Radix Tetrastigma Hemsleyani Flavone Suppresses Cutaneous Squamous Cell Carcinoma A431 Cells via Proteasome Inhibition

Background:
Radix Tetrastigma Hemsleyani Flavone (RTHF) has detoxification and anti-inflammatory activity and is widely used. Here, we report that RTHF inhibits cell proliferation and induces apoptosis in cutaneous squamous cell carcinoma A431 cells and is a potential strategy for cancer therapy.

Material/Methods:
A431 cells were cultured in different concentrations of RTHF. The inhibition of cell proliferation was assessed by MTT assay, cell apoptosis was shown through FCM, and cell invasion was assessed by Transwell methods. Enzyme proteasome assay was used to detect the activity of proteasome and DUB. Expression of apoptosis-related and ubiquitin proteasome pathway-associated proteins were assessed by PCR and Western blot.

Results:
RTHF obviously suppressed the proliferation and induced apoptosis of A431 cells in a dose-dependent manner. Transwell assay showed that RTHF inhibited the cell metastasis significantly. Enzyme proteasome assay show that the RTHF treatment of activity of proteasome and DUB was significantly lower than in control. RTHF increased the expression of Bax and inhibited Bcl-2, pro-caspase3, and pro-caspase9 activity. The expression of USP14, UCHL5, and POH1 decreased and ub-prs increased significantly in the treatment group.

Conclusions:
Our study reveals that RTHF-mediated inhibition of DUBs and proteasome may provide a potential strategy for cancer therapy.

MeSH Keywords:
Apoptosis • Carcinoma, Squamous Cell • Proteasome Inhibitors

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Background
Cutaneous squamous cell carcinoma (cSCC) is the second most common cause of carcinoma in non-melanoma skin [1]. Nowadays, its incidence is continuously increasing due to the aging population and increased ultraviolet exposure [2]. Despite many advances in cancer treatment, there is not always a lasting response, and many drugs have serious adverse effects that limit sustained clinical benefit, so there is a need to assess new therapeutic methods and strategies.

Radix Tetrastigma Hemsleyani Flavone (RTHF) is derived from the root of the Chinese herb Radix Tetrastigma Hemsleyani (RTH), which has been shown to possess detoxification, anti-inflammation, and anti-cancer activities. In vitro, RTHF causes anti-proliferation and apoptosis effects in multiple cancer cell models [3–6]. In vivo, it can inhibit tumor growth in mouse models [7]. However, the mechanism of RTHF is largely unknown. Here, we found that RTHF inhibits proliferation and induces apoptosis of cutaneous squamous cell carcinoma A431 cells. The possible anti-cancer mechanism is proteasome activation.

Material and Methods
Reagents
Radix Tetrastigma Hemsleyani Flavone (RTHF) was synthesized in the Key Laboratory Research and Development of New Drugs of Traditional Chinese Medicine of Zhejiang Province. Our experiments used the following: FBS and RPMI 1640 (HyClone), MTT (Beyotime), Annexin V-FITC Apoptosis Detection Kit (Keygen), Transwell chamber (Corning, USA), Matrigel (BD Biosciences USA), Proteasome-Glo Chymotrypsin-like Cell-Based Assay kit, DUB-Glo Protease Assay kit (Promega, USA), human 19S proteasome (Boston Biochem), and the antibodies to Bax, Bcl-2, Caspase-3/9, ub-prs, USP14, UCHL5, and POH1 (CST).

Cell line
A431 cells were obtained from Zhejiang Province Academy of Medical Science. Cells were maintained in RPMI-1640 at 37°C with 5% CO₂.

Cell viability assay
MTT assay was used to determine A431 cell viability in media containing RTHF for 48 h. We exposed cells to 20 ml MTT for 4 h before culture termination. Finally, the absorbance was read at 570 nm, with cell viability of control as 100%.

Apoptosis assay
A431 cells were cultured in different concentrations of RTHF. Treated and control cells were harvested and resuspended in binding buffer followed by Annexin V-FITC, and PI was analyzed by flow cytometry.

