Knockdown of ubiquitin protein ligase E3A affects proliferation and invasion, and induces apoptosis of breast cancer cells through regulation of annexin A2

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Abstract. The present study used RNA interference (RNAi) to study how the expression of annexin A2 was affected by ubiquitin protein ligase E3A (UBE3A). In addition, the proliferation, apoptosis and invasiveness of BT-549 breast cancer cells was studied following knockdown of UBE3A. Three pairs of small interfering RNA (siRNA) fragments targeting UBE3A were designed and transfected into the BT-549 cells. The effects of silencing UBE3A were detected by reverse transcription-polymerase chain reaction and western blotting, and the same methods were used to detect the expression levels of annexin A2. Cell proliferation was determined using the Cell Counting kit-8, and flow cytometry and a Transwell chamber assay were used to assess the rate of cell apoptosis and invasion, respectively. Following transfection with the three siRNAs targeting UBE3A for 72 h, the mRNA expression levels of UBE3A were downregulated, as compared with those in the untreated groups, and siRNA1 was shown to be the most effective siRNA for silencing UBE3A expression. The protein expression levels were concordant with the mRNA expression levels of UBE3A. In addition, the mRNA and protein expression levels of annexin A2 were downregulated. Cellular proliferation and invasion of the siRNA1 group was inhibited as compared with those in the untreated groups, and apoptosis of UBE3A-siRNA1 cells was increased as compared with that in the untreated groups. The results of the present study indicated that UBE3A may regulate the expression of annexin A2, resulting in promotion of proliferation and invasion and suppression of apoptosis in BT-549 cells.

Introduction

Ubiquitin protein ligase E3A (UBE3A), also known as human papilloma virus E6-associated protein (E6-AP), is an important member of ubiquitin proteasome systems. The E6/E6-AP complex has previously been shown to target p53 for ubiquitination and degradation through the ubiquitin-proteasome pathway (UPP) in cervical cancer (1,2). It has been reported that abnormal expression of UBE3A is associated with various types of tumor, including prostate, cervical and breast cancer (3-5). However, how the loss of function of UBE3A causes disease pathogenesis remains to be elucidated. A previous study by our group reported that UBE3A was overexpressed in breast cancer and associated with its pathological mechanism (6). Therefore, it may be hypothesized that UBE3A has an important role in breast cancer through its ubiquitin-protein ligase activity.

Previously, the degradation of various proteins has been shown to be mediated by UBE3A, including c-Myc (7), human telomerase reverse transcriptase (8), p27 (9), progesterone receptor-B protein and annexin A1 protein (10,11). To date, these cellular substrates of E6-AP have been identified and characterized; however, the significance of these interactions in disease remains to be fully elucidated.

Annexin A2 is a member of the annexin family, which has been reported to be abundantly expressed in numerous types of malignant tissue, and is believed to have an important role in tumorigenesis and cancer progression (12). A previous study demonstrated that annexin A2 could be inhibited by RNA interference (RNAi), which resulted in decreased metastasis and invasion of breast cancer cells (13). Furthermore, a previous study by our group showed that annexin A2 was overexpressed and ubiquitinated in breast cancer tissues (14). These findings suggested that annexin A2 may contribute to the pathogenesis of breast cancer, as it is mediated by ubiquitination, and it may be a novel substrate of E6-AP.
The function of UBE3A in the proliferation, invasion and apoptosis of breast cancer have remained elusive; therefore, the present study used RNAi to silence UBE3A expression in BT-549 breast cancer cells in order to determine its function. The results indicated that UBE3A is involved in carcinogenesis by regulating the levels of annexin A2 in breast cancer cells, which renders it a potential molecular marker of breast cancer and a target of breast cancer therapy.

