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Aromatic turmerone regulates the migration and autophagy of cutaneous squamous cell carcinoma cells through the Igf-1/Pi3k/Akt pathway

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Abstract: In recent years, the incidence of cutaneous squamous cell carcinoma (CSCC) has been increasing year by year. Due to its characteristics of high recurrence and high invasion, CSCC poses a great potential threat to the prognosis of patients. Aromatic turmerone, an active extract of *Curcuma longa*, has been shown to have anti-tumor, anti-inflammatory, and anti-oxidation effects in modern pharmacological research, but its influence on CSCC remains unclear. In this study, we used AT to intervene in human CSCC A431 cell line. The results showed that the growth ability of A431 was significantly inhibited by the intervention of AT. Also, we found that compared to normal A431, the cell invasion number and migration rate of A431 were reduced to (116.00±8.00) and (39.87±4.20) %, respectively, after the intervention of AT. And detecting the inflammatory response in the cells, we found that AT reduced the inflammatory factors and oxidative stress in A431. Meanwhile, aromatic turmerone suppressed the autophagy of A431 and silenced the IGF-1/PI3K/AKT pathway expression, which further reveals the mechanism and pathway of action of aromatic turmerone on CSCC, laying a reliable foundation for the clinical application of aromatic turmerone.

Keywords: Aromatic turmerone, Cutaneous squamous cell carcinoma, IGF-1/PI3K/AKT, Invasion, Migration

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INTRODUCTION

Skin cancer is a malignancy of skin origin, primarily associated with ultraviolet exposure and is therefore more common among outdoor workers, athletes and light-skinned populations (Linares *et al.*, 2015). Cutaneous squamous cell carcinoma (CSCC) accounts for approximately 40% of all skin cancers. Although being less malignant than melanoma, CSCC has the characteristics of aggressiveness, strong migration, and strong destructive power to tissues, resulting in a risk of distant metastasis of more than 16% (Corchado-Cobos *et al.*, 2020). Because of this, a large number of CSCC patients still experience recurrence or metastasis after surgical resection (Winge *et al.*, 2023). Therefore, for CSCC, it is usually difficult to achieve the ideal therapeutic purpose by relying on surgery alone and postoperative chemotherapy is one of the essential components (Hedberg *et al.*, 2022). However, chemotherapy drugs have great toxic and side effects on human normal cells and organs at normal therapeutic doses, and cell resistance is also the major issue of poor chemotherapy effect at present (de Jong *et al.*, 2022).

Aromatic turmerone (AT) is an active extract and one of the main components of *Curcuma longa*, which has been shown to have a variety of positive effects such as anti-tumor, anti-platelet, anti-bacterial and anti-oxidative in modern pharmacological studies (Hori *et al.*, 2021). For tumor cells such as breast cancer, AT has been proven to be effective in inhibiting tumor cell growth and invasion, which is considered a new choice for future tumor treatment (Chen *et al.*, 2018; Li *et al.*, 2018). But its influence on CSCC has not been reported yet. We found that AT inhibited breast cancer invasion through the Insulin-like growth factor 1 (IGF-1)/Phosphatidylinositol 3-kinase (PI3K)/Protein Kinase B (AKT) signaling pathway (Park *et al.*, 2012), and given the established role of this pathway in CSCC (Ferreira Mendes *et al.*, 2020), there may be a potential relationship between AT and CSCC.

Therefore, this study will provide new reference and guidance for the future clinical treatment of CSCC by analyzing the influence of AT on the biological behavior of CSCC and the pathways of action.

MATERIALS AND METHODS

Cell data

The human CSCC A431 cell line cultured in a supporting medium (containing 10% FPS) at 37°C with 5% CO_2 in the air. The cells were subcultured once every 3-4 days, and those grown to the logarithmic phase were used for subsequent experiments. Cells were purchased from BeNa Culture Collection (China).

