# microRNAs in exhaled breath condensate for diagnosis of lung cancer in a resource-limited setting: a concise review

Divyanjali Rai[1], Bijay Pattnaik[1], Sunil Bangaru[1], Jaya Tak[1], Jyoti Kumari[1], Umashankar Verma[1], Rohit Vadala[1], Geetika Yadav[2], Rupinder Singh Dhaliwal[2], Sunil Kumar[3], Rakesh Kumar[4], Deepali Jain[5], Kalpana Luthra[6], Kunzang Chosdol[6], Jayanth Kumar Palanichamy[6], Maroof Ahmad Khan[7], Addagalla Surendranath[1], Saurabh Mittal[1], Pawan Tiwari[1], Vijay Hadda[1], Karan Madan[1], Anurag Agrawal[8], Randeep Guleria[1] and Anant Mohan[1]

[1]Breathomics in Respiratory Diseases Lab, Department of Pulmonary, Critical Care and Sleep Medicine, All India Institute of Medical Sciences, New Delhi, India. [2]Indian Council of Medical Research, New Delhi, India. [3]Department of Surgical Oncology, All India Institute of Medical Sciences, New Delhi, India. [4]Department of Nuclear Medicine, All India Institute of Medical Sciences, New Delhi, India. [5]Department of Pathology, All India Institute of Medical Sciences, New Delhi, India. [6]Department of Biochemistry, All India Institute of Medical Sciences, New Delhi, India. [7]Department of Biostatistics, All India Institute of Medical Sciences, New Delhi, India. [8]Trivedi School of Biosciences, Ashoka University, Sonipat, India.

Corresponding author: Anant Mohan (anantmohan@yahoo.com)

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**This review aims to provide readers with an understanding of the utility of EBC-derived miRNAs for the diagnosis of lung cancer** https://bit.ly/3Ti4Afv

**Cite this article as:** Rai D, Pattnaik B, Bangaru S, *et al*. microRNAs in exhaled breath condensate for diagnosis of lung cancer in a resource-limited setting: a concise review. *Breathe* 2023; 19: 230125 [DOI: 10.1183/20734735.0125-2023].



Received: 24 June 2023
Accepted: 30 Nov 2023

## Abstract

Lung cancer is one of the common cancers globally with high mortality and poor prognosis. Most cases of lung cancer are diagnosed at an advanced stage due to limited diagnostic resources. Screening modalities, such as sputum cytology and annual chest radiographs, have not proved sensitive enough to impact mortality. In recent years, annual low-dose computed tomography has emerged as a potential screening tool for early lung cancer detection, but it may not be a feasible option for developing countries. In this context, exhaled breath condensate (EBC) analysis has been evaluated recently as a noninvasive tool for lung cancer diagnosis. The breath biomarkers also have the advantage of differentiating various types and stages of lung cancer. Recent studies have focused more on microRNAs (miRNAs) as they play a key role in tumourigenesis by regulating the cell cycle, metastasis and angiogenesis. In this review, we have consolidated the current published literature suggesting the utility of miRNAs in EBC for the detection of lung cancer.

## Educational aims

- Provide an understanding of the utility of EBC-derived miRNAs for early detection of lung cancer.
- Describe various identified miRNAs in the EBC of different types of lung cancers.
- To understand miRNAs as the potential therapeutic targets for lung cancer.

## Introduction

Lung cancer is one of the commonest cancers worldwide and is a leading cause of cancer-related deaths. According to the Global Cancer Observatory (GLOBOCAN 2020), 2.2 million new cases of lung cancer were documented worldwide in 2020, with 1.79 million dying from the disease [1]. In 2020, India accounted for nearly 3.3% of new lung cancer cases and 3.7% of lung cancer-related deaths worldwide. In Indian women, the prevalence of lung cancer has risen from 7.9% in 2008 to 27.2% in 2018 [1, 2]. The mortality rate of lung cancer closely mirrors the incidence trend and eventually reflects a 95% case fatality rate [3]. Smoking continues to be the primary risk factor [4]. However, never-smokers account for up to 25% of all lung cancer worldwide and more than 50% of cases in Southeast Asian women [4–6].

