The prognostic impact of microRNA sequence polymorphisms on the recurrence of patients with completely resected non-small cell lung cancer

Kyong-Ah Yoon, PhD,^a Hyekyoung Yoon, MS,^a Sohee Park, PhD,^b Hee-Jin Jang, MD,^c Jae Ill Zo, MD, PhD,^{a,c} Hyun-Sung Lee, MD, PhD,^{a,c} and Jin Soo Lee, MD, PhD^{a,c}

Objectives: MicroRNAs (miRNAs) are widely known for their function as regulators of gene expression via translational repression. Polymorphisms in miRNAs have been shown to affect the regulatory capacity of miRNAs by influencing miRNA processing and/or miRNA-mRNA interactions. The purpose of this study was to investigate the association between 7 single nucleotide polymorphisms (SNPs) commonly found in precursor miRNA (pre-miRNA) and primary miRNA (pri-miRNA) sequences and the recurrence of disease in patients who underwent a complete resection of non-small cell lung cancer (NSCLC).

Methods: Five SNPs found in pre-miRNAs (rs11614913/miR-196a2, rs2910164/miR-146a, rs6505162/miR-423, rs2289030/miR-492, and rs895819/miR-27a) and 2 SNPs found in pri-miRNAs (rs7372209/miR-26a-1 and rs213210/miR-219-1) were genotyped in 388 patients with NSCLC.

Results: Among 388 patients, variants of the rs2910164 SNP were significantly associated with recurrence-free survival (RFS) ($P = .016$, log-rank test). When the results were subdivided by the tumor stage, variants of the rs2910164 and rs11614913 SNPs positively correlated with a better RFS (adjusted hazard ratio [HR], 0.48; 95% confidence interval [CI], 0.28-0.80; adjusted HR, 0.60; 95% CI, 0.38-0.94, respectively) in patients with stage II and stage III disease. Moreover, RFS significantly improved in patients with higher numbers of variant alleles in the rs2910164 and rs11614913 SNPs.

Conclusions: Our findings suggest that polymorphisms in the rs2910164 of miR-146a and the rs11614913 of miR-196a2 are associated with prognosis in patients with completely resected NSCLC. (J Thorac Cardiovasc Surg 2012;144:794-807)

MicroRNAs (miRNA) are endogenous, small, noncoding RNAs that play important roles in the posttranscriptional regulation of messenger RNA (mRNA) expression.^{1,2} Mature miRNAs are generated from precursor miRNAs (pre-miRNAs), which are the processed products of the longer primary miRNA (pri-miRNA) transcripts.^{3,4} MicroRNAs regulate gene expression by translational inhibition or mRNA cleavage, and these mechanisms are dependent on complete or nearly complete sequence complementarity.² Many studies have reported that the dysfunction of miRNAs that target oncogenic or tumor suppressor activity can influence cancer development.⁵⁻⁸ The potential roles of miRNAs in cancer cells have been

suggested by various reports that have characterized the cellular functions of miRNAs as well as by miRNA expression profiling in various cancers. For example, the first miRNA to be associated with lung cancer was *let-7*, and decreased *let-7* expression was shown to lead to an increase in the expression level of its target RAS.^{9,10}

Recently, several miRNA polymorphisms have been reported to modify the regulatory capacity of their respective miRNA by affecting miRNA processing and/or miRNA-mRNA interactions.^{11,12} With respect to cancer, miRNA single nucleotide polymorphisms (SNPs) can be used as genetic markers to predict cancer susceptibility and prognosis. For example, the pri-miRNA SNP rs213210 found in miR-219-1 correlates with an increased risk for esophageal cancer,¹³ and the variants of the rs7372209 SNP found in miR-26a-1 have been associated with the prognosis for colon cancer.¹⁴ The SNPs rs2289030 found in pre-miR-492 and the SNP rs6505162 found in pre-miR-423 have been correlated with an increased risk for bladder cancer in white subjects.¹⁵ The pre-miRNA SNP rs895819 found in pre-miR-27a has also been shown to be related to gastric cancer susceptibility and a reduced risk for familial breast cancer.^{16,17} Two functional polymorphisms, rs11614913 in miR-196a2 and rs2910164 in miR-146a, also appear to contribute to the clinical

From the Lung Cancer Branch, Research Institute and Hospital,^a the National Cancer Control Research Institute,^b and the Center for Lung Cancer, Research Institute and Hospital,^c National Cancer Center, Goyang, Gyeonggi, Republic of Korea. This work was supported by National Cancer Center Research Grant No. 1010040 and No.1110260.

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Address for reprints: Jin Soo Lee, MD, PhD or Hyun-Sung Lee, MD, PhD, Research Institute and Hospital, National Cancer Center, 323 Ilsan-ro, Ilsandong-gu, Goyang, Gyeonggi, 410-769, Korea (E-mail: jslee@ncc.re.kr or thoracic@ncc.re.kr).

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Abbreviations and Acronyms

CI	= confidence interval
HR	= hazard ratio
miRNA	= microRNA
mRNA	= messenger RNA
NSCLC	= non-small cell lung cancer
pre-miRNA	= precursor microRNA
pri-miRNA	= primary microRNA
RFS	= recurrence-free survival
SNP	= single nucleotide polymorphism

outcome of various cancers, suggesting that mature miRNAs have regulatory roles as well.^{17,18} To evaluate the association between miRNA polymorphisms and patient prognosis of non-small cell lung cancer (NSCLC), we evaluated whether 7 SNPs in various pre-miRNAs and pri-miRNAs could predict the clinical outcomes of patients who underwent complete resection for NSCLC.

