Resveratrol affects Proliferation, Migration, Invasion and Apoptosis of Oral Squamous Cell Carcinoma Cells by Inhibiting the WNT Pathway

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Objective: To explore the mechanism by which resveratrol (RES) affects the proliferation, migration, invasion and apoptosis of oral squamous cell carcinoma (OSCC) cells through inhibiting the Wnt pathway.
Methods: Ki-67, matrix metalloproteinase-9 (MMP-9) and B-cell lymphoma-2 (Bcl-2) protein expression levels were detected by immunohistochemical staining. Tca8113 cells were treated with different concentrations of RES (0, 50, 100 and 200 μmol/L) and divided into control, low-dose RES, medium-dose RES and high-dose RES groups. EdU staining, Transwell migration and invasion assays and Annexin V-FITC/PI double-staining were employed to determine cell proliferation, migration, invasion and apoptosis, respectively. E-cadherin and N-cadherin mRNA expression levels in cells were analyzed by RT-qPCR. Wnt1, β-catenin and glycogen synthase kinase-3β (GSK-3β) protein expression levels were detected by Western blotting.
Results: Ki-67, MMP-9 and Bcl-2 were positively expressed in OSCC tissues, with higher rates than those in normal oral mucosal tissues. RES significantly inhibited the proliferation, migration and invasion ability of Tca8113 cells, down-regulated the expression levels of N-cadherin mRNA and Wnt1, β-catenin and GSK-3β proteins, promoted apoptosis, and up-regulated E-cadherin mRNA expression level dose-dependently (P<0.05).
Conclusion: RES can inhibit the proliferation, migration and invasion of OSCC cells and promote apoptosis possibly by inhibiting the Wnt signaling pathway, providing a potential target for targeted therapy of OSCC.

Keywords: resveratrol; oral squamous cell carcinoma; Wnt pathway; proliferation; migration; invasion; apoptosis
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Oral squamous cell carcinoma (OSCC) is the eighth most common malignant cancer worldwide, occurring in the oral and maxillofacial region. The incidence rate of OSCC exhibits an increasing trend annually, and the prognosis is poor. The 5-year survival rate of OSCC patients is low in China [1]. At present, chemotherapy and surgery are mainly used to treat OSCC. Considering that the condition is prone to clinical exacerbations due to tumor invasion, it is essential to search the means to inhibit tumor invasion and metastasis [2]. Resveratrol (RES), serving as a polyphenolic compound, has anti-inflammatory, anti-oxidant, anti-aging and anti-tumor effects, which exists extensively in plants such as peanuts, grapes and knotweed [3]. It has been proven that RES can regulate the occurrence and

progression of tumors by mediating a variety of signaling pathways, including the Wnt signaling pathway, which consists of proteins encoded by oncogenes and tumor suppressor genes, and is of great significance in cell proliferation, differentiation, movement and adhesion. RES can inhibit the Wnt/β-catenin and TGFβ1-Smad2/3 signaling pathways and ameliorate the pathological changes in the kidneys of diabetic nephropathy rats [4]. Besides, RES can activate the Wnt/β-catenin signaling pathway, abate neuronal apoptosis, reduce blood-brain barrier permeability, and ameliorate neurological damage in rats [5]. Moreover, the Wnt signaling pathway also plays an important role in OSCC [6]. MiR-27b suppresses the proliferation activity of OSCC cells through targeted regulation of the FZD7 and Wnt signaling pathways [7]. However, whether RES plays a role in the proliferation and apoptosis of OSCC cells through regulating the Wnt signaling pathway has been rarely reported. In the present study, therefore, the effects of RES on the proliferation, migration, invasion and apoptosis of OSCC cells were analyzed based on the Wnt signaling pathway, so as to provide theoretical bases for the molecular mechanism of RES in the treatment of OSCC.

MATERIALS AND METHODS

Clinical tissues and materials

A total of 60 patients diagnosed as OSCC in our hospital from 2018 and 2020 were enrolled as research objects, including 37 males and 23 females, with an average age of (54±8.41) years old. Biopsy or surgically excised oral mucosal tissue specimens were collected and archived in paraffin-embedded sections. According to the 1997 WHO classification, oral mucosal cancer was divided into three grades: grade I (highly differentiated, n=18), grade II (moderately differentiated, n=33) and grade III (poorly differentiated, n=9). The above grades were confirmed by two pathologists. None of the patients received radiotherapy or chemotherapy before surgery. Meanwhile, 23 healthy subjects with normal oral mucosal tissues were selected as control group. Informed consent was obtained from all participants, and the study protocol was approved by the Medical Ethics Committee of our hospital.