The main risk factors in this group include indoor pollution from burning charcoal for heating and cooking, second-hand smoke, outdoor air pollution, exposure to environmental and occupational carcinogens, infectious factors (bacterial or viral), genetic polymorphisms and hereditary factors. All these factors lead to the accumulation of genetic mutations in cancer-critical genes, which control the normal growth, proliferation and repair mechanisms of DNA in a cell, thereby leading to uncontrolled growth and proliferation of cells forming a tumour [7–9].

Most patients with lung cancer are asymptomatic in the early stages. Even when symptoms do appear, they are nonspecific and resemble more prevalent benign aetiologies notably tuberculosis. This often causes a delay in the diagnosis of lung cancer, which in turn affects prognosis [10, 11]. Conventional diagnostic strategies include computed tomography, positron emission tomography, cytological evaluation of sputum, bronchial suctioning, and histopathological and cytopathological evaluation of biopsies taken during bronchoscopy or by the transthoracic route [2, 12, 13]. However, these tests are typically performed only after the onset of symptoms and have suboptimal diagnostic yield [14–16]. Pathological examination of tissue is currently the gold standard for diagnosing lung cancer, but it requires invasive procedures which may increase the risk of complications.

Despite the high prevalence of lung cancer in developing countries, the concept of early diagnosis is largely limited to developed countries. This could be due to lack of infrastructure, high costs, inability to screen high-risk populations, over-diagnosis, and the resultant psychological impact. In addition, tuberculosis and their sequelae may lead to high false-positive rates on low-dose computed tomography, as was observed in the National Lung Screening Trial. Consequently, developing an effective lung cancer screening programme for early diagnosis is challenging in countries with a high prevalence of tuberculosis [17, 18].

As a result, it is critical to search for noninvasive, easily available and sensitive diagnostic tools for early detection of lung cancer in high-risk populations in resource-limited settings. In this respect, analysis of exhaled breath condensate (EBC) has been a recent focus of study.

## Exhaled breath condensate

EBC analysis is a noninvasive, safe and simple methodology for diagnosis and assessing various aspects of lung diseases. Exhaled breath contains aerosols and vapours that can be obtained and tested for properties that are efficient in illustrating physiological and pathological processes inside the lung without involving invasive procedures [19]. Exhaled breath predominantly consists of water vapour and minor amounts of hundreds of exogenous and endogenous compounds. Some part of the exhaled breath is converted in to the aqueous phase and is referred to as EBC [20].

The fluid which lines the lower respiratory tract contains many non-volatile and volatile substances [21–24]. EBC contains many non-volatile macromolecules like proteins, lipids, oxidation products, nucleotides, ions, cytokines, surfactants, adenosine, histamine, acetylcholine, serotonin, *etc*. [25]. EBC also absorbs many water-soluble volatile compounds, including hydrogen peroxide, ammonia, nitrate, nitrite and hydrochloric acid, and some volatile organic compounds which are divided into different classes, like saturated hydrocarbons (aldehydes, ethane and pentane), unsaturated hydrocarbons (isoprene), oxygen-containing (acetone), nitrogen-containing (dimethylamine, ammonia) and sulfur-containing (ethyl mercaptane, dimethylsulfide) [26].

EBC collection has been performed satisfactorily even in children [19]. Recently, interest has been growing in non-volatile substances in assessing biomarkers of various pathological processes [23]. Through EBC, exogenous agents causing any type of lung injury can be identified and lung diseases can be predicted by analysing biomarkers such as growth factors, cytokines, thrombosis-regulating molecules, proteases, antiproteases, acute phase proteins, novel microRNAs (miRNAs) and progenitor cells linked with different types of biological processes of respiratory diseases. The intensity of these diseases and the response to therapy in these diseases can also be analysed through EBC [27, 28]. The collection of EBC by cooling exhaled air has several advantages compared with many other methods of sampling the airspaces. The main advantage of working on EBC is that it is an easily accessible and noninvasive specimen that is representative of the airway lining fluid. Hence, it could prove to be an ideal source for the discovery and validation of biomarkers of various pulmonary diseases [29, 30].

For EBC collection, several collecting systems are available, such as EcoScreen I/II and EcoScreen Turbo manufactured by Jaeger, Germany; RTube, RTube vent and ALFA manufactured by Respiratory Research, USA [31], Anacon (Biostec, Spain) and Turbo Deccs (Italcil, Italy) [32]. Of these, the RTube system is associated with a greater number of EBC collections and enables for concurrent multiple collections.

