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MicroRNA-374a Expression as a Prognostic Biomarker in Lung Adenocarcinoma

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Background: Lung cancer is the most common cause of cancer-related death, and adenocarcinoma is the most common histologic subtype. MicroRNA is a small non-coding RNA that inhibits multiple target gene expression at the post-transcriptional level and is commonly dysregulated in malignant tumors. The purpose of this study was to analyze the expression of microRNA-374a (miR-374a) in lung adenocarcinoma and correlate its expression with various clinicopathological characteristics. **Methods:** The expression level of miR-374a was measured in 111 formalin-fixed paraffin-embedded lung adenocarcinoma tissues using reverse transcription-quantitative polymerase chain reaction assays. The correlation between miR-374a expression and clinicopathological parameters, including clinical outcome, was further analyzed. **Results:** High miR-374 expression was correlated with advanced pT category (chi-square test, $p=.004$) and pleural invasion (chi-square test, $p=.034$). Survival analysis revealed that patients with high miR-374a expression had significantly shorter disease-free survival relative to those with low miR-374a expression (log-rank test, $p=.032$). **Conclusions:** miR-374a expression may serve as a potential prognostic biomarker for predicting recurrence in early stage lung adenocarcinoma after curative surgery.

Key Words: Lung neoplasms; Adenocarcinoma; Stage; Recurrence; MicroRNAs

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Lung cancer is one of the most frequently diagnosed cancers and is a leading cause of morbidity and mortality globally.¹ Of the various lung cancer types, the most common histologic subtype is adenocarcinoma, which accounts for 40%–50% of diagnoses.² Despite recent advances in immunotherapy and targeted therapy, patients with lung cancer still have a poor prognosis with an average 5-year survival rate of about 55%, even when diagnosed at stage I.¹

MicroRNA (miRNA) is a single-stranded, non-coding, regulatory RNA of 19–25 nucleotides in length.³ miRNAs are one of the components of the RNA-induced silencing complex that inhibits target gene expression either by causing mRNA degradation or suppressing mRNA translation.⁴ As miRNAs can bind mRNA by incomplete complementarity of sequences, a single miRNA can regulate multiple target genes.⁴ Previous miRNA expression profiling studies have shown that up- and down-regulation of miRNA expression is commonly observed in malignant tumor tissues compared to normal counterparts.⁵ This miRNA dysregulation is involved in carcinogenesis and tumor progression.⁵

miRNA expression profiling studies of various human malignancies have identified potential biomarkers for early diagnosis, classification, prognosis, and therapeutic response.⁶

MicroRNA-374a (miR-374a) has been investigated due to its involvement in the carcinogenesis and tumor progression of solid tumors and hematologic malignancies. Previous studies demonstrated that miR-374a promotes cell proliferation and metastasis of osteosarcoma, hepatocellular carcinoma, pancreatic cancer, breast cancer, and esophageal cancer by targeting various tumor suppressors, including Axin2, MIG-6, SRCIN1, LACTB, and ARRB1.^{7–13} However, some studies demonstrated the tumor suppressive role of miR-374a in colon cancer, T-cell lymphoblastic lymphoma, and non-small cell lung cancer (NSCLC) by targeting cyclin D1, PTEN, and AKT.^{14–16}

In this study, miR-374a expression was measured using the quantitative real-time polymerase chain reaction (qRT-PCR) technique with RNA extracted from formalin-fixed, paraffin-embedded (FFPE) tumor tissues of 111 lung adenocarcinoma samples. The expression of miR-374a was correlated with con-

ventional clinicopathologic parameters as well as patient survival.

MATERIALS AND METHODS

Patients and tumor samples

A total of 184 consecutive cases of curative surgery for primary lung adenocarcinoma conducted at Hanyang University Hospital, Seoul, Korea, from 2003 to 2014 were retrospectively selected. Of the total 184 cases, 37 (20.1%) were excluded due to the lack of a suitable FFPE sample and 36 of the remaining patients were excluded because of the poor quality of FFPE RNAs. Finally, qRT-PCR for miR-374a and the U6 control was successful in 111 cases and further statistical analyses were performed. None of the patients received preoperative therapy. Clinicopathological data were collected and reviewed from the medical records and histopathological reports, and additional review of archived pathologic slides was conducted. The clinicopathological parameters included patient age, sex, tumor size, T category, pleural invasion, lymph node metastasis, American Joint Committee on Cancer (AJCC) tumor stage, histological grade, lymphovascular invasion, and perineural invasion. Histologic grading was determined by conventional histological criteria, including architectural abnormalities and cytologic atypia. Most cases with histologic grade 1 were categorized as lepidic predominant subtype, cases with grade 2 were categorized as acinar or papillary predominant subtype, and cases with grade 3 were categorized as solid or micropapillary predominant subtype based on the new 2015 World Health Organization classification. Disease-free survival (DFS) was measured from the date of operation until recurrence or death. Overall survival (OS) was measured from the date of operation until the time of death or the last follow-up.

