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Targeted inhibition of mammalian target of rapamycin (mTOR) signaling pathway inhibits proliferation and induces apoptosis of laryngeal carcinoma cells *in vitro*

Rongrui Li[1,2], Riguang Wang[2], Raosheng Zhai[2], and Zhen Dong[1]

[1]Department of Otorhinolaryngology & Head and Neck Surgery, China-Japan Union Hospital of Jilin University, Changchun; [2]Department of Orthopedic Surgery, the First Affiliated Hospital of JiaMuSi University, Heilongjiang, China

ABSTRACT

Aim and objective. Laryngeal carcinoma is one of the most aggressive cancers of the head and neck region. The survival rate of patients with laryngeal carcinoma is low due to its late metastases and resistance to chemotherapy and radiotherapy. It was reported that mTOR was involved in the growth and apoptosis of various cancer cells. The aim of this study was to detect the effects of mTOR inhibition by mTOR shRNA on the proliferation, apoptosis and invasive ability of Hep-2 human laryngeal carcinoma cells *in vitro*.

Methods and study design. mTOR shRNA was designed and transfected into Hep-2 human laryngeal carcinoma cells. Untreated cells and cells treated with control vector (non-targeted shRNA) were used as control. The proliferation and apoptosis of Hep-2 cells were detected by MTT and flow cytometry. A transwell assay was used to measure the invasive ability of Hep-2. The inhibition effects on the mTOR signaling pathway by mTOR shRNA were studied using RT-PCR and Western blot.

Results. Our results showed that the mRNA and protein expression of mTOR and Akt were high in laryngeal carcinoma cells and could be inhibited by mTOR shRNA. At the same time, low expression of PTEN mRNA and protein was observed in Hep-2 cells. The expression increased when the cells were transfected with mTOR shRNA. This showed that mTOR shRNA could inhibit the proliferation and invasive ability of Hep-2 cells. It also could induce the apoptosis of Hep-2 cells *in vitro*.

Conclusions. The mTOR signaling pathway plays an important role in the development of laryngeal carcinoma. The mTOR shRNA we designed in this experiment effectively inhibited the mTOR signaling pathway. It inhibited the proliferation and invasive ability of the studied laryngeal carcinoma cells and induced their apoptosis *in vitro*. mTOR might therefore be a useful target in the therapy of laryngeal carcinoma.

Introduction

Laryngeal squamous cell carcinoma is one of the most common primary neoplasms of the larynx[1]. It accounts for about 99% of laryngeal malignancies and is characterized by rapid progression, aggressive behavior, late metastases, resistance to chemotherapy and radiotherapy, and poor prognosis[2-4]. The survival rate of patients with laryngeal carcinoma has been low due to its resistance to anticancer drugs and late metastases[5]. It was therefore necessary to find new methods to treat laryngeal carcinoma. With the development of molecular biology, several new strategies have been introduced in the treatment of cancer[6-8]. Gene therapy is one of the new methods to treat cancers and remarkable results have been obtained[9-11]. Targeted regulation of the abnormal gene expression in cancer by molecular biological methods can induce apoptosis and inhibit the proliferation of cancer cells both *in vitro* and *in vivo*, as has been confirmed in various tumor types[12-16].

Key words: mTOR, apoptosis, laryngeal carcinoma.

Correspondence to: Prof Zhen Dong, Department of Otorhinolaryngology & Head and Neck Surgery, China-Japan Union Hospital of Jilin University, Changchun 130033, P.R. China.
e-mail dongzhen91@126.com

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Several studies have demonstrated that the mammalian target of rapamycin (mTOR) signaling, a major component of the phosphoinositide 3-kinase (PI3K)/Akt/mTOR pathway, can regulate several normal cellular functions that are involved in cell proliferation, cell cycle regulation, apoptosis, and angiogenesis[17-19]. Abnormal expression of mTOR signaling was found in several cancers including breast cancer, lung cancer, gastric cancer, ovarian cancer, and prostate cancer and was associated with poor prognosis[20-24]. This suggested that mTOR could be used as a new potent target in the treatment of cancer. Many reports have confirmed that targeted blocking of the expression of mTOR could inhibit the proliferation and induce the apoptosis of cancer cells *in vitro*[25-27]. However, little is known about the expression of mTOR signaling in laryngeal carcinoma and the effects of mTOR inhibition on the proliferation and apoptosis in laryngeal carcinoma cells.