Cell invasion assay
A431 cell invasion assay was determined by Transwell experiments, in which 2×10⁴ cells/well in the upper chamber were added to Matrigel in serum-free medium and cultured with various concentrations of RTHF. FBS was used as a chemottractant in the lower chamber medium. After 12 h, we removed the cells in the upper chamber and the invasive cells were stained with crystal violet.

Proteasome activity assay
The treated A431 cells were detected with a microplate reader (3001, Thermo, USA). The proteasome activity was determined according to the instructions of the Proteasome-Glo Chymotrypsin-like Cell-Based Assay kit.

DUB activity assay
Treated A431 cells were placed in ice-cold DUB buffer, centrifuged at 20 000 g for 10 min, and recorded with a microplate reader (3001, Thermo, USA). The DUB activity was detected according to the instructions of the DUB-Glo Protease Assay kit.

Real-time PCR assay
To determine mRNA expression, total RNA extracted from each sample was used for reverse transcript reaction using the QuantiTect Reverse Transcription kit (Qiagen). The SYBR Green PCR Kit was used for real-time PCR reaction. Gene transcript was normalized using the Ct method, with the control as 1.

Western blot assay
Whole-cell lysates were prepared in RIPA buffer. Protein lysates from cultured cells were fractionated by SDS-PAGE and electrically transferred onto a PVDF membrane. After transfer, primary antibodies and secondary horseradish peroxidase-conjugated antibodies were used to detect the designated proteins. The target proteins were reacted to the ECL detection reagents.

Statistics
All values are presented as the mean ±SD. Statistical differences were determined by t test or one-way ANOVA, with P<0.05 considered significant.
Results

RTHF inhibits the proliferation of A431 cells

The growth inhibition effects of RTHF on A431 cells were detected with MTT assay. As shown in Figure 1, RTHF dramatically inhibited A431 cell growth in a dose-dependent manner (P<0.05). The proliferative inhibition effect of the control group was 0%.

RTHF induced apoptosis of A431 cells

A431 cells were incubated with RTHF for 48 h and cell apoptosis was determined by flow cytometry. As shown in Figure 2, cell apoptosis increased significantly (p<0.05).

RTHF influenced apoptosis-related proteins

Apoptosis-related proteins expression of Bax, Bcl-2, pro-caspase3, and pro-caspase9 were analyzed by Western blotting. Bax was significantly upregulated and Bcl-2 and pro-caspase3/9 were downregulated after treatment with RTHF (Figure 3).

RTHF inhibits invasion of A431 cells

Cell invasion was analyzed by the Transwell culture system. As shown in Figure 4, after culturing with RTHF for 12 h, there was a significant decrease in the cell invasion ability of squamous cell carcinoma A431 cells (P<0.05).

RTHF inhibited the proteasome activity of A431 cells

We detected the effects of RTHF on proteasome CT-like peptidase activity in A431 cells. After incubation for 48 h, RTHF dramatically inhibited the CT-like activity (p<0.05) (Figure 5).
RTHF inhibited the DUB activity of A431 cells

The activities of DUB from cell lysates were detected. As shown in Figure 6, RTHF caused a dose-dependent decrease of DUB activity in A431 cancer cells (p<0.05).

RTHF influenced the ubiquitin proteasome pathway-associated proteins expression, as shown by PCR

We investigated the effect of RTHF on ubiquitin proteasome pathway proteins in A431 cells by real-time PCR assay. As shown in Figure 7, the level of ub-prs increased and levels of USP14, UCHL5, and POH1 decreased after RTHF treatment.

