RESEARCH COMMUNICATION

# Modulatory Effects of Beclin 1 on Expression of Angiopoietin and Tie-2 Receptor in Human Cervical Cancer Cells

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## Abstract

**Aim: To investigate the effect of Beclin 1, an autophagy gene, on the expression of angiopoietin (Ang) protein and the Tie-2 receptor in CaSki human cervical cancer cells. Materials and Methods: Beclin 1 overexpression (pcDNA3.1-Beclin1) and knockdown (pSUPER-Beclin1) plasmids were independently transfected into CaSki cells, and stably transfected cells were selected. Real-time fluorescence quantitative PCR and Western blot analyses were performed to detect the mRNA and protein expression of Beclin 1, Ang-1, Ang-2, and Tie-2. MTT assays were employed to determine cell proliferation rates. Results: In the cells transfected with pcDNA3.1-Beclin1, the expression of Beclin 1, Ang-2, and Tie-2 was markedly increased, but expression of Ang-1 was dramatically reduced. MTT assays revealed that the proliferation of these cells was also significantly suppressed. In the CaSki cells transfected with pSUPER-Beclin1, the expression of Beclin 1, Ang-2, and Tie-2 was inhibited. Conclusion: Overexpression of Beclin 1 can inhibit the proliferation of CaSki cells, which may be attributed to an imbalance among the expression of Ang-1, Ang-2 and Tie-2.**

**Keywords:** Autophagy - Beclin1 - angiopoietin - Tie-2 receptor - cervical cancer

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## Introduction

Autophagy is a physiological process that occurs under the conditions of cell starvation and energy insufficiency in which lysosomes degrade impaired macromolecules and organelles to maintain the balance among synthesis, degradation, and recycling of cellular components. Similar to apoptosis, which has received substantial attention over the last century, programmed cell death via autophagy has become a hot topic in international research. To date, increasing studies have demonstrated that alterations of autophagy activity and autophagy-induced cell death are closely related to the occurrence and development of malignancies (Hotchkiss et al., 2009). The angiopoietin (Ang) family of proteins plays an important role in the regulation of blood vessel formation, maturation, repair, and permeability and are closely associated with cancer cell invasion and metastasis. Ang proteins bind to the Tie-2 receptor and act directly on vascular endothelial cells. In recent years, evidence has shown that alteration of autophagy signals can affect the angiogenesis of tumors (Ramakrishnan et al., 2007).

In the present study, exogenous expression of the Beclin 1 gene was introduced to human cervical cancer cells (CaSki cells), and the effects of both Beclin 1 overexpression and knockdown on cell proliferation and the expression of Ang proteins and the Tie-2 receptor were investigated. Our findings may help elucidate the potential relationship between autophagy and the occurrence and development of malignancies at the level of angiogenesis and also provide a novel target for the treatment of advanced and refractory cervical cancer.

## Materials and Methods

*Cells and reagents*

CaSki cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. The following products were used in the present study: RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA), G418, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), rabbit anti-human Beclin 1, Ang-1, Ang-2, Tie-2 polyclonal antibodies (Cell Signaling Technology, Beverly, MA, USA), biotinylated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), BCA protein quantitation kit (Pierce Biotechnology, Rockford, IL, USA), MTT, and DMSO (Sigma-Aldrich, St. Louis, MO, USA). The Beclin 1 overexpression (pcDNA3.1-Beclin1) and knockdown (pSUPER-Beclin1) plasmids were kindly provided by the Gynecologic Tumor Lab, West China Second Hospital, Sichuan University. The recombinant vectors were confirmed by digestion analysis with restriction endonuclease, and all the constructed plasmids were confirmed by DNA sequencing.

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*Culture of CaSki cells and transfection*

The cells were grown in RPMI-1640 supplemented with heat inactivated 10% fetal bovine serum, penicillin (100 U/ml; Sigma-Aldrich, St. Louis, MO, USA), and streptomycin (100 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) at 37°C with 5% $CO_2$. Cells in the logarithmic phase were digested with trypsin and then seeded in a 6-well plate ($5\times10^5$/well). One day later, pcDNA3.1-Bec and pSUPER-Bec were individually transfected into CaSki cells using Lipofectamine 2000 according to manufacturer's instructions. One day after transfection, G418 was used to screen for positive colonies. The pcDNA3.1-Bec vector presents green fluorescence, and cells carrying green fluorescence were regarded as successfully transfected. One week later, positive colonies were obtained, and cells were resuspended in medium followed by culture, with the aim of acquiring cells with stable expression of Beclin 1. In addition, cells were also transfected with pcDNA3.1 (empty vector) and pSUPER-non (scrambled RNA) as controls. CaSki cells without transfection served as blank controls.

