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Ibrutinib presents antitumor activity in skin cancer and induces autophagy

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Abstract. – OBJECTIVE: Skin cancer is one of the most common malignancies in dermatology. Patient compliance and prognosis of skin cancer are poor. Ibrutinib, a Bruton's Tyrosine Kinase (BTK) inhibitor, is a new anticancer drug used to treat many cancers. Therefore, we aimed to explore the role of ibrutinib in the treatment of skin cancer.

MATERIALS AND METHODS: Cell Counting Kit-8 (CCK8) and plate cloning assay were used to detect cell proliferation. Apoptosis was determined by flow cytometry. Western blotting analysis was used to analyze the expression of key proteins that regulated autophagy. Proliferation and apoptosis of skin cancer cells and induction of autophagy induced by ibrutinib were evaluated.

RESULTS: CCK8 plate cloning assays showed that ibrutinib can gradually inhibit the skin cancer cell proliferation as the treatment time and dose increased. Results of flow cytometry showed that apoptosis in skin cancer cells were induced after ibrutinib treatment. Western blot showed that autophagy in skin cancer cells was found induced by ibrutinib and also related to the time and concentration of ibrutinib treatment. Combination treatment of ibrutinib and 3MA for skin cancer cells can significantly increase apoptosis.

CONCLUSIONS: Ibrutinib has anti-tumor activity in skin cancer and can induce autophagy. Binding to autophagy inhibitors can promote ibrutinib's anti-skin cancer activity. Our experimental results provided new ideas for developing skin cancer drugs.

Key Words:
Ibrutinib, Skin cancer, Autophagy, Proliferation, Apoptosis.

Introduction

Skin cancer is one of the most common malignancies in dermatology. With the rapid development of industrialization and changes of people's living environment, the incidence of skin cancer has been increasing year by year, which has gradually developed into a worldwide public health problem[1]. Skin cancer can be divided into three main types: basal cell carcinoma, squamous cell carcinoma and malignant melanoma[2]. Early specificity of diagnosis and treatment is still lackin; the current common treatment methods are surgery, radiation/chemotherapy, laser and cryotherapy[3]. However, these methods are symptomatic treatments, and patient compliance and prognosis are poor. Therefore, further study of its pathogenesis and looking for new and specific target of treatment has become an important issue in clinical research.

Ibrutinib, an oral inhibitor of BTK, exerts anti-cancer effects by inhibiting BTK required for replication and metastasis of tumor cells. Ibrutinib has been used as an anticancer drug for various cancers such as human lymphoma[4,5], ovary cancer[6], breast cancer[7], lung cancer[8], gastric cancer[9], and glioma[10]. Ibrutinib is a new drug for patients with skin cancer.

Autophagy is a process of entrapment of organelles, cytoplasm, and protein polymers by autophagosomes and subsequent catabolism with lysosomes[11]. Autophagy has been seen as a way of cell death induced by death program. Autophagy is an important adaptive response to extrinsic stimuli, such as hypoxia, nutrient deprivation, pathogen infection and other conditions. It provides not only energy circulation through degradation form amino acids, nucleotides and other substances, but also removes cytoplasmic damaged organelles and metabolites as a defense mechanism to protect cells[12]. According to the transport of cellular material to the lysosome *in vivo*, autophagy can be divided into macro-autophagy, micro-autophagy and chaperone-mediated autophagy. Macro-autophagy: the endoplasmic reticulum-derived monolayer was depressed to form a cup-shaped bilayer membrane-like separation membrane and completely surrounded the material to be degraded (e.g., organelles) to form autophagosomes, which were fused with lysosomes;

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finally cellular material was dissolved by lysosomal enzymes in autophagosomes. Micro-autophagy: lysosomal membranes directly wrapped long-lived proteins and were degraded in lysosomes. Another type of autophagy is Chaperone-mediated autophagy (CMA). In CMA, the intracytoplasmic protein was transported into the lysosome cavity for digestion after binding to the chaperone. The substrate of CMA was a soluble protein selective for protein clearance. However, macro-autophagy ad micro-autophagy had no selectivity[13]. Few research focused on the effect of autophagy in skin cancer. This study firstly investigated ibrutinib-mediated autophagy in skin cancer therapy.

Materials and Methods

Cell Culture

The skin cancer cell lines HS-4 and A431 were cultured in a Roswell Park Memorial Institute 1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS) and penicillin/streptomycin and were maintained in a constant temperature incubator at 37°C and 5% CO_2. Cells were digested with 0.25% trypsin digestion solution containing 0.02% Ethylene Diamine Tetraacetic Acid (EDTA) and passaged for 4 and 5 generations to select cells with good growth and robust proliferation for further study.

