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Original Research

Resveratrol inhibits skin squamous cell carcinoma proliferation, migration and invasion through up-regulating miR-126

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Abstract: To explore the effect and possible mechanism of resveratrol on the proliferation, migration and invasion of skin squamous cell carcinoma cells (HSC-5). Tetramethyl azozo blue (MTT) method, Transwell experiment and real-time fluorescence quantitative PCR (RT-qPCR) were used to detect the effects of resveratrol intervention on the proliferation, migration and invasion of HSC-5 cells and the expression of miR-126. The miR-126 mimics and inhibitors were transfected into HSC-5 cells, and the effects of up-regulation or down-regulation of HSC-5 expression on the proliferation, migration and invasion of HSC-5 cells were detected. The miR-126 inhibitor was transfected into HSC-5 cells, and the effects of resveratrol intervention on HSC-5 cell proliferation, migration and invasion, and β-catenin protein expression were detected. After resveratrol intervention, the growth rate, migration and invasion of HSC-5 cells were significantly reduced, miR-126 expression was significantly increased, and β-catenin protein expression was significantly reduced (p<0.05). After up-regulating the expression of miR-126, the growth rate, migration and invasion of HSC-5 cells were significantly reduced (p<0.05). After down-regulating the expression of miR-126, the growth rate, migration and invasion of HSC-5 cells were significantly increased (p<0.05). Down-regulation of miR-126 expression could reverse the effects of resveratrol intervention on HSC-5 cell proliferation, migration and invasion, and β-catenin protein expression (p<0.05). Resveratrol could inhibit the proliferation, migration and invasion of skin squamous cell carcinoma cells, which may be related to the up-regulation of miR-126 to inhibit the Wnt / β-catenin signaling pathway.

Key words: Resveratrol; Skin squamous cell carcinoma; miR-126; Cell proliferation; Migration and invasion; Wnt / β-catenin signaling pathway.

Introduction

Resveratrol is an antioxidant found in polyphenols. The compound is found in red grapes, blueberries and peanuts. Past studies have shown that the compound can help treat acne, heart disease and cancer. Researchers have shown that the Resveratrol supplement can improve the body's response to diabetes. Taking 10 mg of resveratrol daily reduces insulin resistance in people with type 2 diabetes. Resveratrol has provided scientists with a new and deeper understanding of the mechanism of insulin resistance and oxidative stress. Resveratrol is a potent polyphenol and antifungal chemical and is an active biological agent in red grapes (1-4).

Resveratrol, non-flavonoid polyphenols extracted from medicinal plants such as Polygonum cuspidatum, Cassia tora, mulberry or crops such as grapes, peanut, has antioxidant, anti-inflammatory, antiviral, anticancer and cardiovascular protection effects. Over the years, it has been used as complementary and alternative medicine in the prevention and treatment of cardiovascular disease and cancer like myocardial ischemia, myocarditis, myocardial hypertrophy and heart failure, etc. (1). At present, there have been some studies on the treatment of human cutaneous squamous cell carcinoma (CSCC) using resveratrol. For instance, resveratrol can induce apoptosis of cutaneous squamous cell carcinoma and inhibit the growth of transplanted tumors in nude mice (2-4), but its effect on CSCC migration and invasion and its anti-tumor mechanism is not very clear. miR-126 is a short-stranded, conserved non-coding RNA that affects the post-transcriptional regulation of multiple genes by binding to 3' untranslated region (UTR) of the target mRNA. Studies have shown that miR-126 expression is down-regulated in breast cancer, and up-regulation of miR-126 expression can reduce the proliferation and migration capacity of breast cancer cells via targeted VEGF, thereby inhibiting angiogenesis. Baicalin may have an anti-tumor effect on breast cancer by up-regulating miR-126 expression (5-7). miR-126 has been confirmed to be down-regulated in CSCC (8). In this study, the anti-tumor mechanism of resveratrol is investigated by observing the effects of resveratrol on the proliferation, migration and invasion of CSCC cells and miR-126 expression, to lay the theoretical foundation for the use of resveratrol in prevention and treatment of CSCC.

