RESEARCH COMMUNICATION

Beclin1 Overexpression Inhibitis Proliferation, Invasion and Migration of CaSki Cervical Cancer Cells

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Abstract

The influence of the autophagy-related gene *Beclin1* on proliferation, invasion and metastasis of the cervical cancer CaSki cells and its possible mechanism in vitro were here targeted. After the overexpression vector pcDNA3.1-Beclin1 and RNA interference vector pSUPER-Beclin1 were transfected into CaSki cells in vitro, stable expression cell lines demonstration Beclin1 expression was upregulated, and VEGF and MMP-9 expression were decreased, leading to cell arrest in the G0/G1 phase of the cell cycle. MTT assays further revealed proliferation of cells was significantly inhibited in Beclin1-overexpressing transfectant cells, with invasion and metastasis also being inhibited in Transwell chamber assays. The present results suggest that Beclin1 inhibits invasion and metastasis of cervical cancer CaSki cells in vitro. Mechanisms probably involve Beclin1 inhibition of cell proliferation, and decreased expression of VEGF and MMP-9 proteins.

Keywords: *Beclin1* - autophagy - proliferation - invasion - metastasis - cervical cancer cells

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Introduction

Autophagy is one of the main types of programmed cell death different from apoptosis, and it is a catabolic process involving the degradation of cell's own components (damaged macromolecules and organelles) through lysosomal machinery. The main role of this process is to maintain the balance between the synthesis, degradation, and subsequent recycling of cellular products. As great attention has been paid to apoptosis last century, autophagy has been a hot issue in recent research. The functional relationship between apoptosis and autophagy is complex in the sense that, in several scenarios, autophagy constitutes a stress adaptation that avoids cell death, whereas in other cellular settings, autophagy constitutes an alternative pathway to cellular demise that is called autophagic cell death (Maiuri et al., 2007).

Nowadays, increasing studies have demonstrated the changes in autophagy and/or cell death due to autophagy are closely associated with the occurrence and development of malignancies. Studies also report autophagy may be a promising target for anti-cancer therapy in the future. The present study aimed to investigate the effect of autophagy-related gene Beclin 1 on the proliferation, invasion and migration of cervical cancer CaSki cells. Our results may provide novel therapeutic target in the treatment of cervical cancer.

Materials and Methods

Culture of CaSki cells and transfection

The human cervical cancer CaSki cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were grown in RPMI-1640 (Sigma, St. Louis, MO, USA) supplemented with heat inactivated 10% fetal bovine serum (Gibco, Grand Island, NY, USA), penicillin (100 U/ml; Sigma), and streptomycin (100 μg/ml; Sigma) at 37°C with 5% CO2. Cells in the logarithmic phase were digested with trypsin and then seeded in a 6-well plate (5×10^5/well). PcDNA3.1-Bec (Beclin1 gene overexpressed) and pSUPER-Bec (Beclin1 gene partially-silenced) transfectants were independently used to transfect CaSki cells according to manufacturer's instructions of lipofectamine2000 (Invitrogen, Carlsbad, CA, USA). After 2 days, fresh medium was added to the cells containing the selection reagent, G418 (500μg/ml; Gibco). Selection was continued for 14 days, with the medium refreshed every other day. The cell transfection efficiency was determined using a 200-fold phase contrast microscopic to view 10 regions under fluorescence as a percentage of the total cells. Two weeks later, positive colonies were obtained and cells were dilutely re-suspended in medium followed by culture, aiming to achieve cells with stable expression of Beclin1. Cells successfully transfected with pcDNA3.1-

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Bec and pSUPER-Bec were defined as pcDNA3.1-Bec group and pSUPER-Bec group, respectively. In addition, cells transfected with pcDNA3.1(empty vector) and pSUPER-non(scramble RNA) were defined as pcDNA3.1 group and pSUPER-non group, respectively. CaSki cells without transfection served as blank control. Two plasmids encoding human pcDNA3.1-Bec and pSUPER-Bec were kindly provided by Gynecologic Tumor Lab of West China Second Hospital of Sichuan University. The recombinant vectors were confirmed by digestion analysis of restriction endonuclease, and all the constructed plasmids were confirmed by DNA sequencing.

