SERUM LEVELS OF MIR-146A AND MIR-196-A-2 , EARLY DIAGNOSTIC MARKERS IN NSCLC PATIENTS

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Abstract

Background: One of the most common human cancers in the world is lung cancer. Non-small cell lung cancer (NSCLC) is the most prevalent type representing about 85% of lung cancer cases. Micro RNAs (miRNAs) have been shown to be crucial in the development of cancer, either through their oncogenic or tumor suppressor activities.

Aim of work: to determine whether NSCLC patients' and healthy volunteers' serum expressions of miR-146a and miR-196-a-2 differ to assess the potential of these molecules as noninvasive markers for the early diagnosis of NSCLC. Additionally, to correlate the serum levels with the clinicopathological characteristics in NSCLC patients.

Methods: Eighty Egyptian adults were chosen for this study, and they were as follows; Group I: included 50 newly diagnosed patients with NSCLC. Group II: A control group of 30 people who appeared to be in good health. They were submitted to gene expression analysis for miR-146a and miR-196-a-2 using quantitative Real-Time PCR technique.

Results: both studied miRNAs; were shown to be significantly elevated in the serum of NSCLC patients in comparison to the control group ($p= 0.045$ and <0.001 respectively). It was also found that both serum miR-146a and miR-196-a-2 were statistically significantly substantially higher in the early stages NSCLC patients (stages I+II) than those in the healthy group (group II) (p 0.02 & 0.024). Serum miR-146a showed an AUC of 0.679 with a sensitivity of 70.4 %, a specificity of 76.7 %, and diagnostic accuracy of 73.68%, while serum miR196-a-2 showed an AUC of 0.706 with a sensitivity of 81.5 %, specificity of 56.7%, and diagnostic accuracy of 68.42%.

Conclusion: Serum expressions of miR-146a and miR-196-a-2 could be used as early biomarkers for NSCLC diagnosis.

Keywords: (NSCLC – miRNAs – miR-146a – miR-196-a-2 – gene expression–biomarker).

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1. Introduction

Lung cancer is one of the leading reasons of cancer-related mortalities worldwide, amounting to more than 1.4 million deaths annually [1]**.** According to figures from Globocan 2018, it accounts for a significant portion of cancer-related deaths (18.4% of all cancer deaths) and it is the most prevalent cancer diagnosed globally in both sexes (11.6 percent of all cases) [2].

Non-small cell lung cancer (NSCLC) represents about 85% of cases, and a smaller percentage is represented by small-cell lung cancer (about 15% of cases) [3]. NSCLC has the highest cancer-related mortality rates globally [4]**.**

The major risk factor for lung cancer is tobacco smoking, but there are other variables that may potentially affect risk, such as age, sex, ethnicity, environment, occupational exposures, genetic and epigenetic alterations [5]. MicroRNA is one of the most significant epigenetic factors; its dysregulation is a significant contributor to cancer development [6,7].

Despite advances in early diagnosis and innovative therapy approaches, the prognosis remains poor; the total 5-year survival rate is still between 10 and 20 percent [1]. Regrettably, 50% of individuals who have been diagnosed are at an advanced stage of their illness [8].

The poor prognosis of this malignancy is influenced by several factors, including the lack of manifestations early in the disease, unavailability of effective therapeutic approaches, tumor heterogeneity, and, most critically, a diagnosis at an advanced stage [9].

For NSCLC patients, the prognosis and survival rate are highly correlated with cancer stages, exhibiting a dramatic fall from 68-92% for early stages diagnosed patients to 1-13% for advanced-stages patients [4]**.** A clinical requirement is to increase survival by screening exams and/or testing, and it is crucial to look into the molecular mechanisms behind the onset and spread of NSCLC.

Yet, although conventional biopsy is the gold standard for the diagnosis of NSCLC [10], there is an urgent need to identify blood-based noninvasive markers that can be used for liquid biopsy in patients where conventional biopsy is difficult to perform, or repeated biopsies have not produced enough tissue for pathological diagnosis, as liquid biopsies can be repeated with little discomfort to the patient.

Recent advances in cancer research have revealed a significant association between noncoding RNAs and cancer development [11]**.** The potential of non-coding miRNAs as non-invasive prognostic and diagnostic biomarkers for NSCLC has been established [12]**.**

The microRNAs are noncoding RNA molecules that are very short (20–24 nucleotides), but they are crucial for the growth of malignancies by controlling the cell cycle, metastasis, angiogenesis, metabolism, and apoptosis. Essentially, miRNAs control the expression of genes via post-transcriptionally controlling of its target messenger RNAs (mRNA) [6]. At the post-transcriptional stage, these tiny RNA molecules act as a negative regulator for the genes they target [13] ***(Bracken et al., 2016)***. MiRNA expression patterns are usually changed at distinct developmental stages and in a variety of clinical diseases, such as cancer [14].

