Resveratrol inhibited the progression of human hepatocellular carcinoma by inducing autophagy via regulating p53 and the phosphoinositide 3-kinase/protein kinase B pathway

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Abstract. Resveratrol, a natural product, has been revealed to exert antitumor effects in multiple types of tumors. However, the antitumor effects of resveratrol on hepatocellular carcinoma (HCC) and its potential underlying mechanisms have not yet been elucidated. The present study demonstrated that resveratrol inhibited viability, proliferation, invasion and migration of HCC cells significantly in a time- and dose-dependent manner, indicating that resveratrol exerted antitumor effects in HCC. Furthermore, relative expression of autophagy-related proteins Beclin1 and LC3 II/I ratio was increased while p62 expression was decreased by resveratrol treatment dose-dependently. The $LC3^+$ puncta formation, which represented autophagosome formation was also markedly dose-dependently upregulated by resveratrol treatment, suggesting that resveratrol induced autophagy in HCC cells. In addition, treatment with autophagy inhibitor 3-methyladenine (3-MA) counteracted the inhibitory effect of resveratrol on HCC cell proliferation, invasion and migration, indicating that suppressing autophagy may hamper the antitumor effect of resveratrol in HCC. It was revealed that resveratrol upregulated the expression of p53 while decreasing the ratio of phosphorylated protein kinase B (p-Akt)/Akt in HCC cells. Treatment with p53 inhibitor pifithrin-α and Akt activator insulin-like growth factor-1 decreased the expression of Beclin1 while significantly promoting cell proliferation, invasion and migration compared with the resveratrol treatment group. Taken together, the results of the present study revealed that resveratrol inhibited the proliferation and mobility of HCC cells through inducing autophagy via activating p53 and inhibiting phosphoinositide 3-kinase/Akt. Enhancing autophagy can augment the antitumor effects of resveratrol in HCC. Therefore, combining resveratrol with an autophagy inducer may be a viable option for treating HCC.

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Abbreviations: HCC, hepatocellular carcinoma; 3-MA, 3-methyladenine; IGF-1, insulin-like growth factor-1; SD, standard deviation; EMT, epithelial-mesenchymal transition

Key words: resveratrol, hepatocellular carcinoma, autophagy, p53, phosphoinositide 3-kinase/protein kinase B

Introduction

Hepatocellular carcinoma (HCC) is a common malignant neoplasm and the fourth leading cause of cancer-related mortality in China (1). HCC is the dominant form of primary liver cancer in patients with chronic liver disease and cirrhosis (2). Patients with early-stage HCC can be treated with surgery to remove part of the liver can undergo or transplantation, which contributes toward a 5-year survival rate of >70% (3). However, for the majority of patients with advanced-stage HCC whose disease is too advanced for them to undergo surgical treatment, the 5-year survival rate is extremely low at ~15% (4). Therefore, improving understanding of the molecular mechanism involved in HCC progression and identifying novel approaches to inhibit HCC carcinogenesis are important.

Over recent decades, natural products have served significant roles in the development of anticancer agents (5,6). Resveratrol (trans-3,4',5-trihydroxystilbene) was originally isolated from the roots of white hellebore at 1940 (7). Resveratrol is often used in traditional Chinese medicine to treat skin inflammations, cardiovascular and liver diseases (8,9). Resveratrol exerted a variety of biological effects, including antioxidation, antiproliferation and chemopreventative effects (10,11). Recently, accumulating evidence has indicated that resveratrol also exerts antitumor effects by inhibiting proliferation while inducing apoptosis and autophagy in tumor cells (12,13). For instance, Szekeres *et al* (9) reported that resveratrol induced cell cycle arrest, apoptosis and autophagy in T-cell acute lymphoblastic leukemia cells through inhibiting the protein kinase B (Akt)/mechanistic target of rapamycin (mTOR)/p70S6K/4E-BP1 and activating p38-mitogen-activated protein kinase signaling pathways. Fabre *et al* (10) indicated that resveratrol could induce autophagic and apoptotic death in drug-resistant oral cancer cells and may become

a novel approach for the treatment of oral cancer in the near future (14). However, the antitumor effect of resveratrol in the tumor progression of HCC remains unclear and requires further investigation.

Autophagy is a highly conserved catabolic mechanism in eukaryotes in which intracellular contents, including large protein complexes and dysfunctional organelles, are transported to lysosomes for degradation and reuse (15). Autophagy can prolong the survival of cancer cells and enhance their resistance to apoptosis, and paradoxically, defective autophagy has been associated with increased tumorigenesis, but the mechanism underpinning this remains unclear (16).

The present study aimed to investigate the antitumor effect of resveratrol in HCC. Resveratrol treatment inhibited cell viability, proliferation, invasion and migration of HCC cells through inducing autophagy via regulating p53, phosphoinositide 3-kinase (PI3K)/Akt. The results of the present study suggested that combined treatment with resveratrol and autophagy inducer may serve as effective agents against HCC.

