Aberrant expression of let-7a miRNA in the blood of non-small cell lung cancer patients

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Abstract. Abnormal expression of let-7a microRNA (miRNA) in non-small cell lung cancer (NSCLC) cells and tissue has been previously reported. Our objective was to investigate whether let-7a miRNA is aberrantly expressed in the blood of NSCLC patients. Using real-time PCR (RT-PCR), we analyzed let-7a miRNA in archived whole blood from 65 participants, 35 of whom had NSCLC and 30 of whom did not. Using RT-PCR, we also investigated the expression of let-7a miRNA in NSCLC cell lines (A549 and HCC 1588), a normal human lung fibroblast cell line (WI-38) and in 40 human NSCLC tissues. The 2^{-ddCt} of let-7a miRNA in the blood of normal subjects and those with NSCLC was 3242.49±355.28 and 747.85±177.74, respectively. The relative expression of let-7a miRNA in the A549 and HCC 1588 cancer cell lines was approximately 0.3 and 0.35, respectively, compared to WI-38 cells. The 2^{-ddCt} of let-7a miRNA in the normal human lung tissues and human NSCLC tissues was 42.30±3.98 and 27.73±3.86, respectively. Let-7a miRNAs were under-expressed in the blood of NSCLC patients, as well as NSCLC cells and NSCLC tissues, compared to normal controls. The possibility of using let-7a miRNA as a serologic marker for lung cancer warrants further study.

Introduction

MicroRNAs (miRNAs) are small (18-25 nucleotide in size) non-coding RNA molecules that function in the modulation of the activity of specific messenger RNA (mRNA) targets and play important roles throughout cellular development, proliferation and differentiation. The expression levels of miRNAs vary greatly among species and tissues. Over 700 miRNAs have been identified in human tissues. Some miRNAs are linked to human malignancy and are differentially expressed in human cancer vs. matched normal tissue. There is increasing evidence to support the use of miRNA analysis for the diagnosis, prognosis and therapy of cancer (1-3). Some data from *in vitro* and *in vivo* studies have shown that miRNAs may have roles in the pathogenesis of lung cancer (4,5). miRNA expression was shown to change linearly from normal bronchial tissue, hyperplasia, metaplasia, dysplasia and carcinoma (6). Crawford *et al* reported that miRNA-126 inhibits invasion in non-small cell lung carcinoma (NSCLC) cell lines (7). Fei *et al* reported that antibodies of miRNA-16, miRNA-21 and miRNA-214 inhibited human lung adenocarinoma (A549) cell growth by inducing apoptosis and S-phase arrest (8).

Recent publications have identified several miRNAs that were aberrantly expressed in primary lung cancer tissue (9). Depending on the genes that the miRNAs act on, they may serve to stimulate or suppress tumor formation and growth. Some of these miRNAs have been associated with the survival of NSCLC (10-12).

Let-7 miRNA, the second member of the miRNA family discovered in 2000, was initially found to control developmental timing in *Caenorhabditis elegans*. In humans, the let-7 family consists of 11 related genes and has been reported to be expressed at lower levels in lung cancer, both *in vitro* and *in vivo*. Reduced let-7 miRNA expression has been significantly associated with shortened postoperative survival (13,14).

Recently, cancer-related miRNAs have also been identified in the serum of breast, pancreatic, colon and lymphoma patients (15-18). Microarray data showed the presence of some miRNAs in the serum of NSCLC patients (19). The objective of our study was to investigate the expression of let-7a miRNA in the blood of NSCLC patients compared to normal controls.

Materials and methods

Cell lines, tissues and blood. The established human A549 cell line, squamous cell carcinoma cell line (HCC 1588) and normal human lung fibroblast cell line (WI-38) were cultured under the appropriate conditions: A549 and HCC1588 cells were maintained with RPMI-1640 (Lonza) medium containing 10% fetal bovine serum (FBS; Lonza), streptomycin (100 μg/