MiRNAs are capable of being secreted into extracellular space, and then transported to the peripheral blood and circulating bodily fluids [15]. These miRNAs are detectable in serum or plasma in a very stable state [16]. As a result, these tiny molecules are excellent candidates to be used as biomarkers in liquid biopsies for the detection of cancer. In addition to peripheral blood, certain bodily fluids, including saliva, cerebrospinal fluid, urine, breast milk, and semen, enable the detection of miRNA [17].

One of the most contentious microRNAs, miR-146a has generated controversy over whether it functions as an oncogene or a tumor suppressor [18]. Several research reported its upregulation [19,20,21], and similarly, several others reported its downregulation in cancers [22.23.24]**.**

MiR-196a is another miRNA that was extensively studied, and the majority of the available research indicated that this miRNA plays an oncogenic role and is elevated in a number of malignancies, including NSCLC [25, 26, 27].

The goal of this research was to compare the serum levels of miR-146a and miR-196a-2 in NSCLC patients to those in healthy controls to assess their potential as noninvasive biomarkers for the early diagnosis of NSCLC and to correlate the serum levels with the clinicopathological characteristics in NSCLC patients.

2 Subjects and Methods

The current work involved a total number of 80 subjects. Fifty patients who were newly diagnosed with NSCLC have been recruited from the Chest and Clinical Oncology Departments in Kasr Al-Aini hospital as well as 30 healthy individuals as a control group. Ethical Committee approval was obtained from Kasr Alainy Ethical Committee (Code: MD-68-2020)

2.1 Study population:

The study subjects were divided into two groups:
Group I: It included 50 NSCLC patients who were histopathology and cytology diagnosed with NSCLC at different stages of cancer as follows:
Group Ia: It included 8 individuals in stage I.
Group Ib: It included 19 individuals in stage II.
Group Ic: It included 13 individuals in stage III**.**
Group Id: It included 10 individuals in stage IV.

NSCLC patients who have undergone chemotherapy or radiotherapy, as well as those with a history of a different type of cancer, were excluded from the study.

Group II: It included 30 apparently healthy individuals with ages ranging from 20 to 70 years, whose age, and sex-matched those of the NSCLC patients.

The patient's informed consent was obtained before any specimens were taken.

2.2 Analysis of miRNAs (miR-146a and miR-196-a-2) expression level

Analysis of the expression level of two mature miRNAs (miR-146a and miR- 196-a-2) in serum by quantitative SYBR Green reverse transcription real-time polymerase chain reaction (PCR) and RNU6 was used as a housekeeping gene [28].

2.2.1 Serum sampling and total RNA extraction

Two milliliters of peripheral blood were collected on a serum vacutainer tube and centrifuged for 10 minutes at 1900 xg after being left at room temperature for 30 minutes. The upper yellow serum phase was carefully transferred to tubes with conical bottoms without disturbing the layer of the intermediate buffy coat. The conical tubes containing the serum were centrifuged for 10 minutes at 16,000 xg. The clear supernatant from the previous centrifugation process was carefully transferred to a RNase free tube without disturbing the pellet. Following that, it was kept at -80 °C until the time of RNA extraction. Using miRNeasy Mini

Kit (Catalog no.217004), RNA was extracted from the serum. The quantity and quality of extracted RNA were assessed by measuring the absorbance at 260nm and 280nm using the Quawell Q5000 UV-VIS SPECTROPHOTOMETER. An aliquot of extracted RNA samples was run on a denaturing agarose gel and stained with SYBR Green to assess the integrity of the RNA.

2.2.2 Reverse transcription

The miScript® II RT Kit (Catalogue number 218161) was used to reverse-transcribe complementary DNA (cDNA) from the RNA samples. The reaction tubes were loaded into the Biometra thermal cycler (Analytic Jena, Germany) with thermal profiling conditions, 60 minutes at 37°C, 5 minutes at 95°C, and then 4°C on hold.

2.2.3 Real-time PCR analysis of circulating miRNAs

For the detection of mature miRNA, cDNA prepared in the RT reaction was used as the template for real-time PCR analysis using a miRNA-specific (miR-146a, miR- 196a-2, or RNU6) miScript primer assay (forward primer) and miScript® SYBR® Green PCR Kit *(Catalog no. 218073)* which contains the miScript Universal Primer (reverse primer) and QuantiTect SYBR Green PCR Master Mix.

