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Original article

MicroRNAs as a biomarker in lung cancer

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Abstract. *Introduction:* Lung cancer (LC) is the most common cancer in the world.Well known causes are long term smoking, environmental influences and genetic variations. LC is divided into two main types based on their histological phenotypes; small cell lung cancer (SCLC), and non-small cell lung cancer (NSCLC). The high specificity of these new screening methods, which are non-invasive, safe, inexpensive and simple to perform, is important in the early diagnosis and prognosis of cancer. MicroRNAs are significant biomarkers on the diagnosis metastasis and targeted therapies of NSCLC. In our study, we aimed to investigate the potential of using microRNAs as a biomarker in the early diagnosis of lung cancer. *Patients and methods:* Twenty patients diagnosed with lung cancer and twenty healthy individuals of the same age and gender were selected as the control group. Sixteen microRNAs were studied from blood samples. *Results:* Sixteen miRNAs (Let -7c, Let-7g, miR-1, miR-21, miR-29a, miR-31, miR-34a, miR 103a, miR-141, miR-155, miR-193b, miR-200b, miR-205, miR-340, miR-486, miR-708) were selected for tests and MiR 181 and miR 192 were used as the endogenous control group in line with their binding potentials and gene expression levels. The most specific and sensitive miRNAs were mirR-29a, miR-103a, and miR486 according to endogen controls in patients and healthy volunteer subjects. *Discussion:* A meta-analysis study showed that circulating miRNAs could be promising biomarkers for early diagnosis of lung cancer. Overall, 17 studies were included evaluating 35 miRNA markers and 19 miRNA panels in serum or plasma. *Conclusion:* In conclusion, there is a need for further validation studies for the use of three miRNAs as a biomarker in the early diagnosis and prognosis of lung cancer. (www.actabiomedica.it)

Key words: microRNA, biomarker, lung cancer

Introduction

Lung cancer (LC) is the most common cancer in the world. Well known causes are long term smoking, environmental influences and genetic variations (1,2).

Lung cancer is divided into two main types depending on their histological phenotype; Small cell lung cancer (SCLC) accounts for 15% of lung cancer and is mainly associated with smoking, and non-small cell lung cancer (NSCLC) accounts for 85% of lung cancer. According to histological features, NSCLC cancers are divided into three groups small squamous cell carcinoma (SCC), adenocarcinoma(AD) and large cell carcinoma(LCC) (3,4).

Lung cancer mortality is often associated with late diagnosis, so early diagnosis is important. Low-dose computed tomography or chest X-ray provides early diagnosis. Recent research has shown the great

reliability of new tests such as exhaled breath analysis, and serum biomarkers in the early diagnosis of lung cancer. The new screening methods, which are non-invasive, safe, inexpensive, and simple is important in the early diagnosis and prognosis of cancer (5).

MicroRNAs are single-stranded, non-coding RNA molecules, 18-25 nucleotides in length, with stability, reproducibility and consistency, easily detectable in blood in a non-invasive manner (3,6,7). MicroRNAs have a significant impact on the diagnosis of NSCLC, metastasis, and targeted therapies. The clinical protein markers carcinoembryonic antigen (CEA), cytokeratin fragment 21-1(CF21-1) and cancer antigen-125(CA125) were compared with miRNAs exhibit a higher diagnostic efficacy in NSCLC, they have the potential to be used as diagnostic biomarkers (8,9).

In our study, we aimed to investigate the potential of using MicroRNAs as a biomarker in the early diagnosis of lung cancer.

Materials and methods

Patients

Twenty patients newly diagnosed with lung cancer were recruited from the Department of Oncology of Medicine Faculty of Akdeniz University between 2017-2019 years. Oncology. Twenty people who applied to our center in the same age group with normal physical examination and laboratory findings were selected as the control group. After each individual included in the study was informed and signed a written consent form, 5cc EDTA blood samples were taken from the patients and the control group.

Selected microRNAs

Sixteen miRNAs (Let -7c, Let-7g, miR-1, miR-21, miR-29a, miR-31, miR-34a, miR 103a, miR-141, miR-155, miR-193b, miR-200b, miR-205, miR-340, miR-486, miR-708) were selected for tests and MiR 181 and miR 192 were used as the endogenous control group in line with their binding potentials and gene expression levels.

