



Original Article

Altered expression of microRNA miR-21, miR-155, and let-7a and their roles in pulmonary neuroendocrine tumors

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MicroRNA (miRNA) has a critical effect on tumorigenesis through post-transcriptional modification and is considered to be potential biomarkers for cancer diagnosis and treatment monitoring. We evaluated the expression pattern of three selected miRNAs (miR-21, miR-155, and let-7a) to evaluate their potential roles by quantitative reverse transcription-polymerase chain reaction using formalin-fixed and paraffin-embedded tissues of 63 surgically resected pulmonary neuroendocrine (NE) tumors (19 typical carcinoids (TCs), 6 atypical carcinoids (ACs), 19 large cell NE carcinomas (LCNECs), and 19 small cell lung carcinomas (SCLCs)). Control amplification for U6 small nuclear RNA (U6) was performed in all samples. Normalized Ct values were calculated ($Ct_{\text{Experimental miRNA}} - Ct_{\text{U6}}$) for each case and recorded. The expression levels of miR-21 and miR-155 were significantly higher in high-grade NE carcinomas (LCNECs and SCLCs) than in carcinoid tumors (TCs and ACs) (each $P < 0.001$). The expression level of miR-21 in carcinoid tumors with lymph node metastasis was significantly higher than in carcinoid tumors without lymph node metastasis ($P = 0.010$). To the best of our knowledge, the present study is the first to examine the expression patterns of miR-21 and miR-155 as an adjunctive diagnostic tool or clinically relevant biomarkers for pulmonary NE tumors.

Key words: let-7a, lung, microRNA, miR-155, miR-21, neuroendocrine tumor

Pulmonary neuroendocrine (NE) tumors form a distinct group of neoplasms that share characteristic morphologic, immunohistochemical, ultrastructural, and molecular features. They span a clinical spectrum, from low-grade typical carcinoid (TC) and intermediate-grade atypical carcinoid (AC) to high-grade large cell NE carcinoma (LCNEC) and small cell lung carcinomas (SCLC).¹ Currently, the 2004 World Health Organization (WHO) classification of pulmonary NE tumors is based on combined architectural patterns with the two most relevant parameters, the mitotic index and presence of necrosis, observed by hematoxylin and eosin (H&E) staining, for the purpose of recognizing the four different subtypes.² However, they represent a wide spectrum of phenotypically distinct entities, from which pulmonary NE tumors can sometimes be difficult to differentiate, even for an expert pathologist.^{3,4} Moreover, reproducible and objective pathologic criteria with clinical and prognostic value must be established when comparing the various grades of pulmonary NE tumors. With respect to the difficult issues surrounding the differential diagnosis of pulmonary NE tumors, we think that the molecular data has pathological and clinical relevance.

Since only a few institutions have frozen tissue banks, most molecular studies of these tumors have been retrospective studies performed on formalin-fixed and paraffin-embedded (FFPE) tissue samples. Therefore, the type of molecular studies has been limited. Recently, FFPE tissue using reverse transcription (RT)-polymerase chain reaction (PCR) has become an accurate and robust method for microRNA (miRNA) analysis.⁵

MicroRNA molecules are evolutionarily-conserved, small non-coding RNA molecules (19–24 nucleotides in size) and, unlike mRNA, do not encode amino-acid sequences.^{6,7} The miRNAs can function as endogenous negative gene regulators at the posttranscriptional level by cleavage and/or

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translational repression of their mRNA targets, thereby controlling a wide range of biological functions such as cellular proliferation, differentiation, and apoptosis.^{7,8} Dysregulated expression of miRNAs has been identified in a variety of human malignancies, which suggests that miRNAs can function as potential oncogenes or tumor suppressor genes, depending on the cellular context and the target genes.^{7–11} These observed changes in miRNA also imply that miRNA could potentially be useful diagnostic and prognostic markers in cancer.

Recent studies have documented a relationship between the aberrant expression of a class of miRNA and the pathogenesis of many human cancers, including lung cancer.^{12–14} However, little is known about the expression levels or functions of miRNAs in pulmonary NE tumors. We hypothesized that specific miRNA biomarkers may exist that could accurately and reliably distinguish subtypes of pulmonary NE tumors. In the present study, we selected 3 miRNAs (miR-21, miR-155, and let-7a) from published human miRNA data that are more closely related to tumor development and progression.^{15–17} It is known that miR-21 and miR-155 are overexpressed in a variety of cancers,^{15,16} whereas let-7a is underexpressed in malignancies.¹⁷

Here we report on the expression patterns of miR-21, miR-155, and let-7a to evaluate their potential as diagnostic tools or relevant biomarkers by quantitative RT-PCR using FFPE tissues of 63 surgically resected pulmonary NE tumors including all four subtypes.