RNA extraction and qRT-PCR

To identify areas most representative of tumors and non-necrotic tumor sections, we reviewed and marked histologic slides. Three or four 10- μ m-thick tissue sections from each block were collected. Total RNA was isolated from the FFPE tumor tissues using miRNeasy FFPE kits (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The concentration and purity of extracted RNA was measured using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Waltham, MA, USA).

Universal cDNA synthesis kits (Exiqon, Vedbaek, Denmark) were used to convert RNA into cDNA. qRT-PCR experiments were performed with a mixture of diluted cDNA samples, an miR-374a specific primer set (Exiqon), and an ExiLENT SYBR

Green Master mix (Exiqon). PCR was performed using a CFX96 thermocycler (Bio-Rad, Hercules, CA, USA) under the following conditions: 95°C for 15 minutes followed by 45 cycles of 95°C for 10 seconds and 60°C for 1 minute. After PCR amplification, melting curve analysis was performed to confirm the specificity of PCR products. The expression level of miR-374a was calculated using the 2- Δ Ct method relative to the U6 small nuclear RNA (RNU6B).

Statistical analysis

Statistical analysis was performed using SPSS ver. 21.0 software (IBM Corp., Armonk, NY, USA). Chi-square tests were used to evaluate associations between miR-374a expression and various clinicopathological parameters of pulmonary adenocarcinoma patients. Kaplan-Meier survival curves for both DFS and OS were plotted, and the log-rank test was applied to establish the level of significance. The Cox proportional hazard regression model was employed in both univariable and multivariable survival analyses. A p-value of <.05 was defined as statistically significant.

Ethics statement

This study was approved by the Institutional Review Board (IRB) of Hanyang University Hospital (IRB file no. 2016-07-038) with a waiver of informed consent.

RESULTS

Clinicopathological characteristics of patients enrolled in the study

Two-thirds of patients were over 60 years of age (n = 68, 61.3%) and more females than men (n = 65, 58.6%) were included in the study. Most of the cases were categorized as histologic grade 2 (n = 78, 69.6%) and AJCC stage I (n = 76, 68.5%). Lymph node metastasis was found in 25 cases (22.5%), pleural invasion in 46 cases (41.4%), lymphovascular invasion in 40 cases (36.0%), and perineural invasion in 21 cases (18.9%). The clinicopathological characteristics are summarized in Table 1.

Correlations between miR-374a expression and clinicopathological characteristics in pulmonary adenocarcinoma

The quantitative measures of relative miR-374a expression in all 111 lung adenocarcinoma samples were as follows: mean, 0.048; median, 0.066; standard deviation, 1.585; and range, 0.000–11.794. The patients were divided into two groups (low

Table 1. Summary of clinicopathological characteristics in pulmonary adenocarcinoma patients

Characteristic	No. (%) (n=111)
Age (yr)	
<60	43 (38.7)
≥60	68 (61.3)
Sex	
Male	46 (41.4)
Female	65 (58.6)
Histological grade	
Grade 1	23 (20.7)
Grade 2	78 (70.3)
Grade 3	10 (9.0)
T category	
pT1	51 (45.9)
pT2	53 (47.7)
pT3	5 (4.5)
pT4	2 (1.8)
AJCC stage group	
I	76 (68.5)
II	17 (15.3)
III	18 (16.2)
Lymph node metastasis	
pN0	86 (77.5)
pN1	10 (9.0)
pN2	13 (11.7)
pN3	2 (1.8)
Pleural invasion	
PL0	65 (58.6)
PL1	35 (31.5)
PL2	10 (9.0)
PL3	1 (0.9)
Lymphovascular invasion	
Absent	71 (64.0)
Present	40 (36.0)
Perineural invasion	
Absent	90 (81.1)
Present	21 (18.9)

AJCC, American Joint Committee on Cancer.

vs high) according to the median value of miR-374a expression. We analyzed the associations between miR-374a expression and clinicopathological characteristics, including age, sex, AJCC stage group, primary tumor (T category), lymph node metastasis, pleural invasion, histologic grade, lymphovascular invasion, and perineural invasion. High miR-374a expression was correlated with advanced T category (chi-square test, $p = .004$) and the presence of pleural invasion (chi-square test, $p = .034$) (Table 2).

