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Hydrazinocurcumin Induced Autophagy and Affected Cell Proliferation by Downregulating the JAK/STAT3 Signaling Pathway in Skin Squamous Cell Carcinoma

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This study aims to investigate the relevant mechanism by which hydrazinocurcumin (HC) interferes with A431 cell autophagy by inhibiting the STAT3 signaling pathway. Different concentrations of HC are used to treat A431 cells to study the effects of HC on A431 cell proliferation and apoptosis. Real-time fluorescent quantitative polymerase chain reaction (PCR) is used to further explore the relationship of HC with the JAK signaling pathway and autophagy. Double immunofluorescence staining is used to detect the fluorescence localization of LC3 and STAT3 after HC treatment. With increasing HC concentrations, A431 cell viability decreases in a dose-dependent manner, and the apoptosis rate increases significantly. Laser confocal colocalization reveals that the fluorescence of labeled LC3 protein is significantly increased, and the fluorescence of labeled STAT3 is significantly reduced in this study. HC may induce autophagy in A431 cells and affect cell proliferation by downregulating the JAK/STAT3 signaling pathway.

1. Introduction

Cutaneous squamous cell carcinoma (SCC) that develops in the epidermis or adnexal keratinocytes is one of the most common skin nonmelanoma tumors in China.[1] SCC accounts for 80% to 90% of patients with skin cancer, and its incidence is also increasing.[2,3] Due to the high degree of malignancy, the effectiveness of current nonsurgical treatment is limited, and surgery has a higher recurrence rate than nonsurgical treatment. Thus, it is important to investigate the potential molecular mechanisms in the pathogenesis of SCC and develop novel targeted therapies.

Hydrazinocurcumin (HC) is a synthetic derivative of a plant polyphenolic pigment, curcumin. Current research suggests that HC has anticarcinogenic activity by inhibiting signal transducer and activator of transcription 3 (STAT3). Recent studies have shown that abnormalities in the JAK/STAT3 signaling pathway are associated with the occurrence of various tumors, and STAT3 is the key regulator. The oncogenic function of STAT3 has been demonstrated in mouse tumor models, where STAT3 is constitutively activated in many tumors.[4] The abnormal expression of its ligands and abnormal function of downstream factors in the pathway can also induce tumors.

Autophagy is a basic metabolic mechanism for intracellular degradation, requiring the participation of various autophagy-related proteins (ATG), the most important of which are BECN1/Beclin 1/ATG6, LC3, ULK1/Atg1, ATG5, and others.[5] In studies of human breast, ovarian and prostate cancer, 2/3 of these tumors were associated with loss of the Becn 1 allele;[6] LC3 is the only protein found thus far that is located at the inner membrane of autophagic vesicles and is involved in signal transduction and regulation. LC3 has been demonstrated to be a marker for autophagy occurrence.[7]

The expression level of STAT 3 was found to be closely associated with autophagic activity in several studies of tumors. For example, there are reports confirmed that the downregulation of p-STAT 3 activity increased autophagy, and inhibition of tumor development and progression in the study of ovarian cancer[8] and straight colon cancer,[9] but it has not been confirmed in skin SCC. In our previous research work, it was found that HC could

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reduce the ability of cell invasion, adhesion and proliferation by inhibiting JAK-STAT3 signaling pathway in SCC cells; meanwhile, the expression and positive rate of LC3 were decreased in clinical SCC tissues, and autophagy was inhibited.

Based on our previous research and other studies, we hypothesize that HC can reduce the phosphorylation level of STAT3 or inhibit STAT3 activity to increase autophagy and thus inhibit the occurrence and development of tumors. In this study, we aimed to study the effect of HC on autophagy in SCC cells by inhibiting the STAT3 signaling pathway by detecting changes in cell proliferation, apoptosis, and the expression of related factors in HC-treated SCC cell lines (A431).