MATERIALS AND METHODS

Patients and tissue samples

Formalin-fixed and paraffin-embedded specimens obtained from 63 patients with a histologic diagnosis of pulmonary NE tumor were retrieved from the files at a number of institutions in South Korea. They were registered at the Departments of Pathology of Samsung Changwon Hospital, Gachon University Gil Hospital, Busan National University Hospital, Kosin

University Hospital, Inje University Ilsan Paik Hospital, Keimyung University Hospital, and Dong-A University Medical Center, between 2001 and 2008. The institutional review board of each institute approved our study, and written informed consent was obtained from all the patients before surgery to permit the use of their resected samples for research. To ensure that there would be enough specimens for pathologic examination, only surgically resected cases were considered. To determine an accurate histologic diagnosis of the pulmonary NE tumors, H&E-stained slides from each case were reviewed by three experienced pulmonary pathologists (S.Y.H, C.H.L., M.S.R.)^{18,19} based on the revised WHO classification of 2004,² and only unequivocal cases were used. Combined SCLC with LCNEC, or with other non-small cell lung carcinoma (NSCLC), and combined LCNEC with other NSCLC were not included in this study to determine the precise difference of miRNA expression patterns among four subtypes of pulmonary NE tumors. To demonstrate the NE phenotype, immunohistochemical staining for such general NE markers as chromogranin-A, synaptophysin and CD56 was performed, if that was necessary. Immunohistochemically, the tumor was considered as positive for NE markers if the tumor cells exhibited focal, patchy, or diffuse staining. Finally, the 63 cases enrolled in this study consisted of 19 TCs, 6 ACs, 19 LCNECs, and 19 SCLCs. Representative histologic features for each subtype of pulmonary NE tumor are shown in Figure 1. The clinicopathological characteristics of the 63 pulmonary NE tumors are summarized in Table 1.

RNA extraction

Tumor areas were confirmed and marked on H&E-stained slides under the microscope. A representative FFPE tissue block from each case and four 10 µm unstained sections were used for RNA extraction. The non-tumor areas on the paraffin slides were manually removed with surgical blades, and the remaining tissue on the slide was scraped into an Eppendorf tube for RNA extraction. Deparaffinization was

Table 1 The clinicopathological characteristics of the 63 pulmonary neuroendocrine tumors

Characteristics	TC (<i>n</i> = 19) (%)	AC (<i>n</i> = 6) (%)	LCNEC (<i>n</i> = 19) (%)	SCLC (<i>n</i> = 19) (%)
Age (years)				
Mean	49.6	47.4	65.5	64.2
Range	24–67	36–66	50–77	54–78
Gender (M : F)	12:7	3:3	18:1	14:5
Size (cm)				
Mean	2.9	3.7	4.7	4.5
Range	0.7–5.5	1.4–7.0	1.7–7.3	1.2–10.0
Lymphovascular invasion	3 (15.8)	2 (33.3)	10 (52.6)	12 (63.2)
Lymph node metastasis	2 (10.5)	2 (33.3)	8 (42.1)	10 (52.6)

AC, atypical carcinoid; F, female; LCNEC, large cell neuroendocrine carcinoma; M, male; SCLC, small cell lung cancer; TC, typical carcinoid.

Figure 1 Representative histologic features for each subtype of pulmonary neuroendocrine tumor. (a) Typical carcinoid showed an organoid nesting with fibrovascular septa. The tumor cells were uniform and showed a finely granular nuclear chromatin pattern and a moderate amount of eosinophilic cytoplasm. Neither mitosis nor necrosis was found. (b) Atypical carcinoid showed a punctuate focus of necrosis within tumor nest composed of tumor cells with carcinoid morphology. (c) Large cell neuroendocrine carcinoma consisted of organoid nesting with palisading and rosette-like pattern. The tumor cells were large, polygonal with abundant eosinophilic cytoplasm, coarsely granular nuclear chromatin, and prominent nucleoli (inset). (d) Small cell lung carcinoma consisted of a sheet-like growth pattern with extensive necrosis. The tumor cells were smaller than three lymphocytes (arrow) and had scant cytoplasm, finely granular nuclear chromatin, and inconspicuous nucleoli (inset).

