

# Valosin-containing protein promotes metastasis of osteosarcoma through autophagy induction and anoikis inhibition via the ERK/NF- $\kappa$ B/beclin-1 signaling pathway

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**Abstract.** Valosin-containing protein (VCP) promotes the development of metastasis in osteosarcoma (OS) via the PI3K/Akt signaling pathway. However, inhibition of the PI3K/Akt pathway does not completely reverse VCP-mediated invasion and migration of OS, suggesting that VCP-mediated OS invasion and migration involves additional mechanisms. In the present study, a positive correlation between the expression of VCP and cell autophagy was observed among OS tissues. Inhibiting VCP may decrease the survival of malignant cells; however, an autophagy stimulator may compensate for VCP inhibition and promote malignant cell survival. Altering the level of autophagy did not affect cell invasiveness or migration. ERK, NF- $\kappa$ B and beclin-1 protein expression levels were markedly decreased following VCP inhibition. These findings indicated that VCP may induce autophagy and enhance anoikis resistance without affecting cell invasiveness or migration. Via anoikis resistance, VCP may promote metastasis in OS. Therefore, targeting of the ERK/NF- $\kappa$ B/beclin-1 signaling pathway may be an effective therapeutic strategy for the management of OS.

## Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor which frequently develops in children and adolescents. Although there have been improvements in diagnosis and treatment, the 5-year survival rate of patients with OS remains

unchanged (1). This is due in part to an 80% prevalence of micro-metastases to the lungs at diagnosis (2). Lung metastasis is the leading cause of mortality in patients with OS (2). As with other types of tumor, the development of metastasis in OS is a complex, multi-step and multi-gene process (3). Therefore, clarification of the molecular mechanisms of invasion, and identification of new molecular targets are necessary for the development of effective treatments.

Valosin-containing protein (VCP), also known as p97 in mammals, is involved in a variety of cellular functions. Studies have identified VCP expression in various tumor types, in addition to an association between tumorigenesis and the development of metastasis (4-7). In previous studies, the role of VCP in metastatic OS was investigated. The results illustrated that VCP was involved in the invasion and migration of OS cells, in part through the activation of the PI3K/Akt signaling pathway, and the upregulation of matrix metalloproteinase (MMP)-2 and MMP-9 (8). Furthermore, inhibition of the PI3K/Akt pathway did not completely reverse the VCP-mediated invasion and migration of OS, suggesting that VCP-mediated OS invasion and migration may involve other mechanisms. Upon further investigation, VCP was revealed to inhibit apoptosis by activating the NF- $\kappa$ B signaling. However, the details of this molecular mechanism are yet to be elucidated.

The role of autophagy in tumorigenesis has become a focus of biomedical research (9). The role of autophagy in tumorigenesis and tumor drug resistance is well delineated (10-13); however, the role of tumor cell autophagy in tumor metastasis remains unclear. In the development of metastasis, cells undergo local infiltration and penetration of the vasculature, subsequently entering the circulation for dissemination throughout the body (14,15). Tumor cells must separate from the cell matrix during this process (16). Apoptosis occurs following separation from the matrix in a process called anoikis (17). A growing number of studies have suggested that autophagy provides a mechanism for stromal-isolation of cells to resist anoikis (18,19). In a lung metastasis model of hepatocellular carcinoma, inhibition of autophagy significantly reduced metastasis of hepatoma cells to the lung. Inhibition of autophagy did not affect cell invasiveness, migration or epithelial-mesenchymal transition, but decreased the ability of

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liver cancer cells to resist anoikis and implant in the lung (20). These studies strongly suggested that autophagy promoted metastasis by mediating cellular resistance to anoikis. However, the role of anoikis resistance in the metastasis of OS remains to be investigated.

As a member of the adenosine triphosphate superfamily, VCP is closely associated with energy metabolism. VCP participates in the regulation of protein degradation by inducing autophagy (21), and is also closely associated with the development of numerous diseases. Ozsoy *et al* (22) revealed that VCP-induced autophagy is a prominent mechanism in the development of pre-eclampsia. Whether VCP promotes OS metastasis via autophagy-induced anoikis resistance is unclear, and requires further investigation.

