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Investigation of miR221 and miR222 as Biomarkers in Non-small Cell Lung Cancer

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Abstract. *Background/Aim: MicroRNAs (miRNA) are a class of small non-coding RNAs of 18-25 nucleotides, which regulate gene expression at the post-transcriptional level by disrupting or blocking translation of messenger RNA targets. Non-small cell lung cancer (NSCLC) represents approximately 85% of all lung cancers. Early and accurate diagnosis of the disease affects the probability of success of treatment. The purpose of this study was to investigate the expression levels of serum specific miRNA221 and miRNA222 as a biomarker in NSCLC. Materials and Methods: Thirty-two NSCLC cases and 30 healthy control cases that were diagnosed at Istanbul Yedikule Chest Diseases and Thoracic Surgery Training Hospital were included in this study. miRNAs were detected using miRNA-specific quantitative real-time-PCR. The relative expression of miRNAs was calculated using the* $2^{-\Delta\Delta Ct}$ *method. Results: miR221 and miR222 showed 1.46 and 1.63-fold higher expression in the samples from patients with NSCLC compared to controls, and the difference of expression was statistically significant for miR221 (p=0.000095) but not for miR222 (p=0.084470). In the presence of metastasis in NSCLC patients, miR221 levels were 2.33-fold higher compared to non-metastatic cases (p=0.014), and those of miR221 and miR222 were expressed 1.44 and 1.52-fold higher, respectively, in advanced stage compared to early stage (p=0.000387, p=0.000302). Conclusion: The levels of miR221 and miR222 in the serum of patients could be used as biomarkers for the diagnosis, treatment, and prognosis of NSCLC.*

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Non-small cell lung carcinoma (NSCLC) accounts for approximately 80%-85% of all lung cancer patients and shows an overall five year survival of 15% (1-3). There is no adequate and reliable biomarker for earlier NSCLC detection. Thus, the identification of a minimally invasive and reliable biomarkers is crucial to increase the possibility of earlier NSCLC detection, reduce the potential risk of harm and increase survival (4).

MicroRNAs (miRNAs) are a class of highly conserved family of short single-stranded noncoding RNAs of 18-25 nucleotides that form hairpin structures (5, 6). Specifically, miRNAs function in the regulation of immune responses in epithelial cells and specialized immune cells in response to different environmental factors and respiratory viruses. miRNAs regulate gene expression at the posttranscriptional level as part of the RNA induced silencing complex (RISC) that interacts with the complementary sequences of mRNAs causing their translational inhibition or cleavage. One miRNA may target many mRNAs, within the same pathway (7, 8). miRNAs have been shown to be involved in various cellular and biological processes such as development, proliferation, differentiation, fibrosis, angiogenesis, erythropoiesis, and apoptosis. Deregulated expression of miRNAs can alter cellular responses and contribute to the development of lung diseases (9-11). The detailed cellular function of most miRNAs is still

unknown. However, approximately half of the 22,000 genes in the human genome are controlled by these regulators. miRNAs have been associated with various diseases including cancer and play a critical role in the process of cancer formation. Altered expression levels of cancer-associated miRNAs or 'Oncomirs' have been found in many cancer types (12, 13).

miR221 and miR222 are highly conserved, clustered genes, which contain identical seed sequences and both map to the X chromosome separated by 727 bases. Depending on tumor type, miR221 and miR222 function in cancer development either as oncogenes or tumor suppressors (14, 15). Expression of miR221 and miR222 is significantly increased in solid tumors including thyroid cancer, hepatocarcinoma, estrogen receptor negative breast cells, and melanoma cells.

Early diagnosis of cancer is the most important factor for increasing survival and success of treatment. For this reason, it is very important to detect biomarkers for early diagnosis. Blood serum is a minimally invasive and easy to obtain biofluid that can be searched for potential biomarkers. Serum-based microRNAs have been shown to be differentially expressed in various cancer types, including NSCLC (16).