Res

(Sigma, USA), human OSCC cell line Tca8113 (ATCC, USA), Roswell Park Memorial Institute (RPMI) 1640 medium (Hyclone, USA), fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.), trypsin, penicillin/streptomycin (Gibco, USA), 5-ethynyl-2'-deoxyuridine (EdU) staining kit (Abbiotec, USA), Transwell chamber, 24-well plate (Corning, USA), bicinchoninic acid (BCA) protein assay kit, enhanced chemiluminescence (ECL) luminescence reagent (Shanghai Beyotime Biotechnology Co., Ltd.), Wnt1, β-catenin, glycogen synthase kinase-3β (GSK-3β) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies (Cell Signaling, USA), Ki-67, matrix metalloproteinase-9 (MMP-9), B-cell lymphoma-2 (Bcl-2) and horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG antibodies (Beijing Zhongshan Goldenbridge Biotechnology Co., Ltd.), E-cadherin and N-cadherin primers (BGI, Shanghai), IX71 inverted microscope (Olimpus, Japan), DYCZ 425D dual vertical electrophoresis instrument (Beijing Liuyi Biotechnology Co., Ltd.) and carbon dioxide incubator (Thermo, USA) were prepared.

Detection of Ki-67, MMP-9 and Bcl-2 protein expression levels by immunohistochemical staining

Paraffin-embedded sections were deparaffinized with xylene, dehydrated with different concentrations of ethanol, and incubated with 3% hydrogen peroxide solution for 10 min. Sections were then washed with phosphate-buffered saline (PBS), and dropwise added with goat serum for blocking for 2 h. After adding with Ki-67, MMP-9 and Bcl-2 primary antibodies (1:200), samples were incubated at 4°C overnight. Next, sections were washed with PBS, and added with HRP-labeled goat anti-rabbit IgG secondary antibody, followed by incubation at room temperature for 2 h. Subsequently, sections were washed with PBS, added with DAB development solution, counterstained with hematoxylin, placed in 1% hydrochloric acid for 5 s, dehydrated with different concentrations of ethanol, mounted with neutral resin, and microscopically observed (200×). Finally, brown-

yellow cytoplasm or nucleus was considered as positive staining. Five visual fields were randomly selected under the microscope, and judgement was made according to the proportion of positive cells, that is, the number of positive cells ≤10% indicated a negative result, and the number of positive cells >10% suggested a positive result.

Cell grouping

The frozen Tca8113 cells were resuscitated, inoculated in RPMI 1640 medium containing 10% fetal bovine serum and penicillin/streptomycin (100 U/mL), and placed in an incubator at 37°C with 5% CO_2 and saturated humidity. When the cells were fused to 90%, trypsin was added for digestion, for later use. According to the concentration of RES added to the culture medium, the cells were categorized into control group (routinely cultured, without any drug intervention), low-dose RES group (50 μmol/L), medium-dose RES group (100 μmol/L) and high-dose RES group (200 μmol/L).

Determination of cell proliferation ability by EdU staining

Tca8113 cells in the logarithmic growth phase were selected, and were inoculated into a 24-well plate at a density of 3×10^5 cells/well. The experimental grouping was the same as that in 1.2.1. After incubation for 24 h, staining, fixing and mounting were performed in accordance with the instructions of the EdU staining kit, and the LAS AF Lite image processing software was adopted for statistical analysis.

Determination of cell migration and invasion ability by Transwell assay

A layer of Matrigel (about 50 μL) was spread on the Transwell plate. The single cell suspension of each group in serum-free medium was added to the upper chamber (Matrigel was used in the invasion experiment, not migration experiment), while DMEM medium containing 10% fetal bovine serum was added to the lower chamber, followed incubation in an incubator at 37°C with 5% CO_2 for 24 h. After washing with PBS, samples were fixed with formaldehyde, stained with 0.1% crystal violet, and then observed under a microscope. The cells passing through the membrane were counted statistically.

Determination of cell line apoptosis by Annexin V-FITC/PI double-staining method

Tca8113 cells in the logarithmic growth phase were inoculated into a 6-well plate at a density of 1×10^5 cells/well. After incubation for 48 h, the cells were suspended in 500 μL of binding buffer. After 10 μL of Annexin V-FITC and PI were added, cells were incubated for 10 min in the dark. Then, cell apoptosis was detected by flow cytometry and analyzed by NovoExpress™ software.

