The Effects of UCH-L3 on the Biological Behaviours of Breast Cancer: Correlation with the Expressions of HIF-1α

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Abstract

**Projective:** HIF-1α is highly expressed in the triple negative breast cancer cell line (MDA-MB-231), which is lack of the expression of ER, PR and HER2 and exhibits high invasive and metastatic ability. Previous study detected that siRNA targeting HIF-1α in MDA-MB-231 restrained the cell growth and the abilities of invasion and migration, and promoted apoptosis. UCH-L3 protein was found to be one of the differential proteins detected by Bidirectional gel Electrophoresis and Proteomics in the cells with HIF-1α siRNA cells comparing to no-siRNA cells. The objective of this study is to probe the effect of UCH-L3 on the biological behaviors of the triple negative breast cancer cell line (MDA-MB-231).

**Methods:** Over or blocking expressions of UCH-L3 were established by the transfections with lentiviral constitutive vector and siRNA targeting UCH-L3 respectively. Real time quantitative PCR and Western blot or Co-ip were used to detect the mRNA and proteins. CCK8, and clone formation assays were to evaluate the cell growth and clonality. Matrigel Transwell and Would Scratch assay were used to estimate the cell invasion and mobility.

**Results:** The over-expression of UCH-L3 inhibited the cell growth and clonality, weaken the abilities of cell invasion and migration, and lowered the expression of free and ubiquitined HIF-1α in MDA-MB-231 cells with lentivector comparison to those in the cells with control vector. Application of proteolytic enzyme inhibitor, MG132 increased the protein level of UCH-L3 but still decreased the protein level of HIF-1α in the cells with UCH-L3 over-expression. Blocking UCH-L3 expression by siRNA technique increased the expression of HIF-1α property.

**Conclusion:** high expression of UCH-L3 showed an inhibitory effect on the biological behaviors of triple negative breast cancer cell and negative effect on HIF-1α expression, implying that UCH-L3 likely to be a therapeutic strategy for triple negative breast cancer.

Keywords: Ubiquitin; Ubiquitin C-terminal Hydrolases-L3; Hypoxia-inducible factor-1α; Cell growth; Invasion; Mobility; Oncogenesis

Introduction

Although major advancements have been achieved in breast cancer therapy, such as targeting the receptors of ER or HER2, there still remains a lack of valid strategies for the treatment of the triple negative breast cancer (TNBC) cases. Clinical data also showed those patients have a worse prognosis and a higher mortality than non-TNBC cases. HIF-1 is a type of transcriptional activator, and is induced in response to hypoxia, stress and some growth factors (such as bFGF) via the activations of several signaling pathways [1,2]. More than 100 kinds of HIF-1 target proteins have been reported to play important roles in oncogenesis and malignant progression of tumor. HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits. The expression of the HIF-1α subunit is known to result in HIF-1 activation, further increase angiogenesis and facilitate tumor cell growth and adapts tumor cells to the hypoxic metabolisms, and finally promote malignant progression. Usually, the high expression of HIF-1α in tumor indicates a poor prognosis. A recent study found that HIF-1α expression within hypoxic regions are likely to contribute to the subsequent progression of a tumor by modifying the behavior of cells in the non-hypoxic regions of the local micro-environment [3].

It is known that HIF-1α is not only highly expressed in clinical triple negative breast cancer, but also in the breast cancer cell line MDA-BM-231 in vitro [4]. Like most proteins in cells, HIF-1α is degraded by ubiquitin proteolysis which can be reversed by deubiquitinating enzymes (DUBs). Ubiquitin C-terminal hydrolases 3 (UCH-L3 ) is a type of deubiquitinating enzymes. The objective of this study was to probe the biological effects of UCH-L3 on the triple negative cell line (MDA-MB-231) in order to determine a strategy for slowing down or retarding the progression of triple negative breast cancer.

Materials and Methods

Cell culture and reagents

The human breast cancer cell line MDA-MB-231 was obtained from the Department of Pathology, Peking University Health Science Center. The acquired cells were maintained in DMEM with 10% FCS at 37°C in a humidified environment with 21% O2 and 5% CO2, and were sub-cultured in different sized well plates until 60 to 80% confluent in accordance with this study’s different experimental purposes.

