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Original Article

MiR-21/miR-375 ratio is an independent prognostic factor in patients with laryngeal squamous cell carcinoma

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Abstract: We sought to identify microRNAs that exhibit altered expression in laryngeal squamous cell carcinoma (SCC) and to determine whether microRNA expression is predictive of disease. This study was divided into three steps: (1) The expression of six miRNAs, such as up-regulated miR-223, miR-142-3p, miR-21, miR-16, miR-23a and down-regulated miR-375, was evaluated using total RNA isolated from freshly-frozen primary tumors and non-cancerous laryngeal squamous epithelial tissues and analyzed using quantitative real-time polymerase chain reaction (qRT-PCR). (2) We also investigated the mRNA expression levels of processing elements (RNASEN, DGCR8, and DICER1) that participate in miRNA-biogenesis pathway. (3) We analyzed the relationships between the expression levels of these miRNAs and the clinicopathologic parameters of laryngeal SCC patients. In this study, we found that: (1) A marked difference in the microRNA expression pattern was observed between tumors and non-cancerous tissue. With regard to miRNA-processing elements, the expression level of RNASEN was higher in laryngeal SCC than in normal epithelium ($P<0.01$). (2) The miR-21/miR-375 expression ratio was highly sensitive and specific for disease prediction. Kaplan-Meier analysis revealed a significant association between high expression of miR-21/miR-375 in cancerous tissue and a worse prognosis ($p=0.032$). (3) Furthermore, the expression ratio of miR-21/mir-375 in patients with stage (III-IV) tumors was significantly higher than that in those with stage (I-II) tumors ($p=0.006$). These data suggest that the pattern of microRNA expression in primary laryngeal SCC tissues is exhibiting strong predictive potential.

Keywords: microRNA, microarray, laryngeal squamous cell carcinoma, biomarker, prognosis

Introduction

Laryngeal squamous cell carcinoma (SCC), which originates from the laryngeal epithelium, has the second highest incidence of all head and neck squamous cell carcinomas [1]. In recent years, the incidence of laryngeal cancer has been relatively stable, with about 160,000 new cases diagnosed per year [2]. Despite significant advances in surgery and radiotherapy over the last few decades, no treatment has been shown to achieve a satisfactory therapeutic outcome and the mortality rate of laryngeal SCC is still high, with a 5-year survival rate of 64% [3]. There is very little information about the precise molecular pathways underlying the development of laryngeal SCC. Therefore, there is an urgent need to develop novel and clinically useful markers to distinguish patients with poor prognosis.

Recent work on different cancer types has illustrated the existence of distinct microRNA expression profiles between tumor tissues and