ERK, a member of the mitogen-activated protein kinase (MAPK) family, transmits signals from cell surface receptors to the nucleus, and serves a key role in signal transduction (23,24). The MAPK family has a number of members, including ERK1/2, p38 and c-Jun N-terminal kinase (25). ERK1 (44 kDa) and ERK2 (42 kDa) are the most thoroughly studied (26-29). They are widely expressed and integral to the regulation of cell growth, development and differentiation (30). Phosphorylation of ERK1/2 and activation of the nuclear transcription factor NF- $\kappa$ B exerts a biological effect, such as regulating other proteins. Copetti *et al* (31,32) demonstrated that the autophagy-promoting protein beclin-1 was able to bind to NF- $\kappa$ B. Activated NF- $\kappa$ Bp65 is able to promote beclin-1 expression by binding to the autophagy-promoting protein beclin-1. Numerous studies (33-35) have also confirmed that VCP regulates the ERK/NF- $\kappa$ B signaling pathway, which is involved in a number of biological processes, including cell proliferation, apoptosis, protein degradation and DNA damage repair, in addition to tumorigenesis. Therefore, it is hypothesized that VCP may activate the ERK1/2/NF- $\kappa$ B/beclin-1 pathway, inducing autophagy-mediated anoikis resistance, and subsequently promoting OS metastasis.

The present study aimed to demonstrate that VCP overexpression activates the ERK/NF- $\kappa$ B/beclin-1 pathway, enhances autophagy-mediated anoikis resistance and promotes the metastasis of OS. Successful completion of this study may increase the understanding of VCP in OS metastasis, and ultimately facilitate the development of effective treatments for metastatic OS.

## Materials and methods

**Patient specimens.** A total of 24 paired samples of OS and paraneoplastic tissue were obtained from patients (10 males and 14 females; age range, 9-35 years; mean age, 17 $\pm$ 5 years) with OS who underwent surgery at The First Hospital Affiliated with Nanchang University (Nanchang, China) between January 2010 and December 2017. None of the patients received chemotherapy or radiotherapy prior to surgical resection. Written informed consent was obtained from all patients, and the study was approved by the Ethics Committee of The First Hospital Affiliated with Nanchang University.

**Cell lines.** The human osteosarcoma cell line 143B was purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences. 143B cells were cultured

in Dulbecco's Modified Eagle Medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml of penicillin and 100 U/ml of streptomycin. Cells were cultured at 37°C with 5% CO<sub>2</sub>.

**Lentivirus vector construction and transfection.** To construct siRNA vectors for the downregulation of VCP, reverse complement sequences as well as nonfunctional negative sequences (Invitrogen; Thermo Fisher Scientific, Inc.; Table I) were cloned into the lentivirus vector GV159 (Invitrogen; Thermo Fisher Scientific, Inc.). Fresh medium was administered to 143B cells, and they were cultured to ~80% confluence. Virus particles incorporating lentivirus vector for the downregulation of VCP (LV-down-VCP; MOI=20) and the cell transfection enhancer polybrene (6  $\mu$ g/ml; Invitrogen; Thermo Fisher Scientific, Inc.) were added to the appropriate cultures [LV-down-VCP and negative lentivirus vector (Neg-LV)], and incubated at 37°C, 5% CO<sub>2</sub> for 6-8 h. The medium was collected and replaced with fresh medium at 6 h after transfection. Transfection efficiency was evaluated using a fluorescence microscope 24 h post-transfection. 143B cells transfected with Neg-LV served as the control. A total of 6 independent experiments were performed, and subsequent experiments were started 324 h after transfection.

**Autophagy intervention experiment.** Autophaginib and spermidine trihydrochloride were purchased from Sigma-Aldrich (Merck KGaA). Concentrations were adjusted to 10 mM for storage according to the manufacturer's instructions. For cell treatment, autophaginib and spermidine trihydrochloride were added to the cell culture medium at final concentrations of 20 and 30 nM, respectively, and incubated at 37°C with 5% CO<sub>2</sub> for 6 h. For cells treated with RNA interference and autophagy agonists, the drug treatment was performed 24 h after transfection.

