The tumor suppressor OVCA1 is a short half-life protein degraded by the ubiquitin-proteasome pathway

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Abstract. Ovarian cancer gene 1 (*OVCA1*) is a tumor suppressor associated with ovarian cancer, which is involved in cell proliferation regulation, embryonic development and tumorigenesis. Loss of heterozygosity in the *OVCA1* gene occurs in 50-86% of cases of ovarian cancer; however, the physiological and biochemical functions of OVCA1 are not yet clear. In the present study, the stability and degradation of OVCA1 were investigated in A2780, Hela and 293 cells. The results revealed that the OVCA1 protein was unstable by MG132 inhibiting proteasome mediated degradation, co-immunoprecipitation and half-life measurement experiments. The cellular protein levels of endogenous OVCA1 were too low to be detected by western blotting. In addition, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal inhibited the degradation of OVCA1 in cells. The co-immunoprecipitation assay revealed that the OVCA1 protein interacted with ubiquitin to form a poly-ubiquitinated complex in cells. The half-life of OVCA1, measured by inhibiting protein synthesis with cycloheximide, was <2 h. The present study demonstrated that OVCA1 may be degraded by the ubiquitin-mediated proteasome pathway and may be considered a short half-life protein. In conclusion, the regulation of OVCA1 protein degradation via the ubiquitin-proteasome pathway may represent a novel direction in the development of ovarian cancer therapy.

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Abbreviations: CHX, cycloheximide; ECL, enhanced chemiluminescence; MG132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; UPP, ubiquitin-proteasome pathway; PI, propidium iodide

Key words: co-immunoprecipitation, OVCA1, stability, ubiquitin-mediated protein degradation

Introduction

Ovarian cancer gene 1 (*OVCA1)* is a tumor suppressor gene located on chromosome 17p13.3 (1). Loss of heterozygosity in the *OVCA1* gene occurs in 50-86% of cases of ovarian cancer (1-5). *OVCA1* was originally identified in 1996 (1,6). Since its sequence is highly similar to that of the yeast diphthamide biosynthesis protein 2, the *OVCA1* gene was previously referred to as diphthamide synthesis protein 2-like (7). Previous studies have demonstrated that *OVCA1*, also termed *DPH1* (diphthamide biosynthesis 1), is involved in the biosynthesis of diphthamide by interacting with the eukaryotic translation elongation factor 3 (8-12). OVCA1 has an important role in the regulation of cell proliferation, embryonic development and tumorigenesis (4,13-20). It inhibits the proliferation of epithelial ovarian cancer cells and blocks the cell cycle at G_1 phase (4,19) by decreasing cyclin D1 and increasing p16, a tumor suppressor protein (19). Previous studies have demonstrated that *OVCA1*-mutant mice do not survive during embryonic development or after birth, due to developmental delay and defects in multiple organ systems, and that OVCA1 is involved in p53 deficiency-induced tumorigenesis (14,18). Abnormality in *OVCA1* occurs prior to defects in *p53* and breast cancer 1 gene, and is therefore considered an early event in ovarian tumorigenesis (6,14,18,21). The close link between *OVCA1* and *p53* on human chromosome 17 suggests that they may have synchronized effects in cancer development (14). However, the biological function of OVCA1, and its role in tumor occurrence and development have not yet been determined.

To reveal the biological function of OVCA1 and its association with ovarian cancer, commercial antibodies were used to try and detect OVCA1 in cells. However, the results demonstrated that endogenous OVCA1 could not be observed by western blotting with commercial antibodies,despite the large panel of anti-OVCA1 antibodies tested. The mRNA expression levels of *OVCA1* were detected by reverse transcription-quantitative polymerase chain reaction (data not shown). Currently, it is well established that the stability of tumor suppressors is positively associated with their functions. In order to explain these phenomena and to further understand the functions of OVCA1, the degradation pathway of OVCA1 and its half-life were investigated in this study.