Materials and methods

Reagents and antibodies. Resveratrol, 3-methyladenine (3-MA) and pifithrin-α were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany), and were dissolved in dimethyl sulfoxide. Insulin-like growth factor-1 (IGF-1) was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). Primary antibodies against Beclin1 (1:1,000; cat. no. 3738), LC3 I/II (1:1,000; cat. no. 12741), p62 (1:1,000; cat. no. 88588), p53 (1:1,000; cat. no. 2524), p-Akt (1:2,000; cat. no. 4060), Akt (1:1,000; cat. no. 4685) and GAPDH (1:1,000; cat. no. 5174), as well as horseradish-peroxidase (HRP)-conjugated secondary anti-rabbit (1:1,000; cat. no. 4877) and anti-mouse antibodies (1:1,000; cat. no. 4874) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture. The HCC MHCC97-H cell line with wild-type p53 gene was purchased from Shanghai Institute of Biotechnology and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 complete culture medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% FBS (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 100 μg/ml streptomycin and 100 U/ml penicillin in a humidified atmosphere of 5% CO_2 at 37˚C.

Cell viability assay. MHCC-97H cells ($5x10^3$) were seeded into 96-well plates and treated with different concentrations of resveratrol (0-200 μM) for 24 or 48 h. The purple formazan was dissolved in dimethyl sulfoxide. Cell viability was measured by MTT assay at 450 nm, according to the manufacturer's protocol. All experiments were performed in triplicate and repeated at least twice.

Cell invasion assay. The Transwell invasion chambers coated with Matrigel (1 mg/ml) (BD Biosciences, Franklin Lakes, NJ, USA) were used to perform an invasion assay of MHCC-97H cells. Briefly, 100 μl serum-free Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing $1x10^5$ cells/well was added into the upper chamber, while the lower chamber contained 600 μl DMEM supplemented with FBS (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS. After 24 h of incubation, the cells in the upper chamber were removed and the cells that had invaded through the Matrigel matrix were fixed in 95% ethanol overnight at 4˚C and stained with hematoxylin at 4˚C for 2 h. The cell numbers were counted and images were captured under an inverted microscope (magnification, x400; Olympus Corporation, Tokyo, Japan) on 5 randomly selected fields in each well.

Wound healing assay. Cell migration ability was determined using a wound healing assay. Cells ($1.5x10^6$ cells/well) were seeded into 6-well plates and cultured overnight at 37˚C until the cells reached 90% confluence. Next, a sterile pipette tip was used to create a straight scratch. The destroyed cells were gently rinsed off with PBS 3 times and cultured in MCDB153 medium (Sigma-Aldrich; Merck KGaA) for another 24 h. Cell migration was observed and images were captured under a confocal microscope (magnification, x200; NIKON A1; Nikon Corporation, Tokyo, Japan) at 0 and 24 h.

Western blot analysis. MHCC-97H cells in different groups were lysed and the protein was extracted using radioimmunoprecipitation assay buffer (10X; Cell Signaling Technology, Inc.), containing 1% phenylmethylsulfonyl fluoride. The extracted protein was determined using a bicinchoninic acid protein assay kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Equal amounts of proteins (20 μg) from each sample were separated by 5% SDS-PAGE, prior to being transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Following blocking with 5% skimmed milk at room temperature for 2 h, the membranes were incubated with the aforementioned primary antibodies [diluted in 0.1 mmol/l PBS (PH 7.4) + 1% BSA + 0.1% Na_2N_3] at 4˚C overnight and the corresponding HRP-conjugated secondary antibodies at room temperature for 1 h. The signal was detected using an enhanced chemiluminescence commercial kit (GE Healthcare, Chicago, IL, USA) according to the manufacturer's protocol. Analysis was performed using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA).

Analysis of autophagy. MHCC-97H cells were transfected with green fluorescent protein (GFP)-LC3 plasmid (Invitrogen; Thermo Fisher Scientific, Inc.) using Lipofectamine 3000™ (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Following transfection for 48 h, cells were treated with different concentrations of resveratrol, the intensity of GFP-LC3 fluorescence in cells was determined under a fluorescence microscope (Olympus Corporation). Cells with >5 puncta were considered to have accumulated autophagosomes.

Cell proliferation assay. Cell Counting Kit-8 (CCK-8) assay was used to perform a cell proliferation assay. Cells were seeded into a 96-well plate with $2x10^3$ cells/well in triplicate. CCK-8 reagent was added to each well (5 mg/ml) at indicated time points and then incubated in the dark at 37˚C for 2 h. Absorbance was determined at a wavelength of 450 nm. All the experiments were repeated at least 3 times.

