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Expression levels and clinical significance of miR-203 and miR-133b in laryngeal carcinoma

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Abstract. The present study aimed to investigate the expression levels and clinical significance of microRNA (miR)-203 and miR-133b in laryngeal carcinoma. A total of 154 patients with laryngeal carcinoma (research group) along with 100 healthy individuals (control group) were enrolled in the study. The patients were admitted to Yidu Central Hospital of Weifang (Weifang, China) from February 2016 to October 2018. Fasting venous blood (5 ml) was extracted from all subjects to determine the expression levels of serum miR-203 and miR-133b by reverse transcription-quantitative polymerase chain reaction (PCR) and to compare them among patients with different pathological characteristics. Receiver operating characteristic (ROC) curves were plotted to analyze the diagnostic values of miR-203 and miR-133b for laryngeal carcinoma. The research group showed significantly lower expression levels of miR-203 and miR-133b than the control group (P<0.05). According to ROC curve analysis, when the cut-off value was 0.659, the sensitivity and specificity of miR-203 in diagnosing laryngeal carcinoma were 60.00 and 90.26%, respectively, whereas when the cut-off value was 1.398, the sensitivity and specificity of miR-133b were 55.00 and 87.66%, respectively. The sensitivity and specificity of the joint detection were 70.00 and 83.77%, respectively, when the cut-off value was 0.416. In the research group, miR-203 was expressed significantly different in patients with different pathological stages and tumor types (P<0.050). The expression of miR-133b varied significantly in patients with different pathological stages, differentiation degrees and lymph node metastasis (P<0.050). In conclusion, miR-203 and miR-133b were expressed at low levels in patients with laryngeal carcinoma. The expression of miR-203 was related to tumor-node-metastasis (TNM) stage and tumor type, whereas the expression of miR-133b was related to TNM stage, differentiation degree, as well as lymph node metastasis. Joint detection of miR-203 and miR-133b is expected to be an excellent marker for the diagnosis and treatment of laryngeal carcinoma.

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Introduction

Despite the low incidence rate, laryngeal carcinoma, a common respiratory tract tumor (1), poses a threat to life safety and quality of life of patients if not treated in time (2). According to statistics, the incidence of laryngeal cancer is ~6.0% worldwide (3). The main treatments for laryngeal carcinoma include surgery, chemoradiotherapy and other comprehensive treatment measures (4); however, the remission rate is relatively low, with a 5-year overall survival rate of <67% (5). Moreover, patients with laryngeal carcinoma often have no specific clinical manifestations at the early stages (6). Therefore, early detection and timely treatment are the keys to reducing morbidity and mortality (7).

Autofluorescence endoscopy has been reported to have high sensitivity and accuracy for the early diagnosis of laryngeal carcinoma (8). Topuz *et al* (9) have reported that circulating calprotectin was abnormally expressed in patients with laryngeal carcinoma, suggesting that calprotectin can be used as a biomarker and be useful for the early diagnosis of the disease. Screening of molecular biology-related indicators is the most promising direction to improving the diagnosis of laryngeal carcinoma. MicroRNAs (miRs), a class of short non-coding RNAs with a length of ~22 nucleotides, inhibit the translation and transcription of target genes by binding to the 3'untranslated region of their downstream target miRs, thus changing the expression levels of target genes (10). miRs play a regulatory role in various diseases, such as tumors and cardiovascular diseases (11-13). In a previous study, microarray and quantitative polymerase chain reaction (PCR) were employed to examine different miR expression profiles between cancerous and normal adjacent tissues, and it was shown that miR-203 was downregulated in laryngeal carcinoma (14). Saito *et al* (15) examined the miR expression profiles of 723 patients with laryngeal carcinoma by microarray, and the results revealed that miR-133b

Table I. Primer sequences.

Genes	Forward	Reverse
U6	5'-GCTTCGGCAGCACATATACTAAAT-3'	5'-CGCTTCACGAATTTGCGTGTCAT-3'
miR-203	5'-GTATTCGCACTGGATACGACCGACC-3'	5'-TGCGCTAACAGTCTACAGCCA-3'
miR-133b	5'-GACGATCGATGCTAGCTACGTAGCT-3'	5'-CGAGCTAGCTAGCTAGCTAGTCCAG-3'

miR, microRNA.

was significantly downregulated in laryngeal carcinoma. However, there are still few studies on the clinical value of miR-203 and miR-133b in laryngeal cancer. It is speculated that miR-203 and miR-133b may become effective tools for screening and predicting laryngeal cancer.