Furthermore, the RTube system has added advantages of easy disposal and portability and can easily be prepared for use in a regular freezer. The RTube consists of a T-shaped polypropylene condensation chamber with a non-rebreathing valve and a saliva separator to avoid saliva contamination of the sample and an aluminium condenser tube which is allowed to cool at −70°C before EBC collection [20]. At the time of EBC collection, exhaled breath is passed through a cooling system which results in the accumulation of exhaled breath constituents in the liquid phase [19, 33]. The collection time in most studies is 10–30 min; 10 min of tidal breathing through the system is sufficient to collect ∼1–2 mL of EBC [19, 29, 33, 34]. After collection, the EBC sample can be analysed immediately or can be stored at −70°C to −80°C for future analysis [20, 32].

The microenvironment of a tumour has an important role in cancer progression. The tumour microenvironment is defined as the interactions between tumour cells or cancer cells and the neighbouring non-cancerous cells [35, 36]. Cancer cells can interact with nearby cells and distant cells directly through membrane receptor–ligand interaction and indirectly by releasing cytokines, chemokines and metabolites into the circulatory system [37]. Extracellular vesicles (EVs) have attracted considerable interest in recent years as a new means of intercellular communication. EVs can facilitate molecular communication between cancer cells and stromal cells, modify the local tumour microenvironment, and promote cancer initiation, progression and distant metastasis [38, 39]. Several noncoding RNAs (ncRNA), such as miRNAs, circular RNAs and long noncoding RNAs, are thought to be important regulators of cancer development and progression. MicroRNAs were the first form of ncRNA to be discovered in EVs [40, 41]. These EV-enclosed miRNAs are released by cells into the circulation and are thought to be used for intracellular trafficking and regulating cancer-related molecular mechanisms [42, 43]. These EV-enclosed miRNAs are stable in any condition, can be collected into the EBC, and have emerged as possible biomarkers and an appropriate therapeutic target for cancer [44].

## MicroRNA

miRNAs are indigenous short ncRNAs with ∼20–24 nucleotides that detect target genes by complementary base pairing to mRNA 3′ untranslated regions (UTRs), altering their stability and lowering gene expression [45]. To date, ∼2000 miRNAs have been found in the human genome and this number is continually increasing [46]. miRNA expression alterations have been demonstrated to play an essential role in several forms of cancer. A single miRNA can have multiple target mRNAs, at the same time multiple miRNAs can bind to and regulate the same target. As a result, miRNAs play a role in a variety of biological processes, including gene regulation, haematopoietic development, cell differentiation, maintenance, cell proliferation and apoptosis. miRNAs are thought to regulate one-third of human genes and their dysregulation has been linked to cancer genesis and progression, implying that miRNAs may act as tumour suppressor miRNAs or oncogenic miRNAs in many forms of lung cancer depending on the target gene [45, 47, 48].

## MicroRNAs as a tumour suppressor

### *Let-7 miRNA family*

Initially discovered in *Caenorhabditis elegans*, this miRNA is known to regulate cell fate and was also the first discovered miRNA in humans. It has been confirmed that overexpressing Let-7 in a lung cancer cell line (A549) stopped the cell cycle and prevented A549 cell growth [49, 50]. Let-7 has been shown to inhibit the expression of oncogenes such as RAS (rat sarcoma virus), MYC (master regulator of cell cycle), and HMGA2 (high mobility group AT-hook 2), which are involved lung cancer cell proliferation. Let-7 also inhibits CDK6 (cyclin dependent kinase 6) expression and its reduced expression promotes cell cycle progression [51]. Let-7 suppresses DICER1 (endoribonuclease) expression directly, implying that Let-7 may regulate the overall production of other miRNAs [52]. The Let-7 family is frequently found to be deleted in lung cancer in humans and plays an important role in regulating cancer progression [53].

### *miR-200 family*

miR-200 family members are known to be involved in the epithelial-to-mesenchymal transition (EMT). EMT is defined by the loss of E-cadherin-mediated cell adhesion and an increase in cell motility, which facilitates tumour invasion and metastasis [54]. miR-200 targets the transcriptional repressors of E-cadherin, zinc finger E-box-binding homeobox (ZEB1) and ZEB2. Thus, increased E-cadherin expression and decreased motility of lung cancer cells result from miR-200 upregulation [55].