To explore novel miRNA signatures for the diagnosis of NSCLC, we determined the levels of miR221 and miR222 that had been reported to be frequently deregulated in cancers and correlated with tumorigenesis or metastasis (14). The results of this study will enable us to understand the molecular mechanisms for NSCLC to allow for early diagnosis and treatment.

Materials and Methods

Patients and control individuals. Thirty-two NSCLC patients and 30 healthy control serum samples were obtained from the Istanbul Yedikule Chest Diseases and Thoracic Surgery Training Hospital, from 2010 to 2012. Thirty healthy individuals without any malignancy were selected for the control group, which were matched for age and sex.

All specimens were classified by pathologists according to World Health Organization (WHO) guidelines and TNM Union for International Cancer Control (UICC) pathological staging criteria. Patients were not administered chemotherapy or radiotherapy before the study. The serum samples stored at –80˚C deep freeze until use. Informed consent form was signed by all participants. Ethics Committee approval was obtained from Istanbul University (October 21, 2011; No. 1715).

Total RNA isolation and cDNA synthesis. Total RNA was extracted from serum using Trizol reagent following the manufacturer's protocol (Roche Diagnostics GmbH, Mannheim, Germany). Total RNA concentration and purity were measured at A260 and A280using the NanoDrop-1000 (ND-1000) spectrophotometer. cDNA was generated using the miRCURY LNA™ Universal cDNA Synthesis Kit (Exiqon, Boston, MA, USA).

The reaction mixture contained 4 μl total RNA, 2 μl reverse transcriptase mix, 4 μl reaction buffer, 9 μl RNase-free water, and 1 μl synthetic spike. The cDNA synthesis PCR was set up as follows: 42˚C for 60 min and 95˚C for 5 min.

Table I. *Age, smoking (pack/years) and pulmonary function test values in non-small cell lung cancer (NSCLC) and control groups.*

	NSCLC group		Control group		
	Mean	SD	Mean	SD	*p*-Value
Age	56.25	4.859	54.63	8.109	0.350
Smoking (pack/years)	52.28	34.654	33.87	18.619	0.011
FEV1%	73.63	14.893	98.94	17.760	<0.001
FVC%	73.63	14.641	99.88	14.337	<0.001
FEV1/FVC%	86.13	15.318	96.48	14.334	0.031
DLCO%	92.40	43.048	91.35	23.496	0.944

FEV1: Forced expiratory volume in 1 second; FVC: forced vital capacity; DLCO: diffusion capacity of the lung for carbon monoxide.

Quantitative real-time polymerase chain reaction (PCR). Quantitative real-time PCR was performed using Light Cycler FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH) with specific primers on the Light Cycler 480 RT- PCR (Roche Diagnostics GmbH). The PCR conditions were 95˚C for 10 min, followed by 45 amplification cycles of 95˚C for 15 s, 60˚C for 16 s, and 72˚C for 15 s. A melting curve analysis was performed at the end of each PCR cycle to validate the PCR product specificity.

RT-qPCR analyses were performed using the following miRCURY LNA™ Exiqon miRNA primer sequences: miR221-3p (5'-AGCUACAUUGUCUGCUGGGUUUC-3'), miR222-3p (5'-AGCUACAUCUGGCUACUGGGU-3'). U6 and RNU1A1 were used as reference genes. The relative expression of miRNA to U6 and RNU1A1 was calculated using the $2^{-\Delta\Delta Ct}$ method (17).

Statistical analysis. Expression levels were transformed into fold changes and reported as relative expression. The means of two groups were compared using *t*-test in SPSS 17 (SSPS Inc., Chicago, IL, USA). Differences between groups were estimated using the Mann-Whitney *U*-test. All statistical analyses assumed a two-sided alternative with a 5% level of significance.

Results

There was no statistically significant difference between the mean age of NSCLC cases and the mean age of the control group (p=0.350). However, when examined in terms of smoking (pack/years), there was a significant difference between the study groups (p=0.011). A statistically significant difference was also found between alcohol use of NSCLC patients and the control group [p<0.0001 odds ratio (OR)=1.600, 95% confidence interval (CI)=1.223-2.093].

