Original Article

The diagnostic role of microRNA 21 in patients with nonsmall cell lung cancer: An exploratory study

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ABSTRACT

Background: Although histopathological examination of the biopsy specimen is the gold standard for the diagnosis of non small cell lung cancer (NSCLC), a blood-based noninvasive test (liquid biopsy) may prove to be helpful in patients with repeatedly negative biopsy or for response assessment following neoadjuvant therapy. The present study was conducted to explore the diagnostic value of circulating serum microRNA (miRNA) 21 in patients with NSCLC. **Methods:** This case–control analytical study was carried out in a tertiary care teaching hospital in Northern India. The study consisted of 30 cases of biopsy-proven NSCLC and 30 controls. Serum miRNA-21 expression levels were estimated by extracting total RNA from the serum sample, reverse transcribing it to cDNA and quantified in relation to U6 reference miRNA. **Results:** A total of 30 patients with NSCLC and 30 controls were included in the study. The subjects were comparable in two groups with reference to age, gender, and smoking. Pathological types were adenocarcinoma in 19 (63.3%) and squamous cell carcinoma in 11 (36.6%) patients. Majority of the patients had advanced disease-AJCC stage III in 15 patients and AJCC Stage IV in 13 patients; two patients had stage II disease. There was a significant upregulation of serum miRNA 21 gene expression in the patients with lung cancer compared to controls (median fold change, 3.39 vs. −2.81, $P = 0.00$). A fourfold change in serum miRNA 21 is significantly associated with the diagnosis of NSCLC with a high specificity of 97% and area under curve of 0.84 (95% confidence interval of 0.74–0.94). **Conclusion:** Estimation of serum miRNA 21 expression has potential to be used as liquid biopsy for the diagnosis of NSCLC. Further studies with large sample sizes are warranted to confirm the diagnostic accuracy of serum miRNA 21 expression.

KEY WORDS: Diagnosis, liquid biopsy, lung neoplasms, microRNAs

INTRODUCTION

As per Globocan 2018 estimates, lung cancer is the most frequently diagnosed cancer (11.6% of the total cases)

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worldwide in both genders and is a major cause of cancer-related mortality (18.4% of the total cancer deaths).[1] There are mainly two types of lung cancer: nonsmall cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC constitutes almost 85% of all lung cancer cases, including lung adenocarcinoma, squamous cell carcinoma, and large cell lung cancer.[2] More than half of the patients with NSCLC die within 1 year of diagnosis. Unfortunately, majority of the patients with NSCLC are diagnosed in advanced stage especially in developing countries.[3] Early diagnosis of lung cancer has the potential to improve long-term survival and reduce disease specific mortality. Although traditional biopsy is the gold standard for the diagnosis of NSCLC, there is an urgent need to identify various markers which can be utilized for liquid biopsy in patients where traditional biopsy is difficult to perform or repeated biopsies have not yielded sufficient tissue for pathological diagnosis. Moreover, liquid biopsies may also prove to be indispensable as predictive or prognostic markers for NSCLC as they can be performed repeatedly with minimal discomfort to the patient.

miRNAs are 18–25 nucleotides small RNAs which act by targeting mRNAs and stopping their translation or bringing cleavage of the mRNA to effect. Thus, they control posttranscriptional gene expression. They are estimated to be around 3000 in number and regulate various cellular processes: both metabolic and developmental. A significant amount of nondegraded miRNAs is present in body fluids (for example, blood, plasma, serum, and sputum). Easy nature of availability of miRNAs and their extreme stability in prolonged preservation make them an ideal tool to be used as a biomarker. Several studies have shown that certain miRNAs can be used to diagnose lung cancer and pathological subtype; moreover, they also have the potential to be used as prognostic biomarker for survival outcomes and predictive biomarker for treatment response assessment. A number of miRNAs have been studied as serum biomarkers for the diagnosis of NSCLC. One of the most studied miRNA is 21 which is one of the most abundant and recurring oncogenic miRNA.[4] It is overexpressed in almost every type of solid neoplasms. The present exploratory study was conducted to evaluate the diagnostic role of miRNA 21 in patients with NSCLC.