The PCR cycling conditions included an initial 15-minute phase of PCR activation at 95 °C followed by fifty cycles of three sequential processes: denaturation for 15 seconds at 94 °C, annealing for 30 seconds at 55 °C, and extension for 30 seconds at 70 °C. The qPCR reactions were carried out on StepOne real-time PCR system (Applied Biosystems).

2.2.4 Data Analysis using Comparative cycle threshold Method (ΔΔ CT)

The cycle threshold was used to measure the miRNA expression level **(CT)** [29]**.**

The relative expression (fold change) for each targeted miRNA was computed using the $2^{-\Delta\Delta CT}$ method after the relative gene expression levels of miR146a and miR196-a-2 were normalized to RNU6 (reference gene) [30]**.**

2.3 Statistical Analysis of Results

Advanced statistics version 20 from IBM Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL) was used to analyze the data. When quantitative data were not normally distributed, it was summarised using the median, 25th, and 75th percentiles. Frequency and percentage were used to express categorical data. To investigate the relationship between qualitative variables, the chi-square test was applied. The Mann-Whitney test was used to compare two groups of numerical data that were not regularly distributed (non-parametric t-test). To compare quantitative variables, the non-parametric Kruskal-Wallis and Mann-Whitney tests were utilised. To compare categorical data, an analysis utilising the Chi square ($\chi 2$) test was conducted. An exact test was used in its place when the estimated frequency was less than 5. The correlations between the quantitative variables were determined using the Spearman correlation coefficient. To determine the optimal cutoff value of miR-146a and miR-196a-2 for the detection of cancer cases, receiver operating characteristic curves (ROC) were built utilizing area under curve (AUC) analysis. Statistics were considered significant for P-values under 0.05.

3 Results

The demographic, and clinicopathological data of the NSCLC patients' group (**Group I**) are summarized in table (1).

Table (1): Demographic and clinicopathological data of the NSCLC patients' group **(Group I)**

NSCLC patients' data (Group I)		**Number**	**Percentage**
Age	≤ 50	25	50%
	>50	25	50%
Gender	Male	39	78%
	Female	11	22%
Smoking	Smoker	33	66 %
	Nonsmoker	17	34 %
Family history	Positive	10	20 %
	Negative	40	80 %
Histological type	Adenocarcinoma	31	62 %
	Squamous cell carcinoma	10	20 %
	Others	9	18 %
Lung cancer stage	I	8	16 %
	II	19	38 %
	III	13	26 %
	IV	10	20 %
Tumor size	≤ 5 cm	33	66 %
	> 5cm	17	34 %

Lymph node metastasis	Positive	34	68 %
	Negative	16	32 %
T	T1	16	32 %
	T2	17	34 %
	T3	10	20 %
	T4	7	14 %
N	N0	16	32 %
	N1	13	26 %
	N2	13	26 %
	N3	8	16 %
M	M0	40	80 %
	M1	10	20 %

The relationship between miR-146a and miR-196-a-2 expression and clinicopathological features in NSCLC patients are shown in Table (2).

Table (2): Relationship between miR-146a and miR-196-a-2 expression and clinicopathological features in NSCLC patients.

Characteristics		**miR-146a**	**miR-196-a2**
Age	**< 50**	6.89 (0.67 – 16.83)	4.6 (1.88– 10.63)
	> 50	3.59 (0.17– 8.21)	2.51 (1.81 – 13.46)
	p-value	0.148	0.432
Gender	**Males**	3.93 (0.59 – 16.18)	4.27 (1.77– 11.68)
	Females	3.61 (0.51– 9.48)	4.15 (1.88– 32.89)
	p-value	0.743	0.734
Smoking	**Positive**	4.69 (0.8 – 9.69)	4.27 (1.91– 13.86)
	Negative	3.61 (0.51– 9.48)	4.15 (1.75 – 7.6)
	p-value	0.743	0.660
Family history	**Positive**	3.23 (0.8 – 7.46)	5.15 (1.88 – 13.86)
	Negative	4.27 (0.4 – 12.38)	4.21 (1.59– 10.46)
	p-value	0.990	0.558
Histological type	**Adenocarcinoma**	3.84 (0.29 – 9.48)	4.15 (1.77 – 10.63)
	Squamous cell carcinoma	5.54 (0.08 – 15.07)	8.83 (1.85 – 30.31)
	Others	6.88 (0.8 – 9.69)	4.27 (2.26 – 6.39)
	***p*-value**	0.769	0.868
Tumor size	**≤ 5 cm**	3.93 (0.64- 9.48)	3.44 (1.75- 10.29)
	> 5cm	3.84 (0.21- 17.46)	4.85 (1.91- 15.24)
	P-Value	0.902	0.512
Lymph node metastasis	**Positive**	3.59 (0.37- 8.00)	3.05 (1.83- 10.99)
	Negative	8.19 (0.8- 16.83)	4.91 (1.85- 13.86)
	p-value	0.066	0.382
M/Distant metastasis	**Positive**	2.27 (0.12- 25.91)	9.04 (1.88- 18.45)
	Negative	4.31 (0.66- 9.59)	3.49 (1.81- 9.16)
	p-value	0.784	0.436

All data are presented as median (25th -75th percentiles).
Statistically significant difference at $p<0.05$

Comparison between the median values of fold changes (FC) of miRNAs between group I (NSCLC patients) and group II (control group) are shown in Table (3).