METHODS**Study Population**

This study included 388 patients who had undergone surgical resections for NSCLC at the National Cancer Center in Korea from 2001 to 2008, and follow-up information was available for every patient. The clinicopathologic characteristics of the patients with lung cancer are shown in Table 1. We retrospectively reviewed the medical and pathology records for each patient. Chest computed tomography, positron emission tomography, bronchoscopy, and pulmonary function tests were performed preoperatively. Chest computed tomography scans were obtained postoperatively at 3-month intervals, and positron emission tomography-computed tomography scans were obtained annually to detect recurrence of the tumor. The patients were histologically diagnosed with pathologic stage I, II, or IIIA adenocarcinoma or squamous cell carcinoma after the pulmonary resection and complete lymph node dissection. Criteria for patient exclusion included patients who underwent a limited resection such as a wedge resection or segmentectomy and patients who underwent neoadjuvant chemotherapy treatment. The patients voluntarily donated blood for genetic tests after signing an informed consent form, which was previously approved by the institutional review board.

The recurrence-free survival (RFS) rate was calculated from the date of surgery to the date of recurrence or to the date of the last follow-up. Local recurrences were defined as lesions occurring within the resection margins such as bronchial stumps or staple lines. Regional recurrences were defined as lesions that occurred within the hilar or mediastinal lymph nodes, pleural cavity, or ipsilateral lung. Distant recurrences were defined as lesions occurring within the contralateral lung, brain, liver, adrenal glands, bone, or other locations.

Selection and Genotyping of the miRNA Polymorphisms

The candidate miRNA polymorphisms were selected on the basis of a previously identified association with cancer risk or prognosis by conducting a literature review.¹⁰⁻¹⁹ The minor allele frequency was also considered during the selection of the target SNPs. Data from the International HapMap Project was used to select SNPs that have a minor allele frequency greater than 20% in Asians for our analysis. Five SNPs in pre-miRNAs (rs11614913/miR-196a2, rs2910164/miR-146a,

rs6505162/miR-423, rs2289030/miR-492, and rs895819/miR-27a) and 2 SNPs in pri-miRNAs (rs7372209/miR-26a-1 and rs213210/miR-219-1) fit the aforementioned criteria and were selected for genotyping analysis. Genomic DNA was extracted from the peripheral blood using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, Calif) according to the manufacturer's instructions. Genotyping was conducted with TaqMan probes using a 5'-nuclease assay (Applied Biosystems, Foster City, Calif). To confirm the reproducibility of our method, 50 samples were genotyped in duplicate, and all samples showed concordant results.

Statistical Analysis

Patients who currently smoked or who smoked more than 100 cigarettes during their lifetime were defined as ever-smokers. The Hardy-Weinberg equilibrium of each allele was calculated to ensure that the genotyping results were reliable. The correlation between miRNA polymorphisms and clinicopathologic characteristics was analyzed using the χ^2 test. We analyzed the association between the miRNA polymorphisms and NSCLC prognosis with respect to the cancer stage. The RFS rates were assessed using the Kaplan-Meier product limit method, and the trend among the miRNA polymorphisms was estimated with the log-rank test. The hazard ratios (HRs) and the associated 95% confidence intervals (CIs) were derived from a Cox proportional-hazards regression model. The statistically significant ($P < .05$) covariates from the log-rank test (age, gender, smoking status, histologic tumor type, stage, and postoperative complications) were further analyzed after adjustment in a multivariable Cox proportional-hazards regression model using RFS as the dependent variable. We also analyzed statistically significant polymorphisms that occurred in combination with other polymorphisms, and individuals were represented by categories for the number of variant alleles present in the polymorphisms studied. All of the reported P values were 2-sided. Stata/SE version 11 (StataCorp LP, College Station, Tex) was used for all statistical analyses.

RESULTS**Patient Characteristics**

Tumor recurrence was assessed in 388 patients who had undergone surgical resection of the lung and had clinical information available for review. The ever-smokers and patients with early-stage (stage IA/IB) cancer and adenocarcinoma were dominant in this group. We observed recurrence in 71 patients over a median follow-up period of 36.8 months. By univariate analysis, gender, smoking status, histology, and pathologic tumor stage were all significantly associated with the survival outcome (log-rank $P < .05$) (Table 1). These factors and notable clinical factors such as age and postoperative complication were adjusted as covariates in a multivariate Cox regression analysis. Specifically, the patients with advanced-stage disease had a significantly poorer clinical outcome (HR, 3.56; 95% CI, 2.21-5.74) when compared with the patients with stage I cancer. Adjuvant chemotherapy was not associated with RFS. The patients with lung squamous cell carcinoma had a higher risk of death than did those with adenocarcinoma (HR, 2.00; 95% CI, 1.25-3.20).

The Effects of miRNA Polymorphisms on Clinicopathologic Characteristics

We conducted stratified and interaction analyses of 7 miRNA polymorphisms with the major clinicopathologic

TABLE 1. Demographic characteristics of the patients with lung cancer

Characteristic	Cases		Recurrence		
			No. of events*	HR (95% CI)	Log-rank test, <i>P</i> value
Total, no.	388		71		
Age, y					.633
Mean $\pm$ SD	62.7 $\pm$ 9.2	<63.5‡	36 (19)	Reference	
Median (range)	63.5 (34-85)	$\geq$ 63.5‡	35 (18)	1.12 (0.70-1.79)	
Gender, no.					.017
Male, n (%)	259 (67)		57 (22)	Reference	
Female, n (%)	129 (33)		14 (11)	0.49 (0.27-0.88)	
Smoking status, no.					.047
Never, n (%)	137 (35)		17 (12)	Reference	
Ever, n (%)	251 (65)		54 (22)	1.74 (1.01-3.00)	
Histology, no.					.004
Adenocarcinoma, n (%)	242 (62)		32 (13)	Reference	
Squamous cell carcinoma, n (%)	146 (38)		39 (27)	2.00 (1.25-3.20)	
Pathologic atage (AJCC 7th edition)					<.001
IA, n (%)	135 (35)		7 (5)	Reference	
IB, n (%)	116 (30)		21 (18)	4.31 (1.83-10.16)	
IIA, n (%)	43 (11)		11 (26)	5.79 (2.24-14.94)	
IIB, n (%)	24 (6)		6 (25)	6.24 (2.09-18.59)	
IIIA, n (%)	70 (18)		26 (37)	11.60 (5.02-26.80)	
Postoperative complication					.133
No, n (%)	297 (77)		52 (18)	Reference	
Yes, n (%)	91 (23)		19 (21)	1.50 (0.88-2.55)	
Adjuvant chemotherapy					.495
No, n (%)	303 (78)		54 (18)	Reference	
Yes, n (%)	85 (22)		17 (20)	1.21 (0.70-2.10)	
Follow-up time, mo†					
Median (95% CI)			36.8 (39.5-44.8)		
Range			(0.1-108.9)		

