

RESEARCH ARTICLE

Inhibitory Effect of Curcumin on Invasion of Skin Squamous Cell Carcinoma A431 Cells

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Abstract

Objective: To investigate the regulatory effect of curcumin on expression of signal transducer and activator of transcription 3 (STAT3) in skin squamous cell carcinoma tissues as well as possible mechanisms of curcumin in prevention and treatment of skin squamous cell carcinoma. **Materials and Methods:** Highly invasive A431 cells were treated with curcumin at various doses. The cytotoxic effects of treatment with 5, 10, 15, 20, 25, 30, 35, 40 and 50 $\mu\text{mol/L}$ curcumin for 24, 48 and 72 hours on A431 cells were measured by MTT assay. The invasion capacity of cells treated with 5, 10 and 15 $\mu\text{mol/L}$ curcumin was measured by Transwell test, while adhesive ability was assessed by cell adhesion assay. The effects of 5, 10 and 15 $\mu\text{mol/L}$ curcumin on expression levels of STAT3 were determined by Western blotting and on transcription levels of STAT3 mRNA by RT-PCR. **Results:** Treatment with curcumin at a doses of more than 15 $\mu\text{mol/L}$ for more than 24 hour inhibited the growth of A431 cells in a time- and dose-dependent fashion ($p < 0.001$). The doses of 15 $\mu\text{mol/L}$ and less for 24 hours showed no significant cytotoxic effects on the cells, survival rates being more than 85%. The invasion and adhesive abilities decreased gradually with the increasing curcumin concentration, 15 $\mu\text{mol/L}$ exerting the strongest inhibitory effects ($p < 0.05$). Curcumin showed significant dose-dependent inhibitory effects on the transcription level of STAT3 mRNA ($p < 0.05$). **Conclusions:** Curcumin may reduce the invasive ability of A431 cells by inhibiting the activation of STAT3 signal pathway and expression of STAT3 as a target gene in the pathway.

Keywords: Curcumin - skin squamous cell carcinoma - signal transducer - activator of transcription 3 (STAT3)

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Introduction

In the past 30 years, the morbidity and mortality of cancer has been demonstrating a growing trend (Kraljik et al., 2014; Qian et al., 2014). Skin squamous cell carcinoma (SCC) is a higher incidence of skin malignant tumors, it has a large proportion in the skin malignant tumors, is commonly 80%~90% (Jian et al., 2014). Incidence has increased year by year, especially in the elderly. Compared with the addition of a common skin basal cell carcinoma, squamous cell carcinoma of the skin of the illness development is rapid, destructive, but lymph node metastasis, visceral metastasis can be advanced, so people pay more and more attention to the prevention and control.

The treatment of Western medicine at present no effective and economic means, traditional Chinese medicine because of its extensive pharmacological effects and advantages of the side effects of smaller, in-depth study of traditional Chinese medicine and composition in cutaneous squamous cell carcinoma pathogenesis has important significance in genetic toxicity.

Signal transducer and activator of transcription factor 3 (STAT3) is a key molecule in various oncogenic

signaling pathway, the downstream regulation of many growth, and tumor cell apoptosis, angiogenesis and metastasis related key points, such as CyclinD1, Survivin, Bcl-2, MMP2, STAT3 involved in cell growth (Chen et al., 2013), differentiation, division and development of many physiological process, and plays an important role in the malignant transformation of cells (Qu et al., 2013). STAT3 is the signal transducers and activators of transcription (STAT) is an important member of the family. STAT3, as a transcription factor in the absence of specific stimuli located in the cytoplasm, when cells are stimulated, domain and structure of STAT3 receptor SH2 on residues is tyrosine phosphorylated in combination, at the same time its phosphorylation by JAK (Talbot et al., 2014). STAT3 is EGFR (Aparicio-Siegmund et al., 2014), IL-6/JAK, Src etc (Liu et al., 2014). Focusing multiple oncogenic tyrosine kinase signal channel, in a variety of tumor cells and tissues have had multiple activation, abnormal expression of key genes in STAT3 after the induced activation of the cell proliferation and differentiation, survival, apoptosis, closely related, through various channels to promote cell proliferation, malignant transformation, impede apoptosis expression of carcinogenesis, so at present is defined as a cancer gene

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(Wang et al., 2014).