Prognostic value of miR-374a expression in pulmonary adenocarcinoma

The median follow-up interval of patients for OS and DFS was 27.3 months (range, 0.9 to 118.6 months) and 25.7 months

(range, 0.9 to 85.0 months), respectively. Local recurrence or metastasis was found in 18 patients, and 9 patients died during the follow-up period. On univariate survival analysis, AJCC stage, T category, lymph node metastasis, histologic grade, lymphovascular invasion, and perineural invasion were revealed as prognostic factors for DFS and/or OS (Table 3). Patients with high miR-374a expression showed shorter DFS compared to those with low miR-374a expression, and the difference was statistically significant (log-rank test, $p = .032$) (Fig. 1). However, miR-374a expression level was not an independent prognostic factor in multivariable survival analysis. The OS of the patients with high miR-374a expression was also inferior to those with low expression; however, the difference failed to demonstrate a statistical significance. In addition, stage-stratified survival analyses revealed that high miR-374a expression was significantly associated with shorter DFS only in patients with AJCC stage I (log-rank test, $p = .006$).

DISCUSSION

Fresh frozen tissue has been mostly used for high-throughput miRNA expression profiling for human cancers. However, sampling and long-term storage of fresh tumor tissue is laborious and expensive in daily clinical practice. As tumor specimens are routinely stored as FFPE blocks after diagnostic process in pathology archives and can be linked with medical records, emerging interest has been directed toward whether FFPE tissue can be used for studies of miRNA biomarkers. Interestingly, miRNAs appear to be stable in FFPE samples, even exhibiting near total mRNA degradation.¹⁷ Preservation of miRNA in FFPE tumor samples has been confirmed in previous studies, and the results obtained from qRT-PCR analyses, miRNA expression profiling, and even next-generation sequencing studies are consistent with those of fresh frozen tissue.¹⁸⁻²¹ These results suggest that quantifying miRNA expression level using FFPE tissue can enable the identification of clinically-available miRNA biomarkers in human malignancies.

One of the clinical significances of measuring miRNA expression level in resected tumor tissues is to identify patients at high risk of recurrence or cancer-related death after curative surgery. Zheng et al.²² reported that NSCLC patients with low miR-195 expression showed poor overall survival. In the study of Chen et al.,²³ low miRNA-148a expression was significantly correlated with high histologic grade, frequent lymph node metastasis, and a poor OS in NSCLC. Further, miR-21, as an oncogenic miRNA, has been reported as a biomarker for predicting recur-

rence, metastasis, and resistance of radiation and chemotherapy in NSCLC.^{24–26} In our study, high miR-374a expression correlated with advanced pT category, pleural invasion, and poor DFS in lung adenocarcinoma. Zhao et al.¹⁶ investigated miR-374a expression level in 158 NSCLC tissues by *in situ* hybridization.

They found that miR-374a was highly expressed in NSCLC relative to non-neoplastic tissues and correlated with lymph node metastasis and poor clinical outcome.¹⁶ However, in the study of 38 Estonian NSCLC cases (18 squamous cell carcinomas and 20 adenocarcinomas), low miR-374a expression, as by miRNA

Table 2. Correlation between miR-374a expression and various clinicopathological factors in lung adenocarcinoma (n=111)

Clinicopathological characteristic	No.	miR-374a expression		Chi-square test p-value
		Low	High	
Age (yr)				.437
<60	43	19 (44.2)	24 (55.8)	
≥60	68	36 (52.9)	32 (47.1)	
Sex				.337
Male	46	20 (43.5)	26 (56.5)	
Female	65	35 (53.8)	30 (46.2)	
AJCC stage group				.221
I	76	41 (53.9)	35 (46.1)	
II, III	35	14 (40.0)	21 (60.0)	
Primary tumor				.004
pT1	51	33 (64.7)	18 (35.3)	
pT2, pT3, pT4	60	22 (36.7)	38 (63.3)	
LN metastasis				>.99
Negative	86	43 (50.0)	43 (50.0)	
Positive	25	12 (48.0)	13 (52.0)	
Pleural invasion				.034
Negative	65	38 (58.5)	27 (41.5)	
Positive	46	17 (37.0)	29 (63.0)	
Histological grade				.490
Grade 1	23	13 (56.5)	10 (43.5)	
Grade 2 and 3	88	42 (47.7)	46 (52.3)	
Lymphovascular invasion				.695
Absent	71	34 (47.9)	37 (52.1)	
Present	40	21 (52.5)	19 (47.5)	
Perineural invasion				.234
Absent	90	42 (46.7)	48 (53.3)	
Present	21	13 (61.9)	8 (38.1)	