Materials and methods

Cell lines, antibodies, and plasmids. 293, Hela and A2780 cell lines were purchased from the Institute of Shanghai Biochemistry Cell Biology (Shanghai, China). Mouse monoclonal anti-green fluorescence protein (GFP) antibody (cat. no. TA06), mouse monoclonal anti-GAPDH antibody (cat. no. TA08) and horseradish peroxidase labeled goat anti-mouse immunoglobulin G antibody (catalog no. ZB-2305) were purchased from ZSGB-BIO Company (Beijing, China). Mouse monoclonal anti-ubiquitin antibody (cat. no. sc-8017) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). A GFP-tagged-*OVCA1* plasmid was designed by inserting full-length *OVCA1* into the *Xho*I and *Eco*RI sites of a pEGFP-C1 vector (Clontech Laboratories, Inc., Mountainview, CA, USA).

Cell culture and transfection. Hela and 293 cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% (v/v) newborn calf serum (Gibco; Thermo Fisher Scientific, Inc.) 50 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.), and 50 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.). A2780 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 50 U/ml penicillin (Invitrogen, Thermo Fisher Scientific, Inc.) and 50 μg/ml streptomycin (Invitrogen, Shanghai, China). Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2. Cells were seeded at a density of $3x10^5$ cells/well into 12-well culture plates and were transfected with Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Briefly, 1 μg plasmid DNA was diluted in 50 μl DMED and 4 μl Lipofectamine® 2000 reagent was diluted in 50 μl DMEM. The diluted plasmid was mixed with the diluted lipofectamine® reagent, and was incubated for 15 min at temperature. The mixture was added into a 70-90% confluent cell monolayer. The cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2. GFP and GFP-tagged-OVCA1 protein in the cells transfected with empty vector or GFP-tagged-*OVCA1* were observed directly with a fluorescence microscope.

Cell viability assay. Cell viability was measured by MTT assay. Cells were seeded into 96-well plates (Corning Incorporated, Corning, NY, USA) at $2x10^4$ cells/well. After 24 h, cells were treated with various concentrations of carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132; Merck KGaA, Darmstadt, Germany) for 24 h at 37˚C in a humidified atmosphere containing 5% CO_2. Cell viability was assessed using an MTT assay at 490 nm using a microplate reader.

Flow cytometry. Cells were seeded into 6-well plates (Corning Incorporated, Corning, NY, USA) at $1x10^6$ cells/well. After 24 h, cells were treated with various concentrations of MG132 for 24 h at 37˚C in a humidified atmosphere containing 5% CO_2. Cells were then fixed with 70% ethanol at 4˚C for 12 h, washed with PBS, and resuspended in 500 μl PBS containing 45 μl RNase A and 405 μl propidium iodide (PI) from the Cell Cycle Detection kit (catalog no. KGA512; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China), and incubated at room temperature for 30-60 min in the dark. Cell proliferation was determined by flow cytometry (BD FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

Western blotting. Cells were lysed in lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and centrifuged at 12,000 x g for 5 min. The supernatant was collected as whole cell lysates, and protein concentration was assessed using the Bradford assay (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The whole cell lysate (40 μg) was separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Merck KGaA). After 2 h blocking at room temperature with TBST [20 mmol/l Tris-HCl (pH 7.4), 137 mmol/l NaCl and 0.1% Tween-20] containing 5% (w/v) skimmed milk powder, the membranes were incubated with primary antibody (diluted to 1:1,000 with TBST containing 5% skimmed milk powder) overnight at 4˚C, and were later incubated with horseradish peroxidase-conjugated secondary antibody (diluted to 1:5,000 with TBST containing 5% skimmed milk powder) for 2 h at room temperature. An ECL™ Prime Western Blotting system (catalog no. RPN2232; Merck KGaA) was used to detect protein bands.

Co-immunoprecipitation. Total protein lysate (200 μl) was incubated with 2 μl anti-GFP monoclonal antibody and 40 μl protein A+G agarose (Beyotime, Institute of Biotechnology) at 4˚C for 3 h. After 5 min centrifugation at 2,500 x g, the supernatant was discarded and the precipitate was washed five times with PBS and resuspended in 40 μl 1X SDS-PAGE loading buffer (Beyotime Institute of Biotechnology). After boiling for 5 min, the supernatant was subjected to immunoblotting.