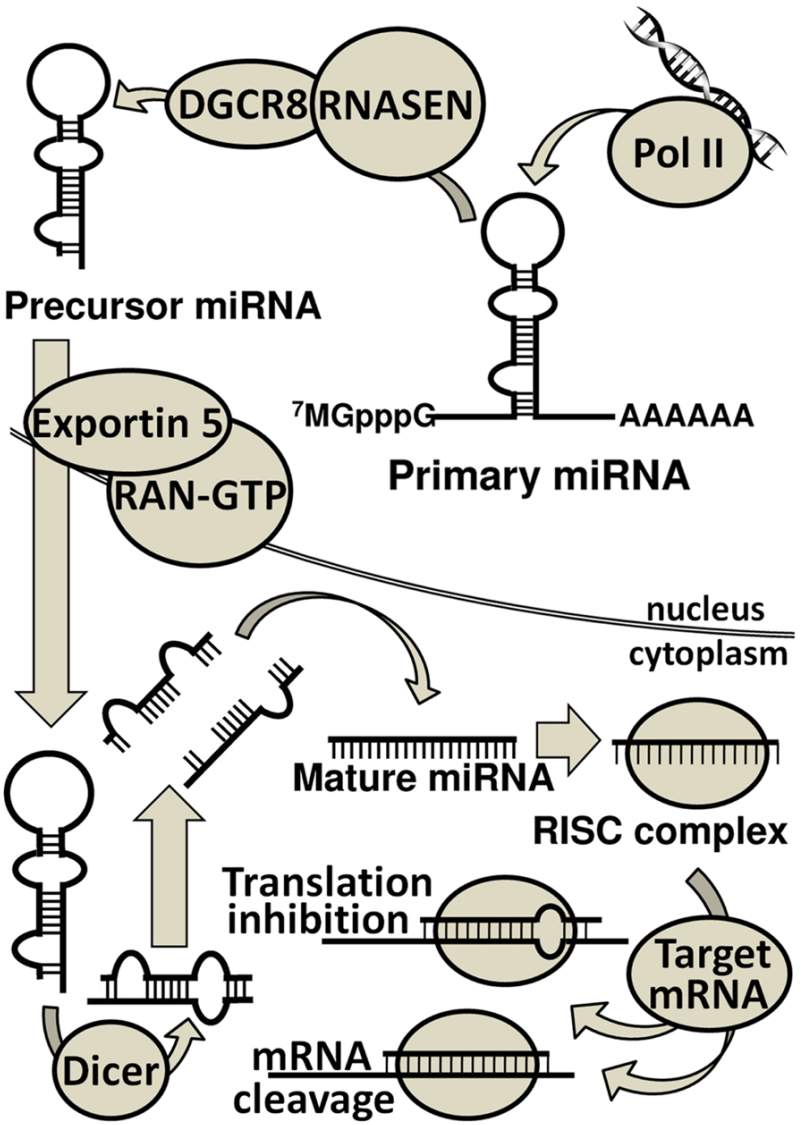

Figure 1. The canonical process of miRNA biogenesis involves the following steps: 1) an miRNA is generated as a long primary precursor miRNA (pri-miRNA) in the nucleus; 2) the long pri-miRNA is excised by the nuclear microprocessor complex formed by ribonuclease 3 (RNASEN) and the DiGeorge critical region 8 (DGCR8) protein to form precursor miRNA (pre-miRNA); 3) the pre-miRNA is exported from the nucleus by guanosine triphosphate-binding nuclear protein Ran (RAN) (Ran-GTP); 4) the RNA-induced silencing complex (RISC) binds to the exported pre-miRNA in the cytoplasm; 5) DICER1 cuts near the hairpin loop to release the mature miRNA duplex after pre-miRNA binding.

their corresponding normal tissue [4]. MiRNAs are small non-coding RNAs of 18 to 24 nucleotides, originally discovered and described in 1993 in the nematode Caenorhabditis elegans [5]. To date, more than 300 miRNAs have been discovered in humans, and computational analyses predict that up to 1,000 miRNAs exist in the human genome [6]. In humans, the canonical process of miRNA biogenesis involves the following steps: in the nucleus, an miRNA is generated as a long primary precursor miRNA (pri-miRNA); the long pri-miRNA is excised by the nuclear microprocessor complex formed by ribonuclease 3 (RNASEN) and the DiGeorge critical region 8 (DGCR8) protein to form precursor miRNA (pre-miRNA); and the pre-miRNA is exported from the nucleus by guanosine triphosphate-binding nuclear protein Ran (RAN) (Ran-GTP). In the cytoplasm, the RNA-induced silencing complex (RISC) binds to the exported pre-miRNA. DICER1 cuts near the hairpin loop to release the mature miRNA duplex after pre-miRNA binding (**Figure 1**). In recent years, gene expression profiling technologies have become increasingly sophisticated, allowing investigators to explore their diagnostic and therapeutic potential as biomarkers in laryngeal SCC and other cancers.

Lately, we reported that miR-21 and miR-375, the expression of which is frequently reported in SCC tissues [7-12], were aberrantly expressed in laryngeal SCC patients [13, 14]. Based on our findings, we selected five oncogenic miRNA, including miR-21, miR-142-3p, miR-223, miR-23a, miR-16, and one tumor suppressor miRNA, miR-375, as candidates for prognostic biomarkers for laryngeal SCC in miRNA assay. We also investigated the mRNA expression levels of miRNA processing elements (RNASEN, DGCR8, and DICER1) in laryngeal SCC. In spite of the undisputed importance of altered expression patterns of miRNAs in various cancers, there is little information on the clinicopatho-

Table 1. The clinicopathologic characteristics in laryngeal SCC patients (N=46)

characteristics	
Age (y)	59.2 ± 7.84
Gender (male/female)	42/4
Differentiation (poor/well)	25/21
Localization(glottic/supraglottic/subglottic)	33/11/2
Lymph node metastasis (negative/positive)	32/14
TNM stage (I-II/III-IV)	31/15
Smoking (negative/positive)	12/31
Alcohol (negative/positive)	22/19

Note: We examined miRNAs by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in 46 consecutive laryngeal specimens from patients with laryngeal SCC (as described below) and compared the results according to the clinicopathological characteristics of the patients. The cohort was composed of 42 males and four females, with a median age of 59.2 years (range, 54-78 years). Among them, no patients were received chemoradiotherapy. The distribution of tumor grades was as follows: Thirty-three patients had glottic tumors, while 11 had supraglottic and 2 hadsubglottic tumors. The pathological tumor TNM stages were I-II in 31 patients and III-IV in 15 patients. There was nodal involvement in 14 patients. The median follow-up period was 38.6 months.

Table 2. PCR primers used to amplify the human protein and miRNA

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
MIR-21	GCTTATCAGACTGATGTTGACTG	CAGCCCATCGACTGGTG
MIR-142-3p	GCCGCTGTAGTGTTTCCTACTT	GTGCAGGGTCCGAGGT
MIR-223	TCGAGGAGCTTCCAGCTGAGCACT	ACGCGTTATTGCGCCCCCATCAGCA
MIR-23a	GCGATCACATTGCCAGGG	GTGCAGGGTCCGAGGT
MIR-16	GCGCACGCGTTGTTTTATCCCAAGT	ATGCATCGATTTCTTTAGGCGCGAT
MIR-375	UUUGUUCGUUCGGCUCGCGUGA	CTTCTGGGCCCCGGCCCGCCG
RNASEN	CAGCAGTGTTGCTTGACACTTAGGA	ACAGTGTAGGTTCGGGCATGG
DGCR8	CTTCAGTGTAGCCCATTCTTGATCC	GCAGCTGCCTTTGACATCCA
DICER1	CAAGTGTCAGCTGTCAGAACTC	CAATCCACCACAATCTCACATG
RUN6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

logic significance of cancer related miRNAs in laryngeal SCC. In this study, the expression of miR-23a and miR-16 did not demonstrate any significant differences between laryngeal SCC tissues and paired normal tissues. Then we examined the expression ratio of miR-21/miR-375, miR-142-3p/miR-375 and miR-223/miR-375 in laryngeal specimens from patients with laryngeal SCC. The use of a microRNA expression ratio of miR-21/miR-375 holds potential as a more useful monitoring marker for laryngeal SCC.