STAT3 not only highly expressed in many tumor cells, but also the continuous high expression in tumor micro-environment in a variety of cells, therefore, inhibit tumor cells and their micro-environment of various stromal cell STAT3 is often considered a target for tumor therapy.

Curcumin is a kind of natural plant polyphenol pigments, extracted from the Zingiberaceae turmeric, there are other Zingiberaceae plants. Widely exists in turmeric rhizome in traditional Chinese medicine. At present, curcumin has been proven to be a safe and effective inhibitors of STAT3, as in T cell leukemia, (Chung et al., 2015). curcumin can inhibit the STAT3 upstream of JAK kinase activity (Uehara et al., 2014), constitutively phosphorylated (Hu et al., 2014). To prevent STAT3, in skin squamous cell carcinoma, whether curcumin can inhibit STAT3 signaling pathway to prevent tumor invasion and metastasis, so far there is no clear reports. The experiments on the effects of different concentrations of curcumin on expression of STAT3 in skin squamous cell carcinoma A431 cells, further explore the mechanism of effect of skin squamous cell carcinoma invasion and metastasis.

Materials and Methods

Cell culture

The human skin squamous cell line A431 cells (Shanghai cell biology Institute, China.) were cultured in RPMI1640(GIBCO company, USA) containing 10%FBS, and incubated under the condition of 37°C and 5% CO₂ in the cell culture box, when the cell growth fusioned to 80%, they were digested with pancreatic digestive enzyme of 0.25%.

Detection of cell killing treated with curcumin

Cells in the logarithmic phase were inoculated in 96 - well plates by 4×10^3 /hole, the edge hole was added sterile PBS as control. Curcumin (Pharmaceutical and biological company, China) were added at the concentrations of 5, 10, 15, 20, 25, 30, 35, 40 and 50 μ mol/L after 24hour. The experimental control group (0.1%DMSO) and zero group were set up, each group had 3 holes. Cultured for 24, 48, 72 hour, they were centrifuged at 1000r/min, discarded the culture medium. Serum free RPMI1640 medium and 20ul MTT (Sigma, American) solution (5mg/ml) were added in per hole with 180 μ L. Cultured for 4 hour, they were centrifuged at 1000r/min, discarded the supernatant, 100 μ L DMSO were added in each hole, shaken for 10min. Optical density at A value was detected by wavelength standard instrument at the wavelength of 490nm, the cell survival rate (%) was calculated according to the following formula.

Cell survival rate (%) = $(A_{490}(\text{experimental group}) - A_{490}(\text{control group}) / A_{490}(\text{experimental group}) \times 100\%$. The experiment was repeated 3 times.

Detection of invasion effect with curcumin by Transwell chamber invasion assay

Cells in the logarithmic growth phase were divided into 4 groups: negative control group (0.1%DMSO) and

curcumin (5, 10, 15 μ mol/L) treatment group. The cells were pretreated for 24 hour. The matrigel was melted overnight, then it was diluted with RPMI1640 culture medium without serum as 1:5, and was joined on the top of the Transwell chamber (Millipore company) by 50ul/hole (69.3ugMatrigel/hole), incubated in the incubator for 4 hour. The cells were digested with pancreatic enzyme of 0.25%, were suspended with serum-free RPMI1640 culture medium containing 0.2% BSA. The cells were adjusted to 5×10^5 /ml. The Transwell Chambers were added into the plate with 24 holes, cell suspension with 200ul were added into the top room, RPMI1640 culture medium containing 10% fetal bovine serum with 500ul were added into the lower room. They were incubated under the condition of 37°C and 5% CO₂ in the cell culture box for 24 hour. After being dried, they were stained with 0.1% crystal violet for 10min, washed 3 times with PBS, fixed with 95% ethanol. Five different field of vision were selected randomly of each chamber, cells were counted under light microscope with 100 times. The mean and variance were calculated.