When the NSCLC cases and control group were evaluated in terms of pulmonary function tests, a significant difference was found for forced expiratory volume in 1 s (FEV1) %, forced vital capacity (FVC)%, and FEV1/FVC% values (p<0.05) (Table I). The distribution of NSCLC histopathological stage results is given Figure 1. Expression levels of miR221 and miR222 are shown in Table II. miR221 showed a 1.46-fold

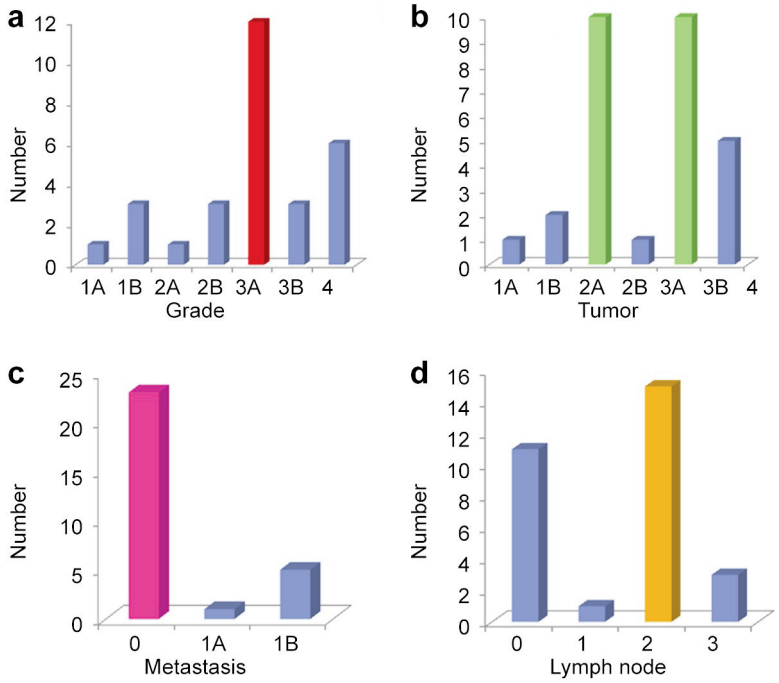

Figure 1. *Non-small cell lung cancer (NSCLC) histopathological grade results. TNM classifications (B, C, D) of NSCLC patients and their Grades (A) determined accordingly are shown.*

Table II. *The fold change of miR221 and miR-222.*

Gene	Mean ΔC_t		$2^{-\Delta\Delta Ct}$		95%CI	Fold change	*p*-Value
	NSCLC	Control	NSCLC	Control			
miR-221	4.63	5.18	0.040444	0.027666	(1.39-1.53)	1.46	0.000095
miR-222	3.16	3.87	0.111545	0.068384	(1.05-2.21)	1.63	0.084470

NSCLC: Non-small cell lung cancer.

Table III. *The fold change of miR221 and miR-222 genes according to stage classification in non-small cell lung cancer.*

Gene	Mean ΔC_t		$2^{-\Delta\Delta Ct}$		95%CI	Fold change	*p*-Value
	Advanced stage	Early stage	Advanced stage	Early stage			
miR-221	7.06	7.59	0.007485	0.005190	(1.36-1.52)	1.44	0.000387
miR-222	1.95	2.56	0.258048	0.169796	(1.43-1.61)	1.52	0.000302

increase and miR221 showed a 1.63-fold increase in patients with NSCLC compared with the control group. This expression difference between the patients and controls was statistically significant for miR221 (p=0.000095) but not for miR222 (p=0.084470). The fold changes of miR221 and miR222 genes are given in Figure 2.

miR221 showed 2.62-fold higher expression in the T3+T4 stage of primary NSCLC tumors compared with the T1+T2 stage of primary tumor (p=0.000165), whereas miR222 showed 4.23-fold higher expression (p=0.007449). In metastatic NSCLC, miR221 levels were 2.33-fold higher than those in non-metastasis (p=0.014).

