

The Expression of MicroRNA-155 in Plasma and Tissue Is Matched in Human Laryngeal Squamous Cell Carcinoma

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Purpose: Tumor-associated microRNAs have been detected in cancer, though whether plasma microRNA-155 (miR-155) could be a potential biomarker for laryngeal squamous cell carcinoma (LSCC) prognosis is unclear. We aimed to determine how miR-155 can be used to predict the clinical characteristics of patients with LSCC and correctly diagnose them.

Materials and Methods: We collected tissue samples and peripheral blood samples before and after treatment from 280 LSCC cases and 560 controls. Real-time quantitative reverse transcription PCR was employed in this study to compare the relative expression of miR-155.

Results: A total of 280 LSCC patients and 560 age- and sex-matched controls were included in the study. The miR-155 level was more up-regulated in LSCC tissue than in the non-tumor tissues (13.6 ± 2.4 vs. 3.1 ± 0.80 , $p < 0.001$). Additionally, a significantly higher miR-155 level in plasma samples from LSCC patients than in those of the controls (8.9 ± 1.25 vs. 1.8 ± 0.8 , $p < 0.001$) was reported. Tissue miR-155 showed an area under the curve (AUC) of 0.933, with a sensitivity of 82.6% and a specificity of 89.2%. The AUC for plasma miR-155 was 0.757, with a sensitivity of 58.4% and a specificity of 69.5%. When early LSCC in TNM I stage was considered, tissue miR-155 showed an area under the curve of 0.804, with a sensitivity of 85.2% and a specificity of 87.3%.

Conclusion: The expression of tissue and plasma miR-155 were significantly up-regulated in patients with LSCC. Our work will serve as a basis for further investigation, preferably large-scale validation in clinical trials.

Key Words: MiR-155, laryngeal squamous cell carcinoma, diagnosis, biomarker

INTRODUCTION

Laryngeal cancer is one of the most common head and neck human cancers (excluding skin cancer) and continues to be a major unsolved health problem.¹ Laryngeal squamous cell carcinoma (LSCC) is the most common histological type of laryn-

geal malignancy.² It composed the most part of laryngeal cancers. Most patients of LSCC were diagnosed firstly between 50 to 75 years of age. The male-to-female sex ratio of laryngeal cancer was from 6:1 to 10:1 in most cases.³ Current methods for the diagnosis of early-stage LSCC were quite unsatisfactory.⁴ Nowadays, only less than 50% of patients with LSCC were in early stage at diagnosis, due to the lack of effective early-diagnosis methods.^{5,6} As effective biomarker is the key of early diagnosis of cancer and therefore the identification of new biomarkers for LSCC is in severe need.

MicroRNAs (miRNAs), an abundant class of 17–25 nucleotides that comprise small noncoding RNAs, post-transcriptionally regulate gene expression by directly binding to the 3' untranslated region (3' UTR) of target miRNAs.⁷ Until now, over 1000 miRNA genes have been identified in mammals; however, revealing their roles in physiology and pathology remains an ongoing process. Recently, it has been suggested that miRNAs

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participate in the regulation of diverse biological processes. Bioinformatics data have indicated that a single miRNA can bind to hundreds of miRNA targets, and these targets could theoretically regulate every biological process.⁸ Each miRNA, though powerful, has also been characterized as being marginal (a given gene can be targeted by several miRNAs, and a given miRNA typically exerts a modest repression).⁹ One miRNA, microRNA-155 (miR-155) is known to regulate multiple aspects of types of cancer progression in addition to demonstrating a correlation with poor prognosis.^{10,11} Located within the noncoding B cell integration cluster (BIC) gene, miR-155 was first discovered through retroviral integrations in B cell lymphomas.¹² Currently miR-155 is found to be amplified in many cancers, irrespective of retroviral integration. As one of the more highly evaluated miRNAs, miR-155 is an established oncogenic miRNA, and many of its targets are known.¹³⁻¹⁵ MiR-155 is known to regulate cellular proliferation and survival through multiple mechanisms, including the mitogen activated protein kinases (MAPKs) signaling pathways. MiR-155 is a positive multi-faceted regulator of MAPK signaling, intervening at heterogeneous points along the signaling cascade to enhance signaling. Through the targeting of inositol polyphosphate-5-phosphatase (SHIP1), miR-155 enhances extracellular signal-regulated kinases (ERK) activation. In a cancer cell line, miR-155 expression enhanced ERK phosphorylation and cellular proliferation; conversely, the loss of miR-155 expression led to decreased ERK activation in B-cells.¹⁶ Downstream MAPK effectors, such as AP-1 complex member c-Jun, are enhanced through miR-155 targeting of DET1. Targeting of DET1 stabilizes c-Jun transcripts and subsequently increases AP-1 activity.¹⁷