*Detection of mRNA expression by real-time PCR*

Total RNA was extracted using Trizol reagent, and 2 μg of RNA was used for reverse transcription. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal reference for the real-time (RT)-PCR analysis. The primers and probes used are shown in Table 1. PCR was performed in triplicate on an FTC 2000 PCR Thermal Cycler (Daehan Medical, Seoul, Korea). The conditions for PCR were as follows: predenaturation 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, with a final extension at 72°C for 5 min. The fluorescence at each cycle was recorded for the delineation of amplification kinetic curves, according to which the Ct value (fluorescence intensity) and △Ct value (difference in the fluorescence intensities of the target and reference genes) of each sample was determined. The data were processed using the $2^{-\triangle\triangle Ct}$ method, and the final data represented the expression of the target gene relative to GAPDH.

*Detection of protein expression by Western blot analysis*

Cells were harvested and washed three times with PBS. These cells were lysed in lysis buffer at 4°C for 30 min and then centrifuged at 14000 rpm/min for 10 min. The supernatant was collected, and the protein concentration was determined. Then, 50 μg of total protein was 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 independently with rabbit anti-human Beclin 1, Ang-1, Ang-2, or Tie-2 antibodies at 4°C overnight under continuous shaking. The membranes were washed three times with TBST containing 0.1% Tween 20 and then incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody at room temperature for 1 h. Specific bands were detected using an enhanced chemiluminescence system. Anti-actin antibodies were used to ensure equal loading. Band intensity was semi-quantified using Photoshop Elements (Adobe Systems, San Jose, CA, USA) software after conversion to a digitized image using an image scanner (GT9700F; Epson, Tokyo, Japan). Western blot analyses were performed in triplicate.

*Delineation of growth curves*

Cells in the pcDNA3.1-Bec, pcDNA3.1, pSUPER-Bec, pSUPER-non, and blank control groups were seeded in a 96-well plate at a density of l×105/well followed by incubation at 370C for 24 h. Then, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) was added into each well, followed by incubation for another 4 h. Subsequently, dimethyl sulfoxide (DMSO, 100 μl/well) was added, followed by gentle vortexing for 15 min. Absorbance was determined at 492 nm using a microplate reader. Cells from each group were added to 5 wells, and the experiment was performed in triplicate. Absorbance was detected at 24–96 h after transfection, and growth curves were delineated with absorbance as the vertical axis and time as the horizontal axis. Growth inhibition rate = $1-(A_{sample}/A_{control})\times100\%$.

*Statistical analysis*

All statistical analyses were performed using SPSS v13.0 (SPSS, Chicago, IL, USA) software. Studies were performed in triplicate with the results expressed as the mean ± SD as appropriate. Results were considered statistically significant at $p<0.05$ obtained with a two-tailed Student's t-test.

## Results

*Beclin 1 expression in transfected cells*

After recombinant plasmid-transfected CaSki cells and monoclonal cells with stable expression were screened, the cell transfection efficiency of a 200-fold fluorescence microscope l0 view cells was counted as the percentage of the total cells. Greater than 80% of the cells exhibited positive fluorescent signals (green fluorescent protein [GFP]) under an inverted fluorescence microscope (Figure1). After RT-PCR and delineation of the kinetic curves following PCR amplification, the relative expression of Beclin 1 was calculated. As shown in Table 2, Beclin 1 expression in the pcDNA3.1-Bec group was significantly higher than in the blank control