Half-life measurement. After 24 h of transfection with *OVCA1*, cells were cultured in medium containing MG132 (20 μmol/l for Hela and 293 cells, and 5 μmol/l for A2780 cells). After 24 h, cells were cultured in fresh medium containing cycloheximide (CHX; Sigma-Aldrich; Merck KGaA) at a final concentration of 50 μg/ml, with or without MG132, for 0, 30, 60, 120, 180, 240 and 300 min, and were subsequently lysed for western blotting. The bands of OVCA1 and GAPDH were scanned, and the intensities of the bands were semi-quantified by ImageJ 1.46r software (National Institutes of Health, Bethesda, Maryland, USA). The relative concentration of OVCA1 at time 0 was defined as 100%. The percentage of OVCA1 at each indicated time point was normalized by comparing the relative concentration of OVCA1 with that at time 0. The protein half-life was calculated by linear regression analysis.

Statistical analysis. Data were from at least three independent experiments were expressed as the mean ± standard deviation. Data were analyzed by one-way analysis of variance followed by Fisher's least significant difference or Tukey's test to compare the means or by bivariate Pearson correlation analysis with SPSS 17.0. (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference. GraphPad Prism version 6 (GraphPad Software, Inc., La Jolla, IL, USA) was used to create the illustrations.

Results

Detection of OVCA1 protein in cells. To the best of our knowledge, only six studies have demonstrated the detection of OVCA1 by western blot analysis (4,8,13,14,18,22). Liang *et al* (18) identified OVCA1 protein in mouse cells by western blotting using a commercial anti-OVCA1 antibody (cat. no. ab40733; Abcam, Cambridge, UK). This antibody and two other anti-OVCA1 antibodies: ab54777 (Abcam), and bs-5714R (BIOSS, Beijing, China) were used in the preliminary experiments. However, no endogenous OVCA1 was observed using these three commercial antibodies by western blotting in any of the cell lines used (Hela, 293 and A2780 cell lines). Subsequently, exogenous GFP-tagged-OVCA1, which was over expressed in Hela cells, was used to assess these three commercial anti-OVCA1 antibodies [cat. nos. ab40733 and ab54777 (Abcam) and BS-5714R (BIOSS)]. After 24 h transfection, only weak signals in the cells transfected with GFP-tagged-OVCA1 were detected by fluorescence microscopy, and the cell transfection score was lower than in the control cells, which were transfected with a GFP vector only (Fig. 1A). After 48 h transfection, the fluorescence intensity of the cells transfected with GFP-tagged-OVCA1 was slightly increased compared with at 24 h (Fig. 1A). Subsequently, the GFP-tagged-OVCA1 protein from these transfected cells was detected by western blotting; however no band was observed after membrane incubation with the aforemention commercial anti-OVCA1 antibodies (data not shown). The membranes were stripped and re-probed with anti-GFP antibody, and a weak band of GFP-tagged-OVCA1 was observed at ~75 kD (Fig. 1B). Due to the low level of OVCA1 protein and the high death rate of transfected cells, which was also described by Bruening *et al* (4), GFP-tagged-OVCA1 was used in this study to help visually estimate the transfection efficiency and the protein expression levels. Small tags, such as hemagglutinin and Myc, cause less disturbance than GFP, but when no or a very weak OVCA1 band is observed, it is difficult to differentiate whether it is a result of high death rate or low transfection efficiency, which may influence the reliability of the results. Therefore in this study, exogenous GFP-OVCA1 was preferentially assessed, and only the results detected with anti-GFP antibody were presented.

OVCA1 degradation is inhibited by MG132. To investigate the pathway of OVCA1 degradation, MG132, a common 26S proteasome inhibitor, was used to inhibit the proteasome degradation pathway, which is the main pathway for cellular protein turnover (23). Since MG132 inhibits cell proliferation and induces apoptosis (24-27), the highest concentrations of MG132 that did not influence cell growth were assessed with MTT assay and determined to be 20 μmol/l in Hela and 293 cells, and 5 μmol/l in A2780 cells (Fig. 2Aa). The effects of MG132 on cell proliferation was dose-dependent (Fig. 2Aa). These results were verified by flow cytometry (Fig. 2Ab).

