Resveratrol Suppresses the Growth and Metastatic Potential of Cervical Cancer through Inhibiting STAT3Tyr705 Phosphorylation

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Abstract
Background: The aberrant STAT3 signaling promotes initiation and progression of human cancers by either inhibiting apoptosis or inducing cell proliferation, angiogenesis, invasion, and metastasis. This study aims to investigate the role of STAT3 and its phosphorylation status in resveratrol-mediated suppression of cervical cancer.

Methods: The effects of resveratrol on cervical tumor growth were also determined by examining the tumor tissues and their histological changes and the volume and weight of tumor tissues grown from Hela cells injected in female athymic BALB/C nude mice following the pretreatment regimen and the treatment regimen. Resveratrol structure and targets interaction virtual screening was performed using molecular docking program Autodock Vina. The phosphorylated STAT3, EMT molecular markers and ECM degradation enzymes protein levels were determined using Western blotting.

Results: Proliferation and the colony formation of Hela cells were inhibited after resveratrol treatment in both dose- and time- dependent manner. Resveratrol inhibited migration and invasion of Hela cells. Molecular docking analysis showed that resveratrol interacted with STAT3 at a pocket composed of 11 amino acidic residues. Treatment with resveratrol resulted in decreases in the level of phosphorylation of STAT3 at Tyr705 but not Ser727, and no obvious changes in the protein level of STAT3 in Hela cells and SiHa cells. Pretreatment or treatment with resveratrol resulted in decreases in the IL-6-induced phosphorylation level of STAT3Tyr705 in Hela cells and SiHa cells, compared with those of treatment with IL-6. Reduced STAT3Tyr705 phosphorylation after STAT3 inhibitors S3I201 enhanced inhibition of invasion potential of Hela cells and SiHa cells treated with Resveratrol. Resveratrol decreases the N-Cadherin, Vimentin, MMP-3, MMP-13 protein level and increases the E-Cadherin protein level in a dose-dependent manner. The tumor size, volume, and weight, and the N-Cadherin, Vimentin, MMP-3, and MMP-13 protein level were significantly decreased, and the E-Cadherin protein level was increased, and the tumors were histologically damaged as revealed by H&E staining in both the resveratrol pretreatment group and the resveratrol treatment group and the magnitude of changes was higher in the former than that of the latter.

Conclusion: Resveratrol inhibits growth and metastatic potential of cervical cancer through blocking
STAT3Tyr705 phosphorylation.

Background

Cervical cancer is one of the most common malignant tumors in the world for woman. Adjuvant chemotherapy and adjuvant radiotherapy (chemoradiotherapy) after radical hysterectomy show similar survival outcomes in cervical cancer patients and also appear to reduce the risk of distant recurrence (1). Neoadjuvant chemotherapy followed by radical surgery also predicts a favorable prognosis for local advanced cervical cancer (2, 3). Platinum-based drugs and taxane are main regimens for chemotherapy of cervical cancer. New drugs are in need of development because of drug resistance and adverse reaction (4, 5).

Resveratrol is a natural stilbene and a non-flavonoid polyphenol. It possesses anti-oxidant, anti-inflammatory, cardioprotective, and anti-cancer properties. It has been reported that resveratrol has anti-cancer effects on breast, cervical, blood, kidney, liver, bladder, thyroid, prostate, brain, lung, gastric, colon, head and neck, bone and cervical cancers (6-8). In addition, resveratrol can reverse multidrug resistance in cancer cells, and sensitize cancer cells to standard chemotherapeutic agents (6-8). Several studies have shown that resveratrol induces autophagy and apoptotic cell death in cervical cancer cells (9, 10) and suppresses migration and invasion of human cervical cancer cells (11). Resveratrol significantly inhibits the occurrence and development of cervical cancer by regulating phospholipid scramblase 1 (12, 13) and exhibits antitumor activity on HPV E6-positive cervical cancer (14, 15). These studies suggest that resveratrol is a potential chemotherapeutic drug for cervical cancer.

Signal transducers and activators of transcription (STATs) is a family of cytoplasmic transcription factors that mediate intracellular signaling from cell surface receptors to the nucleus and trans-activates genes encoding apoptosis inhibitors, cell-cycle regulators and induces of angiogenesis. Many studies have shown that STAT3 is constitutively activated in a wide variety of human tumors, including breast, lung, gastric, hepatocellular, colorectal and prostate cancers. The aberrant STAT3 signaling promotes initiation and progression of human cancers by either inhibiting apoptosis or inducing cell proliferation, angiogenesis, invasion, and metastasis. Suppression of STAT3 activity
induces of apoptosis in tumor cells (13, 16, 17). Studies have shown that resveratrol inhibits IL-6-induced transcriptional activity of STAT3 in human prostate cancer LNCaP-FGC cells (18), and STAT3 axis in primary glioblastoma tumor initiating cells (19). Inhibition of STAT3 signaling plays a critical role in resveratrol-induced suppression of several types of cancer, including ovarian cancer (20, 21), pancreatic cancer cells (22), head and neck tumor cells (23), osteosarcoma (24), colorectal cancer (25), colon cancer (26). In these types of cancers, it has been shown that resveratrol inhibits STAT3\textsuperscript{Tyr705} phosphorylation (22–26) and STAT3\textsuperscript{S727} phosphorylation (23, 25). Zhang et al showed that STAT3 signaling is more critical for cervical cancer cells and is the major target of resveratrol because selective inhibition of STAT3 rather than Wnt or Notch activation commits SiHa and Hela cells to apoptosis (27). However, how resveratrol regulates STAT3 phosphorylation in cervical cancer remains unknown.