In the present study, the expression levels of miR-203 and miR-133b were measured in patients with laryngeal carcinoma to explore whether miR-203 and miR-133b can be used as indicators for the clinical diagnosis of laryngeal carcinoma and to provide reference for clinical diagnosis.

Subjects and methods

General data. A total of 154 patients with laryngeal carcinoma admitted to Yidu Central Hospital of Weifang (Weifang, China) from February 2016 to October 2018 were assigned as the research group. There were 98 males and 56 females, 30-64 years of age in the research group. In addition, 100 healthy individuals receiving physical examinations during the same period were assigned as the control group, including 65 males and 35 females, 30-65 years of age. The study was approved by the Ethics Committee of Yidu Central Hospital of Weifang. Signed written informed consents were obtained from the patients and/or guardians.

Inclusion and exclusion criteria. Inclusion criteria: Patients diagnosed with laryngeal carcinoma based on the diagnostic criteria of laryngeal carcinoma; patients receiving treatment in Yidu Central Hospital of Weifang; patients 18-70 years of age; with elementary school education and above; who cooperated with the research; with no other organ serious disease; patients who they or their immediate family members provided a signed informed consent form; patients who had complete medical records. Exclusion criteria: Patients who died during treatment; patients complicated with diseases of the respiratory or blood system, or other infectious diseases; pregnant or lactating women; patients who had recently received immunosuppressants and hormone drugs.

Detection of serum miR-203 and miR-133b. Fasting venous blood (5 ml) was collected from all subjects in the morning, placed in a vacuum tube and subsequently centrifuged at 1,050 x g at 4˚C for 10 min. Total RNA was extracted from serum using a TRIzol® extraction kit (Invitrogen; Thermo Fisher Scientific, Inc.), and concentration and purity were determined by a NanoDrop 2000 ultraviolet spectrophotometer (KeyuXingye Science and Technology Development Co., Ltd.). Total RNA was reversely transcribed into complementary DNA (cDNA) using a reverse transcription kit (Invitrogen; Thermo Fisher Scientific, Inc.), and the temperature protocol was 42˚C for 60 min, 70˚C for 5 min, storage at 4˚C. The primers were designed and synthesized by Shanghai GenePharma Co., Ltd. (Table I). The reaction was carried out on an ABI PRISM 7500 fluorescence quantitative PCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCR amplification conditions were as follows: 90˚C for 5 min, 90˚C for 5 sec, 60˚C for 30 sec, 72˚C for 5 sec, for a total of 40 cycles. Each sample was repeatedly measured 3 times, with U6 as internal reference. The relative expression levels of genes were quantified using the $2^{-\Delta Cq}$ method (16).

Statistical analysis. SPSS 24.0 software (Shanghai Yuchuang Network Technology Co., Ltd.) was used for statistical analysis. GraphPad Prism 8 software (Shenzhen Softhead Software Technology Co., Ltd.) was used to generate the figures and double check the results. The counting data were expressed as percentage (%) and χ^2 test was used for their comparison between groups. The measurement data were expressed as the mean ± standard deviation (SD) and t-test was used for their comparison between groups. One-way ANOVA, followed by LSD post hoc test, was used for the comparison of the measurement data between multiple groups. Receiver operating characteristic (ROC) curves were plotted to evaluate the diagnostic values of miR-203 and miR-133b for laryngeal carcinoma. P<0.050 was considered to indicate a statistically significant difference.

Results

General data comparison. There was no significant difference between the two groups in terms of age, sex, body mass index (BMI), medical history and marital status (P>0.050). However, the levels of carcinoembryonic antigen (CEA), squamous cell carcinoma antigen (SCC-Ag) and adenosine kinase (AK) were significantly different between the two groups (P<0.050). Details are shown in Table II.

Serum miR-203 and miR-133b expression levels in the two groups. The expression of miR-203 in the research group was significantly lower than that in the control group (0.43±0.20 vs. 0.85±0.29; P<0.05) (Fig. 1A). In addition, the expression of miR-133b in the research group was significantly lower than that in the control group (0.84±0.51 vs. 1.38±1.07; P<0.05) (Fig. 1B).

Table II. Comparison of patients' general data [mean ± SD, n (%)].