Recently, it has been reported that plasma contains sufficiently stable miRNA species that might be useful as non-invasive biomarkers for several cancers, including LSCC.^{18,19} Thus, the aim of the present study was to evaluate the expression of miR-155 as a potential biomarker for LSCC. However, the associations of plasma with tissue miR-155 levels are not yet clear. In this study, we investigated plasma and tissue miR-155 expression in a group of patients with LSCC and controls matched according to age and sex.

MATERIALS AND METHODS

Study designs and participants

We collected tissue samples and peripheral blood samples before and after treatment from LSCC cases and controls. In total, 280 LSCC patients and 560 controls were included in this study. The clinicopathological characteristics (age, gender, smoking status, tumor size, differentiation status, lymph node metastasis, stage classification) of study subjects were recorded by trained investigators. Primary LSCC and adjacent noncancerous tissues were procured from patients undergoing surgical resections. All tissues were snap-frozen immediately after sur-

gery and stored at -80°C until use. Three milliliters of whole blood were collected in an Ethylenediaminetetraacetic Acid Vacutainer (BD Company, San Diego, CA, USA) for plasma. Sample collections were centrifuged at 2500 rpm at 4°C for 10 min. The supernatant fluids were then stored at -80°C until total RNA extraction. This study was approved by the Institutional Review Board (IRB) of the General Hospital of Tianjin Medical University, Tianjin, China.

Plasma and tissue sampling and RNA isolation

Cell-free plasma was isolated via a two-step protocol (2500 rpm at room temperature for 10 min and 14000×g at 4°C for 10 min) within 2 h after collection to prevent contamination of the cellular nucleic acids. The resulting plasma was transferred to new tubes and stored at -80°C. The plasma was transferred to RNase/DNase-free tubes and stored at -80°C until RNA isolation. The total RNA was isolated from the plasma using a mirVana PARIS isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions for plasma samples without enrichment for small RNAs. Briefly, 400 µL of plasma was used to extract the total RNA. Each sample was eluted in 40 µL of RNase-free water. Cancer and non-cancer tissues from LSCC cases were immediately flash frozen in liquid nitrogen, and stored at -80°C until RNA isolation using a mirVana PARIS isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions, which were similar to those described above. The average levels of miR-155 expression in tissues and sera were normalized relative to the average amounts of U6 snRNAm, using the $2^{-\Delta\Delta CT}$ method. RNA quality was measured using a denaturing 15% polyacrylamide gel.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The reverse transcription reaction was carried out using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, America) according to the manufacturer's instructions in a total reaction volume of 7.5 mL. Quantitative polymerase chain reaction (qPCR) was performed in triplicate using TaqMan Universal PCR Master Mix (Applied Biosystems) in an ABI 7500 Real-Time PCR system (Applied Biosystems) with the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The cycle threshold (Ct) values were calculated using SDS 2.0.1 software (Applied Biosystems). Template-free controls for both RT and PCR were included in each experiment to ensure target-specific amplification. In ad-

Table 1. Primers Used in qPCR

MiRNAs		Primer sequence (5'-3')
MiR-155	Forward	TGCCTCCAACCTGACTCCTAC
	Reverse	GCGAGCACAGAATAATACGTA
U6	Forward	GCTTCGGCAGCACATATACTAAAAT
	Reverse	CGCTTCACGAATTTGCGTGCAT

qPCR, quantitative polymerase chain reaction; miR-155, microRNA-155.