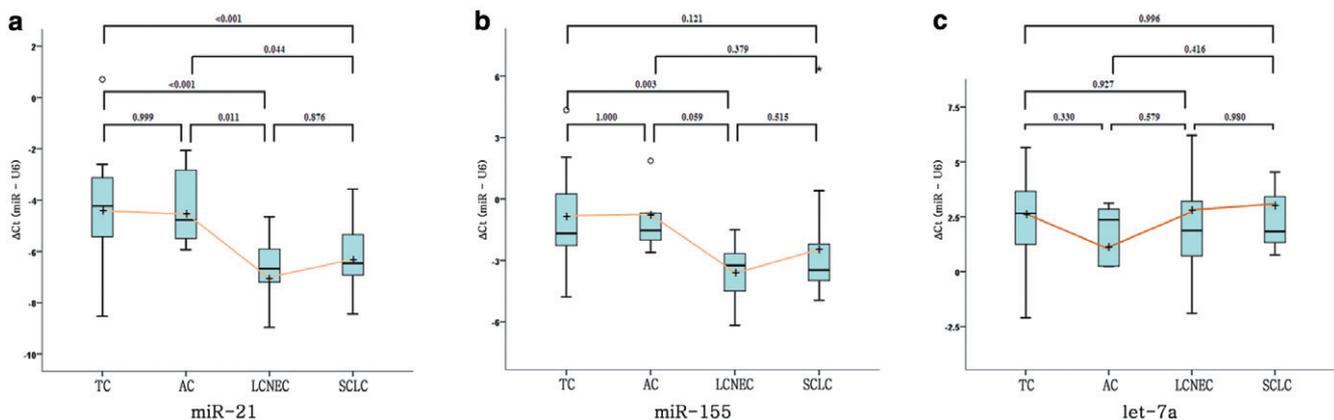
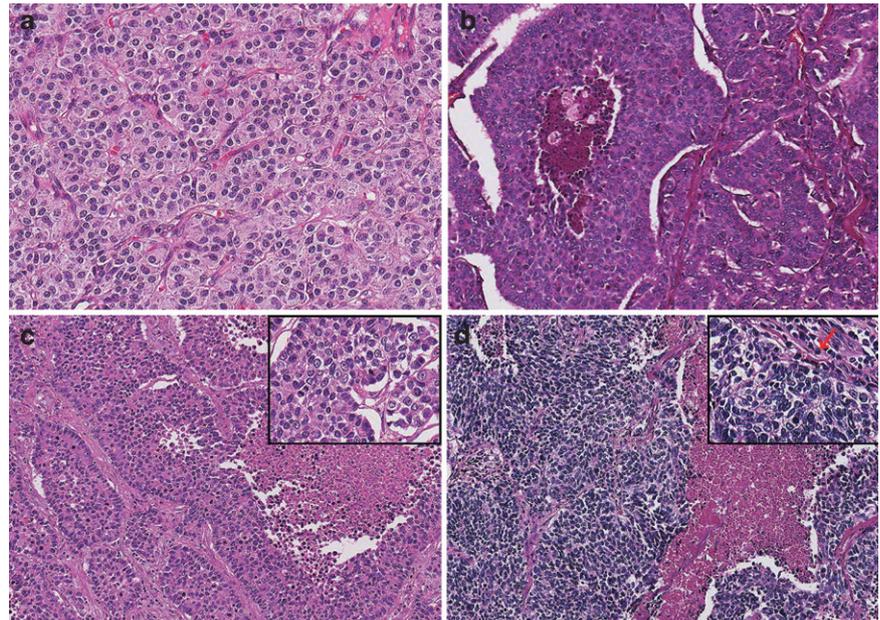


Figure 2 Box-and-whisker plots for comparison of mean normalized Ct (Δ Ct) values of miR-21 (a), miR-155 (b), and let-7a (c) with histologic subtypes of 63 pulmonary neuroendocrine tumors. High-grade neuroendocrine carcinomas (large cell neuroendocrine carcinomas (LCNEC) and small cell lung carcinoma (SCLC)) showed statistically higher expression of miR-21 and miR-155 than carcinoid tumors (typical carcinoid (TC) and atypical carcinoid (AC)), whereas let-7a showed no significant difference.

performed by incubating the sections in xylene three times for 10 min, and then the sections were placed in absolute ethanol for three washes of 10 min each. The samples were then air dried for 30 min at room temperature. Total RNA was extracted from the dried sections using the Ambion RecoverAll Total Nucleic Acid Isolation Kit (Applied Biosystems, Foster City, CA, USA). This procedure involves DNase treatment, purification, and RNA elution. The quality and quantity of isolated total RNA were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Rockford, IL, USA). Purified total RNA samples were stored at -80°C until used for analysis.

Quantitative RT-PCR

Complementary DNA was generated using the TaqMan microRNA RT kit with specifically designed stem-loop RT primers for each mature miRNA (miR-21, miR-155, and let-7a) according to the manufacturer's instructions. We also quantified transcripts of U6 small nuclear RNA (U6) as an endogenous control for normalizing the levels of target miRNA. The RT reactions contained 10 ng of total RNA as the template, 5 μl of gene-specific stem-loop RT primer, 1.5 μl of 10 RT buffer, 0.15 μl of 100 mM dNTPs, 1 μl of MultiScribe RTase, and 4.16 μl of nuclease-free water.