Materials and methods

Patients and samples

Atotal of 46 surgical resection specimens were collected from consecutive patients who underwent partial or total laryngectomy with informed consent after institutional review board approval at the Shanghai Jiaotong University of Medicine between September 2005 and July 2007. None of patients had received preoperative chemotherapy or radiotherapy. Fresh laryngeal SCC tumor specimens were obtained during the tumor survey as described previously [14]. Meanwhile, paired normal laryngeal squamous epithelial tissues derived from laryngectomy specimens that served as normal controls were also assayed. The corresponding adjacent noncancerous tissues were obtained from surgically resected tissues that were located at least 5 cm away from the tumors. Tissue samples were immediately frozen in liquid nitrogen after resection until further processing. The pathological stage, grade, and nodal status of the tumors were defined according to the revised International UICC/TMN Staging System. The clinical and pathological data of the laryngeal SCC patients enrolled in the study are displayed in **Table 1**.

RNA extraction and microarray profiling

Total RNA isolation from tissue samples was performed as described previously [14]. The concentrations and quality of the RNA were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE, USA) and agarose gel electrophoresis. Five micrograms of total RNA was sent for microRNA profiling studies at Asuragen Services using the mirVANA microRNA Bioarrays platform V2 as single-channel format according to the standard operating procedures of the company,

Table 3. Differential expression of miRNAs in LSCC tissues compared to with normal tissue

miRNA name	Score(d)	Fold change	miRNA name	Score(d)	Fold change
hsa-miR-21	8.97	5.71	hsa-miR-451	8.41	2.62
hsa-miR-142-3p	7.64	4.37	hsa-miR-214	5.78	2.60
hsa-miR-23a	7.68	4.22	hsa-miR-151-5p	5.07	2.60
hsa-miR-223	10.58	4.19	hsa-miR-181a	5.40	2.56
hsa-miR-16	6.79	4.03	hsa-miR-365	5.40	2.53
hsa-miR-199b-3p	7.20	3.90	hsa-miR-130a	5.77	2.47
hsa-miR-15b	7.39	3.86	hsa-miR-19b	6.26	2.43
hsa-let-7f	8.08	3.80	hsa-miR-30c	5.06	2.42
hsa-let-7a	8.09	3.69	hsa-let-7c	6.51	2.35
hsa-miR-26b	6.20	3.68	hsa-miR-193b	5.06	2.33
hsa-miR-27a	7.70	3.61	hsa-let-7i	7.00	2.20
hsa-miR-15a	6.72	3.54	hsa-miR-126	6.49	2.09
hsa-let-7g	6.59	3.40			
hsa-miR-107	6.14	3.34			
hsa-miR-34a	6.50	3.33			
hsa-miR-103	6.06	3.29			
hsa-miR-146a	6.01	3.22	hsa-miR-375	1.61	-5.69
hsa-miR-27b	6.37	3.05	hsa-miR-297	2.32	-5.56
hsa-miR-24	7.38	2.94	hsa-let-7f-1	0.65	-5.03
hsa-miR-23b	6.54	2.87	hsa-miR-508-5p	1.11	-4.66
hsa-miR-205	7.04	2.83	hsa-miR-640	1.01	-3.93
hsa-miR-342-3p	5.33	2.83	hsa-miR-646	0.30	-2.89
hsa-miR-106b	5.78	2.77	hsa-miR-923	9.23	-2.00

Note: Positive fold change values are miRNA with higher expression in tumor samples and negative fold change values are for those miRNA having lower expression in tumor samples.

including pre-array qualitative Bioarrays RNA analysis. The Bioarrays platform V2 contains probes specific to microRNA identified in human, mouse, and rat, as well as a number of proprietary microRNAs identified through cloning at Ambion, Inc. The primers used for RNASEN, DGCR8, DICER1, and RNU6 were purchased from Applied Biosystems (**Table 2**).

Quantitative real-Time PCR of miRNAs

The expression of mature miRNAs was assayed using TaqMan miRNA reverse transcription assays (Applied Biosystems) and appropriate primers (Applied Biosystems) following the manufacturer's instructions. In brief, 10 ng of total RNA was used as the template in a 15 μL RT reaction using probes specially designed for specific mature miRNAs. For assessing the expression of each miRNA, reactions were performed in triplicate using the 7900 RT-PCR system (Applied Biosystems). The same reference standard dilution series was repeated on every experimental plate, and RNU6 (also known as U6), which is a small nuclear RNA, was used as an internal control. The reaction conditions indicated in the manufacturer's manual were used and the reaction mixtures were incubated at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The cycle threshold values were calculated with the SDS 1.4 software package. The ΔΔCt method for relative quantization was used to determine miRNA expression levels. The Ct is the fractional cycle number at which the fluorescence of each sample passes the fixed threshold. The ΔCt value was calculated by subtracting the Ct of snRNA U6 from the Ct of the miRNA of interest. The ΔΔCt value was calculated by subtracting the ΔCt of the reference sample (paired non-tumorous tissue for surgical samples) from the ΔCt of each sample. The fold-change was determined as $2^{-\Delta\Delta Ct}$.