### *miR-34 family*

Whenever there is DNA damage, the TP53 (tumour protein P53) gene gets activated and induces the miR-34 family which controls cell cycle arrest and apoptosis in cancer cells [56]. In lung cancer, the miR-34 family is downregulated, which causes upregulation of miR-34 target genes such as *MET*, Bcl2

(B-cell lymphoma 2) and PDGFR (platelet-derived growth factor receptor) [57–59]. Reduced miR-34 expression promotes cell proliferation by upregulating *MET* and Bcl2. Downregulation of PDGFR by miR-34 inhibits tumourigenesis while increasing TRAIL (tumour necrosis factor-related apoptosis-inducing ligand)-induced apoptosis in lung cancer [59].

miR-206 is highly downregulated in clinical specimens of squamous cell lung carcinoma. It seems to serve as a tumour suppressor *via* regulating oncogenic pathways of *MET* and EGFR (epidermal growth factor receptor) by downregulating their signalling. Along with miR-206, miR-133b expression is also lower in cancer cells and is considered tumour suppressive [60, 61].

## Oncogenic miRNAs

### *miR-21*

miR-21, a common oncogenic miRNA, is upregulated in a variety of cancers. miR-21 promotes tumourigenesis and inhibits apoptosis by indirectly stimulating RAS/*MEK*/ERK oncogenic pathway elements. Increased expression of miR-21 inhibits the expression of PTEN (phosphatase and tensin homolog), PDCD4 (programmed cell death protein 4) and TPM1 (tropomyosin alpha-1), resulting in cell multiplication and migration while preventing cell apoptosis [62, 63]. In a recent study, it has been reported that by suppressing SMAD7 (mothers against decapentaplegic homolog 7) expression in lung cancer cells, miRNA-21-5p may promote cell proliferation, migration and invasion [63]. Another *in vitro* study has observed that miR-21 upregulation in lung fibroblasts may cause fibroblasts to transdifferentiate into CAFs (cancer-associated fibroblasts), thereby promoting cancer progression [64].

### *miR-17-92 cluster*

The miR-17-92 polycistronic cluster consists of seven different miRNAs (miR-17-3p, miR-17-5p, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a) and is located at 13q31.3 in intron 3 of the C13orf25 gene [65]. The miR-17-92 cluster is overexpressed in lung cancer, specifically small cell lung cancer [66]. Overexpression of the miR-17-92 cluster suppresses the expression of E2F1, HIF1A (hypoxia inducible factor 1α) and PTEN, thereby promoting cell proliferation and cancer progression [67, 68].

### *miR-221/222*

miR-221 and miR-222 promote the development and progression of lung cancer by repressing the PTEN and TIMP3 (tissue inhibitor metalloproteinase 3) genes [69]. Overexpression of miR-221 or miR-222 can promote cell proliferation and migration by suppressing target genes (PTEN and TIMP3). miR-221 and miR-222 have also been shown to be modulated by epithelial growth factor (EGF) and protooncogene *MET* receptors in tyrosine kinase inhibitors-resistant nonsmall cell lung cancers (NSCLCs).

### *miR-31*

miR-31 is another oncogenic miRNA. It inhibits lung cancer cell growth and tumourigenicity *via* direct repression of the expression of LATS2 (large tumour suppressor kinase 2) and PPP2R2A (Protein Phosphatase 2 Regulatory Subunit B α) tumour suppressors, implying a new regulatory network in lung cancer *via* the miR-31-LATS2-PPP2R2A pathway [70]. According to a recent study, miR-31 is overexpressed in human lung adenocarcinoma, and this overexpression frequently correlates with decreased survival. They discovered that inducing miR-31 caused lung hyperplasia and adenoma and leads to a progression of lung adenocarcinoma. Furthermore, the researchers discovered that miR-31 suppresses the expression of six RAS/MAPK pathway regulators, promoting lung tumourigenesis [71].

