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Migration inhibition of water stress proteins from *Nostoc commune* Vauch. via activation of autophagy in DLD-1 cells

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ABSTRACT

Water stress proteins (WSP1) from *Nostoc commune* Vauch. had been proven to selectively induce colon cancer cells apoptosis. In this study, the effect of WSP1 on migration of human colon cancer cells was investigated. It showed that WSP1 inhibited DLD-1 cell migration, but with an insignificant effect on normal human colon epithelial cells. The data further indicated that WSP1 activated autophagy through down regulation of PI3K/AKT/mTOR pathway. Meanwhile, β catenin was degraded by autophagy, which then restrained epithelial-mesenchymal transition (EMT) of DLD-1 cell and its migration was subsequently suppressed significantly. The same changes occurred in xenografted nude mice according to the obtained immunohistochemical results. Consistently, the application of autophagy inhibitor largely reversed the inhibited migration by WSP1 treatment. Taken together, WSP1 could suppress migration of DLD-1 cells by autophagy inhibited EMT. The results suggested that WSP1 possessed the potential as a selective therapeutic agent against metastatic colon cancer.

1. Introduction

Colon cancer is colonic epithelium malignant changes that caused by carcinogenic factors such as environmental or genetic. It is a common malignant tumor and accounts for approximately 8 million new cases in the global every year [1]. It has been known that the tumor cell metastasis is the leading cause of death in cancer patients [2]. High mortality and low cure rate made target metastasis drugs very much needed to improve treatment outcome of patients.

Natural *Nostoc commune* has been used in traditional medicine in China for centuries and displays wide-ranging properties such as antiviral, anti-bacterial and anti-neoplastic activities [3]. Our previous studies [4] indicated that WSP1 isolated from the *Nostoc commune* Vauch. significantly induced the mitochondrial destabilization and caspase-dependent apoptosis in human colon cancer cells rather than normal cells. The present findings further implied WSP1 had the potential ability to inhibit colon cancer migration.

Autophagy is a lysosomal self-digestion process, which is essential for cellular homeostasis, differentiation and survival to adapt the environment changes [5]. Microtubules associated protein light chain 3 (MAP-LC3) has been widely used to monitor autophagy. It has two forms of mutual conversion. When autophagy occurs, cytoplasmic LC3 will degraded into a short peptide to be LC3-I, then LC3-I is converted to the membrane LC3-II, and forms a double membrane autophagosome around cell organ or nucleus [6]. p62/sequestosome 1(SQSTM1), a ubiquitin like binding protein, interacts with LC3, which regulates autophagosome formation and is degraded by autophagy in turn [7]. Therefore, the expression level of intracellular p62 is negatively correlated with autophagic activity [8]. In addition, autophagy negatively regulates active β catenin and nuclear accumulation of β catenin by promoting disheveled (Dvl) degradation in the late stages of colon cancer development [9]. Besides, β catenin could form a complex with LC3 and p62 and be degraded by autophagy [10]. It has been known that phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B, PKB)/mammalian Target Of Rapamycin (mTOR) signaling pathway plays a critical role in many cellular functions [11]. It is associated with malignant proliferation, growth and survival of human cancers [12]. Moreover, PI3K/Akt/mTOR signaling is a well-known pathway involved in the regulation of autophagy, inhibition of PI3K/Akt/mTOR signaling pathway could promote autophagy [13]. In the study, we have investigated the molecular pathway of autophagy induced by WSP1 and found that PI3K/Akt/mTOR signaling was inhibited.

The epithelial-mesenchymal transition (EMT) is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties. EMT has been shown to occur in the initiation of metastasis for cancer progression. The hallmarks of the EMT include the downregulation of E-cadherin and upregulation of N-cadherin, leading to a mesenchymal-cell-like phenotype and destabilization of adherent junctions [14]. In cancer progression, β catenin often accumulates in the cytoplasm and then translocates to the nucleus as a transcription factor where it binds the TCF/LEF family (T Cell Factor/

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Lymphoïd Enhanced Factor), leading to the expression of a large number of genes, in many of which are transcription factor, such as snail [15,16] which is a inhibitory transcription factor of E-cadherin, and subsequently impacts EMT conversion process.

The contradictory reports [17,18] indicate that autophagy is pro- or anti-tumor depending on the context or the stage of tumor development. In this study, DLD-1 cell line was observed to undergo migration inhibition through EMT by WSP1. The same effect was found in the DLD-1 xenograft in nude mice. At the same time, we also found β catenin was inhibited by WSP1-induced autophagy. Then we used autophagy inhibitor chloroquine (CQ) to address the role of autophagy on the migration effect induced by WSP1 in DLD-1 cells. The result showed that the suppressed effect of β catenin and migration was almost completely conversed. It indicated that WSP1-induced autophagy could inhibit β catenin and alters EMT through increased E-cadherin and decreased N-cadherin, which further interfered DLD-1 cell migration.

2. Materials and methods

2.1. Antibodies and reagents

RPMI-1640 medium, foetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). RNAiso Plus, PrimeScript RT MasterMix and SYBR Green PCR Master Mix were obtained from Takara (Shiga, Japan). E-cadherin, N-cadherin, and p-AKTS473 antibodies were from Bioworld Technology (Minneapolis, MN, USA); β catenin obtained from Abmart (Arlington, MA, USA). LC3, p62, ClassI PI3K and mTOR antibodies were from Proteintech (Wuhan, China); AKT antibody was obtained from Cell Signaling Technology (Danvers, MA, USA); GAPDH and β actin antibodies were from Abmart (Shanghai, China). HRP-conjugated secondary antibodies were obtained from Invitrogen (Carlsbad, CA).