Hela, 293, and A2780 cells were transfected with GFP-tagged-*OVCA1*. In the absence of MG132 treatment, the fluorescence intensities of GFP-OVCA1 levels were low (Fig. 2B). Following treatment with increasing doses of MG132, the fluorescence intensities of GFP-OVCA1 levels

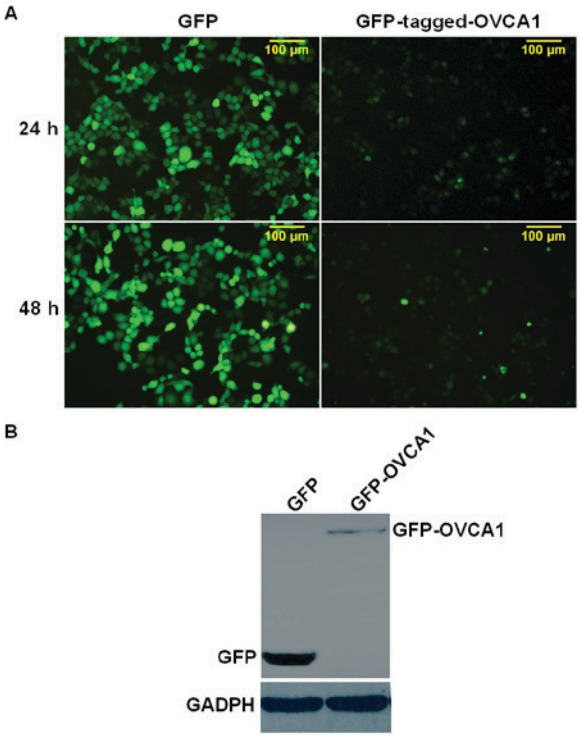

Figure 1. Expression of GFP-tagged-OVCA1 in cells. (A) GFP-tagged-*OVCA1* was transfected into Hela cells, and fluorescence intensity was observed by immunofluorescence microscopy 24 or 48 h post-transfection. (B) GFP-tagged-OVCA1 proteins were detected by western blotting with anti-GFP antibody 48 h post-transfection. Cells transfected with empty vector were used as controls. Scale bar is 100 μm. GFP, green fluorescence protein; *OVCA1*, ovarian cancer gene 1.

were detected and the levels of GFP-tagged-OVCA1 protein in the cells were significantly augmented in a dose-dependent manner (Fig. 2B). The fluorescence intensities and protein levels of GFP in the control cells, which were transfected with empty vector, were not modified following MG132 treatment (Fig. 2C), which was already demonstrated by Gong *et al* (28). These findings demonstrated that OVCA1 degradation may be inhibited by MG132 in various cell lines.

OVCA1 is degraded by the ubiquitin-proteasome pathway (UPP). To confirm that OVCA1 degradation is mediated by the UPP, the interaction between OVCA1 and ubiquitin was determined by co-immunoprecipitation. Hela, 293 and A2780 cells were transfected with GFP-tagged-OVCA1. To overcome the problem of low GFP-tagged-OVCA1 protein detection, cells treated with MG132 were also used (Fig. 3). GFP-tagged-OVCA1 protein in total cell lysates was pulled down with anti-GFP antibody, and the proteins were analyzed with anti-GFP antibody (Fig. 3A, C and E) to check the pull-down effect. The interaction of GFP-tagged-OVCA1 with ubiquitin was checked with anti-ubiquitin antibody (Fig. 3B, D and F). The protein pull down was also attempted with anti-ubiquitin antibody and by analyzing the interaction with anti-GFP antibody; unfortunately, no band was detected (data not shown). This may be due to a low level of OVCA1 proteins in the cell lysates and therefore, a low percentage of ubiquitinated OVCA1 protein among the pulled-down ubiquitinated proteins. The