Small hairpin RNA (shRNA) is a new powerful method to knock down the expression level of specific mRNA. Gene silencing using shRNA has many potential therapeutic applications and has also been applied in cancer research[28]. In this study, we focused on mTOR for its potential role in the development of human laryngeal carcinoma. We knocked down mTOR by shRNA and observed the effects on the proliferation, apoptosis, and ability of migration and invasion of Hep-2 human laryngeal carcinoma cells by methyl thiazolyl tetrazolium (MTT), flow cytometry and transwell invasion assays. RT-PCR and Western blot were used to detect the effects of mTOR shRNA on the mTOR signaling pathway.

Materials and methods

Cell lines and cell culture

All the experiments were performed using the human laryngeal carcinoma cells line Hep-2, which was purchased from the American Type Culture Collection (ATCC catalogue number: CCL-23). Hep-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in humidified air with 5% CO_2. Cells were subcultured with trypsin solution (0.25%) and the effect of trypsin was inhibited by FBS at 37 °C.

mTOR shRNA preparation

The shRNA vector (RNAi-Ready pSIREN-RetroQ Retroviral Vector) was purchased from BD Biosciences Clontech. This vector uses the cell's own RNA polymerase III to transcribe a specifically designed shRNA by using the human U6 promoter which provides a high level of expression in many cell types, resulting in target gene suppression. It was cleaved with BamH I and EcoR I restriction sites. The oligonucleotides encoding the mTOR shRNA were designed as follows: sense, 5'-GAT CCG TGA GAG GAA AGG TGG CAT CTT CAA GAG AGA TGC CAC CTT TCC TCT CAC TTT TTT GGA AG-3'; antisense, 5'-AAT TCT TCC AAA AAA GTG AGA GGA AAG GTG GCA TCT CTC TTG AAG ATG CCA CCT TTC CTC TCA CG-3'. The oligonucleotides were annealed and cloned into pSIREN following its restriction with BamH I and EcoR I to form the mTOR shRNA vector.

Transient transfection with mTOR shRNA

Twenty-four hours before transfection, Hep-2 cells were plated into 6-well or 96-well tissue culture plates and cultured in DMEM supplemented with 10% FBS. After rinsing in PBS, cells were transiently transfected with mTOR shRNA and cultured in DMEM without FBS. Six hours later, the medium was replaced by complete DMEM with 10% FBS and cells were cultured in 5% CO_2 at 37 °C for an additional 24 hours or longer.

MTT assay

The growth inhibition rate of Hep-2 cells was determined by MTT assay. Cells were plated and cultured in 96-well culture plates with a density of 5000 cells per well for 24 hours and then divided into the following groups: cells transfected with mTOR shRNA, untreated cells, and cells treated with control vector (non-targeted shRNA). After transfection was terminated by complete DMEM with 10% FBS, cells were cultured in DMEM supplemented with 10% FBS at 37 °C in humidified air with 5% CO_2. Twenty-four, 48 and 72 hours later, the medium was replaced by 180 μL DMEM and 20 μL MTT reagent (Sigma; 5 mg/mL). The Hep-2 cells were cultured for another 4 hours and then the medium was replaced by 150 μL dimethyl sulfoxide (DMSO). The plate was shaken on a rotary platform for about 10 minutes and the absorbance at 490 nm was monitored by means of a microplate reader. Subsequently, the inhibition rate of Hep-2 cells was calculated according to the absorbance of treated cells and control cells. The experiments were done in triplicate.

Apoptosis assay

Hep-2 cells were plated into 6-well tissue culture plates with a density of 1×10^5 cells per well and divided into 3 groups as described before. After pretreatment for 48 hours, cells were harvested and washed twice in PBS. They were then resuspended and transferred to 70% ice-cold ethanol for 30 minutes at 4 ˚C. The cells were centrifuged again and incubated by Annexin-V FLUOS labeling solution (containing 2 μL Annexin-V-FLUOS labeling reagent and 2 μL propidium iodide solution in 100 μL incubation buffer) at 37 °C for 30 minutes. The apoptotic cells were analyzed by flow cytometry according to the manufacturer's instructions. The experiments were conducted 3 times.