Table (3): Comparison between the median values of fold changes (FC) of miRNAs between group I (NSCLC patients) and group II (control group)

	Group **I (n=50)**	Group **II (n=30)**	***p* value**
miR-146a	3.89 (0.59 – 9.69)	0.93 (0.24 – 3.23)	**0.045***
miR- 196a-2	4.21 (1.85 – 11.68)	1.04 (0.41 – 2.52)	**<0.001***

All data are presented as median (25^{th} -75^{th} percentiles).
The median values of FC are shown relative to reference RNU6.

*Fold change formula: Fold-change = $2^{-\Delta\Delta CT}$

Comparison between the median values of fold changes (FC) of miR-146a and miR-196-a-2 in stage 1 of lung cancer vs the healthy control group are shown in Table (4)

Table (4): Comparison between the median values of fold changes (FC) of miR-146a and miR-196-a-2 in stage 1 of lung cancer vs the healthy control group

	miR-146a	**miR-196-a-2**
Control	0.93 (0.24-3.23)	1.04 (0.41-2.52)
Stage I	16.51 (6.06-29.36)	4.2 (1.85-10.63)
p-value	**0.001***	**0.03***

All data are presented as median (25th -75th percentiles).
The median values of FC are shown relative to reference RNU6
*Fold change formula: Fold-change = $2^{-\Delta\Delta CT}$

Comparison between the median values of fold changes (FC) of miR-146a and miR-196-a-2 in early stage lung cancer (stage I/II) of lung cancer vs the healthy control group are shown in Table (5)

Table (5): Comparison between the median values of fold changes (FC) of miR-146a and miR-196-a-2 in early-stage lung cancer (stage I/II) of lung cancer vs the healthy control group

	miR-146a	**miR-196-a-2**
Control, n=30	0.93 (0.24-3.23)	1.04 (0.41-2.52)
Early-stage group, n=27	6.88 (0.64-15.07)	2.67 (1.4-10.29)
p-value	**0.02***	**0.024***

All data are presented as median (25th -75th percentiles).
The median values of FC are shown relative to reference RNU6
*Fold change formula: Fold-change = $2^{-\Delta\Delta CT}$

ROC curve analysis for serum levels of miR-146a and miR-196-a-2 in NSCLC patients (group I) versus healthy control. Diagnostic sensitivity, specificity, and accuracy are represented in Table (6), Fig.1

Table (6): ROC curve analysis for serum levels of miR-146a and miR-196-a-2 in NSCLC patients (group I) versus healthy control.

	Area Under the Curve	**P-value**	**95% Confidence Interval**							
			Lower Bound	**Upper Bound**	**Cut off**	**Sensitivity %**	**Specificity %**	**PPV %**	**NPV %**	**DA %**
Fold change of miR 146	0.635	0.039*	0.507	0.762	3.4	60	76.7	81.08	53.49	66.25
Fold change of miR 196	0.740	< 0.001*	0.626	0.855	1.745	80	63.3	78.43	65.52	73.75

PVP: Predictive value of positive. NPV: Predictive value of negative. DA: Diagnostic accuracy

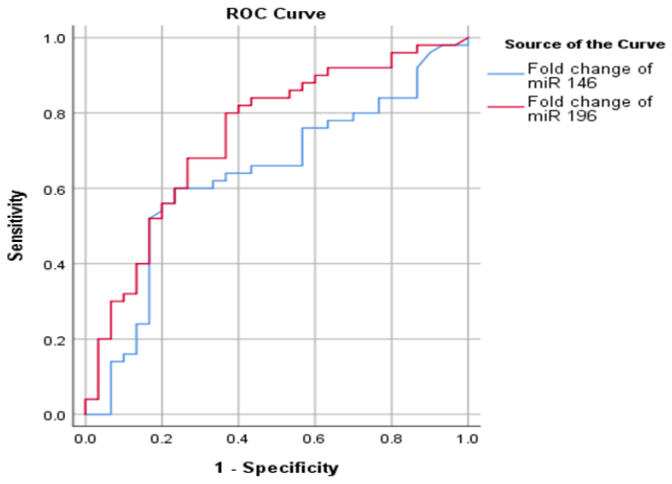

Fig. 1: ROC curve for serum levels of miR-146a and miR-196-a-2 in NSCLC patients (group I) versus healthy control

ROC curve analysis for serum levels of miR-146a and miR-196-a-2 in patient's Stage I vs healthy control. Diagnostic sensitivity, specificity, and accuracy are represented in Fig. 2,3, and Table (7)

Table (7): ROC curve analysis for serum levels of miR-146a and miR-196-a-2 in patient's Stage I vs healthy control.

