

Effects of MiR-194-5p on the proliferation and invasion of laryngeal cancer cells by regulating the expression of Smurf1 and activating the mTOR signaling pathway

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ABSTRACT

Laryngeal cancer has become the focus of research because of its high incidence rate and mortality rate. However, the research on miR-194-5p in laryngeal cancer is quite rare. The purpose of this study is to explore the effect of miR-194-5p on the proliferation and invasion of laryngeal cancer cells in order to find an effective way to treat laryngeal cancer. The results showed that miR-194-5p could activate the mTOR signaling pathway by regulating the expression of Smurf1, which had an effect on laryngeal cancer cells. The results showed that the absorbance of the miR-194-5p group was 0.38 lower than that of the NC group, which indicated that up-regulation of mir-194-5p could weaken the proliferation of laryngeal cancer cells. In addition, the average number of laryngeal cancer cells in NC and the miR-194-5p groups was 125.2 and 53.8, respectively, which indicated that miR-194-5p could reduce the number of laryngeal cancer cells passing through the basement membrane and their invasion ability.

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Introduction

Laryngeal carcinoma main pathological type is squamous cell carcinoma. At present, the treatment of laryngeal cancer is mainly surgery, radiotherapy and chemotherapy. However, there are many complications and adverse reactions in surgery and radiotherapy and chemotherapy. Recurrence and metastasis after treatment will still lead to the death of laryngeal cancer patients. It is very important to find a new and effective treatment for laryngeal cancer. Therefore, the treatment of laryngeal cancer by interfering with the proliferation and invasion of cancer cells has become the focus of this study.

The research on the treatment of laryngeal cancer is the focus of the medical staff and researchers all over the world, and the research results are endless. For example, Xuan, L. studied the expression of circrna in four pairs of matched LSCC tissues and adjacent non-tumor tissues by microarray analysis (1). Further detection of HSA by QRT PCR- circRNA-100855 is the most up-regulated circrna, HSA-circRNA- 104912 is the downregulated circrna (2-4). Peretti, G. studied the reasonable limit of TLM in the treatment of laryngeal cancer (5-7). To highlight the most appropriate and repeatable indications and to integrate this treatment tool into the overall framework of alternative treatment strategies, such as open partial laryngectomy and non-surgical organ preservation protocol (8). Serrano, Manuel introduced in detail the infiltration of laryngeal cartilage tumor through the whole series of organ sections, in which the infiltration was regarded as horizontal infil-

tration (through cartilage) and vertical infiltration (degree of infiltration) (9-10).

After understanding the target gene and regulatory mechanism of miR-194-5p, scholars all over the world are actively studying the corresponding therapeutic effect (11-12). Apoptosis can be detected by fluorescence-activated cell sorting, and insulin-like growth factor-1 receptor protein level of the miR-194-5p target gene can be detected by Western blotting (13-15). Hu, D. the microRNA analysis of 48 breast cancer patients' sera was carried out by exiqon mercury microRNA RT-PCR (16). Seven miRNAs differentially expressed in recurrent and non-recurrent breast cancer patients were identified (17-18). MiR-194-5p and miR-122 were down-regulated in human glioma and GSCs. MiR-194-5p (19-20). Their research provides a reference for this study and avoids mistakes in their research.

The proliferation ability, cycle and invasion ability of laryngeal cancer cells were analyzed by transfection, protein extraction test and cell proliferation test. The results showed that 97% of the experimental samples showed that miR-194-5p could affect the proliferation and invasion of laryngeal cancer cells by regulating the expression of Smurf1 and activating the mTOR signal pathway.

Materials and Methods

Collection of experimental specimens

The laryngeal cancer tissue and adjacent tissue of 36 patients with laryngeal cancer in a cancer hospital were

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collected and stored in liquid nitrogen.

Common reagents and experimental instruments

Mir-194-5p agomir and NC dilution reagent; cell culture reagent, including 0.01M PBS (pH7.4) and 90% serum cell cryopreservation solution; Western blot reagent.

The experimental instruments include a biosafety cabinet, cell incubator, ultra-low temperature refrigerator, spectramax190 full wavelength reader, automatic autoclave, micropipette gun, pure water filter, desktop centrifuge, etc.