Reverse-transcription PCR

The effects of mTOR shRNA on the mRNA expression in Hep-2 cells were detected by reverse-transcription PCR (RT-PCR). Total RNA was isolated by Trizol reagent after pretreatment and then RT-PCR was performed using a 1-step RT-PCR kit according to the instructions. The primer pairs were designed as follows. mTOR (301 bp): sense 5'-GGC CGA GAG CAC CGA GAA-3'; and antisense 5'-CAA GGG TCT GGG CGT ATC AA-3'; AKT (439 bp): sense 5'-CAG GTC GCT ACT ATG CCA TC-3'; and antisense 5'-ACA CCA CGT TCT TCT CGG AG-3'; PTEN (361 bp): sense: 5'-GGGACGAACTG-GTGTAATGA-3'; and antisense: 5'-AGGTAACGGCT-GAGGGAA-3'; and GAPDH (230 bp): sense 5'-ACG GAT TTG GTC GTA TTG GG-3'; antisense 5'-TGA TTT TGG AGG GAT GTC GC-3'). GAPDH was used as the internal control. PCR products were identified by means of ethidium bromide-containing agarose gel electrophoresis.

Western blotting

After pretreatment as described before, Hep-2 cells were collected and the plasmosin was extracted. After estimation of the concentration of protein, immunoblotting was performed. Mouse antihuman monoclonal antibodies against mTOR, p-AKT, PTEN and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated rabbit antimouse antibody was used as secondary antibody. Proteins were visualized by chemiluminescence luminol reagents according to the instructions.

Migration and invasion assay

The effect of mTOR shRNA on the invasive ability of human laryngeal carcinoma cells was detected using a transwell assay. After pretreatment as described before for 48 hours, polycarbonate filters 6.5 mm in diameter and with 8-µm pore size were coated with 50 µL of matrigel and dried in a sterile environment overnight. About 1×10^5 Hep-2 cells were plated on the upper compartment of the chambers in 25 µL of DMEM without serum. 500 µL DMEM with 10% FBS was used as chemoattractant in the lower chamber. Twenty-four hours later, the cells on the upper compartment were removed with a cotton bud and the cells migrating to the lower compartment were stained by Giemsa. The ratio of the migrating cells was then calculated. All the assay tests were performed in triplicate.

Statistical analysis

The data were presented as mean ± SD. Statistical analysis was performed with the SPSS software, version 12.0. P <0.05 was defined as statistically significant.

Results

Effects of mTOR shRNA on cell proliferation

To observe the growth inhibition effects of mTOR shRNA on Hep-2 cells the MTT assay was used. As shown in Figure 1, cell proliferation was significantly inhibited in the group transfected with mTOR shRNA compared with controls. The effects in the transfection group were time dependent. The proliferation inhibitory rate was 24.9 ± 1.25% after transfection for 24 hours and increased notably 48 hours (34.7 ± 1.38%) and 72 hours later (37.2 ± 1.41%) (P <0.05). No significant differences were observed between 48 hours and 72 hours after transfection (P >0.05). These findings indicated that mTOR shRNA could inhibit the proliferation of Hep-2 cells *in vitro*.

Effects of mTOR shRNA on cell apoptosis

After pretreatment for 48 hours as described above, flow cytometry analysis was used to detect the apoptosis rate of Hep-2 cells. As shown in Figure 2, the apoptosis rate of Hep-2 cells transfected with mTOR shRNA was 28.76 ± 3.14%, which was significantly higher than that in controls (1.87 ± 0.21% and 2.03 ± 0.34%) (P <0.05). Several reports have shown that the mTOR signal plays an important role in the development of cancer[20-24]. Our data proved that mTOR inhibition by shRNA can induce the apoptosis of cancer cells.

Effects of mTOR shRNA on inhibition of the mTOR signaling pathway

As shown in Figures 1 and 2, significant changes had occurred 48 hours after transfection. We therefore assessed the mRNA and protein expression of mTOR, Akt

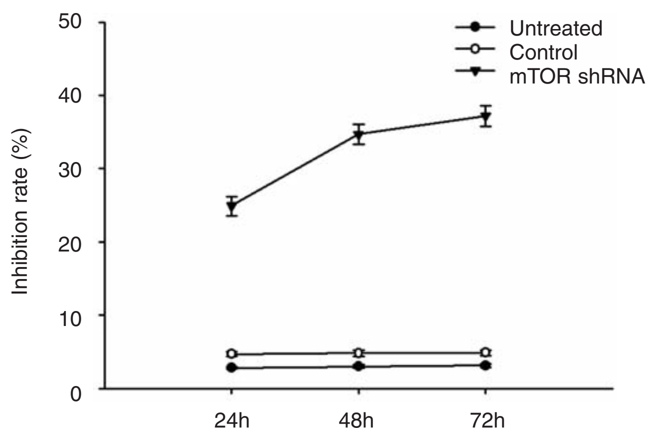

Figure 1 - Growth inhibition in Hep-2 human laryngeal carcinoma cells. Cells were divided into 3 groups: cells transfected with mTOR shRNA, untreated cells, and cells treated with control vector (non-targeted shRNA). The growth inhibition rate was examined by MTT assay and the absorbance measured at 490 nm. Data represent the mean ± SD of 3 independent experiments.