HR, Hazard ratio; CI, confidence interval; SD, standard deviation; AJCC, American Joint Committee on Cancer. *Number of recurrences. †The median follow-up time, 95% confidence interval, and range were calculated among censored observations only. ‡Dichotomized by the median data point.

variables. Age, gender, and smoking were not associated with any of the miRNA polymorphisms (Appendix Table 1). In 108 patients with nodal metastasis, there was no difference between micrometastasis and overt nodal metastasis and any of the miRNA polymorphisms. The CC genotype of the rs7372209 SNP was more evident in squamous cell carcinoma ($P = .014$). The CC genotype of the rs2910164 SNP was associated with more advanced pathologic staging ($P = .036$). In the multivariate analysis (Appendix Table 2), the CC genotype of the rs2910164 SNP was more frequently found in patients with stage II and IIIA disease than in those with stage I disease ($P < .01$; HR, 0.48; 95% CI, 0.28-0.80).

The Effects of miRNA Polymorphisms on RFS

The rates for overall survival and RFS were 84.8% and 74.5% in all patients, respectively. The RFS was selected as the primary end point because additional treatment modalities could affect the survival of the patients after cancer recurrence. The association analysis between RFS and miRNA polymorphisms revealed that variants of the rs2910164 polymorphism in miR-146a were associated

with an improved RFS in all of the patients (adjusted HR, 0.65; 95% CI, 0.45-0.93; $P = .019$). Inasmuch as the pathologic stage of the cancer is an important factor that affects the clinical outcome, the effects of the polymorphisms on the RFS in stage I and higher-stage patients (stage II/III) were further evaluated with stratified analyses. As shown in Table 2, two polymorphisms were significantly associated with the survival of patients with stage II and III disease. Specifically, the rs2910164 allele was associated with an increased RFS in patients with a relatively advanced cancer stage (adjusted HR, 0.48; 95% CI, 0.28-0.80; $P = .005$ in an additive model). The Kaplan-Meier analysis revealed that the CC genotype of the rs2910164 SNP was associated with poor survival in patients with stage II and III disease (log-rank $P = .0047$; Figure 1). Additionally, the rs11614913 allele in miR-196a2 reduced the HR for the RFS in patients with advanced-stage cancer (adjusted HR, 0.60; 95% CI, 0.38-0.94; $P = .026$ in an additive model). The cumulative effects of these 2 SNPs were evaluated in the patients, and the rs2910164 and rs11614913 variant alleles exhibited a trend toward associating with a prolonged RFS in patients with all stages of

TABLE 2. The association between the 7 miRNA polymorphisms and the recurrence-free survival according to the pathologic stages of lung cancer

Genotypes	Total (n = 388)					Stage I (A/B)					Stage II (A/B) and stage IIIA				
	No. of pts.	No. relapsed	Log-rank trend test, Adjusted			No. of pts.	No. relapsed	Log-rank trend test, Adjusted			No. of pts.	No. relapsed	Log-rank trend test, Adjusted		
			<i>P</i> value†	HR	(95% CI)*			<i>P</i> value†	HR	(95% CI)*			<i>P</i> value†	HR	(95% CI)*
<i>mir219-1</i> rs213210															
AA	105	17	.56	Ref.		75	7	.515	Ref.		30	10	.91	Ref.	
AG	182	35		1.16	(0.65-2.08)	113	12		1.14	(0.44-2.95)	69	23		1.13	(0.53-2.41)
GG	100	19		1.18	(0.60-2.31)	62	9		1.20	(0.43-3.38)	38	10		0.91	(0.37-2.24)
AG+GG	282	54		1.17	(0.67-2.03)	175	21		1.16	(0.48-2.81)	107	33		1.06	(0.51-2.18)
Trend for G allele				1.09	(0.78-1.51)				1.10	(0.66-1.83)				0.95	(0.62-1.47)
<i>mir27a</i> rs895819															
TT	185	32	.984	Ref.		127	12	.630	Ref.		58	20	.367	Ref.	
CT	158	32		1.03	(0.62-1.72)	98	13		1.46	(0.65-3.29)	60	19		0.93	(0.48-1.84)
CC	45	7		0.81	(0.35-1.85)	26	3		1.20	(0.33-4.41)	19	4		0.64	(0.21-1.92)
CT+CC	203	39		0.98	(0.61-1.60)	124	16		1.4	(0.65-3.03)	79	23		0.86	(0.45-1.65)
Trend for C allele				0.94	(0.66-0.35)				1.20	(0.69-2.07)				0.84	(0.52-1.35)
<i>mir492</i> rs2289030															
GG	237	41	.961	Ref.		154	16	.776	Ref.		83	25	.869	Ref.	
CG	132	28		1.12	(0.69-1.83)	84	12		1.37	(0.64-2.93)	48	16		1.08	(0.57-2.07)
CC	19	2		0.56	(0.14-2.33)	13	0		NA		6	2		1.09	(0.25-4.69)
CG+CC	151	30		1.04	(0.65-1.69)	97	12		1.17	(0.55-2.48)	54	18		1.08	(0.58-2.02)
Trend for C allele				0.96	(0.65-1.44)				0.95	(0.50-1.78)				1.07	(0.63-1.79)
<i>mir146a</i> rs2910164															
CC	177	43	.016	Ref.		104	12	.891	Ref.		73	31	.006	Ref.	
CG	159	20		0.52	(0.31-0.89)	111	11		0.75	(0.32-1.76)	48	9		0.39	(0.19-0.83)
GG	51	8		0.53	(0.24-1.13)	35	5		1.11	(0.38-3.22)	16	3		0.30	(0.09-1.00)
CG+GG	210	28		0.52	(0.32-0.85)	146	16		0.84	(0.39-1.82)	64	12		0.37	(0.19-0.71)
Trend for G allele				0.65	(0.45-0.93)				0.98	(0.57-1.7)				0.48	(0.28-0.80)
<i>mir423</i> rs6505162															
CC	244	44	.887	Ref.		157	16	.691	Ref.		87	28	.866	Ref.	
AC	120	24		1.18	(0.71-1.95)	78	11		1.41	(0.65-3.08)	42	13		1.02	(0.52-2.00)
AA	21	3		0.69	(0.21-2.24)	14	1		1.02	(0.13-7.97)	7	2		0.81	(0.19-3.41)
AC+AA	141	27		1.09	(0.67-1.77)	92	12		1.37	(0.64-2.94)	49	15		0.98	(0.51-1.87)
Trend for A allele				1.00	(0.68-1.47)				1.24	(0.65-2.33)				0.96	(0.57-1.61)
<i>mir26a-1</i> rs7372209															
CC	204	36	.606	Ref.		128	13	.639	Ref.		76	23	.715	Ref.	
CT	154	28		1.16	(0.70-1.91)	105	13		1.29	(0.59-2.83)	49	15		1.03	(0.53-2.02)
TT	30	7		1.14	(0.50-2.62)	18	2		1.64	(0.36-7.47)	12	5		1.34	(0.50-3.62)
CT+TT	184	35		1.16	(0.72-1.86)	123	15		1.33	(0.62-2.84)	61	20		1.10	(0.59-2.03)
Trend for T allele				1.10	(0.77-1.57)				1.28	(0.70-2.36)				1.12	(0.71-1.76)