Table 2. Clinicopathological Features of 280 LSCC Patients and 560 Controls

Clinical features	Cases	Controls	p value
Mean age (yrs)	64.8±12.1	65.1±9.8	0.162
<60	194	326	0.341
≥60	86	234	
Gender			0.541
Male	245	488	
Female	35	72	
Smoking			<0.001
Yes	212	311	
No	68	249	
Tumor size			
≤3 cm	243		
>3 cm	37		
Differentiation			
Moderate–well	197		
Poor	83		
Lymph node metastasis			
Negative	167		
Positive	113		
Stage classification			
Stage I	69		
Stages II, III, and IV	211		

LSCC, laryngeal squamous cell carcinoma.

dition, if Ct_{U6} did not occur within 32 cycles, the assay was repeated. Samples with low U6 snRNA levels were not excluded from data analyses. Primers used in qPCR are presented in Table 1.

Statistical analysis

We used the paired Wilcoxon nonparametric test to analyze pairs of non-tumor tissue and cancer samples or between cases and controls. The Mann-Whitney U test was used for the analyses of the expression of different miRNAs. Receiver operating characteristic (ROC) curves were drawn, and the areas under the ROC curves (AUCs) were measured to assess the specificity and sensitivity of circulating miR-155 as potential diagnostic biomarkers for LSCC. All statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Differences were considered significant if *p*<0.05. All experiments were performed in triplicate and repeated at least three times.

RESULTS

Clinical characteristics of patients and controls

A total of 280 LSCC patients and 560 age, sex-matched controls were included in the study. The median age of the patients was 64.8±12.1 years, while the median age of the controls was 65.1±9.8 years. No differences were detected between the

Table 3. Associations of Aberrant MiR-155 Expression in Tissue and Plasma with Clinicopathological Data of LSCC Patients

Clinical features	n	Tissue miR-155		p value	Plasma miR-155		p value
		High	Low		Low	High	
Mean age (yrs)	64.8±12.1	64.2±11.4	65.2±10.7	0.492	65.4±9.8	63.9±13.6	0.421
<60	194	101	93	0.365	95	99	0.693
≥60	86	39	47		45	41	
Gender				0.673			0.492
Male	245	123	122		119	126	
Female	35	17	18		21	14	
Smoking				0.676			<0.001
Smoker	212	108	104		120	92	
Never-smoker	68	32	36		20	48	
Tumor size				0.004			0.033
≤3 cm	243	113	130		115	128	
>3 cm	37	27	10		25	12	
Differentiation status				<0.001			0.515
Moderate–well	197	79	118		103	94	
Poor	83	61	22		47	36	
Lymph node metastasis				<0.001			0.007
Negative	167	60	107		64	103	
Positive	113	80	33		76	37	
Stage classification				<0.001			0.001
Stage I	69	19	50		22	47	
Stages II, III, and IV	211	121	90		118	93	

miR-155, microRNA-155; LSCC, laryngeal squamous cell carcinoma.

groups ($p=0.162$). Of the 280 patients, 245 were female, and the others were male. Among the 560 controls, 245 were female, and the others were male ($p=0.541$). However, when tobacco smoking status was considered, the rate of smoking was higher in patients with LSCC ($p<0.001$). The details regarding clinicopathologic features were only collected for the patients, who also came for follow-up treatment. Pathological features were obtained for all cases, among which 243 were in the 0–3-cm group while 37 were in the >3-cm group. Most patients had a tumor of more than stage I (211 of 288, 75.36%). A moderately or well differentiated tumor was seen in 197 cases, while poor differentiated tumors comprised 24.64% of the patient population. Of the patients with lymph node metastasis infor-

mation, 167 had negative lymph node metastasis, and 116 had positive lymph node metastasis (Table 2).

