MicroRNA Expression Profiles in Korean Non-Small Cell Lung Cancer

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Background: MicroRNAs (miRNAs) play an important role in the regulation of cell proliferation, apoptosis, development and differentiation. Several studies have shown that aberrant expression of miRNAs is involved in cancer development and progression by regulating the expression of proto-oncogenes or tumor suppressor genes. In this study, we investigated miRNA expression profiles in Korean patients with non-small cell lung cancer (NSCLC).

Methods: We performed miRNA microarray analysis containing 60~65 bp oligonucleotide probes representing human 318 miRNAs and validated the results of the microarray with Northern blot analysis or quantitative RT-PCR. Next, we examined the correlation between miRNA expression and the target gene transcriptional profile using a human whole-genome-expression microarray.

Results: We showed that 35 miRNAs were expressed differentially in the NSCLCs and corresponding non-malignant lung tissues. We showed that 35 miRNAs were expressed differentially in the NSCLCs and corresponding non-malignant lung tissues. Thirteen of the 35 differentially expressed miRNAs were newly identified in the present study. Of the 35 miRNAs, 2 (*miR-371* and *miR-210*) were over-expressed in lung cancers, and 33 miRNAs, including *miR-145*, were under-expressed in lung cancers. *miR-99b* expression consistently showed a negative correlation with *FGFR3* expression.

Conclusion: Albeit a small number of patients were examined, these results suggest that miRNA expression profiles in Korean lung cancers may be somewhat different from the expression profiles reported on lung cancers in Western populations. The findings suggest that *miR-99b* might be a tumor suppressor through its up-regulation of *FGFR3*.

Key Words: MicroRNAs; Carcinoma, Non-Small-Cell Lung; Korea

Introduction

MicroRNAs (miRNAs) are noncoding, single-stranded RNAs of ~22 nucleotides that repress gene expression

by interacting with messenger RNA (mRNA), either by inhibiting mRNA translation or by inducing mRNA cleavage¹⁻³. To date, more than 400 human miRNAs have been identified and up to 30% of all protein-coding genes are estimated to be regulated by miRNAs^{2,4,5}. Although the precise functions of individual miRNAs have not been characterized, biochemical and genetic studies have revealed that miRNAs regulate a variety of biological processes, such as cell proliferation, apoptosis, development and differentiation⁶⁻⁹.

miRNA genes are frequently located at chromosomal regions, such as fragile sites, and regions of deletion or

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amplification that are genetically altered in human cancer^{10,11}. In addition, it has been reported that some miRNAs are aberrantly expressed or mutated in human cancers, suggesting that they may function as either oncogenes or tumor suppressor genes; depending on the targets they regulate¹²⁻¹⁴. The first evidence of the involvement of miRNAs in cancer came from a study characterizing the 13q14 deletion in chronic lymphocyte leukemia, which revealed that two clustered miRNA genes, *miR-15a* and *miR-16-1*, are the target genes of the 13q14 deletion¹⁵. Subsequently, deregulation of miRNA has been found in various human cancers including lung cancer, such as the down-regulation of *let-7* in lung cancer^{16,17}, up-regulation of the *miR-17-92* cluster in B-cell lymphoma and lung cancer^{18,19}, and down-regulation of *miR-143* and *miR-145* in colorectal cancer²⁰.

Using miRNA microarray technology, several groups have revealed miRNA expression profiles in many human cancers²¹⁻²⁵, and also identified miRNA expression signatures associated with clinical and pathological features as well as disease outcome²¹⁻²³. Although miRNA expression profiles have been studied in lung cancer, the majority of the previous studies were performed on Western populations^{21,22,26}. The epidemiological characteristics of lung cancer in East Asian countries are different from those reported from Western countries. For example, lung adenocarcinoma in women, in East Asian countries, frequently develops in never-smokers^{27,28}. In addition, several recent studies have shown that mutations in the kinase domain of the *epidermal growth factor receptor* gene are more frequent in never-smokers, females and East Asian populations, whereas *KRAS* mutations are more frequent in smokers, males and Western populations^{29,30}. These observations, coupled with the differences in the epidemiological characteristics of lung cancer, suggest that the genetic and environmental factors leading to lung cancer in East Asian populations may be different from those of Western populations^{29,30}. Therefore, it is possible that miRNA expression profiles in lung cancer among East Asian populations may be different from Western countries. In this study, we investigated the miRNA expression profiles in Korean pa-

tients with non-small cell lung cancers (NSCLCs).