Detection of Beclin1 mRNA expression by real time quantitative fluorescence PCR

Total RNA was extracted with Trizol and 2 μg of total RNA were used for reverse transcription. House keeping gene β-actin served as the internal reference. Real time quantitative fluorescence PCR was performed in triplicate. Primers for Beclin1 were 5'-AGGAACTCACAGCTCCATTAC-3' (forward) and 5'-AATGGCTCCTCTCCTGAGTT-3' (reverse). The TaqMan probe was 5'-AGCCCAGGCGAAACCAGGAGA-3' and the expected length of products was 176 bp. The PCR conditions were as follows: pre-denaturation at 94°C for 2 min and 45 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 30 s and extension at 60°C for 40 s followed by a final extension at 72°C for 5 min. The increase index of fluorescence intensity at each cycle was obtained and kinetic curves of PCR amplification were delineated. Then, the Ct value (representing intensity of fluorescence) and ΔCt (difference of Ct between samples and internal reference) were calculated for each sample, and $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of target gene (Beclin1).

Detection of protein expression of Beclin1, VEGF and MMP-9 by western blot

Cells were harvested and washed with PBS thrice. These cells were lysed in lysis buffer at 4°C for 30 min and then centrifugated at 14000 rpm for 10 min. The supernatant was collected and the protein concentration was determined. Then, 50 μg of total protein were subjected to SDS-PAGE and subsequently transferred onto PVDF membranes which were then blocked with 5% non-fat milk for 1 h. These membranes were then treated with rabbit anti-human Beclin1 antibody, VEGF antibody and MMP-9 antibody (Cell Signaling Technology, MA, USA) independently at 4°C overnight under continuous shaking. These membranes were washed with TBST containing 0.1% Tween20 three times and then incubated with horseradish peroxidase conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology, CA, USA) at room temperature for 1 h. Specific bands were detected by an enhanced chemiluminescence system (Pierce, Rockford, IL, USA). Anti-actin was used to ensure equal loading. Band intensity was semi-quantified using Photoshop Elements software after conversion to digitalizing image using an image scanner (GT9700F; Epson, Tokyo, Japan). Western blot assay was performed in triplicate.

Table 1. Real-time RT-PCR for Detecting the Expression of Beclin1 mRNA

Group	ΔCt	$2^{-\Delta\Delta Ct}$
Untransfected cells	5.8±0.12	1.07±0.11
pcDNA3.1-Bec	0.7±0.02	36.27±2.89*
pcDNA3.1	5.9±0.09	0.93±0.13
pSUPER-Bec	7.8±0.11	0.26±0.02*
pSUPER-non	6.1±0.08	0.87±0.09

*significantly different from untransfected cells ($p<0.01$)

Delineation of growth curves

Cells in pcDNA3.1-Bec group, pcDNA3.1 group, pSUPER-Bec group, pSUPER-non group and blank control group were seeded in a 96-well plate at a density of l×105/well followed by incubation at 37°C for 24 h. Then, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT, 5 mg/ml; Sigma) was added into each well followed by incubation for another 4 h, and subsequently dimethyl sulfoxide (DMSO, 100μl/well; Sigma) was added followed by vortexing gently for 15 min. Absorbance (A) was determined at 492 nm with a microplate reader. Cells from each group were added to 5 wells and experiment was performed in triplicate. Absorbance was detected at 24~96 h after transfection, and growth curve was delineated with absorbance as the vertical axis and time as the horizontal axis. Growth inhibition rate = 1-(Asample/Acontrol) ×100%

Flow cytometric analysis of cell cycle

The untransfected or stably transfected cells were harvested by trypsinization, fixed with cold 70% ethanol, and stored at 4°C until analyzed. The cells were resuspended in 100 μl of RNaseA (180 μg/ml) and incubated at room temperature for 30 min. Propidium iodide (final concentration, 50 μg/ml; Merck, Darmstadt, Germany) was added, and cells were incubated at room temperature in the dark for 15 min, and DNA content was detected by flow cytometry (Becton Dickinson FACScan system). The relative proportions of cells in the G0/G1, S, and G2/M phases of the cell cycle were determined from the flow cytometry data.

In vitro invasion

Cells growing in the log phase were treated with trypsin and re-suspended as single-cell solutions. A total of 1×10^5 cells in 0.5 ml of serum-free RPMI 1640 medium were seeded on a 8 μm-pore polycarbonate membrane chambers insert in a transwell apparatus(Costar, Cambridge, MA), coated with Matrigel(BD Biosciences, San Jose, CA). 600 μl RPMI1640 containing 20% FBS was added to the lower chamber.

After the cells were incubated for 12-24 hours at 37°C in a 5% CO2 incubator, cells on the top surface of the insert were removed by wiping with a cotton swab. Cells that migrated to the bottom surface of the insert were fixed in 100% methanol for 2 minutes, stained in 0.5% crystal violet for 2 min, rinsed in PBS and then subjected to microscopic inspection (×200).