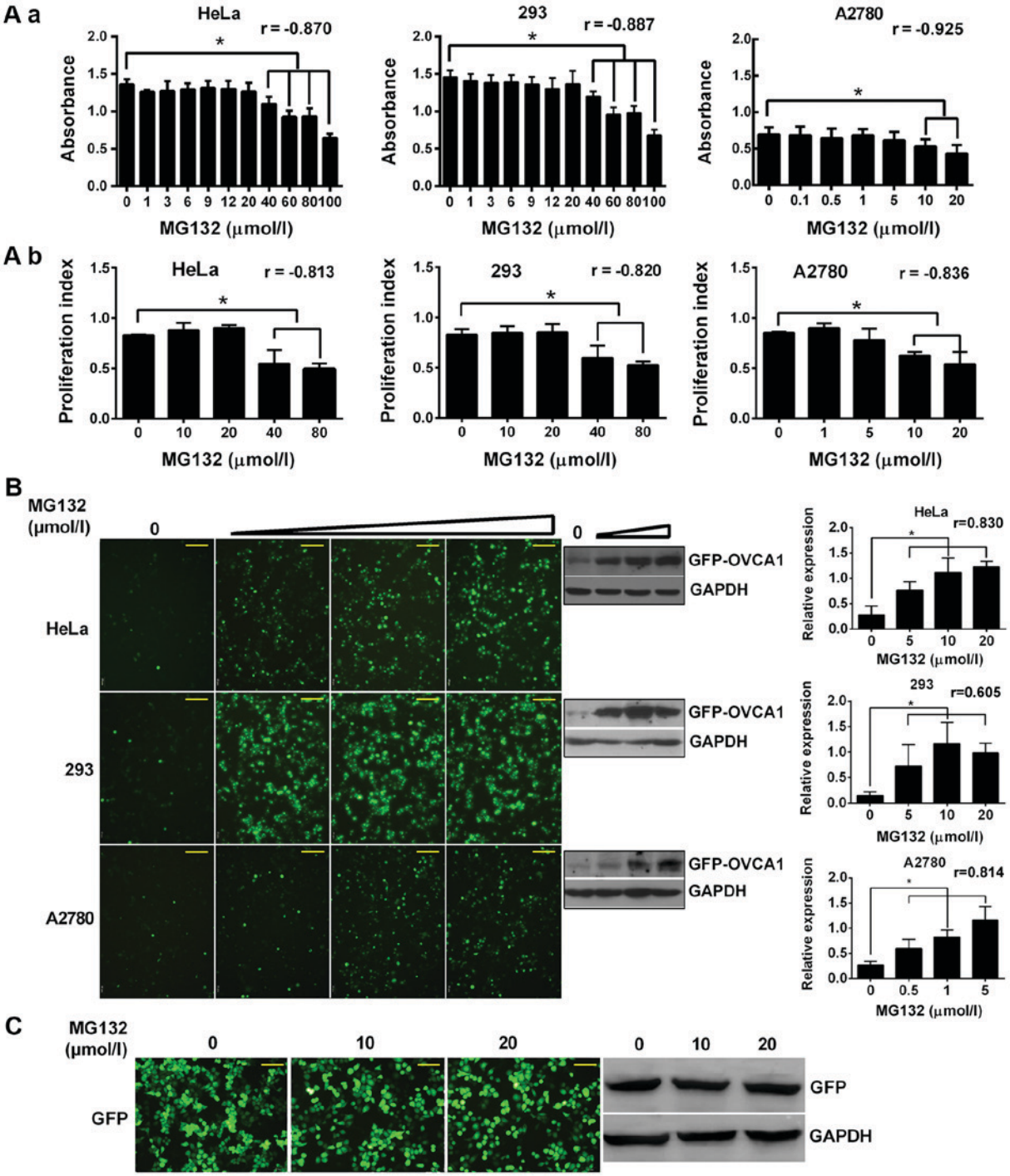

Figure 2. (A) OVCA1 degradation was inhibited by MG132. Cells were seeded and cultured for 24 h and were treated with various final concentrations of MG132, as indicated, for 24 h. The effects of MG132 on cell proliferation were then detected by (Aa) MTT assay or by (Ab) flow cytometry after propidium iodide staining. Pearson correlation coefficients (r) were indicated in the figure. (B) OVCA1 degradation was blocked by MG132 in a dose-dependent manner. Cells were transfected with GFP-tagged-*OVCA1*, as indicated. After 24 h, transfected cells were treated with various concentrations of MG132 (0, 5, 10 and 20 μmol/l for Hela and 293 cells; 0, 0.5, 1 and 5 μmol/l for A2780 cells) for another 24 h. The fluorescence intensities were observed by fluorescence microscopy and OVCA1 levels were detected by western blotting using an anti-GFP antibody. Relative GFP-tagged-OVCA1 expression was normalized to the internal control GAPDH. (C) GFP protein level was not regulated by MG132. Hela cells were transfected with empty pEGFP-C1 vector. After 24 h, cells were treated with various concentrations of MG132, as indicated, for another 24 h. The fluorescence intensities of GFP were observed by fluorescence microscopy, and GFP protein levels were detected by western blotting. Scale bar, 100 μm. *P<0.05. GFP, green fluorescence protein; MG132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; *OVCA1*, ovarian cancer gene 1.

results indicated that OVCA1 binds to ubiquitin and forms poly-ubiquitinated OVCA1.

OVCA1 may be a short half-life protein. To reveal the half-life of OVCA1 degradation in cells, CHX, which inhibits protein synthesis in eukaryotic organisms by disturbing the translocation step (29), was used to block the synthesis of proteins in cells. Hela, 293 and A2780 cells were transfected with GFP-tagged-*OVCA1*. Prior to cell incubation with CHX, cells were treated with MG132, in order to block OVCA1 protein