Materials and Methods

1. Tissue samples

Tumor and corresponding non-malignant lung tissue specimens were obtained from Korean patients with NSCLC that underwent curative resection at the Konyang University Hospital (Daejeon, Korea). Seven NSCLC cases (4 squamous cell carcinomas [SCCs] and 3 adenocarcinomas [ACs]) were included for miRNA microarray analysis. Four NSCLC cases (3 SCCs and 1 AC) among seven NSCLC case were randomly selected and included for Northern blotting and human whole genome expression microarray analysis. Twenty-three NSCLC cases (19 SCCs and 4 ACs) were included for quantitative RT-PCR (qRT-PCR) (Table 1). None of the patients had received chemotherapy or radiotherapy before surgery. Informed consent was obtained from each patient before surgery. This study was approved by the Bioethics Committee of Konyang University Hospital. All of the tumor and macroscopically normal lung tissue samples were obtained at the time of surgery, and were rapidly frozen in liquid nitrogen and stored at -80°C until analysis. Tissue samples were histologically confirmed by hematoxylin-eosin staining.

Table 1. Characteristics of study populations

Characteristics	Microarray	qRT-PCR	p-value
Age	56.57 $\pm$ 13.28	64.69 $\pm$ 9.63	0.085*
Gender			
Male	5	19	0.603 [†]
Female	2	4	
Smoking status			
Smoker	5	20	0.565 [†]
Never smoker	2	3	
Histology			
Squamous cell carcinoma	4	19	0.305 [†]
Adenocarcinoma	3	4	
Pathologic stage			
Stage I	3	10	0.976 [†]
Stage II/III	4	13	

*Student t-test, [†]Fisher's exact test.

2. miRNA extraction

The total RNA of each sample was extracted using the Trizol reagent (Life Technologies, Carlsbad, CA, USA) according to the instructions of the manufacturer. Low molecular weight (LMW) enriched RNA was isolated from 50 μ g of total RNA using the mirVana miRNA extraction kit (Ambion Inc., Austin, TX, USA), and quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

3. miRNA microarray hybridization

The expression of miRNAs was profiled using a miRNA microarray chip (Genomic Tree Inc., Seoul, Korea) containing 60~65 bp oligonucleotide probes representing human 318 miRNAs. Briefly, 1 μ g of LMW RNA was labeled using a ULSTM small RNA labeling kit (Kreatech, Amsterdam, The Netherlands). LMW RNA of an adjacent normal tissue sample was labeled with fluorescent Cy3 and LMW RNA of tumors was labeled with fluorescent Cy5. The fluorescent-labeled LMW RNA was purified using KREApure columns (Kreatech) as per the manufacturer's instructions. The purified target RNA was resuspended in 100 μ L of hybridization solution containing 3.5 $\times$ SSC, 0.3% SDS, and 10 μ g of ssDNA (Invitrogen). The hybridization mixture was heated at 100°C for 2~3 min and hybridized onto a miRNA microarray. The array was hybridized at 42°C for 16 h in a humidified hybridization chamber (Genomic Tree Inc.). The hybridized microarray was washed twice with 2 $\times$ SSC/0.1% SDS for 5 min, 0.1 $\times$ SSC/0.1% SDS for 5 min and 0.1 $\times$ SSC for 2 min. The washed microarray was immediately dried using a microarray centrifuge (Genomic Tree, Inc.).