Detection of E-cadherin and N-cadherin mRNA expression levels by RT-qPCR

Tca8113 cells in the logarithmic growth phase were inoculated into a 12-well plate at a density of 2×10^5 cells/well. After incubation for 24 h, cells in each group were collected. Next, TRIzol reagent was used to extract total RNA, which was transcribed into complementary deoxyribonucleic acid (cDNA) in accordance with the instructions of the reverse transcription kit. A PCR machine was used for amplification, and the relative expression of each mRNA was calculated by $2^{-\Delta\Delta Ct}$, with GAPDH as a reference. The primer sequences were as follows: GAPDH forward primer: 5'-AAGGTGGTGAAGCAGGCAT-3', reverse primer: 5'-GGTCCAGGGTTTCTTACTCCT-3', E-cadherin forward primer: 5'-AACGGGGACGAAGTGCTAAG-3', reverse primer: 5'-CCTCTGCAGGACCTTGATCTC-3', and N-cadherin forward primer: 5'-TGGAGGCCACTATCCGAGAA-3', reverse primer: 5'-GAAGCGCTCAGGCATAAACC-3'.

Detection of Wnt1, β-catenin and GSK-3β protein expression levels by Western blotting

After cells were lysed with protein lysis solution, the total protein was routinely extracted, and protein concentration was determined using BCA protein assay kit. The protein was denatured by heating in boiling water and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred onto

PVDF membrane, blocked with 5% skimmed milk for 2 h, and incubated with primary antibodies (Wnt1, β-catenin and GSK-3β, 1:2,000) at 4°C overnight. Next, they were rinsed with TBST and incubated with HRP-labeled secondary antibody (1:10,000) for 2 h, followed by color development with ECL. Image J software was used for grayscale analysis, with GAPDH as an internal reference for correction [19].

Statistical analysis

SPSS 19.0 software was employed for statistical analysis, and GraphPad Prism 5.01 software was used for plotting. Measurement data were expressed as mean ± standard deviation ($\bar{\chi}$ ± s), and independent *t*-test was employed for comparison between two groups. One-way analysis of variance was applied for comparison among groups. P<0.05 indicated that difference was statistically significant.

RESULTS

Ki-67, MMP-9 and Bcl-2 protein expression levels in OSCC and normal oral mucosal tissues

The results of immunohistochemical staining indicated that Ki-67, MMP-9 and Bcl-2 were positively expressed in tumor tissues, and the positive expression rates were higher than those in normal oral mucosal tissues (Figure 1).

Cell proliferation ability

The results of EdU staining displayed that compared with that in control group, the ratio of EdU-positive cells in low-, medium- and high-dose RES groups was significantly reduced in a dose-dependent manner (P<0.05) (Figure 2).

Cell migration and invasion ability

The results of Transwell migration and invasion assays revealed that compared with those in control group, the number of migrating cells and the number of cells passing through Matrigel in low-, medium- and high-dose RES groups were significantly reduced in a dose-dependent manner (P<0.05) (Figure 3).

Cell apoptosis ability

The results of Annexin V-FITC/PI double-staining demonstrated that compared with that in control group, the apoptosis rate in low-, medium- and high-dose RES groups was significantly increased in a dose-dependent manner (P<0.05) (Figure 4).

E-cadherin and N-cadherin mRNA expression levels

RT-PCR results exhibited that compared with control group, E-cadherin mRNA expression level in the cells of low-, medium- and high-dose RES groups was significantly increased while N-cadherin mRNA expression level was significantly reduced in a dose-dependent manner (P<0.05) (Figure 5).

Wnt1, β-catenin and GSK-3β protein expression levels

The results of Western blotting revealed that compared with those in control group, Wnt1, β-catenin and GSK-3β protein expression levels in the cells of low-, medium- and high-dose RES groups were significantly increased in a dose-dependent manner (P<0.05) (Figure 6).

DISCUSSION

OSCC refers to common oral malignant tumors with various degrees of squamous differentiation and epithelial invasiveness, characterized by high malignancy and strong invasiveness, which is prone to recurrence and metastasis, leading to a poor prognosis and short survival period [8]. The effects of conventional methods such as surgery, radiotherapy and chemotherapy remain unsatisfactory. The roles of RES in diabetes mellitus, gastric cancer and esophageal cancer have been verified in recent years. RES down-regulates Bcl-2 expression by inhibiting the Wnt signaling pathway, thus reducing neuronal apoptosis in diabetic rats, and improving their non-spatial and spatial cognition [9]. In addition, RES suppresses gastric cancer cell growth by down-regulating the Wnt/β-catenin signaling pathway [10]. Moreover, RES suppresses the proliferation and glycolysis of OSCC cells by down-regulating mTOR/PKM2 axis signaling pathway [11]. In this study, EdU staining, Transwell migration and invasion assays, Annexin V-FITC/PI double-staining method were employed to determine cell

proliferation, migration, invasion and apoptosis ability of each group of cells, and the results revealed that RES could prominently inhibit cell proliferation, migration and invasion, and promote cell apoptosis in a dose-dependent manner, indicating that RES can ameliorate OSCC.