Tissues and plasma miRNA-155 levels in cases and controls

Total RNA was isolated from the cancer and control tissues of all LSCC cancer patients. Additionally, plasma miR-155 levels were detected in both cases and controls. In general, the miR-155 levels were quantified using a quantitative reverse transcription PCR assay with U6 as the internal control. In our results, we found that the miR-155 level was more up-regulated in LSCC tissue than in the non-tumor tissues (13.6 ± 2.4 vs. 3.1 ± 0.80 ; $p<0.001$). Moreover, we observed a significantly higher

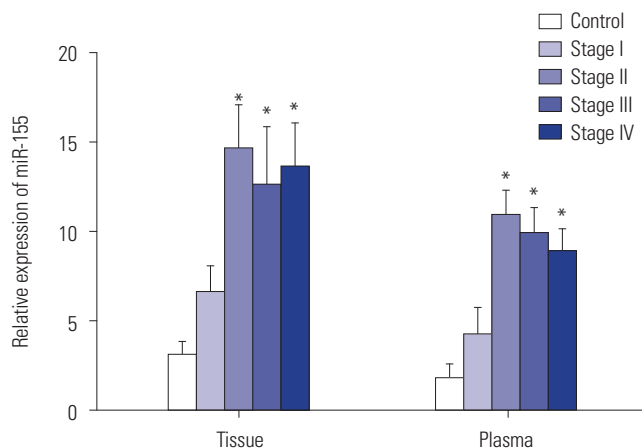


Fig. 1. Relative expression of tissue and plasma miR-155 in LSCC patients. miR-155, microRNA-155; LSCC, laryngeal squamous cell carcinoma.

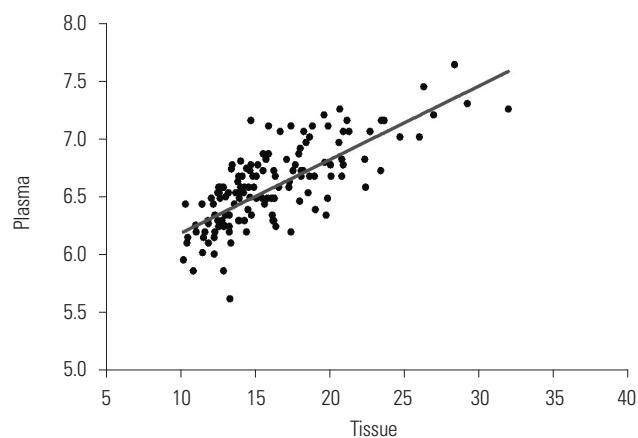


Fig. 2. The association between tissue and plasma miR-155 in LSCC cases. miR-155, microRNA-155; LSCC, laryngeal squamous cell carcinoma.

