

Expression of serum microRNA-378 and its clinical significance in laryngeal squamous cell carcinoma

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Abstract. – **OBJECTIVE:** Studies have demonstrated that miRNA -378 expressed in various malignant tumors. In the present study, we aim to explore the expression of serum miRNA-378 and its clinical significance in laryngeal squamous cell carcinoma (LSCC) patients.

PATIENTS AND METHODS: A total of 127 LSCC patients, 127 vocal cord polyp (VCP) and 130 healthy controls were selected.

RESULTS: The miRNA-378 level in LSCC and VCP group was significantly higher compared to healthy control, and LSCC group has the highest miRNA-378 level. The miRNA-378 level was both decreased significantly in LSCC and VCP group when compared with the same group after surgery. When compared with healthy control, VCP group has a higher miRNA-378 level but has no statistical difference ($p>0.05$) while LSCC group has a statistical difference ($p<0.05$) higher miRNA-378 compare with healthy control. The miRNA-378 expression is correlated with clinical stage and differentiation degree, but did not correlate with patient's age, gender distribution, operation mode, and tumor diameter. The AUC of miRNA-378 was 0.888, 95% confidence interval was 0.849 to 0.929, and AUC hypothesis testing was statistically significant ($p<0.001$).

CONCLUSIONS: miRNA-378 could be used in the diagnosis and the prediction of the postoperative curative effect of laryngeal cancer (LSCC).

Key Words:

Laryngeal squamous cell carcinoma (LSCC), MicroRNA-378, Diagnosis, AUC.

Introduction

Head and neck squamous cell carcinoma is the sixth most common cancer being reported globally, with a mortality rate of about 50%¹. Around 600,000 new cases are being reported each year in the global scenario¹. Of all the head and neck squamous cell carcinomas, nearly 25% of the cas-

es are reported to have laryngeal squamous cell carcinoma (LSCC)². LSCC survival outcomes have not improved in the last three decades in the majority of patients, though the reports indicate that the 5-year survival rate was about 60%³. The key reason for the poor survival outcome is a low rate of diagnosis⁴. LSCC progression undergoes different phases: dysplasia (i.e. mild dysplasia, moderate dysplasia, and severe dysplasia), cancer *in situ* (CIS) and LSCC⁵. Novel biomarkers to identify the early LSCC stages, or specific biomarkers for different individuals, are in urgent need for detecting LSCC in the early stages, which will pave a way to develop individualized therapies. MicroRNAs (miRNAs) role as potential biomarkers and targets for therapy was widely reported in different types of cancer⁶⁻⁸.

miRNA is a non-encoding small RNA molecule which has a loop-stem structure. These miRNAs are known to regulate cell growth and differentiation, which in-turn plays important roles in the life process and the development of diseases⁹⁻¹¹. Studies¹²⁻¹⁴ indicated that miRNA is closely related to the occurrence and development of tumors. Previously reported investigations have shown the miRNA role in tumor regulation in wide varieties of cancers like breast cancer, colon cancer, lung cancer, and prostate cancer. In addition, miRNA also play a key role in LSCC pathogenesis^{15,16}. Wulfken et al¹⁷ indicated that circulating miR-155 could serve as a potential biomarker for LSCC patients. miRNA expression profiling has indicated several specific miRNAs in LSCC, which mainly act by creating a balance between cancer gene and tumor suppressor gene^{18,19}.

Studies²⁰⁻²² have shown that miRNA-378 expressed in various malignant tumors, has a role in the survival, migration, invasion, angiogenesis and growth of tumor cells. Few reports have mentioned the relationship between miRNA-378 and LSCC.

In this study, we detected the expression of miRNA-378 in LSCC patients from northern China, aiming to understand clearly the role of miRNA-378 in LSCC, and investigate the potential diagnosis feasibility of miRNA-378 for LSCC.

Patients and Methods

Patients

The procedures performed in patients were in line with the ethical standards of Department of Otorhinolaryngology, Tianjin Medical University General Hospital, and also in line with the Helsinki Declaration and its later amendments or comparable ethical standards. A signed written informed consent form was received from all the subjects in the study.

In this study, a total of 3 group's patients were recruited: primary LSCC patients; vocal cord polyp (VCP) patients and healthy controls. The LSCC and VCP patients were all recruited from the hospital clinic, they received surgical resection at our department, and their resected tissues were confirmed by pathological examination. Healthy controls were recruited from health adults in the outpatient physical examination. The age of all recruited subjects was limited from 18 to 68.

