Inhibition of laryngeal cancer cell activity by pterostilbene via modulating AKT/mTOR signal pathway and related mechanisms

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Abstract: AKT/mTOR signal pathway participates in growth, proliferation and invasion of tumor cells, and is correlated with onset and occurrence of laryngeal cancer. Resveratrol can modulate proliferation of laryngeal cancer cells. Pterostilbene, as one homolog of resveratrol, has more potent effects for inhibiting tumor cell occurrence and progression, but leaving its role and mechanism for laryngeal cancer cells unillustrated yet. This study investigated the effect of pterostilbene on laryngeal cancer cells and AKT/mTOR signal pathway. Laryngeal cancer cell line Hep-2 was treated with 100 μmol/L or 200 μmol/L pterostilbene, followed by MTT assay for cell proliferation, caspase-3 activity assay for cell apoptosis, and cell invasion assay. Western blot was used to test the change of AKT protein phosphorylation. Real-time PCR was adopted to describe mTOR expression. Pterostilbene significantly inhibited Hep-2 cell proliferation and invasion, and enhanced Caspase-3 activity (p<0.05 compared to control group). 200 μmol/L pterostilbene had more potent effects than 100 μmol/L group. Pterostilbene also suppressed mTOR mRNA of pharyngeal cancer cells, and decreased phosphorylation level of AKT (p<0.05 compared to control group). Pterostilbene can regulate proliferation, apoptosis and invasion of pharyngeal cancer cells via inhibiting AKT/mTOR signal pathway.

Keywords: Pterostilbene, pharyngeal carcinoma, AKT/mTOR, cell proliferation, invasion

Introduction

Laryngeal squamous cell carcinoma (LSCC) or laryngeal cancer, is one common malignant tumor with high malignancy [1]. With transition of life styles and diet habit, plus environmental and social stress, incidence of LSCC is rapidly increasing [2]. Multiple approaches have been developed targeting LSCC with individualized therapy including surgery, radiotherapy, chemotherapy, immune therapy or intervention. The overall prognosis of LSCC, however, had not been radically improved, causing high incidence of metastasis and recurrence, thus bringing heavy burdens for public health [3, 4]. To date, LSCC is still one epidemic malignant tumor worldwide [5]. As a complicated process involving multiple factors, genes and pathways dedicates occurrence and progression of LSCC, the rapid and precise method for early diagnosis of LSCC is still lacking. Moreover, the treatment efficiency for late stage LSCC is unfavorable [6, 7]. Therefore, the establishment of effective way to treat LSCC and inhibit tumor invasion is one major challenge in medicine [8].

Important biological features of LSCC includes invasion and metastasis of cancer cells, which are also major reasons causing death and are correlated with various signal pathways [9]. Protein kinase B (AKT) and mammalian target of rapamycin (mTOR) signal pathway participates in cell proliferation and differentiation or apoptosis [10]. AKT/mTOR signal pathway plays a critical role in tumor cell growth, proliferation and invasion [11]. Previous study indicated the close correlation between AKT/mTOR signal pathway and occurrence and progression of LSCC [12]. Therefore, regulation of AKT/mTOR signal pathway is one research focus for target-
ed inhibition of LSCC progression. Resveratrol can modulate tumor progression [13]. As one homologous derivative of resveratrol, pterostilbene is one type of non-flavonoids polyphenol compound and is widely distributed in multiple plants including grapes, nuts, strawberry, Guangxi dragon's blood and propolis [14]. As one 3,5'-di-methyl-deriviate of resveratrol, pterostilbene (or (E)-3,5-dimethyl-4-hydroxystilbene) has similar pharmaceutical functions as resveratrol. Therefore, pterostilbene also has multiple functions including anti-fungal, anti-cell proliferation, preventing oxidative stress response, anti-inflammation and decreasing blood lipid [15, 16]. However, its function or mechanism in LSCC cells has not been fully illustrated. This study thus investigated the role of pterostilbene on LSCC cells and its effects on AKT/mTOR signal pathway.