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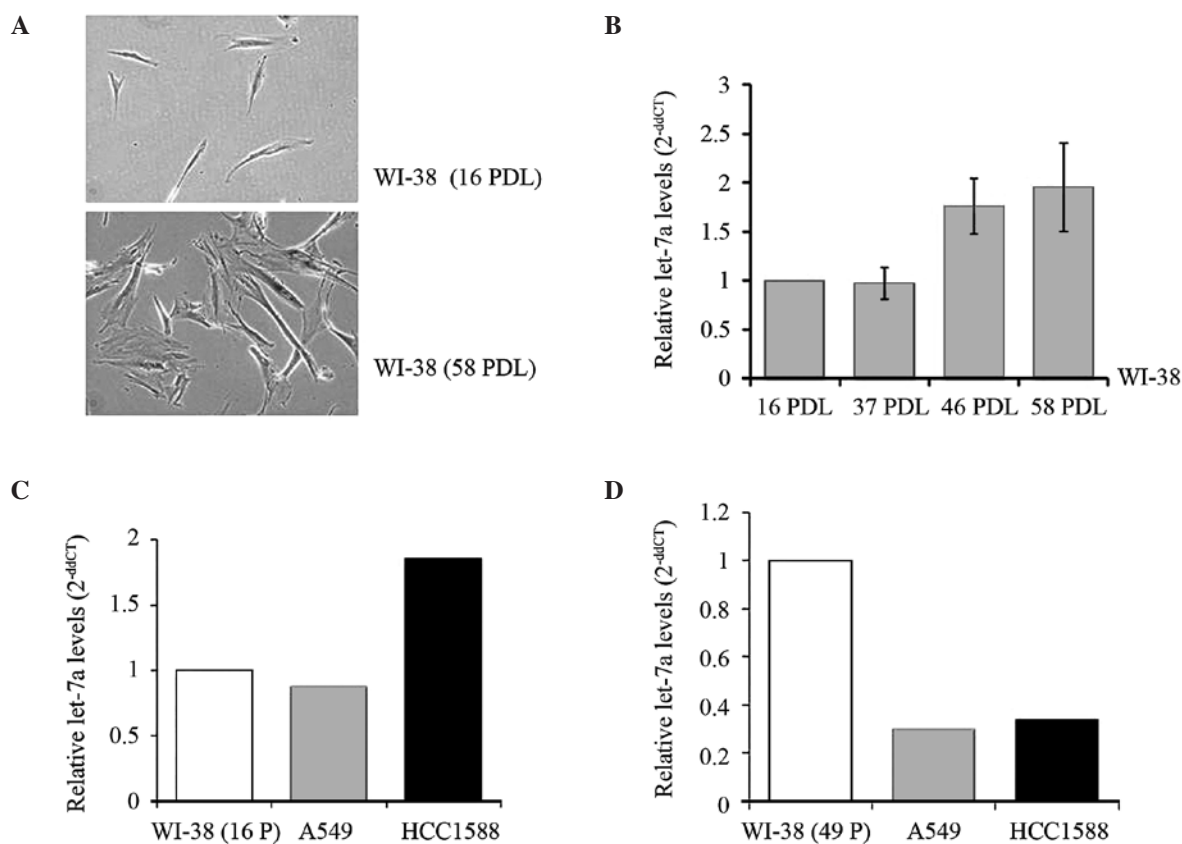E-mail: kyeoung66@hanmail.net

Figure 1. Expression of let-7a miRNA in normal lung fibroblast cells (WI-38) and non-small cell lung cancer cell lines (A549: adenocarcinoma, HCC1588: squamous cell carcinoma). (A) β-galactosidase staining at pH 6.0 on normal WI-38 cells at 16 PDL and senescent WI-cells at 58 PDL. (B) Expression of let-7a miRNA in WI-38 cells shows an increase according to (PDL. (C) Expression of let-7a miRNA in A549 cells was similar to that of WI-38 cells (16 PDL). The expression level of let-7a miRNA in HCC1588 cells was somewhat higher than that of WI-38 cells. (D) Expression of let-7a miRNAs in NSCLC (A549 and HCC1588) cells was lower than that of WI-38 cells (49 PDL). PDL is an intrinsic measure of the 'age' of the particular culture of a cell line.

ml) and penicillin (100 U/mol; Lonza) at 37˚C in a humidified atmosphere containing 5% CO_2 in air. WI-38 cells were cultured in Eagle's minimal essential medium (EMEM; Lonza) with 10% FBS at 37˚C in 5% CO_2.

Forty surgically resected human lung cancer tissues and normal lung tissues were obtained from the National Korea Lung Tissue Bank. Tumor tissues were representative of the two histological types: 20 adenocarcinoma and 20 squamous cell carcinoma (patients did not receive previous treatment).

Venous blood (35 ml) was collected from the antecubital fossa of 35 lung cancer patients prior to treatment, and from 30 normal subjects.

Permission regarding ethics and informed consent were obtained for the use of all samples.

