Original article

Bronchial wash miR-21 as a potential biomarker for non-small cell lung cancer

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ABSTRACT

Background: Lung cancer is one of the major health problems all over the world. Most of cases are discovered at advanced stages because of late appearance of symptoms and the lack of efficient and effective methods for early diagnosis and screening of high risk groups. The microRNA-21(miR-21) was stably present and reliably measurable in all samples of bronchial wash whether positive or negative (control) for lung cancer.

Objectives: to evaluate the expressions of the miRNA-21 as a minimally invasive diagnostic biomarker for non-small cell lung cancer (NSCLC).

Results: Relative quantification of miR-21 gene showed overexpression in samples positive for NSCLC (non-small cell lung cancer) and ROC study yielding 85% sensitivity and 98% specificity in distinguishing NSCLC patients from controls with p-value < 0.05.

Conclusion: altered expressions of the miR-21 in samples of bronchial wash may provide a potential biomarker for detection of non-small cell lung cancer.

Keywords: bronchial wash, miRNA-21, non-small cell lung cancer

Introduction

Lung cancer is one of the leading cause of death from cancer, if not the first among various cancers, all over the world [1,2,3,4,5,6,7,8]. There is increase in the incidence of lung cancer starting from the fifties of the last century as a result of widespread tobacco smoking [9,10,11,12]. There are two types of lung cancer; the non-small cell (NSCLC) and the small cell lung cancer (SCLC) [5,9,10]. NSCLC is the commonest type of lung cancer worldwide, including Iraq, comprising about 85% of whole lung cancer [3,4,5,7,8]. NSCLC consists of three major histological types: squamous cell carcinoma (SqCC), adenocarcinoma (AC), and large cell undifferentiated carcinoma (LCC). The disease is frequently diagnosed at advanced and terminal inoperable stage in more than 75 % of cases [9,10]. Finding of

NSCLC at earlier stage reduces the mortality and improves the outcome [9,10]. Nowadays, the diagnosis of lung cancer depends on the development of symptoms, which are late in the course of disease, especially worrying symptoms such as repeated cough with or without hemoptysis and/or chest pain [9,10]. Therefore, developing a minimally invasive technique by taking advantages of recent developments in molecular genetics for diagnosis of NSCLC at an early stage is important clinically. MicroRNAs (miRNAs) are one of these studied biomarkers and still under investigation [13,14,15,16]. The miRNAs are small non-coding single stranded RNA consisting of 21-23 nts (nucleotides) bases. MicroRNAs exert their effect by post-transcription translation inhibition of target messenger RNAs (mRNAs) by complementary binding to 3'-untranslated regions (3'UTRs) of the target mRNA gene leading to its degradation or translation inhibition, with the resultant decrease in expression of target gene [9,10]. Due to its effect on target mRNAs, miRNAs participate in the physiological processes of proliferation, differentiation, apoptosis, and cell death as well as in pathological processes especially in carcinogenesis [9,10]. They are acting as oncogenes or tumor suppressor genes according to nature of their targeted mRNA genes [9,10]. MiR-21 is now considered as an oncomiR (oncogene) due to its ability of suppressing the actions of several tumor suppressor genes, promoting tumor cell growth, invasion and metastasis [9,10]. It was found to post-transcription down regulate PTEN which is a tumor suppressor gene [9,17]. MiR-21 also promotes growth, invasion, chemo and radio-resistance of NSCLC [9,10,17]. The high expression of miR-21 especially in bronchogenic squamous cell carcinoma has been found to be associated with poor prognosis [9,10,17]. MiR-21 also targets programmed cell death 4 (PDCD4), decreasing apoptosis in lung and breast cancer [18,19].

Materials and Methods

This is a prospective case-control study in which 54 patients were enrolled. Patients were recruited at the Thoracic Surgical Unit in the Specialized Surgery Hospital / Medical City during the period from March 2012 to April 2014.Twenty-four selected patients with NSCLC lung cancer proved by cytopathology on bronchial wash (15 were males and 9 were females).Thirty selected patients with benign pulmonary lesions (control) proved by cytopathology of bronchial wash (20 were males and 10 were females).

