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Effects of Matrine on Autophagy of Esophageal Cancer Ec109 Cells and the Mechanism

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Abstract

Objective: To explore the mechanism of action of matrine for the autophagy of esophageal cancer Ec109 cells.

Methods: Esophageal cancer Ec109 cells were cultured in vitro and treated with matrine at different concentrations, and the cell morphological changes were observed under an inverted phase-contrast microscope. The inhibition rate of cell proliferation was detected using methyl thiazolyl tetrazolium assay. The expressions of light chain 3-II (LC3-II) and phosphatidylinositol 3-hydroxy kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway-related proteins p-mTOR, p-Akt and p-p70 ribosomal S6 kinase (p70S6K) were determined using Western blotting. The mRNA expression of autophagy-related gene Beclin1 was detected by real-time quantitative polymerase chain reaction (RT-qPCR).

Results: It was observed under the inverted fluorescence microscope that after treatment with 0.8 g/L matrine, the cells grew slowly and had irregular shape, and the vacuoles were different in size, leading to the increase in cell volume. After treatment with 1.2 g/L matrine, cytoplasmic vacuolation and cellular swelling were observed, but the cell membrane was intact. When the concentration of matrine reached 1.6 g/L, cellular atrophy became worse, cell debris and suspension cells were significantly increased, and autophagic vacuoles could be seen through acridine orange staining. On the contrary, untreated Ec109 cells were in a long shuttle or cobblestone-like shape with irregular nuclei, and karyopyknosis was rare. In the treatment with matrine at different concentrations for 48 h, the inhibition rate of cell proliferation gradually rose with the increase of matrine concentration ($P<0.05$). Western blotting showed that with rising matrine concentration, the protein expression of LC3-II rose, while those of p-mTOR, p-Akt and p-p70S6K declined. RT-qPCR exhbited that the mRNA expression of Beclin1 was up-regulated by matrine.

Conclusion: Matrine can induce the autophagy of esophageal cancer Ec109 cells, thereby inhibiting their proliferation. The autophagy process may be mediated via the PI3K/Akt/mTOR signaling pathway.

Keywords: matrine; esophageal cancer; PI3K/Akt/mTOR signaling pathway; autophagy.

1. Introduction

Esophageal cancer is one of the top ten cancers seriously threatening human life health. The mortality rate of esophageal cancer ranks 4th among malignant tumors in China.

The prevention and treatment of esophageal cancer remain a major problem in the world, and tumor metastasis, invasion and deterioration are the number one killer of patients [1].

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In the treatment of esophageal cancer, postoperative drug therapy also plays an important auxiliary role. Matrine is an active ingredient of Chinese herbal medicine, which can be extracted from the root of leguminous plant Sophora flavescens. It contains a variety of alkaloid components, among which matrine and oxymatrine are mainly used for clinical medication [2]. *In vivo* and *in vitro* experiments have proven that matrine has a wide range of pharmacological effects, such as anti-inflammation, anti-fibrosis, analgesia, anti-arrhythmia, elimination of various microorganisms, and positive inotropic effect, which has been widely applied in the treatment of hepatic diseases, arrhythmia and skin diseases [3]. In recent years, it has been found in numerous research that matrine has an antitumor effect, mainly manifested as the negative regulation of tumor metastasis and infiltration, interference in cell cycle, promotion of benign development of tumor cells, hindrance of tumor angiogenesis, and influence on drug resistance of tumor cells [4]. Autophagy serves as a lysosome-dependent degradation system in eukaryotic cells, and plays a positive role in regulating cellular immunity and keeping balance of intracellular metabolism. Most researchers have found that autophagy has a special dual effect on tumors. In other words, autophagy can hinder tumor infiltration, while it plays a positive role in cancer cells resisting adverse metabolic stress, thus benefitting tumor survival [5]. Based on this, whether matrine can induce autophagy of esophageal cancer Ec109 cells and its possible mechanism of action were explored in this study.