Materials and Methods

Experimental materials

Human cutaneous squamous cell carcinoma HSC-5 was purchased from Ningbo Mingzhou Biotechnology Co., Ltd. DMEM high glucose medium, fetal bovine

serum and penicillin-streptomycin liquid were purchased from Beijing Solarbio Life Sciences Co., Ltd. Resveratrol (purity 98%) was purchased from Beijing NuoQiYasSheng Biotech Co., Ltd. miR-126 mimic, inhibitor (anti-miR-126) and their corresponding controls (miR-NC, anti-miR-NC), PCR primers were provided by Shanghai Sangon Biotech. Methyl thiazolyl tetrazolium (MTT) cell proliferation detection kit was purchased from Beijing BaiOLaiBo Technology Co., Ltd. Transwell chamber and matrigel were purchased from Corning, USA; Rabbit anti-matrix metalloproteinase 2 (MMP2) antibody, rabbit anti-MMP9 antibody, rabbit anti-β-catenin antibody, rabbit anti-glyceraldehyde phosphate dehydrogenase (GAPDH) antibody and goat anti-rabbit IgG antibody were purchased from Shanghai Aibo Anti-Biotechnology Co., Ltd.

Experimental methods

Cell culture and experimental grouping

After resuscitation of HSC-5 cells, DMEM high glucose medium with 10% fetal bovine serum and 10% penicillin-streptomycin liquid was cultured in a 37℃ thermostatic cell incubator with CO_2 volume fraction at 5%. When the cell density reached 80%-90%, the cells were rinsed with phosphate buffer, added with digestive juice for passage and then transferred to a petri dish containing fresh medium at a proportion of 1:3 after the termination of digestion. Logarithmic phase HSC-5 cells were seeded into 6-well plates, added with culture medium containing 20, 40 and 80μmol/L resveratrol respectively for 24 h culture. Cell proliferation rate was determined by MTT and subsequent experiment concentration was determined. HSC-5 cells were divided into NC group and resveratrol group, and the proliferation, migration and invasion capacity of the cells, as well as the expression levels of related proteins and miR-126, were detected according to the following experimental methods.

In order to investigate the molecular mechanism in the effect of resveratrol on the proliferation, migration and invasion of HSC-5 cells, cell transfection was carried out with reference to Lipofectamine™ 2000 specification. HSC-5 cells were divided into the miR-NC group (transfected with miR-NC), miR-126 group (transfected with miR-126 mimics), anti-miR-NC group (transfected with anti-miR-NC), anti-miR-126 group (transfected with anti-miR-126), and resveratol + anti-miR-NC group (after transfected with anti-miR-NC for 48 h, incubated with medium containing 40 μmol/L resveratrol for 24 h) and resveratol + anti-miR-126 group (after transfected with anti-miR-126 for 48 h, incubated with medium containing 40μmol/L resveratrol for 24 h).

Cell viability detection by MTT assay

HSC-5 cells or transfected HSC-5 cells were seeded into a 96-well plate at 1×10^4cells/well. After overnight culture, resveratrol was added for 24 h reaction according to experimental grouping. The cell culture fluid was discarded, 20 μL MTT reagent was added to each well for 4 hours' further culture. Then liquid supernatant was removed. 150μL dimethyl sulfoxide was added to each well and fully shaken for dissolution. The blank well was set to zero and the absorbance (A) value was detected at 490 nm by a microplate reader. Cell proliferation rate= $(A_{\text{experimental well}}/A_{\text{control well}})\times100\%$

Detection of the number of migrating and invasive cells by Transwell assay

HSC-5 cells in each group were collected, washed with phosphate buffer and added with a serum-free medium to prepare 1×10^8 cell/L single-cell suspension. 200 μL cell suspension was seeded to the upper Transwell chamber, and 500 μL cell culture medium containing 20% fetal bovine serum was added to the lower chamber as an inductor. After incubation in the incubator for 24 h, cells on the filter membrane were wiped off using wet cotton swabs. The filter membrane was then fixed with formaldehyde for 30 min and stained with crystal violet for 15 min. The number of cells that migrated to the lower chamber was counted under the microscope. During the invasion experiment, 70 μL matrigel with a concentration of 1 g/L was uniformly applied to the polycarbonate membrane of the upper Transwell chamber, placed in 37℃ incubator for 1 h to allow the gel to be recombined into basement membrane for use. Other steps were the same as those of the migration experiment.

Detection of MMP2, MMP9 and β-catenin protein expression levels by Western blot

After treatment, HSC-5 cells were added with cell lysate, lysed for 30 min on the ice, centrifuged for 10 min by 4℃ centrifuge at 12 000 r/min. The supernatant was collected and subject to protein quantification for use. An appropriate amount of protein samples was added to isovolumetric 2× loading buffer and boiled for 3 min to denature the protein, followed by polyacrylamide gel electrophoresis. The membrane was transferred using cellulose nitrate membrane, sealed in 5% skim milk at 4℃ overnight, added with corresponding primary antibody (1:500 for MMP2, 1:1000 for MMP9, and 0.25 μg/mL for β-catenin), and incubated at room temperature for 2 h. The second antibody was added (1:300) and incubated for 1 h at room temperature. Chemiluminescence kit was used for development, and the relative expression of target protein was analyzed by scanning grayscale value with β-actin as an internal reference.