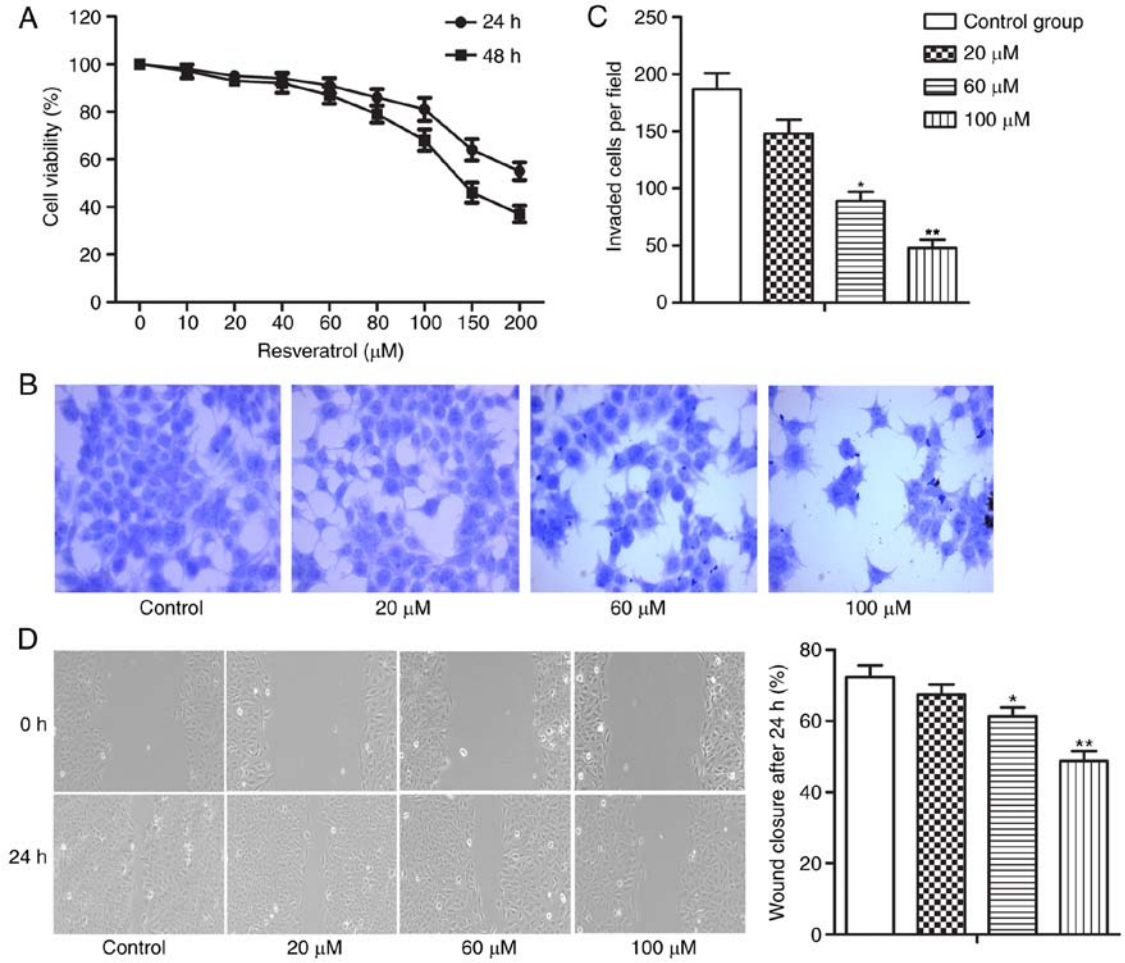

Figure 1. Resveratrol inhibits the viability, invasion and migration of hepatocellular carcinoma cells. MHCC-97H cells were treated with different concentrations of resveratrol (0, 20, 60 or 100 μM). (A) Cell viability was detected using an MTT assay. (B) Images and (C) quantitative analysis of cell invasive ability was detected using Transwell invasion assay. (D) Cell migration ability was detected using a wound healing assay. All data are presented as the mean ± standard deviation of 3 independent experiments. *P<0.05 and **P<0.01, compared with the control group.

Statistical analysis. All data are presented as the mean ± standard deviation. SPSS 19.0 software (IBM Corp., Armonk, NY, USA) was used for analysis. Paired Student's t-tests were used to compare means of two groups and one-way analysis of variance, followed by Bonferroni's post hoc test, was used to compare means of multiple samples. P<0.05 was considered to indicate a statistically significant difference.

Results

Resveratrol inhibits the viability, invasion and migration of HCC cells. To begin with, the present study investigated the potential therapeutic effect of resveratrol in the treatment of HCC. It was revealed that resveratrol treatment suppressed the viability of MHCC-97H cells in a dose- and time-dependent manner (Fig. 1A). Next, the invasion and migration abilities of MHCC-97H cells treated with different concentrations of resveratrol (0, 20, 60 or 100 μM) were compared in the present study. The results demonstrated that resveratrol dose-dependently inhibited the invasion and migration abilities of MHCC-97H cells (Fig. 1B-D; *P<0.05 and **P<0.01). These results indicated that resveratrol exerted antitumor effects in HCC through suppressing the viability, invasion and migration of HCC cells.