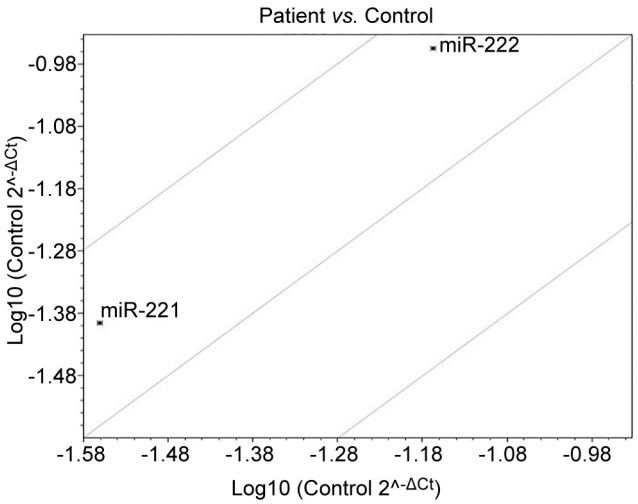

Figure 2. *Scatter plot showing the expression level difference of miR221 and miR-222 genes between patient and control groups.*

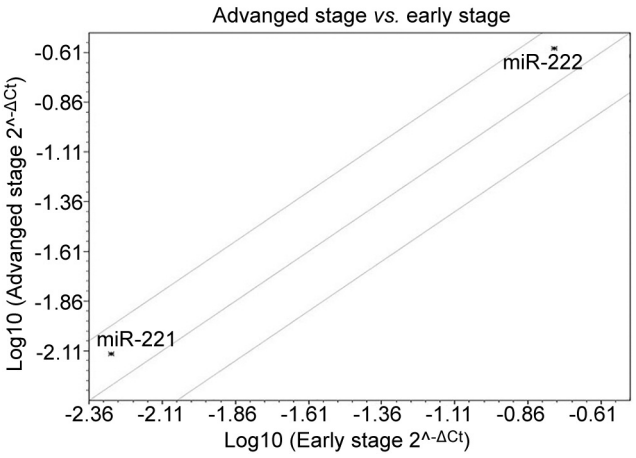

Figure 3. *Scatter plot showing the expression level difference of miR221 and miR-222 genes between early and advanced stages.*

Stage classifications according to miR221 and miR222 genes are shown in Table III. Expression of miR221 and miR222 was 1.44-fold and 1.52-fold higher, respectively, in advanced stage NSCLC than early stage (Figure 3).

Discussion

The differences in expression of various miRNAs have been associated with many diseases such as inflammatory, cardiovascular, neurodevelopmental, autoimmune disease and cancer (18, 19). Many miRNAs are differentially regulated in various types of cancer and miRNAs in oncogenic pathways are thought to have several targets. miRNAs play important roles in carcinogenesis since they act as oncogenes or tumor suppressors (20).

Nowadays, many miRNAs have been identified as regulators of neoplastic transformation, invasion, and metastasis. miR221/miR222 have emerged as key miRNAs dysregulated in many cancer types (21). Despite being the most studied miRNAs, our knowledge regarding their role in signaling pathways is quite limited. Therefore, understanding the signaling pathways involved is important in determining their function in oncogenic processes as well as determining new treatment strategies (14). In addition, several studies have suggested that miRNAs can be used in the treatment of drug resistance in treated patients. Considering these characteristics, miRNAs could be useful diagnostic biomarkers for various cancer types.