Cell Proliferation Assay by CCK8 Method

The skin cancer cell lines HS-4 and A431 were seeded in a 96-well plate at a density of 2×10^3/100 μL. After treatment with different concentrations of ibrutinib, the cells were cultured for 72 hours and then tested by cell counting kit-8 (CCK8). The serum-free medium was replaced when detected. 10 μL of CCK8 were added into each well; after they were incubated at 37°C and 5% CO_2 for 1 h, the OD value was measured at 450 nm. Each measurement was performed in quintuplicate.

Cloning Formation Assay

The treated skin cancer cell lines HS-4, A431, were inoculated in the medium plate at a density of 1×10^4 and maintained in an incubator at 37°C, 5% CO_2, the medium was changed every 2 d and the culture was terminated after 14 d. Medium was removed and cells were washed with the phosphate-buffered saline (PBS) twice, fixed with 5% paraformaldehyde for 30 min. The remaining liquid was removed, 1 mL of 0.1% crystal violet solution per well was added, the crystal violet solution was removed 30 min later, cells were washed until the solution was clear with PBS, the visible colonies was counted.

Flow Cytometry

The supernatant was aspirated to a centrifuge tube, the treated skin cancer cell lines HS-4, A431 were trypsinized and transferred to the appropriate centrifuge tube and centrifuged. The supernatant was removed, washed twice with cold PBS, Annexin V binding solution was used for resuspended, 5 μL of Annexin V-fluorescein isothiocyanate (FITC) and 5 μL of propidium iodide (PI) staining solution were added, protected from light for 15 min, then diluted with 400 μL 1 × binding buffer. Measurement was performed on the specific machine for software analysis, flow cytometry was repeated in triplicate.

Western Blotting

The skin cancer cell lines HS-4 and A431 were inoculated into a 6-well plate at a density of 2 × 103/100 μL, after adhered different treatment was given. After culturing for 72 h, the total protein was extracted and the protein concentration was determined. 40 μg protein were used for gel electrophoresis, using a wet film transfer instrument to transfer the gel protein to polyvinylidene difluoride (PVDF) membrane, blocked with 5% of the skim milk. 1 h later, primary antibodies were used for incubation overnight at 4°C; then, secondary antibodies were used for incubation for 1 h, electrochemiluminescence (ECL) developer was used for visualization.

Statistical Analysis

Statistic package for social science (SPSS) 19.0 statistical software (IBM, Armonk, NY, USA) was utilized for data analysis, flow cytometry results were analyzed using Windows Multiple Document Interface for Flow Cytometry software (WinMDI, Scripps Research Institute, La Jolla, CA, USA). Measurement data were compared with *t*-test and presented as mean ± standard deviation ($\bar{x}\pm s$), categorical data were analyzed by x^2 test. $p<0.05$ indicated significant difference; $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

Results

Ibrutinib Inhibits the Proliferation of Skin Cancer Cells

As to determine the impact of ibrutinib on the viability of skin cancer cells, CCK8 and pla-

te cloning assays were performed in this study. Ibrutinib gradually decreased the viability of skin cancer cells with the increased dose after 72 h of treatment with different concentrations of ibrutinib (Figure 1A). In addition, as ibrutinib treatment time increased, cell viability also decreased. Similarly, plate-cloning assay showed that after treatment with different concentrations of ibrutinib for skin cancer cells, the number of observed colonies was significantly reduced with increasing concentration of ibrutinib (Figure 1B).

Ibrutinib Induces Apoptosis in Skin Cancer Cells

Next, the effect of ibrutinib on the apoptosis of skin cancer cells was focused on, we utilized flow cytometry to determine the apoptosis. Figure 1C showed that ibrutinib gradually induced apoptosis as the treating dose increased. 3MA is an inhibitor of autophagy, and significantly increased apoptosis of skin cancer cells was observed after cells were treated with ibrutinib and 3MA (Figure 3C). These experimental results showed that ibrutinib combined with autophagy inhibitors can increase the apoptosis of skin cancer cells.

