ORIGINAL ARTICLE

Clinical significance of miRNA-195 expression in patients with laryngeal carcinoma

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Summary

Purpose: This study aimed to investigate the expression pattern of microRNA (miRNA)-195 in laryngeal carcinoma and analyze the relationships of miRNA-195 expression with clinicopathological features and prognosis.

Methods: The expression levels of miRNA-195 in pathological cancer tissue and tumor-adjacent normal tissue of 182 patients with laryngeal carcinoma were measured by reverse transcription-polymerase chain reaction (RT-PCR), and the relationships of relative expression levels

of miRNA-195 with clinicopathological features and prognosis of patients with laryngeal carcinoma were analyzed. The invasive ability of cells was measured by Transwell chamber test. Cell proliferation in vitro was determined using 3-(4,5)-dimethylthiahiazo-(-z-y1)-3,5-di-phenytetrazoliumromide (MTT) method.

Results: The expression level of miRNA-195 in laryngeal carcinoma tissue was lower than that in tumor-adjacent normal tissue (p<0.05). In vitro growth, migration and invasion ability of laryngeal carcinoma cells with lower expression

level of miRNA-195 were distinctly higher than those of normal tissue cells. The expression levels of miRNA-195 were not significantly correlated with gender, age, grade of differentiation and tumor site (p>0.05), but were closely correlated with lymph node metastasis and clinical staging (p<0.05). The 5-year survival rate, median survival and progressionfree survival in patients with high expression levels of miR-NA-195 were significantly better than those in patients with low expression levels of miRNA-195 (78 vs. 39%, 57 months vs. 39 months, 40 months vs. 27 months, p<0.05).

Conclusion: These findings indicated that expression of miRNA-195 in laryngeal carcinoma tissue is down-regulated, and the low expression of miRNA-195 may be related to invasion and metastasis of laryngeal carcinoma, which indicates poor prognosis of patients. MiRNA-195 may serve as a potential molecular target for the treatment and prognosis evaluation of laryngeal carcinoma.

Key words: laryngeal carcinoma, miRNA-195, prognosis, survival

Introduction

Laryngeal carcinoma is the twentieth most common cancer in the world, and more than 150,000 new cases are diagnosed each year. World Health Organization in 2012 reported that the total incidence of laryngeal carcinoma in the European Union was 4.4/100,000, and mortality was 1.8/100,000. Incidence and mortality were generally lower in women than in men. Laryngeal carcinoma is a multifactorial disease associated with

various lifestyle-related factors [1]. Smoking and drinking are considered as the major risk factors for laryngeal carcinoma. Satapathy et al. [2] found that microRNAs (miRNAs) are involved in the formation of a variety of head and neck cancers. It is found that the expression levels of miRNA-34a/c [3], miRNA-125b [4], miRNA-125a-5p [5], miR-NA-138 [6] and miRNA-153 [7] are down-regulated in laryngeal carcinoma, which in turn promotes

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tumor proliferation, metastasis and infiltration, increases the risk of laryngeal carcinoma and reduces the survival rate of patients.

The purpose of our study was to investigate the expression pattern of miRNA-195 in laryngeal carcinoma and explore the correlations between miRNA-195 expression and clinicopathological features and prognosis.

Methods

General data

A total of 182 patients with laryngeal carcinoma admitted to Affiliated Hospital of Jining Medical University from January 2011 to January 2012 were enrolled in this study. Cancer tissues and tumor-adjacent normal tissues were collected from those patients.

Inclusion criteria: Patients below 80 years; patients pathologically diagnosed with laryngeal carcinoma; patients without radiotherapy, chemotherapy, molecular targeted therapy, Chinese medicine therapy or immunotherapy before admission; patients signing informed consent and voluntarily participating in this study.

Exclusion criteria: Patients with a history of other types of malignancies or severe cardiovascular, respiratory, psychiatric or neurological diseases; patients who were treated one month before admission. Those patients included 120 males and 62 females; 80 cases aged <60 years and 102 cases \geq 60 years; for clinical staging, there were 130 cases in stage I-II and 52 cases in stage III-IV; 82 cases were classified in the poorly and moderately differentiated group and 100 cases in the well differentiated group; 72 cases with lymph node metastasis and 110 without; for tumor growth site, there were 50 cases with tumor located at the upper segment of esophageal glottis, 95 cases at glottis, and 37 cases at the lower segment of glottis. This study was approved by the ethics committee of Affiliated Hospital of Jining Medical University and informed consent was signed by the patients or their guardians.