METHODS

The present case–control study was conducted in a tertiary care teaching hospital in Northern India after obtaining clearance from Institutional Ethical Committee for Human research (IEC-HR). The study participants were recruited as cases if they had histopathologically proven NSCLC (irrespective of stage). Participants were excluded if they had a synchronous malignancy or history of any previous malignancy or any prior anticancer treatment for current malignancy. Age-matched patients with nonmalignant pulmonary diseases and having no pulmonary mass/nodule on chest computed tomography (CT) were included in the study as controls.

All the participants were of more than 18 years of age and legally eligible to give their consent for participation in the study. The study was commenced only after approval from IEC-HR was obtained. The patients were staged according to the 8th edition of TNM in lung cancer.

MicroRNA 21 quantification

Venous blood samples (5 ml) were collected from each study participant. Blood samples were left at room temperature for 30 min to allow complete coagulation. Samples were then centrifuged immediately after coagulation, and the supernatant were collected in 2 ml microcentrifuge tubes taking care not to disturb the buffy coat. Serum samples were immediately frozen at −280°C until RNA extraction. Total RNA was extracted from serum using the Trizol method according to the manufacturer's instructions. RNA purity and concentration was determined with nanodrop. Samples with an absorbance (A) 260/280 value 1.8–2.0 value were included in subsequent experiments. Extracted total RNA was reverse transcribed to cDNA using miRNA reverse transcription kit according to the instructions of the kit, following which real-time polymerase chain reaction was performed using Master Mix and primers. Relative quantification of the expression of the miRNA 21 was then calculated using U6 miRNA as the reference. MicroRNA (miRNA) expression was quantified as delta Ct values, where Ct = threshold cycle, delta Ct = (Ct target miRNA-21 − Ct miRNA-U6).

Primary outcome measure was serum levels of miRNA-21 expression in patients with NSCLC and controls. Secondary outcome measures were comparison of serum levels of miRNA-21 in histopathological subtypes of NSCLC (squamous cell vs. adenocarcinoma) and in various TNM stages of patients with NSCLC.

Statistical analysis

Microsoft Excel (version 2007) and statistical software SPSS for windows (version 24.0) were used for data presentation and statistical analysis. Categorical variables were measured with frequencies (percentages) and compared using Chi-square/Fisher's exact test in between the groups. Quantitative variables of cases and controls were expressed as mean ± standard deviation values or median (interquartile range) and compared using the student *t*-test for parametric data and Mann–Whitney *U*-test for nonparametric data.[5,6] Mann–Whitney *U*-test and Kruskal–Wallis test were used to compare the fold change in miRNA 21 expression in patients with different pathological types (squamous cell vs. adenocarcinoma) and TNM stages, respectively. A receiver operating curve (ROC) was generated to estimate the diagnostic accuracy of fold change in miRNA 21 expression in patients with NSCLC. Statistical significance was established when $P < 0.05$.

RESULTS

This case–control analytical study was carried out in a tertiary care teaching hospital in Northern India. A total

of 30 patients with NSCLC (either adenocarcinoma or squamous cell carcinoma) and 30 controls were included in the study. The subjects were comparable in two groups with reference to age (cases vs. controls; 59.40 ± 10.64 vs. 60.20 ± 10.46, $P = 0.784$), gender (male to female ratio in cases vs. controls; 23:7 vs. 22:8, $P = 0.766$), and smoking (pack years in cases vs. controls; 21.22 ± 5.37 vs. 21.42 ± 5.15, $P = 0.88$). Majority of the cases had dyspnea ($n = 28$, 93.3%), cough ($n = 19$, 63.3%), weight loss ($n = 23$, 76.6%), and anorexia ($n = 23$, 76.6%). Associated comorbidities in cases were hypertension ($n = 5$, 16.6%), diabetes mellitus ($n = 2$, 6.66%), hypothyroidism ($n = 1$, 3.3%), coronary artery disease ($n = 1$, 3.33%), and hypothyroidism ($n = 1$, 3.33%). Only one patients was a known case of chronic obstructive pulmonary disease ($n = 1$, 3.33%) and was on irregular treatment. Ipsilateral and bilateral pleural effusions were present in 12 (40.0%) and 2 (6.6%) patients. All the patients underwent either bronchoscope or CT guided biopsy for the confirmation of the disease. Pathological types were adenocarcinoma in 19 (63.3%) and squamous cell carcinoma in 11 (36.6%) patients. Majority of the patients had advanced disease-AJCC stage III in 15 patients and AJCC Stage IV in 13 patients; two patients had stage II disease.