RNA isolation and real-time quantitative PCR. Total RNA were isolated from lung cells and lung tissue by TRIzol (Molecular Research Center, Inc.) according to the manufacturer's instructions. Also, total RNA was isolated from blood by the easy-RED™ total RNA extraction kit (Intron biotechnology) as per the manufacturer's protocol. The concentration was quantified using a NanoDrop 1000 Spectrophotometer. Expression of let-7a miRNA was analyzed using quantitative real-time PCR (RT-PCR). Quantitative RT-PCR was performed with the TaqMan microRNA assay (Applied Biosystems Inc.). Normalization was performed with the small nuclear RNA U6 (RNU6B; Applied Biosystems Inc.). The

nucleotide sequences of the primers were as follows: let-7a, 5'-UGAGGUAGUAGGUUGUAUAGUU-3'; RNU6B, 5'-CGC AAGGAUGACACGCAAAUUCGUGAAGCGUUCCAU AUUUUU-3'. All plates were performed in a Roter-gene 6 (Corbett Research).

B-galactosidase staining. The cells ($1x10^5$) were seeded in 6-well plates. After 24 h, the cells were washed with 1X PBS, fixed for 10 min in β-galactosidase fixative (2% formaldehyde; 0.2% glutaraldehyde in PBS), washed again with 1X PBS, and finally stained for 24 h at 37˚C in the absence of CO_2 in staining solution [150 mM NaCl, 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 40 mM citric acid and 12 mM sodium phosphate, pH 6.0], containing 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside. Thereafter, cells were washed in 1X PBS, and the number of galactosidase activity-positive cells (blue staining) was counted under bright field illumination.

Statistical analysis. The dC_t method was used for analysis [dC_t = mean Ct (RNU6B) - mean Ct (let-7a miRNA)] of the RT-PCR outcome. Statistical analysis was performed using SPSS 17.0 (PASW 17.0; Chicago, IL, USA). Statistical significance was evaluated using the Student's t-test for unpaired comparison; $P \leq 0.05$ was considered statistically significant. The area under the receiver operation characteristic (ROC) curve was calculated to verify the accuracy of the expression of let-7a miRNA in the diagnosis of NSCLC.
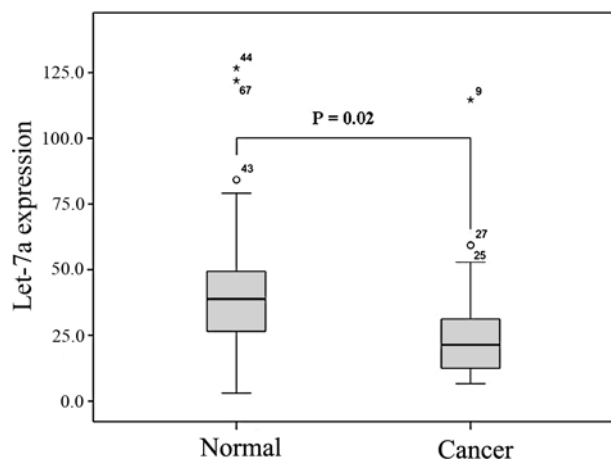
Figure 2. Expression of let-7a miRNA is lower in non-small cell lung cancer tissues than in normal lung tissues.

Results

Expression of let-7a miRNA in normal lung epithelial cell lines and NSCLC cells. Expression of let-7a miRNA in WI-38 cell lines showed an increasing trend according to the population doubling level (PDL). The PDL is an intrinsic measure of the age of the particular culture of a cell line (Fig. 1A). The expression level of let-7a miRNA in senescent WI-38 cells with 58 PDL was doubled compared to that of young WI-38 cells with 16 PDL (Fig. 1B). Therefore, let-7a miRNA may play a role in the senescence of lung fibroblast cells. To date, the expression of let-7a miRNA has been considered to be decreased in cancer cells. However, when we compared young WI-38 cells (16 PDL) to NSCLC cells, we could not find a difference in let-7a miRNA expression. Let-7a miRNA expression was somewhat higher in HCC1588 than in normal WI-38 cell lines (16 PDL) (Fig. 1C). However, the expression of let-7a miRNA was lower in lung cancer cell lines (A549 and HCC1588) than in normal WI-38 cells with 45 PDL, as expected. The relative expression of let-7a miRNA in the A549 and HCC1588 cancer cell lines was approximately 0.3 and 0.35, respectively, as compared to the WI-38 cells (Fig. 1D).

Expression of let-7a miRNA in normal lung tissues and NSCLC tissues. The median age of the NSCLC patients was 62 years. Twenty-nine patients were male and 11 were female. The number of patients with stage I was 10; with stage II, 7; with stage III, 20; and with stage IV, 3. A significant difference in the expression level of let-7a miRNA was observed between 40 normal lung tissues and 40 NSCLC tissues (p=0.02). The expression of let-7a miRNA was down-regulated in NSCLC tissues compared to normal lung tissues (Fig. 2). The 2^{-ddCt} of let-7a miRNA in normal lung tissues and NSCLC tissues was 42.30±3.98 and 27.73±3.86, respectively. No difference in let-7a miRNA expression according to clinicopathologic features (age, gender, smoking status, histology and stage) was observed.