In this study, we investigated STAT3 phosphorylation status in cervical cancer cells and mouse xenograft tumor model after treatment with resveratrol. Our results suggested that resveratrol inhibits growth and metastatic potential of cervical cancer through suppressing STAT3\textsuperscript{Tyr705} phosphorylation. Our findings support that resveratrol is a potential chemotherapeutic drug for cervical cancer.

**Methods**

**Cell culture**

Human cervical carcinoma cell lines Hela cells (HPV18 positive) (28) and SiHa cells (HPV16 positive) (29–31) were purchased from Hunan Fenghui Biological Technology Co., Ltd. (Hunan, China). These cells were certified by STR test. Hela and SiHa cells were cultured with DMEM (Gibco, 11965-092) containing 10% fetal bovine serum (FBS, Capricorn, FBS-HI-11A) and 100 IU/ml of penicillin G sodium and 100 mg/ml of streptomycin sulfate at 37 °C in an incubator with 5% CO\textsubscript{2}/95% air humidified atmosphere. Cells in exponential growth phase were used for experiments.

**Determination Of Cell Proliferation Using CCK-8 Assay**

Hela cells were seeded to 96-well plates at a density 10 000 cells/well. After incubation for 30 minutes
in an incubator at 37 °C, cells were treated with resveratrol (Sigma-Aldrich, 1602105-100MG, USA) or control vehicle and cultured at 37 °C for 24, 48, 72 h. Cells were washed once with 125 µl phosphate-buffered saline (PBS)/well and assayed using a Cell Counting Kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions. The 96-well plates were read at 450 nm on a plate reader (SpectraMax®iD3, Molecular Devices, USA). Cell viability was denoted by the percentage of cell loss which was calculated using the formula: (Drug_{A450}/Control_{A450}) x100, where A450 denotes absorbance at the wavelength 450 nm.

Colony Formation Assay

Suspension of individualized Hela cells were made from cultured cells by trypsin digestion and pipetting. The cell suspension was diluted with DMEM containing 10% FBS and desired concentrations of resveratrol or vehicle control and then aliquoted to 6-well plates at a density 100 cells/well. After culture for 14 days, cells were washed with cold PBS twice and fixed with 3.7% formaldehyde. After cell colonies were stained with Crystal violet (Sinopharm Chemical Re-agent Co., Shanghai, China), the numbers of colonies in each well were counted.

Wound Healing Assay

The effects of resveratrol on migration of Hela cells were examined using wound healing assay. Hela cells were seeded in a 6-well plate at a density 5 × 10^5 cells/well. After 80% confluence, a scratch was made across the cell monolayer on the bottom of plates with a 200 µl sterile pipette tip and washed with PBS. The cultures were then treated with resveratrol at indicated concentrations or untreated (blank control) and incubated for 0, 24, 48, 72 h and images of the cultures were taken with an inverted microscope (Olympus Optical Co., Ltd., IX73P2F, Japan). The scratches across the cell culture were measured using Olympus cellSens software (Olympus, Version 1.5, Japan) based on the images. The experiment was repeated three times.

Invasion Assay

The effects of resveratrol on invasion of Hela cells were examined using Transwell assay. Boyden chambers containing 24-well Transwell plates (Corning Inc., USA) with 8 mm pore size were used.
Hela cells were seeded at a density $1 \times 10^5$ cells/ml in the upper chambers coated with Matrigel. These cells were treated with 0, 10, 20, 40 µM resveratrol dissolved in medium. DMEM medium containing 10% FBS was added to the bottom chamber. After culture for 24 h, the filters in the upper chambers were collected, the cells on the upper side of the filter membrane were wiped out with a cotton swab and the invaded cells on the lower side of the filter membrane were fixed in 4% paraformaldehyde to the slides followed by staining with 0.1% crystal violet for 10 min (Sinopharm Chemical Re-agent Co., Shanghai, China). The cells in the slides were examined and counted in five randomly selected microscopic fields ($\times 400$) using an inverted microscope (IX73P2F, Olympus Optical Co., Ltd., Japan). The number of cells were compared among treatment groups. All experiments were performed in duplicate and repeated three times.

