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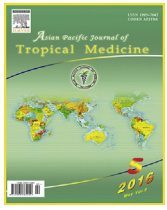

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Effect and mechanism of miR-34a on proliferation, apoptosis and invasion of laryngeal carcinoma cells

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ABSTRACT

Objective: To discuss the effect and mechanism of miR-34a on the proliferation, apoptosis and invasion of laryngeal carcinoma cells.
Methods: The laryngeal squamous carcinoma Hep2 cells were transiently transfected with miR-34a mimics and miR-34a NC. The MTT, colony-forming assay, Hoechst staining and AnnexinV-PI double staining flow cytometry were employed to detect the effect of miR-34a on the viability and apoptosis of laryngeal squamous carcinoma Hep2 cells; Transwell assay to defect the effect of miR-34a on the migration and invasion of laryngeal squamous carcinoma Hep2 cells; western blot and RT-PCR assay to defect the effect of miR-34a mimics on the expression of survivin and Ki-67 mRNA in laryngeal squamous carcinoma Hep2 cells.
Results: Compared with miR-34a NC group, the cell viability in miR-34 mimics group was significantly decreased ($P < 0.01$), the cell apoptosis rate was significantly increased ($P < 0.01$), the abilities of cell migration and invasion were significantly reduced ($P < 0.01$) and the expression of *survivin* and *Ki-67* mRNA was significantly decreased ($P < 0.01$).
Conclusions: The increased expression of miR-34a can induce the apoptosis of Hep2 laryngeal carcinoma cells and inhibit the cell proliferation and invasion, which is related to the down-regulated expression of survivin and Ki-67.

1. Introduction

The laryngeal squamous cell carcinoma is the common malignant tumor in the head and neck in China, where the laryngeal squamous cell carcinoma is the major pathological pattern that accounts for over 90%. The main treatment for laryngeal squamous cell carcinoma is the surgery and it is also accompanied by the chemotherapy and radiation therapy. But the function of swallowing and ventilator phonation for patients in the advanced stage will be greatly affected and those patients will find that 'it is hard to tell about their sufferings'. The prognosis and five-year survival rate for patients in the middle and advanced stage were not good, because the invasion and migration of malignant tumor would cause the death of patients in the end [1,2]. The pathogenesis of laryngeal carcinoma has not been clear. The possible cause might include the smoking, drinking, virus infection, air pollution, activation of oncogenes and inactivation of tumor suppressor genes [2]. Therefore, to seek the effective and specific tumor marker will be of critical significance for the treatment of laryngeal squamous cell carcinoma.

The miRNA is some kind of highly conserved endogenous non-coding small molecule RNA, with the function of oncogenes or tumor suppressor genes, which can be bound with the region of 3′-UTR of target gene mRNA to inhibit the translation of target gene and thus inhibit the expression of target gene. Besides, it is closely related to the development and progression of tumor and plays a key role in the proliferation, apoptosis, invasion and migration of tumor. It is been proved that many kinds of miRNA had the differential expression in laryngeal squamous cell carcinoma, including the miR-34 family [3,4]. The miR-34 family is widely found in the nematodes, arthropods and mammals, including miR-34a, miR-34b and miR-34c. Researches were mainly focused on miR-34a/c. It has been proved that miR-34c had the obvious inhibition effect on the cell cycle and proliferation of laryngeal carcinoma cells Hep2 [5]. But there have been limited researches on the effect of miR-34a in laryngeal squamous cell carcinoma. However,

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Shen *et al.* [6] and Li *et al.* [7] reported that the expression of miR-34a in laryngeal squamous cell carcinoma was significantly lower than that in the paracarcinoma tissues. Besides, miR-34a could affect the proliferation and invasion of cancer cells through the proteins such as targeted survivin. Therefore, in this study, based on previous researches, the liposomes were transfected with miR-34a mimics and miR-34a NC to defect the changes in the proliferation, apoptosis and invasion of laryngeal squamous carcinoma Hep2 cells after the transfection and discuss the related mechanism.