2. Experimental Section

2.1. Cell Culture

The SCC cell line (A431) used in the study was purchased from Shanghai GenePharma Co., Ltd., and HC was purchased from the China National Institute for the Control of Pharmaceutical and Biological Products. A431 cells were thawed quickly in a 37 °C water bath and transferred to centrifuge tubes. After washing, cells were transferred to tissue culture flasks supplemented with Dulbecco's modified Eagle's medium (DMEM, Corning Corporation, USA) and cultured in a 5% CO_2 incubator at 37 °C. Cell growth was observed daily. An appropriate volume of trypsin (Thermo Company, USA) was added for digestion for 30–40 s. The culture flask was shaken to separate the cells completely. The supernatant was washed, and an appropriate amount of medium was added. The cells were gently removed and placed in a 5% CO_2 humidified incubator at 37 °C for cultivation and preservation.

2.2. MTT Assay

A431 cells were suspended in DMEM, and the cell suspension was diluted appropriately and added to a 96-well plate (5000 cells/well). The final volume of each well was ≈100 μL, and each group had 6 duplicate wells. The culture plate was shaken gently to disperse the cells evenly and placed in a 5% CO_2 incubator at 37 °C overnight. The next day, different final concentrations of the drug were added at 0, 0.01, 0.1, 1, 2.5, 5, 10, 20 and 50 μM, and the drug was added for 24 h. After 24 h, 10 μL MTT (Nanjing KGI Biological Company, China) was added and incubated in a 5% CO_2 incubator at 37 °C for 4 h. The medium from the wells was removed. Then, 150 μL DMSO was added to each well. The plate was shaken at low speed for 10 min on a shaker to completely dissolve the crystals. The absorbance value of each well was measured by an immunosensor (Mr-96a, Tecan Corporation, USA) at OD490 nm, and cell viability was calculated.

2.3. Apoptosis Assay

Cell treatment: A total of 2 × 105 cells/well in the log phase were inoculated into 6-well plates and allowed to adhere overnight, and then the drug was added at final concentrations of 0, 5 and 30 μM and treated for 24 h. The supernatant was collected, and collected cells were digested with trypsin and centrifuged at 1000 rpm for 5 min. The supernatant was removed. Then, 1 mL of PBS was added to resuspend the cells. The cell suspension was transferred to a clean 1.5 mL tube and centrifuged at 1000 rpm for 5 min, and the supernatant was removed. Precooled PBS was added to wash the cells, which were centrifuged at 1000 rpm for 5 min, and the supernatant was removed. Then, 100 μL of loading buffer was added to the cells. Then, 5 μL of FITC (Sigma Company, USA) staining solution and 10 μL of PI (Sigma Company, USA) staining solution were added, and the solution was incubated at room temperature for 20 min in the dark. Then, 400 μL of loading buffer was added to form a cell suspension. Cellular apotosis was measured by flow cytometry (CyFlow Cube 6, Beckman Coulter Company, USA).

2.4. Polymerase Chain Reaction (PCR) Experiment

1) Extraction of RNA from samples. The cells were lysed with TRIzol reagent (Thermo Fisher, USA), and 0.2 mL chloroform was added to every 1 mL TRIzol reagent. Samples were centrifuged at 4 °C and 12 000 g × 15 min. The supernatant was collected, and the same amount of isopropanol was added to the sample. The samples were centrifuged again, and the supernatant was discarded. An equal amount of 75% ethanol (75% ethanol is made of DEPC water) was added, and the sample was centrifuged at 4 °C and 7500 g for 5 min. The supernatant was discarded. Finally, the samples were dried, and the proper amount of DEPC H_2O was added to dissolve the pellet. The obtained RNA solution was stored at −80 °C for later use.
2) Reverse transcription and fluorescence quantitative PCR. The concentration and purity of the RNA solution were determined (ND2000, Millipore Company, USA). Synthesis of cDNA was performed by reverse transcription with an RNA kit (Takara, Japan). The expression of target gene mRNA was analyzed by real-time PCR (TL988-48/36, Applied Biosystem Company, USA). The reaction system was as follows: SYBR Premix Ex Taq II(2×), 10 μL; forward primer (10 μM), 1 μL; reverse primer (10 μM), 1 μL; cDNA template, 2 μL; and dH_2O, up to 20 μL. The primers were designed with Primer Premier 6.0 software, and their specificity was assessed. The primer sequences were commissioned by Shanghai Shenggong for synthesis, as shown in **Table 1**.