The hybridization signals were analyzed using a GenePix Pro 6.0 program (Axon Instruments, Foster City, CA, USA). The average fluorescence intensity for each spot was calculated and then the local background was subtracted. All data mining and selection of fold-changed genes were performed using GeneSpringGX 7.3

(Agilent Technologies). Genes were filtered with cut-off values based on the sum of the median of $\geq 1,000$ in each experiment. The averages of the ratios were calculated by dividing the average of the signal channel intensity by the average of the control channel intensity. We considered the results from the Significance Analysis of Microarray 3.0 (SAM) with genes of ratios (Cy5/Cy3) either ≥ 2.0 or ≤ 0.5 from ≥ 5 of 7 tumor samples as significantly different in expression.

4. Northern blotting and qRT-PCR

To validate the microarray analysis results, we carried out Northern blot analysis on two (*miR-145* and *miR-210*) of the 35 differentially expressed miRNAs using a mirVana miRNA detection kit (Ambion Inc.). Briefly, 3 μ g of total RNA was hybridized with 1 pM RNA probe (*miR-145*, biotin-5'-AAAAGGGAUCCUGGGAAAACUG GAC-3'; and *miR-210*, biotin-5'-AAAAUCAGCCGCUGUC ACACGCACAG-3'). The 5S RNA probe (biotin-5'-AAAA UGAUCUCGGAAGCUAA-3') was used to normalize the level of expression. To remove unhybridized RNA, the RNAase A/T1 solution was added, followed by alcohol precipitation. Hybridized RNA was separated on a 15% denaturing polyacrylamide gel and then electroblotted on a Hybond-Nylon membrane (Amersham Biosciences, Buckinghamshire UK). Detection of hybridized RNA was performed using a non-radioactive LightShift Chemiluminescent kit (Pierce Biotechnol Inc., Rockford, IL, USA). The membrane was UV cross-linked, and the biotin end-labeled hybridized RNA was detected with streptavidin-HRP using a luminal enhancer solution. qRT-PCR analysis for miRNAs was performed in duplicate with a TaqMan MicroRNA assay kit (Applied Biosystems, Foster City, CA, USA) according to the instructions of the manufacturer, and 18S RNA was used for normalization.

5. Human whole genome expression microarray

For hybridization onto an Agilent Whole Human Genome 4 $\times$ 44 K 60 mer oligonucleotide array (G4112F; Agilent Technologies), 1 μ g of total RNA was used in the Agilent LIRAK PLUS with the two-color Low RNA

input Linear Amplification method, according to the manufacturer's instructions. Briefly, the total RNA was reverse transcribed into complementary DNA (cDNA) using a T7-promotor primer and MMLV reverse transcriptase. The cDNA was transcribed into complementary RNA (cRNA), during which the cDNA was fluorescently labeled by incorporation of cyanine Cy5-CTP (lung cancer tissue) or Cy3-CTP (normal lung tissue). After purification, using the RNeasy mini kit (Qiagen), the cRNA yield and Cy incorporation efficiency (specific activity) into the cRNA were determined using a NanoDrop Spectrophotometer (NanoDrop Technologies). cRNAs showing a yield >825 ng and a specific activity of $8\sim 20$ pmol/ μ g cRNA were selected for further processing. Equal amounts of the exposed and negative control sample were competitively hybridized onto Agilent Whole 4 $\times$ 44 K human oligonucleotide arrays in a hybridization oven at 60°C for 17 h. Slides were washed according to the manufacturer's instructions with washing buffers and finally dipped in Stabilization and Drying

Figure 1. Unsupervised hierarchical clustering of 315 miRNA expression patterns in non-small cell carcinomas and corresponding non-malignant lung tissues. Ratios of miRNA expression are displayed colorimetrically in lung cancers relative to non-malignant lung tissues. The scale bar at the right side represents the relative level of expression of each miRNA. Columns represent array and miRNAs are represented in rows.

Solution (Agilent Technologies) for protection from environmental ozone. The arrays were scanned on an Agilent scanner (G2565BA) and further processed using Agilent Feature Extraction Software (version 9.5.1).