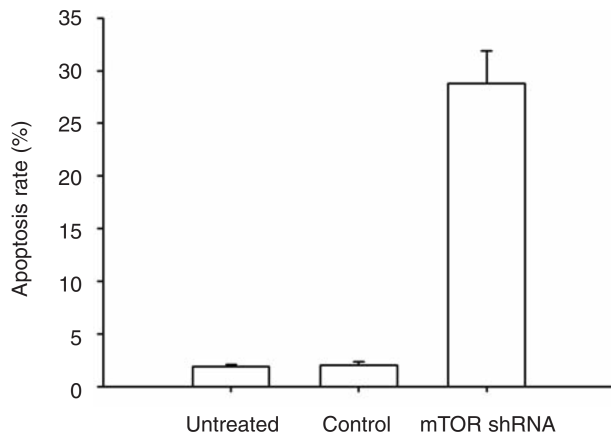

Figure 2 - Apoptosis of Hep-2 cells induced by mTOR shRNA. Cells were divided into 3 groups: cells transfected with mTOR shRNA, untreated cells, and cells treated with control vector (non-targeted shRNA). After treatment for 48 hours, the cells were collected and the apoptosis rates were measured by flow cytometry. Data represent the mean ± SD of 3 independent experiments.

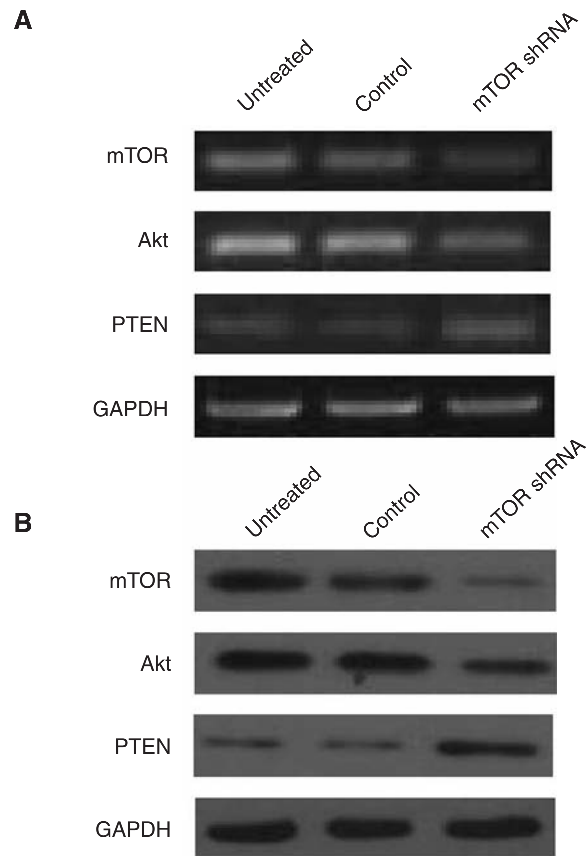

Figure 3 - mRNA and protein expression of mTOR, Akt and PTEN detected by RT-PCR and Western blot after treatment with mTOR shRNA. A) PCR products were electrophoresed on 1.5% agarose gel stained with ethidium bromide. B) Immunochemical detection of expression of mTOR, Akt and PTEN protein in Hep-2 cells by rabbit antihuman monoclonal antibodies.

and PTEN in Hep-2 cells 48 hours after transfection by RT-PCR and Western blotting. As can be seen in Figure 3A, we observed high expression of mTOR and Akt mRNA in the laryngeal carcinoma cells. When the cells were treated with mTOR shRNA, the expression of mTOR and Akt mRNA decreased notably. Conversely, the expression of PTEN mRNA was low in Hep-2 cells and increased significantly in cells transfected with mTOR shRNA. The same results were observed when we assessed the protein expression of mTOR, Akt and PTEN, as shown in Figure 3B. These data suggest that mTOR might play an important role in the development of laryngeal carcinoma and that mTOR shRNA can effectively inhibit the mTOR signaling pathway.