Cell culture

First, 10 ml complete culture medium including 16% fetal bovine serum, 100 U / ml penicillin and 100 U / ml deoxymycin were added to the culture bottle. Then, the cryopreservation tube was quickly transferred from liquid nitrogen to a water bath at 37 °C. The cryopreservation was continuously moved in the water bath, and the cell suspension was thawed within one minute as much as possible. Wipe the cryopreservation tube with alcohol gauze for sterilization, and transfer the cell suspension in the cryopreservation tube to the culture bottle containing the culture medium. After the cell adheres to the wall, the cells are treated with liquid exchange to remove the toxic effect of DMSO on the cells. The resuscitated laryngeal cancer cells were cultured regularly in the medium at saturated humidity. In the process of cell culture, we should pay attention to the color change of the culture medium, observe the change of cell morphology and cell fusion degree with the inverted microscope, and replace the new culture medium in time to keep the cell in a good growth state. When the degree of cell fusion reaches about 80%, cells are propagated.

Screening and transfection of cells

The Hep-2 cells in the logarithmic growth period were inoculated into 24 well culture plates with 1000 cells/ml, 1 ml per well. Under the normal condition, change the solution every 3 days for 10 days. To observe the cell death, select the lowest concentration that can kill cells completely on the 10th day as the screening concentration of the transfected cell resistance gene. The concentration of G418 was 800 μ g / ml.

Lipofectamine 2000 was mixed with the culture medium and then added to the 24 well culture plate. The experiment was continued 24 hours after transfection. The experiment was divided into three groups: blank control group, miR-194-5p group and miR-194-5p NC group.

Protein extraction and detection

The cells of each group were collected by digestion and centrifugation, washed three times with PBS solution; added 100 μ l of cell lysate, split on ice, shook and mixed every 10 minutes, split for 30 minutes; collected the cells in 1.5ml EP tube, centrifuged for 15 minutes at 4 degrees; absorbed the supernatant of protein and stored separately.

The protein concentration was determined according to the instructions of the BCA white concentration test kit. First, the standard protein was prepared at a concentration of 0.5mg/ml, and then the corresponding amount of BCA working solution was prepared according to the number of samples to be tested (the A solution and B solution in the kit were mixed in the proportion of 50:1). Add 100 μ l of

test solution into each hole of 96 hole plate, and mix the diluted standard and the sample to be tested. Incubate in a 37 °C incubator for 30min, cool down, and measure the absorbance with a standard enzyme instrument. Draw the standard curve of protein concentration and calculate the actual protein concentration of each sample.

Cell proliferation experiment

Hep-2, Hep-2 / mock and Hep-2 / Tiaml cells in the logarithmic growth stage were digested and resuspended by trypsin, and then 1000 cells were inoculated into 96 well plates with a volume of 1ml per well. Each group of cells had four subsidiary pores and blank control (only medium was added). Then MTT method was used to detect the growth of cells in each group every 24 hours for 3 consecutive days. At the time point, 20 μ l of 3 mg/ml MTT solution was added to each pore, and the culture was continued in 37 °C cell incubator for 4 hours, and then the culture was terminated. After carefully sucking and discarding the medium in the hole with a 1ml syringe, add 100 μ l DMSO into each hole. Shake the 1min on the wellscan Mk 2 washing machine to make the crystal fully dissolved, adjust the blank control pore to zero, and measure the absorbance value (OD value) of each pore at 570nm of the enzyme-linked immunosorbent detector. The experiment was repeated three times. The average value of the results was taken, and the corresponding OD value was used to represent the cell proliferation capacity, and the growth curve was drawn (21-22).

Cell cycle experiment

After 24 hours of transfection, the laryngeal cancer cells of the mir-194-5p group and NC group were collected in a 1.5 ml EP tube. The cells were pretreated with starvation, and the number of cells could not be less than 1×10^6 . 70% ethanol was fixed at 4 °C overnight, then 100 μ l RNase A was added and incubated at 37 °C for 30min, then PI staining was added at 4 °C for 30min (dark), and then on-board detection was carried out.

Results and discussion

Smurf1 overexpression activates the mTOR signaling pathway

Since mTOR was identified, its related research has been a hot research direction in the field of life science in recent years (23-24). Because the mTOR signaling pathway is involved in the regulation of many physiological functions in the body, its disorder will directly lead to inflammation and even cancer, so its fine regulation is very important for the maintenance of homeostasis, and the in-depth excavation of its regulation mechanism is also very important (25). Therefore, we want to know whether Smurf1, a member of the heat family, can regulate the mTOR signaling pathway. First, we tested whether the overexpressed Smurf1 would affect the activation of mTOR. The E3 ubiquitin ligase Smurf1 (WT and c699a) was overexpressed in laryngeal cancer cells. The effect of Smurf1 on mTOR activation was observed after TNF α -induced activation.