2 Materials and Methods

Materials

Human esophageal cancer Ec109 cell lines were provided by Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, and matrine (purity >97%) was provided by Nanjing KeyGEN Biotech Co., Ltd. RPMI1640 medium and fetal bovine serum (FBS) were purchased from Gibco (USA), trypsin was purchased from Sigma (USA), and methyl thiazolyl tetrazolium (MTT) was purchased from Cell Signaling Technology. Dimethyl sulfoxide (DMSO) was bought from Nanjing GenScript Biotechnology Co., Ltd., skim milk powder was bought from BD, and bicinchoninic acid (BCA) protein concentration assay kits were bought from Suzhou Mindel Biotechnology Co., Ltd. Horseradish peroxidase-labeled goat anti-mouse IgG, goat anti-rabbit IgG, and mouse anti-light chain 3-II (LC3-II) antibodies were from Shanghai Beyotime Biotechnology Co., Ltd., and rabbit anti-p-protein kinase B (Akt), rabbit anti-p-mammalian target of rapamycin (mTOR), and rabbit anti-p-p70 ribosomal S6 kinase (p70S6K) polyclonal antibodies were from Proteintech. Real-time quantitative polymerase chain reaction (RT-qPCR) kits, primers, RNA extraction and reverse transcription kits were obtained from Beijing Nabai Biotechnology Co., Ltd.

Cell culture and experimental grouping

Human esophageal cancer Ec109 cells were cultured with RPMI1640 medium containing 100 mL/L FBS. Upon reaching about 90% confluence of adherent cells, they were digested with 2.5 g/L trypsin and passaged at 1:2. The cell growth status and morphological changes were observed daily, photographed and recorded. The cells were divided into experimental group and control group. 0.8, 1.2 and 1.6 mg/mL matrine was added in experimental group, while the cells in control group were not induced by drugs but only routinely cultured.

Morphological observation

The cells in logarithmic phase were collected, inoculated into a 24-well plate (1×10^6 cells/well), and incubated at 37°C with 5% CO_2. After the cells adhered to the wall, matrine at different concentrations was added. After culture for another 48 h, the old medium was discarded, and the cells were washed with PBS 3 times, stained with 1 μg/mL acridine orange (AO) for 15 min in the dark, and washed again with PBS 3 times. Then the cell morphology was observed and photographed under the inverted fluorescence microscope. The assay was repeated 3 times.

Detection of cell growth inhibition rate

The cells were grouped in the same way as mentioned above. The cell suspension in each group was inoculated into a 24-well plate at a density of 5×10^4 cells/mL (100 μL/well). After 48 h, the medium was replaced, and 10 μL of 5 mg/mL MTT was added in the dark for incubation for 4 h. Then the supernatant was discarded, DMSO solution was added (150 μL/well), and the purple crystals were shaken until they were completely dissolved. Finally, the absorbance (A) of each well at a wavelength of 490 nm was measured using a microplate reader, based on which the inhibition rate of cell proliferation was calculated. The assay was repeated 3 times.

Detection of expressions of autophagy-related proteins by Western blotting

The total protein was extracted in each group, and its concentration was detected according to the

instructions of BCA protein quantification kits. Then 50 μg of total protein was separated through 120 g/L polyacrylamide gel electrophoresis, transferred onto a polyvinylidene fluoride membrane by the wet method, blocked with 50 g/L skim milk powder at room temperature for 1 h, and incubated with anti-LC3-II, p-mTOR, p-Akt, and p-p70S6K primary antibodies (1:1000) at 4°C overnight. After the membrane was washed with TBST 3 times (15 min/time), the protein was incubated with corresponding horseradish peroxidase-labeled goat anti-mouse IgG and goat anti-rabbit IgG secondary antibodies at room temperature for 1 h. Then the membrane was washed again with TBST 3 times (15 min/time), followed by exposure and image development. Finally, the images were taken using an Alpha chemiluminescence gel imaging system and saved.