Materials and methods

Reagents. The BT-549 human breast cancer cell line was obtained from the Cell Bank of Chinese Academy of Sciences (Beijing, China). RPMI-1640 cell culture medium was purchased from HyClone Laboratories, Inc. (Logan, UT, USA). Lipofectamine® 2000 and TRIZol® were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco Life Technologies (Carlsbad, CA, USA). Enhanced Chemiluminescence reagents. as well as goat anti-rabbit horseradish peroxidase-conjugated immunoglobulin G (1:5,000; no. 3053-1-1) secondary antibody were purchased from Epitomics, Inc. (Otsu, Japan). First Strand cDNA Synthesis kit and the polymerase chain reaction (PCR) kit were purchased from Takara Bio, Inc. (Otsu, Japan). Rabbit monoclonal UBE3A (1:1,000; no. 5571-1), rabbit polyclonal β-actin (1:2,000; no. 5779-1) and rabbit polyclonal annexin A2 (1:1,000; no. S0555) primary antibodies, as well as goat anti-rabbit horseradish peroxidase-conjugated immunoglobulin G (1:5,000; no. 3053-1-1) secondary antibody were purchased from Epitomics (Burlingame, CA, USA). Enhanced Chemiluminescence (ECL) Reagent kit, fibronectin and Transwell assay plates were obtained from EMD Millipore (Billerica, MA, USA). Cell Counting Kit-8 (CCK-8) and Annexin V-Fluorescein Isothiocyanate (FITC)/Propidium Iodide (PI) Cell Apoptosis Detection kit were purchased from Nanjing Keygen Biotech., Co., Ltd., Nanjing, China). Negative siRNA and specific UBE3A siRNAs were synthesized by Takara Bio, Inc. Matrigel® was purchased from BD Biosciences (Franklin Lakes, NJ, USA).

Cell culture. The BT-549 human breast cancer cells were cultured in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in an atmosphere containing 5% CO₂.

Western blot analysis. Total protein was extracted from the cells using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China), 72 h
post-transfection. Total protein was quantified using the bicinchoninic acid method (Beyotime Institute of Biotechnology). According to the size of the molecular weight of the protein extracts, the appropriate concentration of SDS-PAGE gel (UBE3A, 8%; annexin A2 and β-actin, 12%). Equal amounts of total protein (30 μg) were separated by SDS-PAGE. The proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (0.45 μm; EMD Millipore) in transfer buffer (Beyotime Institute of Biotechnology) by electroblotting using a Mini-Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Inc.) at 200 mA for 1-2 h. The PVDF membranes were blocked for 1 h at room temperature using blocking solution (Beyotime Institute of Biotechnology), and were then incubated with the specified dilutions of the primary antibodies for UBE3A, annexin A2 and β-actin overnight at 4°C. The membranes were then washed three times with Tris-buffered saline-Tween (15 min/wash), prior to an incubation with the recommended dilution of the labeled secondary antibody at 37°C for 1 h. The goat anti-rabbit alkaline phosphatase-conjugated antibody was detected colorimetrically using ECL Plus. Images were captured using the GelDoc XR system and were analyzed using Image Lab™ 3.0 software (Bio-Rad Laboratories, Inc.). β-actin was used as the internal control. The experiment was repeated three times.

Cell proliferation assay. The CCK-8 assay was performed to examine cell proliferation. The experiment was repeated five times. Cells (2x10⁵/well) in the logarithmic phase were cultured in 96-well culture plates. The cells were divided into the following three groups: siRNA1 group, negative control group and blank control group. The methods of cell transfection were the same as stated above. A total of 10 μl CKK-8 reagent was added at four time-points (24, 48, 72 and 96 h post-transfection) and the plates were incubated at 37°C for 2 h. The absorbance was then measured at a wavelength of 450 nm using a microplate reader (Biochrom Anthos 2010; Biochrom). The experiment was repeated three times.