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Western Blot analysis

The cells were washed with ice-cold PBS and dissolved in a 2% SDS, 100 mM DTT, 60 mM Tris, and pH 6.8 solution. The proteins were then quantified using a Bradford assay (BioRad). The total cell proteins were separated on 10% SDS-PAGE gels and transferred to NC membranes. The specific primary antibodies of the HIF-1α (rabbit monoclonal 3716: Cell Signaling Technology); UCH-L3 (rabbit monoclonal T0638: Abcam); and sheep anti-rabbit or anti-mouse fluorescent secondary antibody were utilized and finally visualized under fluorescence microscopy. Also, mouse anti-human antibody β-tubulin or actin (Beyotime Institute of Biotechnology, China) was used as an internal reference.

Quantitative real-time PCR

In this study, the total RNA was isolated using Takara RNAios Plus (code: D9108A) according to the manufacturers’ protocol. A Prime Script TM RT reagent Kit with gDNA Eraser (TAKARA, Japan RR047A) was used for the cDNA synthesis. SYBR Premix Ex TaqTM II (TAKARA, Japan RR820A) was used for the qRT-PCR. All the procedures were carried out in accordance with each manufacturer’s instructions. The primer sequences for the UCH-L3 were: 5'-TTCAGGGCTATTGACACAGATTC-3'(sense) and 3'-AACAATCAGCAGCTGTGGA-5' (antisense). The primer sequences for the HIF-1α were: 5'-CAACGGTTTAAGGACACATTCTG-3' (sense) and 3'-AACAATCAGCAGCTGTGGA-5'(antisense). The primer sequences for the β-actin were: 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3' (sense) and 3'-TCTGGGTTGAAACTCAAGCAACTG-5' (antisense).

The amplification reactions were performed using Applied Biosystems 7500 Fast Real-Time with the following conditions: 95°C for 30 seconds; followed by 40 cycles at 95°C for 3 seconds; and 60°C for 30 seconds. All of the reactions were performed in triplicate. The relative gene expression was quantified using an ΔΔCt method. The relative values of the UCH-L3 and HIF-1α mRNA for each sample were calculated using β-actin transcript signal as an internal control.

Lentivirus infection

The cells were cultured in a 25 cm² flask to 20 to 30% confluent and washed twice with cold PBS. Then, 50 μL (1 × 10⁵ TU/mL) of lentiviral vectors with UCH-L3 (9572-1) or control vectors (CON220) which had been designed by the Shanghai Genechem Co., Ltd. were added into the enhanced infection solution with final concentration of 10 μg/ mL of Polybrene. After 24 hours, the enhanced infection solution was replaced by DMEM and the efficiency of the transfection was evaluated using inverted fluorescence microscopy. Then, the cells were screened with puromycin (2 μg/mL) for three days.

Cell scratch scratch migration test

The cells in logarithmic growth phase were sub-cultured in 6-well plates until reaching 80% or 90% confluence. Then, 200 μL of sterile pipetting tip was used to scratch the well bottom, and PBS was used to wash off the cell debris three times. Then, a medium containing 10% fresh FCS was added into each well, followed by an incubation period at 37°C in 5% CO₂ for 12 to 24 hour periods before being observed under an inverted microscope. The scratch widths were measured using the built-in ruler of the inverted microscope (10 μM). The scratch migration rates of the cells and were calculated in the formula as followed: (scratch width at the 0 hour minus the scratch width at the measures time)/scratch width at the 0 hour × 100%.

Transwell chamber assay

The cells were added in the upper chamber of a 24-well Transwell unit (1.5 × 10⁵ cells/well) with 8 μm pore size polycarbonate membranes (Corning Costar, Cambridge, MA, USA). The upper chamber contained a serum-free medium, while the lower compartment contained a medium with 10% FBS. Each cell line had three multiple pores. After 20 hours incubation in a humidified atmosphere of 5% CO₂ at 37°C, the cells which were adhering to the lower surface of the filter were fixed with 4% methanol, washed with PBA, and stained with Crystal Violet Solution. For the invasion assay, the upper surface of the filter was pre-coated with a 500 ng/ μL matrigel solution (ABR, USA). Then, the strained cells from at least five random fields were counted.