Detection of miR-126 expression level by real-time fluorescent quantitative PCR (RT-qPCR)

Total RNA was extracted from HSC-5 cells by the TRIzol method. cDNA was synthesized using1 μg total RNA by miR-126 specific reverse transcription primer, and the miR-126 level was detected by RT-qPCR with U6 as an internal reference. The relative expression level of miR-126 was calculated using $2^{-\Delta\Delta Ct}$. miR-126 upstream primer sequence 5'-GTCGTATCCAGTGCAGGGTCCGAG-3', downstream primer sequence 5'-GTATTCGCACTGGATACGAC-3'; U6 upstream primer sequence 5'-CTCGCTTCGGCAGCACA-3', downstream primer sequence 5'-AACGCTTCACGAATTTGCGT-3'.

Statistical analysis

SPSS 20.0 statistical software was used for analysis. Data were expressed as mean ± standard deviation, and the *t*-test was used for comparison between two groups. One-way analysis of variance was taken for comparison of multiple groups, and pairwise comparison between

groups was performed by the SNK-q test. P<0.05 indicates a statistically significant difference.

Results

Effects of different concentrations of resveratrol on the proliferation of HSC-5 cells

As shown in Table 1, compared with the control group, the proliferation rate of HSC-5 cells is significantly reduced in 20, 40 and 80μmol/L resveratrol intervention groups (p<0.05). The next experiment was conducted using 40 μmol/L resveratrol.

Effects of resveratrol on migration and invasion of HSC-5 cells (40μmol/L resveratrol)

As shown in Table 2 and Figure 1, compared with the NC group, the resveratrol group has significantly reduced the number of migrating and invasive HSC-5 cells, and significantly reduced MMP2 and MMP9 protein expression (p<0.05).

Effects of resveratrol on the miR-126 expression

As shown in Table 3, compared with the NC group, the resveratrol group has significantly reduced miR-126 expression in HSC-5 cells (p<0.05).

Effects of miR-126 on the proliferation, migration and invasion of HSC-5 cells

As shown in Table 4 and Figure 2, compared with the miR-NC group, the miR-126 group has significantly reduced HSC-5 cell proliferation, migration and invasion, and significantly reduced MMP2 and MMP9 protein expressions. Compared with the anti-miR-NC group, the anti-miR-126 group has significantly reduced HSC-5 cell proliferation, migration and invasion, and significantly reduced MMP2 and MMP9 protein expressions (p<0.05).

Table 1. Effects of different concentrations of resveratrol on the proliferation of HSC-5 cells (x±s, n=9).

Group	Cell proliferation rate (%)
NC	100.67±9.37
20 μmol/L resveratrol	80.33±7.68*
40 μmol/L resveratrol	44.63±4.39*
80 μmol/L resveratrol	39.16±3.82*
F	60.370
p	0.000

Note: compared with NC, *p<0.05.

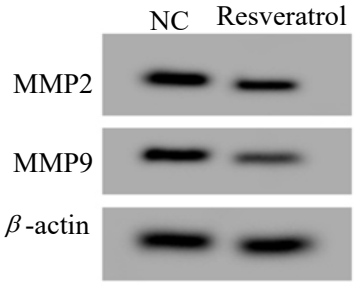

Figure 1. Detection of MMP2 and MMP9 protein expression by Western Blot.

Table 3. Effect of resveratrol on miR-126 expression (x±s, n=9).

Group	miR-126
NC	1.00±0.11
Resveratrol	4.32±0.31*
t	30.279
p	0.000

Note: compared with NC, *p<0.05.

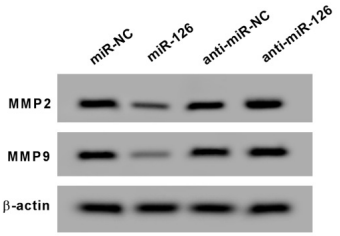

Figure 2. Detection of MMP2 and MMP9 protein expressions by Western Blot.

Down-regulation of miR-126 can reverse the effects of resveratrol on the proliferation, migration and invasion of HSC-5 cells

As shown in Table 5 and Figure 3, compared with the resveratrol + anti-miR-NC group, the resveratrol + anti-miR-126 group has significantly reduced miR-126 expression in HSC-5 cells, significantly increased cell proliferation, migration and invasion, and significantly increased MMP2 and MMP9 protein expressions(p<0.05).

