Resveratrol suppresses malignant progression of oral squamous cell carcinoma cells by inducing the ZNF750 pathway

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Abstract

Deletion or mutation of zinc finger protein 750 (ZNF750) has been linked to oral squamous cell carcinoma (OSCC), but it is not clear whether ZNF750 is a therapeutic target for OSCC. This study examined whether activation of zinc finger protein 750 (ZNF750) pathway may be involved in the ability of resveratrol to inhibit malignant progression of CAL-27 oral squamous cell carcinoma cells. CAL-27 cells were treated with resveratrol and transfected with plasmids expressing a ZNF750 mimic or ZNF750 inhibitor. Cell proliferation was assessed using the CCK-8 assay and a BrdU ELISA, and cell cycle distribution and apoptosis were examined using flow cytometry. Colony formation was also assessed. Western blotting was used to examine the effects of resveratrol on levels of angiogenin, vascular endothelial growth factor (VEGF), prolyl hydroxylase 2 (PHD2), G protein signal-regulated protein 5 (RGS5), integrin A5 (ITGA5), integrin B1 (ITGB1), CD44 and ZNF750. Quantitative PCR was used to examine effects on mRNA levels of platelet derived growth factor (PDGFB) and tumor vascular marker CD105. Resveratrol down-regulated angiogenin, VEGF, RGS5, CD105, and the cell adhesion molecules ITGA5, ITGB1 and CD44 in CAL-27 cells. Conversely, it up-regulated ZNF750, PHD2 and PDGFB. These changes were associated with reduced proliferation, reduced colony formation and increased apoptosis. ZNF750 silencing partly reversed these effects of resveratrol. The ability of resveratrol to suppress progression of oral squamous cell carcinoma may involve activation of the ZNF750 pathway and modification of the tumor vascular microenvironment.

Introduction

Targeted molecular therapies inhibit oncogenic signaling pathways necessary for progression of malignant cancers (Chiorean and Coveler. 2015); for example, monoclonal antibodies may bind key protein actors, and small-molecule kinase inhibitors may inhibit phosphorylation-mediated activation of key proteins. Various genetic variants have been associated with development of human oral squamous cell carcinoma (OSCC) (Zhong et al. 2018; Choi et al. 2018; Junttila et al. 2013), raising the possibility that the corresponding proteins can be targeted to inhibit their involvement in oncogenic pathways.

Deletion or mutation of zinc finger protein 750 (ZNF750) has been linked to OSCC as well as to malignant phenotype in squamous epithelial cell cultures (Lin et al. 2014). Indeed, ZNF750 appears to be a lineage-specific tumor suppressor gene in OSCC (Hazawa et al. 2017) as well as a promising biomarker of poor outcomes in patients with the disease (Nambara et al. 2017; Otsuka et al. 2017). ZNF750 may suppress malignant progression of OSCC by regulating the tumor vascular microenvironment (Pan et al. 2018).

The tumor vascular microenvironment contributes to tumorigenesis by providing oxygen, nutrients and soluble growth factors. Hypoxia and limited nutrients in this microenvironment can trigger production of angiogenic cytokines such as VEGF, triggering vascular abnormality and cancer progression (Pan et al. 2018). The tissue oxygen sensing PHD2 is an important mediator of vascular normalization and regulator of RGS5, which is responsible for vessel maturation. PDGFB is responsible for vessel maturation (Goel et al. 2011). CD44, a transmembrane adhesion receptor for hyaluronan and growth
factors, contributes to the migration and invasion by tumor cells (Cho et al. 2012). Moreover, ITGB1 and ITGA5 mainly participate in cancer cell adhesion, cell migration and invasion (Zhao et al. 2014; Ohyagi-Hara et al. 2013).

Given the ability of the natural phytoalexin resveratrol to reduce the risk of OSCC as well as other cancers (Valenzano et al. 2006; Uzawa et al., 2005), we wondered whether this compound may interact with the endogenous tumor-suppressing activity of ZNF750 in OSCC cells. Therefore, the current study examined whether the inhibitory effects of resveratrol on OSCC malignant phenotype involve activation of ZNF750.