Western Blotting Analysis

Hela cells and SiHa cells were collected and homogenized in RIPA Lysis Buffer (Beyotime Biotechnology, P0013B) after treated with pertinent chemicals. Tissues samples were also homogenized in lysis buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 50 mM NaF, 0.5 mM phenylmethylsulfonylfluoride, 1 mM sodium vanadate, 1% Triton X-100, 0.5% Nonidet P-40, and 1 µg/mL of aprotinin). The homogenized samples were centrifuged at 12,000 $\times$ g for 15 minutes at 4 °C and the supernatants were collected. An aliquot of the supernatant was used to determine the protein concentration using a Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). All samples containing 30 µg protein/sample were aliquoted, mixed with 5x loading buffer, and loaded for electrophoresis in a 10% SDS-polyacrylamide gel. After electrophoresis, the resolved proteins in the gel were transferred onto PVDF (polyvinylidene difluoride) membranes. The membranes were blocked in 5% non-fat milk TBST buffer (20 mM Tris pH7.4, 150 mM NaCl and 0.1% Tween-20) for 2 h at room temperature, then probed with primary antibodies overnight at 4 °C. After washing with TBST buffer for three times, the membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibody (1:10 000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature and developed using the ECL substrate (Thermo Fisher Scientific, Waltham, MA, USA). The relative density of the blots was quantified using Lab Works software (UVP, Upland, CA,
USA). β-actin or GAPDH was used as a loading control. The relative expression of target proteins was normalized by the loading control. The sources and dilution of primary antibodies: E-Cadherin(4A2) Mouse mAb (#14472), N-Cadherin(D4R1H) Rabbit mAb (#13116), Vimentin(D21H3) XP® Rabbit mAb (#5741) were purchased from Cell Signaling Technology, Inc., GAPDH antibody (Absin, abs132004), β-actin antibody (Absin, abs132001), MMP-3 Antibody (Absin, abs135854), MMP-13 Antibody(Absin, abs110501), Rabbit anti-human Phospho-Stat3-Y705 Polyclonal Antibody (Absin, abs118973), Phospho-Stat3(Absin, Ser727) Antibody (Absin, abs130919), STAT3 Antibody (Absin, abs131812) were purchased from Absin Bioscience Co., Ltd, Shanghai, China, and were diluted at 1:1000 when use. ChemiDoc™ MP Imaging System with Image Lab™ Software (Bio-Rad Laboratories, Inc., Version 5.1, USA) was used as image acquisition tools and image processing software packages.

Molecular Docking

Structure-based virtual screening was employed by molecular docking program Autodock Vina (version 1.1.2) (32). The 3D schematic representation of protein-ligand macromolecule was generated by PyMol (version 2.3) (33). The 2D schematic representation of interaction between ligand and other amino acid residues was shown by LigPlus (version 2.1) (34). The structure file of STAT3 protein (PDB ID: 6QHD) was extracted from The Research Collaboratory for Structural Bioinformatics Protein Data Bank (http://www.rcsb.org/) (35, 36), which was a X-ray crystal homodimer structure bound with a DNA at 2.85-Å resolution (37). All hetatoms including double-stranded DNA and crystallographic water were removed and we keep chain A as monomer STAT3. In order to examine weather resveratrol competitively binds to the binding pocket of the pTyr705 peptide, the native pTyr peptide in 6QHD crystal structure of the monomer ligand was removed. The tran-resveratrol molecular structure (PubChem CID: 445154) was obtained in PubChem database (38). The autodock vina tutorial was followed to convert the ligand and the receptor pdb to pdbqt file by AutoDock Tools (39). The grid size in XYZ was set with 96, 66, 118, which is large enough to containing all potential pockets in STAT3 monomer. The pocket with the lowest score that predicted highest binding affinity was chosen as the resveratrol binding site in STAT3.