Statistical analysis

Differential expression of microRNAs by microarray was determined with Significance Analysis of Microarray software. Values are presented as mean ± standard error of the mean (SEM). The difference between experimental and control groups was evaluated using the paired t-test. The relationship between clinicopathological factors in laryngeal SCC patients and the expression of miRNA was analyzed using Fisher's exact or chi-square tests. The last date of follow-up was either the date of death or the last date the patient was contacted. Overall survival was estimated using the Kaplan-Meier method with comparisons between groups made using the log-rank test. A *p*-value less than 0.05 was considered statistically signifi-

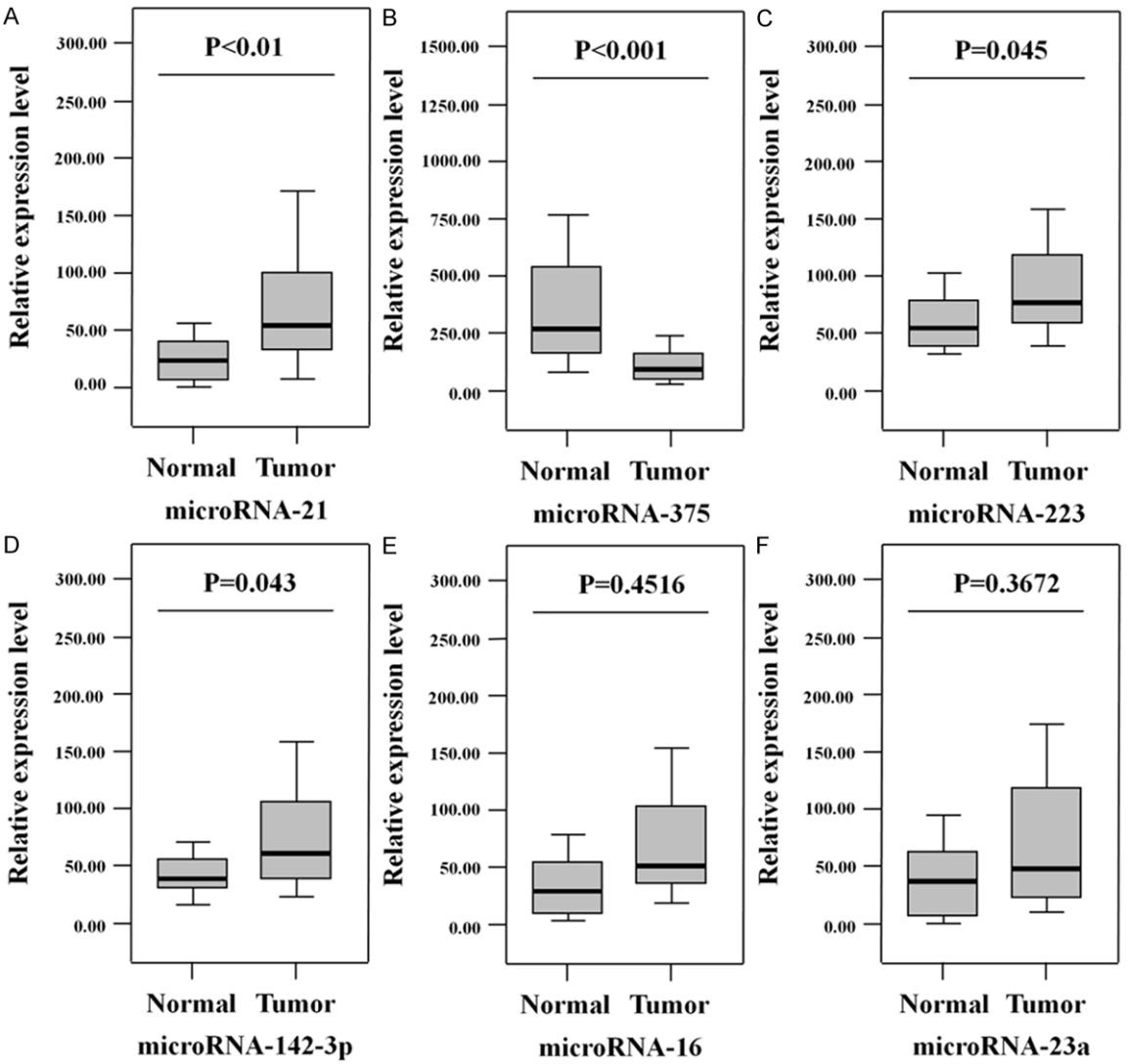

Figure 2. Validation of the differential expression of miR-21 and miR-375 by quantitative real-time PCR in tumor and normal samples (n=46 for tumor samples and n=46 for normal samples).The relative expression level of miR-21 tended to be higher in laryngeal SCC patients than in the controls (p<0.01) (A), while the relative expression level of miR-375 was significantly lower in laryngeal SCC patients than in the controls (p<0.001) (B). The expression of miR-223 and miR-142-3p in laryngeal SCC tissues showed a borderline significant relative to the normal tissues (the value of p were 0.045 and 0.043, respectively) (C, D). However, the expression of miR-16 and miR-23a did not demonstrate any significant differences between laryngeal SCC tissues and paired normal tissues (the value of p were 0.4516 and 0.3672, respectively) (E, F). Boxes indicate the distribution of expression values from the 25th to 75th percentile for microRNAs. The lines inside the boxes indicate the median values and the upper and lower horizontal bars denote the 90th and 10th percentiles, respectively.

cant. Statistical analysis was performed using SPSS 10.0 software program.