**Table 1. Primers and Probes Used in the Real Time Fluorescence Quantitative PCR**

| Target gene | Forward primer (5' to 3') | Reverse primer (3' to 5') | Taqman probe (5' to 3') |
|---|---|---|---|
| Beclin1 | AGGAACTCACAGCTCCATTAC | TTGAGTCCTCTCCTCGGTAA | AGCCCAGGCGAAACCAGGAGA |
| Ang-1 | TCTCTTCCCAGAAACTTCAA | GAGACCCAGGTACTAAATCAAA | ACCATGCTGGAGATAGGAACCA |
| Ang-2 | AGTGGCTAATGAAGCTTGAGA | CAAGTCTCGTGGTCTGATTT | CAGAACCAGACGGCTGTGATGA |
| Tie-2 | CGAGGTCAAGAAGTGTATGT | TGATGTATGGTCCTATGGTGT | AAGGCTCCCAGTGCGCTGGA |

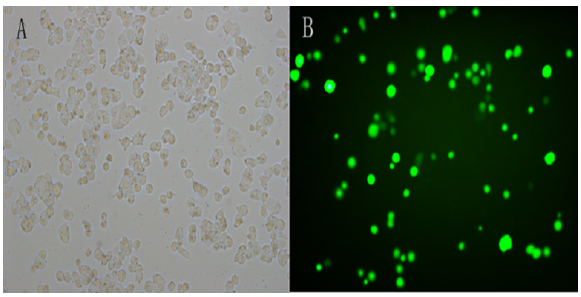



**Figure 1. Expression of GFP on Cervical Cancer CaSki Cells Under a Fluorescent Microscope after Transfection with pcDNA3.1-Bec (×200).** A. Morphology of CaSki cells observed under an ordinary light source. B.Morpholgy of CaSki cells observed under a 492 nm light source.

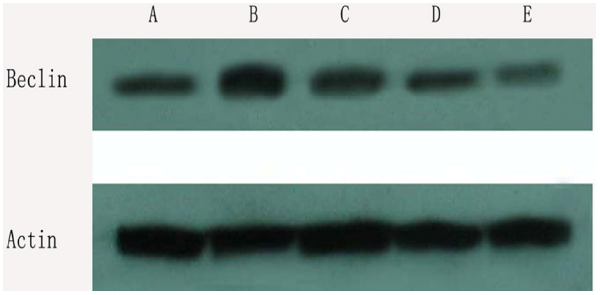



**Figure 2. Beclin1 Protein Expression was Analyzed using Western Blotting.** A. CaSki; B. pcDNA3.1-Bec; C. pcDNA3.1; D. pSUPER-non; E. pSUPER-Bec

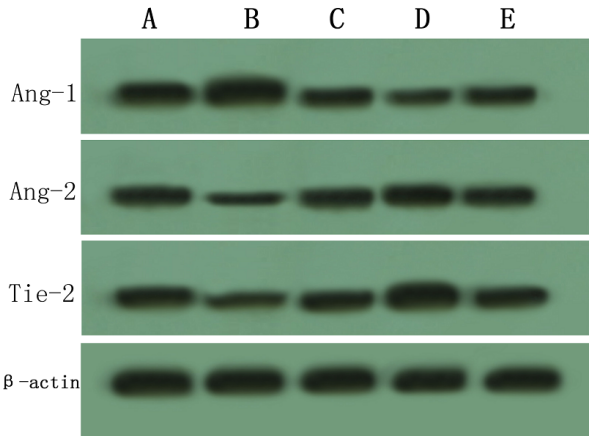
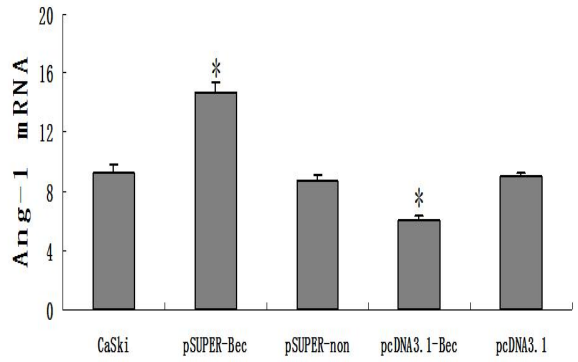



**Figure 3. Ang-1, Ang-2 and Tie-2 Protein Expression were Analyzed Using Western Blotting.** A. CaSki; B. pSUPER-Bec; C. pSUPER-non; D, pcDNA3.1-Bec; E, pcDNA3.1