Animal model and in vivo anti-tumor efficacy of RES
Twenty-four female athymic BALB/C nude mice weighing 14–20 g (4–6 weeks) were purchased from Hunan SJA Laboratory Animal Co., Ltd (Changsha, China). Mice were housed at 20–22°C with a 50–60% relative humidity and fed with a standard laboratory chow and tap water ad libitum. All mice were randomly divided into two groups receiving the pretreatment regimen and the treatment regimen respectively. For the pretreatment regimen, mice were further randomly divided into the control group where mice were treated with vehicle (Normal saline contain 0.1% ethanol, 3 times/week), and the treatment group where mice were treated with resveratrol (30 mg/kg, 3 times/week). After two weeks of treatment, all mice were subcutaneously injected in the right flank with 200 µl Hela cell suspensions containing $5 \times 10^6$ cells in sterile saline. All mice were further treatment with vehicle and resveratrol respectively for 5 weeks. For the treatment regimen, all mice were subcutaneously injected in the right flank with 200 µl Hela cell suspensions containing $5 \times 10^6$ cells in sterile saline. After 10 days of injection, all mice were randomly divided into the control group and the treatment group. Mice in the control group were treated with vehicle (Normal saline contain 0.1% ethonal, 3 times/week), and mice in the treatment group were treated with resveratrol (30 mg/kg, 3 times/week) for 5 weeks. Food and water intake as well as behavioral changes were monitored every day and body weight of animals recorded every three days over the course. Tumor volumes and body weight were measured every three days. Tumor volumes were calculated with the tumor lengths and widths which were measured using a caliper: tumor volume = $(\text{length}) \times (\text{width})^2 \times 0.5$. At the end of treatment, all mice were sacrificed by cervical dislocation. Tumors were isolated, weighed, and aliquoted for Western blotting analysis, HE staining and immunohistochemistry staining assay. This study was approved by the Ethical Committee for Animal Experimentation of Xiangyang No.1 People’s Hospital (NO. 2017DW006).

H&E Staining And Immunohistochemistry (IHC) Assay

Tumor tissues were harvested, fixed with 4% formaldehyde, embedded with paraffin and cut into 4 µm thick sections. For examination of histology of tumor tissues, the paraffin-embedded sections were subjected to H&E staining and examined under an inverted microscope (OLYMPUS IX73). For
immunohistochemistry assay of the expressions of p-STAT3<sup>Tyr705</sup>, E-cadherin, N-cadherin and Vimentin, the paraffin-embedded sections were incubated with anti-human p-STAT3<sup>Tyr705</sup>, E-cadherin, N-cadherin, Vimentin primary antibodies, and a biotinylated goat anti-rabbit antibody was used as secondary antibody. Then the slides were washed with PBS and incubated with diaminobenzidine (DAB) chromogen for 3-5 min to yield a dark brown color. The sections were counter-stained with hematoxylin for microscopic observation (IX73P2F, OLYMPUS OPTICAL CO., LTD., Japan). Cells with moderate and strong brownish cytoplasmic staining were considered as positive, whereas cells with unstained or weakly stained cytoplasm were considered as negative. The expression levels of p-STAT3<sup>Tyr705</sup>, E-cadherin, N-cadherin, and Vimentin were determined by calculating the ratio of the number of positively stained cells to total number of cells in five randomly selected 400 × magnification microscopic field.

Statistical analysis

Data analysis was performed using Stata 7.0 (Stata Corp LP, College Station, TX, USA) and Microsoft Office Excel 2003. All the results are expressed as mean ± standard deviation (SD) for at least three independent experiments. Statistical comparisons between or among groups were performed using Student's t-test or one-way analysis of variance followed by the post hoc StudentNewmanKeuls test, respectively. P < 0.05 were considered statistically significant.

Results

Resveratrol inhibits the proliferation of cervical cancer cells

To examine the effect of resveratrol on the proliferation of cervical cancer cells, we treated Hela cells with resveratrol and determined cell proliferation using CCK-8 assay and colony formation assay. The proliferation of Hela cells was inhibited by resveratrol in a dose-dependent manner (Fig. 1A). The IC<sub>50</sub> of resveratrol on Hela cells was 291.3, 50.09, and 8.73 µM for 24, 48, 72 h treatment respectively (Fig. 1B). The numbers of colonies of Hela cells were decreased with resveratrol treatment in a dose-dependent manner (Fig. 1C and 1D). These results supported that resveratrol inhibits the proliferation of cervical cancer cells in both dose- and time-dependent manner.

Resveratrol Suppresses Migration And Invasion Of Cervical Cancer Cells
To examine the effects of resveratrol on migration and invasion of cervical cancer cells, we treated Hela cells with resveratrol and determined the migration and invasion ability using Wound healing assay and Transwell assay. The results of Wound healing assay showed that treatment with resveratrol resulted in decreases in the width of scratches among Hela cell layers in a dose-dependent manner (Fig. 2A and 2B). The results of Transwell assay demonstrated that resveratrol decreased the number of Hela cells on the lower surface of the filters in the upper chambers of Transwells (Fig. 2C and 2D). These data suggested that resveratrol inhibits migration and invasion of cervical cancer cells.

To examine the effects of resveratrol on EMT and ECM degradation enzymes of cervical cancer cells, we treated Hela cells and SiHa cells with resveratrol and determined the expression of EMT molecular markers such as N-Cadherin, E-Cadherin, and Vimentin and ECM degradation enzymes MMP-3 and MMP-13, which indicate invasion potential of cervical cancer cells. The results showed that treatment with resveratrol resulted in decreases in the N-Cadherin, Vimentin, MMP-3, and MMP-13 protein and increases in the E-Cadherin protein in both Hela cells and SiHa cells in a dose-dependent manner (Fig. 3). The data demonstrated that resveratrol inhibits EMT and invasion potential of cervical cancer cells.