Table 2 Correlation of normalized Ct values of miR-21, miR-155, and let-7a with histologic subtypes of 63 pulmonary neuroendocrine tumors

Diagnosis	Normalized Ct value of miRNA (95% confidence interval)		
	miR-21	miR-155	let-7a
TC	-4.23 (-4.93 to -3.53)	-1.13 (-2.08 to -0.18)	2.51 (1.61 to 3.41)
AC	-4.31 (-5.55 to -3.07)	-1.08 (-2.78 to -0.61)	0.95 (0.66 to 2.55)
LCNEC	-6.59 (-7.28 to -5.89)	-3.58 (-4.53 to -2.63)	2.12 (1.22 to 3.02)
SCLC	-6.22 (-6.91 to -5.52)	-2.65 (-3.60 to -1.70)	2.37 (1.47 to 3.27)
<i>P</i> value†	<0.001	0.003	0.384
Carcinoid tumors	-4.25 (-5.00 to -3.50)	-1.12 (-1.99 to -0.25)	2.14 (1.17 to 3.10)
High-grade NE carcinomas	-6.40 (-6.81 to -5.99)	-3.11 (-3.79 to -2.44)	2.24 (1.68 to 2.81)
<i>P</i> value‡	<0.001	<0.001	0.832

†Difference in the mean normalized Ct values of miRNA among the four subtypes of pulmonary neuroendocrine tumors.

‡Difference in the mean normalized Ct values of miRNA between carcinoid tumors and high-grade neuroendocrine carcinomas.

AC, atypical carcinoid; LCNEC, large cell neuroendocrine carcinoma; NE, neuroendocrine; SCLC, small cell lung cancer, TC, typical carcinoid.

The 15 µl reactions were incubated for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then held at 4°C. Quantitative real time-PCR was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The PCR reaction was performed in 10 µl aliquots with 5 µl TaqMan Universal *MixII*, 0.5 µl TaqMan miRNA for each 2 µl of miRNA. Reactions were incubated at 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 s and at 60°C for 1 min. All experiments were performed in duplicate to confirm reproducibility.

Data normalization

The threshold cycle data (Ct) and baseline were determined using auto settings. The Ct value was defined as the fractional cycle number at which the fluorescence passed the fixed threshold. U6 was used as the endogenous control to normalize expression levels. Following the amplification, the same threshold was set for analyzing all experiments to compare Ct values derived from different experiments. The mean Ct values of each sample were determined from duplicate reactions and then normalized against the corresponding U6 Ct values, calculated as delta Ct ($\Delta Ct = Ct_{\text{Experimental miRNA}} - Ct_{\text{U6}}$). All data presented were normalized Ct (ΔCt) values.

Statistical analysis

In the first part of the experiments, one way analysis of variance was used to determine the association between the expression levels of three candidate miRNAs and histologic subtypes of pulmonary NE tumors. Turkey's test was further used to compare the expression levels of a marker across multiple groups pairwise. Contingency table and Student's t-test were applied to determine the associations between expression levels of miRNAs and clinicopathologic characteristics. A *P*-value < 0.05 was considered statistically significant.

In addition, receiver operating characteristic (ROC) curve analysis was undertaken using the ΔCt value of each miRNA to evaluate their ability in diagnosing the histologic subtype of pulmonary NE tumors. Using this approach, areas under the ROC curve (AUC) identified optimal sensitivity and specificity levels in distinguishing histologic subtypes of pulmonary NE tumors based on the ΔCt value. The AUCs with 95% confidence interval that did not include the 0.5-value signified that the miRNA had some ability to distinguish between the subtypes. To measure the agreement between diagnosis based on the cut-off value of miRNA expression level and histologic diagnosis on H&E section, the generalized kappa value was calculated.

All calculations were performed with SPSS version 18.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Differential expression of miRNAs according to the histologic subtype of pulmonary NE tumors

RNA was successfully extracted from all FFPE samples. All cases showed uniformly high miRNA quality, with Ct values for control U6 control RNA RT-PCR clustering within less than three amplification cycles for all samples. The Ct range for U6 control RNA varied from 25.1 to 27.9 cycles.