Therapy and follow-up

All 182 patients with laryngeal carcinoma were treated with radical tumor resection. After surgery, radiotherapy and chemotherapy were administered based on pathological results and general patient conditions. Patients were followed up from the day of discharge until death or February 2017. Follow-up was performed through outpatient visit and telephone contact to record patients' general status, symptoms and signs, examination data [results of laryngoscopy, larynx computed tomography (CT), and blood tests], etc. On ending the follow-up period , 45 patients were alive without recurrence and metastasis, 40 patients were alive with recurrence and metastasis, 97 cases had died, and no censored case was observed.

Materials and reagents

HEP-2 laryngeal carcinoma cell line was purchased from Cell Resource Center of Chinese Academy of Medi-

cal Sciences (Beijing); polymerase chain reaction (PCR) kits and reverse transcription kits were purchased from Invitrogen (USA); primers and miRNA-195 mimics were provided by Thermo Fisher Scientific Inc. (USA); dimethyl sulfoxide (DMSO) was provided by Shanghai Heyi Chemical Co., Ltd.; 10% fetal bovine serum was purchased from GIBCO (USA).

Experimental methods

Cell culture

TU212 cancer cells were cultured in cell culture medium containing 10% fetal bovine serum, 100 IU/mL penicillin and 10 mg/mL streptomycin. All cells were cultured at 5% CO₂, 37°C and 100% relative humidity. Cells were collected during the logarithmic growth phase for subsequent experiments.

Assessment of cell proliferation ability using 3-(4,5)-dimethylthiahiazo-(-z-y1)-3,5-di-phenytetrazoliumromide (MTT) method

3000 cells were added into each well of a 96-well plate. At 6, 12, 24, 48 and 72 hrs after the beginning of cell culture, 20 μ L MTT reagent was added into each well, followed by cell culture at 37°C for another 4 hrs. Finally, 100 μ L DMSO was added into each well. Subsequently, the absorbance at 570 nm was measured by spectophotometer. Each assay was carried out in triplicate.

Transwell chamber assay

Migration or invasion assays were performed using a 24-well Transwell plate. 3×10^4 laryngeal carcinoma cells in 200 µL in serum-free medium were inoculated into the upper chamber, and 500 µL medium containing 5% serum was added into the lower chamber. Cells were incubated at 37°C for 12 hrs, followed by fixation with 4% paraformaldehyde for 15 min. Finally, cells on the lower surface were stained by hematoxylin and eosin. Ten fields (100x magnification) were randomly selected and cells were counted.

Specimen selection

Histological specimens of cancer tissue examined pathologically were collected by laser capture microdissection (LCM technology) and formed the experimental group, while the adjacent tissues pathologically diagnosed as normal tissues formed the control group. All tissues were stored at -80°C before use.

Determination of miRNA-195 content

Total RNA of laryngeal carcinoma tissue and normal laryngeal tissue was extracted using TRIzol reagent, and reversely transcribed into complementary DNA (cDNA), which was used as the template in reverse transcriptionpolymerase chain reaction (RT-PCR) to detect miRNA-195 content. U6 and GAPDH are endogenous control genes for miRNA and mRNA. Primers used in PCR reactions were: endogenous control U6 gene: 5'-GCTCGTCGGGTCGGGC-CTATTG-3' (upstream primer) and 5'-CCGCCCTATTGCT-TAAGCCCACG-3' (downstream primer). GAPDH endogenous control primers were: 5'-CGCTCTCTGCTCCTCCTGTT-3' (upstream primer), 5'-CCATGGTGTCTGAGCGATGT-3' (downstream primer).

Primers of miR-195 sequence mimic were: 5'-UAGCAGCACAGAAAUAUUGGC-3' (upstream primer) and 5'-CAAUAUUUCUGUGCUGCUAUU-3' (downstream primer). control group primers: 5'-UUCUCCGAACGU-GUCACGU-3' (upstream primer) and 5 '-ACGUGAACGU-UCGGAGAA-3' (downstream primer).