Effects of mTOR shRNA on cell migration and invasion

The invasive ability is an important feature of cancer cells. To investigate the migratory and invasive ability of Hep-2 cells, we used a transwell invasion assay. As shown in Figure 4, Hep-2 cells in the control groups showed high invasive ability and the cells could easily traverse the matrigel barrier. The invasion rates in the control groups were 46.53 ± 5.27% and 45.34 ± 4.98%. By contrast, the cells in the experimental group displayed poor invasive ability and had difficulty traversing the matrigel barrier. The invasion rate in cells transfected with mTOR shRNA was 26.18 ± 3.25%. These findings indicated that targeted inhibition of mTOR by shRNA could reverse the malignant features of human laryngeal carcinoma cells *in vitro*.

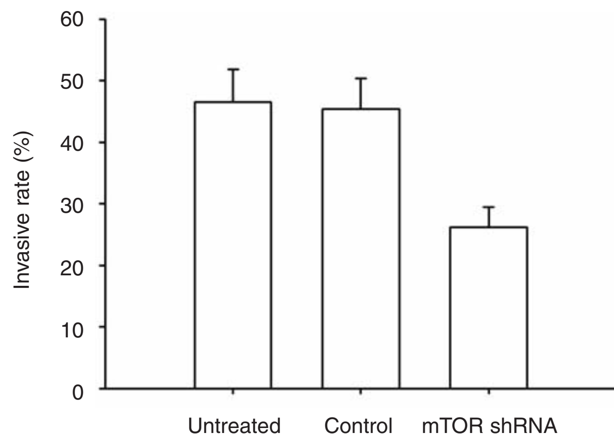

Figure 4 - Effects of mTOR shRNA on the invasive ability of Hep-2 cells assessed by transwell invasion assay. Data from 3 independent experiments.

Discussion

Laryngeal carcinoma is one of the most aggressive cancers of the head and neck region[2]. Most laryngeal cancers are squamous cell carcinomas, which indicates that laryngeal carcinoma mostly originates from the laryngeal epithelium[1]. It is closely correlated with smoking and can spread to adjacent structures and lymph nodes, or migrate more distantly via the bloodstream[29].

Although the traditional therapeutic strategies including surgery, radiotherapy and chemotherapy have had some success, the outcome in many patients with laryngeal carcinoma has been unsatisfactory[5].

mTOR is a serine/threonine kinase that plays an important role in the development of several cancer types[30-32]. It is involved in tumor growth and in the apoptosis of cancer cells, and can control protein translation[33]. Several studies found mTOR to be overexpressed in various cancer types including ovarian cancer, breast cancer, osteosarcoma, and lung cancer[34-37]. Targeted inhibition of mTOR expression was found to induce apoptosis and inhibit the proliferation of cancer cells *in vitro* and *in vivo*[38-40]. These findings suggest that targeted inhibition of mTOR signaling might be a useful strategy in the treatment of laryngeal carcinoma.

To examine the function of mTOR signaling in laryngeal carcinoma, we used mTOR shRNA to knock down the expression of mTOR in a human laryngeal carcinoma cell line and observed the effects of mTOR inhibition on the cells. Growth was significantly inhibited in cells transfected with mTOR shRNA compared with cells treated with control vector and untreated cells. The proliferation inhibition rate increased notably 48 hours after transfection and reached its peak at 72 hours. The results suggested that inhibiting the expression of mTOR by mTOR shRNA could inhibit the proliferation of laryngeal carcinoma cells *in vitro*.

We used flow cytometry to study the effects of mTOR shRNA on apoptosis and found that 48 hours after pretreatment, the apoptosis rate in Hep-2 cells transfected with mTOR shRNA was significantly higher than that in controls. Conversely, the migration and invasion abilities of Hep-2 cells were decreased in cells transfected with mTOR shRNA. These findings indicated that mTOR shRNA could induce apoptosis and reverse the aggressive malignant behavior of human laryngeal carcinoma cells.

We found high mTOR and Akt mRNA and protein expression in the laryngeal carcinoma cells, which decreased notably when the cells were transfected with mTOR shRNA. Conversely, the expression of PTEN mRNA and protein in untreated cells was low but increased significantly when the cells were transfected with mTOR shRNA. These data suggest that the mTOR signaling pathway plays an important role in the development of laryngeal carcinoma and that the mTOR shRNA designed by us can inhibit this pathway effectively.

In conclusion, our data show that mTOR plays an important role in the development of laryngeal carcinoma. The mTOR shRNA we designed in this experiment effectively inhibited the mTOR signaling pathway *in vitro*. It inhibited proliferation and induced apoptosis in the examined Hep-2 laryngeal carcinoma cells. We also found that mTOR shRNA could inhibit the invasive ability of Hep-2 cells. Taken together, these results suggest that mTOR might be a useful target in the therapy of laryngeal carcinoma.

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