Resveratrol attenuates phosphorylation of STAT3 and potentially interacts with STAT3 in cervical cancer cells

To examine the effects of resveratrol on the level of STAT3 protein and phosphorylation status of STAT3 in cervical cancer cells, we treated Hela cells and SiHa cells with resveratrol and determined STAT3 protein and phosphorylation status of STAT3 using Western blotting. As shown in the figure, resveratrol decreased the level of phosphorylation of STAT3 at Tyr705 but not Ser727 while it had no obvious change in the STAT3 protein in Hela cells and SiHa cells (Fig. 4A).

To further examine the role of resveratrol in the regulation of STAT3 phosphorylation in cervical cancer cells, we treated Hela cells and SiHa cells with resveratrol and IL-6 or combination of both, and determined STAT3 Tyr705. As expected, IL-6 indeed increased the phosphorylation of STAT3 Tyr705 in Hela cells and SiHa cells (Fig. 4B). Pretreatment or treatment with resveratrol decreased the
phosphorylation of STAT3 Tyr705 activated by IL-6 in Hela cells and SiHa cells (Fig. 4B).

We performed a structure-based molecular docking study to illustrate the potential interaction between resveratrol and STAT3. The strongest binding sites were shown in Fig. 5A with binding affinity – 7.1 kcal/mol. The pocket was composed with Ser381, Ala377, Val490, Leu438, Asp371, Lys488, Ser372, Asp 369, His437, Leu436 and Leu378. Ser381 and His437 formed hydrogen bonds with resveratrol (Fig. 4C and 4D).

These data demonstrated that resveratrol inhibits phosphorylation of STAT3 and potentially interacts with STAT3 in cervical cancer cells.

Reduced STAT3 phosphorylation enhances the inhibition of invasion potential of cervical cancer cells by resveratrol.

To examine the role of STAT3 phosphorylation in the inhibition of invasion potential of cervical cancer cells by resveratrol, we treated Hela cells and SiHa cells with resveratrol and S3I201 or AG490, and examined the expression of EMT molecular markers N-Cadherin, E-Cadherin, and Vimentin and ECM degradation enzymes MMP-3 and MMP-13. S3I201 inhibits Stat3 dimerization and DNA-binding and transcriptional activity (40). In addition, S3I201 inhibits the phosphorylation of STAT3 at Ser727 (41) and Tyr705 (42). AG490 is a JAK-specific inhibitor and can suppress STAT3 signaling by inhibiting Tyr705 phosphorylation of STAT3 protein (43). The results showed that resveratrol, S3I201, or AG490 inhibited phosphorylation of \text{STAT3}^{\text{Tyr705}} in Hela cells and SiHa cells. Treatment with combination of resveratrol and S3I201 resulted in further decreases in phosphorylation of \text{STAT3}^{\text{Tyr705}} in Hela cells and SiHa cells (Fig. 5). Moreover, resveratrol, S3I201, or AG490 reduced the protein level of N-Cadherin, Vimentin, MMP-3, and MMP-13 and increased E-Cadherin protein level in both Hela cells and SiHa cells. Treatment with combination of resveratrol and S3I201 further decreased the level of N-Cadherin, Vimentin, MMP-3, and MMP-13 protein and increased E-Cadherin protein in both Hela cells and SiHa cells (Fig. 5). The results suggested that reduced STAT3 phosphorylation enhances the inhibition of invasion potential of cervical cancer cells by resveratrol.

Resveratrol Inhibits Cervical Tumor Growth In Mice Model
To determine the effects of resveratrol on cervical tumor growth in vivo, we examined the tumor tissues and their histological changes and the volume and weight of tumor tissues grown from Hela cells injected in female athymic BALB/C nude mice following the pretreatment regimen and the treatment regimen. The results showed that the size (Fig. 6A), the volume (Fig. 6B), and the weight (Fig. 6C) of tumor tissues were significantly decreased in both the resveratrol pretreatment group and the resveratrol treatment group, compared with their respective controls. Both the resveratrol pretreatment regimen and the treatment regimen resulted in damages of tumor mass, as revealed by H&E staining (Fig. 6D). The magnitude of changes in the volume (Fig. 6B), the weight (Fig. 6C), and the histology of tumor tissues (Fig. 6D) was higher in the resveratrol pretreatment regimen group than the resveratrol treatment regimen group. These results suggested that resveratrol inhibits cervical tumor growth in mice model.