The occurrence of tumors is associated with cell malignant proliferation and apoptotic inhibition. Ki-67 is a nuclear non-histone protein that can better reflect cell proliferation, and its overexpression is closely related to the malignant biological behavior and prognosis of OSCC. The high positive expression rate indicates a poor prognosis. Bcl-2, serving as an important anti-apoptotic gene, can prolong cell lifespan and inhibit apoptosis, which plays an essential role in the occurrence and progression of OSCC. OSCC tissues have higher positive expression rate of Ki-67 than adjacent tissues, and Ki-67 is related to TNM staging and lymph node metastasis [12]. The positive expression rates of Ki-67 and Bcl-2 are significantly higher in OSCC mucosal tissues than those in normal tissues, which are related to the degree of OSCC tissue differentiation, tumor size and lymph node metastasis [13]. In this study, Ki-67 and Bcl-2 were positively expressed in OSCC tissues, and the positive expression rates were higher than those in normal oral mucosal tissues, indicating that Ki-67 and Bcl-2 expressions play important roles in OSCC.

Tumor recurrence and target organ metastasis can cause clinical exacerbations and poor prognosis. MMP-9 can destroy extracellular matrix and degrade basement membrane type IV collagen, thus promoting tumor cells to infiltrate through the basement membrane. MMP-9 expression is up-regulated in OSCC tissues, indicating that the increased expression of MMP-9 can promote the migration and invasion of OSCC cells. MMP-9 is not expressed or lowly expressed in normal oral mucosal tissues, but highly expressed in OSCC tissues, revealing that MMP-9 promotes the invasion and metastasis of malignant cells [14]. Epithelial-mesenchymal transition is also of great significance in the infiltration and metastasis of malignant tumors. Absence of E-cadherin expression and enhanced expression of N-cadherin are the hallmark events of epithelial-mesenchymal transition (EMT). E-cadherin is lowly expressed while N-cadherin is highly expressed in OSCC tissues [15]. In this study, the expression levels of MMP-9 and N-cadherin mRNA in OSCC tissues and cells were increased, and E-cadherin mRNA expression level was reduced, indicating that RES can inhibit OSCC EMT and inhibit tumor invasion and metastasis.

Abnormal activation of the Wnt pathway can induce tumorigenesis. Large accumulation of β-catenin is the key to activating the Wnt pathway. GSK-3β can promote β-catenin phosphorylation and act as a switch in the Wnt pathway. A previous study elucidated that the Wnt/β-catenin signaling pathway can promote tumor progression of esophageal squamous cell carcinoma [16]. Xu reported that Nav1.5-siRNA inhibited the proliferation, invasion and metastasis of OSCC cells by suppressing the expressions of key gene β-catenin and downstream target genes cyclinD1 and c-Myc in the Wnt/β-catenin signaling pathway [17]. β-Catenin expression level was significantly higher in OSCC tissues than that in the adjacent tissues, and it is related to TNM staging and lymph node metastasis, which may involve the Wnt/β-catenin pathway [18]. In this study, Wnt1, β-catenin and GSK-3β expression levels were significantly increased in tumor cells, but significantly reduced in a dose-dependent manner after intervention with RES, indicating that RES may exert the effect by inhibiting the Wnt pathway.

In conclusion, RES can inhibit the proliferation, migration and invasion of OSCC cells and promote cell apoptosis possibly by inhibiting the Wnt signaling pathway, providing a potential target for targeted therapy of OSCC. However, whether RES affects cell proliferation, migration, invasion and apoptosis in OSCC through other signaling pathways needs further investigation.

ACKNOWLEDGMENTS

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Figure 1.
Ki-67, MMP-9 and Bcl-2 protein expression levels in OSCC and normal oral mucosal tissues.

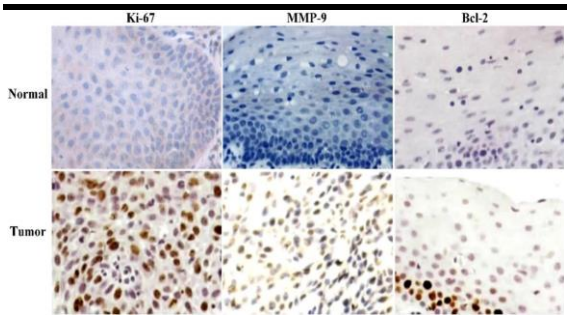

Figure 2.
Cell proliferation ability. *P<0.05 *vs.* control group, #P<0.05 *vs.* low-dose RES group, ΔP<0.05 *vs.* medium-dose RES group.