Flow cytometric assay. The Annexin V-FITC/PI Apoptosis Detection kit was used to detect the rate of cell apoptosis according to the manufacturer's instructions. The experiment was repeated five times. Cells (2x10⁵/ml) in the logarithmic phase were cultured in six-well culture plates. The cells were divided into the same three groups as for the cell proliferation assay. Briefly, the cells were collected 72 h post-transfection, after being washed twice with cold phosphate-buffered saline (PBS). The cells were then suspended in 500 μl 1x binding buffer and subsequently, 5 μl Annexin V and PI were added to the cells. The cells were incubated for 15 min at room temperature in the dark, followed by flow cytometric analysis (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ, USA). The apoptotic rate was calculated as the percentage of Annexin V-positive and PI-negative cells out of the total number of cells. The assay was conducted using a flow cytometer and was analyzed using WinMDI 2.9 software (www.bioo.com).

Cell invasion assay. Transwell chambers were used to perform invasion experiments. Briefly, Matrigel® was thawed at 4°C overnight, and was then diluted with an equal amount of PBS. A total of 80 μl diluted Matrigel® was added to the upper chamber, and 30 μl fibronectin was added to the lower chamber of a 24-well Transwell plate (24-well insert; pore size, 8 μm; EMD Millipore). The Transwell chamber was then incubated at 37°C for 24 h. The cells were harvested from the cell culture plates 72 h post-transfection, and were washed three times with RPMI-1640 supplemented with 1% FBS. The cells (4x10⁴ cells) were seeded into the upper chamber, and 600 μl RPMI-1640 containing adhesive substrate (diluted Matrigel®) was added to the lower chamber. The Transwell chamber was incubated at 37°C for 24 h. The cells that did not invade through the pores were removed using a cotton swab, whereas the cells on the lower surface of the membrane were stained with crystal violet (Beyotime Institute of Biotechnology) and counted using an IX53-F32PH microscope (Olympus Corp., Tokyo, Japan). The experiment was repeated three times.

Statistical analysis. The results are presented as the mean ± standard deviation. Statistical analyses were performed by one-way analysis of variance using SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results
mRNA expression levels of UBE3A and annexin A2 in BT-549 cells. The relative mRNA expression levels of UBE3A and annexin A2 were calculated as UBU3A/β-actin and annexin A2/β-actin, respectively. The UBE3A specific siRNAs

<table>
<thead>
<tr>
<th>Name</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBE3A</td>
<td>324</td>
</tr>
<tr>
<td>Annexin A2</td>
<td>379</td>
</tr>
<tr>
<td>β-actin</td>
<td>594</td>
</tr>
</tbody>
</table>

UBE3A, ubiquitin protein ligase E3A; bp, base pairs.
effectively silenced the mRNA expression levels of UBE3A, as compared with levels in the control groups (n=3, P<0.01). In addition, following UBE3A knockdown with siRNA1, the relative mRNA expression levels of annexin A2 were down-regulated, as compared with those in the control groups (n=3, P<0.05) (Fig. 1A and Table III).

Protein expression levels of UBE3A and annexin A2 in BT-549 cells. The relative protein expression levels of UBE3A and annexin A2 were calculated as \( \frac{\text{UBE3A}}{\beta\text{-actin}} \) and \( \frac{\text{annexin A2}}{\beta\text{-actin}} \), respectively. The protein expression levels were concordant with the mRNA expression levels of UBE3A and annexin A2. siRNA1 was shown to be the most effective siRNA for silencing UBE3A mRNA as well as protein expression; therefore, the siRNA1 group was selected as the experimental group for subsequent assays (Fig. 1B and Table IV).

Cell proliferation. Proliferation of the BT-549 cells in the siRNA1 group was significantly inhibited 24, 48, 72 and 96 h post-transfection, as compared with that of the blank control group (n=5, P<0.01). However, there were no statistically significant differences detected between the negative siRNA group and the blank control group at the four post-transfection time-points (n=5, P>0.05) (Fig. 2 and Table V).