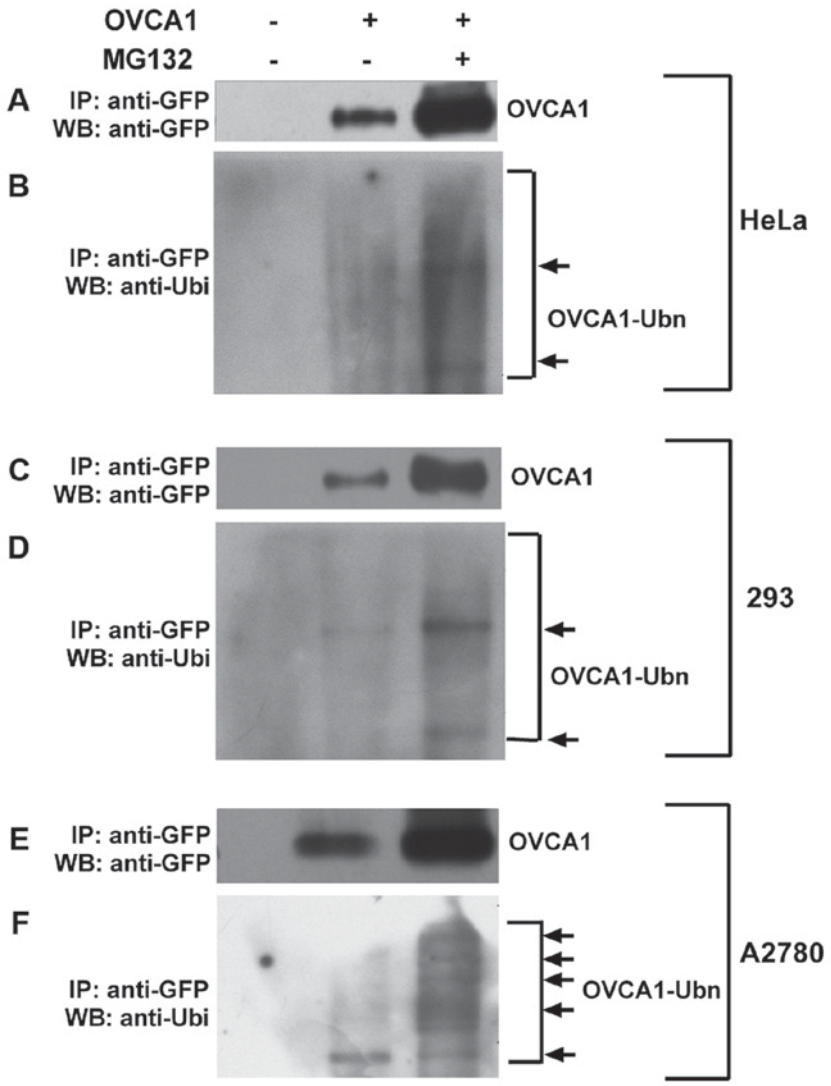

Figure 3. Interaction between OVCA1 and ubiquitin in cells. Cells transfected with GFP-tagged-*OVCA1* were treated with or without MG132 as indicated (final concentration of MG132 was 20 μmol/l for Hela and 293 cells, and 5 μmol/l for A2780 cells). Co-IP was then performed. The proteins in total cell lysates were pulled down with anti-GFP antibody. (A, C and E) Pull-down effect; (B, D and F) interaction between GFP-tagged-OVCA1 and ubiquitin. The poly-ubiquitination ladders are indicated with arrowheads. GFP, green fluorescence protein; IP, immunoprecipitation; MG132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; *OVCA1*, ovarian cancer gene 1; Ubi, ubiquitin; Ubn, polyubiquitin.

degradation and therefore allow the protein detection necessary to carry out the subsequent experiments. After 24 h of treatment with MG132, cells were treated with 50 μg/ml CHX alone for 0, 30, 60, 120, 180, 240 and 300 min. Cells were treated with CHX and MG132 together for 300 min as a control. The levels of GFP-tagged-OCVA1 at these time points were detected. Because A2780 cells died during CHX treatment, this experiment was only performed in Hela and 293 cells. The results demonstrated that the protein expression levels of OVCA1 in cells decreased with time and had completely disappeared after 300 min of CHX treatment. However, when the cells were treated with CHX and MG132 together for 300 min, the protein expression levels of GFP-tagged-OVCA1 remained high (Fig. 4A), thus suggesting that the loss of GFP-tagged-OVCA1 with time may be caused by ubiquitin-mediated protein degradation. The linear regression analysis demonstrated that the GFP-tagged-OVCA1 half-life was of 105 and 118 min in Hela and 293 cells, respectively (Fig. 4B). These results demonstrated that the OVCA1 protein may have a short half-life.

Discussion

The deletion or mutation of tumor suppressor genes has an important role in the development of cancer. *OVCA1* is a tumor suppressor gene, which may be deleted or mutated in ovarian and breast cancer (3,4,6). In addition, the over-expression of *OVCA1* inhibits cell proliferation (4,14,19,20). In the present study, the cellular OVCA1 protein levels were demonstrated to be very low. Controlling the stability of cellular proteins is a fundamental way of regulating cell proliferation, survival and development, particularly tumor suppressors. The assessment of a protein half-life is one of the first steps to check whether the function of a protein is regulated by proteolysis under specific physiological conditions. The half-life of OVCA1 measured in this study was 105 and 118 min in Hela and 293 cells, respectively, thus suggesting that OVCA1 may be a short half-life protein. In the present study, the endogenous protein levels of OVCA1 in cells were too low to be detected by western blotting,