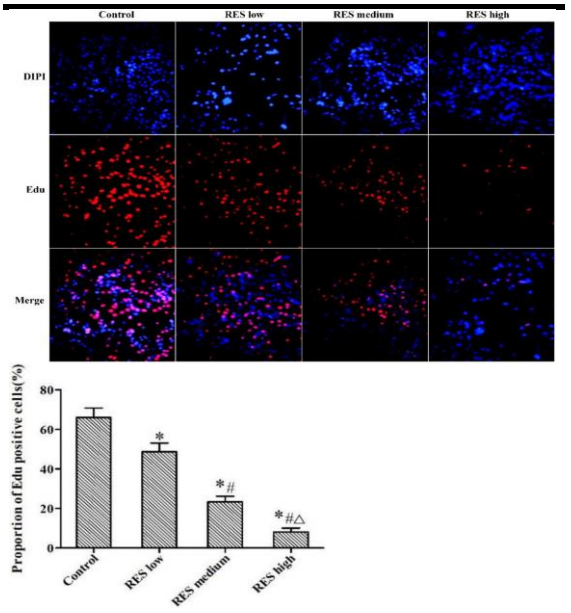

Figure 3.
Cell migration and invasion ability. *P<0.05 *vs.* control group, #P<0.05 *vs.* low-dose RES group, ΔP<0.05 *vs.* medium-dose RES group.

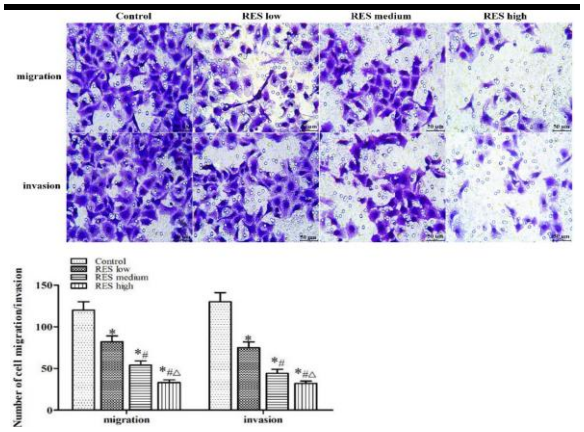

Figure 4.
Cell apoptosis ability. *P<0.05 *vs.* control group, #P<0.05 *vs.* low-dose RES group, ΔP<0.05 *vs.* medium-dose RES group.

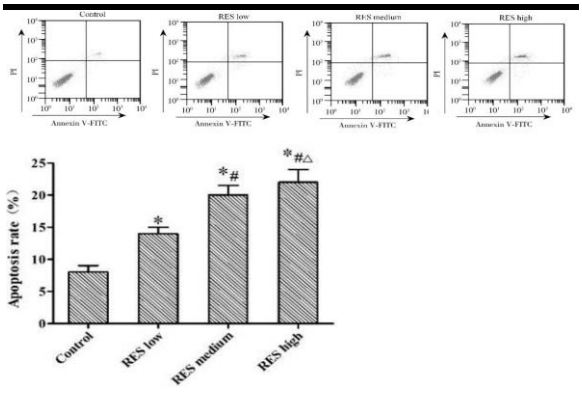

Figure 5.
E-cadherin and N-cadherin mRNA expression levels. *P<0.05 *vs.* control group, #P<0.05 *vs.* low-dose RES group, ΔP<0.05 *vs.* medium-dose RES group.

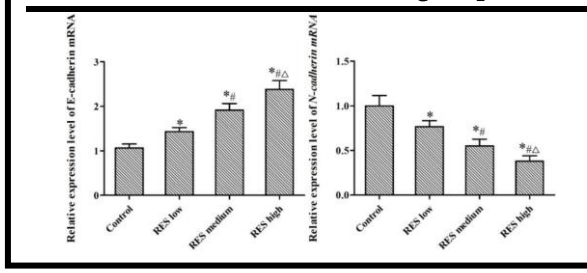

Figure 6.
Wnt1, β-catenin and GSK-3β protein expression levels. *P<0.05 *vs.* control group, #P<0.05 *vs.* low-dose RES group, ΔP<0.05 *vs.* medium-dose RES group.