(Continued)

TABLE 2. Continued

Genotypes	Total (n = 388)					Stage I (A/B)					Stage II (A/B) and stage IIIA					
	No. of pts.	No. relapsed	Log-rank		Adjusted HR	(95% CI)*	No. of pts.	No. relapsed	Log-rank		Adjusted HR	(95% CI)*	No. of pts.	No. relapsed	Log-rank	
			trend test, P value†	Adjusted HR					trend test, P value†	Adjusted HR					trend test, P value†	Adjusted HR
<i>mir196a2</i> rs11614913																
TT	99	24	.087	Ref.			63	7	.747	Ref.			36	17	.019	Ref.
CT	186	32		0.66	(0.39-1.14)		116	12		0.77	(0.30-1.99)		70	20		0.58 (0.30-1.14)
CC	101	15		0.67	(0.35-1.29)		72	9		1.01	(0.37-2.77)		29	6		0.37 (0.14-0.95)
CT+CC	287	47		0.67	(0.40-1.10)		188	21		0.86	(0.36-2.06)		99	26		0.51 (0.27-0.96)
Trend for C allele				0.80	(0.57-1.12)					1.02	(0.60-1.74)					0.60 (0.38-0.94)

mir196a2, MicroRNA; HR, hazard ratio; CI, confidence interval; Ref., reference. *The HRs and 95% CIs were derived from a Cox proportional-hazard regression model controlling for age, gender, smoking status, histologic tumor type, stage, and postoperative complications as covariates. †Trend for an increasing number of allele polymorphisms using the log-rank test.

cancer (adjusted HR, 0.74; 95% CI, 0.59-0.93; $P = .011$). However, the Kaplan-Meier analysis revealed that the effect of the 2 SNPs was only significant for patients with stage II and III cancer (Figure 2). The patients carrying a greater amount of variant alleles of these 2 SNPs had a significantly prolonged RFS (adjusted HR, 0.57; 95% CI, 0.41-0.78; $P = .0005$; Table 3).

Effect of Adjuvant Chemotherapy on Patient Outcome by miRNA SNPs

Platinum-based adjuvant chemotherapy was prescribed in 85 patients at approximately 6 weeks after surgery. The most common treatment modality prescribed was gemcitabine (Gemzar; Eli Lilly, Indianapolis, Ind) plus cisplatin ($n = 32$, 38%). The rs2910164 allele was associated with a significantly altered risk of RFS in patients receiving chemotherapy (Appendix Table 3). In patients who received adjuvant chemotherapy, the recurrence of disease in patients who expressed the variant miRNA allele was 10.6%, whereas the recurrence in the patients carrying the CC genotype was 31.6% ($P = .003$). Interestingly, a similar effect was shown by variant genotypes of rs11614913. In patients who received adjuvant chemotherapy, the recurrence rate in patients harboring the variant genotypes was 12.7%, whereas the survival of the patients carrying the CC genotype was 42.9% ($P = .037$).

The patients who had lymph nodal metastasis ($n = 108$) were dichotomized on the basis of whether micrometastases were discovered after the operation. The Kaplan-Meier analysis revealed that the CC genotype of the rs2910164 allele associates with poor survival in patients with micrometastasis (adjusted HR, 0.117; 95% CI, 0.027-0.495; $P = .004$ in an additive model). However, the sample number was too small to confidently draw conclusions (Appendix Table 4).

DISCUSSION

In this study, polymorphisms in certain miRNAs were significantly associated with lung cancer prognosis in patients who underwent complete resections. Genetic variants in miRNAs have been investigated as genetic markers to predict cancer susceptibility and prognosis. miRNA polymorphisms could have effects on cellular function through the regulation of the primary transcript levels, pre-miRNA and pri-miRNA processing, and the interaction between the miRNA and the target mRNA.¹² Polymorphisms of the miRNA binding site have been suggested to be functional SNPs that affect the binding affinity of miRNAs to their targets. The functional significance of miRNA polymorphisms and the interaction between miRNA and mRNA make it feasible to evaluate the potential associations between miRNA polymorphisms and cancer prognosis.