## Role of miRNAs identified in EBC samples as diagnostic biomarkers in lung cancer

Accumulating evidence suggests that miRNAs in EBC samples could be used as potential diagnostic biomarkers for lung cancer. In one of the earliest reports on the role of miRNAs in lung cancer, Mozzoni *et al.* [72] included 46 control subjects and 54 confirmed NSCLC patients. The NSCLC group comprised 37 adenocarcinoma and 17 squamous cell carcinoma cases, with the majority being early-stage disease. For miRNA analysis, the authors selected miR-21 and miR-486, as both the miRNAs have opposite functional roles (miR-21 as oncogenic and miR-486 as anti-oncogenic). The expression of miR-21 was found to be significantly upregulated and miR-486 levels were downregulated in NSCLC patients. Further, the authors cross-checked their expression in plasma and tissue samples. The details of the studies which have looked at the discrimination of lung cancer by miRNA in EBC are summarised in table 1.

Similarly, in another prospective case–control study, EBC samples were collected from 15 pathologically confirmed, chemotherapy/radiotherapy-naïve lung cancer patients as well as from 15 patients at high risk for lung cancer development using a commercially available condenser EcoScreen (Jaeger, Hochberg, Germany). Quantitative real-time PCR was used to assess the expression of miRNA-155 (RQ or Relative

https://doi.org/10.1183/20734735.0125-2023

**TABLE 1** Discrimination of lung cancer (LC) by microRNA (miRNA) in exhaled breath condensate (EBC)

| Reference | Group | Method | MiRNAs | Result | Interpretation |
|---|---|---|---|---|---|
| Mozzoni *et al.* [72] | NSCLC n=54<br>Control n=46 (26 nodules, seven bronchiectasis, 13 others: emphysema (n=2), inflammatory outcomes (n=2), aspiration pneumonia (n=1), tuberculosis (n=1), rhino-bronchial syndrome (n=1), asthma with allergic rhinitis (n=1), unspecified radiological alteration (n=5)) | qRT-PCR assay | miR-21<br>miR-486 | Upregulated<br>Downregulated | miR-21 was significantly upregulated and miR-486 was downregulated in LC as compared with control<br>Hence, miR-21 was reported as oncogenic and miR-486 as tumour suppressor miRNA |
| Chen *et al.* [80] | NSCLC n=30<br>Control n=30 | qRT-PCR assay | miR-21 | Upregulated | miR-21 was significantly upregulated in NSCLC as compared with control |
| Ibrahim *et al.* [73] | LC n=15<br>Control n=15 | qRT-PCR assay | miR-155 | Upregulated | miR-155 was found to be upregulated in the EBC of LC patients as compared with the controls<br>Reported oncogene and a potential biomarker for early lung detection as well as for prognosis |
| Chen *et al.* [74] | NSCLC n=30<br>Healthy control n=30 | qRT-PCR assay | Let-7 | Downregulated | Levels of Let-7 were downregulated in NSCLC patients relative to the healthy controls<br>Reported as tumour suppressor miRNA and low levels in EBC may serve as a biomarker for the diagnosis and evaluation of NSCLC |
| Xie *et al.* [75] | NSCLC n=62<br>Healthy control n=60 | qRT-PCR assay | miR-186 | Downregulated | Levels of miR-186 were decreased in the EBC of NSCLC patients as compared with healthy controls and can be a potential diagnostic biomarker for NSCLC |
| Pérez-Sánchez *et al.* [76] | LC n=21<br>Healthy donor n=21 | Genome-wide microarray | miR-6865-5p, miR-4707-5p, miR-451a, miR-1469, miR-4507, miR-6780a-5p, miR-668-5p, miR-6794-5p and miR-7855-5p<br>miR-3921, miR-320a and miR-6777-5p | Upregulated<br>Downregulated | Nine microRNAs (miR-6865-5p, miR-4707-5p, miR-451a, miR-1469, miR-4507, miR-6780a-5p, miR-668-5p, miR-6794-5p and miR-7855-5p) were upregulated in EBC of LC patients while three microRNAs (miR-3921, miR-320a and miR-6777-5p) were downregulated compared with the healthy controls |
| Faversani *et al.* [77] | Adenocarcinoma n=14<br>Healthy controls n=9 | qRT-PCR array | miR-597-5p, miR-1260a | Upregulated | miR-597-5p and miR-1260a were upregulated in the EBC of lung adenocarcinoma patients compared with healthy controls |
| Rai *et al.* [78] | LC n=30<br>Healthy controls n=30 | qRT-PCR array | miR-31-3p, Let7i and miR-449c | Upregulated | Expression of miR-31-3p, Let7i and miR-449c were found to be upregulated in EBC of LC patients compared with healthy controls |

qRT-PCR: quantitative, real-time PCR; NSCLC: nonsmall cell lung cancer.