Blood Sample Collection

For LSCC and VCP patients, their blood samples were collected at 2-time points: before surgery and 6 months after the surgery. The fasting serum samples of healthy controls were collected during their physical examination. Blood samples (3 ml) were collected in SSTII advance tubes (Becton Dickinson, Plymouth, UK) and preserved in 4°C refrigerator for 1 hour. The blood samples were centrifuged 3 min at 500 R/min, the upper level of serum was moved to the 1.5 ml EP without RNase. Phenol/chloroform were used to deproteinization; ethanol was used to precipitate RNA, DEPC water was added to dilute these RNA. RNA preparation quality was assessed by electrophoresis using denaturing agarose gel, and purity of extracted RNA was tested by ultraviolet spectrophotometer. The OD260/OD280 ratio value should be greater than 1.8.

The CT or ultrasonography and (or) narrow band imaging (NBI) endoscopy examination were performed to assess tumor recurrence in postoperative LSCC patients after surgery. The imaging or endoscopic examination indicated recurrence of primary tumor site, regional or distant lymph

node metastasis could be considered as recurrence after tumor resection. The follow-up of this study was ended in April 2016.

RT-PCR for miRNA-378

The serum total microRNA was extracted by the microRNA Extraction Kit (miRNeasy Mini Kit, Qiagen, Hamburg, Germany). To measure microRNA-378 level, reverse transcription polymerase chain reaction (RT-PCR) method was employed. The total volume of RT-PCR reaction system (TIANGEN Biotech, Beijing, China) was 20 µl: Reaction system containing 2× microRNA premix reagent 10 µl, self-contained primer 0.4 µl, RT reaction product 2 µl, reverse primer 0.4 µl, and 7.2 µl of DEPC water. SYBR Green I was used as a probe. For quantitative measurement, the CFX96 Touch™ Real-Time PCR Detection System (BioRad, Hercules, CA, USA) was used with the following conditions: 95°C for 10 min, 95°C for 15 s, 60°C for 1 min (40 cycles). The relative expression of microRNA-378 was calculated by methods mentioned by Schmittgen²³: $F = 2^{-\Delta\text{ct}}$, $\Delta\text{ct} = \text{ctmiRNA-378} - \text{ctmiRNA-U6}$. CT indicates the number of cycles experienced by the fluorescent signals reaching the threshold inside the reactor. MIR378 (MIMAT0000731) miRNA qPCR Primer Pairs was purchased from OriGene Technologies, Inc. (Rockville, MD, USA), and U6 primers were synthesized by life technology (Shanghai, China). The U6 RT-PCR primers were following: RT 5'CGCTTCACGAATTTGCGTGTTCAT3'U, and Forward 5'GCTTCGGCAGCACATATACTAAAT3'

Statistical Analysis

The miRNA-378 relative expression level was expressed in means with standard deviations. The data were analyzed by SPSS 17 software (IBM, Chicago, IL, USA) by Wilcoxon rank sum test and H Kruskal-Wallis rank sum test. The sensitivity and specificity of miRNA-378 were calculated by ROC curve (AUC) based on the data collected from healthy control and LSCC patients. Graphpad Prism 5.0 software (GraphPad Software, San Diego, CA, USA) was used for ROC curve analysis. $p < 0.05$ was considered as statistically significant.

Results

Demographic Data

From April 2011 to December 2014, a total of 133 cases of LSCC patients and 127 cases

Table I. Demographic data of selected subjects.

	Healthy	LSCC	VCP
Case number	130	127	127
Age	62.07±3.21	59.72±2.96	61.03±3.67
Gender (M/F)	73/57	78/49	72/55
Tumor diameter (mm)	--	2.7±0.8	
Follow-up period (month)	--	38.6±16.2	1.5±1.2

of VCP were selected successfully; also, blood samples from 130 healthy individuals as control were collected successfully. The demographic data of these patients were listed in Table I. Six patients lost follow-up. The follow-up time of LSCC patients ranged from 8 to 61 months with average follow-up time of 38.6 ± 16.2 months; a total of 18 patients occurred in recurrence up to the follow-up deadline, 8 of them relapsed within 1 year after surgery; 4 of them relapsed within 1 to 3 year after surgery; and 6 of them relapsed within 3 to 5 years.

miRNA-378 Expression Before Surgery

We firstly compared the miRNA-378 expressions in healthy control, VCP and LSCC patients. As seen in Figure 1, the expression of miRNA-378 was significantly different between the 3 groups, the LSCC group have the highest miR-