Values for invasion were obtained by counting five fields per membrane and represent the average of three independent experiments.

In vitro migration

The chambers were not coated with Matrigel and the remaining procedures were those abovementioned.

Statistical analysis

All statistical analyses were performed using SPSS13.0 software. Studies were performed in triplicate with the results expressed as the mean ± SD as appropriate. Results were considered statistically significant at a $p<0.05$ obtained with a two-tailed Student's t-test.

Results

Beclin 1 expression in transfected cells

CaSki cells were transfected with pcDNA3.1-Beclin1, pSUPER-Beclin1, pcDNA3.1 and pSUPER-non independently, and monoclonal cells with stable expression were screened. After RT-PCR and delineation of kinetic curves of PCR amplification, the relative expression of Beclin1 was calculated. As shown in Table 1, the Beclin1 expression in pcDNA3.1-Bec group was 36.3±2.89, which was significantly higher than in CaSki cells without transfection. However, the Beclin1 expression in pSUPER-Bec group was significantly decreased (0.26±0.02) when compared with blank control group ($P<0.01$). Western blot assay also showed CaSki cells in pcDNA3.1-Bec group had increased Beclin1 expression, but pSUPER-Bec effectively inhibited the protein expression of Beclin1 (Figure 1).

Cell proliferation

Cell proliferation was detected in blank control group, pcDNA3.1-Bec group, pcDNA3.1 group, pSUPER-Bec group and pSUPER-non group, and growth curves were delineated. As shown in Figure 2, the proliferation of CaSki cells transfected with pcDNA3.1-Bec was significantly suppressed when compared with blank control group and pcDNA3.1 group ($P<0.05$), and the growth inhibition rate was as high as 31.09% at 96 h after transfection. However, the proliferation of CaSki cells transfected with pSUPER-Bec had dramatically increased proliferation when compared with blank control group and pSUPER-non group ($P<0.05$). In addition, there were no significant differences among pcDNA3.1 group, pSUPER-non group, and blank control group ($P>0.05$).

In the current study, the effect of Beclin1 on the CaSki cell cycle was determined and each assay was performed in triplicate. As shown in Figure 3, the percentage of pcDNA3.1-Bec cells in the G0/G1 phase significantly increased to 64.7% compared with 49.4% of the non-transfected cells, while the percentage of pcDNA3.1-Bec cells in the S phase significantly decreased to 23.1% compared to 37.6% of the non-transfected cells ($P<0.05$), which indicated that overexpression mediated up-regulation of Beclin1 expression-induced cell cycle arrest in the G0/G1 phase.

Effects of Beclin1 on invasion and migration of CaSki cells

As shown in Figure 4, the number of penetrating cells was 52.1±4.9 in pcDNA3.1-Bec group, which was significantly lower than in blank control group ($P<0.05$).

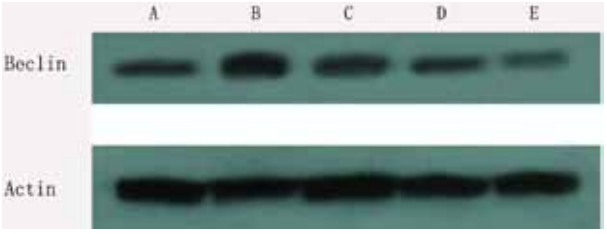

Figure 1. Beclin1 Protein Expression Western Blot. A) CaSki; B) pcDNA3.1-Bec; C) pcDNA3.1; D) pSUPER-non; E) pSUPER-Bec

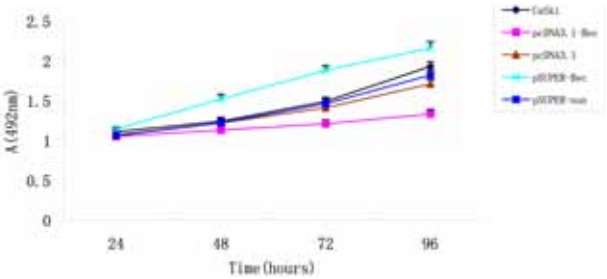

Figure 2. Growth Curves of CaSki cells Transfected with Recombinants

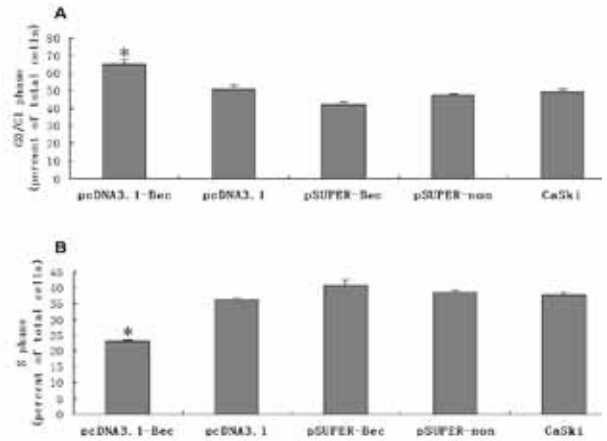

Figure 3. Effect of Beclin1 on the Cell Cycle of CaSki Cells by Flow Cytometry. A) G0/G1 phase ratio; B) The S phase ratio. Mean ± SD; *$p<0.05$ compared with untransfected