Characteristics	Research group (n=154)	Control group (n=100)	χ^2 or t	P-value
Age (years)	48.25±5.39	48.71±6.67	0.604	0.546
Sex			0.049	0.824
Male	98 (63.64)	65 (65.00)		
Female	56 (36.36)	35 (35.00)		
BMI (kg/m^2)	23.71±1.61	23.64±1.73	0.328	0.742
Medical history				
Hypertension	41 (26.62)	24 (24.00)	0.219	0.639
Diabetes	31 (20.13)	18 (18.00)	0.176	0.674
High blood lipid	27 (17.53)	17 (17.00)	0.012	0.912
Marital status			1.208	0.271
Married	116 (75.32)	81 (81.00)		
Unmarried	38 (24.68)	19 (19.00)		
CEA (ng/ml)	3.48±0.32	1.18±1.39	13.291	<0.001
SCC-Ag level	9.13±5.18	0.78±0.34	16.090	<0.001
AK	0.34±0.08	1.14±0.16	52.750	<0.001

BMI, body mass index; CEA, carcinoembryonic antigen; SCC-Ag, squamous cell carcinoma antigen; AK, adenosine kinase.

Table III. Association of miR-203 expression with clinicopathological characteristics [mean ± SD, n (%)].

Characteristics	Cases (n=154)	miR-203 relative expression	t or F	P-value
Age, years			0.300	0.764
≤50	74 (48.05)	0.42±0.19		
>50	80 (51.95)	0.43±0.22		
Sex			0.260	0.795
Male	98 (63.64)	0.42±0.24		
Female	56 (36.36)	0.43±0.21		
Tumor type			0.0065	0.006
Supraglottic carcinoma	33 (21.43)	0.41±0.17		
Glottic carcinoma	29 (18.83)	0.40±0.23		
Subglottic carcinoma	38 (24.68)	0.54±0.18		
Transglottic carcinoma	54 (35.06)	0.41±0.21		
Degree of differentiation			0.552	0.583
Highly differentiated	81 (52.60)	0.44±0.23		
Moderately and poorly differentiated	73 (47.40)	0.42±0.22		
Lymph node metastasis			0.603	0.547
Yes	68 (44.16)	0.41±0.21		
No	86 (55.84)	0.43±0.20		
TNM stage			3.123	<0.02
I-II	89 (57.79)	0.47±0.22		
III-IV	65 (42.20)	0.36±0.21		

miR, microRNA; TNM, tumor-node-metastasis.

Association of miR-203 and miR-133b expression levels with clinicopathological characteristics. The analysis of clinicopathological data and miR-203 and miR-133b expression levels showed that miR-203 expression was related to tumor-node-metastasis (TNM) stages and tumor types (P<0.050; Table III). However, the expression of

Table IV. Association of miR-133b expression with clinicopathological characteristics [mean ± SD, n (%)].

Characteristics	Cases (n=154)	miR-203 relative expression	t or F	P-value
Age, years			0.419	0.675
≤50	74 (48.05)	0.86±0.40		
>50	80 (51.95)	0.83±0.48		
Sex			0.243	0.808
Male	98 (63.64)	0.86±0.48		
Female	56 (36.36)	0.84±0.51		
Tumor type			0.055	0.983
Supraglottic carcinoma	33 (21.43)	0.84±0.57		
Glottic carcinoma	29 (18.83)	0.83±0.46		
Subglottic carcinoma	38 (24.68)	0.81±0.51		
Transglottic carcinoma	54 (35.06)	0.85±0.39		
Degree of differentiation			3.409	<0.001
Highly differentiated	81 (52.60)	0.96±0.44		
Moderately and poorly differentiated	73 (47.40)	0.71±0.47		
Lymph node metastasis			3.623	<0.001
Yes	68 (44.16)	0.68±0.47		
No	86 (55.84)	0.98±0.54		
TNM stage			2.632	0.009
I-II	89 (57.79)	0.93±0.47		
III-IV	65 (42.20)	0.73±0.46		

miR, microRNA; TNM, tumor-node-metastasis.