Blood samples were centrifuged for 15 minutes and MiRNAs isolation (Invitrogen by Thermo Fisher Scientific-mirVana™ miRNA Isolation Kit) was performed from plasma. The obtained miRNA was measured by Qubit 3 Fluorometer device then they was analysed by using Thermal Cycler (Applied Biosystems by Life Technologies-TaqMan Advanced miRNA c DNA Synthesis Kit). Cт values automatically taken from the system are reported in the excel file. The average Ct values of samples were with compared the endogen miR 181 and miR 192. At the end of the study, the ΔCт values of individuals with lung cancer were compared with the ΔCтs of the healthy control group like other study**.** (10).

Ethics committee

Approval was obtained from Faculty of Medicine Clinical Research Ethics Committee of Akdeniz University. Ethics committee approval number and date 471 and 17.08.2016.

Statistical analysis

The data were evaluated using the SPSS (Statistical Package for the Social Sciences) version 23.0 (SPSS Inc., Chicago, IL, USA) program. Descriptive findings are presented as number, percentage, mean ± standard deviation, and median values. Whether the data conformed to the normal distribution was evaluated using the Shapiro-Wilk test and skewness / kurtosis values. Lung cancer and healthy groups were compared with the independent samples t test in cases where the data were in a normal distribution, and with the Mann-Whitney U test when they did not. The limit value for statistical significance was accepted as $p < 0.05$. Receiver operating characteristic (ROC): ROC curve analysis was performed to determine the sensitivity and specificity and diagnostic efficacy of miRNAs among the studied groups. (11).

Results

Nineteen of the twenty patients included in the study were male and one female, the mean age was

60.81 ± 16.3(range: 49-72) years. In the control group, there were nineteen male and one female, the mean age was 60.3± 17.7 (range: 47-72) years.

According to the history of the patients, 12 (60%) patients had a history of smoking 14 - 100 packs (the mean 50 ± 22) of cigarettes per year, while 8 (40%) patients had not a history of smoking. There was no significant difference in miRNA levels between smoking and non-smoking groups in lung cancer patients.

The pathological diagnosis of the patients was as follows; SCLC in six patients, NSCLC (AD) in nine patients, NSCLC (SCC) in three patients and NSCLC (LCC) in two patients (Table 1).

Fifteen of the patients with lung cancer were at stage four, two were at stage three, two were at stage two, and one was at stage one.

Sixteen of the patients had received chemotherapy alone, three had surgery and chemotherapy, and two had chemotherapy and radiotherapy. Eleven of twenty patients died and nine were alive and recurrence was not during the study (Table 1).

The level delta CT (ΔCTs) of microRNAs in the patients were compared as SCLC and NSCLC, miR29a value was significantly lower in SCLC cases than in NSCLC cases. There was no significant difference between SCLC and NSCLC cases in terms of other miRNA levels. No significant difference was found in terms of miRNA levels according to tumor stages of the patients.

Delta CT levels (ΔCTs)according to the endogenous control MiRNA 181 in patients with lung cancer, mir1, mir29a, mir-141, mir193b, mir200b,

Table 1. Characteristics of patients with lung cancer.

No	Age	Sex	Cigarette packet per year	Pathologic Diagnosis	Stage	Therapy	Follow up
1	59	Male	40	NSCLC /AD	3	CT	DIED
2	56	Male	No	SCLC	4	CT	DIED
3	65	Male	40	SCLC /AD	4	CT	DIED
4	64	Male	100	SCLC	4	CT	DIED
5	57	Male	40	NSCLC /SEH	2	CT	ALIVE
6	47	Male	40	NSCLC /LCC	3	ST+CT +RT	ALIVE
7	62	Male	14	NSCLC /LCC	4	CT+RT	ALIVE
8	65	Male	No	NSCLC /AD	4	CT	DIED
9	75	Male	40	NSCLC /AD	4	CT	DIED
10	74	Male	30	SCLC	4	CT	DIED
11	64	Male	No	NSCLC /SEH	4	CT	DIED
12	57	Female	No	SCLC	4	CT	DIED
13	66	Male	No	NSCLC /SEH	4	CT	DIED
14	63	Male	56	SCLC	4	CT	DIED
15	82	Male	40	NSCLC /AD	2	ST+CT	ALIVE
16	78	Male	No	NSCLC /AD	4	CT	ALIVE
17	67	Male	40	NSCLC /AD	1	ST+CT	ALIVE
18	70	Male	50	SCLC	4	CT	ALIVE
19	48	Male	No	NSCLC /AD	4	CT	ALIVE
20	59	Male	No	NSCLC /AD	4	CT	ALIVE