Materials and methods

Major equipment and reagent

LSCC cell line Hep-2 (CCL-23™) was purchased from ATCC cell bank (Manassas, VA, USA). Pterostilbene was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). DMEM culture medium, fetal bovine serum (FBS) and streptomycin-penicillin were purchased from Hyclone (Logan, Utah, USA). DMSO and MTT powders were purchased from Gibco (Waltham, MA, USA). Trypsin-EDTA lysis buffer was purchased from Sigma (St. Louis, MO, USA). PVDF membrane was purchased from Pall Life Sciences (Fajardo, Puerto Rico, USA). Western blotting reagent was purchased from Beyotime (Nantong, Jiangsu, China). ECL reagent was purchased from Amersham Biosciences (Amersham, Buckinghamshire, United Kingdom). Rabbit anti-human pAKT monoclonal antibody, rabbit anti-human AKT monoclonal antibody and goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG secondary antibody were all purchased from Cell Signaling Technology (Danvers, MA, USA). RNA extraction kits and reverse transcription kit were purchased from Axygen (Corning, New York, USA). Caspase-3 activity assay kit was purchased from R&D (Minneapolis, MN, USA). Labsystem Version 1.3.1 microplate reader was purchased from Bio-rad (Hercules, California, USA). ABI7700 Fast fluorescent quantitative PCR cycler was purchased from ABI (Foster City, CA, USA). Ultrapure workstation was purchased from Sutai Engineering (Tianjin, China). Transwell chamber was purchased from Corning (Corning, New York, USA). Thermo Scientific Forma CO₂ incubator was purchased from Thermo Fisher Scientific (Waltham, MA, USA). PCR cycler model 2400 was purchased from PE Gene Amp (Waltham, MA, USA). Other common reagents were purchased from Sangon (Shanghai, China).

Hep-2 cell culture and grouping

Hep-2 cells stored in liquid nitrogen were resuscitated in 37°C water-bath until fully thawing. Cells were centrifuged at 1000 rpm for 3 min, and were re-suspended in 1 ml fresh medium and were removed into 50 ml culture flask containing 2 ml fresh culture medium. Cells were kept in a humified chamber with 5% CO₂ at 37°C for 24~48 h. Cells were seeded in culture dish at 1×10⁷ per cm². The culture medium contained 10% FBS, and 90% high-glucose DMEM medium (containing 100 U/ml penicillin, 100 μg/ml streptomycin). Cells were kept in a humified chamber with 5% CO₂ at 37°C. The medium was changed every other day and cells were passed every 2~3 cells. Cells at log-phase with 2nd to 8th generation were randomly divided into control group, 100 μmol/L pterostilbene and 200 μmol/L pterostilbene for 72 h treatment [17].

MTT for the effect of pterostilbene on Hep-2 cell growth

Hep-2 cells at log-phase were counted, digested and seeded into 96-well plate at 3000 cells per well. Cells were then randomly divided into control, and high- or low-dosage of pterostilbene treatment. Each group was cultured for 72 h in five replicated wells. 20 μl sterile MTT solution (5 g/L) was then added into each test well. With 4 h continuous culture at 37°C, the supernatant was completely removed, with the addition of 150 μl DMSO for 10 min vortex until the complete resolving of crystal violet. Absorbance (A) values was measured at 570 nm in a microplate reader. The proliferation rate was calculated in each group.

Caspase 3 activity assay

Caspase 3 activity in all groups of cells was evaluated using test kit following the manual instruction. In brief, cells were digested by trypsin, and were centrifuged at 600 g for 5 min.
under 4°C. The supernatant was discarded, followed by the addition of cell lysis buffer and iced incubation for 15 min. The mixture was then centrifuged at 20000 g for 5 min under 4°C, followed by the addition of 2 mM Ac-DECD-pNA. Optical density (OD) values at 450 nm wavelength were measured to reflect caspase 3 activity.

**Flow cytometric analysis of cell apoptosis**

After centrifugation, 100 ul blocking buffer was added into the cells and incubated for 10 min at room temperature followed by centrifugation. Then 500 ul buffer containing calcium was added followed by addition of 100 ul Annexin-V antibody and subsequent addition of PI. After that, cell apoptosis was analyzed by flow cytometry.

**Real-time PCR for mTOR expression in Hep-2 cells**

Trizol reagent was used to extract RNA from Hep-2 cells from all groups. Reverse transcription was performed following the manual instruction, using primers designed by Primer-Primer6.0 and synthesized by Invitrogen (China) as shown in **Table 1**. Real-time PCR was performed under the following conditions: 55°C for 1 min, followed by 35 cycles each containing 92°C for 30 s, 58°C for 45 s and 72°C for 35 s. Data were collected and calculated for CT values of all samples and standards based on fluorescent quantification using GAPDH as the internal control. Standard curve was firstly plotted using CT values of standards, followed by semi-quantitative analysis by 2^(-ΔΔCT) method.

