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ORIGINAL ARTICLE

Effect of miR-122a on biological behavior of laryngeal carcinoma cells and its role

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Summary

Purpose: *This study was designed to explore the effect of miR-122a on the biological behavior of laryngeal carcinoma cells and its role.*

Methods: *Thirty-two patients with laryngeal carcinoma who were diagnosed and treated in our hospital from March 2013 to November 2015 formed the research group (RG), and 30 normal people who underwent physical examination in our hospital during the same period formed the control group (CG). We observed the expression of miR-122a in cells and its effect on cell biological function, examined the expression level of miR-122a in laryngeal carcinoma tissues, and further drew receiver operating characteristics (ROC) curve to analyze the diagnostic value of miR-122a in laryngeal carcinoma; we divided them into high and low expression groups according to the expression of miR-122a, and also registered their 3-year survival rate.*

Results: *miR-122a showed low expression in cancer tissues ($p<0.05$). ROC curve analysis revealed that miR-122a had a sensitivity of 82.22%, specificity of 68.75%, and area under the curve (AUC) of 0.770. The 3-year survival rate of the high expression group was 56.25%, and that of low expression group was 25.00%. The survival rate of high expression group was significantly better than that of low expression group ($p=0.024$). The proliferation ability of AMC-HN-8 cells transfected with miR-122a-mimics sequence was obviously inhibited, and its apoptosis rate increased.*

Conclusion: *Upregulation of miR-122a expression can reduce proliferation of laryngeal carcinoma cells and increase their apoptosis, and it can be used as a potential diagnostic index and therapeutic target for laryngeal carcinoma.*

Key words: *miR-122a, laryngeal carcinoma, cell biology*

Introduction

Laryngeal cancer is a common cancer with high morbidity and mortality. In countries with advanced health care systems, the mortality of laryngeal cancer is declining, but the incidence rate in developing countries is still very high [1,2]. Laryngeal carcinoma ranks 11th among the most common cancers among men in the world, and is the second most common malignant tumor in head and neck, posing a great threat to people's life [3]. Due to the evolution of the times and changes of people's lifestyle and improvement of living standards, the morbidity of laryngeal carcinoma is obviously on the rise due to the increase of virus infections, air pollution, smoking, drinking and other factors related to its onset [4]. At present, the best clinical treatment for laryngeal carcinoma is mainly chemotherapy and surgery. Wolfe et al [5] and others believed that a good survival rate was achieved by using single-cycle neoadjuvant chemotherapy for biological selective treatment. Good survival rate was also achieved in patients who chose to undergo primary surgery, both of which were superior to

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concurrent chemoradiotherapy. Because the onset of laryngeal carcinoma is relatively silent, most patients are in advanced stage of disease after being admitted to hospital for diagnosis, and the tumor has metastasized, thus they missed the opportunity of surgical treatment. At present, there is a lack of biomarkers for early diagnosis, comprehensive treatment and disease detection of laryngeal cancer clinically, so it is particularly important to find a key biomarkers.

MicroRNA (miR) is a non-coding RNA with a length of about 22 nucleotides. It can be used as endogenous RNA interference to regulate the expression of target genes and participate in the regulation of a variety of physiological and pathological functions [6]. In recent years, an increasing number of studies have shown that there is a close relationship between miR and tumor [7,8]. In laryngeal carcinoma, abnormal expression of many miRs has been reported in its progress, including miR-210, miR-205, miR-301a-3p, miR- 519a, etc [9-12]. As a member of miR family, previous studies [13,14] have found that miR-122a is closely related to the progression of hepatocellular carcinoma and gastrointestinal cancer, but there is no relevant research to prove whether there is a link with laryngeal carcinoma.

Therefore, this study explored the expression of miR-122a in laryngeal carcinoma and its influence on the biological function of laryngeal carcinoma cells, in order to find new potential diagnostic and therapeutic targets for clinical use.

Methods

Clinical data of patients

In this experiment, 32 patients with laryngeal carcinoma diagnosed and treated in our hospital from March 2013 to November 2015 formed the research group (RG), including 21 males and 11 females, with an average age of 50.8±10.6 years. Another 30 normal people who underwent physical examination in our hospital during the same period formed the control group (CG), including 18 males and 12 females, with an average age of 51.2±10.3 years. This study was approved by the Medical Ethics Committee of our hospital.