Results

Global miRNA profiling of laryngeal SCC samples

The miRNA microarray chip is able to identify 720 microRNAs and determine expression levels of both mature and precursor mirs. For this study, we only examined the mature mirs. Forty-two of the detected miRNAs were significantly differentially expressed between malignant versus normal tissues. Of the 42 differentially expressed microRNAs, 22 were human microRNAs. All human microRNAs identified by Significance Analysis of Microarray method showed >2-fold difference between normal and

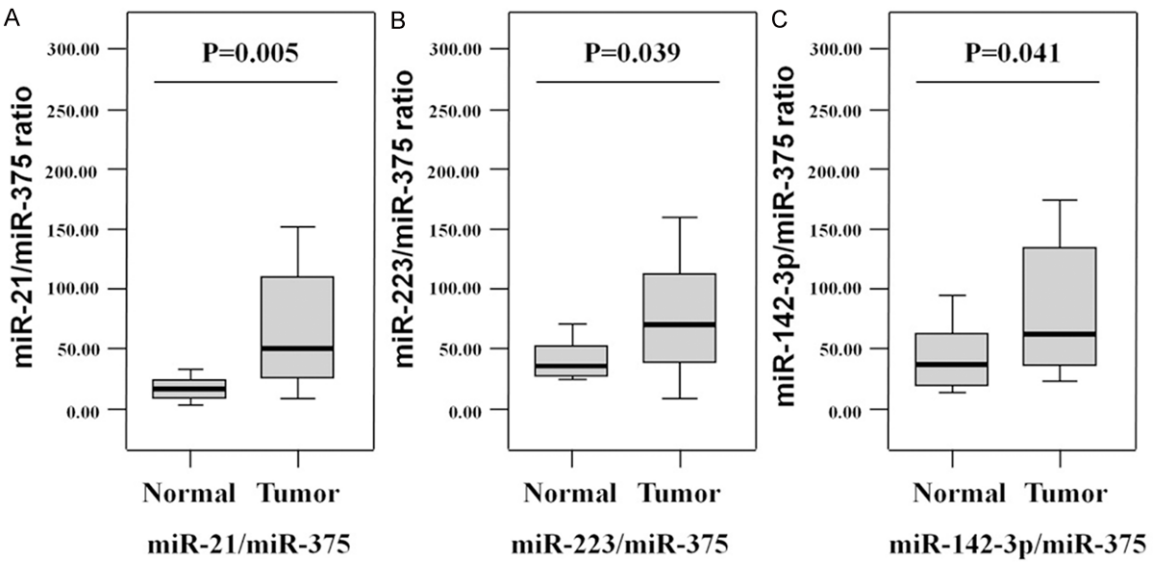

Figure 3. The combined biomarker based on the ratio of miR-21/miR-375 was significantly higher in laryngeal SCC patients than in controls (p=0.005) (A). The ratio of miR-223/miR-375 and miR-142-3p/miR-375 were borderline significant higher in laryngeal SCC patients than in controls (the value of p were 0.039 and 0.041, respectively) (B, C). Boxes, distribution of expression values from the 25th to 75th percentile for microRNAs. The lines inside the boxes indicate the median values. The upper and lower horizontal bars denote the 90th and 10th percentiles, respectively.

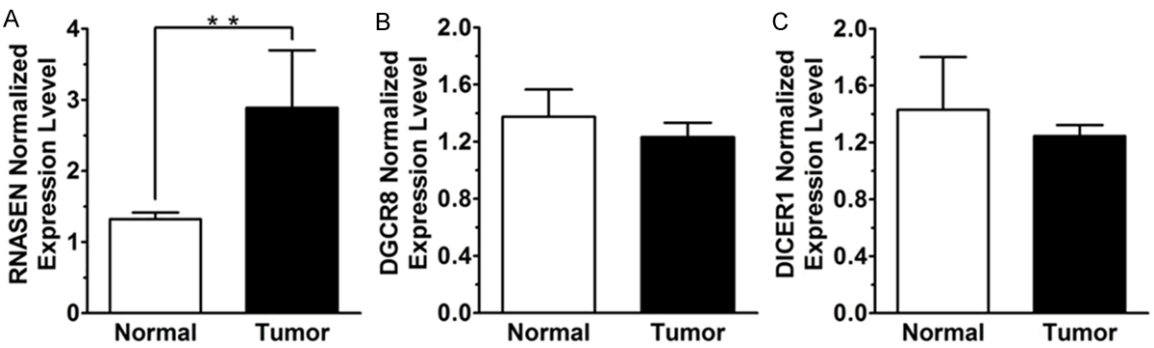

Figure 4. RT-PCR analysis of the mRNA expression of RNASEN, DGCR8, and DICER1 in laryngeal SCC. The mRNA expression level of RNASEN in the laryngeal SCC was higher than that in the normal epithelium (P<0.01) (A). There were nostatistically significant differences in the expression levels of DGCR8 and DICER1 between the laryngeal SCC and the corresponding adjacent non-cancerous tissues (B, C). Values shown are the means SE. **P<0.01, versus control group.

tumor tissue. Concordant with previous reports, over-expression of miR-21, and miR-142-3p and under-expression of miR-375 were detected. Up-regulation of miR-223, miR-23a, and miR-16 were newly observed (**Table 3**).