2. Materials and methods

2.1. Materials

The human laryngeal squamous carcinoma Hep2 cells were purchased from Cell Bank, Chinese Academy of Sciences (Item No. TCHu21).

The rabbit anti-survivin and Ki-67 monoclonal antibody was purchased from Epitomics; the methyl thiazolyl tetrazolium from Gibco; the fetal bovine serum from Hyclone; Hoechst 33258 staining kit and AnnexinV-PI double staining flow cytometry kit from Beyotime Biotechnology (Item No. C1018 and C1063); miR-34a mimics and miR-34a NC from Guangzhou RiboBio; Transwell chamber from Corning (Item No. 3413).

The dual mini vertical electrophoresis apparatus and mini transfer electrophoresis apparatus were purchased from Beijing Liuyi Biotechnology Co., Ltd.; ChemiDocTM XRS gel imaging system from Bio-Rad; TS100 inverted microscope from Nikon; FACS Calibur flow cytometry from BD.

2.2. Methods

2.2.1. MTT to detect cell viability

Cells were seeded onto 96-well plate. When the confluency of cells reached to 50%, Lipofectamine™2000 was transfected with miR-34a mimics and miR-34a NC respectively. A total of 48 h after the transfection, 20 μL 5 mg/mL MTT was added for the continuous culture of 4 h and then the culture medium was sucked out. Afterwards, 150 μL DMSO was added in each well and then it was shaken to fully dissolve the crystals. CD value was measured at 560 nm of analyzer of enzyme-linked immunosorbent assay. The cell relative viability (%) = (OD value of experimental group/OD value of control group) × 100%.

2.2.2. Colony formation assay

The human laryngeal squamous carcinoma Hep2 cells were seeded on 96-well plate. When the confluency of cells reached to 50%, Lipofectamine™2000 was transfected with miR-34a mimics and miR-34a NC respectively. A total of 48 h after the transfection, the staining solution with 10% formaldehyde and 0.1% crystal violet was used for the fixed staining. It was placed at room temperature for 30 min. Afterwards, the staining solution was gently flung off and each well was washed with the distilled water. The culture plate was inverted on the absorbent paper to suck out the water. Then the photo was taken and the analysis was performed.

2.2.3. AnnexinV-PI double staining flow cytometry

Cells were seeded onto 6-well plate. When the confluency of cells reached to 50%, Lipofectamine™2000 was transfected with miR-34a mimics and miR-34a NC respectively. The operation procedure should be in accordance with the manual of kit and it was tested on the machine. According to the manual of Annexin V-FITC/PI cell apoptosis test kit, it was digested with 0.25% trypsin and washed with PBS. After being centrifuged at 2000 r/min for 5 min, cells were collected. Then 500 μL Binding Buffer, 5 μL Annexin V-FITC and 5 μL PI 5 were added in turn. Afterwards, they were mixed and reacted at room temperature and in a dark place for 10 min. The flow cytometry test was performed within 1 h.

2.2.4. Hoechst staining

The human laryngeal squamous carcinoma Hep2 cells were seeded on 6-well plate. When the confluency of cells reached to 50%, Lipofectamine™2000 was transfected with miR-34a mimics and miR-34a NC, respectively. A total of 48 h after the transfection, cells were digested and collected. Then the operation procedure was done in accordance with the manual of Hoechst 33258 staining kit. It was fixed with 4% paraformaldehyde for 15 min and then washed with PBS for 3 times. The Hoechst 33258 staining solution was added for the staining in a dark place for 15 min. After being washed with PBS for 3 times, it was observed under the fluorescence microscope and the photos were taken.

2.2.5. Migration assay

The human laryngeal squamous carcinoma Hep2 cells were seeded on 6-well plate. When the confluency of cells reached to 50%, Lipofectamine™2000 was transfected with miR-34a mimics and miR-34a NC respectively. A total of 48 h after the transfection, the cells were digested and then added in Transwell upper chamber. The DMEM with 5% fetal bovine serum was added in the lower chamber to be cultured for 24 h. Afterwards, the Transwell chamber was taken out and washed. It was then fixed with paraformaldehyde and stained with crystal violet. The stained cytoplasm appeared to be violet. The number of membrane-penetrating cells in five fields was counted under the inverted optical microscope and the mean number of cells for each field was calculated to represent the migration ability of cells. Each experiment had three repeats.