**Table 2. Real-time Fluorescent Quantitative RT-PCR for Detecting the Expression of Beclin1 mRNA**

| Group | △Ct | $2^{-\triangle\triangle Ct}$ |
|---|---|---|
| CaSki | 5.8±0.12 | 1.07±0.11 |
| pcDNA3.1-Bec | 0.7±0.02 | 36.3±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 |

*P<0.01 vs CaSki



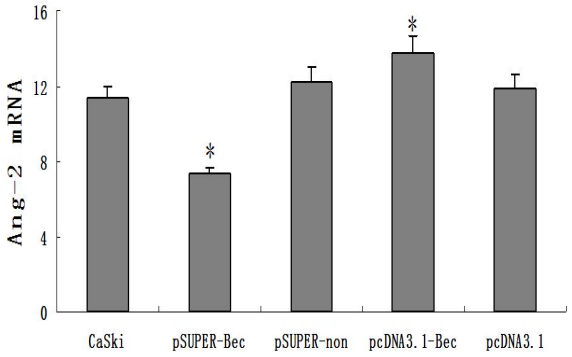




**Figure 4. Ang-1(A), Ang-2(B) and Tie-2(C) mRNA Expression Analyzed using Real-time Flourescent Quantitative RT-PCR.** The results are representative of at least three independent experiments. Data are the mean ± SD; *p<0.05 compared with untransfected CaSki cells

group. However, Beclin 1 expression in the pSUPER-Bec group was significantly decreased when compared with the blank control group. Western blot analysis also showed that the pcDNA3.1-Bec group had increased Beclin 1 expression and that the Beclin 1 shRNA (pSUPER-Bec group) effectively inhibited the protein expression of Beclin 1 (Figure 2).

*Effect of Beclin 1 on the protein expression of Ang-1, Ang-2, and Tie-2 in CaSki cells*

As shown in Figure 3, Western blot analysis indicated that CaSki cells overexpressing Beclin 1 had significantly reduced Ang-1 expression but markedly increased expression of Ang-2 and Tie-2. However, the cells expressing the Beclin 1 shRNA demonstrated dramatically inhibited protein expression of Ang-2 and Tie-2.

*Effect of Beclin 1 on the mRNA expression of Ang-1, Ang-2, and Tie-2 in CaSki cells*

As shown in Figure 4, RT-PCR indicated that the CaSki cells overexpressing Beclin 1 had significantly reduced Ang-1 expressed when compared with the blank control group. However, in the cells expressing the Beclin 1 shRNA, the mRNA expression of Ang-2 and Tie-2 were markedly decreased as compared to the blank control group ($p < 0.05$).

*Cell proliferation*

Cell proliferation was detected in the blank control, pcDNA3.1-Bec, pcDNA3.1, pSUPER-Bec, and pSUPER-non groups, and their photo-absorption values were calculated. As shown in Table 3, the proliferation of CaSki cells overexpressing Beclin 1 was significantly

**Table 3. Photo-absorption Value of CaSki Cells Transfected with Recombinants**

| Ttime(h) | pcDNA3.1-Bec | pcDNA3.1 | pSUPER-Bec | pSUPER-non | CaSki |
|---|---|---|---|---|---|
| 24 | 1.05±0.03 | 1.07±0.05 | 1.14±0.03 | 1.06±0.04 | 1.11±0.04 |
| 48 | 1.13±0.06* | 1.22±0.04 | 1.52±0.06* | 1.22±0.05 | 1.24±0.05 |
| 72 | 1.21±0.05* | 1.41±0.06 | 1.88±0.06* | 1.46±0.07 | 1.49±0.05 |
| 96 | 1.33±0.06* | 1.71±0.05 | 2.16±0.08* | 1.82±0.06 | 1.93±0.06 |

*P<0.05 vs CaSki

suppressed when compared with the blank control and pcDNA3.1 groups ($p < 0.05$), with a growth inhibition rate as high as 31.09% at 96 h after transfection. However, the proliferation of CaSki cells expressing the Beclin 1 shRNA demonstrated dramatically increased proliferation when compared with the blank control and pSUPER-non groups ($p < 0.05$). In addition, there were no significant differences among the pcDNA3.1, pSUPER-non, and blank control groups ($p > 0.05$).

