

## MicroRNA-23a: A Novel Serum Based Diagnostic Biomarker for Lung Adenocarcinoma

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**Background:** MicroRNAs (miRNAs) have demonstrated their potential as biomarkers for lung cancer diagnosis. In recent years, miRNAs have been found in body fluids such as serum, plasma, urine and saliva. Circulating miRNAs are highly stable and resistant to RNase activity along with, extreme pH and temperatures in serum and plasma. In this study, we investigated serum miRNA profiles that can be used as a diagnostic biomarker of non-small cell lung cancer (NSCLC).

**Methods:** We compared the expression profile of miRNAs in the plasma of patients diagnosed with lung cancer using an miRNA microarray. The data from this assay were validated by quantitative real-time PCR (qRT-PCR).

**Results:** Six miRNAs were overexpressed and three miRNAs were underexpressed in both tissue and serum from squamous cell carcinoma (SCC) patients. Sixteen miRNAs were overexpressed and twenty two miRNAs were underexpressed in both tissue and serum from adenocarcinoma (AC) patients. Of the four miRNAs chosen for qRT-PCR analysis, the expression of miR-23a was consistent with microarray results from AC patients. Receiver operating characteristic (ROC) curve analyses were done and revealed that the level of serum miR-23a was a potential marker for discriminating AC patients from chronic obstructive pulmonary disease (COPD) patients.

**Conclusion:** Although a small number of patients were examined, the results from our study suggest that serum miR-23a can be used in the diagnosis of AC.

**Key Words:** MicroRNAs; Gene Expression Profiling; Biological Markers; Lung Neoplasms

### Introduction

MicroRNA (miRNA) is a non-coding, small, single-stranded, endogenous RNAs composed of 20~25 nucleotides that regulate interactions by inhibit messenger RNA translation or induce miRNAs cleavage<sup>1-3</sup>. Several reports suggested that aberrantly expressed miRNA may act as oncogenes or tumor suppressor

genes in lung cancer<sup>4,6</sup>. miRNA regulates gene expression through sequence-specific interaction with 3'UTR in mRNAs<sup>2,3</sup>. Many genes have target sites for interaction with miRNAs and a single miRNA can modulate the expression of multiple genes<sup>7</sup>, hence the miRNA regulatory network is highly complex.

miRNA is known to circulate in a highly stable, cell-free form in the blood. Plasma and serum miRNA is resistant to RNase activity<sup>8,9</sup>. The similarity between circulating miRNA and tumor-derived miRNA patterns suggest that circulating miRNA might be useful as a biomarker<sup>10</sup>. Serum miR-21, miR-210, and miR-155 have been reported to be elevated in lymphoma patients; moreover, miR-21 expression is associated with relapsed-free survival<sup>11</sup>. Serum miR-141 can help dis-

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tinguish patients with prostate cancer from healthy controls<sup>8</sup>. Thus, circulating miRNAs are useful as non-invasive biomarkers for cancers.

For this study, we hypothesize that serum miRNAs profiling could be used as to identify diagnostic biomarkers of non-small-cell lung cancer (NSCLC). To address this hypothesis, we screened serum miRNAs using a miRNA microarray. The results from this assay were validated with quantitative real-time polymerase chain reaction (qRT-PCR) analysis of individual serum samples.

## Materials and Methods

### 1. Tissue and serum samples

Six sets of cancer tissues with matched non-cancerous lung tissue along with serum samples from six cancer patients (three squamous cell carcinoma [SCCs] and three adenocarcinoma [ACs]) and six serum healthy controls were used for the microarray. Eighteen serum samples from NSCLC patients (seven SCCs and eleven AC) who were diagnosed at Konyang University Hospital were used as the training group for qRT-PCR. An additional 36 serum samples from NSCLC patients (19 SCCs and 17 AC) diagnosed at Kyungpook National University

Hospital were used as the validation group for qRT-PCR (Table 1). Fifteen control serum samples were also collected from chronic obstructive pulmonary disease (COPD) patients for the qRT-PCR analysis. None of the patients had received chemotherapy or radiotherapy before sampling. All serum samples were stored at  $-80^{\circ}\text{C}$  prior to use. Written, informed consent was obtained from each patient. This study was approved by the Bioethics Committee of Konyang University Hospital.