**Material And Methods**

**Reagents**

- Resveratrol was purchased from Sigma (St. Louis, MO, USA). Antibodies against the following proteins were obtained from Abcam Signaling Technology (Cambridge, UK): angiogenin (#ab1198321), vascular endothelial growth factor (VEGF, #ab1231474), prolyl hydroxylase 2 (PHD2, #ab1769832), G protein signal-regulated protein 5 (RGS5, #1122176), integrin A5 (ITGA5, #1043208), integrin B1 (ITGB1, #1567432), CD44 (#1431347), and ZNF750 (#1098453). The CCK-8 kit was purchased from Huamei (Wuhan, China). Annexin V-FITC and propidium iodide (PI) kits were purchased from BD (Franklin Lakes, NJ, USA). The BrdU ELISA kit was purchased from Usabio.cn. Trizol reagent was obtained from Thermo Fisher Scientific (Franklin Lakes, MO, USA).

**Cell culture and treatments**

- CAL-27 OSCC cells were provided by the Human Science Research Resources Bank and cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C in an environment of saturated humidity and 5% CO₂. Cells in logarithmic growth phase were used in all experiments. Resveratrol was dissolved in DMSO and added to cells at final concentrations of 10, 20 or 40 µM. Control cells were given the same volume of DMSO vehicle. Cells were incubated with resveratrol for 48 h. Each experiment was performed in triplicate.

**Transfection with plasmids encoding ZNF750 mimic or ZNF750 inhibitor**

To test the effects of ZNF750 overexpression and knockdown, CAL-27 cells were transiently transfected with empty vector (#20190112, Santa Cruz Biotechnology, AB, USA) or the same vector encoding the ZNF750 mimic (#20190123) or the ZNF750 inhibitor (#20190126). Cells were transfected using Lipofectamine 3000 (Invitrogen) based on the manufacturer’s instructions. ZNF750 overexpression and knockdown were confirmed by Western blot.

**Cell viability and proliferation**
After CAL-27 cells had been treated with resveratrol, the culture medium was removed completely, and 100 µL of medium containing 10 µL of CCK-8 reagent was added to each well. Plates were incubated for 2 h, then optical density (OD) at 540 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). Relative cell viability (%) was calculated as OD_{experiment} / OD_{control} × 100%.

Treated cells were lysed in RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) and analyzed by BrdU ELISA based on the manufacturer's instructions. OD at 507 nm was measured using a microplate reader (Bio-Rad).

**Colony formation**

After CAL-27 cells had been treated with resveratrol, numbers of cell colonies were counted under a reversed microscope. For each condition, the numbers of colonies in 10 fields of view were averaged.

**Apoptosis**

After CAL-27 cells had been treated with resveratrol, cells were harvested and adjusted to a concentration of 1×10^6 cells/mL. The cell suspension (0.5 mL) was stained with 1.25 µL Annexin V-FITC at room temperature for 15 min in the dark, then 10 µL PI was added. Cells were sorted by apoptosis stage using a BD flow cytometer.

**Levels of target mRNAs**

After CAL-27 cells had been treated with resveratrol, cells were washed twice with phosphate-buffered saline (PBS) and harvested. Total RNA was extracted using Trizol reagent. RNA concentration and purity were assessed using the NanoDrop 2000 (Thermo Fisher). RNA (1 µg) was reverse-transcribed in a 20-µl reaction using a PrimeScript® RT Kit. Then quantitative PCR was performed using the SYBR® Premix Ex Taq™ II kit (Takara, Dalian, China) on an ABI 7500 Sequence Detection System (Applied Biosystems, NJ, USA). Amplification parameters were 30 s pre-incubation at 95°C, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. The following primers were used: PDGFB forward, 5′-TCCCGAGGAGCTTTATGAGA-3′; PDGFB reverse, 5′-GGGTCATGTTCAGGTCCAAC-3′; CD105 forward, 5′-CCTCTACCTCAGCCCACACT-3′; CD105 reverse, 5′-TCTAACTGGAGCAGGAACCG-3′; β-actin forward, 5′-CTACCTCATAGATGCCACCTCACC-3′; and β-actin reverse, 5′-AGTTGAAGTAGCTTTGGTA-3′. Fold changes in the two target genes were normalized to levels of β-actin using the 2^−ΔΔCT method. Samples were analyzed in duplicate and each experiment was performed three times.