Resveratrol induces autophagy in HCC cells. As resveratrol can act as an autophagy inducer during tumor progression, the present study investigated whether resveratrol induced autophagy in HCC cells. The relative expression of Beclin1 and LC3 II/I, two well-validated biomarkers of autophagy, was increased while the expression of p62, which can clear autophagosomes, was significantly decreased by resveratrol in a dose-dependent manner (Fig. 2A-D; *P<0.05 and **P<0.01). Furthermore, LC3^{+} puncta formation, which represented autophagosome formation, was markedly dose-dependently upregulated by resveratrol treatment (Fig. 2E and F; *P<0.05 and **P<0.01). The aforementioned results suggested that resveratrol can induce autophagy in HCC cells.

Autophagy inhibitor 3-MA counteracts the effect of resveratrol. The present study further investigated the effect of autophagy on the antitumor effect of resveratrol in HCC cells. 3-MA (10 mmol/l) was used to inhibit resveratrol-induced autophagy in MHCC-97H cells. The results demonstrated that suppressing resveratrol-induced autophagy by 3-MA significantly promoted the proliferation, invasion and migration abilities of MHCC-97H cells compared with the 100 μM resveratrol group (Fig. 3A-E; *P<0.05 and $^{\#}$P<0.05). The results elucidated that suppressing autophagy counteracted the