Quantification value) in EBC. The authors found a statistically significant difference with higher EBC miRNA-155 expression in the lung cancer group compared with the control group. This conclusion is consistent with the findings of other research that found miRNA-155 to be one of the miRNAs overexpressed in lung cancer and that it can be used not only as a diagnostic biomarker but also as a prognosis biomarker [73].

In another study by Chen *et al.* [74], the correlation between EBC Let-7 miRNA, NSCLC diagnosis and clinicopathological features was explored. Let-7 expression levels were determined in 180 samples using reverse transcription-quantitative PCR, which included 30 NSCLC patients (lung cancer and para-carcinoma tissues, serum and EBC) and 30 healthy controls (serum and EBC). Let-7 levels in tumour tissues, serum and EBC in NSCLC were significantly lower than in the control group. Let-7 expression in lung cancer tissue, serum, and EBC in NSCLC decreased as the disease progressed (tumour-node-metastasis stage and lymph node metastasis).

A similar study has been performed in Chinese subjects in which the researchers tried to identify the clinical value of miR-186 and interleukin-1β in NSCLC [75]. Blood and EBC were collected from 62 pathologically confirmed treatment naïve NSCLC enrolled patients and 60 age- and sex-matched healthy controls. In serum and EBC, the relative expression of miR-186 was significantly lower in NSCLC patients than in controls ($p<0.05$). Furthermore, patients with stage I–II NSCLC had a lower miR-186 expression when compared with the stage III and IV group ($p<0.05$). The details of the studies which looked at the discrimination of different stages of lung cancer using miRNA in EBC have been summarised in table 2. miR-186 was expressed at a lower level in EBC of adenocarcinoma than in squamous cell carcinoma ($p<0.05$).

Another cross-sectional study of 42 subjects (21 lung cancer and 21 healthy controls) was conducted by Pérez-Sánchez *et al.* [76]. They used GeneChipR miRNA 4.0 array for genome-wide miRNA expression profiling and identified nine upregulated miRNAs (miR-6865-5p, miR-4707-5p, miR-451a, miR-1469, miR-4507, miR-6780a-5p, miR-668-5p, miR-6794-5p and miR-7855-5p) and three downregulated miRNAs (miR-3921, miR-320a and miR-6777-5p) in EBC of lung cancer patients. With the largest area under the curve (AUC of 0.70) in a receiver operating characteristic (ROC) plot, miR-4507 demonstrated the best specificity and sensitivity at the individual level, followed by miR-6777-5p and miR-451a (AUC of 0.66 and 0.63, respectively). After leave-one-out cross validation and ROC curve analyses they found that three microRNAs (miR-4529-3p, miR-8075 and miR-7704) were able to differentiate between adenocarcinoma and squamous cell carcinoma with an AUC of 0.98, 100% specificity and 88% sensitivity [76]. The other three sets of microRNAs (miR-602, miR-551b-5p and miR-1272) were downregulated in stage IV patients and discriminated them from less advanced stages (stage I, II and III) with AUC of 0.88, specificity of 89% and sensitivity of 92%. The miR-6803-5p, miR-548x-3p and miR-1272 distinguished the invasive tumour from the noninvasive one with 100% specificity and sensitivity. The details of the studies which looked at the discrimination of different types of lung cancer by miRNA in EBC have been summarised in table 3.