Resveratrol inhibits phosphorylation of STAT3, EMT and invasion potential of cervical cancer in mice model

To examine the effects of resveratrol on phosphorylation status of STAT3 and EMT and invasion potential of cervical cancer, we tested the p-STAT3 (Tyr705), N-cadherin, E-cadherin, β-catenin, Vimentin, MMP-13, and MMP-3 protein level in the tumor tissues grown from Hela cells were determined using immunohistochemistry and/or Western blotting. The immunohistochemistry results showed that E-cadherin protein was increased and the level of p-STAT3 (Tyr705), N-cadherin, and Vimentin protein was decreased in the tumor tissues grown from Hela cells in both the resveratrol pretreatment group and the resveratrol treatment group, compared with their respective controls (Fig. 7A). The Western blotting results showed that the p-STAT3 (Tyr705), β-catenin, Vimentin, MMP-13, and MMP-3 were decreased in the tumor tissues grown from Hela cells in both the resveratrol pretreatment group and the resveratrol treatment group, compared with their respective controls (Fig. 7B). The magnitude of changes was higher in the resveratrol pretreatment regimen group than the resveratrol treatment regimen group. These results suggested that resveratrol inhibits phosphorylation of STAT3 and EMT and invasion potential of cervical cancer in mice model.

Discussion
In the current study, our data showed that resveratrol inhibits proliferation, migration and invasion of the cervical cancer cell line Hela and SiHa cells. Resveratrol inhibits Hela cells xenograft cervical tumor growth in mice model. This is consistent with previous studies (9–12, 14, 15). In addition, pretreatment with resveratrol also has inhibitory effect on Hela cells xenograft cervical tumor in mice model by even higher magnitude that that of resveratrol treatment regimen, suggesting that resveratrol has constant suppressive effects on cervical cancer independent on treatment regimen, and earlier treatment probably has better outcome.

Metastasis is a complicated biological process that involves primary tumor angiogenesis, cancer cell invasion, vascular intravasation, distant target organ extravasation, and colonization of invaded cells in foreign microenvironment. Epithelial-Mesenchymal transition (EMT) makes primary cancer cells in epithelial nature to acquire ability to migrate and invade (44–46). Matrix metalloproteinases (MMPs) degrade ECM around invasive cancer cells and facilitate vascular intravasation of cancer cells. E-cadherin and N-cadherin are the main biomarkers of EMT (47, 48). In the course of EMT, the level of E-cadherin is decreased, the expression level of N-cadherin is increased (49–51). High levels of β-catenin promote expression of genes that facilitate EMT (52). High Vimentin is a biomarker for mesenchymal state of cancer cells, mediating cytoskeletal organization and focal adhesion maturation (53, 54). MMP-3 and MMP-13 are proteolytic enzymes involved in degradation of ECM around invasive cancer cells (55–57). In the current study, our data demonstrated that resveratrol decreases N-Cadherin, Vimentin, MMP-3, and MMP-13 protein and increases iE-Cadherin protein in both Hela cells and SiHa cells in a dose-dependent manner. The levels of β-catenin, Vimentin, MMP-13, and MMP-3 protein are markedly decreased in the tumor tissues grown from Hela cells in mice model in both the resveratrol pretreatment group and the resveratrol treatment group, compared with their respective control. It is likely that resveratrol inhibits metastatic potential of cervical cancer through inhibiting EMT and ECM enzyme expression.

Inhibition of STAT3 signaling plays a critical role in resveratrol-induced suppression of several types of cancer. It has been shown that resveratrol inhibits STAT3\textsuperscript{Tyr705} phosphorylation in ovarian cancer (20, 21), pancreatic cancer cells (22), head and neck tumor cells (23), osteosarcoma (24), colorectal
cancer (25), colon cancer (22–26) and STAT3$^{S727}$ phosphorylation in head and neck tumor cells and colorectal cancer (23, 25). Similarly, STAT3 signaling is a critical target of resveratrol to induce apoptosis of cervical cancer SiHa and Hela cells (27). In the current study, we found that resveratrol inhibits phosphorylation of STAT3 at Tyr705 but not Ser727. STAT3$^{Tyr705}$ phosphorylation inhibitors AG490 (43) and S3I201 (42) enhance the effect of resveratrol in SiHa and Hela cells. STAT3$^{Tyr705}$ phosphorylation activator IL-6 antagonizes the effect of resveratrol in SiHa and Hela cells.

Pretreatment or treatment with resveratrol decreases in the phosphorylation levels of STAT3$^{Tyr705}$ stimulated by IL-6 in Hela cells and SiHa cells, compared with those of treatment with IL-6. However, pretreatment with resveratrol resulted in a significant reduction in IL-6-induced phosphorylation of STAT3$^{Tyr705}$ in Hela and SiHa cells, compared with the group treatment with resveratrol. In mice model, resveratrol inhibits the expression of other EMT- and ECM-related biomarkers and STST3$^{Tyr705}$, Compared with the treatment group. Further, our structure-based molecular docking study showed that there is a direct interaction between resveratrol and STAT3. Therefore, resveratrol inhibits phosphorylation of STAT3 at Tyr705 but not Ser727 in SiHa and Hela cells probably through direct interaction between resveratrol and STAT3.