MiR-195 irrelevant sequence mimic primer was 5'-GCCAAUAUUUCUGUGCUGCUA-3', control group primer was 5'-CAAUAUUUCUGUGCUGCUAUU-3'. Reaction conditions were 94°C for 30s, followed by 40 cycles



Figure 1. Comparison of relative expression level of miR-NA-195 between laryngeal carcinoma tissue and tumoradjacent normal tissue. RNA is extracted from tissues, and miRNA expressions in different tissues are detected by RT-PCR. Relative expression level of miRNA-195 in laryngeal carcinoma group was significantly lower than that in tumor-adjacent normal tissue (p<0.05).

of 94°C for 15s, 60°C for 15s and 72°C for 20s. The relative expression level of miRNA-195 was normalized by $2^{-\Delta\Delta Ct}$ method.

Statistics

SPSS 20.0 software was utilized for data analysis. Paired *t*-test was adopted to compare the expression levels of miRNA-195 in cancer tissue and tumor-adjacent normal tissue, and chi-square test was used to analyze the correlation between miRNA-195 expression in cancer tissue and clinicopathological features of laryngeal carcinoma. Kaplan-Meier method was applied to calculate and analyze 5-year survival rate, median survival time and progression-free survival. Survival curves were compared using log-rank method. P<0.05 suggested that the difference was statistically significant.

Results

MiRNA-195 was downregulated in cancer tissue compared with tumor-adjacent normal tissue of patients with laryngeal carcinoma

MiRNA-195 was clearly down-regulated in cancer tissues compared with tumor-adjacent normal tissues in 79.67% (145/182) of 182 patients with laryngeal carcinoma. The relative expression level of miRNA-195 in laryngeal carcinoma tissues was 0.76 ± 0.38 , which was significantly lower than that in tumor-adjacent normal tissues (2.69 ± 1.45 , p<0.05) (Figure 1).

Relationships between relative expression level of miR-NA-195 and clinicopathological features

Patients with laryngeal carcinoma were divided into high-expression group (83 cases) and low-expression group (99 cases) according to the



Figure 2. Expression of miRNA-195 in laryngeal carcinoma TU212 cells. Proliferation of laryngeal carcinoma cell was determined by MTT method. Proliferation ability of TU212 cell with miRNA-195 mimics transfection is remarkably lower than that of TU212 cell with no treatment (p<0.05; **A**). There is no significant difference in the proliferation ability between TU212 cell transfected with miRNA-195 inhibitor and cells without treatment (p<0.05; **B**).

average expression level of miRNA-195 in laryngeal carcinoma tissues (0.76). The results showed that there were no significant differences in general data including gender and age between two groups (p>0.05). There were also no significant differences in the grade of differentiation of laryngeal carcinoma and tumor growth site between two groups (p>0.05). Significant difference in lymph node metastasis and larvngeal carcinoma staging was found between the 2 groups. The expression level of miRNA-195 was obviously lower in patients with nodal metastasis than in those with no such metastasis (p=0.009). The expression level of miR-NA-195 in patients with stage I-II was significantly higher than in patients with stage III-IV (p=0.014) (Table 1).

Expressions of miRNA-195 in TU212 cells after transfection with miRNA-195 mimics

Compared with that in control TU212 cells, miR-NA-195 expression level was clearly increased in laryngeal carcinoma cells with miRNA-195 mimics transfection (p<0.05). However, there was no statistically significant difference in miRNA-195 expres-



Figure 3. Invasive ability of laryngeal carcinoma cells. Laryngeal carcinoma cell invasion was detected by Transwell assay. Significant differences in invasive ability were found between TU212 cells transfected with miRNA-195 mimics and cells without treatment (p<0.05). No significant differences in invasive ability were found between TU212 cells transfected with miRNA-195 inhibitor and cells without treatment (p>0.05).

