduced after the AAVC-I intervention. With the increase in time and the longer AAVC-I treatment time, the inhibitory effect became more significant in a time-dependent manner (Figure 1).
AAVC-I inhibits Cal27 cells migration, proliferation, and apoptosis

The cell migration ability was observed using a cell scratch assay. The results showed that, compared with the control group, the cell healing and tumor cell migration abilities were significantly reduced in the experimental group following the AAVC-1 treatment (Figures 2, 3) (*P<0.05).

The extent of the cell invasion was determined using a Transwell assay. The result showed that after the AAVC-1 treatment, the number of crystal violet stained Cal27 cells in the bottom of chamber, and the percentage of the invading cells were significantly reduced compared to the control group (Figures 4, 5) (*P<0.05).

In the apoptosis assay, the effect of AAVC-1 on the apoptosis of the Cal27 cells was determined using an Annexin V-PI double staining kit (Figure 6A): 3.85% vs. 10.79%. Compared to the control group, the percentage of cells undergoing apoptosis was significantly increased following the AAVC-1 intervention (Figure 6B) (*P<0.05).

The effect of AAVC-I on tumor growth revealed by the in vivo assay

After the successful tumor formation, AAVC-1 was injected into the backs of the nude mice. The tumor sizes in the two groups were monitored in real-time. The results showed that tumors in the experimental group exhibited a significantly smaller size compared with the control group. After day 9, the tumors started to decrease with a significant difference (*P<0.05). The difference between the two groups became more significant after 11 days (**P<0.01) (Figure 7A).

The animals were euthanized after the experiment. The tumor mass was isolated, and the
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weight was recorded. The results showed that tumor weight was significantly decreased after the AAVC-1 intervention (Figure 7B) (**P<0.01).

Calculation of the tumor inhibition rate

The tumors were completely dissected from the mice in the experimental and control groups, and their weights were recorded. The tumor inhibition rate was calculated, as shown in Table 1.

Tumor Inhibition rate = \[\frac{(158.97-67.70)}{158.97}\] ×100% = 57.41%.

The results showed that the tumor growth in animals was significantly inhibited after the AAVC-1 intervention, with an IR up to 57.41%.

Discussion

Venomous snakes are widespread all around the world. Ever since the various components of snake venom have been isolated and purified [15-17], new anticoagulant and tumor suppressive effects have been continuously found. Many researchers have explored the tumor suppressive components and have obtained extensive findings. Some researchers found that snake venom can prevent the growth of human cervical cancer cells [18], increase the types of reactive oxygen species to induce the apoptosis of rectal cancer and breast cancer cells [19], and can also be used as a potential...
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Figure 6. A. The inhibitory effect of AAVC-1 on the apoptosis of human tongue cancer Cal27 cells revealed by a flow cytometry assay. B. The role of AAVC-1 in the apoptosis of human tongue cancer Cal27 cells (*P<0.05).

Figure 7. A. The growth curve of tumor size changes in the experimental and control groups (*P<0.05, **P<0.01). B. The differences in the tumor weights between the experimental and control groups (**P<0.01).

Table 1. Tumor weight (**P<0.01)

<table>
<thead>
<tr>
<th>Tumor weight (mg)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>Mean weight (X ± s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>209.7</td>
<td>164.7</td>
<td>162.5</td>
<td>151</td>
<td>143.5</td>
<td>122.4</td>
<td>158.97 ± 29.19</td>
</tr>
<tr>
<td>Experimental group</td>
<td>112.5</td>
<td>78.1</td>
<td>70.2</td>
<td>62.6</td>
<td>44.9</td>
<td>37.9</td>
<td>67.70 ± 24.34**</td>
</tr>
</tbody>
</table>

Note: **indicates P<0.01 as compared with Control group.

treatment for acute lymphocytic leukemia [20]. Other studies have shown that the tumor suppressive component I from the venom of the South Anhui Viper elicits apoptosis and inhibits the proliferation of human gastric cancer cell SGC-7901 in animal models [21]. Other researchers have studied the ability of tumor suppressor component I of Agkistrodon acutus venom to suppress the proliferation and adhesion of ovarian cancer cells A2780 [22]. However, no study has been conducted on oral squamous cell carcinoma.