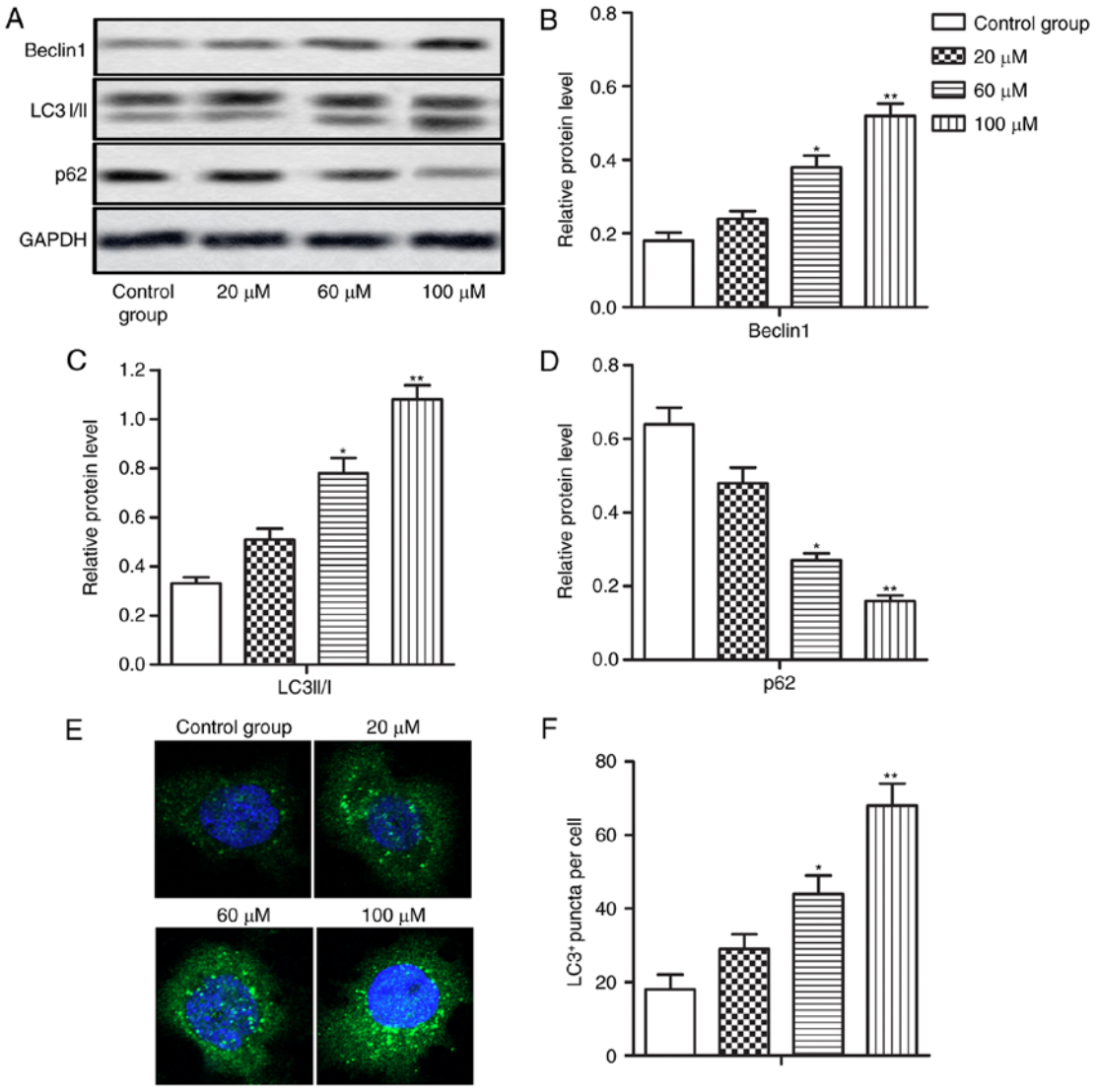

Figure 2. Resveratrol induces autophagy in hepatocellular carcinoma cells. MHCC-97H cells were treated with different concentrations of resveratrol (0, 20, 60 or 100 μM). (A) Relative protein level of Beclin1, LC3 I/II and p62 was detected through western blot analysis. (B) Relative expression level of Beclin1. (C) Relative expression level of LC3 I/II. (D) Relative expression level of p62. (E) Immunofluorescence of MHCC-97H cells was quantified by the number of GFP-LC3 dots, which represent autophagosomes. (F) The number of GFP-LC3 dots were analyzed by bar graph. All data are presented as the mean ± standard deviation of 3 independent experiments. *P<0.05 and **P<0.01, compared with the control group.

inhibitory effect of resveratrol on the proliferation, invasion and migration abilities of HCC cells.

Resveratrol induces autophagy through regulating the p53 and PI3K/Akt pathways. Resveratrol was reported to exert its antitumor effects through the p53 or PI3K/Akt pathways (17,18). To investigate whether these signaling pathways were also involved in resveratrol-induced autophagy in HCC cells, western blot analysis was performed to evaluate the expression of p53, p-Akt and Akt. It was revealed that resveratrol upregulated the expression of p53 while decreasing the ratio of p-Akt/Akt in HCC cells dose-dependently, indicating that resveratrol activates p53 while suppressing the PI3K/Akt pathway in HCC cells (Fig. 4A and B; *P<0.05 and **P<0.01). P53 inhibitor pifithrin-α (30 μM) and Akt activator IGF-1 (50 ng/ml) were used to treat MHCC-97H cells. The results demonstrated that pifithrin-α and IGF-1 significantly decreased the expression of Beclin1, suggesting that inhibiting p53 or activating PI3K/Akt suppressed resveratrol-induced autophagy in HCC cells (Fig. 4C-F; *P<0.05 and $^{\#}$P<0.05). In summary, the results of the present study indicated that resveratrol induced autophagy through activating p53 while suppressing the PI3K/Akt pathway in HCC cells.

Pifithrin-α and IGF-1 counteract the inhibitory effect of resveratrol on proliferation, invasion and migration of HCC cells. The present study then investigated the effects of pifithrin-α and IGF-1 on the motility and proliferation of HCC cells. Pifithrin-α and IGF-1 treatment inhibited the invasion, migration and proliferation abilities of MHCC-97H cells compared with the 100 μM resveratrol group (Fig. 5A-F; **P<0.01 and $^{\#}$P<0.05). The results of the present study suggested that pifithrin-α and IGF-1 counteracted the inhibitory effect of resveratrol on the proliferation, invasion and migration of HCC cells.