Inclusion and exclusion criteria

Inclusion criteria: Patients met the TNM staging standard [15] issued by the American Joint Committee on Cancer (ACJJ) in 2017. After diagnosis, patients received treatment in our hospital, had complete case data, agreed to cooperate with the work arrangement of our medical staff, and patients or their immediate family members signed an informed consent form.

Exclusion criteria were as follows: Patients with injury of important organs; those combined with cardiovascular and cerebrovascular diseases, physical disability or pregnancy; those having autoimmune diseases or other chronic diseases; those transferred from one hospital to another; those with contraindications to surgery, mental diseases and language dysfunction, and diseases affecting the results of this study.

Cell sources, reagents and instruments

TU212, HEp-2 and AMC-HN-8 cells were purchased from Beijing Beina Science & Technology Co,. Ltd. (resource numbers: BNCC340714, BNCC338610, BNCC338377). The main reagents and instruments were as follows: Lipofectamine™ 2000 (Shanghai Mito Biotechnology Co., Ltd., Art. No.:11668019), MMT kit, DMSO reagent (Shanghai Yuanye Biotechnology Co., Ltd., Art. No.:S30860, S24295), RPMI-1640 (Shanghai Yanjin Biotechnology Co., Ltd., Art. No.:31800-022), PBS, bovine fetal serum (Shanghai Hengfei Biotechnology Co., Ltd., Art. No.:P1000, SA133), penicillin-streptomycin double antibody (Beijing Baiaolaibo Technology Co., Ltd., Art. No.: MT0104-SEY), TransScript II Green Two-Step qRT-PCR SuperMix (TransGen Biotech, Beijing, China, AQ301-01), Annexin V/PI apoptosis detection kit (Shanghai Hengfei Biotechnology Co., Ltd., Art. No.:CA1020), microplate reader (Beijing Amygdall Trading Co., Ltd., Art. No.:21261000), PCR instrument (Wuxi MicroSep Biotechnology Co., Ltd., Art. No.:TC9639), flow cytometry (Beijing Image Trading Co., Ltd., Art. No.:AMG0002051). The miR-122a primer sequence was designed and synthesized by Shanghai Sangon Bioengineering Co., Ltd. More details are shown in Table 1.

Cell culture and transfection

Re-purchased HEp-2, AMC-HN-8 cancer cells and human laryngeal carcinoma cells TU212 were transferred to RPMI1640 medium along with penicillin-streptomycin and 10% FBS and cultured at 37°C and in incubator with 5% CO_2 and 95% air. MiR-122a-mimics, miR-122a-inhibitor were transfected using Lipofectamine™ 2000 kit, and the operation steps were strictly in accordance with the kit instructions. All primers were transfected into the cells with the greatest expression difference.

qRT-PCR detection

Collected cells and serum were extracted with TRIzol kit for total RNA, and extracted strictly according to

Table 1. Primer sequence

	Upstream sequence	*Downstream sequence*
U6	5'-TCTCTGCTCCTCGTTCGA-3'	5'-GCGCCCATACGACCAAATC-3'
miR-122a	5'-CAAGCGTTGGAGTGTGACA-3'	5'-CGTCCTACCATTCTCCAGC-3'

the manufacturer's instructions. Purity, concentration and integrity of the extracted total RNA were detected by ultraviolet spectrophotometer and agarose gel electrophoresis. 5x TransScript® II All-in-One SpuperMix for qPCR, TransScript® miR RT Enzyme Mix, and 2×TS miRNA Reaction Mix kit were used for reverse transcription which has performed strictly in accordance with the manufacturer's kit. Then, PCR amplification was carried out. PCR reaction system was as follows: cDNA 1μL, upstream and downstream primers 0.4μL each, 2x TransScript® Tip Green qPCR SuperMix 10μL, Passive Reference Dye (50x) 200μL, ddH2O added to 20μL. PCR reaction conditions were as follows: 94°C pre-denaturation for 30s, 94°C denaturation for 5s, 60°C annealing extension for 30s, with a total of 45 cycles. Each sample was placed in 3 wells, and the experiment was carried out in triplicate. U6 was used as internal reference and $2^{-\Delta ct}$ was used to analyze the data.