Evaluation of quantitative RT-PCR for measuring the miRNAs in laryngectomy specimens and determine the appropriate candidates

To identify more sensitive diagnostic biomarkers in laryngeal SCCs, we selected over-expressed microRNAs such as miR-223, miR-142-3p, miR-21, miR-16, miR-23a and under-expressed microRNA (mir-375) for further analysis, based on our previous findings. The expression of miR-223, miR-142-3p, miR-21, miR-16, miR-23a and miR-375 in laryngeal SCCs and paired, adjacent, histologically normal mucosal samples was determined by qRT-PCR analysis (total n=46). Our data indicate that miR-21 expression is significantly up-regulated (p<0.01) (**Figure 2A**) and miR-375 expression is down-regulated (p<0.001) (**Figure 2B**) in laryn-

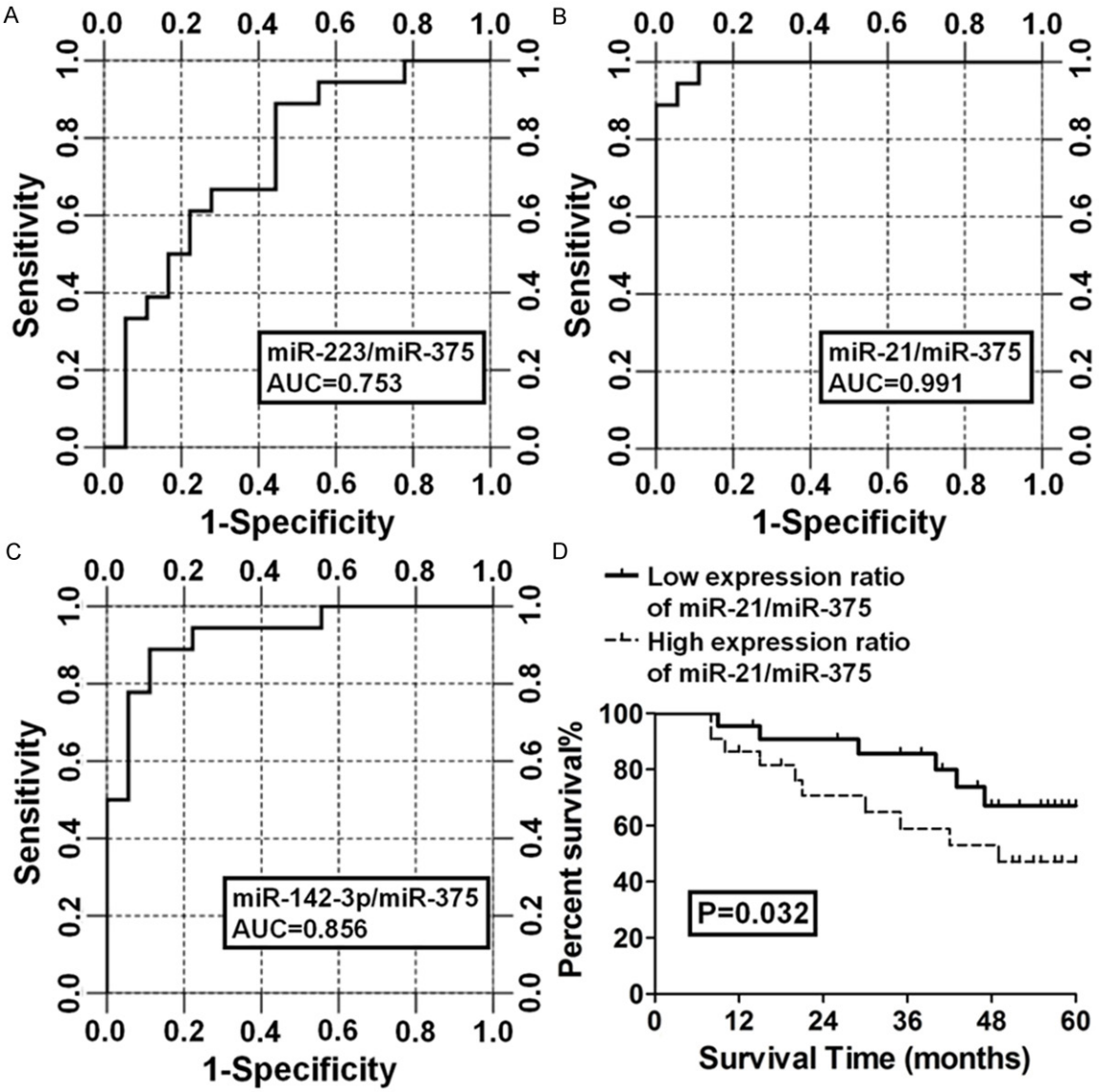

Figure 5. The receiver operating characteristic (ROC) curves for miR-223/miR-375,miR-21/miR-375 and miR-142-3p/miR-375 were plotted and values of the area under the curve (AUC) were 0.753, 0.991, and 0.856, respectively (A-C). Kaplan-Meier analysis revealed a significant association between high expression of miR-21/miR-375 in cancerous tissue and a worse prognosis (p=0.032) (D).

geal SCC tissue relative to the paired normal tissues. The expression of miR-223 and miR-142-3p in laryngeal SCC tissues showed a borderline significant relative to the normal tissues (the value of p were 0.045 and 0.043, respectively) (**Figure 2C** and **2D**). However, the expression of miR-16 and miR-23a did not demonstrate any significant differences between laryngeal SCC tissues and paired normal tissues (the value of p were 0.4516 and 0.3672, respectively) (**Figure 2E** and **2F**). Therefore we excluded two miRNAs such as miR-16 and miR-23a and selected miR-21, miR-223, miR-142-3p and miR-375 as candidates for further study.