Cell grouping and intervention

After adjusting the logarithmic-growth-phase cells to 1×10^5 cells/mL, 50μg was planted into a 96-well plate,

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with 8-10 duplicate wells set. AT (Med Chem Express, USA) and DMSP (AT: DMSP= 100:1) were prepared into a 1g/L AT culture solution and then added into the wells to cultivate cells according to the concentrations of 0, 2.5, 5, 10, 20, 40, 80 and 160 g/L, respectively. Following 48 hours of routine culture, add 10μL of CCK-8 solution and the absorbance (450 nm) was measured to calculate the IC50. The optimal intervention concentration of AT was selected based on the half maximal inhibitory concentration (IC_{50}), with cells in this group set as the intervention group. In addition, normally cultured A431 cells were set as the control group.

Cell proliferation assay
Cells in the intervention and control groups were inoculated into 96-well plates, with 4 duplicate wells set in each group, into which CCK-8 solution was added at 0, 24, 48 and 72 hours of culture, respectively. The absorbance was detected and the cell growth curve was drawn, tested on a Varioskan LUX enzyme marker (Thermo Fisher, USA).

Cell invasion ability determination
The prepared Matrigel glue was evenly spread in the Transwell chamber (Corning, USA). Cells were concentrated in the upper chamber after 24 hours of starvation treatment, while 600μL of complete medium with 15% serum was added to the lower chamber. Following 48 hours of continuous cultivation, the transmembrane cells were fixed with 4% paraformaldehyde and stained with crystal violet for cell counting under the microscope.

Scratch-wound assay
After seeding the cell suspension in a 6-well plate and allowing the cells to be tiled to 80% of the area, a 1-μL pipette tip was used to scratch the wells. The exfoliated cells were washed off with PBS. After 48 hours of uninterrupted culture, the scratch areas before and after culture were calculated by Image J software 1.8.0 (National Institutes of Health, USA) and the area ratio was calculated. Cell migration rate = (0h scratch area - scratch area after 48h)/0h scratch area × 100%.

Measurements of inflammatory factors and oxidative stress responses
After 48 h of normal culture of the two groups of cells, their cell culture liquid was obtained and the supernatant was obtained after centrifugation (3000 rpm/min) for 10 min. The enzyme linked immunosorbent assay (ELISA) kit detects interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α), super oxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA). All the kits were purchased from Amyjet Scientific Inc (China).

Western blot detection of protein expression
The cells were lysed and separated by by 10%SDS-PAGE (Thermo Fisher, USA), followed by blotting onto a PVDF membrane (Thermo Fisher, USA). Incubation was performed with anti-IGF-1, PI3K, AKT, Beclin1 and LC3-II antibodies (1:1000, Abcam, USA). The electro generated chemiluminescence (ECL) reagent (Med Chem Express, USA) was used for membrane development in a dark room and the intensity of the bands was normalized to β-actin.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS 23.0 software (IBM, USA). The results were statistically described as ($\bar{\chi}\pm s$) and analyzed with the independent sample t-test between groups, with the presence of statistical significance indicated by $P<0.05$.

RESULTS

Cytotoxicity test
CCK-8 test showed that the activity of A431 decreased with the increase of AT concentration ($P<0.05$, fig. 1). The IC_{50} of AT for A431 was calculated to be (22.16±1.87) g/L, so AT at a concentration of 20 g/L was selected for subsequent experiments.

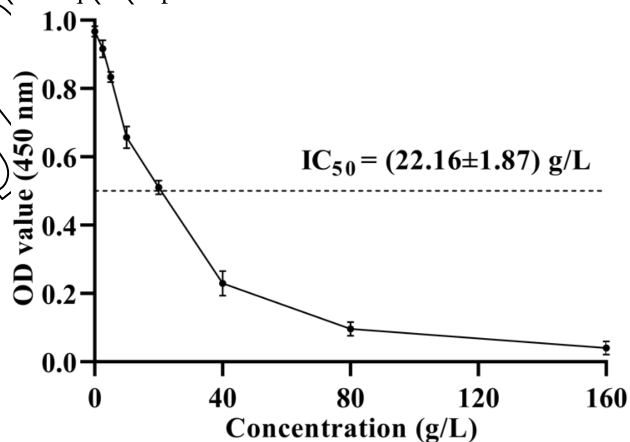

Fig. 1: Cytotoxicity test. the IC_{50}=(22.16±1.87) g/L. Half maximal inhibitory concentration, IC_{50}.