RTHF influenced ubiquitin proteasome pathway-associated proteins expression, as shown by Western blot

We investigated RTHF on ubiquitin proteasome pathway proteins in A431 cells by Western blot assay. As shown in Figure 8, RTHF increased the level of ub-prs but decreased levels of USP14, UCHL5, and POH1 in a concentration-dependent manner. In addition, we investigated the effect of RTHF in 19S RP by Western blot. As shown in Figure 9, expression of USP14, UCHL5, and POH1 were significantly decreased in a dose-dependent manner compared to control.
Discussion

Recently, several herbal medicines have been investigated for their potential as major sources of pharmaceutical agents with various activities [8–10]. Flavonoids are effective anti-cancer agents without toxicity to normal cells and tissues, making them ideal in cancer therapy [11]. Radix Tetrastigma Hemsleyani Flavone (RTHF) has a surprisingly wide range of beneficial properties, including anti-inflammation, anti-cancer, and improving immune response activities. In this study, we showed that RTHF significantly inhibited the growth and induced cell apoptosis of A431 cells.

The ubiquitin proteasome pathway (UPS) is the major pathway that controls the expression of proteins in cell cycle, proliferation, and apoptosis [12]. It plays an important part in the regulation of numerous cellular functions [13,14]. In numerous cancer types, UPS over-expression is often associated with chemo-resistance and poor prognosis [15–18]. In fact, the proteasome has chymotrypsin-like, trypsin-like, and peptidyl-glutamyl peptide-hydrolyzing (PHGH)-like activity. In this study, we showed the ability of RTHF to significantly decrease the chymotrypsin-like activity. The addition of ubiquitin molecules to targeted proteins can be reversed, called deubiquitination, and is performed by proteases termed deubiquitinases (DUBs) [19]. Recent findings indicate several DUBs are considered to be oncogenes [20–22]. Several inhibitors of DUB activity have been reported to increase the expression of poly-Ub proteins and enhance cancer cell apoptosis, indicating that DUB inhibitors are potential anti-cancer agents [23,24]. In this report, we showed that RTHF significantly inhibits the activity of DUB compared to control.

Figure 8. (A) Effect of RTHF on the activities of ub-prs, USP14, UCHL5, and POH1 in A431 cells. (B) Proteins were subsequently quantified by densitometric analysis with control being one-fold.

Figure 9. (A) Effect of RTHF on the activities of USP14, UCHL5, and POH1 in 19S RP. (B) Proteins were subsequently quantified by densitometric analysis with that of control being one-fold.
The 26S proteasome is comprised of a 20S core catalytic component (20S proteasome) capped at one or both ends by a 19S regulatory component. Recent research has revealed the functions of 19S RP include binding ubiquitinated proteins, recycling ubiquitin, and unfolding proteins, and is considered a novel anti-cancer drug target [25,26]. In humans, 19S RP is associated with 3 deubiquitinases (DUBs), USP14/Ubp6, and UCHL5/Uch37, which belong to the ubiquitin-specific proteases (USP) and ubiquitin C-terminal hydrolases (UCH) families, respectively. POH1 is a Zn21-dependent protease of the JAMM family [27,28]. USP14 has been shown to be related to cancer progression. Several studies have shown that the expression USP14 is negatively correlated with patient survival in some carcinomas, such as colorectal, liver, and lung cancer [29]. Recent observations show that USP14 may be associated with metastasis. A recent study reported that UCHL5 can promote cell growth, in part by altering the expression of apoptotic mediators. UCHL5 is a subunit of 19S RP and has a role by suppressing or promoting the degradation of specific proteasome substrates in eukaryotic cells [30,31]. Knockdown of UCHL5 results in alterations in the mitochondrial apoptosis pathway and reduced cell survival in non-small cell lung carcinoma [32]. POH1 is essential for viability, and POH1 knockdown inhibits proteasome activity and reduces cell survival [33]. In addition, POH1 over-expression promotes resistance to several anti-cancer agents currently in clinical use. Indeed, it has been reported that knockdown of both UCHL5 and USP14 but (not either one alone) leads to defects in UPS and impairs proteasome activity [30]. RTHF significantly decreases USP14, UCHL5, and POH1 expression in cutaneous squamous cell carcinoma A431 cells.

Conclusions

We found that RTHF significantly mediates inhibition of growth and induction of apoptosis in A431 cells, and showed that inhibition of DUBs might be one of the major mechanisms by which RTHF inhibits tumor cells.

Conflict of Interests

None.

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