Values are presented as number (%).

AJCC, American Joint Committee on Cancer; LN, lymph node.

Table 3. Univariate Cox regression analysis of clinicopathological parameters and patient survival in lung adenocarcinoma

	Disease-free survival			Overall survival		
	HR	95% CI	p-value	HR	95% CI	p-value
Age (<60 yr vs ≥60 yr)	1.129	0.607–1.129	.702	2.231	0.856–5.817	.101
Sex (male vs female)	0.694	0.376–1.282	.244	0.469	0.200–1.099	.081
AJCC stage (I vs II, III and IV)	4.975	2.535–9.764	<.001	4.606	1.800–11.789	.001
T category (pT1 vs pT2, pT3, and pT4)	2.661	1.391–5.089	.003	4.663	1.713–12.694	.003
LN metastasis (absent vs present)	4.314	2.313–8.045	<.001	3.087	1.331–7.157	.009
Pleural invasion (absent vs present)	1.397	0.702–2.782	.341	3.488	1.406–8.656	.007
Histologic grade (grade 1 vs grade 2 and 3)	3.667	1.130–11.898	.030	2.367	0.552–10.149	.246
LVI (absent vs present)	3.969	2.053–7.673	<.001	3.677	1.488–9.085	.005
Perineural invasion (absent vs present)	2.363	1.172–4.762	.016	3.855	1.524–9.751	.004
miR-374a expression (low vs high)	2.740	1.054–7.125	.039	1.485	0.395–5.590	.559
miR-374a (low vs high), stage I only	5.676	1.434–22.462	.013	2.844	0.464–17.433	.250

HR, hazard ratio; CI, confidence interval; AJCC, American Joint Committee on Cancer; LN, lymph node; LVI, lymphovascular invasion.