Table 1. Relationships between relative expression level of miRNA-195 and clinicopathological features

Clinicopathological features	Expression level of miRNA-195		x ²	р
	Low n (%)	High n (%)	_	
Gender				
Male	65 (65.7)	55 (66.3)	0.284	0.865
Female	34(34.3)	28(33.7)		
Age, years				
<60	44(44.4)	36(43.4)	0.725	0.343
≥60	55(55.6)	47(56.6)		
Clinical staging				
I-II	50(50.5)	80(96.4)	3.152	0.014
III-IV	49(49.5)	3(3.6)		
Grade of differentiation				
Poorly and moderately differentiated	45(45.5)	37(44.6)	0.114	0.634
Well differentiated	54(54.5)	46(55.4)		
Tumor growth site				
Supraglottic type	25(25.3)	25(30.1)	0.497	0.536
Glottic type	50(50.5)	45(54.2)		
Subglottic type	24(24.2)	13(15.7)		
Lymph node metastasis				
Yes	60(60.6)	12(14.5)	7.354	0.009
No	39(39.4)	71(85.5)		

transfected with miRNA-195 inhibitor (p>0.05). miRNA-195 mimics was remarkably lower than

Detection of proliferation of laryngeal carcinoma cells using MTT method

The result of MTT assay showed that at 6, 12, 24, 48 and 72 hrs after transfection, the pro-

sion level between control TU212 cells and cells liferation ability of TU212 cell transfected with that of TU212 cell with no treatment (Figure 2a). There was no distinct difference in the proliferation ability between TU212 cells treated with miRNA-195 inhibitor and cells without treatment (Figure 2b).



Figure 4. Relationship between expression level of miRNA-195 and prognosis of patients with laryngeal carcinoma. A: Difference in 5-year survival rate between high and low miRNA-195 expression groups is statistically significant (p<0.05). B: Difference in median survival time between high and low miRNA-195 expression groups is statistically significant (p<0.05). C: Difference in progression-free survival between high and low miRNA-195 expression groups is statistically significant (p<0.05).

Detection of invasive ability of laryngeal carcinoma cell using Transwell method

The invasive ability of TU212 cell transfected with miRNA-195 mimics was 15.4 ± 2.3 , which was significantly lower than that of cells without treatment (49.7±3.6, p<0.05). There was no significant difference in invasive ability between TU212 cells (50.37±3.5) with no treatment and TU212 cells treated with miRNA-195 inhibitor (Figure 3) (p>0.05).

Relationship between expression level of miRNA-195 and prognosis of patients with laryngeal carcinoma

Survival curves of patients with laryngeal carcinoma were plotted using Kaplan-Meier method. The 5-year survival rate in high miRNA-195 expression group was 78%, significantly higher than that in low expression group (39%, x^2 =6.571, p=0.015) (Figure 4a). The median survival of patients in the high expression of miRNA-195 group was 57 months, significantly longer than that in the low expression group (39 months, x^2 =8.194, p=0.001) (Figure 4b). In addition, progression-free survival of patients in the high expression of miR-NA-195 group was 40 months, was significantly lower than that in the low expression group (27 months, x^2 =3.260, p=0.040) (Figure 4c).

Discussion

Laryngeal carcinoma is one of the most common head and neck tumors, and squamous cell carcinoma (SCC) is the most common type of laryngeal carcinoma. Laryngeal carcinoma accounts for 1-5% of all malignant tumors, ranking third in head and neck tumors. This carcinoma has a significant impact on the physiological and psychological conditions of patients. Most patients with laryngeal carcinoma have a long history of smoking and drinking. At present, laryngeal carcinoma is usually treated with combined therapy, that is, surgery combined with radiotherapy and chemotherapy. However, complications caused by surgery as well as difficulties in laryngeal function reconstruction after surgery lead to high rates of recurrence and metastasis. In order to further improve the survival rate and early diagnosis of laryngeal cancer patients and prevent the genesis of laryngeal carcinoma, it is necessary to understand the molecular mechanism of this disease.