Ibrutinib Induces Autophagy in Skin Cancer Cells

Western blot results showed that expressions of LC3A/B-I, LC3A/B-II, and Atg7 significantly increased at a time- and concentration- dependent manner after ibrutinib treatment in the skin cancer cell lines HS-4 and A431 (Figure 2A and B). We found that expressions of Atg7, LC3A/B-II were increased after treatment with ibrutinib and were downregulated after 3MA treatment (Figures 3A and 3B), and 3MA inhibited ibrutinib-induced autophagy. To further investigate the effect of ibrutinib and autophagy on skin cancer cells, ibrutinib combined with 3MA were used for treating skin cancer cell line HS-4. The plate clone assay showed (Figure 3D) that autophagy was

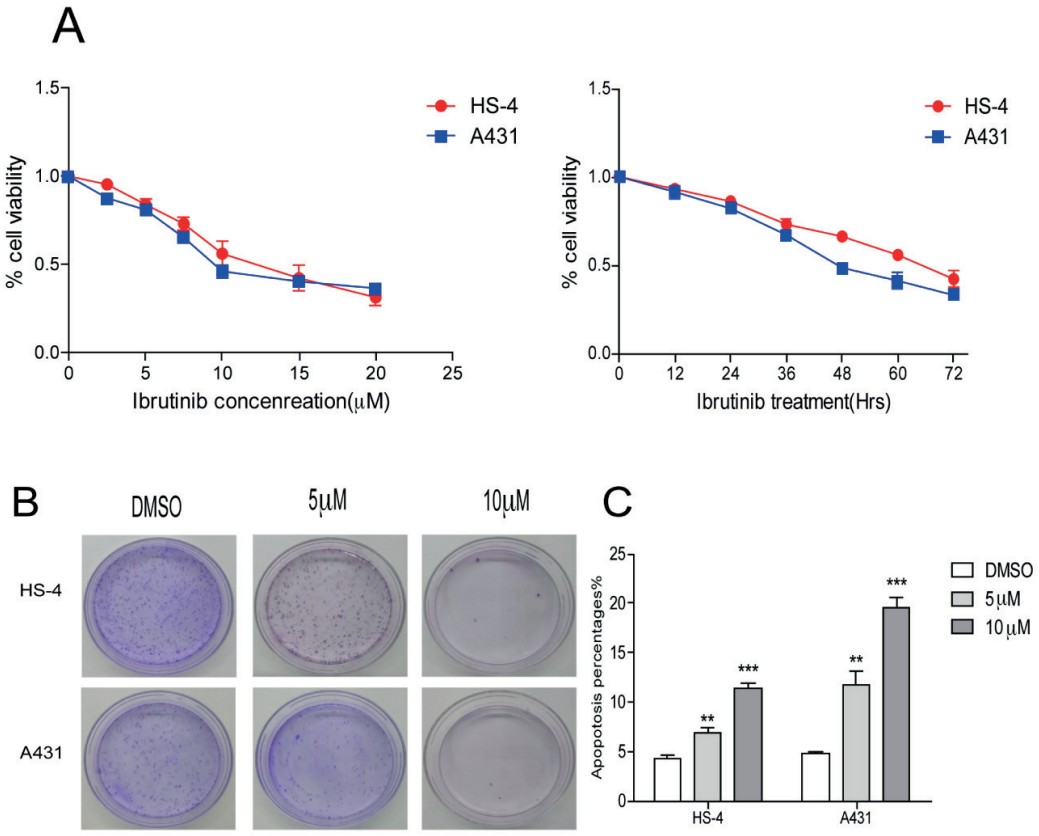

Figure 1. Ibrutinib inhibits proliferation of skin cancer cell lines. ***A,*** CCK8 assay showed the proliferation of skin cancer cell lines (HS-4, A431) (n = 4) after treatment with ibrutinib at different concentrations (left), and different time points (right). ***B,*** The expression of plate clone in HS-4, A431 cells treated with different concentrations of ibrutinib (0.5 μM, 10 μM) for 10 days. ***C,*** Cell apoptosis in HS-4, A431 cells treated with different concentrations of ibrutinib.