Cell apoptosis. The rate of apoptosis of the cells in the siRNA1 group, negative control group and blank control group

Table III. Relative mRNA expression levels of UBE3A and annexin A2 in BT-549 human breast cancer cells.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>UBE3A/( \beta\text{-actin} )</th>
<th>Annexin A2/( \beta\text{-actin} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA1 group</td>
<td>3</td>
<td>0.15±0.016</td>
<td>0.942±0.032</td>
</tr>
<tr>
<td>siRNA2 group</td>
<td>3</td>
<td>0.32±0.026</td>
<td>1.009±0.014</td>
</tr>
<tr>
<td>siRNA3 group</td>
<td>3</td>
<td>0.23±0.016</td>
<td>0.99±0.02</td>
</tr>
<tr>
<td>Negative siRNA</td>
<td>3</td>
<td>0.59±0.014</td>
<td>1.333±0.022</td>
</tr>
<tr>
<td>Lipofectamine 2000 group</td>
<td>3</td>
<td>0.587±0.021</td>
<td>1.326±0.018</td>
</tr>
<tr>
<td>Control group</td>
<td>3</td>
<td>0.592±0.027</td>
<td>1.336±0.026</td>
</tr>
</tbody>
</table>

\( n \), number of experiments.

siRNA, small interfering RNA; UBE3A, ubiquitin protein ligase E3A; n, number of experiments.

Figure 1. BT-549 human breast cancer cells were transfected with siRNA1, siRNA2, siRNA3 and negative control siRNA, using Lipofectamine® 2000. The cells treated with Lipofectamine® 2000 only were considered the control. Lane 1, siRNA1 group; lane 2, siRNA2 group; lane 3, siRNA3 group; lane 4, negative control group; lane 5, Lipofectamine® 2000 group; lane 6, blank control group. (A) Total RNA was extracted from the cells 72 h post-transfection. Reverse transcription polymerase chain reaction was performed to measure the relative mRNA expression levels of UBE3A and annexin A2. The PCR products were separated by 1.5% agarose gel electrophoresis. The mRNA expression levels of \( \beta\text{-actin} \) served as a loading and normalization control. (B) Protein expression levels of UBE3A and annexin A2 protein expression levels were determined by western blotting. The protein expression levels of \( \beta\text{-actin} \) served as a loading and normalization control. Data were averaged from three independent experiments. siRNA, small interfering RNA; UBE3A, ubiquitin protein ligase E3A.
was 11.104±1.1935%, 2.852±0.7213% and 2.788±0.3954%, respectively, 72 h post-transfection. The rate of apoptosis was significantly higher in the siRNA1 group, as compared with the control groups (n=5, P<0.05) (Fig. 3).

Cell invasion. The number of invasive cells in the siRNA1 group, negative control group and blank control group were 53.96±4.55, 93.16±3.91 and 94.43±3.71, respectively, 72 h post-transfection. The number of invasive cells in the siRNA group was significantly lower as compared with that in the blank control group (n=10, P<0.01) (Fig. 4).

Discussion

The UPP is an important pathway of protein degradation for numerous proteins vital to cellular regulation and function. Furthermore, the ubiquitin-proteasome system is a crucial regulator of the cell cycle, and it is well-known that abnormal cell cycle control may lead to oncogenesis (15-17). A previous study demonstrated that UPP dysregulation has an important role in mammary tumorigenesis (18). UBE3A is an important member of the ubiquitin-ligase family and is one of the key enzymes associated with the maintenance of normal cellular physiological functions (15). Abnormal alterations of UBE3A may cause the development of various diseases.

RNAi has recently been identified as being capable of gene silencing. RNAi is mediated by double-stranded RNA, which can degrade specific target mRNAs (4). The present study used chemically synthesized siRNAs to suppress the expression of UBE3A. Chemical synthesis of siRNA has previously been shown to effectively block gene expression (19). According to the principle of RNAi, specific siRNAs were synthesized in vitro, and were then transfected into BT-549 human breast cancer cells. The interference effects were detected using semi-quantitative RT-PCR and western blotting. The results of the present study demonstrated that three specific siRNAs were able to effectively inhibit the expression of UBE3A. siRNA1 was shown to exhibit the best interference effect. Following effective UBE3A gene knockdown, the expression levels of annexin A2 post-transfection. The number of invasive cells in the siRNA group was significantly lower as compared with that in the blank control group (n=10, P<0.01) (Fig. 4).
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were downregulated. This may have an adverse effect on certain proteins that are degraded in an E6-AP-dependent manner. Shimoji et al (10) previously reported that RNAi-mediated downregulation of endogenous E6-AP increased the levels of endogenous annexin A1 protein, but had no effect on the accumulation of endogenous annexin A2 protein.