MiRNAs is a group of small non-coding RNAs that can regulate gene expression after transcription. Through the regulation of tumor cell proliferation, metastasis, invasion and apoptosis, different miRNAs act as oncogenes or tumor suppressors

in the pathogenesis of laryngeal carcinoma [8]. MiRNA-195, as a tumor suppressor gene, is downregulated in breast cancer [9], gastric cancer [10], prostate cancer [11], osteosarcoma [12], esophageal cancer [13], etc. In this study, the expression level of miRNA-195 was clearly down-regulated in cancer tissues than in tumor-adjacent normal tissues in 79.67% (145/182) of 182 patients with laryngeal carcinoma. The relative expression level of miR-NA-195 in laryngeal carcinoma tissue group was 0.76±0.38, which was significantly lower than in tumor-adjacent normal tissues (2.69±1.45,p<0.05). In addition, proliferation and invasion of laryngeal carcinoma cells with miRNA-195 overexpression were weakened. Our findings were consistent with previous studies [9,11], further demonstrating that miRNA-195 may inhibit the growth and proliferation of cancer cells in the process of tumor formation.

In this study we found that miRNA-195 expression is correlated with lymph node metastasis and staging, suggesting that the invasion and metastasis of larvngeal carcinoma cells may depend on the down-regulation of miRNA-195. This conclusion was further verified by Transwell assay on laryngeal carcinoma cell invasion. Wang et al. [14] found that miRNA-195 overexpression inhibited proliferation, diffusion and distant invasion of colorectal cancer cells by targeting CAR-MA3 gene. MiRNA-195 inhibited vascularization and metastasis of hepatocellular carcinoma cells by downregulating the expression of vascular endothelial growth factor (VEGF), guanine nucleotide exchange factor 2 (VAV2) and cell division cycle 42 (CDC42) [15]. Singh et al. [16] also showed that miRNA-195 inhibited the proliferation, invasion and metastasis of breast cancer cells by targeting fatty acid synthase (FASN), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), acetyl-CoA carboxylase alpha (ACACA) and 25-hydroxyvitamin D 1-alpha hydroxylase (CYP27B1) genes. Song et al. [17] found that miRNA-195 targeted heparin binding growth factor (HDGF) could inhibit the occurrence and development of cervical cancer. A most recent study [18] revealed that miRNA-195 targeted cullinneddylation 1 domain containing 1 (DCUN1D1) could inhibit the growth and invasion of laryngeal carcinoma cells. More studies are needed to identify more targets of this miRNA.

We found that miRNA-195 expression is closely associated with 5-year survival rate, median survival time and progression-free survival. Qu et al. [19] revealed that miRNA-195 can act on B-cell lymphoma-2 like protein 2 (BCL2L2) mRNA to increase the sensitivity of colorectal cancer cells to doxorubicin, thereby enhancing treatment efficacy. Zhu et al. [20] found that miRNA-195 overexpression in breast cancer cells enhanced the sensitivity to radiotherapy through inhibiting the proliferation of B-cell lymphoma-2 (Bcl-2). Therefore, miRNA-195 may serve as a potential therapeutic target for cancer.

Target cells are obtained directly from frozen or paraffin-embedded tissue sections without destroying the tissue structure. Although this technique is highly accurate, it still requires the pathologist to distinguish between cancer tissue and normal tissue, so errors cannot be avoided. In this experiment, normal tissues were collected from the regions far away from cancer tissue to reduce errors. QRT-PCR was performed in triplicate to determine the difference in expression of miRNA-195 in tumor tissues and adjacent healthy tissues and in HEP-2 cells with and without miRNA-195 mimic. The relationship between relative expression level of miRNA-195 and clinicopathological features of laryngeal cancer patients was analyzed. Different primers were used in the detection miRNA-195

expression in tissues and cells. Different grouping methods were used. Therefore, only intra-group comparisons were performed and comparisons between groups were not performed. In addition, radiochemotherapy used during follow-up was not included in the analysis. We will solve those problems in our future studies. However, we failed to identify the downstream targets of miRNA-195 in laryngeal cancer tissue, which is a limitation of our study.

In summary, the expression of miRNA-195 in laryngeal carcinoma tissue is down-regulated, and the low expression level of miRNA-195 may be related to invasion and metastasis of laryngeal carcinoma, which is related to poor prognosis of patients. MiRNA-195 may serve as a potential molecular target for the treatment and prognosis evaluation of laryngeal carcinoma.

Conflict of interests

The authors declare no conflict of interests.

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