In another study specifically aimed at adenocarcinoma, miRNA profiling of 754 unique miRNAs was done in EBC collected from 14 early stage (I–II) lung adenocarcinoma patients and nine healthy subjects. Of all the miRNAs tested, 11 were found to be associated with adenocarcinoma and were further validated in plasma and tissue samples *via* quantitative PCR. When compared with another cohort of pleural mesothelioma and asbestos-exposed non-mesothelioma subjects, it was found that miR-597-5p and miR-1260a were significantly upregulated in the EBC of adenocarcinoma patients. The levels of

**TABLE 2** Discrimination of lung cancer (LC) stages based on microRNA (miRNA) in exhaled breath condensate (EBC)

| Reference | LC stages | Samples, n | MiRNAs | Result | Interpretation |
|---|---|---|---|---|---|
| Xie *et al.* [75] | I–II | 23 | miR-186 | Downregulated | miR-186 was downregulated in the early stages compared with later stages |
| | II–IV | 29 | miR-186 | | |
| Pérez-Sánchez *et al.* [76] | I–III | 10 | miR-548ae, miR-548ac, miR-1272, miR-4529-3p, miR-3124-5p, miR-602, miR-4787-5p and miR-551b-5p | Upregulated | Eight miRNAs (miR-548ae, miR-548ac, miR-1272, miR-4529-3p, miR-3124-5p, miR-602, miR-4787-5p and miR-551b-5p) were upregulated in early stages (I–III) while downregulated in stage IV |
| | IV | 11 | miR-548ae, miR-548ac, miR-1272, miR-4529-3p, miR-3124-5p, miR-602, miR-4787-5p and miR-551b-5p | Downregulated | |

**TABLE 3** Discrimination of lung cancer (LC) types based on microRNA (miRNA) in exhaled breath condensate (EBC)

| Reference | LC types | Samples, n | miRNAs | Result | Interpretation |
|---|---|---|---|---|---|
| Pérez-Sánchez *et al.* [76] | Adenocarcinoma | 11 | miR-4529-3p, miR-8075 and miR-7704 | Upregulated | Levels of 3 microRNAs (miR-4529-3p, miR-8075 and miR-7704) were highest in EBC of adenocarcinoma patients and lower in SCC patients |
| | SCC | 10 | miR-4529-3p, miR-8075 and miR-7704 | Downregulated | |

SCC: squamous cell carcinoma.

miR-1260a and miR-518f-3p in the plasma of adenocarcinoma patients were high, with the low levels of Let-7f-5p in comparison with healthy subjects. These circulating microRNAs were analysed in pleural mesothelioma and are predominantly expressed in lung adenocarcinoma tissue only [77].

Recently, our group published the results of differential expression of miRNAs in EBC of 30 lung cancer patients [78]. We identified 78 miRNAs which were differentially expressed in lung cancer patients compared with healthy controls. Based on the fold change and literature review, six miRNAs were shortlisted for further validation in 10 EBC samples from each group. In the validation set, three miRNAs including Let-7i, miR-449c, and miR-31-3p were found to be significantly upregulated in the EBC of lung cancer compared with healthy controls. The AUC of Let-7i expression was 0.955, with a sensitivity and specificity of 75% and 95%, respectively; corresponding values for miR-449c were 0.980, 90% and 100%, and for miR-31–3p expression 0.865, 70% and 95%, respectively. Interestingly, all three miRNAs are known to have oncogenic properties. Hence, the identification of these three miRNAs in EBC of lung cancer patients could be used as a potential biomarker for lung cancer diagnosis.

## Advantages of using EBC over peripheral blood samples for analysis of miRNA expression

EBC contains viable material from the lungs and lower respiratory tract and is thus directly representative of the local respiratory milieu. It has been demonstrated that miRNAs are enclosed in exosomes and can be trapped by EBC [79]. However, peripheral blood samples give systemic levels of miRNAs. The expression of miRNAs in peripheral blood (including in serum and plasma) correlates with that in EBC. Mozzoni *et al*. [72] quantified the expression of miR-21 and miR-486 in plasma and EBC obtained from patients with NSCLC and healthy controls and found that miR-21 was elevated and miR-486 was suppressed in both plasma and EBC of NSCLC subjects. Subsequently, Chen *et al.* [80] reported elevated levels of miR-21 in both EBC and serum samples of NSCLC patients compared with healthy controls. Both the samples showed positive correlation with good sensitivity and specificity. In 2020, Xie *et al.* [75] and Chen *et al.* [74] estimated and compared the levels of miR-186 and Let-7 miRNAs, respectively, in both serum and EBC of NSCLC patients and found that both miRNAs were markedly decreased compared with healthy subjects, with a good correlation between levels obtained in plasma and in EBC. Recently, miR-1260a was shown to be elevated in both plasma and EBC of adenocarcinoma patients compared with healthy controls [77]. However, miRNAs miR-597-5p and miR-518f-3p were found to be elevated in EBC and plasma, respectively. Interestingly, Let-7f-5p was suppressed in plasma of adenocarcinoma patients in comparison with healthy controls. These results indicate the correlation of miRNAs expression in EBC and peripheral blood. To focus on miRNA signatures in lungs, EBC could be a reliable specimen to identify various miRNA signatures in various clinical settings.