Inclusion Criteria

1. Patients were presented for the first time complaining from chest problem (cough, sputum, hemoptysis, tightness, chest pain ...), proved to be due to

pulmonary diseases malignant or benign.

2. Patients underwent Fiberoptic bronchoscopy with bronchial wash.

Exclusion Criteria

1. All cases that have received any form of specific cancer treatment (radical surgery, chemotherapy and/or radiation therapy) prior to sample collection were excluded from the study.
2. All cases known to have a second primary tumor other than lung cancer were excluded from the study.
3. All cases with uncertain diagnosis, whether benign or malignant were also excluded from the study.

Ethical approval for this work was obtained from Baghdad Medical College Ethics Committee. Every patient participated in the study have received a written information sheet explaining to them the aim of the study, and a signed consent form was taken from each one before participating in the study and the right was given to them to withdraw from this study at any time.

Samples collection

In a labeled nuclease free tube (2 – 3) milliliters of bronchial wash was taken directly from bronchoscope and kept on ice until transferred to the laboratory for separation. The separation is performed by centrifugation at a speed of 1500 g for 15 minutes at a temperature of 4 degree centigrade. Then, the supernatant fluid was discarded and the sediment was re-suspended in a 5 times volume of RNA Later solution is kept in a deep freeze at -80 degree centigrade (Ć) until RNA extraction. The rest of the bronchial wash specimen was taken for cytopathological study.

Cytopathological diagnosis

The smears that were taken from deposit of bronchial wash samples are stained by Papanicolaou's stain and/or Hematoxlin and eosin stains. The following criteria were used for cytopathological diagnosis of the main types of NSCLC lung cancer.

1. Squamous cell bronchogenic carcinoma

The cells are enlarged with a raised nucleo-cytoplasmic ratio (N/C ratio), the nucleus of malignant cell exhibits hyperchromatism, abnormal chromatin pattern, and irregular nuclear membrane. The cytoplasm of malignant cells is abundant and dense, in the well differentiated type it is cyanophilic, while basophilic in less mature type. Other features of malignant cells are also seen such pleomorphism, bizarre shaped cells and, giant tumor cells [9,10,20,21].

2. Bronchogenic adenocarcinoma

There is moderate hyperchromasia of their nuclei with fine granular chromatin pattern, prominent nucleoli with occasional mitotic figures. The cytoplasm is

amphophilic with fine or coarse vacuoles which are due to degeneration rather than mucus secretion, and cells are never ciliated. The presence of papillary clustering or three dimensional appearances may be seen as well [9,10,20,21,22,23].

3. Large cell bronchogenic carcinoma

LCC is characterized by syncytial clusters of cell and dispersed cells, the cells have irregular nuclei with striking chromatin clearing, prominent, often multiple nucleoli and ill-defined, feathery cytoplasm [9,10,20,21,22].

qRT-Realtime PCR

The extractions was performed by the use of extraction kit (mirVana™ miRNA Isolation Kit, with phenol) according to the manufacturer's instructions [24]. The extracted total RNA then, was treated by DNA-free kit to get rid of any contaminant DNA using DNase and according to manufacturer's instructions [25]. The purity and concentration of RNA was measured by Nano-drop spectrophotometer and a samples with a ratio of A260/A280 (1.9 – 2.0) were taken for gene quantification [25]. The extracted total RNAs were stored at a temperature of (– 80 C) for reverse transcription into corresponding cDNAs.