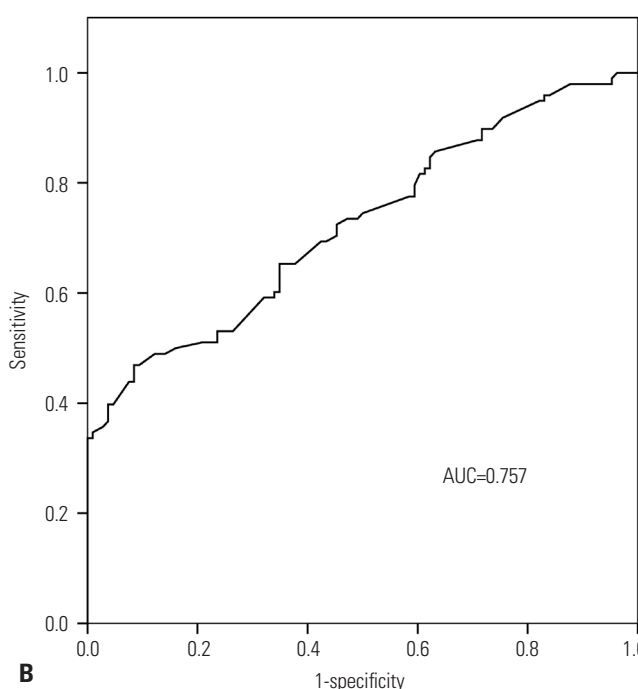
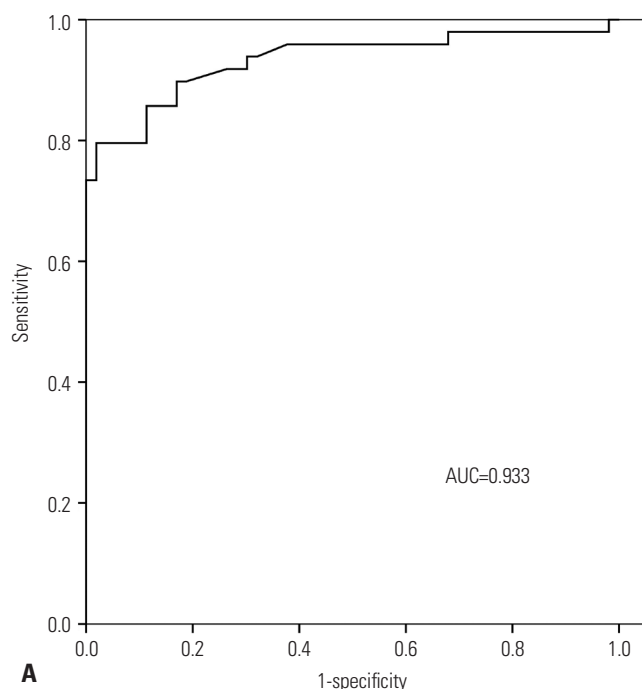


Fig. 3. Receiver operating characteristic curves of tissue and plasma miR-155 for the diagnosis of LSCC. (A) Tissue miR-155. (B) Plasma miR-155. AUC, area under the curve; miR-155, microRNA-155; LSCC, laryngeal squamous cell carcinoma.

miR-155 level in plasma samples from LSCC patients than in those from the controls (8.9 ± 1.25 vs. 1.8 ± 0.8 ; $p < 0.001$). We also reported the miR-155 levels of the LSCC cases for each different stage. It was found that both tissue and plasma miR-155 levels were higher in stage I LSCC cases. Additionally, the levels of miR-155 in stage II, III, and IV were higher than that of stage I (Table 3). However, no increasing trend was observed as the stage increased. The expression data for miR-155 in tissue and plasma are presented in Fig. 1.

We also conducted advanced analyses to detect a possible relationship between the tissue and plasma levels of miR-155. Both tissue and plasma samples were used in the detection of the miR-155 level. On Pearson correlation analyses, a significant association was detected ($p < 0.001$; $r = 0.725$) (Fig. 2).

Diagnostic performance of tissue and plasma miR-155 for LSCC

The diagnostic performances of tissue and plasma miR-155 for LSCC were detected. Cutoff points were determined using the highest sum of sensitivity and specificity. The cutoff points for tissue and plasma miR-155 were 7.34 and 3.12, respectively. Tissue miR-155 showed an AUC of 0.933, with a sensitivity of 82.6% and a specificity of 89.2% (Fig. 3A). However, the AUC for plasma miR-155 was 0.757, with a sensitivity of 58.4% and a specificity of 69.5% (Fig. 3B).

We also conducted advanced analyses on the diagnostic performances for early TNM I stage LSCC. As well, the cutoff points were determined using the highest sum of sensitivity and specificity. The cutoff points for tissue and plasma miR-155 were 7.34 and 3.12, respectively. Tissue miR-155 showed an AUC of 0.804,

with a sensitivity of 85.2% and a specificity of 87.3% (Fig. 4A). However, the AUC for plasma miR-155 was 0.824, with a sensitivity of 72.4% and a specificity of 76.9% (Fig. 4B).