The results for the expression level of miR-21, miR-155, and let-7a according to the histologic subtype of the pulmonary NE tumors are shown in Table 2 and Figure 2. Results showed miR-21 to be the most consistently overexpressed in all subtypes of pulmonary NE tumors. The mean ΔCt values for miR-21 were -4.23 (95% confidence interval (CI): -4.93 to -3.53) in TC, -4.31 (95% CI: -5.55 to -3.07) in AC, -6.59 (95% CI: -7.28 to -5.89) in LCNEC, and -6.22 (95% CI: -6.91 to -5.52) in SCLC. The expression level of miR-21 was clearly different between the histologic subtypes of pulmonary NE tumors (*P* < 0.001). In pairwise comparison, the expression level of miR-21 was significantly higher in high-

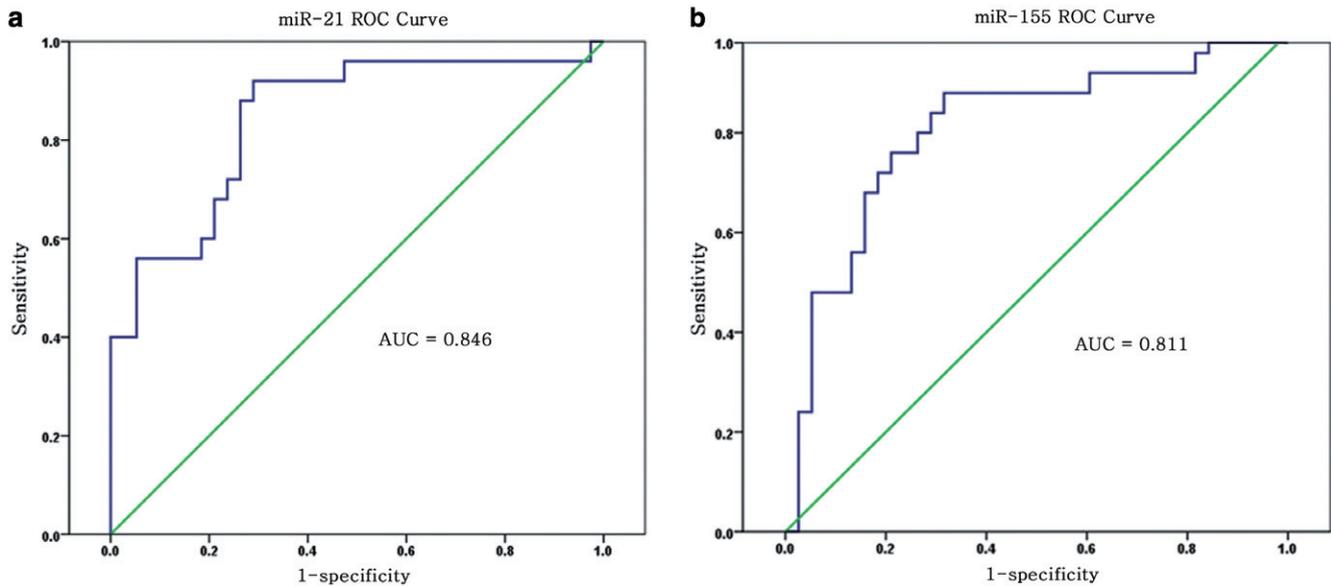


Figure 3 Receiver operating characteristic (ROC) curve analysis using normalized Ct (Δ Ct) values of miRNA expression for discriminating carcinoid tumors and high-grade neuroendocrine (NE) carcinomas. (a) Δ Ct value of miR-21 for carcinoid tumors yielded areas under the ROC (AUC) of 0.846 (95% CI: -0.744 to -0.948). At the cut-off Δ Ct value at -5.93 (carcinoid tumor ≥ -5.93 , high-grade NE carcinoma < -5.93), the expression level of miR-21 had 92.0% sensitivity and 71.1% specificity in discriminating carcinoid tumors from high-grade NE carcinomas. (b) Δ Ct value of miR-155 for carcinoid tumors yielded an AUC of 0.811 (95% CI: -0.696 to -0.925). At the cut-off Δ Ct value at -2.32 (carcinoid tumor ≥ -2.32 , high-grade NE carcinoma < -2.32), the expression level of miR-155 had 80.0% sensitivity and 73.7% specificity in discriminating carcinoid tumors from high-grade NE carcinomas.

grade NE carcinomas (LCNECs and SCLCs) than in carcinoid tumors (TCs and ACs) ($P < 0.001$) (Table 2). Furthermore, the expression level of miR-21 was significantly different between TC and LCNEC ($P < 0.001$), between TC and SCLC ($P < 0.001$), between AC and LCNEC ($P = 0.011$) and between AC and SCLC ($P = 0.044$). However, no difference was found between TC and AC ($P = 0.999$) and between LCNEC and SCLC ($P = 0.876$) (Fig. 2a).

For miR-155, the mean Δ Ct values for miR-155 were -1.13 (95% CI: -2.08 to -0.18) in TC, -1.08 (95% CI: -2.78 to -0.61) in AC, -3.58 (95% CI: -4.53 to -2.63) in LCNEC, and -2.65 (95% CI: -3.60 to -1.70) in SCLC. The expression level of miR-155 was different between the histologic subtypes of pulmonary NE tumors ($P = 0.003$). In particular, the expression level of miR-155 was significantly higher in high-grade NE carcinomas than in carcinoid tumors ($P < 0.001$) (Table 1). Although a significant difference was found between TC and LCNEC ($P = 0.003$), no difference was found between TC and AC ($P = 1.0$), between TC and SCLC ($P = 0.121$), between AC and LCNEC ($P = 0.059$), between AC and SCLC ($P = 0.379$), and between LCNEC and SCLC ($P = 0.515$) (Fig. 2b).