Detection of Beclin1 mRNA expression by RT-qPCR

The cells were grouped in the same way as mentioned above. After treatment for 48 h, the total mRNA was extracted in each group and reversely transcribed according to the instructions of the TaKaRa kits and reaction conditions of primers, and the target gene Beclin1 was amplified, with GAPDH as an internal control. The primer sequences were as follows: Beclin1: F: 5'-GTGGCTTTCCTGGACTGTGT-3', R: 5'-CACTGCCTCCTGTGTCTTCA-3'. GAPDH F: 5'-ACAGTCCATGCCATCACT-3', R: 5'-AGTAGAGGCAGGGATGATG-3'. The RT-qPCR system was 10 μL in total, with 3 replicate wells. The relative expression levels of target genes in each group were calculated using $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All data were expressed as mean ± standard deviation ($\bar{\chi} \pm s$), and SPSS21.0 software was used for statistical analysis. Differences among groups were compared using one-way analysis of variance, and independent-samples *t* test was performed for comparison between two groups. $P<0.05$ was considered to be statistically significant.

3. Result

Morphological changes of matrine-treated Ec109 cells

The Ec109 cells treated with matrine at different concentrations for 24 h were observed under the inverted fluorescence microscope. It was found that after treatment with 0.8 g/L matrine, the cells grew slowly and had irregular shape, and the vacuoles were different in size, leading to the increase in cell volume. After treatment with 1.2 g/L matrine, cytoplasmic vacuolation and cellular swelling were observed, but the cell membrane was intact. When the concentration of matrine reached 1.6 g/L, cellular atrophy became worse, cell debris and suspension cells were significantly increased, and autophagic vacuoles could be seen through AO staining. On the contrary, untreated Ec109 cells were in a long shuttle or cobblestone-like shape with irregular nuclei, and karyopyknosis was rare (Figure 1).

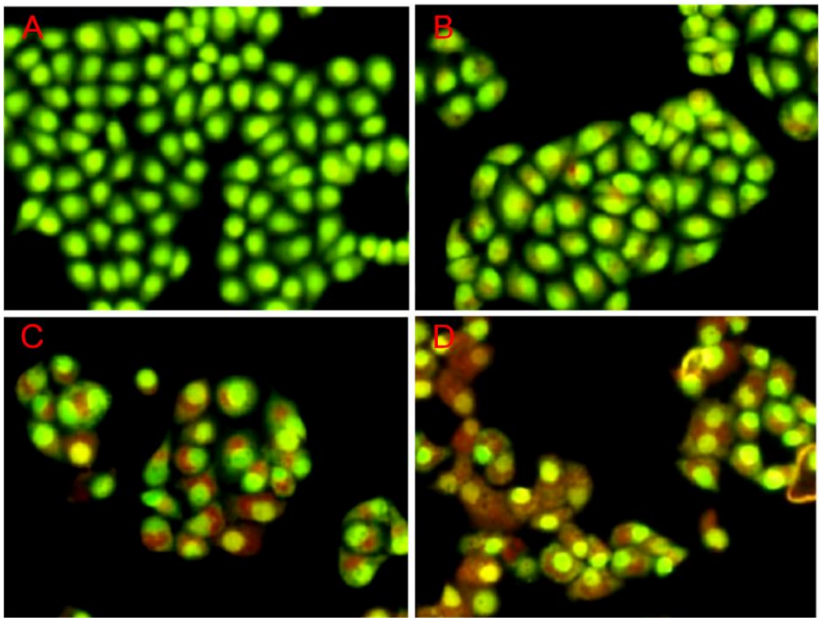

Figure 1. Matrine induced autophagy of Ec109 cells. A: Control group; B: 0.8 g/L matrine group; C: 1.2 g/L matrine group; D: 1.6 g/L matrine group.

Effects of matrine on Ec109 cell proliferation

In the treatment of Ec109 cells with 0.8, 1.2 and 1.6 mg/mL matrine for 48 h, the inhibition rate of cell proliferation gradually rose with the increase of matrine concentration in an obvious dose-dependent manner, namely (31.28 ± 2.41)%, (50.86 ± 2.68)% and (85.43 ± 2.23)%, respectively ($P<0.05$) (Figure 2).