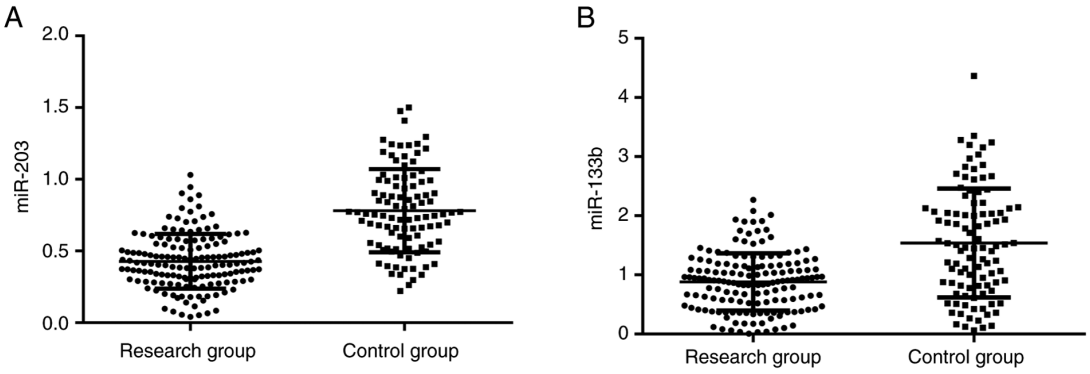

Figure 1. Comparison of serum miR-203 and miR-133b expression levels between the two groups. The expression levels of (A) miR-203 and (B) miR-133b in the research group were significantly lower than those in the control group (P<0.05). miR, microRNA.

miR-133b varied in patients with different pathological stages, differentiation degrees and lymph node metastasis (P<0.050; Table IV).

Diagnostic efficacy of miR-203, miR-133b and their combination in laryngeal carcinoma. ROC curve analysis showed that the area under curve (AUC) of serum miR-203 was 0.788 [95% confidence interval (CI): 0.728-0.848, P<0.001] and when the cut-off value was 0.659 the sensitivity and specificity were 60.00 and 90.26%, respectively. The AUC of serum miR-133b was 0.712 (95% CI: 0.642-0.782, P<0.001) and when the cut-off value was 1.398 the sensitivity and specificity were 55.00 and 87.66%, respectively. In addition, when the cut-off value was 0.416 the sensitivity and specificity of the joint detection were 70.00 and 83.77%, respectively. Details are shown in Fig. 2 and Table V.

Discussion

Although great progress has been achieved in the treatment of laryngeal carcinoma with surgery, radiotherapy and chemotherapy (17), the long-term prognosis of patients is still unsatisfactory due to various factors (18). Seeking tumor markers with high sensitivity and accuracy has become a

Table V. ROC curve analysis.

Index	miR-203	miR-133b	Joint detection
AUC	0.788	0.712	0.838
95% CI	0.728-0.848	0.642-0.782	0.787-0.890
Cut-off	0.659	1.398	0.416
Sensitivity (%)	60.00	55.00	70.00
Specificity (%)	90.26	87.66	83.77
P-value	<0.001	<0.001	0.001

andROC, receiver operating characteristic; miR, microRNA; AUC, area under curve; 95% CI, 95% confidence interval.

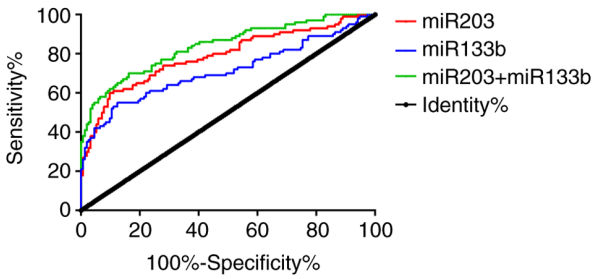

Figure 2. ROC curves of serum miR-203 and miR-133b in diagnosing laryngeal carcinoma. ROC curve analysis of serum miR-203 revealed that, when the cut-off value was 0.659, the sensitivity and specificity were 60.00 and 90.26%, respectively. When the cut-off value was 1.398, the sensitivity and specificity of serum miR-133b were 55.00 and 87.66%, respectively. In addition, when the cut-off value was 0.416, the sensitivity and specificity of the joint detection were 70.00 and 83.77%, respectively. miR, microRNA; ROC, receiver operating characteristic; miR, microRNA.

research hotspot (19,20). In the present study, the expression levels of miR-203 and miR-133b were detected to explore their potential role as indicators for the diagnosis of laryngeal carcinoma.