Expression of Wnt/β-catenin signaling pathway-related proteins

As shown in Table 6 and Figure 4, compared with the NC group, the resveratrol intervention group has significantly reduced β-catenin protein expression in HSC-5 cells. Compared with resveratrol + anti-miR-NC group, resveratrol + anti-miR-126 group has significantly increased β-catenin protein expression in HSC-5 cells (p<0.05).

Discussion

CSCC, the most common cutaneous malignant tumor, features strong invasiveness and poor prognosis

Table 2. Effects of resveratrol on migration and invasion of HSC-5 cells(x±s, n=9).

Group	MMP2	MMP9	Number of migrating cells	Number of invasive cells
NC	0.82±0.08	0.73±0.07	179.14±16.05	152.07±14.16
Resveratrol	0.40±0.04*	0.32±0.03*	105.73±9.07*	88.05±8.27*
t	14.087	16.151	11.946	4.678
P	0.000	0.000	0.000	0.000

Note: compared with NC, *P<0.05.

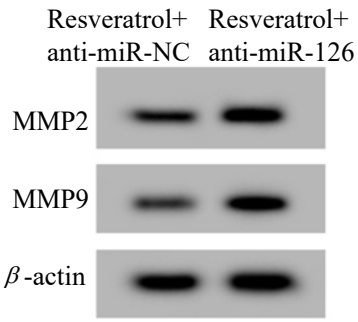

Figure 3. Detection of MMP2 and MMP9 protein expression by Western Blot.

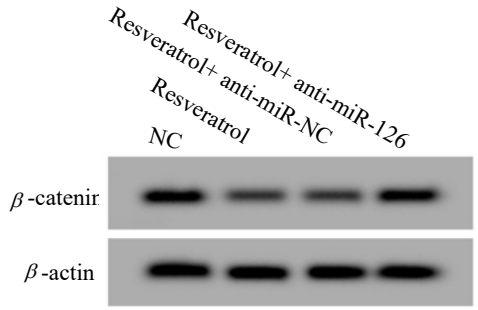

Figure 4. Detection of β-catenin protein expression by Western Blot.

Table 6. Expression of Wnt/β-catenin signaling pathway-related proteins (x±s, n=9).

Group	β-catenin
NC	0.84±0.08
resveratrol	0.43±0.03*
resveratrol +anti-miR-NC	0.40±0.04
resveratrol +anti-miR-126	0.76±0.07#
F	132.065
p	0.000

Note: Compared with NC,*p<0.05; compared with resveratrol +anti-miR-NC, #p<0.05.

in the late stage. Surgery is the preferred therapy for CSCC, but strong invasiveness and poor resistance to chemotherapy of tumor cells often lead to tumor recurrence and distal metastasis. Resveratrol is a natural polyphenol compound with a potential role in the treatment of cancer by regulating cell proliferation, cell cycle and apoptosis via the regulation of cell signal transduction pathways (9,10). In this study, we investigated the anti-tumor effect of resveratrol in CSCC, finding that resveratrol inhibited HSC-5 cell proliferation in a dose-dependent manner. Further studies have found that resveratrol can also reduce the migration and invasion capacity of HSC-5 cells, and inhibit the expression of MMP2 and MMP9 proteins that promote migration and invasion, which is consistent with the anti-tumor effect of resveratrol reported at home and abroad (11,12). In addition, the present study found that miR-126 expression level could be significantly increased by resveratrol. The above studies indicate that the inhibitory effect of resveratrol on the proliferation, migration and invasion of CSCC cells may be related to the up-regulation of miR-126 expression.

Located on human chromosome 9, miR-126 is encoded by intron 7 of epidermal growth factor-like domain-containing 7 (egfl7) gene, which is closely related to the occurrence and development of multiple human tumors. It has been reported that the low expression level of miR-126 is associated with poor overall survival rate, clinicopathological staging and tumor recurrence in patients with liver cancer (13). Low miR-126 also promoted the occurrence of tongue squamous cell carcinoma (14). miR-126 has decreased expression in colon cancer, and the low expression of miR-126 can promote the expression of the mammalian target of rapamycin (mTOR). Restoration of miR-126 expression will activate the mTOR pathway, reduce the viability of colon cancer cells, and induce autophagy and apoptosis (15). Up-regulation of miR-126 expression can also reduce the proliferation and metastasis of prostate cancer and osteosarcoma cells, and reverse the epithelial-mesen-

Table 4. Effects of miR-126 on the proliferation, migration and invasion of HSC-5 cells(x±s, n=9).