FIGURE 1. Recurrence-free survival (RFS) of patients according to the rs2910164 genotypes by cancer stage. A, All. B, Stage I. C, Stages II and III. CI, Confidence interval.

In the current study, we analyzed the genotype frequencies and the clinical information from 388 patients to test the effects of 7 published miRNA polymorphisms on lung cancer recurrence. Among the miRNA SNPs analyzed, variants of the rs2910164 polymorphism in miR-146a were strongly associated with RFS in patients with NSCLC. Patients with advanced-stage NSCLC who expressed the variant allele of the rs2910164 locus correlated with an increased RFS. Regarding survival, an association

between the rs2910164 polymorphism and cancer has been reported in esophageal cancer, hepatocellular carcinoma, breast cancer, and thyroid cancer.²⁰⁻²³ The rs2910164 polymorphism represents a functional polymorphism in miR-146a that is located in the 3' miRNA passenger strand and alters the expression of the mature sequence. The variant G allele of the rs2910164 locus was associated with increased expression of mature miR-146a, and increased expression of miR-146a was found to suppress breast cancer metastasis.²³ Recently, a potential therapeutic application of miR-146a was suggested for treating cancer. The inhibitory function of miR-146a on cancer invasion has also been reported in pancreatic cancer cells that overexpress epidermal growth factor receptor and its target, the nuclear factor- κ B regulatory kinase interleukin-1 receptor-associated kinase 1.²⁴ Our results revealed a significant association between the rs2910164 polymorphism and lung cancer prognosis in patients with advanced-stage disease, suggesting that the variant allele and the resultant increase in mature miR-146a levels might be involved in the inhibition of recurrence.

Variants of the rs11614913 polymorphism found in miR-196a2 also exhibited a positive association with the RFS in patients with stage II/III disease. The association of rs11614913 with prognosis has been reported for various types of cancer. For example, patients who harbor the variant TT genotype have significantly reduced overall survival among patients with pharyngeal tumors.²⁵ However, our data show that the TT genotype is associated with a poor RFS in early-stage patients only.

Given that the prognosis of cancer patients likely involves multistep, multigenic pathways, it is unlikely that any single miRNA polymorphism would have a dramatic effect on the survival outcome. Therefore, it is important to conduct pathway-based analyses that assess the combined effects of polymorphisms that might interact in the same pathway. To this end, we used a multigenic approach to evaluate the associations of miRNA SNPs with the survival and outcome of patients with surgically resected NSCLC. Our data reveal that 2 SNPs and their combined genotypes were associated with the survival outcome of patients with NSCLC. These findings suggest that SNPs in miRNAs, and particularly the combined genotypes of several SNPs, can be used as prognostic factors for patients with surgically resected NSCLC.

The most significant finding of this study was that the combined analysis of multiple SNPs in miRNAs that regulate the same or similar pathways had a much higher potential value in terms of revealing polymorphisms of prognostic importance. Although 2 SNPs were associated with the survival outcome in individual SNP analysis, considering the borderline CI and multiple comparisons, the impact of any individual miRNA SNP on survival outcomes is probably minimal. However, when these 2 SNPs were

FIGURE 2. Kaplan-Meier curves showing the recurrence-free survival (RFS) probability according to the number of variant alleles from the combination of the rs2910164 (G allele) and rs11614913 (C allele) polymorphisms by cancer stage. A, Stage I. B, Stages II and III. CI, Confidence interval.

TABLE 3. The additive effects of the combination of rs2910164 (G allele) and rs11614913 (C allele) on the recurrence-free survival according to the clinical stages of lung cancer patients

Pathologic stage	rs2910164 (G allele) & rs11614913 (C allele)		No. of patients	No. relapsed	Recurrence rate, %	Log-rank trend test, P value*	Adjusted		P value
	combined						HR	(95% CI)†	
All	0 variant alleles	49	15	31	.0039	Ref.	Ref.		
	1 variant allele	117	27	23			0.82	(0.43-1.55)	.5368
	2 variant alleles	142	19	13			0.48	(0.24-0.95)	.0351
	3 variant alleles	60	7	12			0.36	(0.15-0.88)	.0258
	4 variant alleles	17	3	18			0.63	(0.18-2.22)	.4755
	Trend						0.74	(0.59-0.93)	.0113
Stage I	0 variant alleles	28	4	14	.7667	Ref.	Ref.		
	1 variant allele	71	7	10			0.95	(0.27-3.31)	.9324
	2 variant alleles	97	10	10			0.79	(0.24-2.60)	.6976
	3 variant alleles	40	4	10			0.68	(0.17-2.79)	.5959
	4 variant alleles	14	3	21			1.56	(0.34-7.13)	.5688
	Trend						1.00	(0.70-1.43)	.9922
Stages II and IIIA	0 variant alleles	21	11	52	.0003	Ref.	Ref.		
	1 variant allele	46	20	43			0.82	(0.39-1.75)	.6126
	2 variant alleles	45	9	20			0.35	(0.14-0.85)	.0208
	3 variant alleles	20	3	15			0.22	(0.06-0.79)	.0207
	4 variant alleles	3	0	0			NA		.9875
	Trend						0.57	(0.41-0.78)	.0005

HR, Hazard ratio; CI, confidence interval; Ref., reference. *Trend for an increasing number of variant alleles. †The HRs and 95% CIs were derived from a Cox proportional-hazard regression model controlling for age, gender, smoking status, histologic tumor type, stage, and postoperative complications as covariates.

combined, the RFS correlated significantly with a higher number of polymorphisms that confer an increased RFS when analyzed individually.

Our study was limited to patients who underwent complete resection without prior treatment and focused on the patient RFS to avoid heterogeneity in the interpretation of the results. However, further biological and/or functional evidence is needed to confirm the genetic effects of these polymorphisms on lung cancer prognosis in these patients. Despite the importance of the protective effect of these miRNA polymorphisms on the RFS of NSCLC patients who underwent complete resections, this study only considered the Korean population, which may limit the application of these findings to other ethnicities. Furthermore, the subset analysis with respect to cancer stage was limited by the sample size, particularly for patients with stages II and III NSCLC.