**1) RNA extraction and cel-miRNA spike in:** Total RNA containing small RNA was isolated from serum using Trizol reagent (Ambion Inc., Austin, TX, USA) according to the manufacturer's protocol with the following modifications. The Trizol reagent was mixed at a 3 : 1 ratio with serum. After the addition of chloroform, the tubes were shaken and centrifuged to separate the upper aqueous phase which was carefully transferred to a fresh tube. Isopropanol was then added to the aqueous phase followed by centrifugation at  $12,000\times g$  for 10 minutes. The precipitated RNA was then washed with 75% ethanol and centrifuged at  $7,500$  for 5 minute. Each sample was spiked with 2 ng of cel-miR-39 after adding phenol and guanidine thiocyanate to serve as an external processing control<sup>8,9</sup>.

**2) miRNA microarray:** The miRNA assay probes correspond to 470 well-annotated human miRNA sequences (miRBase version 9.1, February 2007; <http://microrna.sanger.ac.uk/>) and 265 miRNAs recently identified<sup>12</sup>. Assay probes were designed with a  $T_m$  of  $60\pm 8.6^{\circ}\text{C}$  and a length of 17~21 nt (average 18 nt). To maximize the specificity, candidate probes were collectively examined to minimize sequence similarity, particularly at the 3'-ends. Briefly, 15  $\mu\text{L}$  of the cDNA from the synthesis reaction was added to 5  $\mu\text{L}$  of the multiplexed MSO pool (MAP; Illumina Inc., San Diego, CA, USA). Next 30  $\mu\text{L}$  of a reagent containing streptavidin paramagnetic particles (OB1; Illumina Inc.) was added, then the mixture was heated to  $70^{\circ}\text{C}$  and allowed to anneal at  $40^{\circ}\text{C}$ . All 735 human miRNAs were assayed simultaneously.

After binding and washing, the annealed MSOs were extended with the cDNA primer, forming an amplifiable product. The extended oligos were eluted from the

Table 1. Characteristics of the study populations

Characteristics	Microarray	qRT-PCR	
		Training group	Validation group
Age*, yr	69.00 $\pm$ 7.01	65.72 $\pm$ 9.66	66.33 $\pm$ 8.51
Gender			
Male	4	14	26
Female	2	4	10
Histology			
Squamous cell carcinoma	3	7	19
Adenocarcinoma	3	11	17
Pathologic stage			
Stage I/II	2	4	11
Stage III/IV	4	14	25

\*Ages are represented as mean age $\pm$ standard deviation. qRT-PCR: quantitative real-time polymerase chain reaction.

streptavidin beads and added to a PCR reaction in which one of the universal primers was fluorescently labeled and the other universal primer was biotinylated. The PCR products were captured by streptavidin paramagnetic beads, washed, and denatured to yield single-stranded fluorescent molecules for hybridizing to the arrays. The universal arrays used for fluorescent reporting consisted of captured oligos immobilized on beads and randomly assembled into wells etched in a Sentrix universal bead chip (1536 bead array). The identity of each bead was determined before hybridization to the miRNA assay product, and the same arrays were used to report the results of similar assays using the address sequence technique (GoldenGate\_Genotyping Assay, and DASL Gene Expression Assay, GoldenGate Methylation Assay). Arrays were scanned on the BeadArray Reader, and software for automatic image registration and intensity extraction was used to derive intensity data per bead type corresponding to each miRNA. The quality of hybridization and overall chip performance were monitored by visual inspection of both the internal quality control checks and raw scanned data. Raw data were extracted using the software provided by the manufacturer (Illumina BeadStudio version 3.1.3 (Gene Expression Module version 3.3.8). Array data were filtered by a detection  $p$ -value  $< 0.05$  (similar to signal to noise) for all samples. Selected gene signal values were transformed by a logarithm and normalized by the quantile method. Comparative analysis between the Test and Control groups was carried out using a  $t$ -test (adjusted Benjamini-Hochberg false discovery rate [FDR] was 5% controlled) and expressed as fold change. Hierarchical cluster analysis was performed using complete linkage and Euclidean distance as a measure of similarity. All data analysis and visualization of differentially expressed genes was conducted using ArrayAssist<sup>®</sup> (Stratagene, La Jolla, CA, USA) and R statistical language version 2.4.1.

## 2. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from each sample using

Trizol reagent (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions. miRNA qRT-PCR analysis was performed in duplicate with a TaqMan MicroRNA assay kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The relative fold-changes of miRNAs expression were calculated by the standard curve method and cel-miR-39 was used for normalization.

## 3. Statistical analysis

SPSS version 12.0 software was used for statistical analysis (SPSS, Chicago, IL, USA). The optimal cut-off was chosen according to receiver operating characteristic (ROC) curve analysis. The area under the ROC curve (AUC) value was calculated to measure of the accuracy of the test.