On the other hand, substantial overlaps were seen between the let-7a distribution ranges among the histologic subtypes. The mean Δ Ct values for let-7a were 2.51 (95% CI: 1.61 to 3.41) in TC, 0.95 (0.66 to 2.55) in AC, 2.12 (1.22 to 3.02) in LCNEC, and 2.37 (1.47 to 3.27) in SCLC (Table 1).

The expression level of let-7a was not statistically different between the histologic subtypes of pulmonary NE tumors ($P = 0.384$) (Fig. 2c).

Predicting value based on miRNAs expression in differential diagnosis of pulmonary NE tumors

Since we found that two miRNAs, miR-21 and miR-155, can differentiate carcinoid tumors from high-grade NE carcinomas, the cut-off Δ Ct values of miR21 and miR-155 were chosen from the ROC curve analysis in order to maximize sensitivity and specificity. The ROC curves with corresponding AUCs for miR-21 and miR-155 expression level in pulmonary NE tumors are shown in Figure 3.

The ROC curve analysis indicated that the Δ Ct value of miR-21 for carcinoid tumors yielded an AUC of 0.846 (95% CI: -0.744 to -0.948). At the cut-off Δ Ct value at -5.93 (carcinoid tumor ≥ -5.93 , high-grade NE carcinoma < -5.93), the expression level of miR-21 had 92.0% sensitivity and 71.1% specificity in discriminating carcinoid tumors from high-grade NE carcinomas. The kappa value for comparing the diagnosis based on the cut-off Δ Ct value of miR-21 and histologic diagnosis on H&E section was 0.594 (Table 3).

For miR-155, the Δ Ct value of miR-155 for carcinoid tumors yielded an AUC of 0.811 (95% CI: -0.696 to -0.925). At the cut-off Δ Ct value at -2.32 (carcinoid tumor ≥ -2.32 ,

Table 3 Comparison of diagnosis based on the cut-off Δ Ct value of miRNA expression and histologic diagnosis on hematoxylin and eosin section in 63 pulmonary neuroendocrine tumors

miRNA	Histologic diagnosis	Diagnosis based on the cut-off Δ Ct value†		Kappa value	P value
		Carcinoid tumor	High-grade NE carcinomas		
miR-21	Carcinoid tumor	23	2	0.594	<0.001
	High-grade NE carcinomas	11	27		
miR-155	Carcinoid tumor	20	5	0.519	<0.001
	High-grade NE carcinomas	10	28		

†Cut-off Δ Ct value of miR-21 = -5.93, cut-off Δ Ct value of miR-155 = -2.32.
NE, neuroendocrine; Δ Ct, normalized Ct.

Table 4 Correlation between miRNA expressions and clinicopathologic characteristics in 25 carcinoid tumors

Clinicopathologic characteristics	No. of patients (n = 25)	Normalized Ct value of miRNA (mean \pm standard deviation)		
		miR-21	miR-155	let-7a
Sex				
Female	10	-3.71 \pm 1.48	-0.30 \pm 2.79	1.59 \pm 1.81
Male	15	-4.61 \pm 1.96	-1.67 \pm 1.31	2.50 \pm 2.62
P value		0.229	0.172	0.353
Age (year)				
\leq 50	14	-3.73 \pm 1.76	-0.92 \pm 1.78	1.65 \pm 2.79
>50	11	-4.91 \pm 1.73	-1.37 \pm 2.51	2.76 \pm 1.49
P value		0.108	0.606	0.247
Tumor size (cm)				
\leq 2	8	-4.29 \pm 2.65	-1.39 \pm 2.58	1.85 \pm 3.25
2< and \leq 3	15	-4.32 \pm 1.31	-0.97 \pm 2.01	2.18 \pm 1.70
>3	2	-3.55 \pm 2.09	-1.14 \pm 1.22	2.96 \pm 3.83
P value		0.858	0.908	0.841
Lymphovascular invasion				
No	19	-4.10 \pm 1.96	-1.09 \pm 2.35	1.79 \pm 2.54
Yes	6	-4.72 \pm 1.24	-1.24 \pm 1.10	3.22 \pm 1.02
P value		0.475	0.883	0.197
Lymph node metastasis				
No	21	-3.86 \pm 1.61	-0.86 \pm 2.12	1.88 \pm 2.44
Yes	4	-6.29 \pm 1.52	-2.49 \pm 1.55	3.46 \pm 1.08
P value		0.010	0.160	0.225

high-grade NE carcinoma (<-2.32), the expression level of miR-155 had 80.0% sensitivity and 73.7% specificity in discriminating carcinoid tumors from high-grade NE carcinomas. The kappa value for comparing the diagnosis based on the cut-off Δ Ct value of miR-155 expression and histologic diagnosis on H&E section was 0.519 (Table 3).