Detection of adhesion ability by adhesion test

Cells in the logarithmic growth phase were divided into 4 groups: negative control group (0.1%DMSO) and curcumin (5, 10, 15 μ mol/L) treatment group. The cells were pretreated for 24 hour. The 96-well plate was packaged with fibronectin solution, which were prepared to a final concentration of 10 ug/ml with filter double distilled water. It was saved at 4°C overnight after being sealed. It was washed 3 times by PBS. Serum-free RPMI1640 culture medium containing 0.2% BSA were added into each hole of 50 ul/hole. It was closed for 2 hour at 37°C. The cells were digested with pancreatic enzyme of 0.25%, were suspended with serum-free RPMI1640 culture medium containing 0.2% BSA. The cells were adjusted to 2×10^4 /hole. The cells were vaccinated in 96-well plates, each group was settled up for 5 holes. The 96-well plates were incubated in cell cultured box for 1 hour at 37°C. After being incubated, they were washed for 3 times by PBS, which was warmed in advance. MTT(2mg/ml) was added into each hole by 50ul/hole. Then it was incubated in cell cultured box for 3 hour at 37°C. After being incubated, MTT was discarded, DMSO was added into each hole with 150ul/hole. Being shaken for 10min, the Optical density at A value was detected by wavelength standard instrument at the wavelength of 490nm, the cell adhesion amount was calculated according to the following formula.

Cell adhesion amount = $(A_{490}(\text{experimental group}) - A_{490}(\text{control group}))$.

Detection of the expression of STAT3, p- STAT3 by using Western blot

A431 cells were inoculated into the culture bottle by 1×10^4 /cm². After being adhered, the medicines were added into the bottle respectively, according to the following group: the negative control group (0.1%DMSO) and curcumin (5, 10, 15 μ mol/L) treatment group. After being incubated for 24h, the total protein of the cells were extracted. It was separated by 10% SDS-PAGE, then it

Table 1. Primer Sequences for RT-PCR Analysis

Primers	Primer sense	Primer sequences 5'-3'	Product size
STAT3	Forward	CCTTCCTGCGGTTTCAGT	344 bp
	Reverse	GCTGCAGGTCGTTGGTGCTAC	344 bp
β-action	Forward	ATCGTGCGTGACATTAAGGAGAAG	231 bp
	Reverse	AGGAAGGAAGGCTGGAAGTC	231 bp

was transferred onto PVDF membrane. The membrane was closed by 5% skim milk at room temperature for 1h. Then it was washed by TBST for 3 times(5min/time). The first antibody of STAT3 mouse monoclonal antibody and P-STAT3 mouse monoclonal antibody (Cell Signaling Technologies company,USA)was added on the membrane, it was incubated at 4°C overnight. It was washed by TBST for 3 times(5min/time) again. The second antibody was added on the membrane, shaken for 1h at room temperature. Then it was washed by TBST for 3 times(5min/time). It was imaged by ECL imaging system.

Detection of transcription level of STAT3 gene by RT-PCR

Cells in the logarithmic phase were inoculated in 6-well plates by $2 \times 10^5/\text{cm}^2$. After being adhered, the medicines were added into the bottle respectively, according to the following group: the negative control group (0.1%DMSO) and curcumin (5, 10,15 umol/L) treatment group. After being incubated for 24h, the total RNA of the cells were extracted. The concentration of total RNA was detected, 10ug of total RNA was reverse transcribed into cDNA by ReverTraAce- alpha -RT-PCR kit (TOYOBO company, China).The volume of total system was 20ul, with the condition of 99°C for 5 min, 4°C for 5 min. The cDNA was stored at -20°C. The sequences of primers(SANGON, China) were showed in Table 1. Beta action gene was used as an internal matter.

Thermal cycling conditions were as follows: pre-denaturation at 94°C for 3 min; 26 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min, and re-extension at 72°C for 10 min. PCR amplification products were separated on 2% agarose gel. The imaging acquisition was collected by BIO-RAD system, and analyzed by Quantity One software.

Statistical methods

SPSS13.0 statistical software was used for statistical analysis. Data are given as mean and standard deviation (Mean±SD). Between the two groups were compared using independent samples T-test, single factor analysis of variance for comparison of multiple groups of data, with $p < 0.05$ for the differences have statistical significance.