There was a significant upregulation of serum miRNA 21 gene expression in the patients with lung cancer compared to controls (median fold change, 3.39 vs. -2.81, $P = 0.00$) [Figure 1]. Although there seems to be an upregulation of miRNA 21 expression with the advancing stage [Figure 2] and adenocarcoma pathological type, sample size was small to detect a statistical significance [Figure 3].

A ROC curve generated to identify the diagnostic value of serum miRNA 21 revealed that a fourfold change (4.12) is significantly associated with the diagnosis of NSCLC with a high specificity of 97% and area under curve of 0.84 (95% CI of 0.74–0.94) [Figure 4].

DISCUSSION

Liquid biopsy is the term given to diagnostic procedures performed on cancer derived material, captured from blood sample. Sources of tumor material that can be assessed by liquid biopsy include circulating tumor cells, circulating free DNA, circulating small-RNA, miRNA, extracellular microvesicles (including exosomes) containing small-RNA, mRNA, and DNA. Advantages of liquid biopsy are that it is minimally invasive, avoids the complications of traditional invasive biopsy procedures, provides fresh tumor derived material which is unhampered by preservatives, being useful where traditional tumor biopsy is difficult (deep seated mediastinal tumor), can be repeatedly performed for treatment monitoring and follow-up, and thus can be used as a guide for prognosis of patients. Circulating miRNAs are being investigated extensively in various solid neoplasms as liquid biopsy markers because they are
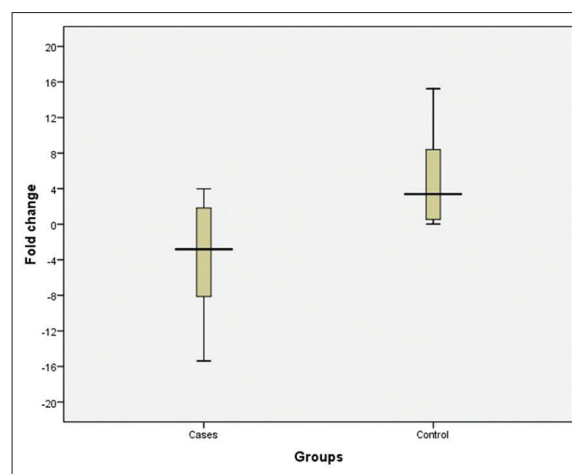

Figure 1: Box plot displays serum microRNA 21 gene expression in the patients with lung cancer compared to controls
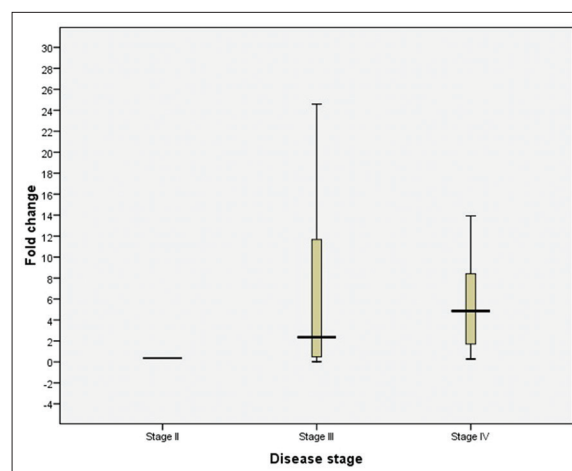