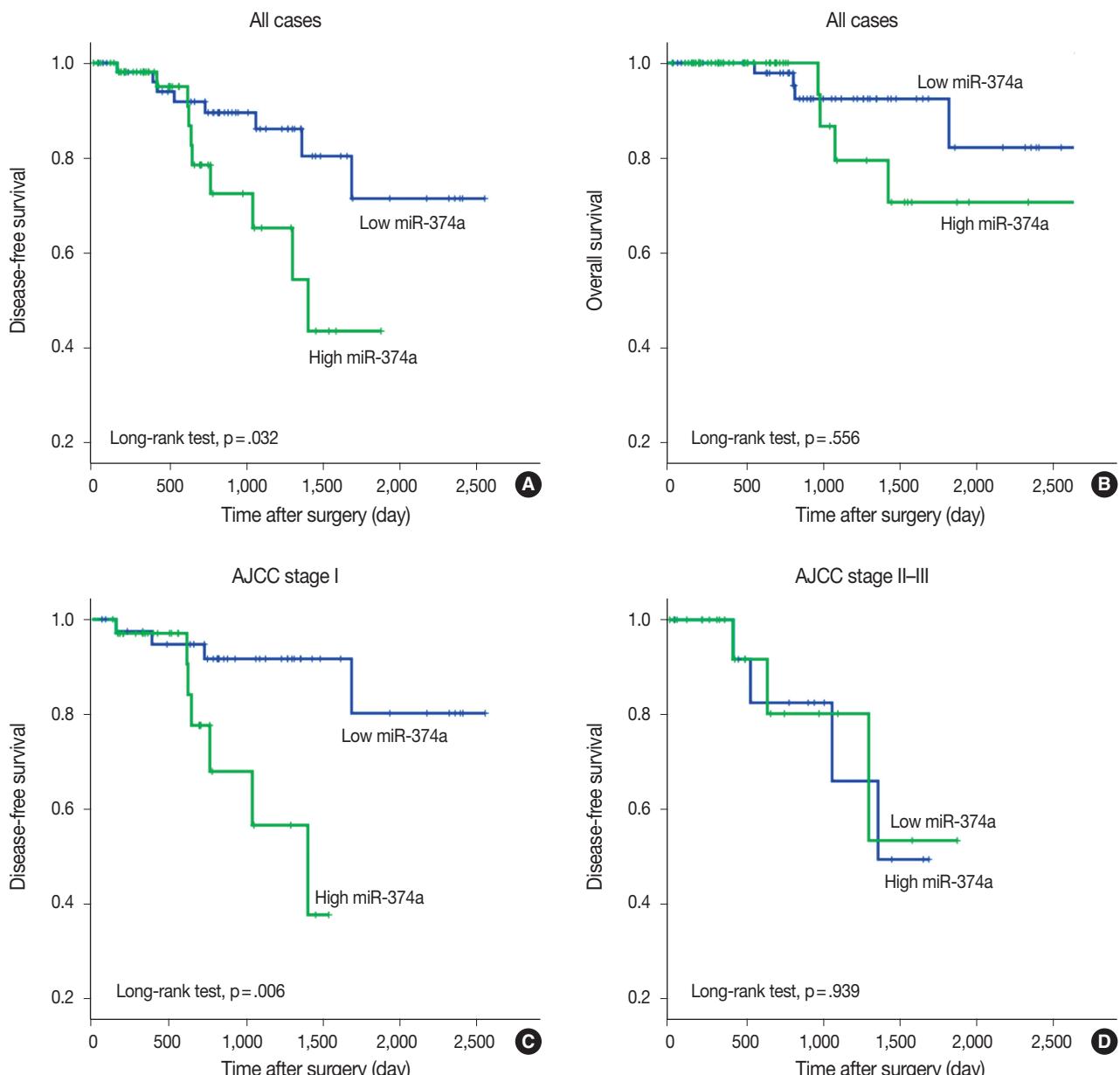


Fig. 1. Kaplan-Meier survival curves of lung adenocarcinoma patients stratified based on miR-374a expression level. (A) Disease-free survival according to miR-374a expression (log-rank test, $p=.032$). (B) Overall survival according to miR-374a expression (log-rank test, $p=.556$). (C) Disease-free survival according to miR-374a expression only in American Joint Committee on Cancer (AJCC) stage I patients (log-rank test, $p=.006$). (D) Disease-free survival according to miR-374a expression in AJCC stage II-III (log-rank test, $p=.939$).

microarray, was associated with poor survival. This discrepancy may result from the difference in miRNA detection methods and study population, such as ethnicity and histologic type. Lack of reproducibility between studies suggests that further validation with a larger case series and optimization of detection methods is needed for clarification.

Most previous studies have reported miR-374a as an oncogenic miRNA in various cancer types. In studies with osteosarcoma

cell lines, tumor cells transiently transfected with miR-374a mimic showed an increase in cell proliferation and colony formation by downregulating FOXO1 and/or Axin2.^{7,27} Downregulation of miR-374a inhibited cell proliferation, migration, and invasion in breast cancer cell lines (MDA-MB-23a and MCF-7) by targeting LACTB.¹¹ In triple-negative breast cancer, ARRB1 was a direct target of miR-374a, and knockdown of miR-374a attenuated cell proliferation and migration in vitro.¹³ Down-

regulation of miR-374a inhibited tumor growth and decreased Ki-67 proliferation index in xenograft mice.¹³ However, some studies have reported miR-374a as a tumor suppressive miRNA. Induced overexpression of miR-374a in colon cancer cell lines inhibited cell proliferation and invasion based on in vitro assays and a xenograft model by targeting cyclin D1.¹⁴ Nasopharyngeal cancer cells transfected with miR-374a mimic inhibited cell proliferation and invasion in vitro, and showed a decreased number of metastatic lesions in a mouse model.²⁸ These results suggest that miR-374a may have dual roles in tumor progression, and the role depends on the major target pathway in a given cancer subtype.

In conclusion, our data show that high miR-374a expression correlates with advanced pT category and pleural invasion. High expression of miR-374a may serve as a potential prognostic biomarker for predicting recurrence in early stage lung adenocarcinoma after curative surgery. Further validation studies for a prognostic biomarker and related mechanism should be explored.

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Conflicts of Interest

The authors declare that they have no potential conflicts of

interest.

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