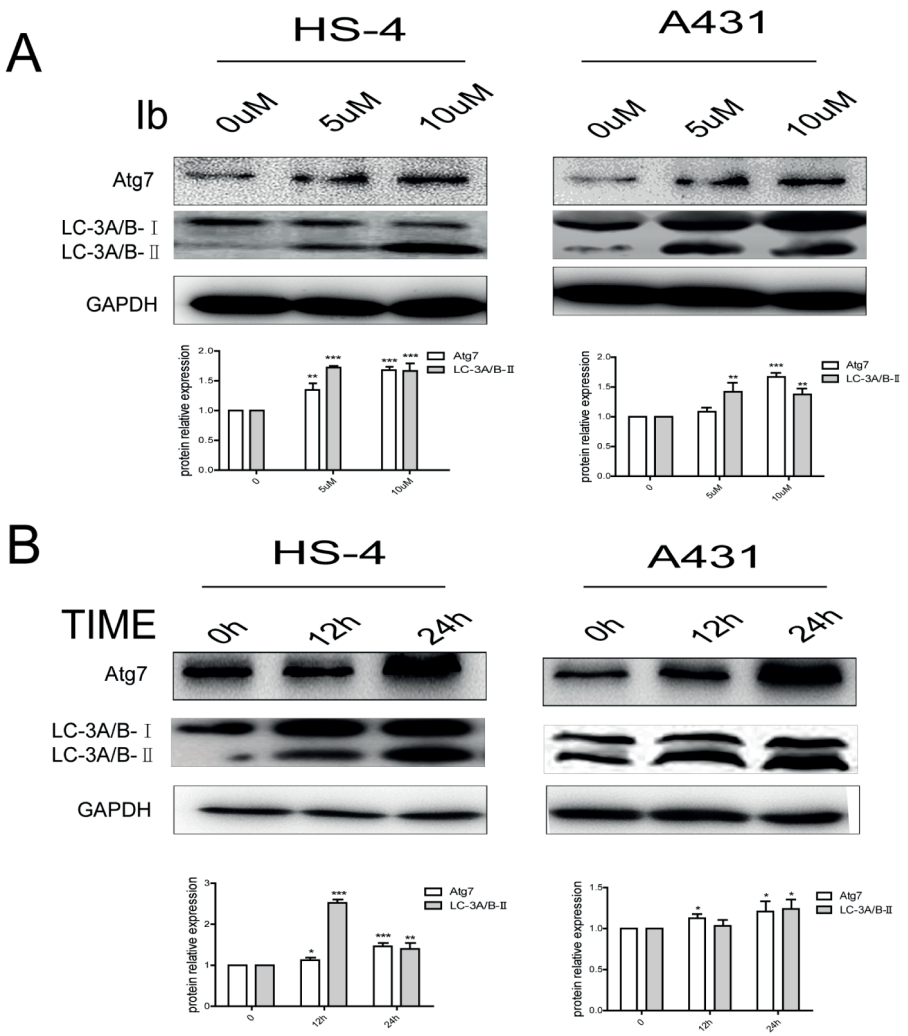

Figure 2. Ibrutinib induces autophagy in skin cancer cells. Western blot results showed that protein expressions of Atg7, LC3A/B-II and GAPDH in skin cancer cells treated with different concentrations of ibrutinib ***(A)*** and treated with 10 μM ibrutinib at different time points ***(B)***.

inhibited, colony formation amount was reduced. These experimental results showed that ibrutinib induced autophagy in skin cancer cells and thus reducing its cancer-killing effect.

Discussion

In countries rich in sunshine, the prevalence of skin cancer is extremely high, including basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and cutaneous melanoma (CMM). Skin cancer is a malignant tumor with a good prognosis. Timely and effective treatment is necessary for skin cancer. The development of new therapeutic drugs has become necessary.

Ibrutinib has the advantages of high efficiency, high selectivity and irreversibility, which have achieved encouraging results in clinical trials of various B-cell malignancies, such as CLL, diffuse large B-cell lymphoma, follicular lymphoma, lymphoma mantle cell and waldenstrom macroglobulinemia[14,15]. Ibrutinib has been approved by the FDA for MCL and relapsed/refractory CLL in November 2013 and February 2014, respectively[16,17]. Farooqui et al[18] considered that ibrutinib had the potential to replace chemical immunotherapy.

Three forms of programmed cell death in tumor cells were generally accepted, namely apoptosis (type I), autophagy (type II) and necrosis (type III) [19]. Autophagy is a double-edged sword, which has an important effect on the development of tumors[20]. The study found that autophagy cannot only inhibit the occurrence of tumors, but also promote the survival and metastasis of tumor