Figure 1: Basic and constitutive expressions of UCH-L3: The (A) represents the basic and constitutive levels of both UCH-L3 and HIF-1α mRNA in the MDA-MB-231 as indicated in the methods. The relative values of the UCH-L3 mRNA for each sample were calculated using β-actin transcript signal as an internal control and the p<0.01. (1B) represents the protein levels of the UCH-L3 and HIF-1α with UCH-L3 lentiviral or control vector. In summary, the total cell proteins from the different groups were separated on 12% SDS-PAGE gels, and then transferred to NC membranes. The specific primary antibodies of the UCH-L3 or HIF-1α, and the sheep anti-rabbit fluorescent secondary antibody, were utilized and finally visualized under a fluorescence microscope. Then, mouse anti-human antibody β-tubulin was used as an internal reference. The experiment was repeated three times.
Cell proliferation and clone formation assay

Cell proliferation assay was performed using a Cell Count Kit-8 (CCK-8, Beyotime Institute of Biotechnology, Jiangsu, China) in this study. To summarize, the cells were digested and seeded in 96-well plates at a density of 800/well, and then cultured for five days. Following this, 10 µL of CCK-8 were added into each well of three in each group each day and then incubated at 37°C for four hours. The absorbance was measured at 450 nm. For the cell clone formation, the cells were digested and seeded into flasks at a density of 300/flask and cultured for fourteen days in a humidified atmosphere of 5% CO₂ at 37°C, and the media was changed every other day. The cells were treated as Transwell chamber assay, and the cell clones were counted, with each cell line containing three flasks.

Small-interfering RNA (siRNA) interference

Transfection agents and siRNA duplexes that targeted UCH-L3 mRNA or control were purchased from Santa Cruz Biotechnology Inc., USA. Cells were transfected with either siRNA duplexes that targeted UCH-3 (siRNA: sc-42306) or with control siRNA that did not target any known genes. According to the manufacturer’s protocol, siRNA duplex (80 pmol) and 18 μl siRNA transfection reagent were added into cells with 600 μl siRNA transfection medium in 6-well plate for incubation of 7 hours, followed by the replacement with normal culture medium containing 20% normal serum and antibiotics.

Co-IP

Co-IP was performed essentially as the manual instruction. Rabbit

Figure 2: Cell growth abilities tested by CCK8: MDA-231 cells (1 × 10⁵/ml) with a UCH-L3 lentiviral or control vector were seeded into 96-well plates and incubated in culture medium for 4 to 5 days. The live cell numbers were tested each day using a CCK8 test kit. Each condition was set in quadruplicate, and three tests were performed per replicate, with a *p<0.01 versus the control vector. The experiment was repeated three times.
IgG was used to pre-clear the unspecific protein binding. Cell lysates were pre-cleared for 2 hours and subsequently incubated overnight at 4°C with HIF-1α or ubiquitin antibody respectively. While rabbit IgG was used as negative control. The lysis was washed and analyzed by immuno-blot with ubiquitin or HIF-1α antibody consequently.

Statistical analysis

The data were expressed as the mean ± standard deviation (SD), and a statistical analysis was performed using a Student’s t-test. All of the results from the three independent experiments were considered to be significant if p < 0.05.

Results

Basic and constitutive expressions of the mRNA and proteins of the UCH-L3 in Triple Negative Breast Cancer cell line (MDA-MB-231)

UCH-L3 lentiviral or control vectors were transfected into the MDA-MB-231 for 24 hours. Then, puromycin was used for screening for 72 hours. The total cells RNA and proteins were extracted from the serum-starved cells for 24 hours and were analyzed by real-time qPCR and Western Blot methods, respectively. The basic and the constitutive expression of both mRNA and protein of UCH-L3 were tested. It was observed that the UCH-L3 lentiviral vector had up-regulated the UCH-L3 mRNA level nine times (Figure 1A) compared with the control vector group. The Western Blot detected a new band at approximately 26 kDa in the UCH-L3 lentiviral vector group when compared with that in the control vector group (26 kDa) as showed in Figure 1B.

Inhibitory effects of the UCH-L3 on the malignant behaviors in MDA-MB-231 cells.

In order to determine the effects of the UCH-L3 on the biological behaviors, the control or UCH-L3 over-expression cells were cultured for different time in DMEM with 10% FCS according to the experiment purpose. The CCK8 tests indicated that the over-expression of UCH-L3 significantly suppressed the growth of the MDA-MB-231 cells as showed in Figure 2. Also, the scratch assay (Figure 3) and Transwell experiment showed that the cell mobility and invasion be slow down in UCH-L3 over-expression group comparing to that in control group (Figure 3B). Finally, it was determined that the UCH-L3 over-expression depress the clone formation in the MDA-MB-231 cells (Figure 3B).