In the current study, our data showed that treatment with resveratrol, S3I201, or AG490 resulted in decreases in N-Cadherin, Vimentin, MMP-3, and MMP-13 protein and increases in E-Cadherin protein in both Hela cells and SiHa cells. Treatment with combination of resveratrol and S3I201 further decreases N-Cadherin, Vimentin, MMP-3, and MMP-13 protein and increases E-Cadherin protein in both Hela cells and SiHa cells. Studies have shown that the activation of STAT3 signaling promotes metastasis of cervical cancer (58–60). We showed that resveratrol and S3I201 or AG490 consistently decreases phosphorylation of STAT3$^{Tyr705}$ in Hela cells and SiHa cells. Treatment with combination of resveratrol and S3I201 further decreases the level of phosphorylation of STAT3$^{Tyr705}$ in Hela cells and SiHa cells. These profiles correspond to those of EMT and ECM enzyme biomarkers. Further, similar correspondence between the level of phosphorylation of STAT3$^{Tyr705}$ and EMT and ECM enzyme
biomarkers is observed in tumor tissues grown from Hela cells in mice model. Therefore, it is likely that resveratrol inhibits growth and metastatic potential of cervical cancer through inhibiting STAT3\textsuperscript{Tyr705} phosphorylation.

**Conclusion**
The current study supports that resveratrol inhibits growth, epithelial mesenchymal transition and extracellular matrix degradation of cervical cancer through inhibiting STAT3\textsuperscript{Tyr705} phosphorylation. Resveratrol is a potential chemotherapeutic and chemopreventive natural compound for cervical cancer.

**List Of Abbreviations**
CCK-8 assay, Cell Counting Kit-8
DAB, diaminobenzidine
ECL, Electrochemiluminescence
EMT, Epithelial-Mesenchymal Transition
ECM, extracellular matrix
FBS, Fetal bovine serum
GAPDH, Glyceraldehyde 3-phosphate dehydrogenase
H&E, hematoxylin-eosin staining
IHC, immunohistochemistry
MMP-3, matrix metalloproteinase 3
MMP-13, matrix metalloproteinase 13
qRT-PCR, quantitative reverse transcription-polymerase chain reaction
RES, resveratrol
STATs, Signal transducers and activators of transcription
STR, Short Tandem Repeat

**Declarations**

**Ethics approval and consent to participate**
Mouse xenograft experiments in this study were complied with the ARRIVE guidelines and were conducted in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated
guidelines. This study was approved by the Ethical Committee for Animal Experimentation of Xiangyang No.1 People’s Hospital (NO. 2017DW006).

**Consent for publication**

Not applicable.

**Availability of data and materials**

All data generated or analysed during this study are included in this published article and its supplementary information files. The original data are available upon request to the corresponding author.

**Competing interests**

The authors declare no competing financial interests.

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**Author’s contributions**

SXD was responsible for designing the study, data collection and analysis, and preparing the graphs, and was a major contributor to writing the manuscript; XQQ, ZL and XLX were responsible for performing the experiments; ZQ was responsible for the statistical analysis; XHX processed the charts and tables in the revision process of the later articles; WXB and JN contributed to the critical review of the manuscript; SM and FP supervised and contributed to the critical review of the manuscript. All authors have read and approved the manuscript.

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Figures

Figure 1

Resveratrol inhibited proliferation of cervical cancer cells. (A) Hela cells were treated with resveratrol at indicated concentrations for 24, 48, 72 h. The cell proliferation was determined using a CCK-8 kit. (B) IC50 of resveratrol in inhibiting Hela cell growth. (C) Colony formation of Hela cells treated with RES. Hela cells were treated with resveratrol at indicated concentrations for 14 days. (D) Quantitative analysis of colony formation of Hela cells treated with RES. *, compared with the control, P<0.05.
Figure 2

Resveratrol inhibited migration and invasion of cervical cancer cells. (A) The effects of resveratrol on migration potential of Hela cells were examined using wound healing assay.

Hela cells were seeded, scratched, and then treated with resveratrol at indicated concentrations for 0, 24, 48, 72h. The cells in the dished were examined and the scratches were measured using a light microscope. (B) Quantitative analysis of the scratch sizes in the wound healing assay. (C) The effects of resveratrol on invasion potential of Hela cells were examined using Transwell assay. Hela cells were seeded in the Transwell Boyden chambers and then treated with resveratrol at indicated concentrations for 24h. The cells passed the transwell chamber were stained with Crystal violet and examined using a light microscope.