Effect of AT on A431 activity
AT intervention led to significantly inhibited cell growth of A431 in the intervention group ($P<0.05$, fig. 2A). In addition, the number of invasive cells and migration rate in the intervention group were (116.00±8.00) and (39.87±4.20) %, respectively, which were reduced compared with the control group ($P<0.05$, fig. 2B and C), confirming that AT has an inhibitory effect on A431 activity.

Influence of AT on inflammatory responses in A431
The IL-1β, IL-6 and TNF-α in the intervention group were (26.65±2.93) pg/mL, (23.80±1.21) pg/mL and (31.17±3.41) pg/mL, respectively, all of which were decreased compared to the control group ($P<0.05$, fig. 3), indicating that AT can inhibit inflammatory responses in A431.

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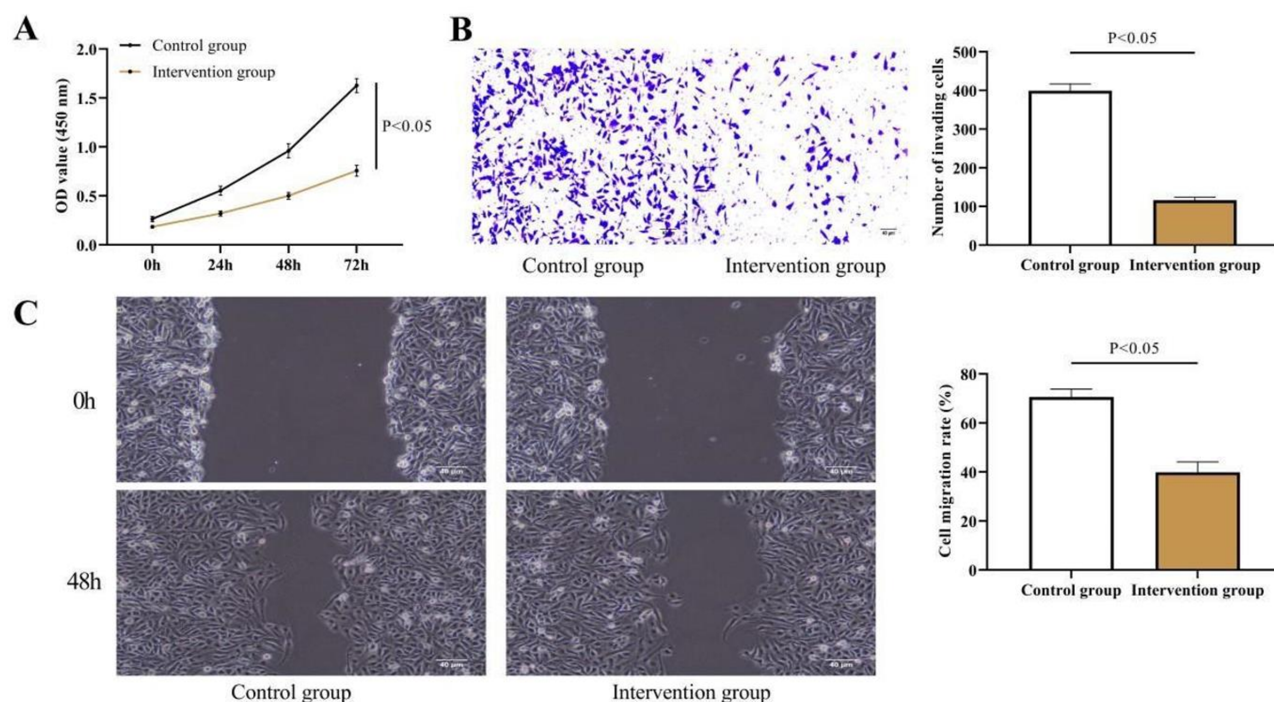

Fig. 2: Effect of AT on A431 activity. A: The effect of AT on the growth ability of A431 cells was detected by CCK-8.B: The effect of AT on the invasive ability of A431 cells was detected by Transwell. C: The effect of AT on the migratory ability of A431 cells was detected by cell scratch assay.