## Results

### 1. Expression profiling of serum miRNA

We compared the miRNA profiles of lung cancer tissue versus non-cancerous lung tissues profiles, and those of serum from lung cancer patients versus serum from healthy controls. Six miRNAs were overexpressed and three miRNA were underexpressed in both tissue and serum from SCC patients (Table 2). 16 miRNAs were over-expressed and 22 miRNA were down-expressed on both tissue and serum of AC patients (Table 3).

Table 2, miRNAs differentially expressed in tissue and serum from squamous cell carcinoma patients

miRNA	Fold change		Type
	Tissue	Serum	
HS_31.1	2.82	2.44	Up
hsa-miR-1268	2.35	99.52	Up
hsa-miR-17-5p:9.1	5.27	2.26	Up
hsa-miR-200a*	2.29	3.51	Up
hsa-miR-450a	2.22	7.14	Up
hsa-miR-876-3p	3.55	3.36	Up
hsa-miR-150	-1.29	-9.64	Down
hsa-miR-29c*	-2.43	-2.56	Down
hsa-miR-486-3p	-2.42	-4.62	Down

\*Passenger microRNA.

**Table 3.** miRNAs differentially expressed in tissue and serum from adenocarcinoma patients

miRNA	Fold change		Type
	Tissue	Serum	
hsa-miR-324-5p	2,39	2,16	Up
hsa-miR-30d	2,32	73,80	Up
hsa-miR-191	3,74	284,14	Up
HS_303_a	2,28	51,69	Up
hsa-let-7b	4,93	51,92	Up
hsa-miR-16	2,52	100,72	Up
hsa-miR-1228*	4,37	15,31	Up
hsa-miR-23a	3,10	213,35	Up
hsa-miR-196b	6,01	4,01	Up
hsa-miR-27a	2,63	58,80	Up
hsa-let-7g	8,51	30,48	Up
HS_19	5,49	2,39	Up
hsa-miR-146a	3,46	288,64	Up
hsa-miR-1249	2,76	150,51	Up
hsa-miR-130b*	4,05	18,62	Up
hsa-miR-335*	9,35	49,55	Up
hsa-miR-103	-3,03	-2,75	Down
hsa-miR-518b	-2,25	-41,17	Down
HS_276,1	-7,19	-9,28	Down
HS_239	-2,67	-8,30	Down
hsa-miR-1233	-5,32	-2,53	Down
HS_202,1	-2,46	-42,21	Down
HS_89	-2,04	-10,87	Down
HS_217	-34,38	-26,98	Down
hsa-miR-189:9,1	-3,29	-3,37	Down
hsa-miR-193a-3p	-5,00	-2,25	Down
hsa-miR-181c*	-2,71	-4,17	Down
hsa-miR-1254	-2,13	-11,78	Down
hsa-miR-1224-3p	-2,80	-5,61	Down
hsa-miR-1182	-2,35	-9,84	Down
hsa-miR-187*	-2,25	-2,66	Down
hsa-miR-1225-5p	-2,55	-3,23	Down
hsa-miR-1197	-2,85	-3,20	Down
hsa-miR-1282	-14,15	-77,39	Down
hsa-miR-1284	-2,74	-5,88	Down
hsa-miR-18b*	-2,25	-13,52	Down
hsa-miR-24-2*	-4,94	-41,64	Down
hsa-miR-1293	-9,29	-70,99	Down

\*Passenger microRNA.