2.5. Double Labeling Cellular Immunofluorescence Method

The cells in the log phase were inoculated onto glass slides in a 24-well plate, incubated overnight, and treated with the drug at final concentrations of 0 and 30 μM for 24 h. The medium was discarded, and PBS was used for cell washing. The cells were fixed with 4% paraformaldehyde for 10–20 min, and washed 3 times with PBS for 10 min each time. Then, 0.2% Triton X-100 (prepared in PBS) was added and incubated for 30 min. Normal goat serum was added at room temperature for 30 min and then removed. Fluorescent antibodies (Rabbit, Cell Signaling Technology, Inc.; USA) were added and incubated at room temperature for 1–2 h (diluted with PBS). The cells were washed with PBS

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Table 1. Primer sequences used in this study.

Gene names	Primer sequences (5′→3′)
GAPDH	Forward: GGTGAAGGTCGGTGTGAACG Reverse: CTCGCTCCTGGAAGATGGTG
STAT3	Forward: CAGTGACCAGGCAGAAGA Reverse: ACTCCATCGCTGACAAAA
JAK2	Forward: TCAGCAATTCAGCCAATG Reverse: AAGGACCACTTCCAGGTT
LC3	Forward: CCTCAGACCGGCCTTTCA Reverse: GCTGCTTCTCACCCTTGTAG
Beclin-1	Forward: CGTGGAATGGAATGAGAT Reverse: CGTAAGGAACAAGTCGGTAT
Bax	Forward: TTTTGCTTCAGGGTTTCATC Reverse: GACACTCGCTCAGCTTCTTG
Bcl-2	Forward: GGAGGATTGTGGCCTTCTTT Reverse: TGTGCAGGTGCCGGTTCAG
CDK4	Forward: TGGAGTGTTGGCTGTATCTT Reverse: AGCCCAATCAGGTCAAAG
CDK6	Forward: ACCAGCAGCGGACAAATA Reverse: GCTCTGTACCACAGCGTGA

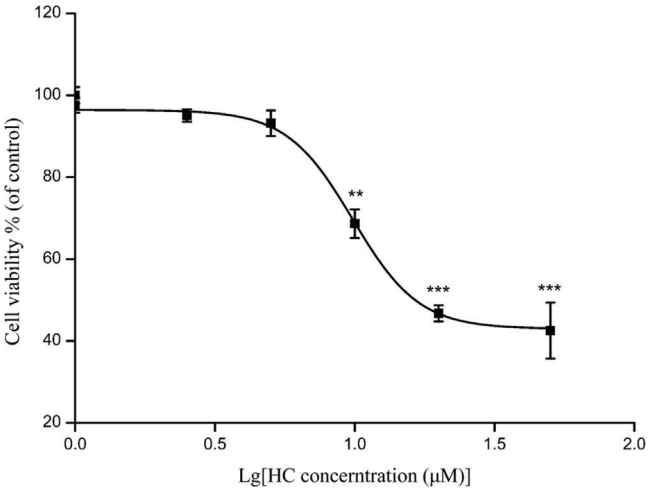


Figure 1. The effect of hydrazinocurcumin on cell proliferation as assessed by MTT assay. A431 cell viability was not significantly decreased upon exposure to HC concentrations of 0.01, 0.1, 1, 2.5 and 5 μM but was significantly decreased when HC concentrations were increased to 10, 20 and 50 μM. $^{**}p < 0.01$ and $^{***}p < 0.001$.

thrice for 10 min each time. Fluorescent antibodies (mouse; Cell Signaling Technology, Inc.; USA) were added and incubated at room temperature for 1–2 h (diluted with PBS). The cells were washed with PBS thrice for 10 min each time. The FITC (rabbit)- and TRITC (mouse)-labeled secondary antibodies (the two were mixed) were diluted at 1:500 with PBS and incubated at room temperature for 1 h. The cells were washed with PBS thrice for 10 min each time. The slides with (DAPI)-containing mounting medium were mounted, and pictures were obtained (Leica Camera AG, USA) as soon as possible after drying.