The expression of tissue and plasma miR-155 after surgery

A total of 280 LSCC cases were obtained to detect the change of plasma miR-155. Compared to preoperative expression, plasma miR-155 demonstrated significant depression. As shown in Fig. 5, the expression of tissue miR-155 changed from 8.9 to 4.3 ($p = 0.002$). In the smoking group, the expression of plasma miR-155 was 11.5 ± 1.5 , while the postoperative level was 5.2 ± 0.9 ($p = 0.001$). The level of miR-155 in the non-smoking group changed from 8.1 ± 0.9 to 4.0 ± 1.3 , and a significant difference

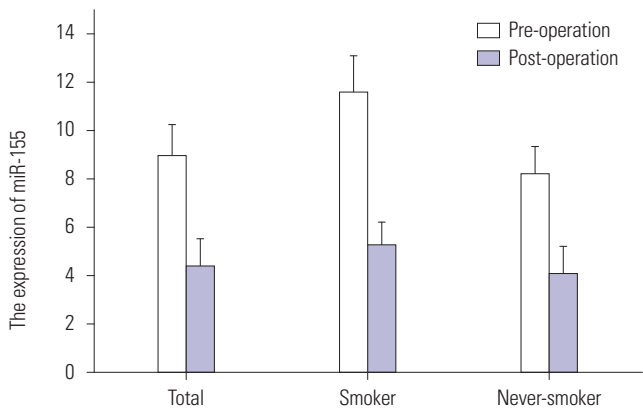


Fig. 5. Preoperative and postoperative plasma miR-155 levels of LSCC patients. miR-155, microRNA-155; LSCC, laryngeal squamous cell carcinoma.

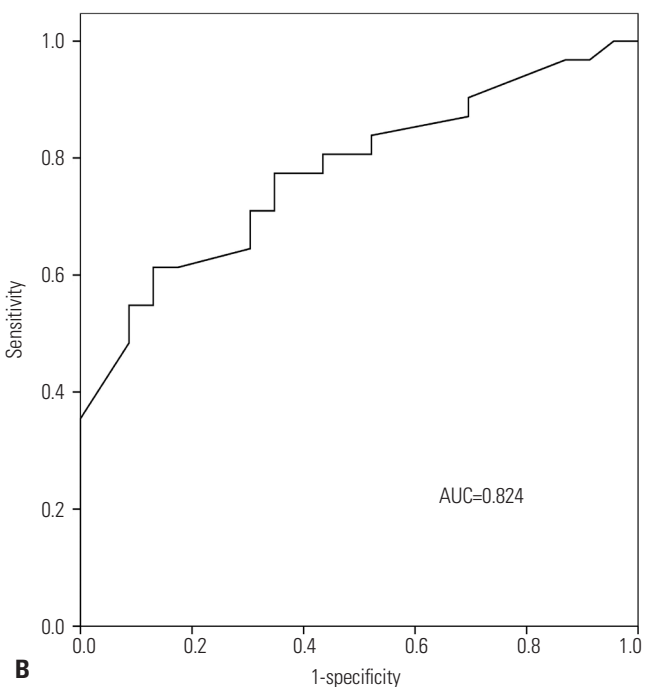
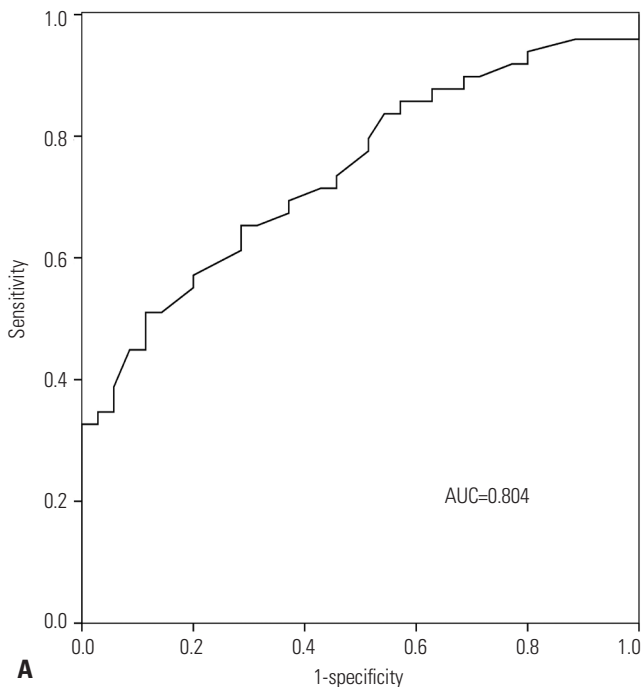