Figure 2: Box plot displays serum microRNA 21 gene expression in the patients with different stages of lung cancer
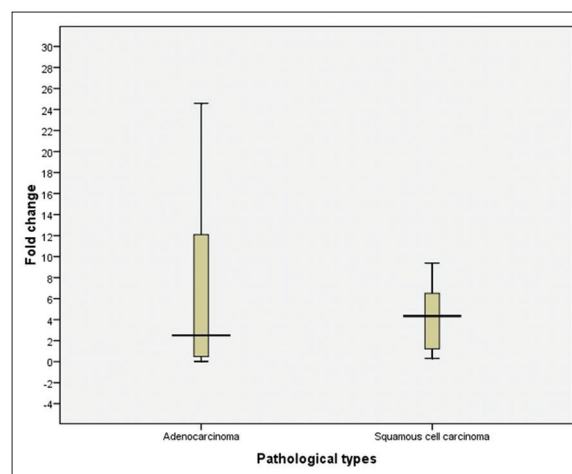

Figure 3: Box plot displays serum microRNA 21 gene expression in the patients with different pathological types of lung cancer

exceedingly stable in circulation and resistant to RNAse degradation.[7] They have displayed remarkable stability

in severe conditions such as extreme pH or temperature levels and extended storage. Close to 3000 different types of miRNAs have been reported till date.

miRNA 21 is overexpressed in almost all types of solid tumors. It modulates tumorigenesis by inhibiting negative regulators of the RAS/MEK/ERK pathway. miRNA-21 causes posttranscriptional downregulation of expression of tumor suppressor PTEN and results in growth and invasion of tumors in patients with NSCLC.[8-10] miRNA 21 expression is also inversely correlated with expression of PDCD4 and maspin and, thus, prolonging cell proliferation and migration while inhibiting apoptosis.[11] miRNA 21 also regulates the epithelial–mesenchymal transition (EMT) through Akt/GSK3β downstream targets including

Figure 4: A receiver operating curve displays the diagnostic value of serum microRNA 21 with the diagnosis of nonsmall cell lung cancer area under curve of 0.84 (95% confidence interval of 0.74–0.94)

regulation of E-cadherin and phosphorylation of β-catenin, which, in turn, regulates the EMT proteins including downregulation of E-cadherin as well as increased expression of vimentin, snail, and slug.[8]

Our study supports the hypothesis that serum miRNA 21 is overexpressed in patients with NSCLC and a fourfold rise is highly specific for the diagnosis. A Chinese study involving 20 patients with NSCLC and 10 healthy individuals highlighted that overexpression of miR-21 showed a highly discriminative ROC curve profile with an area under the curve of 0.91 ± 0.04, sensitivity of 78.8%, and specificity of 100%.[12] A case–control study[13] involving 80 patients with NSCLC and 80 healthy controls also confirmed that circulating serum miRNA-21 can diagnose NSCLC reliably with a sensitivity and specificity of 80% and area under ROC curve of 0.89 (95% CI: 0.83–0.94). The authors highlighted that mean relative expression of plasma miRNA 21 was significantly higher in patients with NSCLC compared to healthy individuals (2.32 ± 1.7 vs. 0.715 ± 0.48, $P = 0.0001$). In order to further improve the diagnostic accuracy, attempts have also been made to combine multiple miRNAs into a test panel to diagnose NSCLC. A study to assess the accuracy of a panel of 34 serum miRNAs to diagnose early stage NSCLC in asymptomatic high-risk individuals (heavy smokers, aged over 50) showed a high overall accuracy of 80%.[14] In addition, the authors suggested that these diagnostic miRNA signatures could distinguish benign lung nodules from invasive NSCLC. Another study[15] identified that a panel of four serum miRNA signatures (miRNA-21,-126,-210, and 486-5p) can reliability differentiate patients with NSCLC from the healthy controls with a sensitivity of 86.2% and specificity of 96.5%. Moreover, it could identify stage I NSCLC with a sensitivity of 73.3% and specificity of 96.5%.
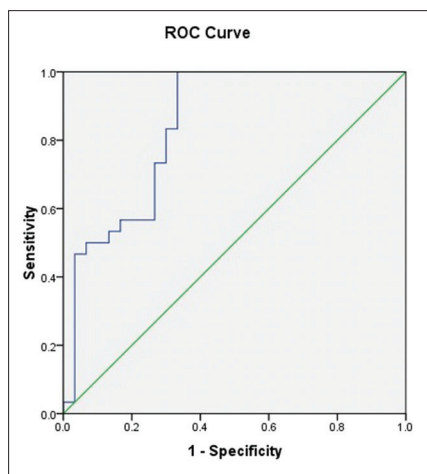
Table 1: Published studies highlighting the diagnostic role of miRNA-21 in nonsmall cell lung cancer either alone or in combination of other miRNAs