	Area Under the Curve	**P value**	**95% Confidence Interval**							
			Lower Bound	**Upper Bound**	**Cut off**	**Sensitivity %**	**Specificity %**	**PPV %**	**NPV %**	**DA %**
Fold change of miR 146	0.793	<0.001*	0.640	0.947	4.95	80	83.3	61.5	92.6	82.5
Fold change of miR 196	0.777	<0.001*	0.630	0.924	1.23	90	56.7	40.9	94.4	65

PVP: Predictive value of positive. NPV: Predictive value of negative. DA: Diagnostic accuracy

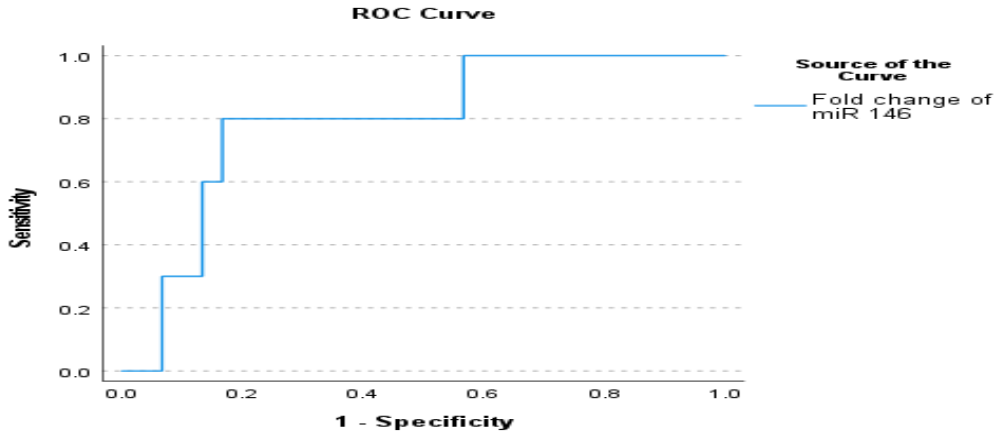

Fig. 2: ROC curve for serum levels of miR-146a in patient's Stage I vs healthy control

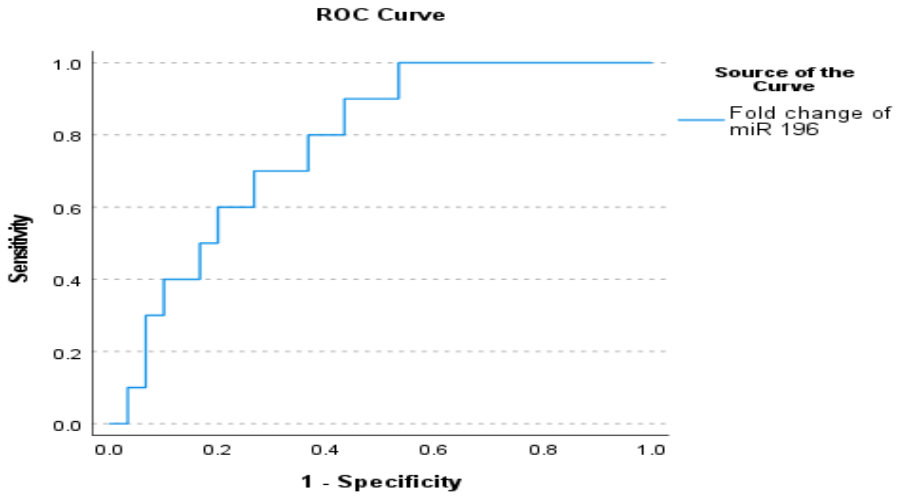

Fig. 3: ROC curve for serum levels of miR-196-a-2 in patient's Stage I vs healthy control

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ROC curve analysis for serum levels of miR-146a and miR-196-a-2 in early NSCLC patient's (Stage

I+II) vs healthy control group. diagnostic sensitivity, specificity, and accuracy are represented in Fig. 4, and Table (8)

Table (8): ROC curve analysis for serum levels of miR-146a and miR-196-a-2 in early NSCLC patient's (Stage I+II) vs healthy control group