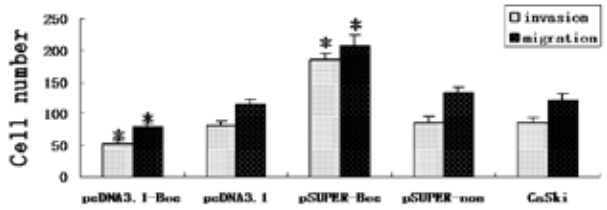

Figure 4. Influence of Beclin1 on invasion and metastasis of CaSki cells. Mean ± SD; *$p<0.05$ compared with untransfected CaSki cells

In the detection of migration, results showed the number of migrating CaSki cells was 79.3±6.1 in pcDNA3.1-Bec group, which was also markedly lower than in blank control group ($P<0.05$). The number of penetrating cells and migrating cells in pSUPER-Bec group were remarkably increased when compared with blank control group ($P<0.05$).

Effects of Beclin1 on the protein expressions of VEGF and MMP-9 in CaSki cells

As shown in Figure 5, Western blot assay showed transfection with pcDNA3.1-Bec could markedly decrease

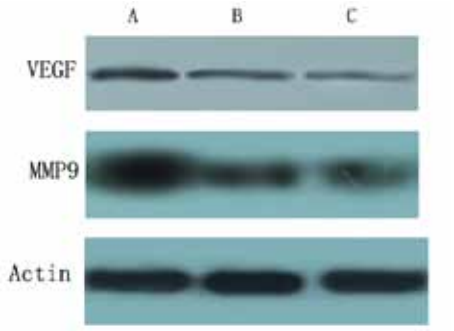

Figure 5. VEGF and MMP-9 Expression Analyzed using Western Blotting. A) pSUPER-Bec; B)CaSki; C) pcDNA3.1-Bec

the protein expressions of VEGF and MMP-9, but pSUPER-Bec transfection effectively elevated the protein expressions of VEGF and MMP-9.

Discussion

Cervical cancer is the most prevalent malignancy of the female reproductive system. Although great progress has been achieved in the platinum containing neoadjuvant chemotherapy and concurrent chemoradiotherapy, the 5-year survival rate of advanced cervical cancer patients is still unfavorable. Therapeutic failure is characterized by uncontrolled local cancer, recurrence and/or distal metastasis. Nowadays, biotherapy has been a hot issue in the treatment of cancers, but most studies aim to induce apoptosis of cancer cells. Given the fact that most cancer cells have defects in the response to induction of apoptosis, it would be desirable if therapeutic agents could kill cancer cells resistant to apoptosis through alternative mechanisms. In recent years, autophagy was introduced to the anti-cancer therapy and it has been a new topic in the treatment of cancers. Cervix squamous cancer CaSki cell line contains a large number of copies of the HPV 16 viral genome established from cells metastatic to the small bowel mesentery. The invasive capacity of CaSki cells is stronger than that of HeLa cells and SiHa cells, and CaSki cells exhibit more chemoresistance to anti-cancer drugs than C33A and SiHa cervical cancer cells based on cell viability (Padilla et al., 2002). Therefore, CaSki cells were recruited in the present study which makes our results more clinical.