Authors	Year of publication	Sample size	Pathological type	Results
Abdollahi *et al.*[16]	2019	43	NSCLC	Sensitivity and specificity of miRNA 21-90% and 67%, respectively; Sensitivity and specificity of combined miRNA panel (21-5p, 638, 1481-3p, 152-3p) - 96.4% and 86.7%
Abu-Duhier *et al.*[13]	2018	80	NSCLC	Both sensitivity and specificity of miRNA 21-80% at the expression level of 1.207
Qiu *et al.*[17]	2018	58	Undifferentiated	Sensitivity and specificity of miRNA 21-86.2% and 76.2%, respectively, at the expression level of 3.89; Sensitivity and specificity of combined miRNA panel (21 and 19) - 86.6% and 97.6%
Sun *et al.*[18]	2018	28	Adenocarcinoma	Sensitivity and specificity of miRNA 21-82.1% and 96.4%, respectively; Sensitivity and specificity of combined miRNA panel (21 and 339-5p) - 92.9% and 92.9%
Arab *et al.*[19]	2017	72	NSCLC	Sensitivity and specificity of miRNA 21-61.1% and 80.3%, respectively; Sensitivity and specificity of combined miRNA panel (21, 141, 328, 375) to diagnose early NSCLC - 61.5% and 80.3%
Qi *et al.*[20]	2014	30	Stage I NSCLC	Increased expression levels of miRNA 21 in the stage I NSCLC patients compared to healthy volunteers (20.4 vs. 3.8, *P*=0.001)
Yang *et al.*[21]	2015	152	NSCLC	Sensitivity and specificity of miRNA 21-69% and 71%, respectively; Sensitivity and specificity of combined miRNA panel (148a, 148b, 152, 21) - 96% and 91%
Liu *et al.*[22]	2012	70	NSCLC	Increased (twofold change) expression level of serum miR-21 in 42 out of 70 (60.0%) patients compared with that in normal volunteers
Li *et al.*[12]	2011	20	NSCLC	Sensitivity and specificity of miRNA 21-78.8% and 100% respectively
Shen *et al.*[15]	2011	58	NSCLC	Sensitivity and specificity of combined miRNA panel (21, 126, 210, 486-rp) - 86.22% and 96.55%

NSCLC: Nonsmall cell lung cancer

Table 1 enlists the previously published studies highlighting the diagnostic role of miRNA-21 in NSCLC, either alone or in combination of other miRNAs.[12,13,15-22]

Though our study could not identify a difference in expression of miRNA 21 in different pathological types and clinical stages, we believe it might be due to a small sample size of our study. Shen *et al*.[15] highlighted that serum miRNA signatures can diagnose lung adenocarcinoma with a higher sensitivity compared to squamous cell carcinoma (91.6% vs. 82.3%, $P < 0.05$). Over expression of the miRNA 21 also causes progression of lung adenocarcinoma by suppressing apoptosis through Hippo signaling pathway.[23] Serum miRNA 21 has also been investigated for its role as prognostic/predictive maker in patients with NSCLC.

The present study was an exploratory study to assess the diagnostic role of miRNA 21 in Indian patients with NSCLC. As the results of the study are encouraging, we believe that further studies are warranted to assess the diagnostic accuracy of a panel of serum miRNA signatures in a large cohort of patients.

CONCLUSION

Estimation of serum miRNA 21 expression has the potential to be used as liquid biopsy for the diagnosis of NSCLC. Further studies with large sample sizes are warranted to confirm the diagnostic accuracy of serum miRNA signatures in patients with NSCLC.

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Conflicts of interest
There are no conflicts of interest.

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