The results of the present study revealed that the expression levels of serum miR-203 and miR-133b in the research group were significantly lower than those in the control group, in agreement with the relevant literature results (21,22), suggesting that miR-203 and miR-133b may have key biological functions in the occurrence and progression of laryngeal carcinoma. Subsequently, the association of miR-203 and miR-133b expression levels with the patients' clinicopathological data in the research group was analyzed. The results showed that miR-203 expression was related to TNM stage and tumor type, but not lymph node metastasis, indicating that miR-203 might play an antitumor role in early laryngeal carcinoma. In addition, the expression of miR-133b was shown to vary significantly in patients with different pathological stages, differentiation degrees and lymph node metastasis. Tian *et al* (23) have reported that miR-203 was downregulated in laryngeal squamous cell carcinoma and was closely related to poor differentiation, advanced clinical stage (III-IV), tumor stages (T3-4), lymph node metastasis and 5-year overall survival reduction. These results are contrary to our findings, i.e., that miR-203 has no association with lymph node metastasis in laryngeal carcinoma, suggesting that miR-203, like many other miRs, might have a biphasic effect on human cancers and act as an oncogene or tumor suppressor depending on the cellular environment of the tumor. Finally, in the present study, ROC curve analysis showed that the AUC of miR-203 and miR-133b was 0.788 and 0.712, respectively, indicating that both miRs are valuable in the diagnosis of laryngeal carcinoma and could be useful screening and prediction tools for this disease. There have been numerous studies on the diagnostic value of single serum markers in laryngeal carcinoma, pointing out that single marker detection tends to cause missed diagnosis and misdiagnosis, as well as delay in treatment, whereas the joint detection has a better diagnostic effect (24-26). Therefore, the ROC curve of the joint detection of miR-203 and miR-133b was also plotted in the present study, and it was shown that the AUC and cut-off value were 0.838 and 0.416, respectively, whereas the sensitivity and specificity for diagnosing laryngeal carcinoma were 70.00 and 83.77% respectively, indicating better effectiveness compared with that of the single detection. Thus, the joint detection of miR-203 and miR-133b could be useful for the diagnosis of laryngeal carcinoma. In the past, laryngeal cancer blood markers, such as CEA and SCC-Ag, have been commonly used in clinical practice. Because of the significant degree of response to tumor lesions and injuries, these markers have in general extremely high sensitivity and low specificity. In clinic, the detection of the aforementioned markers can be used to determine whether the patient has a tumor; however it is not ruled out that some highly specific diseases could also cause the rise of CEA. Therefore, to determine the patient's disease, follow-up inspections are still needed. Compared with conventional laryngeal cancer tumor markers, the advantages of miR-203 and miR-133b detection in peripheral blood are as follows: i) The detection is convenient and the cycle is short. Only peripheral blood is needed. ii) The test results are intuitive and the results do not need to be interpreted compared with imaging techniques. iii) The sample is easy to maintain, which can be conducive to the long-term treatment of patients. iv) In addition to the significant sensitivity and the excellent specificity, it can effectively assist doctors to quickly and effectively judge whether the patient has laryngeal cancer. Previous studies have shown that traditional tumor diagnostic markers, such as CEA and SCC-Ag, are highly sensitive to the occurrence of laryngeal cancer, although the specificity is low (27,28). Studies have also shown that CEA and SCC-Ag levels increase during the occurrence of uremia and inflammatory reactions (29,30). The combined detection of miR-203 and miR-133b has better specificity, is more effective and accurate in identifying laryngeal cancer and more conducive to early clinical screening, improving the early detection rate of laryngeal cancer and prognosis.

Previous studies have reported that miR-203 and miR-133b are effective in the prognosis of patients with osteosarcoma and bladder cancer (31,32). In the present study, due to the short experimental period, the patients were not followed up and whether miR-203 and miR-133b have an impact on the prognosis of patients remains unknown. In addition, due to the lack of support from basic experiments, the specific impact mechanism of miR-203 and miR-133b on laryngeal cancer is not clear yet. The extended experimental time, the expanded sample size and *in vitro* experiments will be included in

our future study to further explore the effects of miR-203 and miR-133b on laryngeal cancer and provide reference for clinical practice.

In conclusion, miR-203 and miR-133b were expressed at low levels in patients with laryngeal carcinoma. The expression of miR-203 was related to TNM stage and tumor type, whereas the expression of miR-133b was related to TNM stage, differentiation degree and lymph node metastasis. The joint detection of miR-203 and miR-133b is expected to be an excellent marker for the diagnosis and treatment of laryngeal carcinoma.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

NZ conceived and designed the study, and drafted the manuscript. HL acquired, analyzed and interpreted the experimental data. AZ and MW performed serum miR-203 and miR-133b detection. NZ wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Yidu Central Hospital of Weifang (Weifang, China). Signed written informed consents were obtained from the patients and/or guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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