Detection of cell proliferation

Cells harvested 24h after transfection were collected, and the cell density was adjusted to 3×10^4 cells/well; they were inoculated on 96-well plates and incubated at 37°C for 24, 48, 72 and 96h respectively. Then, 20μL MTT solution (5 μmg/mL) was added at each time point, cells were cultured at 37°C for 4h, 150μL DMSO was added to each well, and the optical density (OD) value of cells in each group was measured at 450nm absorbance using an enzyme reader.

Detection of apoptosis

Cells transfected for 24h were digested with 0.25% trypsin, washed twice with PBS after digestion, added with 100μL of binding buffer, prepared into 1×10^6 cell/mL suspension, sequentially added with AnnexinV-FITC and PI, incubated in the dark at room temperature for 5min, detected with FC500MCL flow cytometry system, and the experiment was repeated for 3 times to take the average value.

Follow-up of patients

Patients were followed up for 3 years, and their survival was recorded by telephone and outpatient medical records. The follow-up time was 4, 8 and 12 months per year, respectively.

Observation indicators

Main observation indicators: the expression level of miR-122a in laryngeal carcinoma tissues; the expression level of miR-122a in cells and its effect on cell biological function.

Secondary observation indicators: the diagnostic value of miR-122a for laryngeal carcinoma. According to

Table 2. Clinical basic data [n(%)]

	Research group (32)	*Control group (30)*	x^2 *or t*	*p*
Age, years	50.8±10.6	51.2±10.3	0.151	0.881
Gender, n (%)			0.209	0.647
Male	21 (65.63)	18 (60.00)		
Female	11 (34.38)	12 (40.00)		
BMI (kg/m^2)	22.26±0.37	22.21±0.25	0.618	0.538
Marital status, n (%)			0.242	0.623
Married	29 (90.63)	26 (86.67)		
Unmarried	3 (9.38)	4 (13.33)		
Nationality, n (%)			0.011	0.915
Han	22 (68.75)	21 (70.00)		
Ethnic minorities	10 (31.25)	9 (30.00)		
Place of residence, n (%)			0.501	0.479
Cities and towns	18 (56.25)	19 (63.33)		
Countryside	14 (43.75)	11 (36.67)		
History of smoking, n (%)			29.370	0.001
Yes	30 (93.75)	8 (26.67)		
No	2 (6.25)	22 (73.33)		
History of drinking, n (%)			25.930	0.001
Yes	4 (12.50)	23 (76.67)		
No	28 (87.50)	7 (23.33)		
Exercise habits, n (%)			0.047	0.829
Yes	13 (40.63)	13 (43.33)		
No	19 (59.38)	17 (56.67)		

the expression level of miR-122a, patients were divided into high and low expression groups, and their 3-year survival rate was registered.

Statistics

In this study, SPSS 20.0 software package was used to carry out statistical analysis on the collected data, GraphPad 7 software package was used to draw the required pictures, and Kolmogorov-Smirnov test was used to analyze the distribution of dose data, in which normal distribution data were expressed by mean±standard deviation (mean±SD), inter-group comparison was conducted by independent-sample T-test, intra-group comparison was conducted by paired T-test, and the counting data were expressed as percents, conducted by x^2 test, the diagnostic value of miR-122a in laryngeal carcinoma was drawn by ROC analysis, the 5-year survival of patients using Kaplan-Meier method, and Log-rank test was used for survival comparison between two groups. A p value lower 0.05 was considered to show statistically significant difference.

Results

Clinical data of patients in the RG and CG included age, gender, BMI, marital status, nationality, place of residence and exercise. There was no significant difference between the two groups, which were comparable (p>0.05), while there was difference in the classification of smoking and drinking history (p<0.05). More details were shown in Table 2.

Expression level of miR-122a in laryngeal carcinoma tissues

The expression level of miR-122a was 3.56±3.62 in cancer tissues and 7.52±2.01 in adjacent tissues, and the expression level of miR-122a in adjacent tissues was significantly higher than that in cancer tissues (p<0.05), as shown in Figure 1.

Diagnostic value of miR-122a for laryngeal carcinoma

According to ROC curve analysis, when cut-off value was 1.125, miR-122a had a sensitivity of 82.22%, specificity of 68.75% and AUC of 0.774 (p<0.001), as shown in Figure 2 and Table 3.