The effect of UCH-L3 on HIF-1α expression

In order to probe the effects of the UCH-L3 on the HIF-1α expression, the total cell RNA and protein extractions from the control and UCH-L3 over-expression groups were tested using real-time qPCR and Western Blot. It was found that the HIF-1α protein but not mRNA in the UCH-L3 lentivirus group was obviously decreased in the MDA-MB-231 cells (Figure 4A) compared with the control. Further, siRNA targeting UCH-L3 increased the protein level of HIF-1α to some extent (Figure 4B). Those findings implied that the effect of UCH-L3 on the malignant behaviors to be consistent with its effect on the expression of HIF-1α protein in MDA-MB-231 cells. HIF-1α protein is degraded in the cell cytoplasm via proteolytic enzymatic hydrolysis under normoxic condition and accumulated in the cytoplasm and entry into the cell nucleus to combine with the subunit of HIF-1β, and then to activate the transcription of its target proteins due to inactivation of its degradation under hypoxic condition. Proteolytic enzyme inhibitor, MG132 can inhibit the degradation of HIF-1α protein and results in HIF-1α protein accumulation in the cytoplasm. In this study, addition of MG132 (Sigma) with final concentration of 0.5 µM in UCH-L3 over-expression cells for 1 to 3 days failed to result in HIF-1α accumulation, but increased the protein level of UCH-L3 (Figure 4C), suggesting that UCH-L3 probably be degraded by proteolytic enzyme and up-regulated by MG132.
Figure 4: The effect of UCH-L3 on the expression of HIF-1α protein (A) represents protein level of HIF-1α with UCH-L3 lentiviral in MDA-MB-231 (B) represents the effect of UCH-L3 blocking with siRNA on HIF-1α protein as described in the methods. C represents the effect of MG132 (final concentration of 50 µM) on the changes of HIF-1α and UCH-L3 protein. In summary, the total cell proteins after appropriate treatment from the different groups were separated on 12% SDS-PAGE gels, and then transferred to NC membranes. The specific primary antibody of the UCH-L3 or HIF-1α, and the sheep anti-rabbit or anti-mouse fluorescent secondary antibody, were utilized and finally visualized under a fluoresce microscope. Then, mouse anti-human antibody β-tubulin was used as an internal reference, and the p<0.01 when compared to those of the controls cells. The experiment was repeated three times.
UCH-L3 is mainly related with protein degradation via promoting detachment of ubiquitin from polyubiquitinated complex and increasing ubiquitin recycle. In order to understand the possible mechanism for the effect of UCH-L3 on HIF-1α expression, Co-ip was performed using anti-ubiquitin or anti-HIF-1α as IP and western blot antibodies respectively. The result detected that both free and ubiquitinated HIF-1α were decreased in UCH-L3 over-expression group comparing to those in control group (Figure 5), providing a possibility that UCH-L3 promote the detachment of ubiquitin from polyubiquitinated HIF-1α complex and the degradation of HIF-1α.

Discussion

HIF-1 has been proved to play an important role in promoting the growth, invasion, metastasis and suppressing apoptosis of almost all cancer including breast cancer. While the siRNA of targeting HIF-1α repressed the cell growth and invasion, and promoted apoptosis had been reported [5,6]. This research team’s previous study had probed the target proteins of HIF-1α in MDA-MB-231 cells after HIF-1α siRNA using bi-directional gel electrophoresis combined with Flight Mass Spectra. It was found that 21 differential proteins, including 19 down-regulated proteins and 2 up-regulated proteins, had been detected in the differential proteomics analysis comparing with no-siRNA cells. The majority of those proteins had been previously reported to be HIF-1 targets. However, it was determined that UCH-L3 was one of the two up-regulated differential proteins and had not previously been reported to be a HIF-1 target, which implied that UCH-L3 may be a novel direct or indirect target of the HIF-1.