Expression of let-7a miRNA in the blood of normal subjects and NSCLC patients. There were no differences in baseline characteristics (gender, age and smoking status) between

Table I. Baseline characteristics of study populations.

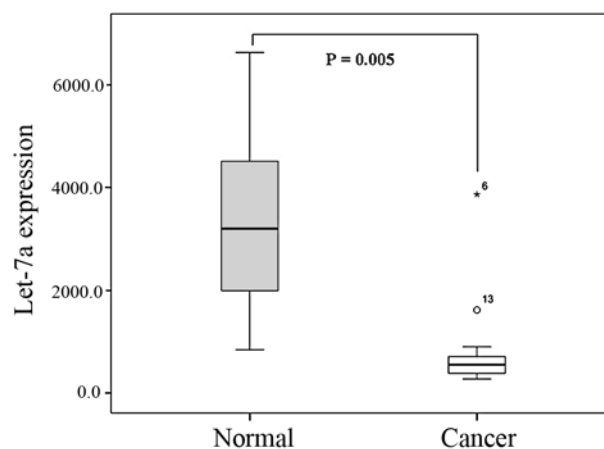|	No. (%)	
	Lung cancer (n=35)	Normal subjects (n=30)
Gender		
Male	28 (80)	16 (53)
Female	7 (20)	14 (47)
Age (years)		
Median (range)	67 (48-84)	60 (37-80)
Smoking		
Current	20 (57)	16 (53)
Ex-smoker	9 (25)	10 (33)
Non-smoker	6 (17)	4 (13)
Stage		
I	5 (14)	
II	1 (3)	
III	16 (46)	
IV	13 (37)	
Histological subtype		
Adenocarcinoma	18 (51)	
Squamous cell carcinoma	15 (43)	
Bronchioloalveolar cell carcinoma	1 (3)	
Poorly differentiated carcinoma	1 (3)	

Figure 3. Blood level of let-7a miRNA is lower in non-small cell lung cancer patients (n=35) than in healthy subjects (n=30) (p=0.005).

normal subjects and NSCLC patients (P>0.05) (Table I). Most of the NSCLC patients had advanced stage disease. Among the 35 patients, 29 (83%) were stage III and IV. The expression level of let-7a miRNA in whole blood samples from NSCLC patients (n=35) was compared to those from healthy controls (n=30). The 2^{-ddCt} of let-7a miRNA in NSCLC was 747.85±177.74 and 3242.49±355.28 in normal subjects. The level of let-7a miRNA was down-regulated in the blood of NSCLC patients (p=0005) (Fig. 3). The area under the ROC curve of let-7a miRNA expression in the whole blood was 0.951. Sensitivity and specificity were 90.3 and 90.3%,
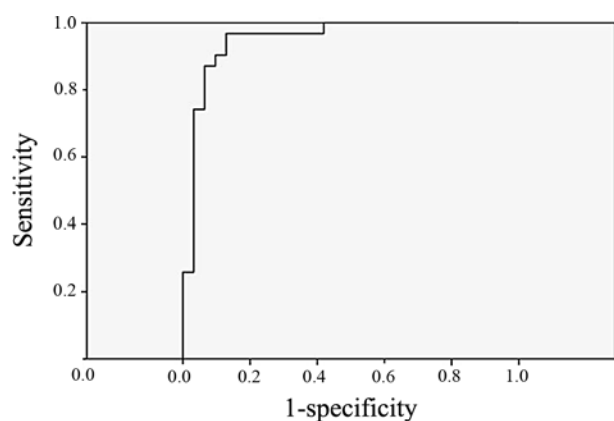
Figure 4. ROC curve of blood let-7a miRNA for the diagnosis of non-small cell lung cancer. The area under the ROC curve was 0.951 (P<0.0001).

respectively, at a cut-off value of 868.5 (Fig. 4). We investigated associations between let-7a miRNA and the clinicopathologic features of the NSCLC patients (age, gender, smoking history, histology and stage), but found no significant correlation.

Discussion

In the present study, we examined the expression of let-7a miRNA in the cells, tissues and blood of NSCLC. The degree of down-regulation was somewhat different in each sample. Among the miRNAs, blood let-7a miRNA was markedly decreased in the NSCLC samples.