2.6. Statistical Analysis

SPSS 24 software was used for statistical analysis. A paired sample *t* test was used for two paired samples. One-way ANOVA was used for multigroup comparisons. The LSD test was used to assess for homogeneity of variance between groups. The Dunnett T3 test was used to assess for heterogeneity of variance. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of HC on A431 Cell Proliferation

The viability of A431 cells treated with HC was detected using the MTT assay to evaluate the cell proliferation ability. The results showed that HC inhibited A431 cell proliferation ($p < 0.05$). A dose-dependent change was noted in which cell viability decreased as the HC concentration increased, as shown in **Figure 1**.

3.2. Effects of HC on A431 Cell Apoptosis

Flow cytometry was used to detect the apoptosis rate. Compared with the control group, the results showed that the apoptosis rates of A431 cells treated with 5 and 30 μM HC significantly increased. The apoptosis rate was positively associated with the concentration of HC, indicating that HC can promote cell apoptosis. The effect was dose dependent, as shown in **Figure 2**.

3.3. JAK Signaling Pathway and Autophagy-Related Gene Expression after HC Treatment

To further investigate the effect of HC on the JAK-STAT3 signaling pathway and autophagy-related gene expression, A431 cells were treated with DMEM containing 30 μM HC. Real-time fluorescence quantitative PCR showed that: a) apoptosis-related Bax mRNA expression was increased ($p < 0.01$), and the Bcl-2/Bax ratio was downregulated; b) JAK pathway-related JAK2 mRNA and STAT3 mRNA were decreased ($p < 0.05$); and c) autophagy-related LC3 mRNA was significantly increased ($p < 0.001$), and Beclin-1 mRNA expression was enhanced ($p < 0.05$). No significant changes were found in cell cycle-related CDK6 mRNA and CDK4 mRNA. The results suggested that HC inhibits the expression of the JAK/STAT3 pathway, promoting the transcription and expression of both autophagy-related and apoptosis-related genes (**Figure 3**).

3.4. Localization of LC3 and STAT3 Detected by Double Immunofluorescence Staining after HC Treatment

To further investigate the effect of HC on A431 cells and the interaction of JAK-STAT3 signaling and autophagy, STAT3 protein was labeled with red fluorescence, and LC3 protein was labeled with green fluorescence. The direct effect of HC on the JAK pathway-related protein STAT3 and the autophagy-related protein LC3 was shown by the colocalization of STAT3 and LC3 through confocal microscopy. Compared with the control, the green fluorescence intensity of A431 cells in the HC treatment group was signifi-