**Western blotting.** Total protein was extracted from the OS tissues and cell lines using radioimmunoprecipitation assay lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.) containing 60  $\mu$ g/ml phenylmethylsulfonyl fluoride. Protein concentrations were determined using a Bradford assay. Proteins (15  $\mu$ g/lane) were separated by SDS-PAGE using a 5% concentrated gel and 15% separating gel. Following electrophoresis, the protein was transferred to a nitrocellulose membrane and the membrane was blocked with 5% skimmed milk for 30 min at room temperature. Western blot analysis was conducted using primary antibodies against VCP (cat. no. ab36047), ERK (cat. no. ab79853), NF- $\kappa$ B (cat. no. ab16502), beclin-1 (cat. no. ab62557) and LC3 (cat. no. ab62721; all Abcam; dilution, 1:2,000) and GAPDH (cat. no. sc-48166, Santa Cruz Biotechnology, Inc.; dilution, 1:5,000) and horseradish peroxidase-conjugated secondary antibodies (cat. nos. sc-2004 and sc-2020, Santa Cruz Biotechnology, Inc.; dilution, 1:5,000). Membranes were incubated with primary antibodies at 4°C for ~12 h (overnight), and subsequently with secondary antibodies at room temperature for 2-3 h. Immune complexes were detected using a pro-light HRP kit (Pierce; Thermo Fisher Scientific, Inc.). The strip gray value was determined using ImageJ software (version 1.46; National Institutes of Health). A total of 6 independent experiments were performed.

Table I. Small interfering RNA sequence targeting valosin-containing protein and non-targeting negative control sequence.

Oligomer	Sequence (5'→3')
siRNA-F	TGCTGTACAGGTCATCATCATTGTCTGTTTTGGCCACTGACTGACAGACAATGGATGACCTGTA
siRNA-R	CCTGTACAGGTCATCCATTGTCTGTTCAGTCAGTGGCCAAAACAGACAATGATGATGACCTGTAC
Negative-F	TGCTGAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT
Negative-R	CCTGAAATGTACTGCGTGGAGACGTCAGTCAGTGGCCAAAACGTCTCCACGCAGTACATTTTC

**Migration assay.** Cell migration was assessed using a wound healing assay to determine the ability of cells to move into a cellular space in two-dimensions, *in vitro*. In brief, cells were cultured to confluence in six-well tissue culture dishes, to a density of  $\sim 5 \times 10^6$  cells/well. The wound was created by dragging a rubber policeman (Thermo Fisher Scientific, Inc.) across the center of the plate. Cultures were rinsed with PBS and replaced with fresh medium alone or containing 10 g/l BSA (Gibco; Thermo Fisher Scientific, Inc.), and incubated at 37°C for 24 h. BSA was only used in place of FBS in the wound healing experiments. Images were captured using a light microscope at 0 and 24 h, and the migration distance was determined using ImageJ Software (National Institutes of Health).

**Transwell invasion assay.** The invasiveness of OS cells was assessed using the BD BioCoat™ BD Matrigel™ Invasion Chamber (BD Bioscience) according to the manufacturer's protocol. Cells ( $2 \times 10^5$ ) were resuspended in serum-free DMEM and added to the upper chambers; the medium in the lower chambers contained 5% FBS as a chemo-attractant. At 24 h, cells that had migrated through the Matrigel-coated membrane were stained with Diff-Quik (Sysmex Corporation, Kobe) at room temperature in Diff-Quik A for 10-20 sec, Diff-Quik B for 5-10 sec and Diff-Quik C for 5-10 sec, and images were captured under a light microscope (magnification, x200).

**MTT assay.** Osteosarcoma 143B cells ( $2 \times 10^6$  cells) were cultured in suspension for 7 days and subsequently cultured for 6 h. MTT solution (5 mg/ml; 500  $\mu$ l/well) was added to a 24-well plate, incubated for 4 h at 37°C, 1 ml DMSO was added to each well, and the plate was agitated for 10 min to completely dissolve the crystals. The absorbance at 490 nm was measured using a microplate spectrophotometer.