Abbreviations: SCLC: Small Cell Lung Cancer, NSCLC: Non-Small Cell Lung Cancer LCC: Large Cell cancer, SEH: Squamous Epithelial Cell, CT: Chemotherapy ST: Surgical Treatment RT: Radiotherapy. Stage 1: Good 2: Moderate 3: Mild 4: Severe

Table 2. MiRNA comparison of lung cancer and healthy subject according to endojen (Delta181CT).

MiRNA	Mean ± SD	Median	p
Let7c			
Lung ca (n=14)	0.938 ± 2.55	0.345	0.257*
Healthy (n=14)	-0.678 ± 2.05	-0.688	
Let7g			
Lung ca (n=14)	-1.337 ± 2.44	-1.321	0.660*
Healthy (n=14)	-0.954 ± 3.30	-0.455	
mir1			
Lung ca (n=16)	-5.162 ± 2.65	-4.817	**<0.001***
Healthy (n=17)	-0.265 ± 3.96	-0.661	
mir21			
Lung ca (n=17)	-1.906± 2.67	-1.115	**0.020***
Healthy (n=16)	-4.928 ± 3.28	-4.695	
mir31			
Lung ca a (n=17)	2.268± 2.48	2.233	0.697*
Healthy (n=12)	2.966 ± 3.24	3.190	
mir29a			
Lung ca (n=16)	-4.970 ± 2.24	-4.647	**<0.001***
Healthy (n=17)	-0.022 ± 3.02	0.556	
mir34a			
Lung ca (n=10)	-1.085 ± 1.66	-0.860	0.156*
Healthy (n=14)	0.506 ± 3.28	1.305	
mir103a			
Lung ca (n=17)	1.149 ± 1.62	1.065	**0.001***
Healthy (n=16)	-2.305 ± 3.08	-2.451	
mir141			
Lung ca (n=16)	-7.430 ± 3.39	-6.979	**0.008***
Healthy (n=8)	-3.186 ± 3.34	-2.387	
mir155			
Lung ca (n=14)	4.071 ± 2.07	4.145	**0.001***
Healthy (n=6)	0.350 ± 1.86	1.098	
mir193b			
Lung ca (n=17)	-7.095 ± 3.90	-7.353	**<0.001***
Healthy (n=10)	-0.864± 3.33	0.612	
mir200b			
Lung ca (n=6)	-3.355 ± 2.76	-3.808	**0.043***
Healthy (n=6)	2.666 ± 5.75	3.096	
mir205			
Lung ca (n=17)	-4.108 ± 2.62	-3.866	**0.003**†
Healthy (n=15)	-0.924± 3.00	0.070	
mir340			
Lung ca (n=17)	-3.663 ± 2.92	-4.460	**0.005***
Healthy (n=16)	-0.552 ± 3.04	-0.056	
mir486			
Lung ca (n=16)	-5.545± 2.11	-6.100	**<0.001**†
Healthy (n=16)	-8.904 ± 2.74	-8.110	
mir708			
Lung ca (n=16)	0.483 ± 2.37	0.635	**0.042**†
Healthy (n=14)	2.385 ± 5.29	3.550	

* Independent samples t test. † Mann-Whitney U test

mir-205, mir-340, mir-708 values were significantly lower than the healthy controls, mir-21, mir103a, mir155 and mir486 were higher than healthy controls (Table 2).

Delta CT levels (ΔCTs) according to the endogenous control MiRNA 192 in patients with lung cancer, mir1, mir29a, mir-141, mir193b, mir200b, mir-205 and mir486 values were significantly lower than the healthy controls, mir21, mir103a and mir155 were higher than healthy controls (Table 3).

Diagnostic values of microRNAs were evaluated by ROC Analysis. The significantly higher AUCs of the microRNAs ΔCTs 181 and ΔCTs 192 in the ROC analysis curve are shown in Table 4 and 5, respectively. According to ΔCTs 181, the most specific miRNAs are miR29a and miR486. According to ΔCTs 192, the most specific miRNAs are miR-29a and miR103a.