High and low expression groups divided by the expression of miR-122a and 3-year survival rate of patients

In this experiment, patients were divided into 16 cases of miR-122a high expression group (≥2.420) and 16 cases of miR-122a low expression group (< 2.420) according to the median value of

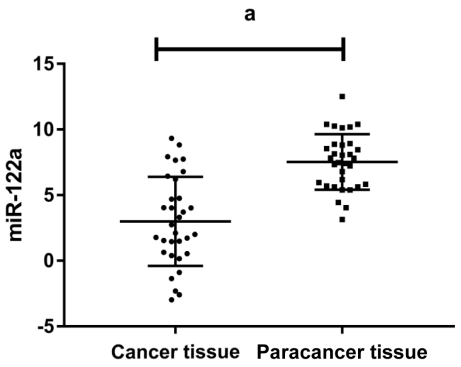

Figure 1. Expression level of miR-122a in laryngeal carcinoma tissues. The expression level of miR-122a in paracancer tissues was significantly higher than that in cancer tissues (p< 0.05).

Table 3. ROC diagnosis

	miR-122a
AUC	0.770
Std.error	0.057
95%CI	0.659-0.881
P	0.001
Cut-off	4.105
Sensitivity (%)	82.22
Specificity (%)	68.75

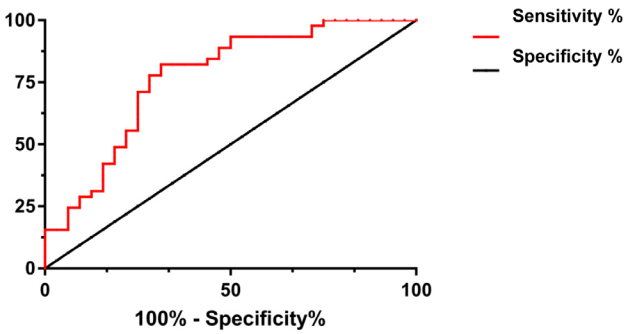

Figure 2. Diagnostic value of miR-122a for laryngeal carcinoma. MiR-122a had a sensitivity of 82.22%, specificity of 68.75% and AUC of 0.764 (p<0.001).

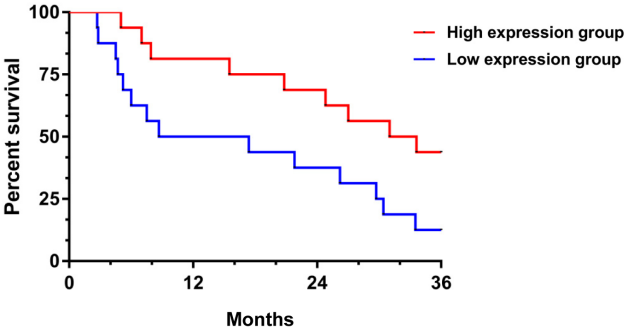

Figure 3. High and low expression groups divided by the expression of miR-122a and their 3-year survival rate. The 3-year survival rate was 56.25% in the high expression group and 25.00% in the low expression group. The survival rate of high expression group was significantly better than that of low expression group (p=0.030).

miR-122a expression level detection results. All the follow-up patients were evaluated. There were 7 dead patients in the high expression group, with a 3-year survival rate of 56.25% and 12 dead patients in the low expression group, with a 3-year survival rate of 25.00%. The survival rate of high expression group was significantly better than that of low expression group (p=0.024). More details are shown in Figure 3.

Expression of miR-122a in cells and its effect on cell biological function

By detecting the relative expression of miR-122a in cells of each group, it was found that its expression in AMC-HN-8 cells significantly decreased in TU212 compared with HEp-2 (p<0.05). AMC-HN-8 cells were selected for transfection, and the results showed that miR-122a expression of AMC-HN-8 cells in miR-122a-mimics group and miR-122a-inhibiting group was significantly higher than that in miR-NC group (p<0.05), while miR-122a expression in miR-122a-inhibiting group was lower than miR-NC group (p<0.05). MTT test indicated that the proliferation ability of AMC-HN-8 cells in the miR-133a-mimics group was significantly lower than that of miR-NC group (p<0.05), while miR-122a-inhibiting group was higher than that of miR-NC group (p<0.05). Flow cytometry showed that the apoptosis rate of AMC-HN-8 cells in the miR-122a-inhibiting group was significantly lower than that in the miR-NC group, while the miR-133a-mimics group was higher than that in the miR-NC group (p<0.05). More details are shown Figure 4.