**Statistical analysis.** All statistical analyses were conducted using SPSS software (version 13.0; SPSS Inc., Chicago, IL, USA). Data are expressed as the mean  $\pm$  standard deviation. Bivariate correlation analysis (Spearman's Rho) was performed to evaluate the association between VCP expression and autophagy in OS tissues. One-way analysis of variance followed by a Fisher's Least Significant Difference test was used to analyze multiple samples.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Correlation between VCP expression and autophagy in OS tissues.** To investigate the association between VCP expression and autophagy in OS, VCP and autophagy-associated proteins

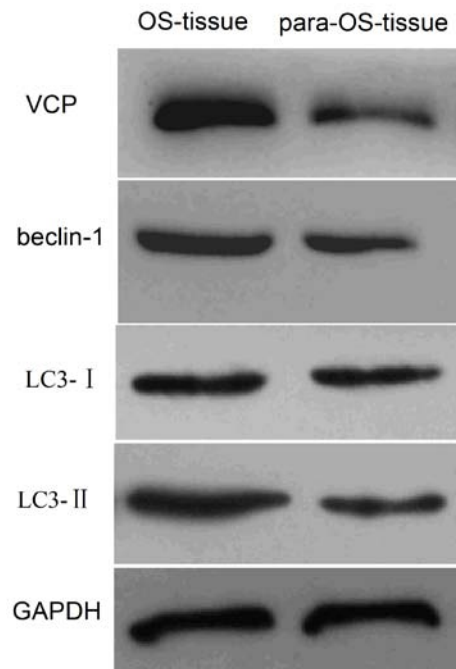


Figure 1. Western blot analysis of the expression levels of VCP, beclin-1 and LC3-I/II. Expression levels in OS tissues were higher than those in paracancerous tissues. VCP, valosin-containing protein; LC3-I/II, microtubule-associated protein 1A/1B-light chain 3-I/II; OS, osteosarcoma.

beclin-1 and microtubule-associated protein 1A/1B-light chain 3 (LC3)-I/II were analyzed in 24 tissue samples from patients with OS, using western blot analysis. The association between VCP and autophagy-associated proteins was evaluated by bivariate correlation (Spearman's Rho). The results revealed that VCP, beclin-1 and LC3-I/II expression was greater in OS tissues compared with paracancerous tissues (Fig. 1). There was a positive correlation between increased expression levels of VCP and autophagy (Spearman's Rho=0.658; data not shown). Therefore, increased expression levels of VCP may contribute to cell autophagy in OS.

**VCP induces autophagy to enhance anoikis resistance.** To determine if VCP was able to induce autophagy and anoikis resistance, VCP expression was inhibited in 143B cells by RNA interference. After 7 days in suspension culture, LC3-II/I expression levels and cell survival were analyzed (Fig. 2). The results illustrated that VCP inhibition lead to a significant decrease in LC3-II/I expression (Fig. 2A) and cell survival (Fig. 2B). Further studies performed with an autophagy inhibitor autophinib and autophagy stimulator