Analyze the ratio by dividing the up-regulated miRNAs by the down-regulated miRNA, as a combined biomarker.

Because miR-223, miR-142-3p, miR-21 showed consistent up-regulation and miR-375 consistent down-regulation, we next sought to determine if expression ratios constructed between these microRNA could improve their predictive potential for differentiating laryngeal SCC

Table4. Correlation between the expression ratio of miR-21/miR-375 and clinicopathological factors in laryngeal SCC patients

		Expression ratio of miRNA-21/miRNA-375		
	N	High	Low	*p*-value
Total	46	22 (47.8%)	24 (52.2%)	
Gender				
Male	42	21 (50.0%)	21 (50.0%)	
Female	4	1 (25.0%)	3 (75.0%)	0.665
Age (y)				
<65	22	10 (45.5%)	12 (54.5%)	
≥65	24	12 (50.0%)	12 (50.0%)	0.990
T stage				
T0/1/2	21	6 (28.6%)	15 (71.4%)	
T3/4	25	16 (64.0%)	9 (36.0%)	0.036
N stage				
Negative	32	17 (53.1%)	15 (46.9%)	
Positive	14	5 (35.7%)	9 (64.3%)	0.443
Stage				
Low (I–II)	31	10 (32.3%)	21 (67.7%)	
High (III–IV)	15	12 (80.0%)	3 (20.0%)	0.006
Localization				
Glottic	33	13 (39.4%)	20 (60.6%)	
Supraglottic	11	8 (72.7%)	3 (27.3%)	0.117
Subglottic	2	1 (50.0%)	1 (50.0%)	
Smoking[a]				
Negative	12	8 (66.7%)	4 (33.3%)	
Positive	31	12 (38.7%)	19 (61.3%)	0.191
Alcohol[b]				
Negative	22	12 (54.5%)	10 (45.5%)	
Positive	19	9 (47.4%)	10 (52.6%)	0.885

[a]Data missing in 3 samples. [b]Data missing in 5 samples. $p<0.05$. Note: Correlation between the expression ratio of miR-21/miR-375 and clinicopathological factors in laryngeal SCC patients. *p* values, as calculated using the χ^2 or Fisher's exact test, are shown, with $p<0.05$ considered statistically significant.

tumor from paired normal tissues. Following the methods of Gordon [15], we analyzed the ratio by dividing the relative expression value of each of the up-regulated miRNAs by the expression value of the down-regulated miRNA, as a combined biomarker. The data indicate that the miR-21/miR-375 ratio is significantly higher in laryngeal SCC patients than in controls (p= 0.005) (**Figure 3A**). The ratio of miR-223/miR-375 and miR-142-3p/miR-375 were borderline significant higher in laryngeal SCC patients than in controls (the value of p were 0.039 and 0.041, respectively) (**Figure 3B** and **3C**).

Expression levels of RNASEN, DGCR8, and DICER1 in laryngeal SCC

We investigated the mRNA expression levels of RNASEN, DGCR8, and DICER1 to evaluate the differences in miRNA processing between tumor tissues and adjacent non-cancerous tissues. The expression levels of RNASEN in the laryngeal SCC were higher than the levels in the corresponding adjacent non-cancerous tissues ($P<0.01$) (**Figure 4A**). There were no statistically significant differences in the expression levels of DGCR8 and DICER1 between the laryngeal SCC and the corresponding adjacent non-cancerous tissues (**Figure 4B** and **4C**).

Validation study on clinical application as a diagnostic monitoring biomarker

Receiver operating curve analysis was done to determine which of their ratios showed the greatest predictive power. The receiver operating characteristic (ROC) curves for miR-223/miR-375,miR-21/miR-375 and miR-142-3p/miR-375 were plotted and values of the area under the curve (AUC) were 0.753, 0.991, and 0.856, respectively (**Figure 5A-C**). The miRNA expression ratio of miR-21/miR-375 was dichotomized based on a within-cohort median cutoff. Kaplan-Meier analysis revealed a significant association between high expression of miR-21/miR-375 in cancerous tissue and a worse prognosis (p=0.032) (**Figure 5D**).

Correlation between the clinicopathological characteristics and the expression ratio of miRNAs

To determine whether there was any correlation between the expression ratio of miR-21/miR-375 level and the clinicopathological features of the laryngeal SCC patients, the miRNA expression levels from the qRT-PCR analysis were dichotomized based on a within-cohort median cutoff. The major characteristics of the patients are shown in **Table 4**. When characteristics of the patients were compared, after stratification based on the expression levels of miR-21/miR-375, no correlation was observed between miR-21/miR-375 levels and the age, sex, differentiation, tobacco consumption, and alcohol use of the patient. Patients with T3-4 tumors exhibited a significant upper-expression of miR-21/miR-375 level, the difference was statistically significant (p=0.036). The expres-

sion ratio of miR-21/mir-375 in patients with stage (III-IV) tumors was significantly higher than that in those with stage (I-II) tumors (p=0.006).