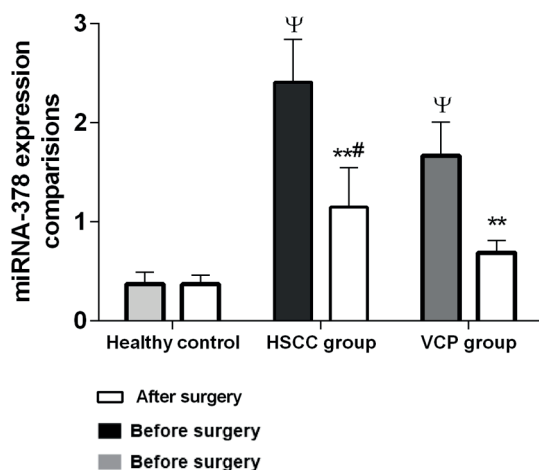


Figure 1. Comparison of miRNA-378 expressions among healthy control, VCP and LSCC patients before surgery and 6 months after surgery. ** $p < 0.01$, compared with before surgery in the same groups; # $p < 0.05$, compared with VCP group post surgery. $\Psi p < 0.01$, compared with healthy subjects.

NA-378 level (2.41 ± 1.43) when compared with VCP (1.67 ± 1.46) and healthy control (0.37 ± 0.22 , $p < 0.05$); and the miRNA-378 level in VCP group was obviously higher ($p < 0.01$) than healthy control. These results suggest that high expression of miRNA-378 may be associated with laryngeal lesions.

miRNA-378 Expression After Surgery

Six months after surgery, the expressed miRNA-378 in LSCC and VCP group decreased significantly when compared with the same groups before the surgery ($p < 0.05$). The expressed miRNA-378 decreased 53% in LSCC group and 59% in VCP group 6 months after surgery, but still higher than healthy control. When compared with healthy control, VCP group has a higher level but not statistically different ($p > 0.05$) while LSCC still had a higher level with statistical difference ($p < 0.05$) (Figure 1).

miRNA-378 Expression is Correlated with Clinical Stage and Differentiation Degree

We further studied the relationship between miRNA-378 expression level and clinical pathological parameters in LSCC ($p < 0.05$, Table II), results showed the miRNA-378 expression is correlated with clinical stage and differentiation degree, patients with higher clinical stage and low differentiated degrees have much higher expressed miRNA-378 (3.73 ± 1.44 vs. 1.62 ± 1.16 in stage IV vs. stage I; 3.98 ± 1.66 vs. 1.51 ± 1.18 in low differentiation vs high differentiation degree), but not correlated with patient's age (≤ 50 vs. > 50), gender distribution, operation mode (Partial nephrectomy vs. Radical nephrectomy) and tumor diameter (≤ 2 mm vs. > 2 mm).

miRNA-378 in Relapsed Patients:

According to the image or endoscopic examination results, we compared miRNA-378 expressions in patients with tumor recurrence and patients without tumor recurrence (blood sample of LSCC patients 6 months after the surgery). Results showed that the patients with tumor recurrence had significantly higher miRNA-378 level than patients without tumor recurrence (3.86 ± 2.16 vs. 1.95 ± 1.03 , $Z = 26.26$, $p = 0.000$).

Diagnostic Efficiency of Serum miRNA-378 in LSCC:

We used miRNA-378 as a biological indicator for LSCC patients and healthy people. As a sensitivity curve, the higher the ROC curve closer

Table II. The relationship between miRNA-378 expression level and clinical pathological parameters in LSCC patients (n=127).

Pathological parameter	Cases	miRNA-378 (Mean±SD)	t-value	t-value
Age (yr)				
≤50	57	2.213±1.38	-1.067	0.135
>50	70	2.56±1.22		
Gender				
Male	78	2.38±1.08	-0.324	0.747
Female	49	2.45±1.34		
Clinical stages				
I	46	1.62±1.16	13.268 (F)	0.000
II	35	2.34±1.24		
III	28	2.94±1.52		
IV	18	3.73±1.44		
Operation mode				
Partial laryngectomy	89	2.38±1.24	-0.317	0.751
Radical laryngectomy	38	2.47±1.89		
Differentiation degree				
High	41	1.51±1.18	35.22 (F)	0.001
Middle	63	2.42±1.24		
Low	23	3.98±1.66		
Tumor diameter (mm)				
≤2	72	2.23±1.15	-1.909	0.058
>2	55	2.64±1.26		