Reverse Transcription of total RNAs to cDNAs

Two samples from DNA free total RNA (each containing 1 µg of total RNA) were taken for reverse transcription of miRNAs into cDNA using (TaqMan® MicroRNA reverse Transcription Kit and primers) according to manufacturer's instructions [26]. A Primer pool for reverse transcription of miRNAs into cDNAs were created from miRNA specific primers and a mix of reagent was also used to reduce pipetting errors. The concentration and purity of cDNA was also checked with Nano-drop spectrophotometer. The quality of cDNA as a template for real time PCR amplification of miRNA was assessed by qRT-PCR amplification of (miR-RNU-48 housekeeping gene) with a non-template sample as a control (NTC). The optimum concentration of primers and the optimum annealing temperature was assessed by serial dilutions of primers and the temperature also investigated by test changing annealing temperature and performing PCR runs. The optimum primer concentrations and annealing temperature in table 1

Table1: Properties and amounts of primers used in real-time qRT-PC assays of microRNAs.

Property	microRNA-21	microRNA-RNU-48
Forward primer	5'GCCCGCTAGCTTATCAG ACTGATG-3'	5'TCTGAGTGTCTTCGCTGA CG-3'
amount of use	10 pmol	15 pmol
Reverse Primer	5'GTGCAGGGTCCGAGGT-3'	5'GAGGTATTCGCACCAGA GGA-3'
amount of use	10 pmol	15 pmol
Optimized annealing temp.	55 C	56.5C

Realtime qRT-PCR amplification of cDNA

The realtime PCR amplification of miRNAs (21, & housekeeping gene RNU48) in the samples taken from bronchial wash was performed in duplicate using TaqMan® MicroRNA Master Mix II, no UNG Kit with primers and probes from Applied Biosystems [27]. The assays were performed according to the manufacturer's instructions with the use of master mix pool to reduced pipetting errors with a non-template control (NTC). The thermal prolife was designed including hold at 95Ć for 10 minutes followed by forty cycles of 15 seconds at 95 C followed by annealing and extension at 60Ć for forty cycles. The mean of Ct value for each specimen was taken for gene expression study.

Statistical analysis

Analysis of the present results were carried out using IBM SPSS-22 statistical software .Frequency of positive results were studies as number and percentage. Differences between frequencies were calculated by applying Chi square. Results were considered significant when p value < 0.05.Mean and standard deviation (SD) were calculated for total RNA concentration and purity by SPSS-21 software. Mean and SD of calculated Ct values were subjected to student *t*-test, ANOVA, and LSD statistical tests. Results of differences were considered significant when p value is < 0.05.The ΔCt and ΔΔCt

for each gene were calculated according to the equations:

a. The ΔCt of target gene = [Ct of target gene – Ct of housekeeping gene].

b. The ΔCt of control gene = [Ct of control gene – Ct of housekeeping gene].

c. Expression = ($2^{-\Delta\Delta Ct}$), the result of expression = $2^{-\Delta\Delta C}$ {[Ct of target gene – Ct of housekeeping gene]-[Ct of control – Ct of housekeeping gene]} [28].

ROC area (Receiver Operator Characteristic) for sensitivity and specificity of expression of each gene were calculated with significant being *p*-value <0.05.

Results

The cytopathological findings of bronchial wash samples that were positive for NSCLC summarized in figure1. Bronchogenic squamous cell carcinoma was the most frequent type of lung cancer accounting 19/24 (79.17 %) of whole NSCLC cases, subdivided according to sex into 13/24(54.17%) in men and 6/24 (25 %) in women from whole non-small cell lung cancer cases. Bronchogenic adenocarcinoma subtype of NSCLC were 4/24 (16.67 %) also subdivided into 1/24 (4.17 %) in males and 3/24 (12 %) in females, while large cell bronchogenic carcinoma accounting for 1/24 (4.16 %) of whole lung cancer. The results showed cancer was more common in the age group (60 – 69 years), and followed by the age group (70 – 79 years) and in most age groups the males were more common than females or equal to them figure 2.