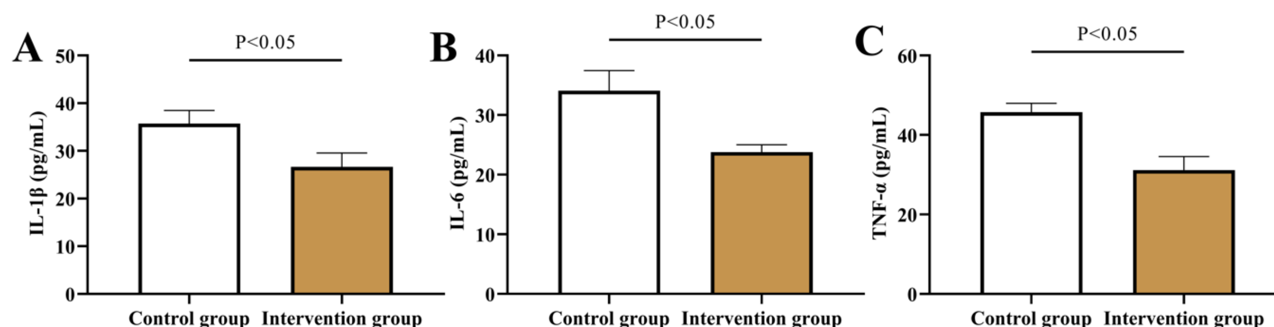

Fig. 3: Influence of AT on inflammatory responses in A431. A: Effect of AT on IL-1β in A431. B: Effect of AT on IL-6 in A431. C: Effect of AT on TNF-α in A431. Aromatic turmerone, AT; Interleukin-1β, IL-1β; Interleukin-6, IL-6; Tumor necrosis factor-α, TNF-α.

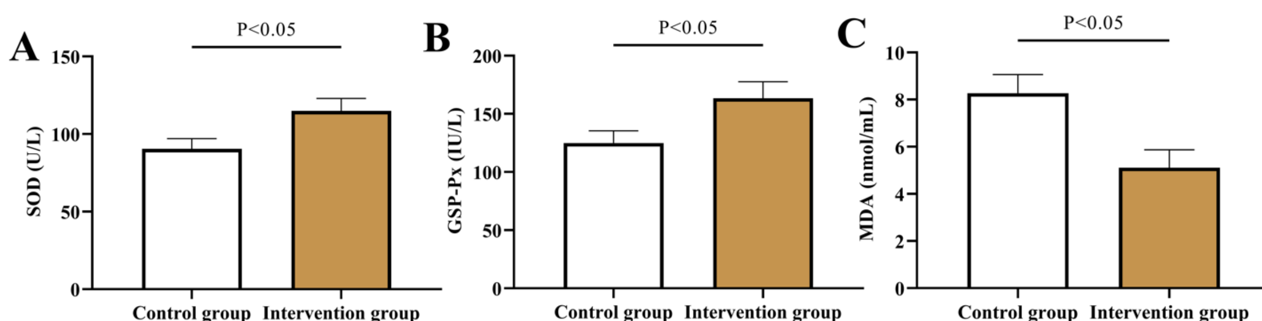

Fig. 4: Impact of AT on oxidative stress in A431. A: Effect of AT on SOD in A431. B: Effect of AT on GSH-Px in A431. C: Effect of AT on MDA in A431. Aromatic turmerone, AT; Super oxide dismutase, SOD; Glutathione per oxidase, GSH-Px; Malondialdehyde, MDA.