2.2. Preparation and purification of WSP1

Desiccated colonies of NCV were collected from Shanxi, China in 2012. The data of technological processes for preparation of *Nostoc commune* Vauch. and purification of WSP1 were carried out as previously described [4].

2.3. Cell culture

Human colon cancer cell line DLD-1 was purchased from American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS. The human colon epithelial cell line FHC was obtained from the Chinese Type Culture Collection and cultured in DMEM/F12 1:1 medium supplemented with 10% (v/v) FBS. Cultures were incubated at 37 °C in 5% CO_2 and 95% humidity.

2.4. Wound healing assay

Wound healing assay was carried out as previously described [19]. FHC and DLD-1 cells were seeded in a 24-well plate at a density of 1×10^4 cells per well. Twenty-four hours after treatment when cells have formed a confluent monolayer, a wound was administered across the cell monolayer using a sterile pipette tip. Cells were washed twice with PBS so as to remove any cell debris and then incubated in RPMI1640 free serum for 24 h to exclude the effect of the serum on cell growth. WSP1 of 0.04, 0.05, 0.08 and 0.10 μg/μl was added into the according wells respectively, with the control being replaced with PBS. Photographs were recorded at zero time and WSP1-treatment for 24 h by inverted microscope (OLYMPUS CKX41). The percentage of wound healing was calculated by the equation: percent wound healing = average of [(gap area: 0 h) − (gap area: 24 h)] / (gap area: 0 h). All assays were repeated in three independent experiments.

2.5. Transwell

DLD-1 cells were seeded in a transwell litter room at a density of 1×10^4 cells per well. Twenty-four hours after treatment when cells have formed a confluent monolayer, remove the transwell chamber and discard the upper chamber medium and wash it twice with PBS. The immunostain fixation solution was fixed for 30 min, washed twice with PBS, wiped off the cells on the upper chamber surface with a cotton swab, and then stained with 0.1% crystalline violet for 15 min. Finally, it was observed and counted by inverted microscope.

2.6. Quantitative real-time PCR

Total RNA was extracted from cells using Trizol reagent. cDNA was synthesised from 500 ng RNA with PrimeScript RT Master Mix. Quantitative real-time PCR was performed using 2× SYBR Green PCR Master Mix under the following conditions: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 64 °C for 34 s, and a melt curve step using a Step One Plus Real-Time PCR System (Applied Biosystems). The expression levels of target gene for each experiment were normalised to GAPDH. The relative gene expression levels were detected and calculated using the ΔΔCt comparative method. The RT-PCR primers used were as follows:

E-cadherin, 5′-GAACGCATTGCCACATACAC-3′ (forward) and 5′-AACTCTCTCGGTCCAGCCCAG-3′ (reverse);

N-cadherin, 5′-TTTTGCCCCCAATCCTAAGA-3′ (forward) and 5′-CAGCGTTCCTGTTCCACTCAT-3′ (reverse);

MMP-2, 5′-TGGATGATGCCTTTGCTCGTGC (forward) and 5′-ATCGTC ATCAAAATGGGAGTCT-3′ (reverse);

MMP-9, 5′-CACCCTTGTGCTCTTCCCT-3′ (forward) and 5′-AAGTCT TCCGAGTAGTTTTGG-3′ (reverse);

STAT3, 5′-TCTCAACTTCAGACCCGTCAACA-3′ (forward) and 5′-ACAG CTCCACGATTCTCTCCTCC-3′ (reverse);

UPA, 5′-GCTGCTGACCCACAGTGGAA-3′ (forward) and 5′-AAAGTC ATGCGGCCTTGGAG-3′ (reverse);

β Catenin, 5′-CAACTAAACAGGAAGGGATGGAAGG-3′ (forward) and 5′-CAGATGACGAAGAGCACAGATGG-3′ (reverse);

GAPDH, 5′-GCACCGTCAAGGCTGAGAAC-3′ (forward) and 5′-TGGT GAAGAACGCCAGTGGA-3′ (reverse).

2.7. Immunofluorescence staining analysis

The level of autophagy is characterized by immunofluorescence analysis. DLD-1 cells were plated in 6-well plates with glass slides (1×10^6 cells/well). After treatment with 0.10 μg/μl WSP1 for 24 h, the cells were fixed with immunostaining fixative for 60 min at 4 °C. Fixed cells were washed twice with PBS, then the coverslips were fixed with 4% paraformaldehyde in PBS for 20 min, permeabilized with 0.2% Triton X-100 in 0.1 M PBS for 5 min, blocked in 10% goat serum for 30 min and incubated overnight at 4 °C with polyclonal antibodies to LC3. After washing three times with 0.1 M PBS (pH 7.4), the cells were incubated with fluorescence-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO, USA) for 90 min at room temperature. After washed three times by PBS, the cells were incubated with DAPI for 1 h at 4 °C in the dark. The slides were washed with PBS to remove the excess DAPI. Then the cells nuclei and the fluorescence of LC3-labelled vacuole formation (autophagosomes) were viewed under Delta Vision Elite.

In order to assess the levels of autophagy per cell precisely, the percentage of LC3-positive cells that had 5 or more LC3 puncta in the cytoplasm were determined. The number of LC3 puncta per LC3-positive cell was determined. 100 cells per sample were counted for triplicate samples [20].