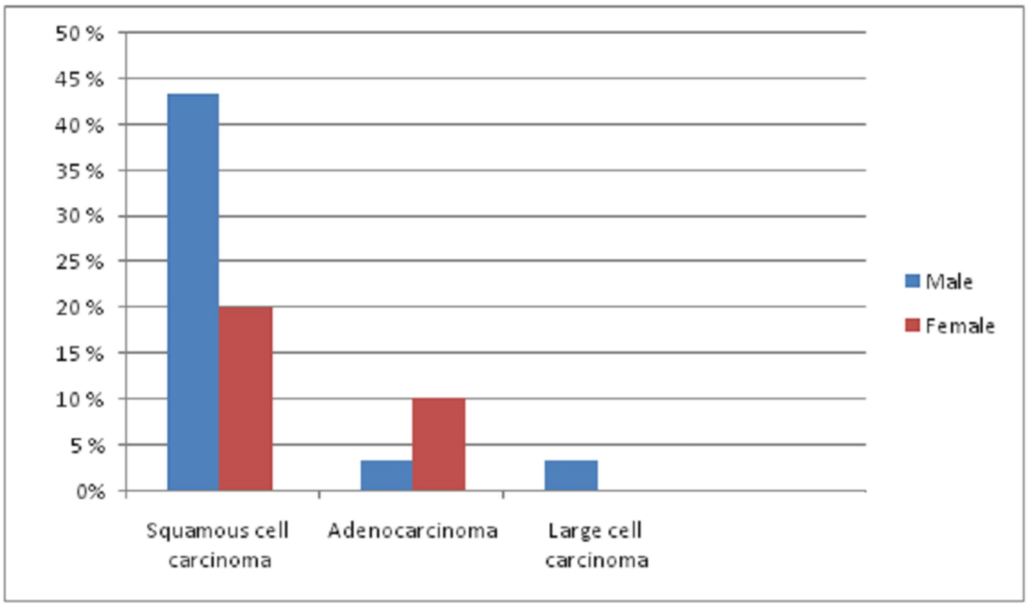

Figure 1: Cytopathological results of bronchial wash positive for malignant cells, frequency of the cytopathological type according to sex.

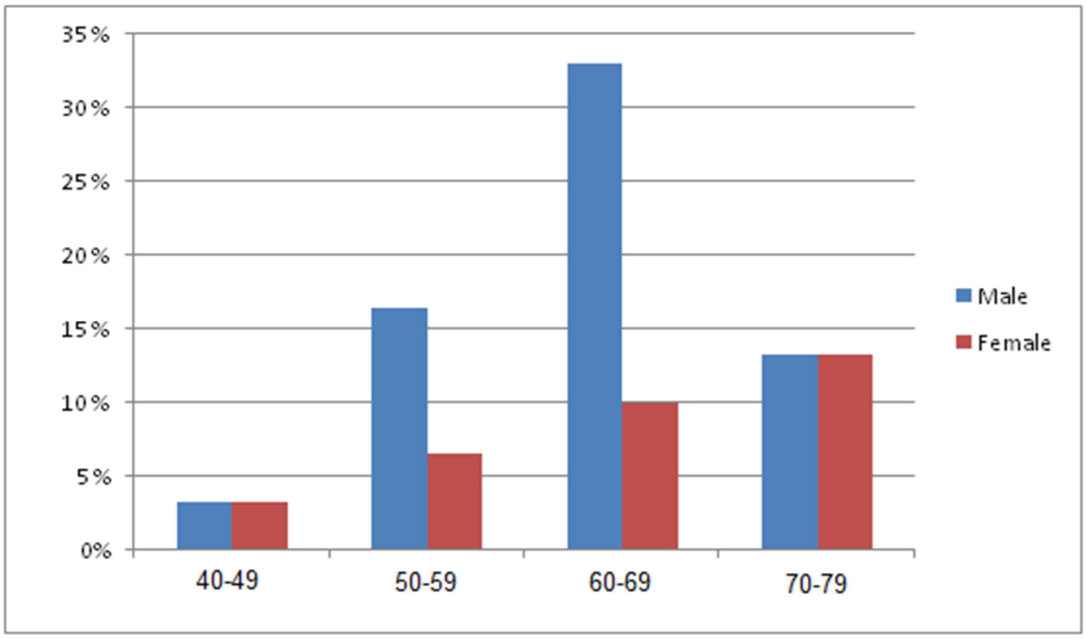

Figure 2: Cytopathological results of bronchial wash positive for malignant cells, the frequency according to the age groups and sex.