Fig. 4. Receiver operating characteristic curves of tissue and plasma miR-155 for the diagnosis of early-stage LSCC. (A) Tissue miR-155. (B) Plasma miR-155. AUC, area under the curve; miR-155, microRNA-155; LSCC, laryngeal squamous cell carcinoma.

was detected ($p=0.012$).

DISCUSSION

Sensitive and specific biomarkers were capital in developing preventive screening kinds of cancers, including LSCC. However, current methods were insufficient in detecting LSCC in the early stage. Recent achievements in imaging technology have greatly improved the diagnosis of LSCC; however, the high cost made it unavailable in developing countries. In recent years, miRNAs have been regarded as new diagnostic tools for the detection of kinds of cancers. As miRNA expression was tissue-specific and quite stable, miRNAs may be used as a marker to resolve tumor tissue from normal tissue or as a diagnostic tool. Although several groups have investigated the potential application of miRNA as a biomarker for LSCC,^{20,21} most of the reports have used histological specimens and thus have limited clinical significance. Given that miRNAs are stable in circulation, we propose that they may serve as ideal noninvasive biomarkers for the diagnosis of LSCC. Therefore, we analyzed both tissue and plasma levels of miR-155 in patients with LSCC and controls and evaluated their diagnostic performance as diagnostic markers of LSCC.

To our knowledge, this is the first study to explore the diagnostic impact of miR-155 in a case-control study with 500 participants. Previous reports indicated that miR-155 was up-regulated in several malignancies, such as Hodgkin lymphoma,²² breast cancer,²³ and lung carcinoma,²⁴ and perceived an oncogene, although the diagnostic impact was not addressed in these studies. Similarly, in LSCC, miR-155 has been interpreted as an oncogene. In a global miRNA profiling study with 51 formalin-fixed archival LSCC samples using a quantitative reverse transcription-PCR approach, miRNA expression was correlated with patients' clinical parameters. Thirty-eight of 117 (33%) consistently detected miRNAs were significantly differentially expressed in malignant tissues when compared to normal tissues, and overexpression of miR-155 in LSCC patients was detected.²⁵

As in most previous studies, the diagnostic effects of miR-155 on different cancers were analyzed in the tissues.^{26,27} It has been reported that serum miRNAs were quite stable and could hardly be degraded by enzymes. It has also been demonstrated that miRNAs are resistant to cancers. However, as the importance of circulating miRNAs is considered in the diagnosis of cancer, the application of plasma or serum miR-155 in the diagnosis of breast cancer,²⁸ glioma,²⁹ lung adenocarcinoma,²⁴ and Hodgkin lymphoma.²² In a previous study, the levels of miR-155 in cancer tissues or cell lines were significantly higher than in benign tissue or non-cancer cells.³⁰ However, miR-155 levels in plasma were not reported in previous studies. In our study, we conducted a case-control study to identify the expression and association of both tissue and plasma miR-155 in LSCC cases.