2.2.6. Invasion assay

Matrigel gel was evenly paved on the micromembrane (8 μm) of Transwell chamber to be prepared as the gel for further use. Other operations were the same as ones in section 2.2.5. The number of membrane-penetrating cells in five fields was counted under the inverted optical microscope and the mean number of cells for each field was calculated to represent the migration ability of cells. Each experiment had three repeats.

2.2.7. RT-PCR

The total RNA was extracted according to the manual of trizol kit (Invitrogen) in the situation without RNAase. The micro UV spectrophotometer was employed to detect the absorption value of samples at 260 nm and 280 nm to analyze the purity of RNA. The OD260/OD280 ratio within 1.8–2.0 indicated the qualified purity of RNA. The primers were as follows. *Survivin* forward primer: 5′-GTTGCGCTTTCCTTTCTGTC-3′, reverse primer: 5′-TCTCCGCAGTTTCCTCAAAT-3′; *Ki-67* forward primer: 5′-ACTCCAGTTGCCAGTGAT-3′, reverse primer: 5′-GACTTCGGCTGATAGACA-3′; *GADPH* forward primer: 5′-AGCCACATCGCTCAGACA-3′, reverse primer: 5′-TGGACTCCACGACGTACT-3′. The one-step RT-PCR kit was employed to reversely transcribe RNA into cDNA and then

perform the PCR amplification. The collected 5 uL amplification product was used for the further testing with 2% agarose gel. The primers were added in 25 μL PCR system. The reaction conditions included the denaturation at 94 °C for 45 s, renaturation at 59 °C for 45 s and extension at 72 °C for 60 s, with 35 cycles in total.

2.2.8. Western blot

The RIPA lysis buffer with the certain quantity was added in the collected cells. It was put in Vortex instrument for 30 s of shaking every 10 min. After 40 min, it was centrifuged at 4 °C and 10 000 r/min for 10 min. The supernatant was sucked carefully to obtain the total protein. The protein concentration was measured with BCA kit. The protein loading buffer was treated with SDS gel electrophoresis and then it was transferred with the wet method. Then the film was immersed into the primary antibody solution (Survivin and Ki-67, with the dilution ratio of 1:100) for the incubation at 4 °C over night. After being washed, it was immersed into the secondary antibody solution (1:100) for the incubation at the room temperature for 1–2 h. Afterwards, the film was taken out and washed, while ECL reagent was added on the film for the exposure in the gel imaging system. Statistics was performed on the gray value of each antibody band using 'Quantity one' software.

2.3. Statistical analysis

The results were expressed as mean ± SD, with three repeats for each set of data at least. The *t* test was employed and $P < 0.05$ was meant to be significant difference. All data were treated using SPSS 17.0.

3. Results

3.1. Effect of miR-34a on viability and number of clones of laryngeal squamous carcinoma Hep2 cells

It was shown that the viability of laryngeal squamous carcinoma Hep2 cells in miR-34a mimics group [(52.39 ± 5.96)%] was significantly reduced compared with miR-34a NC group [(94.48 ± 8.67)%] ($P < 0.01$). The results showed that compared with miR-34a NC group (125.49 ± 9.68), the number of clones in miR-34a mimics group was significantly reduced (48.37 ± 4.95) ($P < 0.01$).

3.2. Effect of miR-34a on proliferation and apoptosis of laryngeal squamous carcinoma Hep2 cells

According to the results of Hoecsht staining, the apoptosis rate of laryngeal squamous carcinoma Hep2 cells in miR-34a mimics group [(43.38 ± 5.67)%] was significantly increased compared with miR-34a NC group [(5.06 ± 0.63)%] ($P < 0.01$). According to the results of flow cytometry, compared with miR-34a NC group [(9.10 ± 0.71)%], the apoptosis rate of laryngeal squamous carcinoma Hep2 cells in miR-34a mimics group [(44.47 ± 3.58)%] was significantly increased ($P < 0.01$).