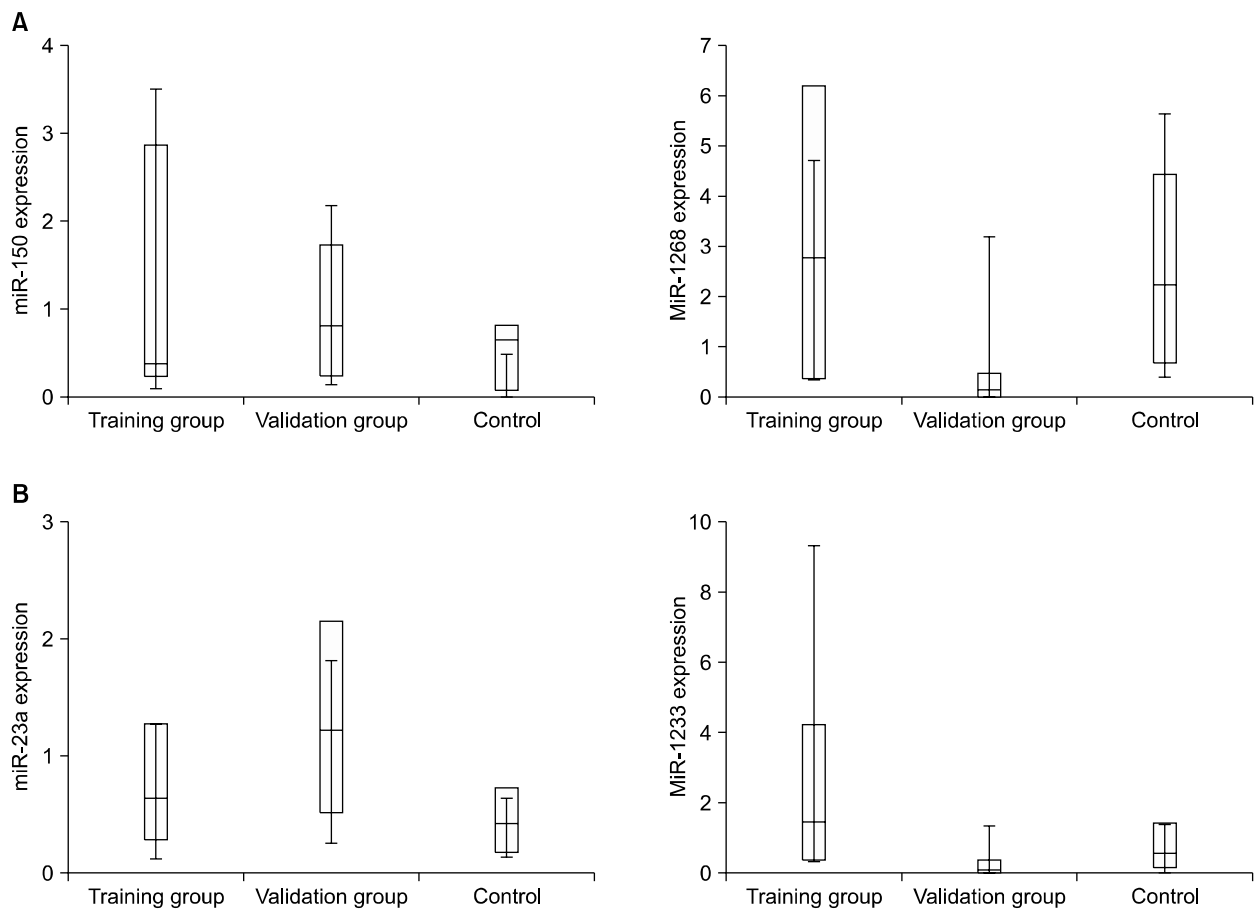
## 2. qRT-PCR analysis of five miRNAs

Of the four miRNAs chosen for further analysis, two miRNA (miR-150 and miR-1268) were chosen in case of SCC and two miRNA (miR-23a and miR-1233) were chosen in AC cases. The miRNAs most differentially ex-

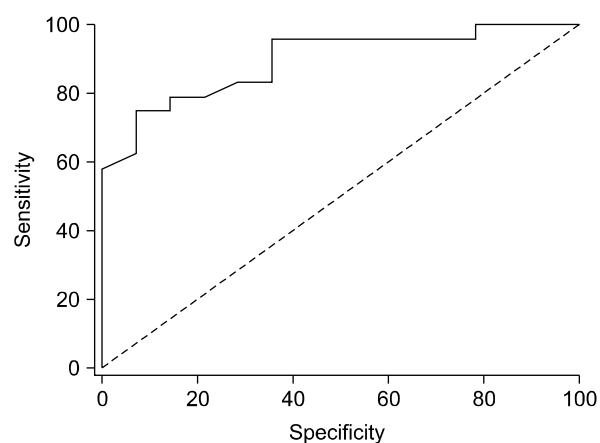
pressed in serum from patients with NSCLC versus the controls were selected based primarily on fold-change followed by p-values and false discovery rates (data not shown). Expression levels of miR-150 and miR-1268 shown by qRT-PCR were not consistent with the microarray results for cases of SCC (Figure 1A). Interestingly, the qRT-PCR results for miR-150 conflicted with those from microarray in both training and validation groups. For ACs, the results from qRT-PCR of miR-1233 was not consistent with the results from microarray for training and validation groups, but only the results from qRT-PCR of miR-23a were concordant with those from microarray for training and validation groups (Figure 1B). To evaluate whether serum miR-23a can be used as a potential marker for diagnosing ACs, ROC curve analyses were performed on all patients in training and validation group. These analyses revealed that the level of serum miR-23a was a potential marker for discriminating AC from COPD patients with a ROC curve area of 0,896 (95% CI, 0,75~0,97) (Figure 2). At a cut-off value of 0,35, the sensitivity and specificity for miR-23a was 75% and 92,7%, respectively.