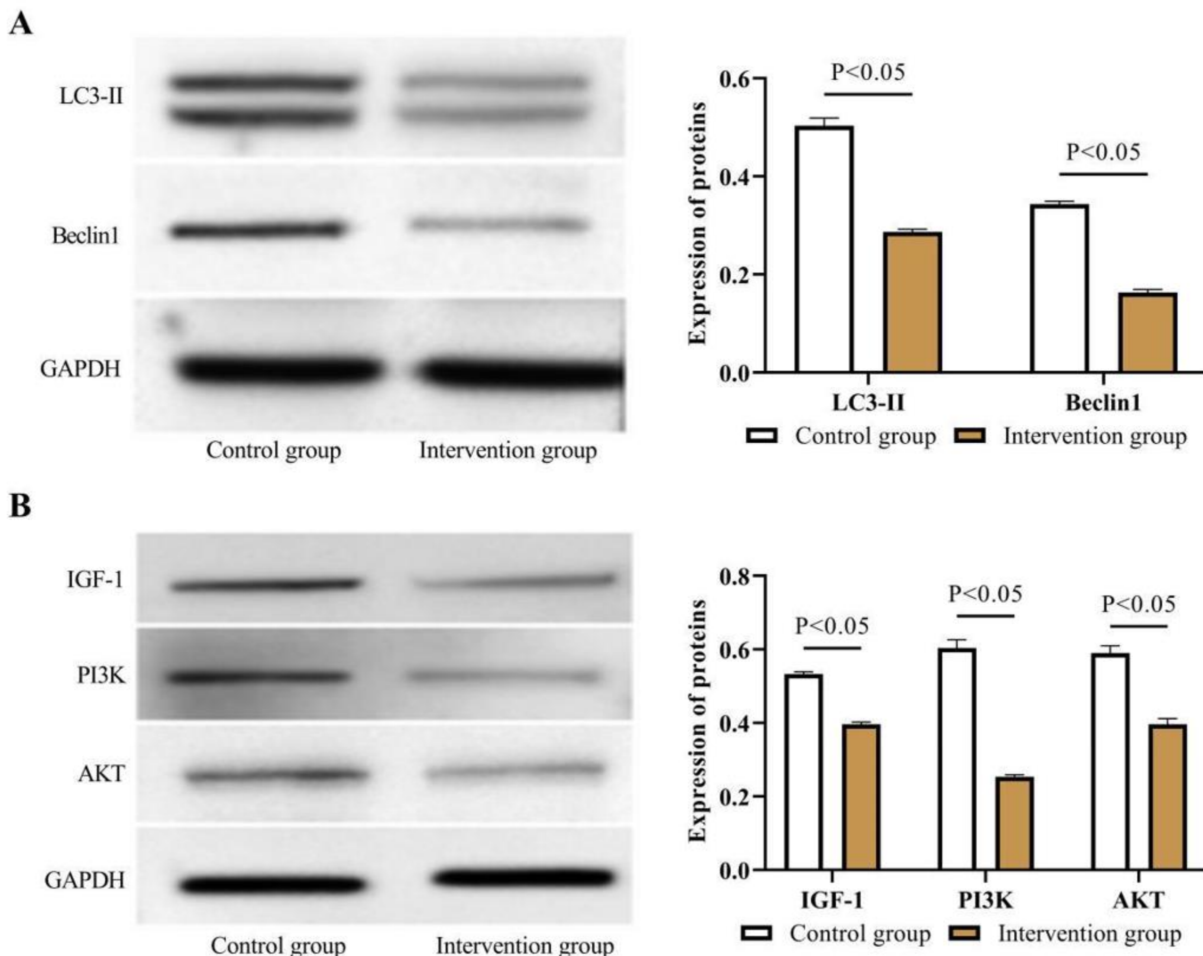

Fig. 5: Effect of AT on autophagy and IGF-1/PI3K/AKT of A431. A: Effect of AT on autophagy of A431. B: Effect of AT on the IGF-1/PI3K/AKT. Aromatic turmerone, AT; Insulin-like growth factor 1, IGF-1; Phosphatidylinositol 3-kinase, PI3K; Protein Kinase B, AKT; Microtubule-associated protein light chain 3II, LC3-II.

Impact of AT on oxidative stress in A431

According to the subsequent detection of oxidative stress, SOD and GSH-Px were elevated while MDA was reduced in the intervention group versus the control group (P<0.05, fig. 4), suggesting the ability of AT to inhibit oxidative stress damage in A431.

Effect of AT on autophagy and IGF-1/PI3K/AKT of A431

After examination, the LC3-II and Beclin1 in the intervention group were (0.29±0.01) and (0.16±0.01), respectively, which were lower than those in the control group (P<0.05, fig. 5A), suggesting that AT can inhibit the autophagy of A431. Finally, IGF-1, PI3K and AKT were higher in the intervention group than in the control group (P<0.05, fig. 5B), which indicates that AT has the effect of inhibiting the IGF-1/PI3K/AKT in A431.