The total RNA concentration and purity of each sample, after total RNA extraction by mirVana Isolation kit and treatment with DNase kit, was measured by nano-drop and the ratio of A260 / A280 and samples results 1.9 – 2.0 were considered as good samples and taken for further steps. The mean ± SD for concentration of whole samples of bronchial wash positive for lung cancer cells was 4346 ± 53.147µg/ml and for males the mean concentration was 4785.88±65.174 µg/ml and for females it was 3466.96±23.201µg/ml. The mean ± SD of concentration of whole samples of bronchial wash that were negative for lung cancer cells was 3230.45±22.404 µg/ml and for males 3205.995±23.908 µg/ml and for females it was 3279.36±19.078 µg/ml.

The mean of purity ± SD of whole samples of bronchial wash that were positive for lung cancer cells was 1.9602±0.03616 and for males it was 1.9553±0.0386606 and for females it was 1.970±0.0299629. The mean of purity ± SD of whole samples of bronchial wash that were negative for lung cancer cells was 1.95510±0.0372479 and for males it was 1.95035±0.395731 and for females it was 1.96460±0.0318441.

Results for raw Ct values of all studied microRNA genes compared between NSCLC cases and control by *t*- test as studied from bronchial wash specimens

Table 2: Recorded results for raw Ct values of all studied mRNA and microRNA genes compared between lung cancer cases and control as studied from bronchial wash specimens.

	Study group		
	Control group (C)	**Cases (lung Ca)**	***p*- (*t*-test)**
Ct value - miR-RNU-48			0.02
Range	(22.19 to 25.96)	(23.2 to 26.6)	
Mean	24.35	24.99	
SD	1.10	0.90	
SE	0.20	0.16	
N	30	24	
Ct value - miR-21			<0.001
Range	(25.96 to 30.16)	(22.68 to 29.97)	
Mean	28.87	26.14	
SD	0.90	1.79	
SE	0.16	0.33	
N	30	24	

Comparison between different histological types and control cases in mean raw Ct value of markers studied in bronchial wash. The mean raw Ct value was compared between different histological types of lung cancer and control cases for each of the studied markers in bronchial wash using ANOVA test. The *p*–LSD (Least significant difference) for difference in mean raw Ct values was studied different types of lung cancer themselves. In miR-RNU-48, ANOVA testing for difference in mean raw Ct value between different histopathological types of lung cancer

and control was not significant; the *p*-value was > 0.05. The *p*-(LSD) for difference in mean raw Ct value was significant the *p*-value was < 0.05, on comparing between C (control) and SqCC (squamous cell carcinoma) only. However, it was not significant, *P*-value > 0.05, when comparing between C and AC (adenocarcinoma), and between SqCC and AC. In miR-21, ANOVA testing for difference in mean raw Ct value between different histopathological types of lung cancer and control was significant; the *p*-value was < 0.05. The *p*-(LSD) for difference in mean raw Ct value was significant; the *p*-value was < 0.05, on comparing between C and SqCC, and between C and AC. However, it was not significant, *p*-value was > 0.05, when comparing between SqCC and AC, table 3

Table 3: ANOVA and LSD for differences in means of raw Ct values of different markers studied in bronchial wash according to histological types.