**Transwell chamber assay for cell invasion**

Following the instruction of test kit, serum-free DMEM medium was used for another 24 h cell culture. Transwell chamber was pre-coated using 1:5 50 mg/L Matrigel dilutions on the bottom and upper layer of the membrane, followed by 4°C air-dry. 500 μl DMEM medium containing 10% FBS was added into the interior chamber, whilst 100 μl tumor cell suspension in serum-free DMEM medium was added into the exterior of chamber. Each group was tested in triplicate. Chambers were placed into 24-well plate. Control cells were cultured in Transwell chamber without Matrigel. After 48 h, PBS was used to rinse Transwell chamber to remove membrane-fixed cells. Chambers were then fixed in cold ethanol and stained by crystal violet for 20 min. Cells at the lower surface of the micro-pore membrane were observed and counted under. Each experiment was repeated for more than three times.

**Western blot for AKT phosphorylation level**

Total proteins were extracted from Hep-2 cells. In brief, cells were mixed with lysis buffer on ice for 15~30 min, with ultrasound treatment (5 s, 4 times). After centrifugation at 10000 g for 15 min at 4°C, the supernatant was saved, quantified by Bradford method and was stored at -20°C for Western blot assay. Proteins were separated in 10% SDS-PAGE, and were transferred to PVDF membrane by semi-dry method (100 mA, 1.5 h). Non-specific binding sites were removed by 5% defatted milk powder for 2 h. Anti-pAKT monoclonal antibody or anti-AKT monoclonal antibody (1:1000) was added for 4°C overnight incubation. After PBST washing, goat anti-rabbit secondary antibody (1:2000) was added for 30 min incubation at room temperature. ECL reagent was then added for developing the membrane for 1 min after PBST rinsing, followed by exposure under dark. The film was scanned and analyzed by protein imaging system and Quantity One software for measuring band density. Each experiment was replicated for four times (N=4) for statistical analysis.
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Statistical processing

SPSS 11.5 software was used for analyze all data, which were presented as mean ± standard deviation (SD). Comparison of means among groups was performed using analysis of variance (ANOVA), whilst between-group comparison of means was finished using student t-test. A statistical significance was defined when p<0.05.

Results

Effects of pterostilbene on Hep-2 cell proliferation

MTT assay was used to test the effect of different concentrations of pterostilbene on proliferation of LSCC cell Hep-2. Results showed that 72 h of pterostilbene treatment significantly inhibited proliferation of Hep-2 cells (p<0.05 compared to control group). With elevated concentration, such inhibitory effects on Hep-2 proliferation were further enhanced, as 200 μmol/L pterostilbene had significantly higher inhibitory effects on Hep-2 cell proliferation (p<0.05 compared to 100 μmol/L pterostilbene, Figure 1). These results indicated that pterostilbene could inhibit Hep-2 cell proliferation in a dose-dependent manner.

Effects of pterostilbene on the apoptosis of Hep-2 cells

Caspase 3 activity was examined to examine the effect of different concentrations of pterostilbene on apoptosis protein levels in Hep-2 cells. Results showed that after pterostilbene treatment, Caspase 3 activity was significantly enhanced in Hep-2 cells (p<0.05 compared to control group). With higher dosage, Caspase 3 activity was further enhanced in Hep-2 cells, as 200 μmol/L pterostilbene caused higher Caspase 3 activity (p<0.05 compared to 100 μmol/L pterostilbene), indicating that the potency of pterostilbene to induce apoptosis was enhanced with higher concentration (Figure 2A). Consistent with higher caspase-3 activity, increased cell apoptosis in Hep-2 cell was also observed after pterostilbene treatment in a dose-dependent manner as demonstrated by flow cytometry (Figure 2B). Taken together, these results supported that pterostilbene could regulate Hep-2 cell apoptosis in a dose-dependent manner.