(D) Quantitative analysis of the migrated number of cells in the Transwell assay. *, compared with the control, P<0.05.
Figure 3
Resveratrol inhibits the expression of EMT molecular markers and ECM degradation enzymes of cervical cancer cells. Hela cells (A) and SiHa cells (B) were seeded in 6-well plates at 1×10^6 cell/well and cultured for 24 h, and then treated with resveratrol at indicated concentrations in fresh media for 24 h. The protein levels were determined using Western blotting, the samples derive from the same experiment and that blots were processed in parallel (cropped blot images are combined into graph, full-length blots are presented in Supplementary Figure 1). Photoshop CC 2019 (Adobe, v20, USA) was used as cropping and drawing tools. *, compared with the control, P<0.05.
Resveratrol potentially interacted with STAT3 and inhibited phosphorylation of STAT3 in
cervical cancer cells. (A) Resveratrol inhibited phosphorylation of STAT3 in cervical cancer cells. Hela cells and SiHa cells were seeded in 6-well plates at 1×10^6 cell/well and cultured for 24 h, and then treated with resveratrol at indicated concentrations in fresh media for 24 h. The protein levels were determined using Western blotting, the samples derive from the same experiment and that blots were processed in parallel (cropped blot images are combined into graph, full-length blots are presented in Supplementary Figure 2). Photoshop CC 2019(Adobe, v20, USA) was used as cropping and drawing tools. *, compared with the 0 group, P<0.05. (B) Hela cells and SiHa cells were seeded in 6-well plates at 1×10^6 cell/well and cultured for 24 h, and then divided into four groups: the control group where the cells were treated with vehicle, the IL-6 treatment group where the cells were treated with IL-6, the RES+ IL-6 group where the cells were treated with Resveratrol for 24 h, then IL-6 were added and incubated for another 24 h, the IL-6+ RES group where the cells were treated with IL-6 for 3 h, then resveratrol were added and incubated for another 24 h. After treatment, cells were collected and the protein levels were determined using Western blotting, the samples derive from the same experiment and that blots were processed in parallel (cropped blot images are combined into graph, full-length blots are presented in Supplementary Figure 3). Photoshop CC 2019(Adobe, v20, USA) was used as cropping and drawing tools. RES, resveratrol 40mM[IL-6, 50μg/ml. *, compared with the control, P<0.05. #, compared with the IL-6 group, P<0.05. [], compared with the RES+IL-6 group, P<0.05. (C) Molecular docking between resveratrol and STAT3. The docking model was generated using Autodock Vina (version 1.1.2). (D) The docking pocket of STAT3 was composed of Ser381, Ala377, Val490, Leu438, Asp371, Lys488, Ser372, Asp 369, His437, Leu436, and Leu378. Ser381 and His437 formed hydrogen bonds with resveratrol.
Reduced STAT3 phosphorylation enhanced inhibition of the expression of EMT molecular markers and ECM degradation enzymes of cervical cancer cells treated with resveratrol.

Hela and SiHa cells were seeded in 6-well plates at 1×10^6 cell/well and cultured for 24 h, and then treated with S3I201, AG490 in fresh media for 2 h. Afterwards, resveratrol was added at indicated concentrations. After 24 h culture, cells were collected and the protein levels were determined using Western blotting, the samples derive from the same experiment and that blots were processed in parallel (cropped blot images are combined into graph, full-length blots are presented in Supplementary Figure 4). Photoshop CC 2019(Adobe, v20, USA) was used as cropping and drawing tools. The results were
quantitatively analyzed. *, compared with the control, P<0.05. #, compared with AG490 treatment, P<0.05. Δ, compared with S3I201 treatment, P<0.05.

**Figure 6**

Resveratrol inhibited cervical tumor growth in mice model. Hela cells were injected into female athymic BALB/C nude mice which were treated with resveratrol following the pretreatment regimen and the treatment regimen (n=6/group). The size (A), the volume (B), and weight (C) of the tumor tissues grown from Hela cells were examined and measured.

The histology of the tumor tissues was examined using H&E staining (D). *, P<0.05.
Pretreatment or treatment with Resveratrol resulted in inhibition of the expression of EMT molecular markers and ECM degradation enzymes and phosphorylation of STAT3 in cervical cancer tissues of mice model. (A) p-STAT3 (Tyr705), N-cadherin, E-cadherin, and Vimentin in the tumor tissues grown from Hela cells were determined using immunohistochemistry. (B) p-STAT3 (Tyr705), β-catenin, Vimentin, MMP-13, and MMP-3 protein levels in the tumor tissues grown from Hela cells were determined using Western blotting. The samples derive
from the same experiment and that blots were processed in parallel (cropped blot images are combined into graph, full-length blots are presented in Supplementary Figure 5).

Photoshop CC 2019 (Adobe, v20, USA) was used as cropping and drawing tools. The results were quantitatively analyzed. *, P<0.05.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

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