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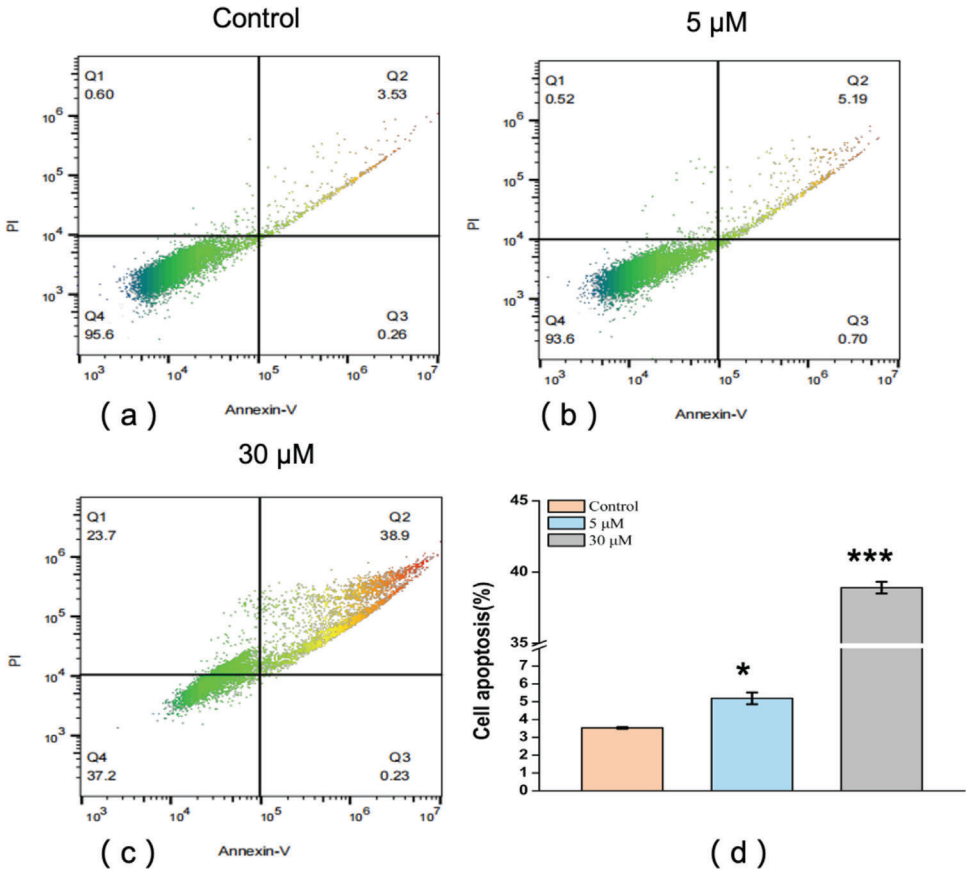


Figure 2. Effects of hydrazinocurcumin treatment on apoptosis. a) Results of apoptosis detection in the control group; b) results of apoptosis detection in the HC (5 μM) group; c) results of apoptosis detection in the HC (30 μM) group; and d) histogram of apoptosis results of the three groups. The apoptosis rate of A431 cells in the HC (5 μM) and HC (30 μM) groups was significantly greater than that in control group. $*p < 0.05$ and $***p < 0.001$.

cantly increased, whereas the red fluorescence intensity was significantly reduced. This finding indicates that HC inhibits the expression of the JAK/STAT3 pathway, promoting autophagy, as shown in **Figure 4**.

4. Discussion

Currently, autophagy has become one of the hot spots in tumor research. Autophagy plays a role in tumor occurrence and development.[10] Research has found that curcumin exhibits various pharmacological actions with low toxicity, including inhibiting the growth of tumor cells through different mechanisms; it has great potential in the treatment and prevention of tumors.[11] Previous studies have shown that curcumin blocks the DAMP-TLR4 signaling pathway and inhibit melanoma cell proliferation and metastasis.[12] Curcumin also induces apoptosis and reduce the survival rate and proliferation of gastric cancer cells.[13] These findings suggest that HC may be effective in SCC cells by inhibiting cell proliferation and inducing autophagy. The results of our study showed that HC significantly inhibits A431 in a dose-dependent manner.

Current studies found that overactivation of STAT3: a) directly promotes tumor growth and b) regulates the antitumor immune response between the tumor stroma and immune system to indirectly promote tumor growth.[14] In early malignant transformation and cancer progression, STAT3 is involved in selectively inducing and maintaining the carcinogenic inflammatory microenvironment.[15] STAT3 overactivation in tumor cells and other related immune cells plays a role in the negative regulation of neutrophils, natural killer cells, effector T cells and dendritic cells (DCs), which may downregulate antitumor immunity.[16] Overexpression of STAT3 happened in tumor tisses can increase immune escape or establish immune tolerance through several mechanisms, and the inflammatory microenvironment further promotes tumor angiogenesis and tumor cell growth, invasion and metastasis.[17]

STAT3 is an oncogene, and its constitutive activation is found in tissues and cells of many malignant tumors. STAT3 is mainly located in the cytoplasm. However, after JAK is activated, the SH domains of STAT3 bind to phosphotyrosines, and p-STAT3 is formed and translocated into the nucleus. Active STAT3 induces and regulates the high expression of genes related to cell proliferation, differentiation, and apoptosis, showing carcinogenesis. For example, high STAT3 expression was found in skin angiosarcoma and Kaposi's sarcoma.[18,19]