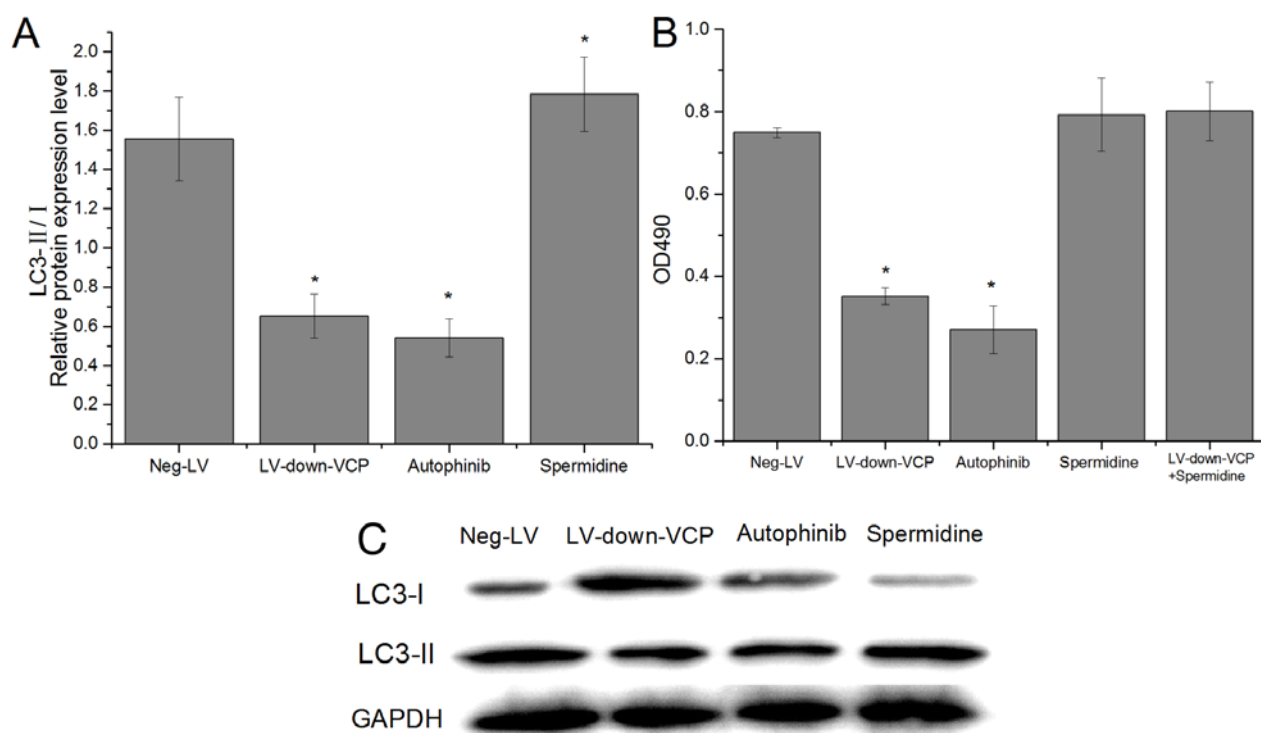


Figure 2. Autophagy-related protein expression levels and cell survival in each group. (A) Downregulation of VCP expression can inhibit autophagy-related protein expression levels. LC3-II/I expression represents the level of autophagy in a cell. (B) MTT assay was used to detect cell survival after 7 days in suspension culture. (C) Representative images of LC3-I and LC3-II levels in each group according to western blotting. N=6. Data are presented as the mean  $\pm$  standard deviation. \*P<0.05 vs. Neg-LV. Neg-LV, 143B cells transfected with negative lentivirus vector; LV-down-VCP, 143B cells transfected with lentivirus vector-downregulating VCP; VCP, valosin-containing protein; LC3-I/II, microtubule-associated protein 1A/1B-light chain 3-I/II; OS, osteosarcoma; OD, optical density.