Expression of miRNA can be measured by real-time quantitative PCR, microarray hybridisation (microarray) and next-generation sequencing (NGS) [81–83]. The high-throughput NGS and microarray technologies are used to explore miRNAs expression with higher sensitivity in various lung diseases. This is cost-effective, allows a much larger data coverage and can detect novel miRNAs regulating disease pathogenesis. However, real-time quantitative PCR is used to examine known miRNAs present in a clinical specimen. This is also a cost-effective method and can detect specific miRNAs differentially expressed in the specimen.

## Limitations of EBC

A major limitation of using EBC to identify and study miRNAs is its variable replicability. Various studies involving lung cancers of similar stage and type have produced different miRNA signatures. In addition, since EBC comprises >99.9% water vapour and also contains nebulised fluid droplets from the alveoli,

bronchi and mouth, it is prone to contamination by fractions derived from non-affected or healthy areas of the lung and airways [84]. In addition, sometimes EBC-derived miRNA levels can be close to or below the detection limit of the appropriate technique. Thus, the expression of miRNAs with sufficient sensitivity and specificity are needed to effectively measure biomarkers in EBC. Currently, there is no standardised method to assess EBC dilution. Besides this, miRNA concentrations in EBC may be affected by several other confounding factors [85–87]. Due to these drawbacks, the methodologies and interpretation of EBC studies are still evolving.

## Conclusion

There is a need to develop a point-of-care technology for cost-effective and early diagnosis of lung cancer. The biogenesis of miRNAs has been studied; however, there are numerous undiscovered miRNAs whose functions may be crucial in lung cancer pathogenesis. The expression of some miRNAs is significantly abnormal in tumour cells compared with normal cells, indicating miRNAs may have biological roles in lung cancer pathogenesis. Many clinical results have demonstrated that miRNAs function as potential biomarkers for diagnosis, prognosis and therapy of lung cancer. Nevertheless, a multicentric large cohort study is needed to establish the clinical use of EBC-derived miRNAs across the globe.

### Key points

- Assessment of miRNAs in EBC is a potential biomarker for early diagnosis of lung cancer.
- A multidimensional evaluation of various noninvasive diagnostic modalities including clinical and radiological approaches combined with EBC-derived miRNAs could be a robust composite tool for early lung cancer detection.
- Further focused research is required to identify lung cancer-specific miRNAs and their role in lung cancer pathophysiology.

### Self-evaluation questions

1. What are the EBC-derived miRNAs which could be used as potential diagnostic biomarkers for lung cancer detection in different populations?
2. What are their molecular mechanisms for the progression of lung cancer?
3. Can the EBC-derived miRNAs be used to identify lung cancer types without using any invasive procedure?
4. Can we predict the response to treatment modalities in lung cancer patients using EBC-derived miRNAs at different stages of the disease?

Conflict of interest: The authors have no conflicts of interest or disclosures.

Support statement: This work was supported by Indian Council of Medical Research (ICMR/I-1061), India vide letter no 55/4/7/CARE-EBC/2018-NCD-II.

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## Suggested answers

1. Based on the existing data, it can be suggested that integrating the identification of miRNAs such as miR-21, Let-7 and miR-31-3p as biomarkers, in conjunction with established screening techniques, holds promise for detecting lung cancer across different populations.
2. Oncogenic miRNAs promote tumourigenesis by directly inhibiting tumour suppressing factors or prevent them exerting their anti-tumour effect by preventing proliferation, promoting apoptosis, inhibiting angiogenesis and reducing immune surveillance.
3. Yes, EBC-derived miRNAs could be used to discriminate different types of lung cancer. However, further research is needed.
4. Yes, response to treatment modalities could be predicted using EBC. However, further research is needed.