Correlation of miRNAs expression with clinicopathologic characteristics in carcinoid tumors and high-grade NE carcinomas

To further determine the clinical significance of dysregulated expressions of miRNAs, we evaluated expression levels of miR-21, miR-155, and let-7a in relation to various clinicopathologic characteristics in carcinoid tumors and high-grade NE carcinomas, respectively. We found that the mean Δ Ct value for miR-21 in carcinoid tumors with lymph node metastasis (-6.29 ± 1.52) was quite different from that in carcinoid tumors without lymph node metastasis (-3.86 ± 1.61) ($P =$

0.010). Other clinicopathologic characteristics were not correlated with the miR-21 expression level. On the other hand, the expression levels of miR-155 and let-7a showed no significant differences according to clinicopathologic characteristics in carcinoid tumors (Table 4). In addition, the expression levels of miR-21, miR-155 and let-7a showed no significant differences according to further histopathological prognostic features beyond necrosis and mitoses, including nuclear pleomorphism, aerogeneous spreading, palisading, papillary formation, and pseudoglandular patterns suggested by the 2004 WHO classification² in carcinoid tumors. In high-grade NE carcinomas, no significant correlations between the expression levels of miR-21, miR-155, and let-7a and clinicopathologic characteristics were found in high-grade NE carcinomas (Table 5).

DISCUSSION

Since pulmonary NE tumors represent a distinct neoplastic entity that shares a wide spectrum of morphologic character-

Table 5 Correlation between miRNA expression and clinicopathologic characteristics in 38 high-grade neuroendocrine carcinomas

Clinicopathologic characteristics	No. of patients (<i>n</i> = 38)	Normalized Ct value of miRNA (Mean ± Standard deviation)		
		miR-21	miR-155	let-7a
Sex				
Female	6	-5.67 ± 1.63	-3.13 ± 2.02	2.73 ± 1.39
Male	32	-6.57 ± 1.12	-3.11 ± 2.09	2.14 ± 1.78
<i>P</i> value		0.087	0.986	0.415
Age (year)				
≤50	1	-4.87	-0.4	0.97
>50	37	-6.44 ± 1.25	-3.21 ± 2.00	2.28 ± 1.72
<i>P</i> value		.	.	.
Tumor size (cm)				
≤2	3	-6.38 ± 0.72	-2.49 ± 3.13	1.55 ± 0.52
2< and ≤3	25	-6.58 ± 1.26	-3.63 ± 1.33	2.45 ± 1.60
>3	10	-5.82 ± 1.35	-2.73 ± 1.19	1.81 ± 2.29
<i>P</i> value		0.334	0.201	0.508
Lymphovascular invasion				
No	16	-6.30 ± 1.35	-3.14 ± 1.34	2.61 ± 1.21
Yes	22	-6.50 ± 1.19	-3.09 ± 2.57	2.82 ± 1.43
<i>P</i> value		0.636	0.945	0.067
Lymph node metastasis				
No	20	-6.35 ± 1.18	-3.17 ± 2.65	2.34 ± 1.76
Yes	18	-6.46 ± 1.36	-3.05 ± 1.14	2.14 ± 1.70
<i>P</i> value		0.802	0.854	0.730

istics, these tumors have been a difficult challenge for pathologists to diagnose. Additionally, there are no specific immunohistochemical or molecular markers that allow for separation of these tumors. In this study, miR-21 showed the best separation between high-grade NE carcinomas and carcinoid tumors in pairwise comparison. In addition, the expression level of miR-155 was significantly higher in high-grade NE carcinomas than in carcinoid tumors. However, most of the difficulties in diagnosing pulmonary NE tumors come from problems in distinguishing LCNEC from SCLC and TC from AC.^{3,4} Unfortunately, no difference was found between TC and AC and between LCNEC and SCLC in the expression levels of miR-21 and miR-155. Moreover, the kappa values for comparing the diagnosis based on the cut-off Δ Ct values of miR-21 and miR-155 expression and histologic diagnosis on H&E section were 0.594 and 0.519, respectively. These kappa values just made it into the moderate agreement category, which is not yet efficient for routine clinical application. Hence, it cannot be used as a substitute of accurate morphologic characterization of tumors, and careful correlation with routine morphology is essential in all cases. However, miRNAs are well preserved in formalin-fixed tissue, making them ideal candidates for molecular markers used in routinely processed material. Therefore, miR-21 and miR-155 could be employed as an adjunctive maker for the differential diagnosis of pulmonary NE tumors in surgical pathology specimens.