	Final diagnosis			*p*-value (ANOVA)
	SqCC	**AC**	**C (Control)**	
Ct value miR-RNU-48			0.13[NS]	
Range	(23.2 to 26.6)	(24.4 to 25.65)	(22.19 to 25.96)	
Mean	25.03	25.07	24.35	
SD	1.03	0.52	1.10	
SE	0.24	0.26	0.20	
***p*-(LSD) for difference in mean between:**				
C X SqCC = 0.028				
C X AC = 0.2[NS]				
SqCC X AC = 0.95[NS]				
Ct value - miR-21			<0.001	
Range	(24.32 to 29.56)	(25.21 to 29.6)	(25.96 to 30.16)	
Mean	26.05	27.08	28.87	
SD	1.43	1.85	0.90	
SE	0.33	0.93	0.16	
***p*-(LSD) for difference in mean between:**				
Cont X SqCC <0.001				
Cont X AC = 0.022				
SqCC X AC = 0.19[NS]				

On studying the sensitivity and specificity of each marker, the P value for ROC curve was statistically significant, P value < 0.05, in all microRNAs.

Table 4: The ROC area occupied by each test and its *p*-value of markers studied from bronchial wash samples.

	ROC area	*P*-value
Ct value - miR-21	0.871	<0.001
Ct value - miR-RNU-48	0.658	0.036

The differences in the mean normalized Ct values between malignant and control cases were statistically significant, *P*-value was < 0.05 with miR-RNU-48 & miR-21, table 5.

Table 5: Recorded results for normalized Ct values of studied markers (miR-RNU-48 & miR-21), compared between lung cancer cases and control, as obtained from bronchial wash specimens.

	Study group		
	Control group	**Cases (lung Ca)**	***P*- (*t*-test)**
Standardized Ct value mir-RNU-48			<0.001
Range	(0.78 to 0.93)	(0.85 to 0.98)	
Mean	0.87	0.92	
SD	0.04	0.03	
SE	0.01	0.01	
Standardized Ct value - miR-21			<0.001
Range	(0.94 to 1.07)	(0.85 to 1.11)	
Mean	1.03	0.96	
SD	0.03	0.06	
SE	0.01	0.01	

Comparison between different histopathological types of lung cancer and control cases in mean normalized Ct values of markers (miR-RNU-48 & miR-21) studied in bronchial wash samples. The mean of normalized Ct values were compared between different histopathological types of lung cancer and control cases for each of the studied markers in bronchial wash samples using ANOVA test. The *P*-(LSD) also studied for differences in mean between control and different histopathological types of non-small cell lung cancer and also between different histopathological types themselves. ANOVA testing for miR-RNU-48 between all histopathological types of lung cancer and control was statistically significant, *P*-value was <0.05. The *P*-(LSD) for difference in mean normalized Ct values of miR-RNU-48 was statistically significant; *P*-value was < 0.05, between C and SqCC, and between C and AC. The *P*-(LSD) for differences between the types of lung cancer was not significant, *P*–value was > 0.05, between SqCC and AC.ANOVA testing for miR-21 between all histopathological types of lung cancer and control was significant, *P*-value was <0.05. The *P*-(LSD) for difference in mean normalized Ct values of miR-21 was statistically significant; *P*-value was < 0.05, between C and SqCC, and between SqCC and AC. The *P*-(LSD) for differences between the types of lung cancer were not significant, *P* – value was > 0.05, between C and AC.

Table 6: ANOVA and LSD for differences in means of normalized Ct values of different markers as studied in bronchial wash samples, according to histopathological types of lung cancer.