Results

The cell viability of MTT detection

When the concentration of curcumin is more than 15 umol/L, the action time was more than 24h, the growth inhibition of cells in a time and dose dependence, and the difference was statistically significant ($p < 0.001$); when the concentration of curcumin is less than or equal to 15umol/L, the reaction time was 24h, there is no obvious toxic effect on the cells, the cell survival rate > 85%(Table

Table 2. The Concentration Gradient of Curcumin Inhibition of A431 Cells after rate($\bar{x} \pm s$, n=3)

Group (umol/L)	Inhibition(%)		
	24h	48 h	72 h
5	4.30±3.30	16.66±4.49	27.36±3.18
10	12.30±0.53#	32.11±2.51#	56.73±0.31#●
15	20.62±0.62 [▽]	54.12±2.12 [▽]	61.23±2.48 [△] ●
20	63.33±0.84*	71.79±0.64*■	75.00±1.09 [△] ●
25	61.11±1.27*	69.65±0.98*	67.57±10.98 [△]

*vs 5,10,15umol/L, $p < 0.05$; vs 5,10umol/L, $p < 0.05$; vs 5,10,20,25umol/L, $p < 0.05$; #vs 5,15,20,25umol/L, $p < 0.05$; vs 24h, $p < 0.05$; vs 48h, $p < 0.05$

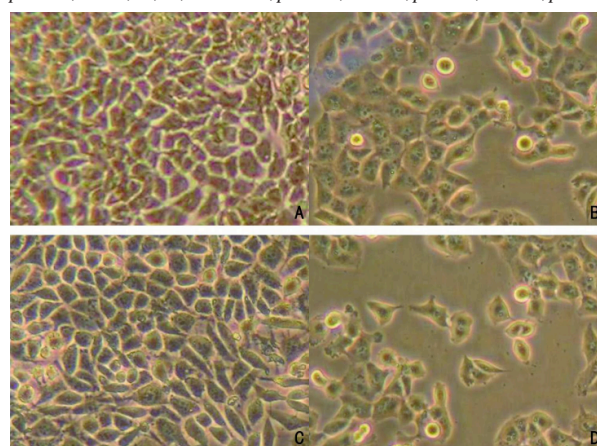


Figure 1. Effect of Curcumin on the Invasion Ability of A431 Cells in vitro (×100). A) negative control group (0.1%DMSO); B) curcumin (5,umol/L) treatment group; C) curcumin (10umol/L) treatment group; D) curcumin (15umol/L) treatment group

2). the choice the concentration of curcumin is less than 15 umol/L and the action time is less than or equal to 24h are selected for the test conditions of invasion and adhesion.

The invasive ability of cells

The numbers of transmembrane cell are (472.33±37.17), (309.33±38.99), (187±17.06) and (126.33± 8.62)/ vision of the negative control group and curcumin (5, 10, 15umol/L) treatment group (Figure 1). Compared with the negative control group, the number of transmembrane cell gradually decreased with the drug concentration of curcumin gradually increased, the treatment by 15 umol/L has the most obvious effect. The differences between each group was statistically significant ($F=85.66$, $p < 0.05$).

Cell adhesion ability

The amount of cell adhesion are (1.357±0.094), (0.878±0.026), (0.482±0.032) and (0.3036±0.030) of the negative control group and curcumin (5, 10, 15umol/L) treatment group. Compared with the negative control group, the ability of cell adhesion gradually decreased with

the drug concentration of curcumin gradually increased, the treatment by 15 $\mu\text{mol/L}$ has the most obvious effect. The differences between each group was statistically significant ($F=381.74$, $p<0.01$).

Expression of STAT3, P-STAT3 protein

The result of Western blot shows that the inhibition of the expression with P-STAT3 gradually enhanced, while the the concentration of curcumin gradually increased. It is in a dose dependent. The treatment by 15 $\mu\text{mol/L}$ has the most obvious effect. The differences between each group was statistically significant ($F=116.07$, $p<0.05$), while there is no significant difference in the level of STAT3 protein (Figure 2 and Figure 3).

STAT3 gene transcription level of mRNA

The results of RT-PCR show that the inhibition of the transcription level of STAT3 gradually enhanced, while the the concentration of curcumin gradually increased. It is in a dose dependent. The transcription level of STAT3 are (0.781+0.135), (0.515+0.174), (0.268+0.062), (0.045+0.003) of the negative control group and curcumin (5, 10, 15 $\mu\text{mol/L}$) treatment group (Figure 4). Compared with the negative control group, the transcription level gradually decreased with the drug concentration of curcumin gradually increased, the treatment by 15 $\mu\text{mol/L}$ has the most obvious effect ($t=9.445$, $p<0.01$). The differences between each group was statistically significant ($F=23.022$, $p<0.05$).