The availability of a reliable marker will be of value, not only for a more reliable and robust subclassification of cases, but also in identifying patients to determine an individual's risk-benefit ratio in future clinical trials. Pulmonary NE tumors do not constitute a single, uniform entity but build up a variable

clinical behavior. In particular, confusion frequently arises regarding the biologic behavior of carcinoid tumors. Although carcinoid tumors are considered to have a low metastatic potential, lymph node metastases are present in 4–14% of TC cases and 35–64% in AC cases.²⁰ The 5- and 10-year disease-free survival rates of patients who had regional lymph node metastasis were 74% and 53%, respectively, compared with 96% and 84%, respectively, in those without lymph node metastasis.^{21,22} Interestingly, in this study, four carcinoid tumor patients diagnosed with lymph node metastasis had high expression levels of miR-21, which would suggest the potential practical use of miR-21 in future clinical management of patients with carcinoid tumors to predict lymph node metastasis. Knowledge of a specific molecular alteration associated with metastasis would enable assessment of the risk of tumor dissemination by investigating biopsy material prior to surgery. Due to the relatively small number of events, however, further confirmation of these results is required.

In addition to diagnostic and clinical considerations, the biological implications of our findings in tumorigenesis of pulmonary NE tumors should also be discussed. Both miR-21 and miR-155 are well known as 'oncomirs'.¹¹ In this study, miR-21 and miR-155 levels were high in almost all the samples, although with a moderate degree of variability. In this regard, it is intriguing that miR-21 and miR-155 overexpression appears to be a common event in both carcinoid tumors and high-grade NE carcinomas. miR-21, located in chromosome 17q23.1, is the most commonly overexpressed oncogenic miRNA among human cancers from various organs, including the lung.²³ Although the detailed molecular mechanisms underlying such oncogenic functions of

the overexpressed miR-21 remain to be clarified, previous studies have identified several tumor suppressors as molecular targets of miR-21, including tropomyosin 1, programmed cell death 4, PTEN, tissue inhibitor of metalloproteinase 3, and Maspin.^{24–26} It is notable that a high miR-21 expression was associated with a poorer prognosis of pancreatic ductal adenocarcinoma patients.²⁶ In this study, we found a significant correlation between the overexpression of miR-21 and lymph node metastasis of carcinoid tumors. Furthermore, the expression level of miR-21 was significantly higher in high-grade NE carcinomas than in carcinoid tumors. Our finding would suggest that the downstream effects of miR-21 overexpression could be pivotal in establishing a complete malignant phenotype. It is known that miR-155 is a downstream effector of transforming growth factor β (TGF- β) induced RhoA expression, contributing to TGF- β -induced epithelial-mesenchymal transition, tight junction dissociation, cell migration, and invasion.²⁷ In this context, our study revealed that the expression level of miR-155 was higher in invasive, high-grade NE carcinomas than in carcinoid tumors. Further functional analysis of the target molecules regulated by miR-21 or miR-155 could also be of help to understand the tumorigenesis of pulmonary NE tumors.

In contrast, let-7 is considered to be a tumor suppressor gene that reduces cancer cell growth, and it is underexpressed in malignant tumors.¹⁷ Enhanced let-7 expression leads to decreased high-mobility group A2 expression, a chromatin-remodeling protein that activates proinvasive and prometastatic genes.²⁸ Loss or reduction of let-7 also leads to Ras overexpression, thus, promoting cellular growth and contributing to tumorigenesis.²⁹ Unfortunately, the expression level of let-7a was not statistically different between the histologic subtypes of pulmonary NE tumors and showed no significant differences according to clinicopathologic factors both in carcinoid tumors and high-grade NE carcinomas in this study. These results can be explained by a previous study, in which the same miRNAs had oncogenic or tumor suppressor activity, depending on the tissue or cell type in which they were expressed.^{10,11}

In conclusion, the miR-21 and miR-155 were differentially expressed according to the histologic subtypes of pulmonary NE tumors, and the expression level of miR-21 was significantly higher in carcinoid tumors with lymph node metastasis than in carcinoid tumors without lymph node metastasis. To the best of our knowledge, the present study is the first to examine the expression patterns of miRNAs as an adjunctive diagnostic tool or clinically relevant biomarkers for pulmonary NE tumors. Taking into account the great importance of miRNAs in tumors, we believe that our results will be of importance for both clinical researchers and those who design miRNA-based novel therapeutics. However, further studies with a large number of cases, especially ones including more ACs, and identification of additional miRNAs whose

aberrations can be specific to pulmonary NE tumors are needed to validate our findings.

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