Results

To generate miRNA expression profiles for NSCLC, we analyzed the expression of 315 human miRNAs using oligonucleotide microarray hybridization chips in 7 pairs of primary lung cancers and corresponding non-malignant lung tissues (Figure 1). We identified 35 miRNA genes that were differentially expressed in the NSCLCs and corresponding non-malignant lung tissues with ratios (Cy5/Cy3) either ≥ 2 fold or ≤ 0.5 fold from at least 5 of 7 tumor samples (Table 2). Among the 35 differentially expressed miRNAs, two (*miR-371* and *miR-210*) were over-expressed in lung cancers, and 33 miRNAs, including *miR-145*, were under-expressed in lung cancers. Among the 35 differentially expressed miRNAs, 13 of the miRNAs have not been previously reported to be altered in lung cancer; *miR-376b*, *miR-144*, *miR-520d-5p*, *miR-520e*, *miR-520f*, *miR-23a*, *miR-296-5p*, *miR-133a-2*, *miR-99b*, *miR-497*, *miR-425*, and *miR-338-3p* were down-regulated, and *miR-371-3p* was up-regulated.

To validate the microarray results, the expression of representative miRNAs were determined using Northern blot analysis (*miR-210* and *miR-145*) and qRT-PCR (*miR-210* and *miR-29c*). Consistent with the results of the microarray analysis, *miR-210* was up-regulated in the lung cancers, and *miR-145* and *miR-29c* were down-regulated in the lung cancers (Figure 2).

We predicted tumor-associated target genes of the new 13 miRNAs using 3 public algorithms (MIRAND³¹, TARGETSCAN³², and PICTAR³³). The list of putative target genes of 5 miRNAs (*miR-144*, *miR-23a*, *miR-99b*, *miR-133a-2*, and *miR-296-5p*), which were predicted by at least 2 of the 3 programs, are shown in Table 3. We next examined the correlation between miRNA expression and target gene transcriptional profiles. Of the 5 miRNAs, *miR-99b* expression consistently showed a negative correlation with *fibroblast growth factor re-*

Table 2. MiRNAs differentially expressed between lung cancers and their corresponding normal lung tissues

miRNA	Location	Type	Fold	q-value	Results from previous studies
hsa-mir-145	5q32	Down	0.19	0	Down
hsa-mir-181a	1q31.3	Down	0.41	0	Down: 181c-prec
hsa-mir-181c	19p13.12	Down	0.37	0	Down: 181c-prec
hsa-mir-30a-3p	6q13	Down	0.37	0	Down
hsa-mir-30a-5p	6q13	Down	0.46	0	Down [†] : 30a-3p
hsa-mir-30c	6q13	Down	0.34	0	Down: 30a-3p
hsa-mir-30d	8q24.22	Down	0.31	0	Down [†] : 30a-3p
hsa-mir-30e-3p	1p34.2	Down	0.23	0	Down: 30a-3p
hsa-mir-140-5p	16q22.1	Down	0.31	0	Down
hsa-mir-143	5q32	Down	0.38	0	Down
hsa-mir-125b	11q24.1	Down	0.37	0	Down: 125a,125a-prec*
hsa-mir-99a	21q21.1	Down	0.34	0	Up: 99b prec [†]
hsa-mir-376b	14q32.2	Down	0.51	0	
hsa-mir-126	9q34.3	Down	0.37	0	Down [†]
hsa-mir-101	1p31.3	Down	0.37	0	Down: 101-1
hsa-let-7f	9q22.31	Down	0.46	0	Down: let-7a-2-pre Up: let-7a and -7g [†]
hsa-mir-95	4p16.1	Down	0.48	0	Down
hsa-mir-144	17q11.2	Down	0.27	0	
hsa-mir-520d-5p	19q13.43	Down	0.51	0	
hsa-mir-520e	19q13.43	Down	0.46	0	
hsa-mir-520f	19q13.43	Down	0.37	0	
hsa-mir-23a	19p13.2	Down	0.43	0	
hsa-mir-296-5p	20q13.32	Down	0.46	0	
hsa-mir-133a-2	18q11.2	Down	0.41	0	
hsa-mir142-3p	17q22	Down	0.49	0	Up: 142-as [†]
hsa-mir-9	15q25.3	Down	0.44	0	Down, Up: 9-1 [†]
hsa-mir-27a	19p13.2	Down	0.51	0	Down: 27b
hsa-mir-29c	1q32.1	Down	0.59	0	Down: mir-29b-2
hsa-mir-99b	19q13.43	Down	0.58	0	
hsa-mir-497	17p13.1	Down	0.51	0	
hsa-mir-125a	19q13.43	Down	0.43	0	Down
hsa-mir-425	3p21.31	Down	0.56	0	
hsa-mir-338-3p	17q25.3	Down	0.37	0	
hsa-mir-371-3p	19q13.43	Up	2.63	0.93	
hsa-mir-210	11p15.5	Up	2.51	0.93	Up*, [†]