Group	MMP2	MMP9	Cell proliferation rate (%)	Number of migrating cells	Number of invasive cells
miR-NC	0.83±0.08	0.70±0.07	100.58±10.06	174.23±16.40	150.11±14.29
miR-126	0.48±0.05*	0.36±0.03*	64.55±6.07*	124.15±12.42*	98.02±9.61*
anti-miR-NC	0.86±0.07	0.72±0.07	100.36±9.86	169.04±15.66	146.27±14.09
anti-miR-126	1.11±0.11#	0.94±0.08#	127.14±12.32#	187.13±17.53#	172.33±16.83#
F	129.935	120.702	61.393	27.702	45.293
P	0.000	0.000	0.000	0.000	0.000

Note: compared with NC,*p<0.05; compared with anti-miR-NC, #p<0.05.

Table 5. Anti-miR-126 can reverse the effects of resveratrol on the proliferation, migration and invasion of HSC-5 cells(x±s, n=9)

Group	miR-126	MMP2	MMP9	Cell proliferation rate (%)	Number of migrating cells	Number of invasive cells
Resveratrol +anti-miR-NC	1.00±0.11	0.38±0.04	0.30±0.03	46.33±4.41	111.27±10.05	82.34±8.16
Resveratrol +anti-miR-126	0.42±0.04*	0.76±0.08*	0.75±0.07*	82.14±8.26*	161.03±16.02*	158.33±14.63*
t	14.866	12.746	17.726	11.473	7.894	13.609
p	0.000	0.000	0.000	0.000	0.000	0.000

Note: compared with resveratrol+anti-miR-NC group, *p<0.05.

chymal transformation in vitro (16-20), demonstrating the potential application value of miR-126 in cancer treatment. To investigate whether miR-126 has anti-tumor effects on CSCC, the present study up-regulated or down-regulated miR-126 expression levels in HSC-5 cells by transfection with miR-126 mimics and miR-126 inhibitors, respectively. The results indicate that the up-regulation of miR-126 can significantly reduce the proliferation, migration and invasion capacity of HSC-5 cells, inhibit MMP2 and MMP9 protein expression with anti-tumor effects. However, the down-regulation of miR-126 significantly increased the proliferation and migration of HSC-5 cells. In addition, down-regulation of miR-126 expression can also reverse the inhibitory effect of resveratrol on the proliferation, migration and invasion of HSC-5 cells. It suggests that the inhibitory effect of resveratrol on the proliferation, migration and invasion of HSC-5 cells might be related to the up-regulation of miR-126 expression. In the end, it can be said that the discussion of gene expression and control is of particular importance. In this regard, new technologies in genetic engineering can be very effective in the future in relation to changes in gene expression (21-33). Non coding RNAs serve as particular targets of genomic lesions, colorectal primary lesions, in cancers, and even in pathologic diminution of the rate of fetal growth such as intrauterine growth restriction (IUGR), have been associated with alterations in expression of tumor suppressor genes or activation of specific oncogenes (34-36). These associations emphasized the importance of miRNAs. In the current research, the miR-126 expression was studied.

Studies have shown that Wnt/β-catenin signaling pathway is abnormally activated in a variety of malignancies including CSCC, inhibition of activation of the Wnt/β-catenin pathway can accelerate CSCC cell apoptosis and inhibit cell migration and invasion. Studies have also shown that resveratrol inhibits colon cancer cell growth by inhibiting Wnt/β-catenin pathway. miR-126-3p can improve the drug resistance of glioma cells to temozolomide by blocking the Wnt/β-catenin pathway. However, it is not known whether the anti-tumor effect of resveratrol in CSCC is related to the inhibition of the Wnt/β-catenin pathway. Further analysis showed that resveratrol intervention could reduce β-catenin expression, inhibit the activation of the Wnt/β-catenin signaling pathway, while down-regulation of miR-126 could reverse the inhibitory effect of resveratrol on Wnt/β-catenin signaling pathway. It is suggested that inhibition of the Wnt/β-catenin signaling pathway by up-regulation of miR-126 expression may be the mechanism of resveratrol in inhibiting proliferation, migration and invasion of HSC-5 cells.

To sum up, resveratrol can inhibit the proliferation, migration and invasion of CSCC cells, and its mechanism may be related to the up-regulation of miR-126 expression to inhibit Wnt/β-catenin signaling pathway. This will lay a theoretical foundation for the use of resveratrol in prevention and treatment of CSCC.

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