The present study aimed to determine why the expression of annexin A2 was downregulated following knockdown of UBE3A expression. The results suggested that UBE3A may have a role in controlling the functions of annexin A2 in breast cancer cells. It may be hypothesized that ubiquitinated annexin A2 was not degraded, but its function, activity or position may have been altered, which was regulated by UBE3A in BT-549 breast cancer cells. Another hypothesis may be that following knockdown of UBE3A, annexin A2 may be degraded via UPP, or another pathway. Previous studies have suggested that polyubiquitinated protein may be generally degraded by the 26S proteasome; however, if polyubiquitinated proteins are not connected to the target protein 48 or 63-bit lysines, but to the 6, 11, 27, 29 and 33 lysines, the ubiquitinated proteins may not be degraded, but their functions may be altered (20,21). Furthermore, monoubiquitinated proteins may not be degraded; however, their activity, positioning or structure may be modified, thus regulating the endocytosis pathway, histone modification, gene transcription and nuclear protein localization (22). Future studies may detect the ubiquitinated position or numbers of ubiquitinated annexin A2 in the BT-549 breast cancer cells prior to as well as following UBE3A RNAi, in order to further study the association between annexin A2 and UBE3A. Exploring the role of UBE3A in the transcriptional regulation of annexin A2 or other genes may be an interesting area of research.

Treatment and prognosis of breast cancer are complex and are associated with numerous factors in vivo and in vitro. Cell proliferation, apoptosis and invasion are considered the most important factors affecting the treatment and prognosis of breast cancer. In the present study, a CCK-8 assay, flow cytometry and a Transwell assay were used to detect the proliferation, apoptosis and invasion of BT-549 cells following silencing of UBE3A. The results demonstrated that the proliferation of BT-549 cells was restrained following transfection with UBE3A siRNA. The rate of apoptosis of the cells in the siRNA1 group was increased, and the invasiveness of the cells was decreased. All of these differences were statistically significant when comparing the siRNA1 group with the control groups.

The present study aimed to determine how UBE3A could influence proliferation, apoptosis and invasion of breast cancer cells. A previous study demonstrated that UBE3A was able to impact the function of cells, including transcription, signal transduction, cell survival, cell cycle control and DNA repair (23). In addition, it has been reported that UBE3A can promote proliferation and inhibit apoptosis of cervical cancer cells (4). In prostate cancer cells, UBE3A has been shown to regulate the phosphoinositide 3-kinase-Akt pathway, which
results in increased prostate cell growth, proliferation and decreased apoptosis (24). Therefore, it was hypothesized that in breast cancer cells, UBE3A may affect cell signaling pathways and influence cell biological behavior.

The present study also demonstrated that following knockdown of UBE3A, the expression of annexin A2 was downregulated. Annexin A2 has previously been demonstrated to promote the development, invasion and metastasis of breast cancer (13), which may explain the simultaneous influence on proliferation, apoptosis and invasion of breast cancer cells. Furthermore, UBE3A may regulate other cancer-associated proteins, which have important roles in the pathogenesis of breast cancer and directly or indirectly lead to cancer through regulating cell proliferation, migration, invasion and apoptosis. E6-AP has also been reported to promote the degradation of p53 (3,25,26). Altered degradation of p53 may also affect cell cycle progression and induce apoptosis. A future aim of our group is to use two-dimensional polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry incorporated with online database research to identify the effect of knockdown of UBE3A expression on the levels of various proteins.

In conclusion, the results of the present study indicated that UBE3A has a role in regulating the functions of annexin A2 and a positive role in the development of breast cancer. These results suggested that UBE3A may be a potential marker for treatment of breast cancer. However, the specific mechanisms underlying the functions of UBE3A in breast cancer require further research.

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