*Ref. 22, [†]Ref. 26.

ceptor 3 (FGFR3) expression (Table 3).

Discussion

We performed miRNA expression profiling using microarray analysis in Korean patients with NSCLC and identified a number of miRNAs that were differentially expressed in NSCLCs. To date, two large studies on miRNA expression profiles in lung cancer have been re-

ported^{22,26}. In a study of 104 matched pairs of lung cancers and non-cancerous lung tissues, Yanaihara et al²² identified a set of 43 differentially expressed miRNAs; 28 were down-regulated and 15 were up-regulated. The majority of the miRNAs detected in the present study were the same miRNAs that were identified by Yanaihara et al²². For example, *miR-145*, *miR-181c*, *miR-30 family*, *miR-140*, *miR-143*, *miR-125*, *miR-126*, *miR-101*, *miR-95*, *miR-9*, and *miR-125a* were down-regulated and

Figure 2. (A) Northern blot analysis for has-miR-145 and has-miR-210 in four paired lung cancers and corresponding non-malignant lung tissues. Has-miR-210 was up-regulated in lung cancers, and has-miR-145 was down-regulated in lung cancers. (B) Quantitative RT-PCR (qRT-PCR) of miRNA expression utilized total RNA isolated from cancer tissues and non-malignant lung tissues. The expression of miRNA was normalized to 18S rRNA expression and was performed in duplicate. Has-miR-29c was down-regulated in lung cancers ($p=0.0001$, Mann-Whitney's U test), whereas has-miR-210 was up-regulated in lung cancer ($p=0.1295$), although statistically not significant.

miR-210 was up-regulated in both studies (Table 2). In addition, we found that *let-7f* was down-regulated in lung cancer tissues, which was also consistent with the results of Yanaihara et al²², where *let-7a-2* was down-regulated in lung cancer. Volinia et al²⁶ also found that lung cancer cells showed distinct miRNA profiles compared to normal cells in a study of 80 lung cancers and 40 normal lung tissues. They reported that 3 miRNAs were down-regulated and 35 miRNAs were over-expressed in lung cancers. When our data were compared with the results of the study reported by Volinia et al²⁶, several miRNAs showed the opposite results of up-regulation or down-regulation. For example, they reported that *let-7a*, *let-7g*, *miR-142* and *miR-9-1* were up-regulated in lung cancers. However, these miRNAs were down-regulated in lung cancers in the present study, as well as in the study reported by Yanaihara et al²². This discrepancy may be due to differences in the methods used in the studies. Volinia et al²⁶ measured the ex-

pression of both the pri-miRNAs and the active mature miRNAs simultaneously. Considering that the pri-miRNAs are transient products in miRNA biogenesis, and presumably are rapidly further processed after synthesis³⁴, the expression status of the pri-miRNA and the mature miRNA is not necessarily the same. Therefore, the profiles of the combined expression of the pri-miRNAs and the mature miRNAs may be different from the expression of the mature miRNAs²⁴.