Discussion

Laryngeal carcinoma is one of the major causes of cancer mortality in the world, and its morbidity is increasing year by year [16]. In 2013 12,260

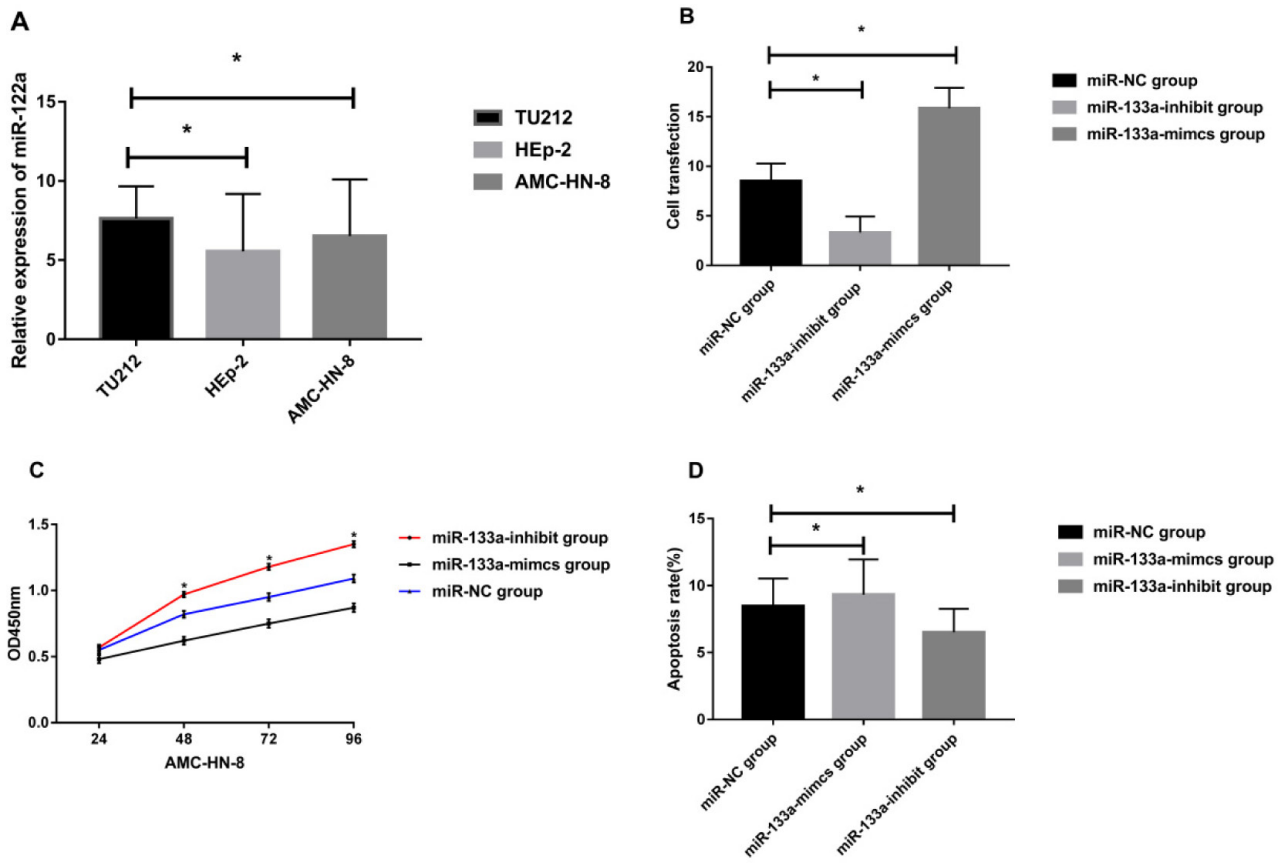

Figure 4. Expression of miR-122a in cells and its effect on cell biological function. **A:** Compared with HEp-2, the expression of miR-122a in AMC-HN-8 cells decreased significantly in TU212 (*p<0.05). **B:** AMC-HN-8 cells were selected for transfection, and the results showed that miR-122a expression of AMC-HN-8 cells in the miR-122a-mimcs group and miR-122a-inhibit group was significantly higher than that in miR-NC group (*p<0.05), while miR-122a expression in miR-122a-inhibit group was lower than that of miR-NC group (*p<0.05). **C:** MTT test indicated that the proliferation ability of AMC-HN-8 cells in miR-133a-mimcs group was significantly lower than that of miR-NC group (*p<0.05), while proliferation ability of miR-122a-inhibit group was higher than that of miR-NC group (*p<0.05). **D:** Flow cytometry showed that the apoptosis rate of AMC-HN-8 cells in the miR-122a-inhibit group was significantly lower than that in the miR-NC group, while apoptosis rate in miR-133a-mimcs group was higher than that in the miR-NC group (*p<0.05

new laryngeal carcinoma cases were reported in the United States, of which 3,630 died [17]. The treatment of laryngeal carcinoma has improved in the past 20 years, but the survival rate of patients with this disease is far from the clinical expected effect [18]. At present, the root cause of laryngeal carcinoma is still uncertain and its onset is difficult to detect [19]. Highly specific diagnostic markers are lacking. Therefore, it is extremely important to find an effective biomarker for the diagnosis of laryngeal cancer.

MiR is endogenous non-coding short-chain RNA, which can degrade target genes and inhibit target gene translation, thus achieving gene silencing after transcription [20]. A study [21] showed that nearly 30% of the encoded proteins in human body are affected and regulated by miRs. MiRs are differentially expressed in tumors, and can inhibit or promote the occurrence and development of tumors by regulating target genes. As a member of miR family, miR-122a has been proved to exert tumor inhibition effect in liver cancer [22], but whether miR-122a has effect in laryngeal carcinoma has not been studied so far. Therefore, we studied the clinical diagnostic value of miR-122a in laryngeal carcinoma patients and the effect of miR-122a on the biological function of laryngeal carcinoma cells in order to provide information for early laryngeal carcinoma screening.