Ubiquitin is a 76-amino acid protein and ubiquitously distributed and highly conserved throughout eukaryotic organisms. Ubiquitination is related to post-translational regulation, in which proteins undergo ubiquitin conjugation and then degradation by proteolytic enzyme system. The conjugation of ubiquitin is known to regulate the intracellular activities of target proteins by altering their stability, localization, and/or activities, and also their dynamics. However, ubiquitination can be reversed by deubiquitinating enzymes (DUBs) which are subdivided into UCHs and ubiquitin specific processing proteases (USPs). In summary, ubiquitination and deubiquitination are two processes which control the balance of ubiquitin and the degradation of target proteins in cells and are involved in variety of processes such as cell growth, differentiation, transcription and oncogenesis [7]. Therefore, targeting these two pathways has the potential to treat a broad range of devastating diseases, including cancer [8-15]. Studies showed that USPs are increasingly involved in tumor development. For example, USP2a rescued FAS from degradation, and thereby prevents the apoptosis of prostate cancer cells [9]. UPS14 negatively regulated toll-like receptor 4-mediated signaling and autophagy induction by inhibiting the ubiquitination of TaK1- Binding Protein 2 and Beclin 1, while down regulation of UPS14 has been found to significantly inhibit breast cancer cell proliferation and metastasis [16]. USP10 had the ability to regulate the stability of the EMT-transcription factor Slug/SNAI2 [17] and USP11 also has the abilities for deubiquitlation and stabilization of p21 protein resulting in the inhibition of proliferation in the cell-cycle progression and DNA damage responses [18].

UCHs, another family of deubiquitinating enzymes includes four members: UCH-L1/PGP9.5 (protein gene product 9.5); UCH-L3; UCHL5/UCH37; and the BRCA1-associated protein-1 (BAP1). BAP1 is considered to be a tumor suppressor [8]. It remains controversial whether UCH-L1 is a tumor promoter or suppressor. The results of previous studies have shown that the UCH-L1 enzyme influence several signaling pathways which play crucial roles in oncogenesis, tumor invasion, migration and prognosis [13-15]. UCH-L1 mRNA levels showed significantly a positive correlation with histological grades and negative correlation with estrogen and progesterone receptor status in breast cancer [19]. UCH37 are generally considered to be tumor promoters. It is known that UCH37 is required for proper cell cycle progression via interactions with an ubiquitin receptor hRpn13 [20]. In addition, UCH37 regulates the transcriptional activities of the E2 promoter binding factor [21] and the Tcf7 DNA binding for the activation of Wnt signaling [22]. Therefore, UCH37 has been taken as a target for cancer therapy [23].

UCH-L3 is capable of cleaving small molecules and amino acids which are linked by ester, thioester and peptide bonds to the C-terminus of ubiquitin. Study showed that UCH-L3 modulate the proteasome degradation of targeted proteins involved in a variety of processes including proliferation, differentiation, apoptosis [10] and EMT, [12] as well as prognosis [19,20]. Early study showed that UCH-L3 mRNA level
was significantly up-regulated in breast cancer tissue compared to adjacent normal breast tissue and had significantly a positive correlation with histological grades and negative correlation with estrogen and progesterone receptor status, also suggesting the possible involvement of UCH-L3 in the pathogenesis and progression of breast cancer [19]. UCH-L3 hydrolysis is significantly influenced by thermal stability [24]. A recent study showed that UCH-L3 might play a role in the regulation of autophagic death of leukemia [25]. This study showed that UCH-L3 exhibit an inhibitory affection on the biological behaviors in MDA-MB-231 cells.

The HIF-1 activities are dependent on the expressions of the HIF-1α subunit, which is mainly degraded via the ubiquitin proteasome pathway. Previous studies have shown that UCH-L1 has a deubiquitinating effect on HIF-1α [26].

Conclusion

This study confirmed that UCH-L3 over-expression by lentivirus decreased the expression of HIF-1α, in consistent with its inhibition on the abilities of MDA-MB-231 cell growth, immigration and invasion as well as clone formation. UCH-L3 modulates the levels of ubiquitin and target protein in the cell by removing ubiquitin from polyubiquitin-protein complex and promoting protein degradation. The ubiquitination of HIF-1α is known to be a prerequisite step for the subsequent degradation by the proteolytic enzyme systems. This study demonstrated that the levels of free and ubiquitinated HIF-1α protein were lower in UCH-L3 lentiviral group than those in the control group. The findings in this study suggested that UCH-L3 not only promote the detachment of ubiquitin from polyubiquitinated HIF-1α and then the degradation of HIF-1α, but also counteract the effect of proteolytic enzyme inhibitor (MG231) on HIF-1α protein degradation in MDA-MB-231 cell. In summary, the previous and this study of this research suggest that UCH-L3 exhibit an inhibitory affection on the biological behaviors in MDA-MB-231 cells, implying an alternatively therapeutic strategy for triple negative breast cancer.

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