3.3. Effect of miR-34a on migration and invasion abilities of laryngeal squamous carcinoma Hep2 cells

The number of migrated cells in miR-34a mimics group (62.28 ± 5.98) was significantly lower than that in miR-34a NC group (159.32 ± 8.96) ($P < 0.01$). The number of invaded cells in miR-34a mimics group (69.26 ± 6.94) was significantly lower than that in miR-34a NC group (188.45 ± 10.95) ($P < 0.01$).

Table 1
Effect of miR-34a on expression of survivin and Ki-67 mRNA and protein in laryngeal squamous carcinoma Hep2 cells.

Group	mRNA expression		Protein expression	
	Survivin/GAPDH	*Ki-67/GAPDH*	*Survivin/GAPDH*	*Ki-67/GAPDH*
MiR-34a NC group	0.32 ± 0.04	0.09 ± 0.01	0.78 ± 0.08	0.28 ± 0.03
MiR-34a mimics group	0.49 ± 0.04**	0.10 ± 0.02**	1.06 ± 0.11**	0.37 ± 0.04**

Compared with miR-34a NC group, **$P < 0.01$.

3.4. Effect of miR-34a on expression of survivin and Ki-67 mRNA in laryngeal squamous carcinoma Hep2 cells

As shown in Table 1, compared with miR-34a NC group, the expression of *survivin* and *Ki-67* mRNA in miR-34a mimics group was significantly reduced ($P < 0.05$); as shown in Table 1, compared with miR-34a NC group, the expression of survivin and Ki-67 protein in miR-34a mimics group was significantly reduced ($P < 0.05$).

4. Discussion

The miRNA is some kind of highly conserved endogenous non-coding small molecule RNA, which can be bound with the region of 3′-UTR of target gene mRNA to regulate the translation of target gene and be involved in the development and progression of tumor. It is been proved that miRNAs could regulate over 60% human protein-coding genes and had the significant impact on the expression of genes. Besides, the abnormal expression miRNA in tumors can be divided into two types. One is called as 'tumor suppressor miRNA'. Its expression is reduced or the function is lost, playing the role like tumor suppressor genes. The other one is 'oncogene miRNA'. Its expression is increased, playing the role like oncogenes [8,9]. Therefore, the abnormal expression of miRNA can be treated as the marker of tumor risk, diagnosis and prognosis, even the targeted therapy based on miRNA. miR-34a is the member of miR-34 family, which is located in 1p36.23, with the low expression in many tumors such as the cervical carcinoma, gastric carcinoma and hepatic carcinoma. It was also involved in the development and progression of tumors and the lymph node metastasis [10–12]. Shen *et al.* [6] performed RT-PCR assay and proved the low expression of miR-34a in laryngeal squamous cell carcinoma. The expression of miR-34a was negatively related to the pathological distribution, lymph node metastasis and clinical staging of patients with laryngeal squamous cell carcinoma, while positively related to the survival rate. miR-34a mimics could significantly inhibit the cell proliferation and down-regulate the expression of survivin protein and also block the cell cycle at the stage of G_0/G_1. Li *et al.* [7] proved that the expression of miR-34a was decreased in the laryngeal squamous cell carcinoma and the exogenous supply of miR-34a could significantly inhibit the proliferation and migration of cells. It indicated that miR-34a played the role of tumor suppressor gene in the laryngeal squamous cell carcinoma and it was also closely related to the development and