In conclusion, the results indicated that there is a significant association between miRNA polymorphisms and NSCLC prognosis. These results suggest that the presence of variant alleles in miR-146a and miR-196a2 may serve as prognostic markers for the recurrence of completely resected lung cancer.

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APPENDIX TABLE 1. Association between miRNA polymorphisms and clinicopathologic characteristics

	Age (n = 388)			Gender (n = 388)			Smoking (n = 388)			Histology (n = 388)			P staging (n = 388)			Postop. complication (n = 388)			Nodal metastasis (n = 108)		
	χ^2			χ^2			χ^2			χ^2			χ^2			χ^2			Overt		
	<63.5	≥63.5	P value	M	F	P value	Never	Ever	P value	Adeno	Squamous	P value	I	II/IIIa	P value	No	Yes	P value	Micrometa	meta	P value
rs213210																					
AA	46	59		64	41		45	60		72	33		75	30		85	20		8	15	
AG	93	89		121	61		63	119		113	69		113	69		139	43		20	34	
GG	54	46	.311	74	26	.137	28	72	.082	56	44	.178	62	38	.230	72	28	.319	12	19	.957
AG+GG	147	135	.180	195	87	.161	91	191	.069	169	113	.149	175	107	.111	211	71	.259	32	53	.993
rs895819																					
TT	98	87		129	56		62	123		118	67		127	58		135	50		15	31	
CT	77	81		97	61		59	99		101	57		98	60		125	33		17	28	
CC	19	26	.398	33	12	.160	16	29	.760	23	22	.253	26	19	.259	37	8	.259	8	9	.568
CT+CC	96	107	.309	130	73	.280	75	128	.548	124	79	.657	124	79	.147	162	41	.143	25	37	.536
rs2289030																					
GG	118	119		156	81		81	156		146	91		154	83		178	59		23	39	
CG	68	64		88	44		52	80		86	46		84	48		106	26		15	26	
CC	8	11	.741	15	4	.505	4	15	.248	10	9	.532	13	6	.910	13	6	.366	2	3	.989
CG+CC	76	75	1.00	103	48	.707	56	95	.634	96	55	.777	97	54	.968	119	32	.474	17	29	1.000
rs2910164																					
CC	91	86		116	61		65	112		107	70		104	73		139	38		23	35	
CG	77	82		104	55		59	100		106	53		111	48		121	38		14	23	
GG	25	26	.854	39	12	.298	12	39	.175	28	23	.255	35	16	.087	36	15	.494	3	10	.531
CG+GG	102	108	.649	143	67	.671	71	139	.623	134	76	.566	146	64	.036	157	53	.453	17	33	.684
rs6505162																					
CC	117	127		170	74		80	164		151	93		157	87		183	61		28	37	
AC	61	59		75	45		47	73		75	45		78	42		92	28		9	26	
AA	14	7	.250	13	8	.344	8	13	.466	14	7	.909	14	7	.974	19	2	.276	2	5	.206
AC+AA	75	66	.376	88	53	.178	55	86	.262	89	52	.895	92	49	.946	111	30	.481	11	31	.117
rs7372209																					
CC	103	101		144	60		66	138		115	89		128	76		153	51		24	38	
CT	73	81		96	58		57	97		106	48		105	49		120	34		13	26	
TT	18	12	.441	19	11	.239	14	16	.263	21	9	.037	18	12	.485	24	6	.729	3	4	.816
CT+TT	91	93	.919	115	69	.114	71	113	.239	127	57	.014	123	61	.461	144	40	.524	16	30	.829
rs11614913																					
TT	57	42		64	35		35	64		66	33		63	36		80	19		11	17	
CT	94	92		127	59		66	120		112	74		116	70		129	57		15	37	
CC	42	59	.076	66	35	.788	36	65	.999	64	37	.555	72	29	.301	86	15	.005	14	12	.098
CT+CC	136	151	.103	193	94	.727	102	185	1.000	176	111	.408	188	99	.831	215	72	.292	29	49	1.000

miRNA, MicroRNA; postop, postoperative; M, male; F, female.

APPENDIX TABLE 2. Multivariate analysis of clinicopathologic characteristics with the miRNA polymorphisms

P staging (n = 388)				Stage I		Stage II/IIIA	
rs2910164	I	II/IIIA	χ^2 P value	HR (95% CI)	χ^2 P value	HR (95% CI)	χ^2 P value
CC	104	73		1		1	
CG	111	48		0.75 (0.32-1.76)	.5141	0.39 (0.19-0.83)	.0143
GG	35	16	.087	1.11 (0.38-3.22)	.8512	0.30 (0.09-1.00)	.0494
CG+GG	146	64	.036	0.84 (0.39-1.82)	.6649	0.37 (0.19-0.72)	.0033
Trend				0.98 (0.57-1.70)	.9504	0.48 (0.28-0.80)	.0054

Histology (n = 388)				Adeno		Squamous	
rs7372209	Adeno	Squamous	χ^2 P value	HR (95% CI)	χ^2 P value	HR (95% CI)	χ^2 P value
CC	115	89		1		1	
CT	106	48		0.83 (0.39-1.77)	.6238	1.55 (0.79-3.02)	.1993
TT	21	9	.037	1.82 (0.59-5.57)	.2970	0.93 (0.27-3.25)	.9101
CT+TT	127	57	.014	0.95 (0.47-1.93)	.8960	1.41 (0.74-2.67)	.2940
Trend				1.13 (0.64-1.98)	.6728	1.59 (0.73-1.85)	.5395

Postop. complication (n = 388)				No		Yes	
rs11614913	No	Yes	χ^2 P value	HR (95% CI)	χ^2 P value	HR (95% CI)	χ^2 P value
TT	80	19		1		1	
CT	129	57		0.57 (0.30-1.08)	.0819	0.62 (0.20-1.93)	.4065
CC	86	15	.005	0.61 (0.29-1.27)	.1841	0.77 (0.17-3.45)	.7325
CT+CC	215	72	.292	0.58 (0.33-1.04)	.0664	0.65 (0.22-1.94)	.4372
Trend				0.76 (0.51-1.11)	.1559	0.84 (0.37-1.88)	.6638

HR, Hazard ratio; CI, confidence interval.