In this study, increased levels of both miR221 and miR222 were detected in patients with NSCLC compared to control subjects. miR221 and miR222 showed a 1.46- and 1.63-fold increase in NSCLC patients, respectively. This expression difference between the patient and control was statistically significant for miR221 (p=0.000095), but not for miR222 (p=0.084470). A study of patients with aggressive NSCLC by Garofalo *et al*. found that miR221/222 were over-expressed and induced resistance to TRAIL by suppressing PTEN and TIMP3. *PTEN* is a tumor suppressor gene that plays a significant role in cell development and apoptosis; it converts phosphatylilinositol-3,4,5-triphosphate (PIP3) to phosphatidylinositol-4,5-bisphosphate (PIP2), thereby directly antagonizing the activity of PI3 kinase (PI3K).

Suppression of PTEN results in continuous PI3K/Akt pathway activation, leading to stimulation of cell proliferation and survival (22, 23). Garofalo *et al*. also found that MET oncogene controlled the activation of miR221/222 *via* the c-Jun transcription factor and they suggested that miR221/222 expression levels could be a prognostic factor for predicting TRAIL resistance (22).

Pu *et al*. showed that miR221 plasma levels were different between colorectal cancer patients and healthy controls and suggested that miR221 could be a prognostic factor (24). However, Chen *et al*. demonstrated that miR221/222 was over-expressed in papillary thyroid carcinoma (25).

Up-regulation of miR221 and miR222 leads to the proliferation of endothelial cells and suppression of angiogenesis. In addition, an increase in cell proliferation targeted by the p27 cell cycle inhibitor was found. This suggests that the function of miRNAs is cell type specific. The fact that more than half of the cancer-related fragile regions at a chromosomal location consist of genes that encode miRNAs demonstrates that miRNAs are involved in regulating apoptosis and cell growth (26, 27).

In a study on serum samples from NSCLC patients, miR222 serum level was found to be approximately two-fold down-regulated, but this was not statistically significant (28). In

another study, which we reached a parallel result, the over-expression of miR222 was suggested to correlate with advanced papillary thyroid carcinoma (29).

According to the study published by Foss *et al*., the diagnosis of NSCLC may be predicted months ago by evaluating the levels of different miRNAs (4).

miRNAs also have a regulatory role in metastasis and invasion. According to the results of our study, the levels of miR221 were 2.33-fold higher in the presence of metastasis in NSCLC patients (p=0.014585). miR222 was also expressed at higher levels in patients with metastases, and this difference was statistically significant (p=0.006675). The most common histological subtype of lung adenocarcinoma in NSCLC shows a heterogeneous structure. Histological subtyping of lung adenocarcinoma consists of a non-invasive bronchioalveolar carcinoma (BAC), a pure invasive adenocarcinoma (IAC) and a mixed spectrum of adenocarcinoma (AC-mix). Invasion of tumor cells is a pathological characteristic that allows identification of histological subtypes and, thus, characterizes the first stage of the metastatic process (30). Several studies have shown that the risk of death from non-invasive BAC is significantly lower than the risk of death from invasive tumors and tumors with a linear invasion of more than 0.5 cm, which confirms the clinical significance of invasion in lung adenocarcinoma (31-35).

According to the results of meta-analyses, mir221 and mir222 over-expression was significantly associated with poor overall survival of patients with cancer, including NSCLC. Also, the elucidation of the mechanism of deregulation of the miR-221/222 cluster is expected to improve the understanding of tumor development and promote the identification of biomarkers for cancer prognosis. The miR-221/222 cluster might be used as a potential therapeutic target (36-38).

In conclusion, changes in the expression of miR221 and miR222 genes can be used as a biomarker for the diagnosis, treatment, and prognosis of NSCLC.

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Conflicts of Interest

The Authors have no conflicts of interest to disclose in relation to this study.

Authors' Contributions

Concept: Y.M.O., E.C.İ.Y.; Supervision: Y.M.O., E.C., İ.Y.,; Materials: E.A., P.Y.; Data Collection and/or processing: Y.M.O.; Analysis and/or interpretation: Y.M.O., E.C., P.Y., E.A., A.K., İ.Y.; Literature search: Y.M.O., E.C., İ.Y..; Writing: Y.M.O, E.C., A.K.; Critical reviews: Y.M.O, E.C., A.K.

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