	Final diagnosis			
	SqCC	**AC**	**Control**	***P*- (ANOVA)**
Standardized Ct value - miR-RNU-48				<0.001
Range	(0.85 to 0.97)	(0.93 to 0.96)	(0.78 to 0.93)	
Mean	0.91	0.94	0.87	
SD	0.04	0.01	0.04	
SE	0.01	0.01	0.01	
P (LSD) for difference in mean between:				
Control X SqCC <0.001				
Control X AC <0.001				
SqCC X AC = 0.12[NS]				
Standardized Ct value - miR-21				<0.001
Range	(0.88 to 1.07)	(0.96 to 1.11)	(0.94 to 1.07)	
Mean	0.95	1.02	1.03	
SD	0.05	0.07	0.03	
SE	0.01	0.03	0.01	
***P*-(LSD) for difference in mean between:**				
Control X SqCC <0.001				
Control X AC = 0.47[NS]				
SqCC X AC = 0.009				

The P-value for ROC curves was statistically significant, *P*-value < 0.05, in both studied microRNAs, table 7

Table 7: The ROC area occupied by each test and the p-value of markers studied from bronchial wash samples.

	ROC area	***P*-value**
Standardized Ct value - miR-RNU-48	0.833	<0.001
Standardized Ct value - mir-21	0.821	<0.001

Studying the sensitivity of and specificity of each marker, the P-value for ROC curves was statistically significant, *p*-value < 0.05, all microRNAs, figure 3

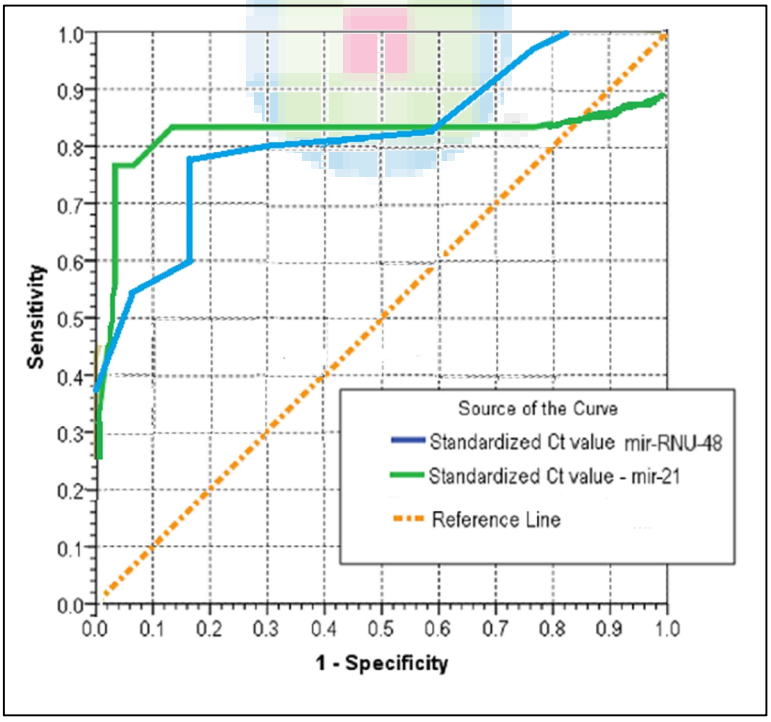

Figure 3: The ROC curves for sensitivity and specificity of studied markers (miR-RNU-48 and miR-21), in samples of bronchial wash.

Comparative CT Method

Calculation of expression by comparative Ct method using the formula, expression = ($2^{-\Delta\Delta C}$), the result of expression = $2^{-\Delta\Delta C}$ {[Ct of target gene – Ct of housekeeping gene]-[Ct of control – Ct of housekeeping gene]} [28].

The gene is overexpressed when its comparative Ct value is over (1), and under expressed when its comparative Ct value is less than. MiR-21 was (10.4) times more overexpressed in samples positive for NSCLC when compared to negative samples (control), table 8

Table 8: Mean Ct value with SD of cancer cases and control cases with comparative Ct values of studied markers and their P-values, in samples of bronchial wash.

	Ca cases		Controls	Comparative Ct- value		*P*- value
	Mean	SD	Mean	SD		
Ct-miR-RNU-48	Control gene					
Ct-miR-21	0.591	0.992	0.057	0.094	10.4	<0.001

The expression of miR-21 gene in samples of bronchial wash positive for bronchogenic squamous cell (SqCC) compared to controls cases using comparative Ct method was (6.8) times more than control, table 9

Table 9: Comparative Ct values with mean and SD of mir-21 gene in samples of bronchial wash positive for lung squamous cell carcinoma by cytopathology.