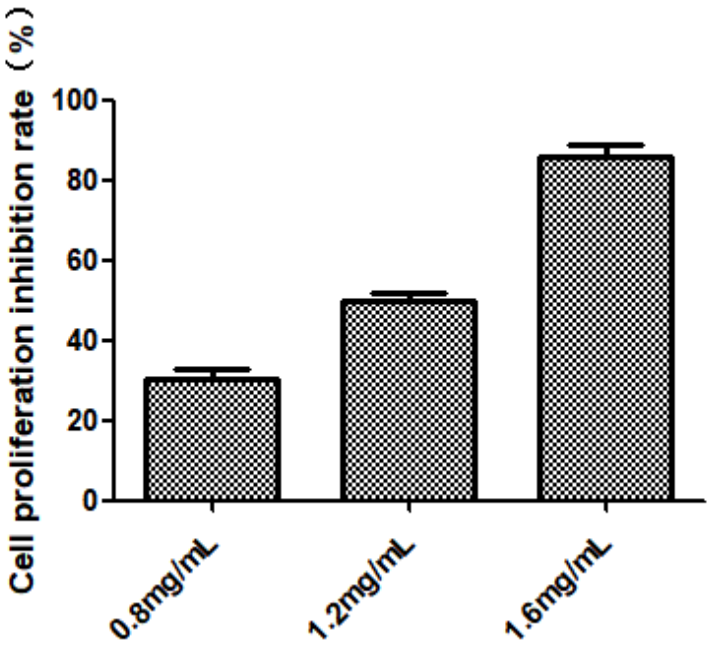

Figure 2. Effects of matrine at different concentrations on proliferation of Ec109 cells.

Expressions of autophagy-related proteins in Ec109 cells

The results of Western blotting showed that in the treatment with 0.8, 1.2 and 1.6 mg/mL matrine for 48 h, the expression of LC3-Ⅱ in Ec109 cells rose compared with that in control group, while the expressions of p-mTOR, p-Akt and p-p70S6K in the PI3K/Akt/mTOR pathway declined with the increase of matrine concentration (Figure 3). It can be inferred that the effect of matrine on Ec109 cells may be realized through promoting autophagy, and the changes in PI3K/Akt/mTOR pathway-related molecules may be involved in this regulatory process.

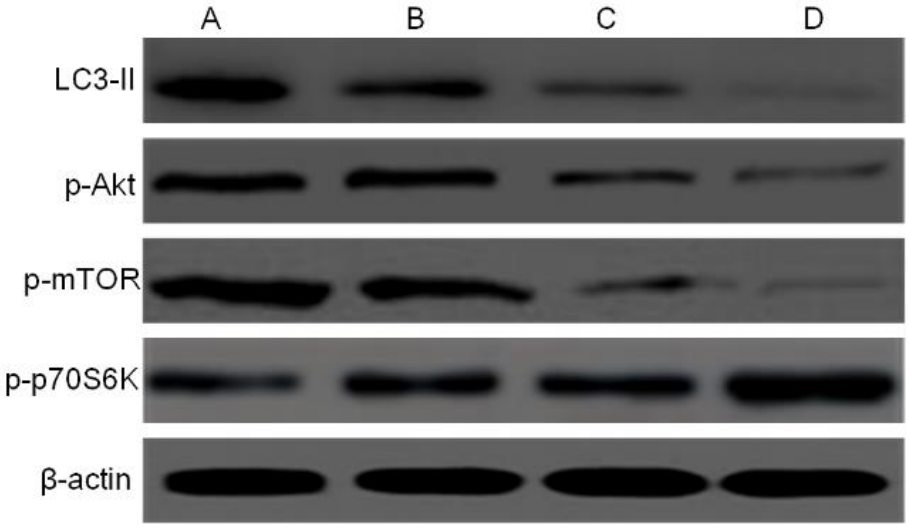

Figure 3. Expressions of autophagy-related proteins in Ec109 cells detected using Western blotting. A: Control group; B: 0.8 g/L matrine group; C: 1.2 g/L matrine group; D: 1.6 g/L matrine group.

Beclin1 mRNA expression

The results of RT-qPCR revealed that the relative mRNA expression of Beclin1 in Ec109 cells after the intervention of matrine gradually rose with the increase of matrine concentration, and the difference was statistically significant compared with that in control group ($P<0.05$) (Figure 4).