Autophagy is a process of physiological cell death during the development (Berry et al., 2008). In the autophagy, some macromolecules including proteins, RNA, glycogen and other damaged organelles due to physiological or pathological reasons are degraded and recycled. This process helps to maintain numerous physiological functions including cell stability and differentiation. Autophagy exists in eucaryotic cells of from yeast to mammals. In addition, researchers have identified autophagy in some pathological conditions including tumors, neurodegenerative diseases, microbial infection and aging (Mizushima et al., 2008). Currently, increasing evidence reveals autophagy plays dual roles (promotion and inhibition) in the occurrence and development of tumors. On one hand, rapid growth of cancers causes insufficiency of nutrient supply, and autophagy can maintain the cell's energy balance though recycling macromolecules and organelles; on the other hand, autophagy exerts anti-cancer effects, which are opposite to its cancer promotion effects (Hait et al., 2006). Although the exact mechanism underlying the suppression of cancer growth by autophagy is still unclear, some tumor suppressors such as DAPK, PTEN and P53 have been found to stimulate autophagy (Sun et al., 2008). Beclin1 is also known as autophagy-related gene (Atg) 6 in yeast and was first identified in viral encephalitis. About 24.4% of amino acid in Beclin1 are identical to Atg6, and murine Beclin1 gene had 93% homology with human's. These findings suggest Beclin1 is a highly conservative gene in evolution. Researchers have found the relationship between the autophagy and tumor. Human Beclin1 gene is located on chromosome 17q21 and encodes a peptide containing 450 amino acid with molecule weight of 60 kD. Study has demonstrated the relationship between Beclin1 and Bcl-2, an anti-apoptotic factor (Germain et al., 2009). Evidence shows Beclin1 expression is an early event in the occurrence and development of human cervical squamous cell carcinoma and is also closely related to the pelvic lymph node metastasis and histological stages of cancers (Wang et al., 2006). In the present study, CaSki cells were transfected with eukaryotic expression vectors carrying Beclin1 gene which was mediated by liposome, and the mRNA and protein expressions of Beclin1 were markedly increased in transfected cells when compared with CaSki cells without transfection. Furthermore, cell proliferation was determined by MTT assay, showing significantly suppressed proliferation and induced cell arrest in the G0/G1 phase in transfected cells. However, in cells transfected with pSUPER-Beclin1, the Beclin1 expressions were decreased accompanied by increased cell viability. These findings suggest Beclin1, an autophagy gene, involve in the occurrence and development of cervical cancer, and over-expression of Beclin1 may effectively inhibit the in vitro proliferation of CaSki cells.

Invasion and metastasis are the important biological behaviors of malignancies and are the main causes of death in patients with advanced cervical cancer. Active cell migration is a critical factor in the invasion and metastasis of cancers. Highly invasive cancer cells usually have more active cell migration. In the present study, the effects of Beclin1 transfection on the abilities of CaSki cells to invade and migrate were determined by Transwell assay. The upper chamber was coated with Matrigel which is derived from mouse EHS sarcoma and rich in matrix components including laminin, type IV collagen, etc. This Matrigel is similar to natural basement membrane. Our results showed the number of penetrating cells in pcDNA3.1-Bec group was significantly lower than in blank control group. On the contrary, after down-regulation of Beclin1, the number of penetrating cells increased significantly. These results imply Beclin1 is closely related to the invasion and migration of CaSki cells and high Beclin1 expression can inhibit the invasion and migration.

The main components of extracellular matrix and basement membrane include collagen, laminin, fibronectin, etc. The degradation or destruction of these

components is the crucial steps in the invasion and metastasis of cancers in which matrix metalloproteinases (MMPs) play an important role. In addition, MMPs also involve in the peripheral capillary formation and formation of vascular endothelial basement membrane. Evidence shows MMP9 is involved in the angiogenesis in cancers through regulating vascular endothelial growth factor (VEGF) (Kim et al., 2006). Newly generated capillaries are indispensable for the growth and proliferation of cancer cells, and are necessary for the invasion and metastasis of cancers. Many experiments have demonstrated stimulation of autophagy signals can inhibit angiogenesis in cancers (Shinohara et al., 2005). Ramakrishnan et al found angiogenesis inhibitor induced autophagy in endothelial cells could be initiated in the presence of endothelial cell specific growth factor such as VEGF, which was independent of nutritional deficiency or hypoxic stress. This study indicated autophagy response could be used a potential strategy to enhance the efficacy of angiogenesis inhibitor (Ramakrishnan et al., 2007). Our results showed Beclin1 over-expression could significantly decrease the protein expressions of VEGF and MMP-9 when compared with CaSki cells without transfection. But down-regulation of Beclin1 expression increased the protein expressions of VEGF and MMP-9. These findings suggest the anti-cancer effects of Beclin1 are related to not only the decreased proliferation of cancer cells by Beclin1 but the down-regulation of VEGF and MMP-9 by Beclin1.

Taken together, with further understanding of the relationship between autophagy and growth, invasion and metastasis of cancers, the exact mechanism underlying the anti-cancer effects of autophagy will be clarified. Based on the different stages in cancer development and differences in the differentiation of cancer cells and signalling pathways, malignancies can be treated by inducing autophagy (such as induction of Beclin1 overexpression), which may be a novel therapeutic strategy.

Acknowledgements

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