	Area Under the Curve	P value	95% Confidence Interval		Cut off	Sensitivity %	Specificity %	PPV %	NPV %	DA %
			Lower Bound	Upper Bound						
Fold change of miR 146	0.679	0.016*	0.533	0.825	3.42	70.4	76.7	73.08	74.19	73.68
Fold change of miR 196	0.706	0.003*	0.568	0.843	1.23	81.5	56.7	62.86	77.27	68.42

PVP: Predictive value of positive. NPV: Predictive value of negative. DA: Diagnostic accuracy

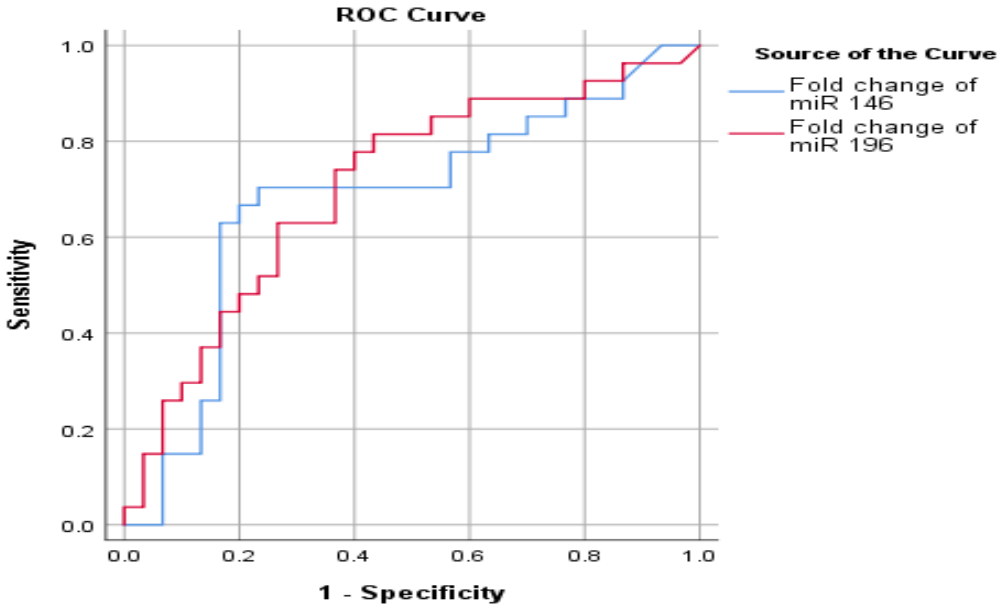

Fig. 4: ROC curve for serum levels of miR-146a and miR-196-a-2 in early NSCLC patient's (Stage I+II) vs healthy control group

4 Discussion

MicroRNAs were initially identified in 1993, in studies on Caenorhabditis elegans' developmental time [31]**.** Since then, the characteristic function and modes of action of miRNAs have generated interest on a global scale, offering a fresh perspective on the fundamental principle of molecular biology [32,33]**.** In this study, both studied miRNAs; miR-146a and miR-196a-2 were found to be significantly elevated in the serum of NSCLC patients compared to the control group (p= 0.045 and <0.001 respectively).

Regarding miR-146a, our finding was similar to those done by ***Chen et al., (2008)*** and ***Wang et al., (2015),*** who found that serum miR-146a along with other miRNAs were significantly upregulated in lung cancer patients when compared to the normal controls. ($p < 0.0001$) [34,35]. In addition, ***Lv et al., (2017)*** also discovered that lung adenocarcinoma patients had significantly higher serum levels of miR-146a compared to healthy controls ($p < 0.0001$) [19]. According to another subsequent study of ***Liu et al., (2020)***, miR-146a was considerably overexpressed in NSCLC patient blood and tissue samples as compared to healthy controls, additionally, they found that three NSCLC cell lines had considerably elevated levels of this miRNA than in the normal bronchial epithelial cell lines, and that higher expression of miR-146a in NSCLC tissues was five times higher than that in the comparable paracancerous tissues [21].

Contradictory to the results of this study, ***Wu et al., (2014)*** found that miR-146a blood and tissue levels were considerably lower in NSCLC patients than those in healthy controls and that these low levels of this miRNA in NSCLC patients were significantly correlated with greater TNM stage, LNM, and bad prognosis [23]. In the same line, their results are similar to the report by ***Mohamed et al, (2019)***, who found that lung cancer patients had considerably

lower serum levels of miR-146a than did normal controls ($p < 0.001$) [24].

Also different from the current study, studies done by ***Dezfuli et al., (2021)*** and ***Vafadar et al., (2021)*** have found that NSCLC patients' peripheral blood mononuclear cells have significantly lower levels of miR-146a than those in normal controls ($P \leq 0.001$) [36,37].