## Discussion

The potential use of miRNAs as biomarkers for lung cancer has been demonstrated. Several studies have identified pathognomonic or tissue-specific miRNA expression profiles in lung cancer and other cancer<sup>5,13,14</sup>. Specific microRNA biomarkers may also accurately and reliably distinguish squamous from nonsquamous NSCLC. miR-205 is a highly accurate marker for lung cancer with squamous histology<sup>15</sup>. miRNAs regulate tumor activities such as tumor progression and metastasis; hence, miRNA profiling has been associated with clinical outcomes. The expression of let-7 is down-regulated in NSCLC patients and is associated with poor prognosis<sup>5,16</sup>. Saito et al.<sup>17</sup> reported that high levels of miR-21 are associated with worse cancer-specific mortality and relapse-free survival rates independent of other clinical factors in stage I patients. Moreover, the expression of certain miRNAs is associated with chemo-



**Figure 1.** Box plot representing the expression of four miRNAs assessed by qRT-PCR. (A) Expression analysis to compare miR-150 and miR-1268 levels in the serum of SCC patients to serum samples obtained from COPD patients. (B) Expression analysis to compare miR-23a and miR-1233 levels in the serum of AC patients to serum samples obtained from COPD patients.



**Figure 2.** Receiver operating characteristic curves for miR-23a in COPD control subjects and patients with adenocarcinoma. Curves were generated using data for miR-23a from qRT-PCR normalized to cel-miR-39.

resistance in lung cancer<sup>18,19</sup>.

More recent methods have improved the threshold sensitivity of miRNA detection down to a few nanograms of total RNA<sup>9</sup>. This amount can easily be obtained in clinical specimens from fine needle aspiration biopsies (FNAB). The measurement of miRNA by qRT-PCR on FNAB samples has been reported<sup>20</sup>. miRNAs stability is high in both fresh and formalin-fixed paraffin-embedded tissues. This property makes miRNAs in clinical samples potentially useful diagnostic and prognostic biomarkers.

In recent years, miRNAs have been found in body fluids such as serum, plasma, urine, and saliva. Three different routes have been proposed to explain how miRNAs enter the circulation<sup>21</sup>. First, free miRNAs may

be directly secreted by cells in a fashion similar to cytokines and hormones. Second, miRNAs may be selectively packed into microparticles and then released by cells via the shedding of microvesicles. Finally, miRNAs could be released via cell-derived exosomes.

Circulating miRNAs are highly stable and resistant to RNase activity as well as extreme pH and temperatures in serum and plasma<sup>8,22</sup>. The exact mechanism underlying the stability of circulating miRNAs remains largely unknown; however, a couple hypotheses have been proposed. First, miRNAs may be modified by methylation, adenylation, and uridylation. These modifications have a role of in stabilizing miRNAs<sup>23</sup>. Second, circulating miRNAs could be protected by specific proteins. RNA-binding protein could protect miRNA from degradation<sup>24</sup>.

The results of this study showed that only the expression of miR-23a was consistent with the results of the microarray analysis. In contrast, the qRT-PCR results for miR-150, miR-1268 and miR-1233 was not consistent with those of the microarray analysis. There are two possible explanations for disagreement between the microarray and qRT-PCR results. First, the sample size for the microarray analysis was too small and may have resulted in false positives. Second, this discrepancy might be explained by the fact that these two methods used different control groups. The profile of circulating miRNAs could be also influenced by the presence of other diseases or even subtle physiological condition changes. Wang et al.<sup>25</sup> reported that serum miR-146a and miR-223 levels are significantly reduced in septic patients compared to SIRS patients and healthy controls. In particular, the level of liver-specific miR-122 was elevated in plasma or serum from not only hepatocellular carcinoma patients but also individuals with hepatitis B virus infection, cirrhosis, and general liver injury<sup>26</sup>. These data suggested that the miRNAs profiles of healthy controls and COPD patients might be different.

miRNA expression analysis has found that the miR-23a family is up-regulated in various types of cancer<sup>27-29</sup>. Additionally a previous study showed that miR-23a functions as an oncogene in gastric cancer,

prostatic cancer and HCC. miR-23a promotes the growth of the gastric cancer and has anti-apoptotic effects on HCC cells through the regulation of target genes<sup>30,31</sup>. More importantly, the interleukin-6 receptor (IL-6R) tumor suppressor gene is directly regulated by miR-23a<sup>30</sup>.

The findings of this study have provided information about the serum miRNA profiles of lung cancer patients although only a small number of patients was examined. Moreover, miR-23a expression could be a potential serum-based biomarker used for minimally invasive procedures to diagnose AC. Additional large scale studies needed to validate the use of circulating miRNAs as novel biomarkers for lung cancer.

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