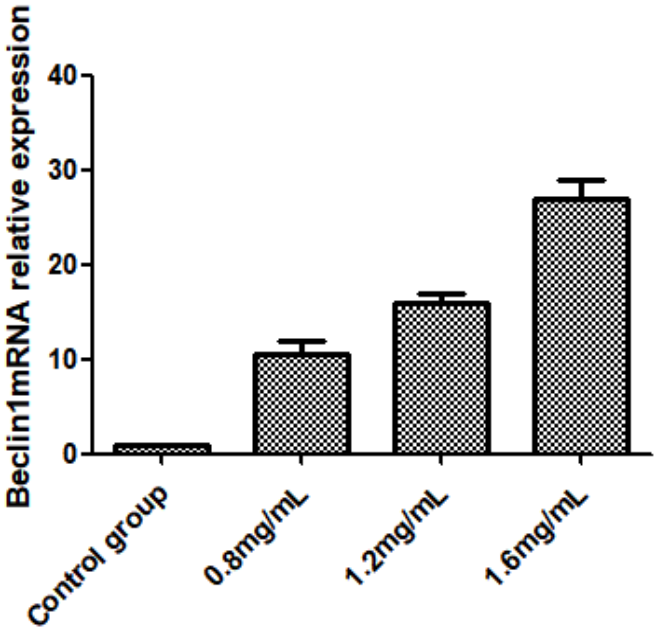

Figure 4. Beclin1 mRNA expressions in Ec109 cells of control group and different matrine concentration groups.

4. Discussion

Esophageal cancer is a disease of abnormal cell proliferation manifested as space-occupying protrusion due to local hyperplasia in the esophageal mucosa, which is a multi-stage complex process mediated by multiple genes. The incidence rate of esophageal cancer displays a significant difference in geographical distribution around the world [6]. In recent years, with the continuous R&D of novel chemotherapeutic drugs and the constant development of therapeutic regimens, the efficacy of chemotherapy on esophageal cancer has been highly improved, but drug resistance remains one of the main problems, which greatly affects the efficacy of chemotherapy and the prognosis of patients. Therefore, it is urgent to deeply explore the mechanism of the occurrence and metastasis of esophageal cancer, and try to find new antitumor drugs with higher anti-cancer activity and smaller side toxic and side effect.

At present, traditional Chinese medicine has been regarded as a potential antitumor resource with great potential in resisting tumors. The effective ingredients of many Chinese herbal medicines have been widely applied in clinical treatment. Matrine, one of the main ingredients of the traditional Chinese medicine Sophora flavescens, possesses the effects of improving eyesight, nourishing liver and gallbladder, expelling wind-dampness, killing insects, removing blood stasis and relieving heat dysentery. According to a large number of studies, the anti-cancer activity of matrine can be realized by blocking cell cycle progression, inhibiting telomerase activity, blocking angiogenesis in tumor tissues, negatively regulating tumor cell infiltration and migration, and reversing drug resistance of tumor cells [7]. Xie treated hepatic oval cells in rats with matrine at different concentrations, and found that matrine could induce cell autophagy, leading to enhanced cell death [8]. Fang *et al.* found in the *in vitro* experiment on lung cancer A549 cells that cell autophagy occurred under the action of matrine, thereby interfering with the cellular energy cycle process, and ultimately hindering lung cancer cell proliferation [9].

Autophagy, namely self-digestion, refers to a way for eukaryotic organisms to obtain nutrients and energy necessary for survival in the case of extreme environments such as high temperature, hypoxia and outright hunger. Autophagy process is a lysosome-dependent degradation method in cells. After rearrangement of the cytoplasmic inner

membrane, senescent proteins and organelles in cells are wrapped to be autophagosomes, and then autophagosomes bind to lysosomes to produce autophagic lysosomes, which can selectively degrade or reuse senescent, excess and damaged organelles and proteins in cells, so that the mass and homeostasis of organelles can be controlled in this way [10,11]. In recent years, autophagy has been increasingly studied in the fields of tumors and skin diseases, and it may become a key target for clinical treatment. Autophagy itself has a special dual effect. On one hand, autophagy can inhibit cell proliferation or prevent DNA mutations through inducing programmed cell death, ultimately suppressing tumor formation. One the other hand, autophagy protects cancer cells from the destruction of chemotherapeutic drugs through keeping the intracellular metabolic stability and renewing some organelles, ultimately delaying the tumor cell apoptosis [12]. Therefore, the exact role of autophagy in the formation, development and metastasis of tumors remains controversial. It has been found that autophagy can prevent the progression of bladder cancer [13]. However, YING *et al.* [14] found that initiating autophagy could protect bladder cancer cells from apoptosis. In this study, the results manifested that when the concentration of matrine reached 1.6 g/L, cellular atrophy became worse, cell debris and suspension cells were significantly increased, and autophagic vacuoles could be seen through AO staining. It was confirmed in MTT assay that matrine could obviously inhibit the proliferation of Ec109 cells, and such an effect became more significant with the increase of matrine concentration.