As shown in Figure 1, only smurf1-wt activated TNF α induced mTOR, while smurf1-c699a had no significant effect. Moreover, our experiment further confirmed that the activation effect will be more significant with the increase

of Smurf1 expression.

Analysis of the proliferative ability of laryngeal cancer cells

Cancer cells can proliferate infinitely. They ignore the signals of normal proliferation control and only follow their own internal proliferation standards. They can even move and invade adjacent organizations.

MiR-194-5p can affect the activation of the mTOR signaling pathway by regulating the expression of Smurf1, thus affecting the proliferation of laryngeal cancer cells. Rapid quantification of miRNA discovery is the key to the elimination of miRNA in gene regulation. At present, the detection methods of miRNA mainly include northern blotting, microarray analysis gene chip technology (microarray), and real-time fluorescence quantitative PCR. High throughput analysis cannot be performed due to the low sensitivity of northern blotting. In general, real-time quantitative PCR is used to confirm the predicted relative performance level of miRNA. Gene chip has the characteristics of high throughput, high precision and fast detection. It is widely used in gene diagnosis and experimental research. The stronger the proliferation of laryngeal cancer cells, the more difficult it is to inhibit, and the higher the harm to the human body.

As shown in Figure 2, compared with NC group and blank control group, the absorbance of miR-194-5p group cells decreased significantly at 24h, 48h and 72h.

Effect of MiR-194-5p on cell cycle of laryngeal carcinoma

Cancer cells also have a cell cycle. Different cytotoxic drugs act on different stages of cancer cell division to prevent its division so as to play an anti-tumor role. There are two mechanisms of cell cycle regulation, including the cell cycle driving mechanism and the regulatory mechanism. When the regulatory mechanism of the cell cycle is destroyed and transformed into tumor cells, the cell cycle can be divided into four successive periods. That is, G1, s, G2 and m. G1 phase refers to the gap period from the completion of the last mitosis to the DNA replication, that is, the preparation period for cell growth and DNA synthesis. The length of this phase varies with cells. In the cell cycle, the genetic information is precisely duplicated by DNA replication of the genome and equal separation of genetic information of M phase and transferred from mother cell to two daughter cells. G2 stage and mitosis entry: G2 stage refers to the time interval from the completion of DNA replication to the beginning of mitosis. Mitosis: usually divided into prophase, metaphase, anaphase, telophase and cytokinesis

Flow cytometry was used to detect and record the cell cycle of laryngeal cancer in the NC group and miR-194-5p group. The results are shown in Table 1. The percentage of G0 / G1 cells in the mir-194-5p group was higher than that

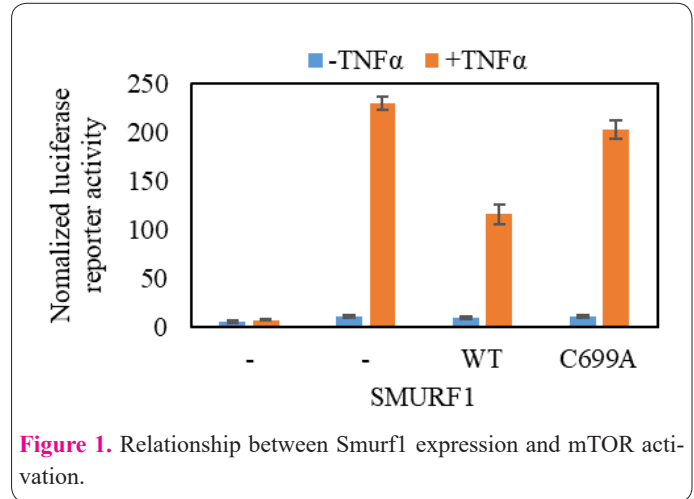


Figure 1. Relationship between Smurf1 expression and mTOR activation.