Many studies have shown that the JAK2/STAT3 pathway is highly activated in various cancers and inhibits the antitumor immune response.[20] In liver cancer, the tumorigenic effect of

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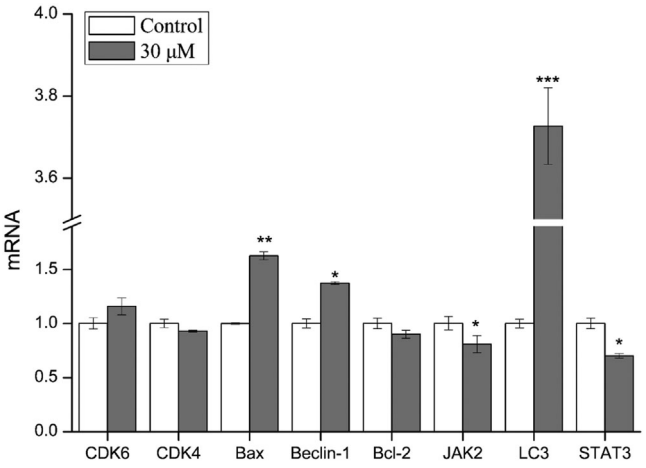


Figure 3. Related gene expressions after hydrazinocurcumin treatment. Compared with the control group, the transcription levels of the Bax, LC3 and Beclin-1 genes in A431 cells of the HC (30 μM) group were significantly increased, JAK2 and STAT3 were significantly decreased, and CDK6 and CDK4 were not significantly changed. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

STAT3 is mediated by continuous stimulation of IL-6, which leads to the downstream phosphorylation of JAK2 and further phosphorylation of STAT3. Phosphorylated STAT3 may homodimerize or heterodimerize, resulting in nuclear translocation and DNA binding. It also regulates the transcription of many downstream genes, including cytokines involved in autocrine signaling; thus, a negative feedback loop that promotes tumorigenesis, cell proliferation and anti-apoptosis is established.[21]

In cancer treatment, the JAK2/STAT3 signaling pathway is of interest given its potential effect in inhibiting tumor growth and restoring antitumor immunity. Recently, numerous studies have shown that drug therapy targeting the JAK2/STAT3 pathway is effective for many cancers.[22] With the development of a variety of treatments, cancer patients have started choosing natural products, such as ginseng, instead of chemotherapy.[23] Songhee et al.[24] discovered the roles of ginsenosides 20(S)-Rh2 and 20(R)-Rh2 in colorectal cancer cell invasion and signaling cascades. Compared with 20(R)-Rh2, 20(S)-Rh2 is more effective in cancer cell invasion and activation of STAT3 and its signaling cascade. In addition, 20(S)-Rh2 is a STAT3 inhibitor that inhibits the phosphorylation of tyrosine residues but not serine residues, thereby inhibiting cancer cells. A study showed that iron depletion therapy can be effective for inhibiting cancer development.[25] For example, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT) can be used to treat certain cancers by inhibiting IL-6 expression and secretion through iron chelation and the generation of cytotoxic free radicals. Dp44mT Can treat cancer through various ways, such as Dp44mT suppresses the proliferation of pancreatic cancer, breast cancer and prostate cancer cells by activating 5'AMP signaling; Dp44mT can inhibit cell proliferation and induce apoptosis of lung cancer and melanoma by activating mitogen-activated protein kinase signaling pathway; Dp44mT also suppresses IL-6/JAK2/STAT 3 pathway by stimulating transcription of NDRG 2, promote cell apoptosis, and promote apoptosis, thus inhibiting the proliferation of glioma cells.[26] Dp44mT also inhibits cell proliferation and induce apoptosis in lung cancer and melanoma by activating mitogen-activated protein kinase signaling.[27] In glioma cells, Dp44mT inhibits the IL-6/JAK2/STAT3 pathway by stimulating the transcription of NDRG2, thereby inhibiting the proliferation of glioma cells and promoting apoptosis.[27,28] Pantoprazole inhibits the activation of the JAK2/STAT3 pathway. Thus, the use of high concentrations of pantoprazole to regulate the JAK2/STAT3 pathway can affect muscle atrophy caused by cancer cachexia.[29] Targeted therapy is the cornerstone of precision medicine. STAT3 activation is strongly associated with drug resistance, so inhibiting STAT3 can restore tumor sensitivity to targeted drugs. lncRNAs promote drug resistance and the development and metastasis of tumors; they also exhibit high tissue-specific expression