## Discussion

Cervical cancer is the second leading malignancy occurring in women. Although great progress has been achieved in radiotherapy, chemotherapy, and platinum-based adjuvant chemotherapy for the treatment of middle- and late-stage cervical cancer, the 5-year survival rate of these patients is still unacceptable. Therapy failure is mainly attributed to uncontrolled local cancer, recurrence, and distant metastasis. The invasion of malignancies is closely related to angiogenesis, and the ability to acquire the potential for angiogenesis is a basic characteristic of cancer cells. Currently, anti-angiogenic therapy is a major focus for the treatment of cancers.

Autophagy is a type of physiological cell death that occurs during development (Levine et al., 2011). During this process, some proteins, RNA, glycogen, and other macromolecules as well as senescent or impaired organelles involved in pathological and physiological processes are degraded and recycled, and this is critical for the maintenance of many physiological functions, including cellular stability and cell differentiation. With the introduction and innovation of techniques for gene delivery, gene therapy has become an important topic in the treatment of cervical cancer.

In the present study, we investigated a new potential target for the treatment of cervical cancer at the level of angiogenesis. CaSki cells are derived from cervical cancer cells that had metastasized to the small bowel mesentery. The invasiveness of CaSki cells is higher than that of HeLa cells and SiHa cells, and when compared with SiHa cells and C33A cells, CaSki cells are also more resistant to chemotherapeutics (Padilla et al., 2002). Therefore, our findings may be beneficial for the treatment of advanced or recurrent cervical cancer.

In recent years, increasing studies have demonstrated that autophagy plays the dual roles of promotion and suppression of the occurrence and development of malignancies. On one hand, autophagy recycles intracellular macromolecules and organelles, which is crucial for the maintenance of energy balance (Sun et al., 2008). On the other hand, in contrast to the potential cancer-promoting effect of autophagy, substantial evidence has demonstrated its anti-cancer role in different cancer cell types (Yang et al., 2011). The autophagy-related Beclin 1 gene was first identified in a study on viral encephalitis, and the encoding protein has 24.4% homology to Apg6, a yeast autophagy-related protein. In addition, the human Beclin 1 gene has 93% homology to that of mice and rats. These findings suggest that Beclin 1 has been highly conserved during evolution. It is in the study of Beclin 1 that researchers identified the correlation between autophagy and cancer. Human Beclin 1 is located at 17q21, a site of susceptibility to cancers, and the encoded protein consists of 450 amino acids with a corresponding molecular weight of 60 kDa. Moreover, Beclin 1 is closely related to Bcl-2, an apoptosis suppression factor (Germain et al., 2009). There is evidence showing that Beclin 1 is an early event in the occurrence and development of human cervical cancer, and Beclin 1 is also associated with pelvic lymph node metastasis and the histological grade of cervical cancer (Wang et al., 2006).

In the present study, the Beclin 1 gene was transfected into CaSki cells and the overexpression of Beclin 1 was confirmed by Western blot analysis and RT-PCR. In addition, our findings also demonstrated that the proliferation and survival rate of CaSki cells were significantly suppressed following overexpression of Beclin 1. However, in CaSki cells expressing the Beclin 1 shRNA, Beclin 1 expression was reduced and cell survival was increased significantly. These findings suggested that Beclin 1 plays an important role in the occurrence and development of cervical cancer and that overexpression of Beclin 1 can effectively suppress the in vitro proliferation of CaSki cells.

Angiogenesis refers to a physiological process involving the growth of new blood vessels from pre-existing vessels. The Ang family of proteins is a group of recently identified regulators that specifically function on vascular endothelial cells. To date, four structurally similar Ang proteins have been identified, including Ang-1, Ang-2, Ang-3, and Ang-4 (Kim et al., 2007). The molecular weight of both Ang-1 and Ang-2 is 75 kDa. Ang-1 is a homologous hexamer and widely expressed in embryos and mature tissues. Ang-2 has 60% homology to Ang-1 and forms a homodimer. Ang-2 is widely expressed in embryos and some mature tissues, including the placenta, uterus, and ovaries, all of which undergo evident vascular remodeling. The Ang receptors belong to the receptor tyrosine kinase (RTK) family and includes Tie-1 and Tie-2, of which Tie-2 is a shared receptor of the Ang family. Tie-2 is mainly expressed in vascular endothelial cells and early hematopoietic cells and is involved in the stability of mature vessels and proliferation of endothelial cells. Tie-2 is located at 9q21, is about 109 kb in length,

and encodes 1122 amino acids. The Ang proteins bind their receptors and are involved in the initiation and development of angiogenesis during malignancy, which can affect cancer growth, metastasis, and invasion. Thus, the Ang proteins are a major focus for the treatment of cancer (Liao et al., 2007).