As conclusion, twelve microRNAs (miR-1, miR-21, miR-29a, miR-103a, miR-141, miR-155, miR-193b, miR-200b, miR-205 miR -340, miR-486, miR-708) out of sixteen microRNAs studied in lung cancer patients were found miR103a, miR29a and miR486 to be specific and sensitive in statistical analysis when compared to both endogenous controls and healthy individuals.

Discussion: In recent years, MicroRNAs have become the center of attention as they play an extraordinary role in the tumorigenesis process by regulating nucleotide molecules, cell cycle, metastasis, angiogenesis, metabolism and apoptosis. They also play a role in the regulation of cancer cell metabolism and resistance or susceptibility to chemotherapy and radiotherapy.

Table 3. MiRNA comparison of lung cancer and healthy subject according to endojen (Delta 192CT).

MiRNA	Mean±SD†	Median	p
Let7c			
Lung ca (n=18)	4.058 ± 2.35	3.931	0.050*
Healthy (n=11)	2.036 ± 2.92	2.314	
Let7g			
Lung ca (n=15)	2.031± 2.70	1.406	0.578*
Healthy (n=12)	2.708 ± 3.52	2.477	
mir1			
Lung ca (n=16)	-1.032 ± 1.21	-0.939	**0.001***
Healthy (n=13)	2.916 ± 3.25	3.094	
mir21			
Lung ca (n=18)	1.937 ± 2.13	2.135	**0.001***
Healthy (n=13)	-2.162 ± 3.75	-3.625	
mir31			
Lung ca (n=18)	6.863 ± 2.70	7.440	0.164†
Healthy (n=11)	5.450 ± 2.53	6.483	
mir29a			
Lung ca (n=17)	-0.857 ± 1.53	-0.979	**<0.001***
Healthy (n=13)	2.944± 2.18	2.962	
mir34a			
Lung ca (n=9)	3.740 ± 2.65	3.201	0.513*
Healthy (n=13)	2.884 ± 3.15	2.689	
mir103a			
Lung ca (n=17)	5.447± 2.33	5.167	**<0.001***
Healthy (n=13)	1.039± 2.67	0.376	
mir141			
Lung ca (n=15)	-3.211 ± 1.78	-2.727	**<0.001***
Healthy (n=4)	3.057 ± 2.07	3.442	
mir155			
Lung ca (n=14)	8.256 ± 2.96	8.826	**0.043***
Healthy (n=5)	4.440 ± 4.35	6.190	
mir193b			
Lung ca (n=18)	-3.262 ± 2.67	-3.808	**0.001**†
Healthy (n=10)	1.585 ± 3.33	1.397	
mir200b			
Lung ca (n=5)	1.411± 1.26	1.736	**0.014**†
Healthy (n=4)	7.937± 2.73	6.774	
mir205			
Lung ca (n=18)	0.039 ± 1.76	0.2895	**0.004***
Healthy (n=12)	1.988 ± 1.47	2.571	
mir340			
Lung ca (n=17)	0.704± 2.05	0.466	0.071*
Healthy (n=13)	2.577± 3.04	3.265	
mir486			
Lung ca (n=18)	-1.287 ± 2.61	-1.262	**0.001***
Healthy (n=13)	2.577 ± 3.04	3.265	
mir708			
Lung ca (n=17)	4.205± 4.24	4.696	0.975*
Healthy (n=10)	4.253 ± 2.89	4.147	

* Independent samples t test. † Mann-Whitney U test

Therefore, it is important to investigate the expression of miRNA and understand its relationship to lung cancer and the development of anti-cancer strategies (3,6,7,12).

Extensive studies are being conducted on the diagnostic potential of microRNAs. Since microRNA molecules are defined in blood and sputum, they constitute an excellent diagnostic material especially for patients with NSCLC (13,14).

Inamura et al. have evaluated miRNAs according to lung cancer types, six miRNAs in NSCLC type (Let 7, miR34, miR 21, miR 200b, miR34a, miR29), four miRNAs in LCC type (miR-205, miR-93, miR-221, and miR-30e), five miRNAs of the AD type (miR-29b, miR-29c, let-7e, miR-100, and miR-125a-5p) have reported to show high levels of expression (3,15).

We compared the serum 16 microRNAs levels in our study in patients with SCLC and NSCLC, miR29a was significantly lower in patients with NSCLC. There was no significant difference between NSCLC and NSCLC in terms of other microRNA levels.