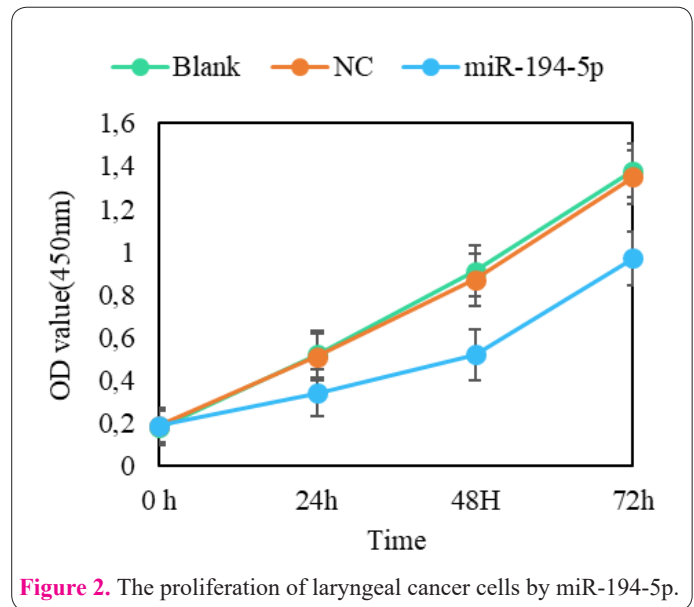


Figure 2. The proliferation of laryngeal cancer cells by miR-194-5p.

in the NC group (P < 0.01). This suggests that up-regulation of miR-194-5p can slow down the progression of the laryngeal cancer cell cycle.

Analysis of invasion ability of laryngeal cancer cells

The invasion in the late stage of the tumor is the result of high deterioration of tumor lesions and high development of malignant behavior. It is a sign of an advanced malignant tumor. Cancer cells are unicellular-like organisms whose invasion includes invasive tumor cells invading other adjacent tissues, degeneration and necrosis of the invading tissues, and progressive changes in space and time (26). The reason for the invasion is first because cancer cells have the ability of amoeba movement. They can extend pseudopodia to insert into the surrounding tissue gap, run and destroy the surrounding tissue, or even replace it. Another reason is that many cancer cells can also secrete some special molecular substances to help cancer

Table 1. Effect of miR-194-5p on cell cycle of laryngeal carcinoma.

| Group | n | The proportion of cells in each stage (%) | | |
|------------------|---|---|------------|-----------|
| | | G0/G1 | S | G2/M |
| NC Group | 5 | 50.88±2.11 | 41.71±1.23 | 7.41±0.2 |
| miR-194-5p Group | 5 | 62.74±2.36 | 30.35±1.98 | 8.91±0.19 |

cells adhere to the surrounding tissues. If the number of cells is small, it indicates that the invasion ability is weak. Otherwise, it indicates that the invasion ability is strong.

As shown in Figure 3, the number of cells passing through the basement membrane in the miR-194-5p group was significantly lower than that in the NC group, which indicated that up-regulating the expression of miR-194-5p could inhibit the overexpression of Smurf1, activate the mTOR signal pathway, and reduce the invasion ability of laryngeal cancer cells.

Laryngeal cancer is one of the common tumor diseases. Early diagnosis and treatment can improve the survival rate of patients, but for late patients, laryngeal cancer often occurs through metastasis, the metastasis of cancer cells is mainly related to the proliferation and invasion of cancer cells. In this paper, the relationship between miR-194-5p and laryngeal cancer was studied and discussed, and the proliferation, cycle and invasion ability of mir-194-5p and pachytene cells were compared. MiR-194-5p can not only be used as a new diagnostic marker of laryngeal cancer to detect the possibility of metastasis of laryngeal cancer but also be used to open up a new way of treatment of laryngeal cancer. Through miR-194-5p, the proliferation of laryngeal cancer cells can interfere, the invasion ability can be reduced, and the disease of patients with laryngeal cancer can be alleviated. This brings a new dawn for the treatment and research of laryngeal cancer and other tumor diseases.

Acknowledgments

Not applicable.

Interest conflict

The authors declare that they have no conflict of interest.

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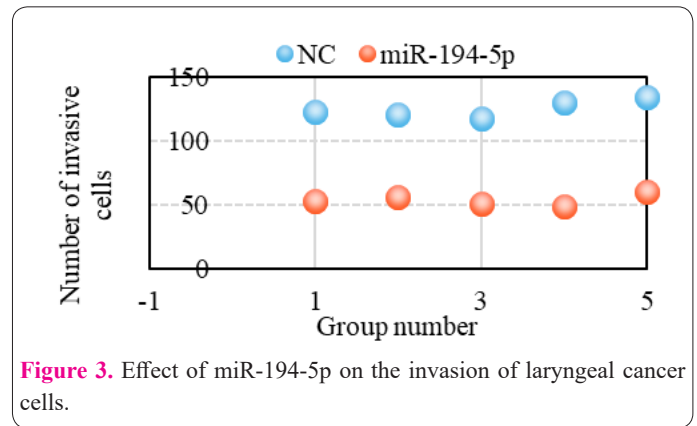


Figure 3. Effect of miR-194-5p on the invasion of laryngeal cancer cells.

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