In order to ensure the comparability and accuracy in this experimental study, we observed the basic clinical data of patients and health check-ups and found that there were differences in smoking and drinking. Mayne et al [23] and others explained that the risk of primary tumor development caused by smoking and drinking was 2.1 times that of non-smoking and non-drinking, and the overall survival rate was also poor. We detected the expression level of miR-122a in cancer tissues of 32 patients with laryngeal carcinoma by qRT-PCR, and found that the expression level of miR-122a in laryngeal carcinoma tissues was significantly lower than in adjacent tissues, which indicated that miR-122a might become a potential diagnostic and treatment target for laryngeal carcinoma. For this reason, we further found through ROC curve analysis that the area under miR-122a curve was 0.770 with high specificity and sensitivity, which was a potential clinical diagnostic index for laryngeal carcinoma. Kim et al [24] and others thought miR-122a played an important role in physiological processes such as lipid metabolism, stress response and tumor inhibition, and its expression level in normal hepatocytes was very high. We further divided the patients into high and low expression groups according to the median value of miR-122a expression level results in laryngeal carcinoma. Observing the 3-year survival rate of patients, we found that the 3-year survival rate of high expression group was 56.25%; the 3-year survival rate in the low expression group was 25.00%, and the survival rate in the high expression group was significantly better than in the low expression group, suggesting that miR-122a could be used as an indicator for predicting the survival rate of patients. This research preliminarily proved the clinical value of miR-122a. Finally, in this study, we chose to upregulate the expression of miR-122a in AMC-HN-8 cells to observe its effect on laryngeal carcinoma cells. The results revealed that the proliferation ability of AMC-HN-8 cells transfected with miR-122a-mimics sequence was significantly inhibited and the apoptosis rate increased, which indicated that miR-122a can be expected to be a potential target for laryngeal carcinoma treatment.

However, we still have certain limitations in this study. Firstly, we did not observe the cell invasion. Secondly, we did not conduct mice experiments. It is not clear how many concentrations of miR-122a can be injected to achieve the best target control effect. We will conduct more in-depth experimental analysis as soon as possible to provide clinical answers.

In summary, upregulation of miR-122a expression can reduce the proliferation of laryngeal carcinoma cells and increase apoptosis, and it can be used as a potential diagnostic index and therapeutic target for laryngeal carcinoma.

Conflict of interests

The authors declare no conflict of interests.

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