APPENDIX TABLE 3. The association between the 7 miRNA SNPs and RFS according to adjuvant chemotherapy

	Adjuvant chemotherapy No (n = 303)									Adjuvant chemotherapy Yes (n = 85)								
	N	No. relapsed	RFS rate, %	Median RFS (mo)	Log-rank trend test,		HR	lower CL	HR upper CL	N	No. relapsed	RFS rate, %	Median RFS (mo)	Log-rank trend test,		HR	lower CL	HR upper CL
					<i>P</i> value	Prob χ^2								<i>P</i> value	Prob χ^2			
rs213210																		
AA	79	12	85	N.R.	.382		Ref.			26	5	81	N.R.	.691		Ref.		
AG	143	26	82	N.R.		0.920	1.036	0.519	2.069	39	9	77	55.12		0.915	0.938	0.291	3.027
GG	80	16	80	N.R.		0.693	1.168	0.541	2.523	20	3	85	57.26		0.618	0.680	0.149	3.093
AG+GG	223	42	81	N.R.		0.816	1.080	0.563	2.072	59	12	80	55.12		0.790	0.858	0.279	2.640
Trend						0.686	1.083	0.735	1.596						0.630	0.836	0.403	1.733
rs895819																		
TT	135	25	81	93.76	.688		Ref.			50	7	86	N.R.	.540		Ref.		
CT	131	23	82	N.R.		0.372	0.763	0.422	1.381	27	9	67	55.12		0.031	3.299	1.113	9.779
CC	37	6	84	N.R.		0.436	0.693	0.276	1.740	8	1	88	57.26		0.800	0.750	0.082	6.907
CT+CC	168	29	83	N.R.		0.314	0.748	0.425	1.316	35	10	71	55.12		0.094	2.420	0.859	6.819
Trend						0.329	0.809	0.529	1.238						0.354	1.378	0.700	2.715
rs2289030																		
GG	184	31	83	N.R.	.964		Ref.			53	10	81	57.26	.769		Ref.		
CG	102	21	79	N.R.		0.845	1.058	0.598	1.873	30	7	77	N.R.		0.640	1.297	0.437	3.853
CC	17	2	88	N.R.		0.635	0.705	0.167	2.980	2	0	100	N.R.		0.993		NA	
CG+CC	119	23	81	N.R.		0.965	1.013	0.581	1.764	32	7	8	N.R.		0.679	1.257	0.426	3.705
Trend						0.874	0.963	0.606	1.530						0.725	1.204	0.429	3.377
rs2910164																		
CC	139	31	78	N.R.	.136		Ref.			38	12	68	52.92	.003		Ref.		
CG	123	16	87	N.R.		0.178	0.657	0.356	1.211	36	4	89	55.12		0.030	0.228	0.060	0.864
GG	40	7	83	N.R.		0.448	0.724	0.315	1.666	11	1	91	N.R.		0.992		NA	
CG+GG	163	23	86	N.R.		0.161	0.676	0.391	1.168	47	5	89	57.26		0.006	0.137	0.034	0.559
Trend						0.243	0.789	0.530	1.174						0.003	0.152	0.043	0.530
rs6505162																		
CC	194	33	83	N.R.	.600		Ref.			50	11	78	N.R.	.115		Ref.		
AC	91	19	79	96.22		0.442	1.259	0.700	2.266	29	5	83	57.26		0.188	0.419	0.115	1.530
AA	15	2	87	N.R.		0.895	0.907	0.212	3.880	6	1	83	N.R.		0.284	0.309	0.036	2.644
AC+AA	106	21	80	N.R.		0.507	1.211	0.688	2.131	35	6	83	N.R.		0.130	0.391	0.116	1.319
Trend						0.639	1.118	0.703	1.777						0.147	0.493	0.189	1.284
rs7372209																		
CC	153	26	83	N.R.	.582		Ref.			51	10	80	57.26	.494		Ref.		
CT	121	21	83	N.R.		0.791	1.082	0.604	1.938	33	7	79	55.12		0.439	1.501	0.537	4.195
TT	29	7	76	N.R.		0.812	1.110	0.470	2.621	1	0	100	N.R.		0.993		NA	
CT+TT	150	28	81	N.R.		0.758	1.089	0.634	1.870	34	7	79	55.12		0.463	1.471	0.525	4.122
Trend						0.763	1.062	0.720	1.565						0.529	1.373	0.512	3.681

(Continued)

APPENDIX TABLE 3. Continued

	Adjuvant chemotherapy No (n = 303)									Adjuvant chemotherapy Yes (n = 85)								
	Log-rank									Log-rank								
	No.	RFS	Median	trend test,	Prob	HR			No.	RFS	Median	trend test,	Prob	HR				
	N	relapsed	rate, %	RFS (mo)	P value	χ ²	HR	lower CL	upper CL	N	relapsed	rate, %	RFS (mo)	P value	χ ²	HR	lower CL	upper CL
rs11614913																		
TT	78	15	81	N.R.	.527		Ref.			21	9	57	52.92	.037		Ref.		
CT	144	26	82	N.R.		0.562	0.826	0.432	1.579	42	6	86	57.26		0.094	0.384	0.125	1.175
CC	80	13	84	N.R.		0.797	0.905	0.421	1.945	21	2	90	N.R.		0.043	0.181	0.035	0.944
CT+CC	224	39	83	N.R.		0.600	0.850	0.463	1.559	63	8	87	57.26		0.020	0.301	0.109	0.831
Trend						0.774	0.944	0.637	1.399						0.019	0.411	0.196	0.863

miRNA, MicroRNA; SNP, single nucleotide polymorphism; RFS, recurrence-free survival; HR, hazard ratio; CI, confidence interval; N.R., not reached.