Not surprisingly, MiRNA-146a is one of the most controversial microRNAs, its role as a tumor suppressor or oncogene has been the topic of debate [18]. The discrepancy in the results may be explained by a common single nucleotide variant rs2910164 (G > C) in precursor miR-146a, several researchers reported that this variant alters miR-146a expression level and/or maturation [38, 39, 40, 41, 24]**.**

Another factor that may contribute to such variation in the results is the linkage between miR-146a expression and the inflammatory environment accompanying the carcinogenesis process. Inflammatory mediators like tumor necrosis factor (TNF) upregulate miR-146a, which acts as an anti-inflammatory agent that downregulates these mediators by targeting their mRNAs [42,43]. So, the final expression level might be the net result of the interplay between the oncogenic/tumor suppressor role and its anti-inflammatory protective response by the host.

Concerning miR-196a-2, the results of this study are in line with previous findings that miR-196a-2 is an oncogenic factor and is up-regulated in various malignant tumors. Studies done by ***Vinci et al, (2011), Liu et al, (2012), and Toraih et al, (2016)*** found that miR-196a-2 is significantly upregulated in NSCLC tissue compared to the healthy neighboring lung tissue [44, 45, 46].

Furthermore, the study by ***Bao et al., (2018)*** in China revealed that miR-196a-2 may be a noninvasive biomarker that can be potentially used for the clinical diagnosis of NSCLC as serum miR-196a-2 expression levels were significantly higher in NSCLC patients compared to the normal controls (P<0.0001) [47]. Also, ***Mohamed et al. (2019)***, reported that miR-196a-2 serum expression was significantly upregulated in lung cancer patients in comparison to normal controls ($p < 0.001$) [24].

Moreover, the pooled data from a meta-analysis study that included 23 articles, revealed that miR-196a expression is elevated in tumor tissues or serum/plasma of patients than in healthy people, in addition, elevated miR-196a expression was an unfavorable prognostic biomarker [48] ***(Xiong et al., 2021)***.

In contrast to this study, ***Vafadar et al., (2021)*** reported miR-196a-2 down-regulation in the peripheral blood of NSCLC patients in case-control research [37].

ROC analysis was plotted to evaluate the utility of using the studied circulating miRNAs (miR-146a, miR-196-a-2) for distinguishing NSCLC patients (group I) from the normal control group (group II), ROC analysis revealed that both studied miRNAs were useful serum biomarkers for distinguishing between NSCLC patients and normal controls, where miR-146a showed AUC of 0.635 with a sensitivity of 60%, specificity of 76.7 %, and diagnostic accuracy of 66.25, while miR-196-a-2 showed AUC of 0.740 with a sensitivity of 80%, specificity of 63.3%, and diagnostic accuracy of 73.75%.

The ROC curve findings of miR-196-a-2 in the present study were very close to that reported by ***Bao et al., (2018)*** that the AUC of serum miR-196-a-2 to diagnose NSCLC was 0.785. Optimal sensitivity and specificity were 67.86% and 77.57%, respectively in diagnosing NSCLC [47]. Whereas in a different study, miR-146a displayed an AUC of 0.78 with a sensitivity of 84.06 and a specificity of 58.57 in diagnosing NSCLC [35]**.**

Since lung cancer early detection frequently results in additional treatment options and improved overall survival [49], that is why in the current study a closer look at miR-146a and miR-196-a-2 expression in stage I cancer patients was warranted. Both miRNAs exhibited a discriminating ability between stage I lung cancer patients and the healthy control group, as both were statistically significantly upregulated in the stage I lung cancer patients' group when compared with the healthy control group (group **II**) (p-value; 0.001, 0.03 respectively).

Moreover, when NSCLC patients of the I / II cancer stages were regrouped into one group (early-stage group), the diagnostic ability of both studied miRNAs was investigated again. Both were statistically significantly higher in the early-stage patients' group than in the control group (p-value; 0.02, 0.024 respectively).

In agreement with this finding, ***Wu et al., (2020)*** concluded that the serum level of miR-146a in the early NSCLC group at stages I/II was significantly increased than those in the normal control group ($P < 0.05$) [50].

Both miRNAs chosen for investigation in this study have been documented to be strongly linked with NSCLC diagnosis [35, 47, 48] however, there are fewer publications on using them for NSCLC early diagnosis. Since both miRNAs in the current study were significantly elevated in the serum in early-stage lung cancer patients (stages I and II) than in the healthy controls, therefore the potential of using them as early diagnostic biomarkers for lung cancer was explored. ROC analysis revealed that either miR-146a or miR-196-a-2 has demonstrated a discriminating ability between stage I lung cancer patients and healthy controls. Serum miR146a showed an AUC 0.793, sensitivity of 80 %, specificity of 83.3 %, and diagnostic accuracy of 82.5%, while serum miR196-a-2 showed an AUC of

0.777, sensitivity of 90 %, specificity of 56.7%, and diagnostic accuracy of 65%.