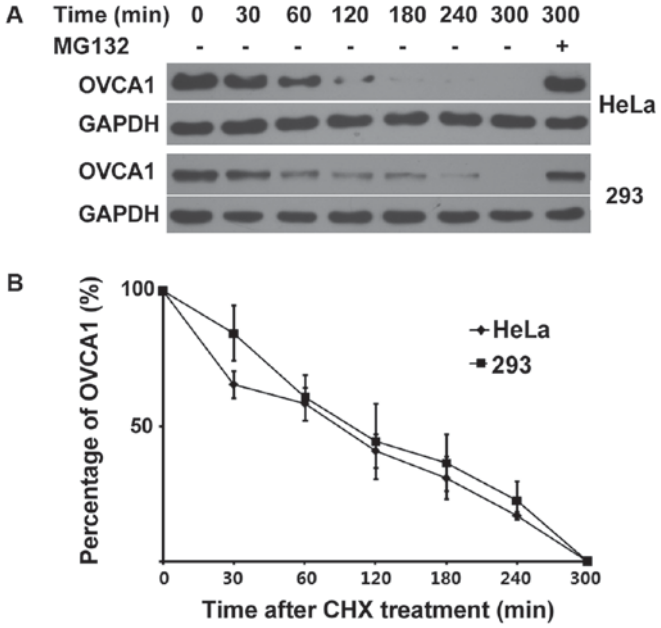

Figure 4. Half-life of OVCA1. Cells were transfected with GFP-tagged-*OVCA1* and were treated with MG132 at a final concentration of 20 μmol/l for 24 h, prior to treatment with CHX, for the indicated time. (A) Total cell lysates were used for western blotting. (B) Relative concentration of OVCA1 at each indicated time point was calculated. Data were from three independent experiments. CHX, cycloheximide; MG132, carbobenzoxy-L-leucyl-L-leucy l-L-leucinal; *OVCA1*, ovarian cancer gene 1.

transfection efficiency was also low and the death rate of *OVCA1*-transfected cells was high (data not shown). Consequently, the GFP-fused OVCA1 protein was used to observe transfection efficiency and to avoid alterations in OVCA1 protein levels caused by low transfection efficiency or cell death. Although the stability of GFP is not affected by MG132 (28), and it is often used as a tag protein for studies on ubiquitin-mediated protein degradation (30,31), being able to measure either endogenous OVCA1 or a suitable smaller tag fusion protein would be more convincing.

Cellular proteins are degraded through various pathways, including the lysosomal pathway, the UPP and the caspase pathway (32,33). The UPP is a specific protein degradation pathway, which is essentially responsible for the degradation of most intracellular proteins (32). Ubiquitin first attaches to a target protein, involving three critical enzymes, the ubiquitin activating enzyme, the ubiquitin-conjugating enzyme and the ubiquitin ligase, and forms protein complexes. The protein complexes are then recognized by the 26S proteasome, a large multi-enzyme complex responsible for protein degradation (34-36). MG132 is an inhibitor commonly used to block the proteolytic activity of the 26S proteasome complex (35). In the present study, cellular OVCA1 protein levels were significantly increased following MG132 treatment, thus indicating that OVCA1 may be degraded by the UPP. The subsequent co-immunoprecipitation experiment confirmed that OVCA1 can interact with ubiquitin in cells. These results suggested that the UPP may be one of the pathways allowing the OVCA1 protein degradation.

Modification of ubiquitination serves an important role in regulating protein stability and activity, and is closely associated with the regulation of biological processes and the development of numerous diseases, such as cancer, neurodegenerative diseases and autoimmune diseases (37). The ubiquitin-proteasome system has therefore become an important target for drug screening, and research on the ubiquitination process has become crucial in drug development (38,39). In this study, the OVCA1 was demonstrated to be a short half-life protein that was degraded by the UPP. The molecules involved in the UPP-associated OVCA1 degradation are currently being investigated. Novel molecules targeting the UPP, and hence, regulating the stability or degradation of tumor suppressors have already shown great promise in the treatment of some types of cancer (38,39). Regulating OVCA1 protein degradation may therefore represent a novel target in the treatment of ovarian cancer.

In conclusion, the present study demonstrated that the OVCA1 protein was degraded by the UPP and may be a short half-life protein. These findings provided a potential novel direction for ovarian cancer therapy by regulating OVCA1 protein via UPP. Since endogenous OVCA1 levels were too low to be detected, GFP-tagged OVCA1 was determined in the study. The development of novel techniques for the detection of endogenous OVCA1 is therefore crucial. The signaling pathways of OVCA1 degradation through the UPP will be further investigated in order to provide potential novel targets for the treatment of ovarian cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YWL and FDK participated in the design of the study, the writing and revising of the manuscript, the generation of the figures, cloning of the gene and flow cytometry. YL performed MTT, immunoprecipitation, cloning of the gene and western blotting experiments. YHW partly performed the statistical analysis and flow cytometry, and LS partly performed western blotting experiments. CYZ contributed to the conception and design of the study, was involved in drafting and revising the manuscript, and gave final approval of the manuscript to be published. All authors read and approved the final manuscript.

Ethical approval and consent to participate

Not applicable.

Patient consent for application

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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