**Levels of target proteins**

After CAL-27 cells had been treated with resveratrol, cells were harvested and total protein was extracted using RIPA lysis buffer. Total protein concentration was estimated using the Bradford method (Thermo Fisher Scientific). Proteins (50 µg) were fractionated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Nonspecific binding sites were blocked with 5% skim milk for 1.5 h at room temperature on a shaking table. Then blots were incubated overnight at 4 °C with rabbit anti-mouse monoclonal antibodies (all diluted 1:1000) against the
following proteins: angiogenin, VEGF, PHD2, RGS5, ITGA5, ITGB1, CD44, and ZNF750. Subsequently blots were washed three times with PBS-Tween 20, and blots were incubated for 2 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit antibody (diluted 1:4000). Proteins were detected using luminol reagent and peroxide solution (Millipore, Billerica, MA, USA). Densitometry of images was performed using Image J software.

Statistical analysis Data were reported as mean ± SD. Inter-group differences were assessed for significance using Welch’s t test (comparisons of two groups) or ANOVA (comparisons of three or more groups). P < 0.05 was considered significant.

Results

Impact of resveratrol on expression of genes related to vascular normalization and metastasis

As shown in the Fig. 1, after treatment with different concentrations of resveratrol, it could significantly inhibited expression of angiogenin and VEGF in a concentration-dependent manner (Fig. 1A). It also down-regulated RGS5, CD105 and MMP3, while up-regulating PHD2, PDGFB and E-cadherin in CAL-27 cells (Fig. 1B-D).

Impact of resveratrol on expression of cell adhesion molecules ITGA5, ITGB1 and CD44

We further assess the effects of resveratrol on the expression levels of cell adhesion molecules in CAL-27 cells. The results from Fig. 2 showed that resveratrol significantly down-regulated ITGA5, ITGB1 and CD44, which are involved in cell adhesion and cell migration, in a concentration-dependent manner.

Impact of resveratrol on proliferation and apoptosis

Resveratrol significantly decreased the viability of CAL-27 cells in a concentration-dependent manner based on the CCK-8 assay (Fig. 3A), which coincided with significant up-regulation of apoptosis based on flow cytometry (Fig. 3B). Resveratrol also significantly decreased numbers of colonies and BrdU-positive cells in a concentration-dependent manner (Fig. 3C-D).

Impact of resveratrol on ZNF750 expression

In order to assess the mechanism in the inhibitory effect of resveratrol on the growth of CAL-27 cells, we further observe the expression level of ZNF750 in cells after treatment with resveratrol. As shown in Fig. 4, we found that resveratrol significantly up-regulated ZNF750 in a concentration-dependent manner.
Regulation of OSCC cell growth by resveratrol via ZNF750 induction

Our experiments above suggested that resveratrol up-regulated the tumor supressor ZNF750 in OSCC cells, leading us to ask whether this activation might help explain the compound's ability to inhibit the malignant phenotype in these cells. We transfected CAL-27 cells with plasmids expressing a ZNF750 mimic or inhibitor and then treated the cells with resveratrol (Fig. 5A-B). Expression of the mimic led to significantly lower viability and greater apoptosis than expression of the empty vector, while expression of the inhibitor led to significantly higher viability and less apoptosis (Fig. 5C-D). These results suggest that resveratrol inhibits the growth of OSCC cells by activating the ZNF750 pathway.

Discussion

Drugs derived from medicinal herbs show various potent biological functions against multiple cancers (Treasure 2005; Cao et al. 2017). Resveratrol is derived from certain plants and shows anti-inflammatory, anti-oxidative stress, and anti-tumor properties (Rauf et al. 2017; Thaung et al. 2017; Xia et al. 2017; Wu et al. 2010; Jiang et al. 2017; Yousef et al. 2017). It can modify the tumor microenvironment and sensitize cancer cells to chemo- and radiotherapy (Jiang et al. 2017). It can also inhibit proliferation and induce apoptosis in several tumor cell types (Kim et al. 2017; D’Asti et al. 2016). The present study extends these activities of resveratrol to OSCC cells, and it suggests that the anti-tumor efficacy against this disease involves activation of ZNF750-dependent tumor-suppressive pathways.