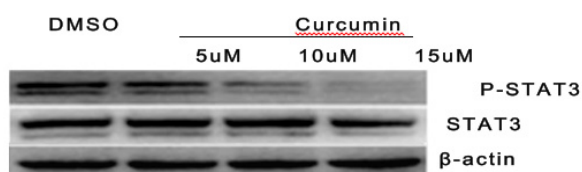


Figure 2. Effect of Curcumin on Expression of STAT3 and P-STAT3 Protein in A431 Cells

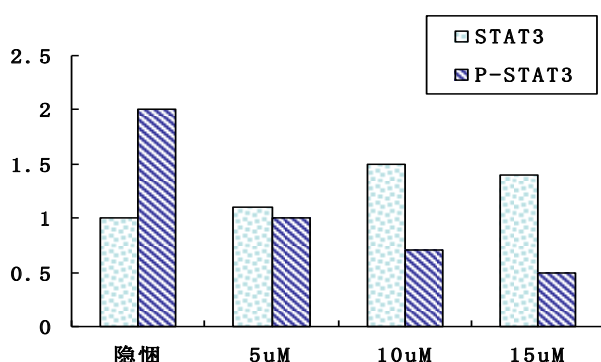


Figure 3. The Relative Expression of STAT3 and P-STAT3 Protein in A431 Cells Western Blot detection after Curcumin Treatment

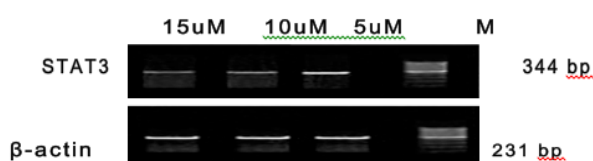


Figure 4. Effect of Curcumin on STAT3 Cells A431 Gene Transcription Level of mRNA

Discussion

In the past 30 years, China's cancer disease incidence rate and the mortality rate has been a growing trend. Cutaneous squamous cell carcinoma is higher incidence rate of malignant skin tumors, which occupies a great proportion in the skin malignant tumor occurrence and ultraviolet radiation, the skin squamous cell carcinoma is closely related with the environment, and the destruction of the ozone layer increased, its incidence has been rising trend, generally 80% to 90%, especially in the elderly. With the addition of a common skin basal cell carcinoma (basal, cell carcinoma, BCC) compared with squamous cell carcinoma of the skin, illness development is rapid, destructive, but lymph node metastasis, visceral metastasis can be advanced, so people pay more and more attention to the prevention and control. The treatment of Western medicine at present no effective and economic means, traditional Chinese medicine because of its extensive pharmacological effects and advantages of the side effects of smaller, in-depth study of traditional Chinese medicine and its components in skin squamous cell carcinoma pathogenesis has important significance in genetic toxicity. In recent years there has been a lot of research shows that many Chinese traditional medicine has strong antitumor effect, and in the induction of apoptosis, promoting differentiation and improve immune function has its unique advantage. Jiang Huang is commonly used in traditional Chinese medicine, and its main bioactive components for the curcuminoids and essential oil. The former has the function of reducing blood lipid, antioxidant, anticoagulation, choleric, anticancer and so on; the latter mainly anti-inflammatory, antibacterial and cough relieving effect. As a kind of anticancer drugs with good development prospect, has become a hot spot of research. Recent studies showed that curcumin, in animal models of skin cancer, liver cancer, colon cancer, lung cancer, ovarian cancer, breast cancer, gastric cancer and other tumor cells have obvious inhibition, and induce apoptosis of tumor cells. Current studies suggest that curcumin, by inducing malignant tumor cell differentiation (Kang et al., 2015), inducing apoptosis of tumor cells (Toden et al., 2015). and exerts its anticancer effect (Fiala et al., 2015). On tumor growth inhibitory effect of each period, a wide range of clinical applications (Zhou et al., 2015).