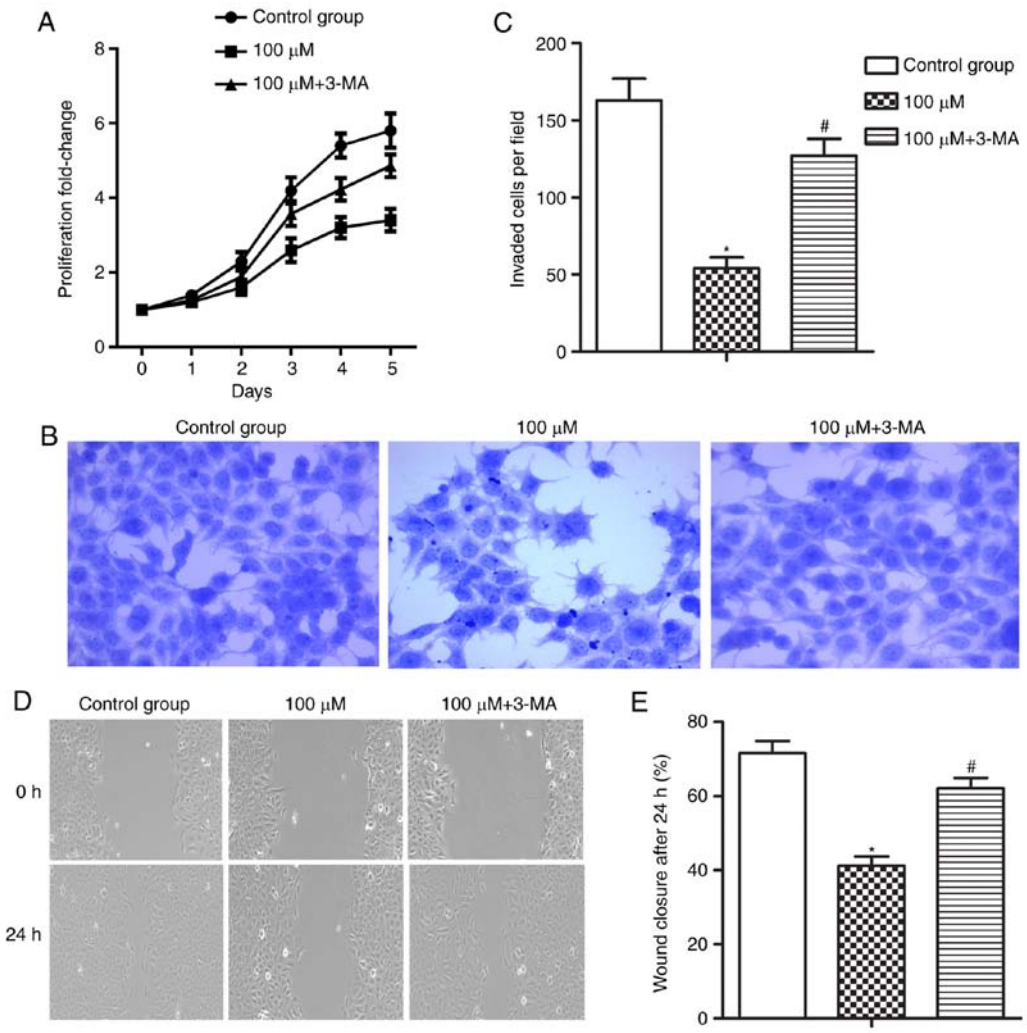

Figure 3. Autophagy inhibitor 3-MA counteracts the effect of resveratrol. MHCC-97H cells were treated with 100 μM resveratrol with or without 3-MA treatment. (A) Cell proliferation was detected using a CCK-8 assay. (B) The cell invasion ability was detected using a Transwell invasion assay. (C) Invaded cells per field. (D) The cell migration ability was detected using a wound healing assay. (E) Wound closing rate. All data are presented as the mean ± standard deviation of 3 independent experiments. *P<0.05, compared with the control group; $^{\#}$P<0.05, compared with the 100 μM resveratrol group.

Discussion

Resveratrol, as a natural product compound, has been demonstrated to have multiple potential chemoprotective activities in various types of cells and animal models (19,20). In addition to antioxidation and chemopreventive effects, resveratrol has also been demonstrated to exert antitumor effects through suppressing proliferation, and inducing apoptosis and autophagy in a variety of cancer cells (21). However, the mechanism of the antitumor effect of resveratrol in HCC remained unclear.

The present study was designed to investigate the antitumor effect of resveratrol in HCC and its potential mechanism. The results demonstrated that resveratrol treatment inhibited cell viability, proliferation, invasion and migration through inducing autophagy in HCC cells via activating p53 while suppressing PI3K/Akt pathway in a time- and dose-dependent manner.

Numerous studies have reported that resveratrol inhibited cell proliferation and suppressed cell mobility in various types of cancer cells. For instance, Chai *et al* (18) reported that resveratrol induced mitochondrial apoptosis and decreased cell migration, invasion and epithelial-mesenchymal transition (EMT)-inducing transcription factor in oral squamous cell carcinoma cells. Harikumar *et al* (19) indicated that resveratrol suppressed invasion, colony forming capacity and cell proliferation in colorectal cancer cells by modulation of focal adhesion molecules. The inhibitory effects of resveratrol on the adhesion, migration and invasion had also been observed in human bladder cancer cells (22). In agreement with the results of these studies, it was observed that resveratrol inhibited cell proliferation, invasion and migration in HCC cells in a time- and dose-dependent manner, suggesting that resveratrol exerted an antitumor effect in HCC.

Autophagy is a major degradation system that promotes the lysosomal digestion of organelles and cytoplasmic components (23). The role of autophagy in cancer cells is diverse: On the one hand, autophagy can promote cancer progression through driving cell metabolism (24); on the other hand, autophagy itself can induce cell death, which is known as autophagic cell death and induction of autophagy is also reported to facilitate the activation of apoptosis (25,26).