There is a large amount of evidence indicating that the let-7 miRNA family is associated with the regulation of cell proliferation and differentiation during development. Let-7 miRNA has been reported to induce cell cycle arrest in the G1 phase by repressing the target genes involved in the traversion of the cell cycle (20). Let-7f miRNA and let-7g miRNA expression changed according to the stage of cell cycle arrest in WI-38 human fibroblasts (i.e., premature senescence, replicative senescence and quiescence) (21). Let-7 miRNA was undetectable in human and mouse embryonic stem cells; however, the level of let-7 increased upon differentiation (22). Let-7 miRNA has been associated with the neural differentiation of embryonal stem cells (23). Let-7 miRNA was also previously known as a regulator of the aging mechanism in *Caenorhabditis elegans* (24). In the results, we first described an increase in let-7a miRNA according to the age of the WI-38 cell line. The expression of let-7a miRNA in WI-38 cells with 58 PDL was over 2-fold more than that of WI-38 cells with 16 PDL (Fig. 1A). To the best of our knowledge, this phenomenon is an unknown finding. If we had not checked the PDL, we would not have been able to make a precise comparison of the expression of let-7a miRNA between WI-38 and NSCLC cells. These findings also suggest that let-7a miRNA may play a role in aging and senescence in the pathogenesis of fibrogenic lung reactions (25). The examination of the expression of let-7a miRNA in patients with fibrogenic lung disease, such as idiopathic pulmonary fibrosis or chronic obstructive pulmonary disease, is warranted.

The deregulation of let-7 miRNA has been shown to be a feature of many types of cancer, including lung cancer

(26). To date, the decreased expression of let-7 miRNA in NSCLC tissues has been reported in only a few studies. Most of these have investigated NSCLC tissues in the early stages. In this study, we investigated the expression of let-7a miRNA (among the let-7 family) in Korean NSCLC tissues that were in early and advanced stages. As expected, let-7a miRNA was down-regulated in all stages of NSCLC. The rate of decrease of let-7a was approximately 64% in 40 NSCLC tissues compared to 40 normal lung tissues. Notably, the rate of the decrease of let-7a miRNA was 23% in the blood of NSCLC patients compared to normal subjects. The sensitivity and specificity of blood let-7a miRNA in the diagnosis of NSCLC were high.

Aberrantly expressed miRNAs in the blood of lung cancer patients have been reported rarely. Microarray analysis of miRNAs in the serum of NSCLC patients only has recently been reported. The expression of some miRNAs differed between patients with longer and shorter survival. Serum miRNA-486, miRNA-30d, miRNA-1 and miRNA-499 were independent predictors of postoperative survival in NSCLC patients (27). Our report is the first to compare the down-regulated expression of let-7a miRNA in the blood of NSCLC patients to that of normal subjects. To date, clinicians have not had an effective serologic marker for lung cancer. When lung cancer is suspected, they may check let-7a miRNA levels in the blood. If the 2^{-ddCt} is lower than 800, this indicates the need for invasive biopsy. These findings may be applied to the detection of early lung cancer. Variable clinical factors affect let-7 miRNA expression in the blood of normal subjects and lung cancer patients. Therefore, the findings of this study must be confirmed in a large scale sample.

The physiologic role of the depressed expression of let-7a in NSCLC patients remains uncertain. Many genes (i.e., RAS, Myc, Hmga2 and Cycin D1) may be targets of let-7a miRNA. The epidermal growth factor receptor (EGFR) gene has also been also regarded as candidate target gene. Zhong *et al* reported that let-7a miRNA inhibited the growth of NSCLC cells and enhanced the cytotoxicity of targeted agents (EGFR tyrosine kinase inhibitor) *in vitro* (28). Observation of the change of let-7a miRNA expression in the blood of NSCLC patients after EGFR tyrosine kinase inhibitor therapy would be informative.

There are some limitations to our study, including small sample size. Due to the short follow-up time, we could not prove the prognostic value of let-7a miRNA in the blood and tissue of NSCLC patients. Despite the above limitations, our study provided initial data on down-regulated blood let-7a miRNA in NSCLC patients, and suggested the diagnostic value of the analysis of blood let-7a miRNA in NSCLC patients. The development of minimally invasive tests for the detection and monitoring of cancer patients has long been a goal of cancer research. Circulating let-7a miRNA may have clinical utility for the diagnosis, monitoring and follow-up of patients with NSCLC. This should be explored by further studies conducted with larger sample numbers.

In conclusion, let-7a miRNA was down-regulated in the blood of NSCLC patients compared to normal subjects. This data suggests that blood let-7a miRNA has potential as a clinically useful non-invasive biomarker in lung cancer.

Acknowledgements

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