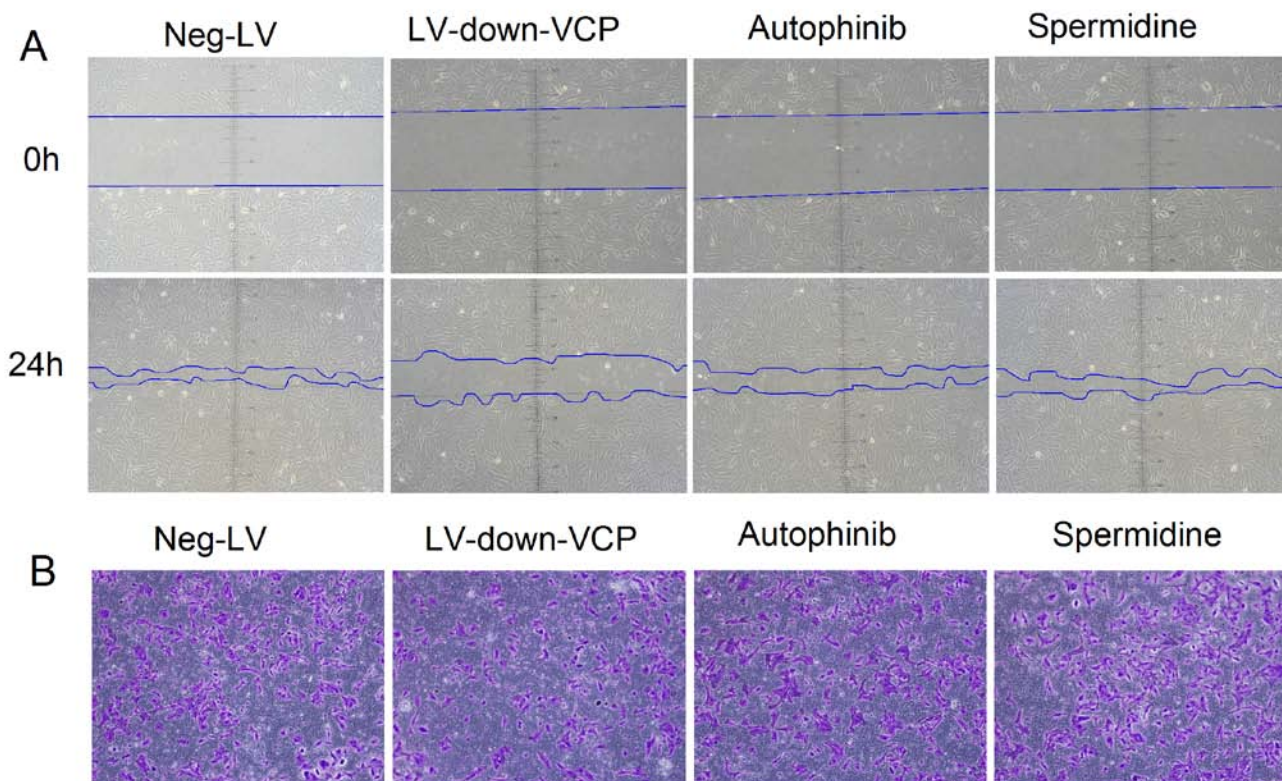


Figure 3. Invasion and migration potential of osteosarcoma cells was determined using Matrigel and wound-healing assays, respectively. (A) Cell migration. (B) Cell invasion. VCP, valosin-containing protein; Neg-LV, 143B cells transfected with negative lentivirus vector; LV-down-VCP, 143B cells transfected with lentivirus vector-downregulating VCP.



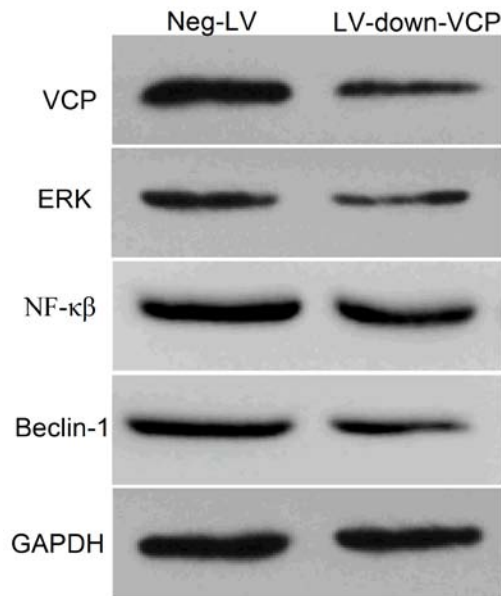


Figure 4. Western blotting was used to detect proteins of the ERK/NF- $\kappa$ B/beclin-1 signaling pathway. VCP inhibition resulted in markedly decreased expression levels of ERK, NF- $\kappa$ B (p65) and beclin-1. ERK, extracellular signal-regulated protein kinase; NF- $\kappa$ B, nuclear factor  $\kappa$ B; VCP, valosin-containing protein; Neg-LV, 143B cells transfected with negative lentivirus vector; LV-down-VCP, 143B cells transfected with lentivirus vector-downregulating VCP.

spermidine trihydrochloride revealed that autophagy inhibitors and stimulator could cause relative changes in the levels of autophagy-related proteins in cells (Fig. 2A), and inhibiting autophagy decreased cell survival; conversely, autophagy stimulation promoted cell survival and counteracted the changes caused by VCP inhibition (Fig. 2B). Collectively, these results indicate that VCP induced autophagy to enhance cell survival and possibly anoikis resistance.

*VCP promotes migration and invasion in OS cells via enhanced cell survival induced by autophagy.* To identify whether VCP expression effects autophagy, and may therefore alter the malignant phenotype of OS cells, lentivirus vectors and autophagy-inhibitory and stimulating agents were used to treat OS cells. Wound-healing and Matrigel-invasion assays were used to evaluate the malignant phenotype of OS cells. The results demonstrated that after 24 h, the migration rate and number of invaded cells were reduced in cells where VCP was downregulated, compared with those transfected with Neg-LV. By contrast, there was no marked difference in the migration rate and invasive ability of cells treated with autophagy inhibitor or stimulator, compared with that of Neg-LV cells (Fig. 3). Collectively, the results revealed that VCP can induce cell autophagy and promote OS cell invasiveness or migration, but altering autophagy levels *in vitro* did not affect cell invasiveness or migration.