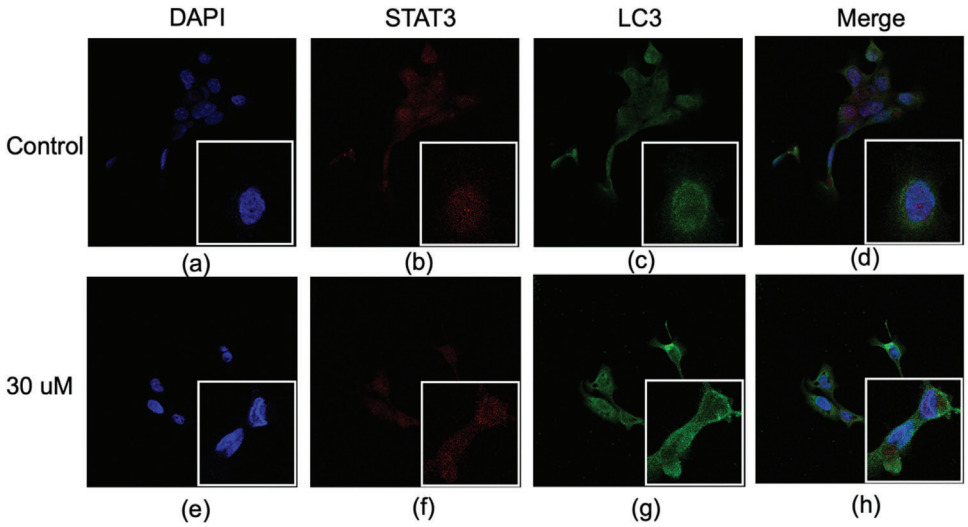


Figure 4. Immunofluorescence localization of LC3 and STAT3 proteins after hydrazinocurcumin treatment. Negative control cells are labeled with DAPI with blue fluorescence a), STAT3 with red fluorescence b), LC3 with green fluorescence c) and channels Merge d). HC (30 μM) treated cells are are labeled with DAPI with blue fluorescence e), STAT3 with red fluorescence f), LC3 with green fluorescence g) and channels Merge h).

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Figure 5. A schematic figure of hydrazinocurcumin in skin squamous cell carcinoma.

in tumors. For example, lncRNAs H19 and PCA3 are biomarkers for gastric and prostate cancer, respectively, and HOTAIR overexpression in breast cancer is associated with metastatic potential and poor prognosis.[30] Guan et al.[31] found that the expression of lncRNA HOTAIR was significantly increased in primary multiple myeloma cell lines. The results of our study showed that the inhibitory mechanisms of HC on A431 cells include promoting apoptosis, inhibiting the JAK/STAT3 pathway, and inducing autophagy. HC affects A431 cell proliferation potentially by downregulating the JAK/STAT3 pathway and inducing autophagy.

In conclusion, HC inhibits the JAK/STAT3 signaling pathway and induces A431 cell autophagy to inhibit cell proliferation, suggesting that HC is a potential candidate for SCC treatment. With the increasing understanding of the antitumor activity and antitumor mechanism of HC, HC is very likely to become an anticancer drug. Future research can use HC to induce tumor cells to further confirm its effects on the occurrence and development of tumors (**Figure 5**).

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Q.M. and J.X. performed the experiments and wrote the manuscript; Z.S., H.L., L.J., Y.W., and Y.L. performed the experiment; J.Z. and X.X. analyzed the data; Q.M. and J.X. revised the manuscript; Z.S. and J.X. conceived the topic and revised the manuscript. All authors approved the submitted version.

Data Availability Statement

Research data are not shared.

Keywords

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