Previous work has demonstrated that ZNF750 can suppress the malignant progression of OSCCs by regulating the tumor vascular microenvironment (Goel et al. 2011). We confirmed and extended those findings by showing that, in OSCC cells, resveratrol down-regulates angiogenin, VEGF, RGS5, CD105, and the cell adhesion molecules ITGA5, ITGB1 and CD44 (D’Asti et al. 2016). At the same time, it up-regulates PHD2 and PDGFB. These results support the idea that resveratrol inhibits malignant progression in OSCC by regulating the tumor vascular microenvironment. Our observation that resveratrol also up-regulates ZNF750 suggests that this endogenous tumor suppressor mediates at least some of the changes in the tumor microenvironment. It is well known that suppressed genes have been enriched for terms related to cell proliferation. Earlier researches reported that ZNF750 is typically mutated or deleted in squamous cell carcinoma (Hazawa et al. 2017; Nambara et al. 2017; Otsuka et al. 2017). The loss of ZNF750 is related to impaired differentiation and failure to fully inhibit the proliferative genetic program, both of which are important markers of tumor (Okuno et al. 2014). Previous study have demonstrated that ZNF750 could suppress the malignant progression of OSCCs by regulating tumor vascular microenvironment (Goel et al. 2011). Thus, this study further observe the role of ZNF750 in the inhibitory effect of resveratrol on the proliferation of OSCC CAL-27 cells. Western blot analysis displayed that resveratrol induced the up-regulation of ZNF750 expression. Then OSCC CAL-27 cells were transfected with ZNF750 knockdown partially reversed the effects of resveratrol on OSCC cell growth. This idea is strengthened by our observation that ZNF750 knockdown partially reversed the effects of resveratrol on OSCC cell growth.
Our results justify further work into how resveratrol may induce ZNF750 expression and how this affects downstream signaling and gene expression pathways. This future research may help develop novel therapeutic targets against OSCC and potentially other cancers in which resveratrol shows anti-tumor efficacy.

In summary, resveratrol could regulate the tumor vascular microenvironment to suppress the oral squamous cells carcinoma malignant process through the activation of ZNF750 pathway. These results of the present study provide a reference for clinical treatment of oral squamous cell carcinoma.

**Declarations**

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**Authors' contributions**

YJD: investigation (lead), Writing-original draft (equal); YJW: curation (equal); XJY: curation (equal); YX: conceptualization (lead), writing-original draft (equal); YJD: investigation (equal) YX: methodology (supporting); YX: Resource (supporting); YJW: methodology (supporting); XJY: visualization (supporting), writing original draft (supporting). All authors read and approved the final manuscript.

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**Availability of data and materials**

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

**Ethics approval and consent to participate**

This article does not contain any studies with human or animal subjects.

**Competing interests**

The authors declare that they have no conflicts of interest with respect to the work described in this manuscript.

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Figures

**Figure 1**

Effects of resveratrol (Res) on expression of genes associated with vascular normalization and metastasis: (A) angiogenin and VEGF, (B) PHD2 and RGS5, (C) PDGFB and CD105, and (D) E-cadherin and MMP3. *P < 0.05 vs. Res 0 μM control.

**Figure 2**

Effects of resveratrol (Res) on expression of cell adhesion and migration molecules ITGA5, ITGB1 and CD44. *P < 0.05 vs. Res 0 μM control.
Figure 3

Effects of resveratrol (Res) on malignant phenotype of OSCC cells. Treated cells were assayed for (A) viability, (B) apoptosis, (C) colony-forming ability, and (D) proliferation based on BrdU incorporation. *P < 0.05 vs. Res 0 μM control.

Figure 4

Effects of resveratrol (Res) on ZNF750 expression. *P < 0.05 vs. Res 0 μM control.
Figure 5

Effects of ZNF750 overexpression and silencing on growth of OSCC cells cultured in the presence of resveratrol (Res). Control cells were transfected with empty plasmide. (A-B) Confirmation by Western blot that ZNF750 was up-regulated in cells transfected with plasmid expressing a ZNF750 mimic, and down-regulated in cells transfected with plasmid expressing the inhibitor. (C) Viability and (D) apoptosis levels were assayed by flow cytometry. *P < 0.05 vs. control group; #P < 0.05 vs. Res group.