The key point of the treatment of OSCC is to inhibit cancer cell proliferation and elicit the cells’ apoptosis. A variety of in vitro experimental methods were used by our research group to explore the effects of tumor suppression component I of Agkistrodon acutus venom on the invasion, migration, proliferation, and apop-
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Figure 8. The morphological differences between the control and experimental groups under HE staining (×400).

Figure 9. The expressions of Ki-67 in the control and experimental groups (×200).

Figure 10. The expressions of the cleaved caspase 3 protein in the control and experimental groups (×200).

tosis of human OSCC Cal27 cells. Cal27 cells were implanted in tumor-bearing mice. After successful tumor formation, AAVC-1 was used to interfere with the tumor growth. Dynamic changes in tumor size were measured and growth curves were plotted. Finally, the nude mice were killed to calculate the tumor weight and the tumor inhibition rate. The tumors were sectioned onto slides, and the morphological changes of the tumor tissue were observed using HE staining. The expressions of the Ki-67 antigen and the apoptosis-associated protein CC3 were determined using the immunohistochemical SP method. The results showed that the proliferating cells in the experimental group were significantly reduced, and the inhibitory effect was increased with the increase of time and a longer AAVC-I intervention time, indicating a time-dependent manner. In the scratch test, after the AAVC-I intervention, the healing of the cell scratches was significantly suppressed, and the cell migration was inhibited. In the Transwell experiment, the percentage of Cal27 cells invading to the bottom of the chamber in the experimental group was significantly lower than it was in the control group, suggesting that AAVC-I can inhibit the invasion of OSCC Cal27 cells.

The results of the apoptosis assay showed that the percentage of apoptotic cells increased significantly after the AAVC-I intervention, and the difference was statistically significant. In the animal experiments, AAVC-I was injected into nude mice through the tail veins to interfere with the tumor growth. The tumor size was monitored in real time. The results showed that the tumor size in the experimental group began to decrease starting on day 7. The tumor sizes of the two groups began to show significant differences starting on day 9, and the tumor growth rate was significantly inhibited. After the 11th day, the differences between the two groups were more obvious. Finally, nude mice were euthanized, and the tumor masses isolated. The tumor sizes of the experimental group were significantly smaller than of the sizes in the control group (**P<0.01). The calculated tumor inhibition rate IR was 57.41%, showing a significant tumor inhibition effect. The tumor was sectioned onto slides for observation. Following HE staining, it
was found that mitosis in the experimental group was reduced and apoptosis was observed.

Ki-67 is a type of cell proliferation-related antigen. The expression of Ki-67 can be used to determine the cell proliferation activity. In this study, the SP method was used to determine the expression of the Ki-67 antigen, and the results showed that the Ki-67 antigen expression in the control group was reduced, and the number of cells in the proliferation state was significantly decreased. AAVC-I significantly inhibited the proliferation of OSCC cells. Caspase3 is the most important terminal cleaving enzyme in the process of apoptosis and participates in the killing mechanism of CTL cells. In order to investigate tumor apoptosis, the apoptosis-associated protein CC3 level was measured. In the control group, the ratio of positive cells was significantly reduced. Abundant brown-yellow positive cells confirmed that AAVC-I induced the apoptosis of the OSCC cells.

Taken together, AAVC-I exhibits a significant inhibitory effect on the growth of OSCC, which may be associated with the inhibition of cell proliferation and the induction of apoptosis. Further research is expected to explore the detailed mechanism.

Through in vitro and in vivo assays, this study confirmed the inhibitory effect of AAVC-I on OSCC cells, explored the use of tumor suppressing components in snake venom, and provided a theoretical basis for the medical treatment of OSCC.

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Disclosure of conflict of interest

None.

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