Effects of pterostilbene on invasion ability of Hep-2 cells

Transwell chamber assay was used to test the effect of different concentrations of pterostilbene on Hep-2 cell invasion potency. Results showed that pterostilbene treatment significantly inhibited invasion ability of Hep-2 cells (p<0.05 compared to control group). With higher concentration, inhibition on Hep-2 cells was potentiated, as 200 μmol/L pterostilbene led to higher inhibitory effects on Hep-2 cell invasion (p<0.05 compared to 100 μmol/L pterostilbene, Figure 3). These results supported that pterostilbene could inhibit Hep-2 cell invasion.

Effects of pterostilbene on AKT/mTOR signal pathway phosphorylation level in Hep-2 cells

Western blot was used to test the effect of pterostilbene on phosphorylation level of AKT/mTOR signal pathway in Hep-2 cells. Results
showed that pterostilbene treatment significantly inhibited phosphorylation level of AKT signal pathway of Hep-2 cells (p<0.05 compared to control group). With higher concentration, phosphorylation level was further suppressed but without significant difference (p>0.05) compared to 100 μmol/L pterostilbene (Figure 4).

Effects of pterostilbene on mTOR mRNA expression of AKT/mTOR signal pathway in Hep-2 cells

Real-time PCR was used to test the effect of pterostilbene on mTOR mRNA level of AKT/mTOR signal pathway in Hep-2 cells. Results showed that pterostilbene treatment significantly inhibited mTOR mRNA level of AKT/mTOR signal pathway of Hep-2 cells (p<0.05 compared to control group). With higher concentration, mTOR mRNA level was further suppressed (p<0.05 compared to 100 μmol/L pterostilbene, Figure 5). These results showed that pterostilbene could regulate AKT/mTOR signal pathway via multiple ways, thus functioning on LSCC occurrence and progression.

Discussion

LSCC is mainly manifested as hoarse vocalization, breath distress, cough, swallowing difficulty and metastasis of neck lymph node. The demonstration of early stage LSCC benefits the improvement of 5-year survival rate of patients [18]. Early diagnosis and treatment are critical for alleviating LSCC threaten. However, due to atypical early symptoms, LSCC is frequently misdiagnosed with laryngitis or vocal cord polyp [19]. Therefore, the identification of LSCC related pathogenesis mechanism and molecular targets, and development of effective treatment plan are major challenge currently. Pterostilbene has been shown to induce tumor cell apoptosis, and regulate tumor cell cycle to suppress tumorigenesis. Meanwhile, it can also exert anti-tumor effects and suppress tumor angiogenesis via activating autoimmune response [20]. As one novel drug, pterostilbene...
has been shown to have significant effects in treating Alzheimer’s disease, cardiovascular disorder, brain injury, tumor and hypercholesterolemia. It has more potent pharmaceutical role than resveratrol in arresting tumor cells, plus higher specificity to inhibit DNA triplex polymerase or replication of simian virus 40 DNA [21, 22]. This study treated in vitro cultured LSCC cells with different concentrations of pterostilbene and demonstrated that pterostilbene inhibited tumor cell proliferation and enhanced apoptotic protein activity or LSCC cell invasion, thus suggesting that pterostilbene could reduce probability of recurrence or metastasis of LSCC via decreasing cell proliferation, facilitating apoptosis and suppressing cell invasion, thus retarding LSCC occurrence and progression.

AKT/mTOR signal pathway is closely correlated with occurrence, progression and prognosis of LSCC [15, 16]. As one important downstream factor of AKT signal pathway, mTOR participates in regulating tumor cell proliferation, growth, survival and angiogenesis. AKT/mTOR signal pathway frequently facilitate tumor survival via inhibiting apoptosis signal and activating anti-apoptosis factors [23]. Activated AKT can initiate various enzymes, kinase and transcription factors via phosphorylation, further activating effector protein mTOR to exert anti-apoptotic and facilitating proliferation effects. Previous studies showed that regulation of AKT/mTOR signal pathway can inhibit LSCC cell proliferation and facilitate cell apoptosis [24, 25]. This study demonstrated that pterostilbene can inhibit phosphorylation level of AKT signal pathway to reduce expression of downstream target protein mTOR. These results illustrated the molecular mechanism underlying the regulation of LSCC cells by pterostilbene, providing evidences for illustrating detailed mechanism.

Conclusion
Pterostilbene can inhibit AKT/mTOR signal pathway, thus exerting regulation of LSCC cell proliferation, apoptosis and invasion.

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Disclosure of conflict of interest
None.

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