Bica-Pop et al. have evaluated miRNA-21 has been extensively studied in many types of cancer, including NSCLC. In this study, increased miR-21 expression was associated with a worse outcome in NSCLC patients. (16) In our study, miR21 and mir103 and mir155 were found to be significantly higher in lung cancer cases when compared with endogenous controls. It can be considered more significant (6) as it supports cell growth and invasion by suppressing miR-21 expression and tumor suppressor PTEN.

Table 4. Area under curve (AUC), cut-off value, sensitivity, specificity, and p value of miRNAs by ROC analysis (ΔCTs 181).

miRNAs	AUCs	Cut-off value	Sensitivity (%)	Specificity (%)	P-value
Mir-29a	0.904	-2.44	93.8	76.5	<0.001
Mir-155	0.893	2.32	78.6	100.0	0.006
Mir-486	0.887	-7.81	93.8	68.8	<0.001
Mir-193b	0.865	-4.89	82.4	90.0	0.002
Mir-103a	0.853	-0.69	88.2	75.0	0.001
Mir-1	0.827	-3.29	75.0	76.5	0.001
Mir-141	0.820	-3.18	93.8	62.5	0.012
Mir-205	0.808	-0.70	94.1	66.7	0.003
Mir-340	0.776	-1.37	82.4	75.0	0.007
Mir-21	0.761	-3.71	82.4	81.3	0.011
Mir-708	0.719	3.14	87.5	57.1	0.042

Table 5. Area under curve (AUC), cut-off value, sensitivity, specificity, and p value of miRNAs by ROC analysis (ΔCTs 192).

miRNAs	AUCs	Cut-off value	Sensitivity (%)	Specificity (%)	P-value
Mir-141	1.000	-0.23	100.0	100.0	0.003
Mir-200b	1.000	4.39	100.0	100.0	0.014
Mir-29a	0.923	1.54	100.0	69.2	<0.001
Mir-193b	0.889	-1.88	83.3	90.0	0.001
Mir-103a	0.882	1.88	94.1	69.2	<0.001
Mir-1	0.832	0.71	93.8	76.9	0.002
Mir-21	0.825	-1.63	100.0	69.2	0.002
Mir-486	0.821	1.34	83.3	69.2	0.003
Mir-205	0.810	1.35	83.3	75.0	0.005

Sheervalilou et al. evaluated miR-10b, miR-1 and miR-30a in 47 NSCLC patients and 41 healthy plasma samples for investigating the effects of tobacco on MicroRNA expression, patients were divided into non-smokers and smokers. MiR-1 and miR-30a expression levels were significantly decreased in smokers, while miR-10b level was found to be significantly higher. Their findings showed that smoking had significant effects on microRNA levels. It has been published that miR 1 is excellent and miR-10b and miR-30a are good markers for detection of lung cancer (4).

The smoking history of the patients in our study was as follows; while 12 (60%) patients smoked between 14 and 100 packs of cigarettes per year, 8 (40%) patients had no smoking history. There was no significant difference between smokers and non-smokers in lung cancer cases in terms of miRNA levels.

Nadal et al. 60 selected miRNAs studied in 70 patients with NSCLC and 22 healthy individuals. Four miRNAs (miR-193b, miR-301, miR-141 and miR-200b) were found significant in ROC analysis (17). In our study, we found 12 microRNAs out of 16 microRNAs to be significant and our common miRNAs with this study were miR193, miR-141 and miR-200b.

A meta-analysis study showed that circulating miRNAs could be promising biomarkers for early diagnosis of lung cancer. Overall, 17 studies were included

evaluating 35 miRNA markers and 19 miRNA panels in serum or plasma. The potential role of circulating miRNAs for non-invasive lung screening has been highlighted (18).

The recent findings with the role of miRNAs in lung cancer, and discusses the potential and challenges of developing miRNA-targeted therapeutics in this dreadful disease (19).

The small number of cases in our study was the most important limitation. In addition, miRNAs were not separated according to the pathological diagnoses of the cases. In the validation study, it is planned to increase the number of patients and to evaluate the pathological diagnoses in detail.

In conclusion, there is a need for further validation studies for the use of three miRNAs (miR103a, miR29a and miR486) which we found significant in our study, as a biomarker in the early diagnosis and prognosis of lung cancer, as well as for therapeutic purposes, when compared with the literature studies.

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