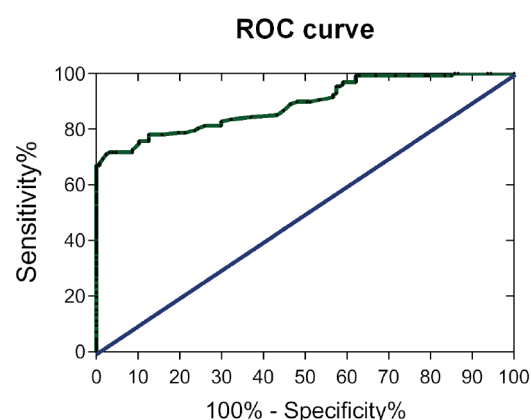
to the upper left corner, is higher the accuracy of the tested methods; when AUC value is between 0.7 to 0.9, it indicates normal accuracy; and when AUC is more than 0.9, it indicates higher accuracy in the tested methods. The AUC of miRNA-378 was 0.888 with a standard error of 0.0001; 95% confidence interval of ROC curve was 0.849 to 0.929, and AUC hypothesis testing was statistically significant ($p<0.001$, Figure 2).

Discussion

The miRNA gene accounts for about 1% of the whole genome, controlled by the complementary pair of target RNA gene; miRNA resulted in the degradation of mRNA and the inhibition of translation. miRNA and its target mRNA molecules constitute a complex regulatory network involving wide varieties of biological processes like apoptosis, cell proliferation, cell differentiation, development, stress response and other biological activity²⁴.

miR-378 is highly conserved miRNA which is expressed in a variety of tumors, especially in liver cancer²⁵, non-small-cell lung cancer²⁶, and colorectal cancer²⁷. The abnormal expression of miRNA-378 could involve in the development of tumor and its expression may be used as a marker of clinical diagnosis, pathological clas-

sification, and prognosis. Wang et al²⁸ used gene chips to screen 8 cases of laryngeal cancer tissue and its adjacent normal tissue. They identified 47 different miRNAs and the candidate miRNA was verified by RT-qPCR, and concluded that the miRNA-378 is highly expressed in laryngeal carcinoma and it might serve as a potential biomarker for early detection of laryngeal carcinoma. Lee et al²¹ reported that miRNA-378 could inhibit the expression of SuFu and Fus-1, two tumor suppressor genes, and promote cell survival, tumor growth, and angiogenesis.

**Figure 2.** The AUC of diagnostic efficiency of serum miRNA-378 in LSCC patients.

Our research revealed the expression of miRNA-378 in LSCC was higher than that in VCP group and healthy control, the relative expression of miRNA-378 in serum can effectively distinguish LSCC patients and normal population, our result is consistent with Renova's report. In addition, our research demonstrated that the expression of miRNA-378 was correlated with the tumor-differentiation degree and clinical stage, but not correlated with patients' age, and gender. In this study, the difference of hypothesis testing in AUC was statistically significant, which indicates that miRNA-378 can effectively distinguish benign and malignant lesions of the larynx. Our study showed that the relative expression of serum miRNA-378 in LSCC group was significantly decreased at 6 months after LSCC surgery, and similar condition was noticed as well as VCP group, which means miRNA-378 could be used as a biological indicator for the curative effect of lesions in the larynx.

The blood sample has the characters of easy to obtain, noninvasive, repeatable, and the serum miRNA has good stability, it is not easy to be affected by temperature, and pH value. Therefore, miRNA-378 can be used as a convenient biological indicator for the diagnosis of LSCC. However, it is not clear whether the high expression of miRNA-378 in plasma is consistent with expression in cancer tissues for LSCC patients. Previous articles have proved that the expression of miRNA is matched in plasma and LSCC tumors, such as MicroRNA-155¹⁷ and seven micro-RNAs²⁹. Li et al³⁰ summarize microRNAs in laryngeal cancer and their implications for diagnosis, prognosis and therapy, some of them shows promising potential for the diagnostic accuracy. Wu et al⁸ also reported that combination of miR-148a and miR-375 serum levels enables the sensitive detection of LSCC.

Conclusions

Our work also has certain limitations: we did not compare the miRNA-378 level in serum and LSCC (VCP) tissue, with a still limited case number; we did not perform correlation analysis. But our research provides useful data in the diagnosis of LSCC. At present, the mechanism of miRNA-378 in the occurrence and development of LSCC is not clearly elicited. A larger population was needed to elicit the role of miRNA-378 in the diagnosis of LSCC, targeting therapy, curative effect evaluation, and prognosis.

Conflict of interest

The authors declare no conflicts of interest.

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