On grouping stages I and II into an early group, both miR-146a and miR-196-a-2 displayed good discriminating ability between the early-stage group of NSCLC patients and healthy controls. Where AUC, sensitivity, specificity, and diagnostic accuracy were 0.679, 70.4 %, 76.7 %, and 73.68% respectively for serum miR-146a and were 0.706, 81.5 %, 56.7%, and 68.42% respectively for serum miR196-a-2, showing that miR-146a & miR-196-a2 may serve as biomarkers for early detection of NSCLC.

This finding regarding miR-146a supports what was found by ***Wu et al., (2020)*** that miR-146a could be a preferable biomarker for NSCLC patients at early stages, they reported that the AUC of this serum miRNA for early stages lung cancer diagnosis was 0.813 with a sensitivity of 68.75 %, specificity of 90 % [50].

In the current study, miR-196-a-2 showed no statistically significant difference among the 4 subgroups of lung cancer when compared to each other (p-value > 0.05), while miR-146a was statistically significantly upregulated in stage I lung cancer patients' group when compared to stages II, III and IV (p-value; 0.025, 0.03, 0.042 respectively). The explanation for this finding could possibly be attributed to the previously mentioned miR-146a single nucleotide variant that alters its expression [41] or due to the dual role of miR-146a as an anti-inflammatory agent and controversial oncogenic / tumor suppressive role [18].

In the current study, no statistically significant associations were found between the studied miRNAs and clinicopathological traits of the NSCLC patients regarding age, sex, smoking, family history, tumor pathology, or metastasis.

In the same line, no significant associations were reported between miR-146a expression levels and NSCLC patients' clinicopathological characteristics [35]**.** While miR-196-2 expression levels were positively associated with higher tumor stage and LNM, no other association was reported between this miRNA level and other clinicopathologic characteristics, including age, sex, smoking, or histological type [47].

Due to its non-invasive nature, circulated miRNA serum testing is a cutting-edge and essential screening technique. In addition, it has a number of other benefits as serum samples are much simpler to get and less uncomfortable than lung biopsy, it is significantly less expensive, and more readily available than low-dose computed tomography (LDCT) especially on a wider scale of cancer screening. The candidate miRNAs can be optimized into a limited set for the tumor-specific origin and act as possible biomarkers for human cancers [51, 12, 52]. Circulating miRNA expression patterns displayed extraordinary stability and disease or tissue specificity [53, 12]**.**

Moreover, during the genesis and development of cancerous processes, fluctuations in the expression level of molecular markers typically take place far earlier than the visible signs and symptoms. The development of such biomarkers for early diagnosis may increase the efficacy of treatment modalities and improve patient survival.

5 Conclusion

As mentioned before, early cancer detection improves the patient's survival and prognosis. In this study, it was demonstrated that the serum of early stages NSCLC patients has over-expressed miR-196a-2 and miR-146a compared to healthy controls making it possible to employ these non-invasive serum miRNAs as early diagnostic biomarkers for NSCLC, especially in the high-risk population. More explorations in more diverse geographical regions on a wider scale with recruiting more patients in the early cancer stages are expected to further verify the findings in the future.

List of abbreviations

AUC: area under the curve
cDNA; Complementary DNA
CT: Cycle threshold
FC: fold change
LDCT: Low-dose computed tomography
miRNAs: Micro RNAs
mRNA: Messenger RNAs
NSCLC: Non-small cell lung cancer
PCR: Polymerase chain reaction
ROC: receiver operating characteristic curves
TNF: tumor necrosis factor
ΔΔ CT: Comparative cycle threshold Method

Declarations

Ethics approval

Ethical Committee approval was obtained from Kasr Alainy Ethical Committee (Code: MD-68-2020)

Consent to participate.

Informed consent was obtained from all individual participants included in the study.

Competing Interests:

The authors declare that they have no competing interests.

Consent to Publish

Not applicable

Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author

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Author contributions

MS: Conception of study design, participants' sample collection, practical work, literature search, data analysis, interpretation, co-writing and critically revising the manuscript.
MH: Conception of study design, supervision of practical work, data interpretation, critically revising the manuscript.
NG: Conception of study design, data analysis and interpretation, critically revising the manuscript
HA: Conception of study design, patient recruitment and interpretation of clinical data, and critically revising the manuscript.
RZ: Conception of study design, data analysis and interpretation, co-writing, and critically revising the manuscript.

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