Angiogenesis is indispensible for the proliferation and growth of cancer cells. Angiogenesis not only depends on the presence of pro-angiogenic factors (such as vascular endothelial growth factor, etc.), but it also disrupts the process of vascular stability factors, including separation of peripheral cells, interruption of the angiogenesis microenvironment, and promoting the proliferation and migration of endothelial cells. In vitro and in vivo experiments show that Ang-1, Ang-2, and Tie-2 compose a system that can regulate vascular quiescence and endothelial stability and, subsequently, maintain the maturation and stability of vessel networks and the proliferation of endothelial cells (Karamysheva et al., 2008). It is considered that Ang-1 is an anti-angiogenic factor that can phosphorylate and activate Tie-2, which then regulates the interaction between endothelial cells and between endothelial cells and the extracellular matrix to promote vascular maturation and maintain vascular structure (Arai et al., 2004). Ang-2 is an antagonist and competitive inhibitor of Ang-1, thus promoting vascular stability. Studies have demonstrated that Ang-2 plays dual roles in angiogenesis (Fiedler et al., 2006). Evidence shows that an imbalance between Ang-2/Ang-1 may be attributed to the interruption of pre-existing vascular stability, and this pathological process plays an important role in the early initiation and activation of angiogenesis during malignancy (Oliner et al., 2004). Oliner et al. used antibodies to inhibit the interaction between Ang-2 and Tie-2, and the results showed that the growth of cancer cells was markedly suppressed and was accompanied by a reduction of endothelial cell proliferation(White et al., 2003). Other studies on prostate cancer, liver cancer, gastric cancer, and oral squamous cell carcinoma revealed upregulation of the protein and mRNA expression of Ang-2 in these tumors. The findings in these studies indicate that Ang-2 could be used as a factor to predict the prognosis of malignancies and that it also may serve as a potential target to inhibit angiogenesis for the treatment of cancer (Hu et al., 2009). Therefore, alteration of the expression of Ang-1/Ang-2 and their receptors may provide a promising new direction for cancer treatment.

There is evidence that autophagy signals can inhibit angiogenesis in tumors (Shinohara et al., 2005). To treat cancers by blocking angiogenesis has been a major focus in antitumor therapy. Our findings suggested that the expression of Ang-2 and Tie-2 were markedly upregulated following overexpression of Beclin 1 when compared with the blank controls, but knockdown of Beclin 1 markedly increased Ang-1 expression. These findings suggested the anti-cervical cancer effect of Beclin 1 in vitro might be attributed to an imbalance between the expression of Ang proteins and their receptors. Ang-1 and Ang-2 might competitively bind to Tie-2 in two ways: one would be that Ang-2 binds to Tie-2 on endothelial cells and blocks the phosphorylation of Tie-2 by Ang-1. The other is that Ang-2 is overexpressed but Ang-1 is downregulated, which interrupts the balance between these regulators of angiogenesis. Both models involve alterations in the angiogenesis of malignancies. Our previous studies show that both apoptosis and autophagy play important roles in chemotherapy-induced death of cervical cancer cells. The overexpression of Beclin 1 promotes chemotherapy-induced apoptosis of cancer cells, enhancing the antitumor effect (Sun et al., 2010, 2011). In the present study, our results further suggest that changes in the expression of Ang proteins and/or their receptors might also exert an effect on chemotherapy. According to these findings, it is necessary to include autophagy as a potential novel target for enhancing anti-angiogenic effects for the treatment of cancer.

These results, together with our developing understanding of autophagy and the processes of proliferation and cell invasion, highlight the role of autophagy and its related genes in preventing tumor progression. Based on the different tumor stages and the differences in the state of cell differentiation and signaling pathways, we expect that induction of autophagy (e.g., overexpression of Beclin 1) may provide a new approach for the treatment of cancers.

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