	SqCC cases		Controls		
	Mean	SD	Mean	SD	Comparative Ct Value
Ct-miR-21	0.39	0.378	0.057	0.094	6.8

The expression of miR-21 gene in samples of bronchial wash positive for squamous cell lung (AC) compared to controls cases using comparative Ct method was (9.7) times more than controls, table 10.

Table10: Expression of mir-21gene in bronchial wash samples positive for lung adenocarcinoma.

	AC cases		**Controls**		
	Mean	SD	Mean	SD	Comparative Ct value
Ct-miR-21	0.551	0.703	0.057	0.094	9.7

When the standardized Ct value 0.935, the sensitivity is 30%, specificity is 100.0% and accuracy is 65%. When standardized Ct value 1.065, the sensitivity is 86.7%, the specificity is 6.7%, and the accuracy is 46.7%, table 11

Table 11: Specificity, sensitivity, accuracy, positive predictive values and negative predictive values.

					PPV at pretest probability =		
Positive if > cut-off value	Sensitivity	Specificity	Accuracy	Optimal if minimum	50%	90%	NPV at pretest probability = 10%
Standardized Ct value - miR-21							
0.935	30.0	100.0	65.0	0.700	100.0	100.0	92.8
0.945	53.3	96.7	75.0	0.468	94.1	99.3	94.9
0.955	60.0	96.7	78.3	0.401	94.7	99.4	95.6
0.965	73.3	96.7	85.0	0.269	95.7	99.5	97.0
0.975	76.7	96.7	86.7	0.236	95.8	99.5	97.4
0.985	76.7	93.3	85.0	0.243	92.0	99.0	97.3
0.995	80.0	90.0	85.0	0.224	88.9	98.6	97.6
1.005	83.3	86.7	85.0	0.213	86.2	98.3	97.9
1.015	83.3	76.7	80.0	0.287	78.1	97.0	97.6
1.025	83.3	66.7	75.0	0.373	71.4	95.7	97.3
1.035	83.3	56.7	70.0	0.464	65.8	94.5	96.8
1.045	83.3	43.3	63.3	0.591	59.5	93.0	95.9
1.055	83.3	23.3	53.3	0.785	52.1	90.7	92.6
1.065	86.7	6.7	46.7	0.943	48.1	89.3	81.8

Discussion

In the present study, we found that the miRs (RNU-48 & 21) were stably present and readily measurable in the bronchial wash samples. Additionally, miR-21 was significantly over-expressed in specimens of cancer patients compared with those in normal controls. The data produced from our present study imply that miR-21 in bronchial wash could serve as a biomarker for diagnosis of lung cancer and miR-21 also could serve as a target for future therapy. The current study was in agreement with other studies. Shen et al. [29] have found that the expression of miR-21 may help in diagnosis of lung cancer in solitary lung nodule; Schwarzenbach et al. [30] suggested that miR-21 is a marker for early diagnosis of lung cancer. Cortez et al. [31] who reported the presence of miR-21 in body fluids as a marker for diagnosis and prognosis. Gao et al. [32] reported that over expression of miR-21 is associated with poor prognosis of lung cancer.

Inspite of our result appears to be promising, there are some limitations in this study. First, the sample sizes of the two study groups were too small, so further launching of miR-21 in large sample seize and in an independent studies is clearly required. Secondly, most of the studied cases were in advanced stages, and we were not sure of miR-21 expression in early stages of lung cancer, however, investigations in the future may give an answer.

In conclusion, we found that the expressions of the miRNA-21 in samples of bronchial wash could readily and specifically measured to be used as a minimally invasive diagnostic biomarker for NSCLC. Nonetheless, further independent cohort is required for validating the utility of this potential biomarker.

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