At present, curcumin has been proven to be a safe and effective inhibitors of STAT3, as in T cell leukemia, curcumin can inhibit the STAT3 upstream of JAK kinase activity, constitutively phosphorylated to prevent STAT3, in H-RS cells, curcumin can inhibit the phosphorylation of JAK2 through to block phosphorylation STAT3, STAT3 is the signal transducers and activators of transcription (STAT) is an important member of the family. STAT3. Focusing multiple oncogenic tyrosine kinase signal channel, in a variety of tumor cells and tissues have had multiple activation, abnormal expression of key genes in STAT3 after the induced activation of the cell proliferation and differentiation, survival, apoptosis, closely related, through various channels to promote cell proliferation, malignant transformation, impede apoptosis expression

of carcinogenesis, so at present is defined as a kind of oncogene (Kim et al., 2013), STAT3 not only highly expressed in many tumor cells (Xu et al., 2014), but also the continuous high expression in tumor micro-environment in a variety of cells, therefore, inhibit tumor cells and their micro-environment of various stromal cell STAT3 is often considered a target for tumor therapy. In order to clarify the curcumin through inhibition of STAT3 signaling pathway and inhibition of STAT3 gene expression on the invasion of skin squamous cell carcinoma A431 cells, Several signaling channel of carcinogenic tyrosine kinase converge into the point of STAT3, such as EGFR, IL-6/JAK, Src, and the over activation occurs in diverse kinds of cancer cells and tissues. After the activation, STAT3 induces this anomalous expression of pivotal gene related to cell activities, then speeds up cell proliferation and vicious transformation, inhibit anti-carcinogenesis function expressed by apoptosis by the various ways, therefore, it is described as a kind of oncogene (Snyder Met al., 2014). Our research shows that compared with normal skin tissues, positive rate of SCC on skin of phosphorylated STAT3 is raised obviously, and expression in SCC are all in nuclear type, which implies that the abnormal activation of STAT3 cascaded ingredient plays a significant role in the occurrence of skin SCC. The staining intensity of *p*-STAT3 in skin SCC of level II and III notably increases, which lead to the speculation that the over activation of STAT3 may be closely relevant to invasive growth of SCC.

In order to clarify the curcumin through inhibition of STAT3 signaling pathway and inhibition of STAT3 gene expression on the invasion of skin squamous cell carcinoma A431 cells, in this study, high invasive cutaneous squamous cell carcinoma cell line A431 as the research object, using different doses of curcumin treatment, we examined the influence of Jiang Huang element to the skin squamous cell carcinoma tumor cells invasion. The study of toxic effect on the cells by the method of MTT to detect different concentrations of curcumin in cells, affect the Transwell chamber invasion assay method for the detection of different concentrations of curcumin after cell invasion ability, ability to detect the change of cell adhesion experiments, results showed curcumin concentration, 15 $\mu\text{mol}/24\text{h}$, the role of time, the growth inhibition of cells in a time and dose dependence, when curcumin concentration less than 15 $\mu\text{mol}/\text{L}$, the reaction time was 24hour, no obvious toxic effect on the cells, the cell survival rate >85%; with the increase of the concentration of curcumin, cell invasion and adhesion ability decreased gradually with concentration of 15 $\mu\text{mol}/\text{L}$, the most obvious effect of curcumin effect.

This study using the Western blot method to detect the effect of different concentration of cells after STAT3 and P-STAT3 protein levels, but also by real-time fluorescent quantitative PCR detection effect of curcumin in different concentration plant cells after STAT3 gene transcription level of mRNA, indicated that curcumin could significantly inhibit the STAT3 gene transcription level of mRNA, the inhibition was dose dependence of it.

The study found that, the activation of STAT3 in

skin squamous cell carcinoma (P-STAT3), promoting tumor cell invasion and metastasis, cyclin D1 STAT3 as a downstream gene expression, reported that cyclin D1 is to influence the process of invasion skin squamous carcinoma cells regulates the activation of STAT3. The research group through inhibition of P-STAT3 expression by curcumin, interference of *p*-STAT3 signal transduction pathway in cutaneous squamous cell carcinoma, attempts to prevents the *p*-STAT3 signal transduction pathway induced by over expression of cyclinD1 resulting in skin squamous cell carcinoma effect generated at the source, which will be anti tumor for clinical drug therapy and gene therapy offers a possible interference, open a new important way.

In summary, curcumin can significantly weaken the skin squamous cell carcinoma A431 cells by inhibiting expression of the activity of the STAT3 signaling pathway and the pathway target gene STAT3 on the invasion and metastasis ability.

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