Discussion

The dismal outcome of laryngeal squamous cell carcinoma (SCC) patients highlights the need for novel prognostic biomarkers. MiRNAs control the expression levels of particular genes. Therefore, dysregulation of miRNAs, which occurs in certain diseases, such as cancers, may be attributed to aberrant gene expression. In the present study, we demonstrate altered processing of miRNA genes in laryngeal SCC as compared with normal laryngeal epithelium. We also evaluated the correlation between potentially cancer-related miRNAs and the clinicopathologic characteristics of patients with laryngeal SCC. The involvement of microRNAs in cancer and their potential as biomarkers of diagnosis and prognosis are becoming increasingly appreciated. Many studies have reported significant associations between miRNA expression profiles and important clinical features of tumors, as well as patient survival rates [16-22]. By elucidating the presence of miRNA expression signatures and their contribution to the development and progression of tumors in patients with laryngeal SCC, the present study has extended our knowledge of the role of miRNAs in the pathogenesis of this malignancy.

In this study, the permutation-based software Significance Analysis of Microarray was used to identify differentially expressed genes by pairwise comparisons between groups of interest. Of the 42 human microRNAs in this group, six microRNAs such as up-regulated miR-223, miR-142-3p, miR-21, miR-16 and miR-23a as well as down-regulated miR-375were used for in-depth examination of a larger population of fresh-frozen laryngeal SCC and normal laryngeal tissue.

Some of the microRNAs identified as differentially expressed in HNSCC compared with normal tissue have been characterized in past reports, particularly in relation to cancer. MiR-21 has attracted more attention than any other miRNA as it is one of the most highly up-regulated genes in various cancers and has been implicated in multiple malignancy-related processes, including cell proliferation, apoptosis, invasion, and metastasis [23, 24]. MiR-21 has been previously identified as a putative oncogene in oesophageal cancer [25], The notion that miR-21 functions as an oncogene is strengthened by the recent discovery that suppression of miR-21 inhibits tumor growth through down-regulation of the tumor suppressor tropomyosin I. Similar to the findings for miR-21 in other tumor types, miR-21 expression in the laryngeal SCC samples were high in both tumor as well as in matched normal tissues, but the expression of miR-21 was twice as high in tumor samples as they were in the normal samples. A recent study [26] of human gastric cancers, published during the preparation of our manuscript, showed that the expression of miR-21 in cancer tissues was significantly higher than that in non-cancerous tissues, consistent with our data. The biological significance of regulatory interactions between miR-21 and their target genes may lie in their functions in various cellular activities critical to the outcome of tumorigenesis. Previous studies have demonstrated that the novel tumor suppressor protein programmed cell death 4 (PDCD4) is down-regulated in several human solid cancers and is suppressed by miR-21 [27]. Phosphatase and tensin homolog (PTEN) was shown to be a direct target of miR-21 in cholangiocarcinoma cells [28]. Thus, the role of miR-21 is likely to be the inhibition of apoptosis. Some growth factors regulate the expression of miR-21; a recent report revealed that transforming growth factor-B (TGF-b) increases the expression of miR-21 through the SMAD signal transduction pathway by promoting the transition from pri-miR-21 to pre-miR-21 [29].

MiR-375 was the only down-regulated microRNA found when comparing tumors and normal tissues. Validation experiments also showed it to be sharply and significantly down-regulated in tumors relative to normal tissues. These findings appear to be consistent with the observations made in recent studies on miR-375. Though miR-375 has mainly been studied in the context of diabetes, as it influences beta-cell mass and insulin levels [30], its expression has been shown to be decreased in a number of malignancies, including esophageal squamous cell and adenocarcinomas [25, 31]. Additionally, the recent identification of a target for miR-375, phosphoinositide-dependent protein kinase-1, suggests a feasible role for miR-375 as a tumor suppressor since phos-

phoinositide-dependent protein kinase-1 is crucial for the activation of anti-apoptotic AKT protein [32]. In our study, lower expression of miR-375 was also found in laryngeal tumors, compared with normal tissues.

Although the functional roles of RNASEN require further elucidation, it is likely that RNASEN over-expression is a key mechanism in the aberrant miRNA biogenesis in laryngeal SCC. Our results are consistent with previous findings that RNASEN expression levels are often increased in esophageal and cervical SCCs as compared with the levels in adjacent non-cancerous tissues. We also investigated the expression ratio as a diagnostic biomarker in laryngeal SCC to improve the predictive potential of alterations in the expression of individual microRNAs. The ratio of miR-21/miR-375 showed a highest discriminatory potential, with a sensitivity of 94% and specificity of 94%, in distinguishing tumor from normal tissue. These data suggest that the ratio of these microRNAs may hold significant clinical potential, but further validation is necessary in an independent series of laryngeal SCC tumors.

In conclusion, global miRNA profiling of archival fresh laryngeal SCC samples as identified that approximately 42 of the miRNAs are dysregulated in this disease. The use of a microRNA expression ratio miR-21/miR-375 holds potential as a more useful monitoring marker for laryngeal SCC. Our results may imply that miRNA biogenesis is aberrantly accelerated in laryngeal SCC. Analysis of the expression levels of miRNAs should provide useful information for evaluation of the staging, prognosis, and treatment of laryngeal SCC patients.

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Disclosure of conflict of interest

None.

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