In general, our data showed that the expressions of miR-155 in both tissue and plasma were significantly higher. Moreover, we found that the expression levels of miR-155 in tissue and plasma were significantly associated. This result was accordant with several previous studies, such as microRNA-375 in colorectal cancer³¹ and miR-106b~25 expressions in patients with gastric cancers.³² MiR-155 in tissue and plasma has been used as a diagnostic marker for LSCC. According to the requirements for biomarkers in diseases, we speculated that both tissue and plasma miR-155 could be used as diagnostic markers for LSCC. Therefore, all of the data in our study were sufficient for describing diagnostic markers for the diagnosis of LSCC in all stages. Our study was the first to detect both tissue and plasma miR-155 in LSCC patients by using qPCR. This technology could be used to discover new biomarkers for LSCC from 280 LSCC cases and 560 controls with reasonable reliability. In the current study, we analyzed the expression of miR-155 in LSCC tissues and plasma samples in order to determine their potential value in the diagnosis of LSCC patients and early-stage LSCC. Our data showed that levels of tissue and plasma miR-155 expression were more up-regulated in LSCC than in the corresponding non-cancerous laryngeal tissues or the controls, and the ROC data showed that the AUC for tissue and plasma miR-155 was 0.933 and 0.757, respectively. In stage I LSCC patients, the ROC data indicated that the AUC for tissue and plasma miR-155 was 0.804 and 0.824, respectively. Based on these ROC analyses, we found that the tissue and plasma miR-155 levels were potential important diagnostic markers for LSCC. A total of 280 LSCC cases were obtained to detect the change of plasma miR-155. Compared to the preoperative expression, plasma miR-155 demonstrated significant depression. The changes in plasma miR-155 demonstrated the potential application of miR-155 for the prognosis of LSCC. In this paper, we found that only plasma miR-155 was differently expressed in the LSCC cases. It is an interesting result that suggests that miR-155 level was more related with the pathological characteristics. When the plasma miR-155 level was considered, the expression of circulating miR-155 was modified after tobacco smoke exposure. In a study conducted by Herberth, et al.,³³ it was reported that blood miRNAs are sensitive to environmental stressors, including tobacco smoke. In conclusion, we found that plasma miRNA was sensitive to different clinical and environmental factors.

The tumor promoter roles of miR-155 in different cancers are determined by both *in vitro* and *in vivo* study, and an up-regulated level of miR-155 expression would increase cell viability, prevent cell apoptosis, and increase tumorigenicity. As an important regulatory molecule, miR-155 played a key role in kinds of oncogenic processes. It could down-regulate B-cell lymphoma 6 (BCL6) protein, which could modulate the interleukin 4 responses of B cells.³⁴ Additionally, miR-155 could act to enhance transcription and contribute to the pathogenesis of leukemia. MiR-155 could reduce the expression of BCL6 and

then leads to up-regulation of certain BCL6 targets, including inhibitor of differentiation (Id2), interleukin-6 (IL6), cMyc, and Mip1 α /Ccl3, all of which promote cell survival and proliferation.³⁵ In an *in vitro* and *in vivo* study, it was found that the expression levels of miR-155 in LSCC were significantly higher than those in the control mucosa tissues.³⁶ Down-regulation of SOCS1 expression and elevated expression of STAT3 were also observed in LSCC. The relevance of the three factors was statistically significant. Moreover, knockdown of miR-155 elevated SOCS1 expression level, suppressed STAT3 expression, and inhibited hep-2 cell growth, migration, and invasion, whereas overexpression of miR-155 resulted in the opposite effects. STAT3 protein level in poor or moderate cell differentiation was significantly higher than that in a higher degree of cell differentiation. These data demonstrated the aberrant expression and function of miR-155 and its downstream targets in LSCC. These findings suggest that miR-155 plays a promoting role during the development of LSCC, and it may be a useful marker for the prognosis and assessment of therapeutic effects.³⁰

In conclusion, the expressions of tissue and plasma miR-155 were significantly up-regulated in patients with LSCC. Due to its reasonable sensitivity and specificity for LSCC, tissue and plasma miR-155 could serve as potential biomarkers for determining both LSCC stage and early cases. Our work will serve as a basis for further investigation, preferably large-scale validation in clinical trials, before plasma miR-155 can be used as a routine screening tool for LSCC.

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