APPENDIX TABLE 4. The association between the 7 miRNA SNPs and RFS according to the pattern of nodal metastasis

	Micrometastasis (n = 40)									Overt nodal metastasis (n = 68)								
	No. N	No. relapsed	RFS rate, %	Median RFS (mo)	Log-rank trend test, P value	Prob χ^2	HR	HR lower CL	HR upper CL	No. N	No. relapsed	RFS rate, %	Median RFS (mo)	Log-rank trend test, P value	Prob χ^2	HR	HR lower CL	HR upper CL
rs213210																		
AA	8	4	50	52.92	.774		Ref.			15	5	67	N.R.	.784		Ref.		
AG	20	7	65	N.R.		0.778	0.803	0.174	3.699	34	12	65	41.98		0.988	0.991	0.336	2.930
GG	12	4	67	42.38		0.551	1.768	0.272	11.494	19	5	74	N.R.		0.687	0.765	0.207	2.823
AG+GG	32	11	66	N.R.		0.963	0.966	0.230	4.067	53	17	68	N.R.		0.872	0.918	0.323	2.608
Trend						0.595	1.301	0.492	3.439						0.677	0.874	0.463	1.648
rs895819																		
TT	15	5	67	N.R.	.710		Ref.			31	13	58	40.24	.083		Ref.		
CT	17	8	53	42.38		0.247	2.355	0.552	10.040	28	7	75	N.R.		0.024	0.310	0.113	0.855
CC	8	2	75	N.R.		0.837	1.204	0.206	7.056	9	2	78	N.R.		0.108	0.280	0.059	1.320
CT+CC	25	10	60	42.38		0.343	1.829	0.525	6.369	37	9	76	N.R.		0.014	0.303	0.117	0.782
Trend						0.577	1.246	0.576	2.694						0.030	0.426	0.198	0.919
rs2289030																		
GG	23	8	65	52.92	.669		Ref.			39	13	67	57.26	.711		Ref.		
CG	15	6	60	N.R.		0.431	0.592	0.161	2.180	26	8	69	N.R.		0.519	0.731	0.281	1.897
CC	2	1	50	N.R.		0.267	4.212	0.332	53.428	3	1	67	N.R.		0.885	0.856	0.104	7.046
CG+CC	17	7	59	N.R.		0.730	0.813	0.251	2.635	29	9	69	N.R.		0.529	0.742	0.293	1.877
Trend						0.974	1.017	0.370	2.798						0.593	0.802	0.356	1.803
rs2910164																		
CC	23	13	43	19.25	.015		Ref.			35	12	66	N.R.	.426		Ref.		
CG	14	2	86	N.R.		0.013	0.139	0.029	0.659	23	7	70	N.R.		0.581	0.766	0.297	1.976
GG	3	0	100	N.R.		0.994	NA			10	3	70	N.R.		0.452	0.609	0.167	2.219
CG+GG	17	2	88	N.R.		0.003	0.089	0.018	0.443	33	10	70	N.R.		0.431	0.711	0.304	1.660
Trend						0.004	0.117	0.027	0.495						0.402	0.776	0.429	1.404
rs6505162																		
CC	28	13	54	42.38	.139		Ref.			37	9	76	N.R.	.177		Ref.		
AC	9	2	78	N.R.		0.301	0.421	0.082	2.166	26	11	58	57.26		0.199	1.849	0.724	4.723
AA	2	0	100	N.R.		0.993	NA			5	2	60	38.63		0.554	1.625	0.325	8.116
AC+AA	11	2	82	N.R.		0.177	0.321	0.061	1.674	31	13	58	40.24		0.195	1.806	0.739	4.416
Trend						0.144	0.333	0.076	1.458						0.257	1.454	0.761	2.776
rs7372209																		
CC	24	8	67	N.R.	.356		Ref.			38	12	68	N.R.	.723		Ref.		
CT	13	5	62	42.38		0.942	0.956	0.280	3.265	26	9	65	N.R.		0.982	1.011	0.402	2.540
TT	3	2	33	18.27		0.177	3.721	0.552	25.103	4	1	75	N.R.		0.321	0.351	0.044	2.782
CT+TT	16	7	56	42.38		0.732	1.216	0.397	3.725	30	10	67	N.R.		0.700	0.839	0.344	2.045
Trend						0.398	1.474	0.600	3.621						0.440	0.764	0.386	1.513

(Continued)

APPENDIX TABLE 4. Continued

Micrometastasis (n = 40)										Overt nodal metastasis (n = 68)								
	N	No. relapsed	RFS rate, %	Median RFS (mo)	Log-rank trend test, <i>P</i> value		Prob χ^2	HR		N	No. relapsed	RFS rate, %	Median RFS (mo)	Log-rank trend test, <i>P</i> value		Prob χ^2	HR	
								lower CL	upper CL								lower CL	upper CL
rs11614913																		
TT	11	6	45	36.09	.316		Ref.			17	7	59	38.63	.096		Ref.		
CT	15	4	73	N.R.		0.184	0.349	0.074	1.651	37	14	62	57.26		0.549	0.743	0.281	1.964
CC	14	5	64	N.R.		0.032	0.166	0.032	0.859	12	1	92	N.R.		0.088	0.158	0.019	1.312
CT+CC	29	9	69	N.R.		0.050	0.238	0.057	1.002	49	15	69	N.R.		0.269	0.583	0.224	1.519
Trend						0.035	0.410	0.180	0.938						0.075	0.525	0.258	1.066

miRNA, MicroRNA; *SNP*, single nucleotide polymorphism; *RFS*, recurrence-free survival; *HR*, hazard ratio; *CI*, confidence interval; *N.R.*, not reached.