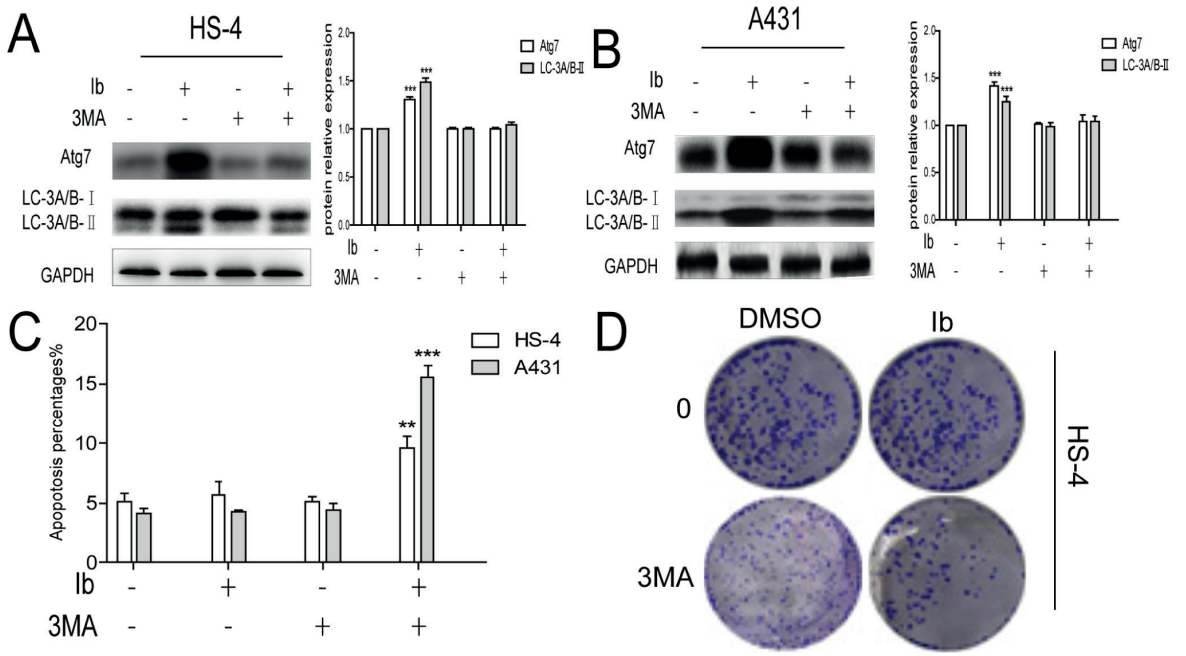

Figure 3. Inhibitors can inhibit ibrutinib-induced autophagy in skin cancer cells. Under treatment with ibrutinib (10 μM) and the inhibitor 3MA (2 μM), Western blot results showed that in HS-4 ***(A)*** and A431 ***(B)*** cell lines, protein expressions of Atg7, LC3A/B-II and GAPDH. ***C,*** Apoptosis of HS-4, A431 cells in different treatments of ibrutinib (10 μM) and the inhibitor 3 MA (2 μM) during cell culture. ***D,*** Apoptosis of HS-4 cells in different treatments of ibrutinib (10 μM) and inhibitor 3MA (2 μM) during cell culture.

cells[21]. Many evidence indicated that autophagy pathway was a potential target therapy for many diseases including cancer[22-24], diabetes mellitus[25], infection[26], etc. Therefore, autophagy has a significant effect on cell homeostasis, development and disease progression. Recently, many cancer treatment studies have shown that autophagy can be activated to protect tumor cells upon targeted therapy such as Philadelphia chromosome-positive cells and imatinib[27], glioblastoma and ibrutinib[15]. Autophagy-related genes, namely ATGs, LC3 (Atg8) -I (LC3-I) and LC3-II, play a role in autophagy formation. Therefore, we hypothesized that ibrutinib could induce autophagy, thus reducing the anticancer efficiency of skin cancer.

Based on this problem, CCK8, plate cloning assays and flow cytometry were used to verify the anti-tumor activity of ibrutinib. The findings showed that ibrutinib can gradually inhibit cell proliferation, increase apoptosis of skin cancer cells as the treating time and dose increased. Western blot was used to investigate the correlation between ibrutinib and autophagy. The results suggested that ibrutinib can induce autophagy in skin cancer cells in a time- and dose-dependent manner. Combination treatment with 3MA resulted in the decrease of autophagy. Further research found that, ibrutinib combined with 3MA skin cancer cells can significantly increase apoptosis and reduce proliferation. It was suggested that ibrutinib did have an obvious anti-cancer potential in skin cancer. Its combination with autophagy inhibitors enhanced anticancer activity.

Conclusions

Ibrutinib has a profound anti-tumor effect on skin cancer cells. We firstly demonstrated that ibrutinib induced autophagy in skin cancer cells. Our results provide important insight into the use of anticancer and autophagy inhibitors in the treatment of skin cancer and will help to develop new drugs.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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