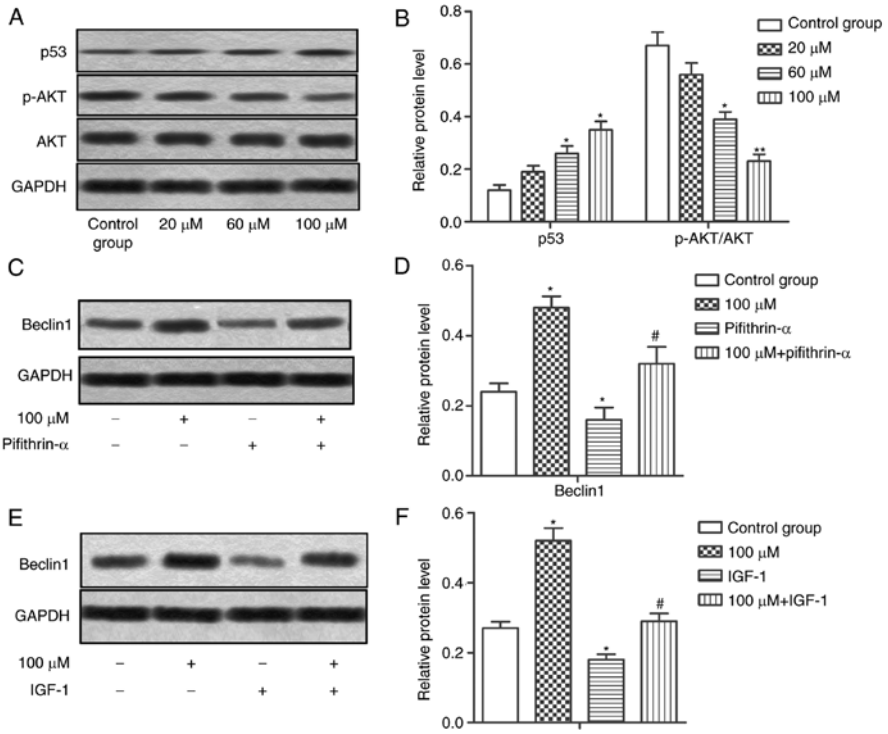

Figure 4. Resveratrol induces autophagy through regulating the p53 and phosphoinositide 3-kinase/Akt pathways. MHCC-97H cells were treated with different concentrations of resveratrol (0, 20, 60 or 100 μM) with or without p53 inhibitor pifithrin-α or Akt activator IGF-1. (A) Relative protein expression level of p53, p-Akt and Akt was detected through western blot analysis. (B) Densitometric analysis of the relative expression level of p53, p-Akt and Akt. (C) Relative protein expression of Beclin1 treated with resveratrol (100 μM) with or without p53 inhibitor pifithrin-α was detected through western blot analysis. (D) Densitometric analysis of the relative protein expression of Beclin1. (E) Relative protein expression of Beclin1 treated with resveratrol (100 μM) with or without Akt activator IGF-1 was measured by western blot. (F) Densitometric analysis of the relative protein expression of Beclin1. All data are presented as the mean ± standard deviation from 3 independent experiments. *P<0.05 and **P<0.01, compared with the control group, $^{\#}$P<0.05, compared with the 100 μM group. Akt, protein kinase B; IGF-1, insulin-like growth factor-1.