Autophagy-related protein LC3 is an indispensable molecule for the formation of autophagosomes, involved in each stage of autophagosome formation, which can be used as a reliable marker for detection of autophagy. LC3 can be classified into LC3-I and LC3-II. When autophagy does not occur, LC3 is processed into soluble LC3-I expressed in the cytoplasm. When autophagy occurs, LC3-I is further activated by autophagy-related gene 7 (Atg7), and binds to phosphatidylethanolamine on the surface of autophagic membrane after ubiquitin-like processing and modification, forming LC3-II [15]. Generally, the higher level of autophagy corresponds to the higher expression of LC3-II [16]. In this study, the results of Western blotting showed that the expression of LC3-Ⅱ significantly rose in Ec109 cells treated with matrine at different concentrations in a concentration-dependent manner. It is speculated that autophagy may play a key role during the action of matrine on Ec109 cells. Atg refers to the genes that play roles in autophagy [17]. The Atg1/ULK1 complex composed of Atg1 and Atg13 is an important positive factor inducing autophagy. The mechanism of autophagy in regulating cell growth and apoptosis is rather complicated. The PI3K/Akt/mTOR signaling pathway is one of the important regulatory pathways, and it has corresponding changes at each stage of tumor development. Akt is a highly conserved serine/threonine protein kinase consisting of 480 amino acid residues, which can be activated with the activation of its upstream PI3K. In addition, the downstream mTOR can be activated with the activation of Akt, and plays a negative role in regulating autophagy. Atg13 phosphorylated by mTOR cannot bind to Atg1, thereby reducing the formation of autophagosomes. Moreover, the adhesion between ribosomes and endoplasmic reticulum is enhanced with the increase of mTOR activity, thus reducing the formation of autophagosome membranes from the shedding of endoplasmic reticulum membrane [18]. With the enhanced activity of mTOR, its key downstream substrate p70S6K will also be activated. Then activated p70S6K can mediate the translation process of key mRNAs in the cell cycle progression, thereby exerting positive effects in keeping intracellular metabolic homeostasis, resisting apoptosis factors, and promoting cell growth. In this study, the results of Western blotting manifested that the expressions of p-mTOR, p-Akt and p-p70S6K in the PI3K/Akt/mTOR pathway were down-regulated in Ec109 cells treated with matrine. It is speculated that autophagy of esophageal cancer cells induced by matrine may be mediated by the PI3K/Akt/mTOR signaling pathway. At the same time, the difference in the mRNA expression of Beclin1 in Ec109 cells between experimental group and control group was also detected using RT-qPCR. Beclin1 is involved in the regulation at the initial stage of autophagy, and its expression varies from tumor to tumor. Moreover, it is also the first cancer suppressor gene related to autophagy found in mammals [19]. The results of RT-qPCR proved that matrine was crucial in the chemotherapy of esophageal cancer, and it worked through inducing the expression of Beclin1 and enhancing autophagy. Beclin1 gene was also present in the PI3K/Akt/mTOR signaling pathway. Therefore, the experimental results demonstrate that the PI3K/Akt/mTOR

signaling pathway may be involved in the autophagy of Ec109 cells induced by matrine.

In conclusion, matrine can induce autophagy of esophageal cancer Ec109 cells, thereby inhibiting the proliferation of Ec109 cells. Moreover, the autophagy process may be mediated through the PI3K/Akt/mTOR signaling pathway. The research results provide new ideas for the prevention and treatment of esophageal cancer, that is, matrine can be used as an ideal anti-cancer drug to regulate autophagy and assist the clinical treatment of esophageal cancer.

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