DISCUSSION

Recent years have seen the rising incidence of CSCC, posing a great potential threat to patient prognosis and health due to its high risk of recurrence and metastasis (Heppt & Leiter, 2023). Finding new therapeutic drugs for CSCC is therefore a hotspot and a difficulty in modern clinical research. Nowadays, natural compounds have attracted much attention because of their excellent safety. Among them, curcumin and a variety of curcuminoids have been clinically validated for their killing effect on tumor cells (Saga *et al.*, 2020; Kim *et al.*, 2012). The application potential of AT, the main active ingredient of curcuma oil, cannot be ignored. In this study, we found that AT effectively inhibited the activity of CSCC cells, laying a foundation for the future therapeutic application of AT in CSCC.

Although there have been studies indicating the effects of AT on glioma, ulcerative colitis, etc. (Cao *et al.*, 2023; C. Li *et al.*, 2022), research on its application in CSCC is still relatively rare. Therefore, we first need to determine the optimal dosage of AT by cytotoxicity testing. The activity of A431 was found to show a gradual decreasing trend as the AT concentration increased, and reached the lowest value at 160 g/L, suggests that AT inhibits the

growth of A431. Using the IC_{50}, we determined that the optimal concentration of AT to be used was 20 g/L and then proceeded to the next step of research. After AT intervention, the cancer cell growth curve of the intervention group was reduced compared to the control group and the cell invasion and migration rate were also decreased, confirming that AT can effectively inhibit the proliferation, invasion, and migration capacities of A431. These results also preliminarily confirm that AT has important therapeutic potential for CSCC. Similarly, in previous studies, AT also showed an excellent inhibitory effect on gastric cancer cell activity (Aratanechemuge *et al.*, 2002), which can also support the results of this study.

On the other hand, in the previous research on AT, its excellent anti-inflammatory and antioxidant effects have also been widely concerned (Yang *et al.*, 2020). It can also activate the immune response of lymphoblastomas and resist oxidative stress damage (Kim *et al.*, 2013). The inflammatory infiltration effect of tumor cells and oxidative stress damage to organs and tissues are also typical pathological processes (Liang *et al.*, 2023). Therefore, we further tested the changes in A431 inflammation and oxidative stress responses under AT intervention. The results showed lower IL-1β, IL-6, TNF-α, and MDA in the intervention group versus the control group, as well as higher SOD and GSH-Px, indicating significantly inhibited inflammation and oxidative stress in the intervention group, which once again confirms the important potential of AT in inhibiting the pathological progression of CSCC. This is also consistent with the findings of Doan CC et al (Doan *et al.*, 2022).

Furthermore, autophagy is also one of the important links in CSCC that cannot be ignored. As a programmed cell death process, autophagy is also one of the processes involved in the progression of apoptosis under normal conditions. However, autophagy of tumor cells is usually over-activated, which in turn enhances the adaptability and life cycle of tumor cells through continuous self-renewal, ultimately leading to the continuous progression of tumors (Zheng *et al.*, 2023). Moreover, for chemotherapy, the enhancement of autophagy is also a key process that leads to the development of drug resistance in cells (J. Li *et al.*, 2022). In the current study, LC3-II and Beclin1 were lower in the intervention group than in the control group, indicating that AT also has an inhibitory effect on autophagy in CSCC cells. This also suggests that the use of AT in the future may help reduce chemotherapy resistance in CSCC.

Finally, as mentioned earlier, the effect of AT on CSCC may be related to the IGF-1/PI3K/AKT. After detecting the expression of proteins related to this pathway, it was found that AT could also inhibit the function of the IGF-1/PI3K/AKT. The IGF-1/PI3K/AKT is a classic pathway in tumor research, which has an important influence on activating the activity and malignant infiltration ability of tumor cells. Joshi P et al. also believe that the anti-tumor pathway of AT is related to the PI3K/AKT pathway (Joshi *et al.*, 2021). This also confirms our viewpoint that AT mainly exerts anti-tumor effects by inhibiting the expression of IGF-1/PI3K/AKT in CSCC.

However, we need to conduct a more comprehensive analysis of the pathways and mechanisms of action of AT, in order to provide more comprehensive references for clinical practice.

CONCLUSION

AT inhibited the activity, invasion and migration of CSCC cells and exerted excellent anti-inflammatory and anti-oxidative stress injury effects, and its mechanism was related to the inhibition of autophagy in CSCC cells by silencing the expression of IGF-1/PI3K/AKT pathway.

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