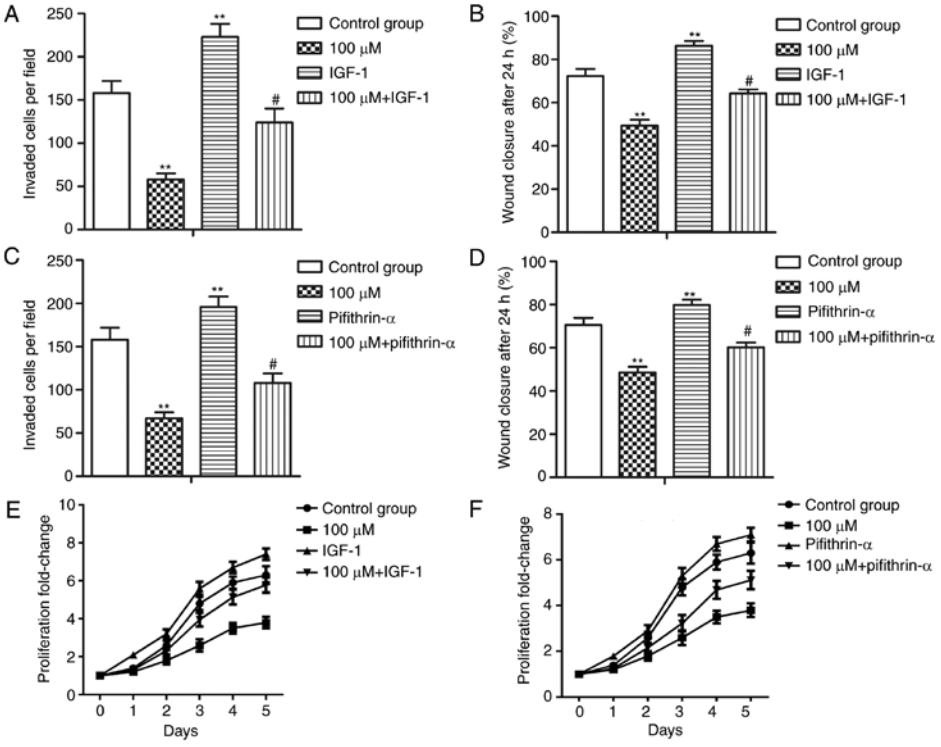

Figure 5. Pifithrin-α and IGF-1 counteract the inhibitory effect of resveratrol on the proliferation, invasion and migration of HCC cells. MHCC-97H cells were treated with 100 μM resveratrol with or without pifithrin-α or IGF-1. (A) The invasion ability of MHCC-97H cells treated with 100 μM resveratrol with or without pifithrin-α was detected using Transwell invasion assay. (B) Cell migration ability was detected using a wound healing assay. (C) The invasion ability of MHCC-97H cells treated with 100 μM resveratrol with or without IGF-1 was detected using a Transwell invasion assay. (D) Cell migration ability was measured using a wound healing assay (E) The proliferation of MHCC-97H cells treated with 100 μM resveratrol with or without pifithrin-α was detected using a CCK-8 assay. (F) The proliferation of MHCC-97H cells treated with 100 μM resveratrol with or without IGF-1 was detected using a CCK-8 assay. All data are presented as the mean ± standard deviation of 3 independent experiments. **P<0.01, compared with the control group; $^{\#}$P<0.05, compared with the 100 μM group.

Although the relevance of autophagy with therapeutic efficacies of antitumor agents has been discussed, the conclusions are contradictory. For instance, Lamy *et al* (25) indicated that autophagy promoted cancer cell growth, which may counteract the effect of resveratrol in Ishikawa endometrial cancer cells. However, Mariño *et al* (26) determined that inhibition of autophagy attenuated resveratrol-induced ovarian cancer cell death. Autophagy-related proteins were detected through western blot analysis and autophagosomes were quantified in the present study. Beclin1 is a specific marker of autophagy and LC3 II, generated by enzymatic cleavage, indicates the initiation of autophagy while p62 is reported to clear the autophagosome (27,28). In agreement with the results of a study undertaken by Zhu *et al* (29), the present study observed dose-dependently enhanced autophagic activity in the resveratrol treatment group. Furthermore, inhibiting autophagy with 3-MA counteracted the inhibitory effects of resveratrol on proliferation, invasion and migration of HCC cells. The results indicated that resveratrol inhibited HCC progression through inducing autophagy, and enhancing autophagy can augment the antitumor effect of resveratrol in HCC.

As resveratrol can induce apoptosis and autophagy in cancer cells, the present study investigated the potential signaling pathway involved in these processes. Nuclear translocation of p53 is important for apoptotic induction. It was reported that p53 was crucial for resveratrol-induced apoptotic cell death in human ovarian cancer cells (30). The PI3K/Akt pathway is an important signaling pathway associated with autophagy (31,32). He *et al* (31) suggested that resveratrol suppressed renal cell carcinoma viability and migration while promoting apoptosis via the p53/AMPK/mTOR-induced autophagy signaling pathway. Deng *et al* (32) also reported that resveratrol-induced autophagy was regulated by the PI3K/Akt/mTOR pathway. Similarly, the present study demonstrated that resveratrol treatment activated p53 while inhibiting the PI3K/Akt pathway in HCC cells. In addition, p53 inhibitor pifithrin-α (30 μM) and Akt activator IGF-1 treatment decreased the expression of Beclin1 and significantly inhibited the proliferation, invasion and migration of HCC cells. The aforementioned results demonstrated that resveratrol suppressed the proliferation and mobility of HCC cells through inducing autophagy via activating p53 while suppressing the PI3K/Akt signaling pathways. As mutant p53 cancer cell lines were more resistant to imiquimod-induced apoptosis than wild-type p53 cancer cell lines (33), our future studies will investigate the effect of resveratrol in HCC cells containing p53 mutant genes. It is worth investigating the role of resveratrol in converting mutant p53 protein to transcriptionally active wild-type p53.

Taken together, the results of the present study revealed that resveratrol inhibited the viability and mortality of HCC cells through inducing autophagy via regulating the p53 and PI3K/Akt pathways. Enhancing autophagy can augment the antitumor effects of resveratrol in HCC. Therefore, combined treatment with resveratrol and an autophagy inducer may be a viable option for treating HCC. To the best of our knowledge, the present study was the first to elucidated that autophagy was involved in the effect of resveratrol on HCC cells. Specifically, resveratrol could inhibit the progression of HCC through inducing autophagy via regulating the p53 and PI3K/Akt pathways.

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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

BZ analyzed and interpreted the data regarding autophagy. XY was responsible for conducting western blot and statistical analysis. SS was responsible for the design and drafting of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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