progression of laryngeal squamous cell carcinoma and lymph node metastasis. Therefore, in this study, the laryngeal squamous carcinoma Hep2 cells was transiently transfected with miR-34a mimics and miR-34a NC and The MTT, colony-forming assay, Hoechst staining, AnnexinV-PI double staining flow cytometry and Transwell assay were employed to detect the effect of miR-34a on the proliferation, apoptosis, migration and invasion of laryngeal squamous carcinoma Hep2 cells. The results showed that the cell viability, number of cell cloning, number of cell migration and invasion in miR-34a mimics group were significantly lower than that in miR-34a NC group, while the cell apoptosis rate was significantly higher than that in miR-34a NC group. It indicated that the increased expression of laryngeal squamous carcinoma Hep2 cells could significantly induce the cell apoptosis and inhibit the cell proliferation, migration and invasion. Meanwhile, Nie *et al.* [13] and Zhao *et al.* [14] proved that the increased expression of miR-34a in esophageal squamous cell carcinoma and osteosarcoma cells could significantly induce the apoptosis of cancer cells and inhibit the colony-formation, migration and invasion of cells, which were in line with the findings of this study.

The development and progression of tumor is a process that involves multiple factors and steps, being presented as the activation of oncogenes and inactivation of tumor suppressor genes finally. By regulating the expression of oncogenes and tumor suppressor genes, miRNAs play a key role in the development and progression of tumors. The survivin is also named as the apoptosis inhibitor-5, as the new member of apoptosis inhibitor protein family. It is situated in 17q25 and the strongest apoptosis inhibitor found so far, with the functions of inhibiting the cell apoptosis and maintaining the mitosis. The survivin can affect the apoptosis pathway of caspase 3 through the indirect or direct way to inhibit the cell apoptosis and promote the cell proliferation. Survivin had the high expression in most of human tumor tissues, but nearly had no expression in the normal mature tissues [15]. According to the immunohistochemistry performed by Wang *et al.* [16], the expression of survivin and Ki-67 was gradually increased in the vocal cord polyp tissues, laryngeal precancerous lesions and laryngeal carcinoma tissues. The expression of them was positively correlated. Ki-67 is some kind of nuclear antigen related to the cell proliferation, which was firstly found by Gerdes *et al.* in 1983. It is closely related to the cell mitosis. Except no expression at G_0 phase of cell cycle, it is expressed in other phases. Its expression is highest at M phase and can be regarded as the marker of cell proliferation. It can reliably reflect the proliferation rate of tumors and it is related to the development, progression, metastasis and prognosis of many tumors. Acikalin *et al.* [17] reported the results of immunohistochemistry and also proved the over-expression of Ki-67 in the laryngeal carcinoma tissues, which was closely related to the tumor staging and lymph node metastasis. It indicated that the down-regulated expression of survivin and Ki-67 could significantly induce the cell apoptosis and inhibit the proliferation and invasion of cells.

Therefore in this study, the western blot and RT-PCR assay were employed to detect the expression of survivin and Ki-67 protein and mRNA in cells after the transfection of miR-34a mimics and miR-34a NC. The results showed that, compared with miR-34a NC group, the expressions of survivin and Ki-67 protein and mRNA were all significantly decreased in miR-34a mimics group. The previous research also proved that the over-expression of miR-34a could reduce the viability and invasion of human papillomavirus positive cervical carcinoma cells through the targeted regulation of expression of E23F and survivin [10]. The increased expression of miR-34a in the gastric cancer cells could significantly inhibit the proliferation, migration and invasion of gastric cancer cells, which was related to the decreased expression of survivin [11]. It indicated that the increased expression of miR-34a in laryngeal carcinoma Hep2 cells could significantly reduce the expression of survivin and Ki-67 protein and mRNA and thus induce the cell apoptosis and inhibit the proliferation and invasion of cells. Meanwhile, a research also proved that miR-34a could significantly increase the sensitivity of gastric cancer cells to the cisplatin, which was related to PI3K/AKT/survivin pathway [18]. Besides, the effect of miR-34a on the proliferation, apoptosis, migration and invasion of tumors was related to many signaling pathways [19,20]. Accordingly, the further studies will discuss the specific signaling pathways regarding the effect of miR-34a on the proliferation and apoptosis and invasion of Hep2 gastric cancer cells.

In conclusion, miR-34a mimics could significantly reduce the viability of Hep2 cells, induce the cell apoptosis and inhibit the migration and invasion of cells, which were realized by down-regulating the expression of survivin and Ki-67.

Conflict of interest statement

We declare that we have no conflict of interest.

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