*VCP induces autophagy via the ERK/NF- $\kappa$ B/beclin-1 signaling pathway.* To investigate the mechanism by which VCP induced autophagy, 143B cells were treated with LV-down-VCP vector, and the expression levels of VCP, ERK, NF- $\kappa$ B and autophagy-associated protein beclin-1 were determined using western blotting. The results illustrated that VCP inhibition led

to a marked decrease in the expression levels of ERK, NF- $\kappa$ B and beclin-1 (Fig. 4).

## Discussion

OS lung metastases are frequently present at diagnosis and confer high rates of mortality in patients with OS. Despite the emergence of a number of novel chemotherapeutic regimens, the clinical outcome for patients with metastatic OS remains poor (36). Therefore, clarification of the molecular mechanism of invasion, and identification of new molecular targets are imperative to the development of effective treatments for OS.

VCP is involved in the development of various types of tumor (37-40), and thus, may serve as a potential tumor marker. Previous studies have revealed that VCP expression levels in OS samples with pulmonary metastasis were higher compared with those without pulmonary metastatic disease. Inhibition of VCP expression may suppress OS metastasis by modulating the Akt/NF- $\kappa$ B signaling pathway (8). However, our previous study revealed that inhibition of the PI3K/NF- $\kappa$ B pathway does not completely reverse VCP-mediated invasion and migration of OS (Long *et al*, unpublished data). Other mechanisms of VCP-mediated OS invasion and migration may be involved.

Further investigation into autophagy and anoikis resistance may highlight novel mechanisms involved in OS metastasis. In the late stages of metastasis, autophagy may compensate for the loss of external signals that promote and maintain metabolism, and delay apoptosis, therefore allowing cells to reconnect with the extracellular matrix, and ultimately increase viability. Autophagy supports the adjustment of metastatic cells to the altered matrix environment, and allows cells to enter a dormant state (41). In the present study, the expression levels of VCP and autophagy-associated proteins beclin-1 and LC3-II/I were detected in 24-paired samples of OS and paracancerous tissues. The results revealed a positive correlation between the expression of VCP and autophagy-associated proteins. Therefore, an increase in VCP expression levels may promote autophagy in OS. Silencing VCP expression in 143B cells using an RNA interference technique resulted in significantly decreased expression levels of LC3-II/I, suggesting that VCP is able to induce autophagy. Furthermore, VCP inhibition resulted in reduced cell survival following 7 days in suspension culture, suggesting that VCP may also enhance anoikis resistance. Autophagy inhibition decreased cell survival after seven days in suspension culture, and conversely, autophagy stimulation promoted cell survival and counteracted the decrease caused by VCP inhibition. Autophagy did not affect cell invasiveness or migration. Collectively, the results indicate that VCP induced autophagy and enhanced cell survival, to promote OS metastasis, but altering autophagy levels *in vitro* did not affect cell invasiveness or migration.

The ERK/NF- $\kappa$ B signaling pathway induces anoikis and promotes autophagy (42,43). Specifically, the inhibition of autophagy by beclin-1 silencing reduces liver cancer metastasis, by reducing the resistance of malignant cells to apoptosis (44). In the present study, VCP inhibition resulted in decreased levels of ERK, NF- $\kappa$ B and beclin-1 protein expression, in addition to decreased migration and invasiveness compared with the Neg-LV group. VCP may increase the metastatic potential of OS through the promotion of autophagy via the ERK/NF- $\kappa$ B/Beclin-1 signaling pathway.

In conclusion, in the present study, VCP promoted migration and invasion in OS by inducing autophagy and possibly inhibiting anoikis via the ERK/NF- $\kappa$ B/beclin-1 signaling pathway. These results may enhance the development of novel treatment strategies for patients with OS.

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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

XHL organized and analysed the data, and wrote the manuscript. YFZ and ML completed the cell experiments. SHH and ZLL performed surgery, specimen collection and tissue experiments. YS contributed to the project design and experiment management.

### Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The First Hospital Affiliated with Nanchang University. Written informed consent was obtained from all participants.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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