Racial differences in miRNA expression were reported in uterine leiomyomas. Wang et al³⁵ found that substantial number of miRNAs that are significantly differently regulated between black and white women. Black women had a greater than 2-fold overexpression in *miR-23a/b*, *let-7s*, *miR-145*, *miR-197*, *miR-411*, and *miR-412*. In the present study, we found that *miR-376b*, *miR-144*, *miR-520d-5p*, *miR-520e*, *miR-520f*, *miR-23a*, *miR-296-5p*, *miR-133a-2*, *miR-99b*, *miR-497*, *miR-425*, and *miR-338-3p* were down-regulated and *miR-371-3p*

Table 3. Correlation between microRNA expression profiles and target gene transcriptional profiles in NSCLC

miRNA	Target gene	Target gene transcriptional profiles*			
		Case1	Case2	Case3	Case4
miRNA-144	MYBL1	0.69	0.82	0.52	0.57
	MAP3K8	0.11	0.30	0.49	0.29
	MYCN	1.20	1.09	1.24	1.32
	MET	1.12	1.25	0.95	0.41
	ETS1	0.36	0.79	0.38	0.51
	TAL1	0.74	1.23	0.93	1.42
	HOXA10	0.88	0.96	0.77	1.98
	PIM1	0.65	0.34	0.75	1.10
miRNA-23a	ARHGEF5	2.11	0.77	4.15	1.56
	CTNNBIP1	0.86	0.70	0.58	0.52
	MET	1.12	1.25	0.95	0.41
	MEIS1	0.46	1.85	0.91	0.32
	CCND1	1.91	2.14	0.73	0.90
	IRF2	0.80	1.57	1.04	0.91
	TEAD1	0.56	1.17	0.47	0.44
miRNA-99b	FGFR3	2.84	1.64	9.48	6.23
miRNA-133a-2	BCL2L2	0.98	0.77	0.72	0.66
	EVI1	0.30	0.90	1.41	0.87
	FGFR1	0.82	1.12	1.34	0.79
	CRK	1.54	1.20	1.82	1.15
	MLLT3	0.46	1.88	0.64	0.61
	YES1	1.20	0.74	1.87	1.37
	USP6	1.05	0.62	1.19	1.11
	ELF3	0.13	0.63	4.16	1.22
miRNA-296-5p	FGFR1	0.82	1.12	1.34	0.79

*Ratios of transcription was cy5 (lung cancer tissue)/Cy3 (normal lung tissue) in human whole genome expression microarray.

was up-regulated. To characterize these miRNA, we analyzed their chromosomal location. *miR-520d-5p*, *miR-520e*, *miR-520f*, *miR-99b*, *miR-371-3p* were located in 19q13.43.

The results of this study showed that *miR-99b* was down-regulated in NSCLCs and the expression was negatively correlated with *FGFR3* expression. *FGFR3* belongs to a family of structurally-related tyrosine kinase receptors (FGFR1~4) involved in many aspects of embryogenesis and tissue homeostasis. These receptors regulate various biological processes, including proliferation, differentiation, angiogenesis, migration, and apoptosis³⁶. *FGFR3* has been demonstrated to be involved in the RAS/RAF/MEK/MAPK pathway through activation of p90 ribosomal S6 kinase³⁷. In addition, it has been reported that *FGFRs* are frequently overexpressed in

NSCLC cell lines, suggesting that an *FGFR*-dependent autocrine signaling pathway may operate in a subset of NSCLCs³⁸. Based on these observations, it is possible that *miR-99b* may be involved in lung tumorigenesis through up-regulation of *FGFR3*.

Albeit a small number of patients were examined, these results suggest that miRNA expression profiles in Korean lung cancers may be somewhat different from the expression profiles reported on lung cancers in Western populations. The findings suggest that *miR-99b* might be a tumor suppressor through its up-regulation of *FGFR3*.

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