# Non-small Cell Lung Cancer Detection Using MicroRNA Expression Profiling of Bronchoalveolar Lavage Fluid and Sputum

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**Abstract**. *Aim: To assess if miRNA expression profiling of bronchoalveolar lavage (BAL) fluid and sputum could be used to detect early-stage non-small cell lung cancer (NSCLC). Materials and Methods: Hierarchical cluster analysis was performed on the expression levels of 5 miRNAs (miR-21, miR-143, miR-155, miR-210, and miR-372) which were quantified using RNA reverse transcription and quantitative real-time polymerase chain reaction in sputum and BAL samples from NSCLC cases and cancer-free controls. Results: Cluster analysis of the miRNA expression levels in BAL samples from 21 NSCLC cases and sputum samples from 10 cancer-free controls yielded a diagnostic sensitivity of 85.7% and specificity of 100%. Cluster analysis of sputum samples from the same patients yielded a diagnostic sensitivity of 67.8% and specificity of 90%. Conclusion: miRNA expression profiling of sputum and BAL fluids represent a potential means to detect early-stage NSCLC.*

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related mortality worldwide, largely because most patients (70%) are diagnosed with disease either at locally advanced or metastatic stages which are associated with poor survival (1). Detection of NSCLC at the early stage is, therefore, preferable. Bronchoscopy is a standard component of the diagnostic work-up of patients with suspected NSCLC (2). False-negative bronchoscopic examinations are a commonly experienced clinical conundrum which has led to a strategy of using multiple simultaneous tests from the same bronchoscopic procedure [*e.g.* bronchial brushing, bronchoalveolar lavage (BAL), endobronchial ultrasound-guided biopsy] in order to boost the probability of obtaining a diagnosis. Despite this, the diagnostic accuracy of bronchoscopic examinations is sub-optimal (3), with sensitivities ranging from 30-69% (4-6) depending on the size of the primary tumor and the number of parallel tests performed per bronchoscopy. False-negative bronchoscopies often result in repeated bronchoscopic exams or image-guided biopsies, which can lead to diagnostic delays and expose patients to complications, including pneumothorax and pulmonary hemorrhage (7).

Similarly, the diagnosis of NSCLC using standard cytological analysis of sputum can be a tedious endeavor, prone to unsatisfactory sample collection and poor sensitivity [as low as 8% for a single spontaneously expectorated sputum sample (8)], which necessitate either repeated sputum collection or escalation to more costly and invasive testing methods in order to achieve a diagnosis.

MicroRNAs (miRNAs) are a group of small, non protein-coding RNA molecules that have myriads of roles in the regulation of cellular processes (9) and that have been observed to be over- or underexpressed in malignant and non-malignant diseases (10). MiRNAs are attractive as potential biomarkers as they are expressed in a tissue-specific manner and have been shown to be present in the cellular milieu and bodily fluids (11), thus diagnostic tests using miRNAs do not require the discrete presence of malignant cells (12). Furthermore, miRNAs obtained from the human respiratory tract have been shown to be surprisingly robust against degradation by RNAse enzymes for relatively long

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Table I. *Baseline clinical and pathological characteristics of study participants.*

| Characteristic | NSCLC cases (n=21) | Controls (n=10) |
|---|---|---|
| Median age (range), years | 70 (51-82) | 59 (30-77) |
| Gender, M:F | 81%:19% | 80%:20% |
| Histology, n (%) | | |
| Adenocarcinoma | 13 (62%) | |
| Squamous cell carcinoma | 5 (24%) | N/A |
| Large cell carcinoma | 3 (14%) | |
| Stage (AJCC 7th ed. (24)) | | |
| I | 12 (57%) | N/A |
| II | 9 (43%) | |
| Smoking status | | |
| Current smoker | 13 (62%) | 4 (40%) |
| Previous smoker | 7 (33%) | 3 (30%) |
| Never smoker | 1 (5%) | 3 (30%) |
| Smoking history, median pack-years (range) | 44 (0-108) | 40 (0-77) |
| Median primary tumour diameter (range), cm | 1.6 (0.6-4.5) | N/A |

NSCLC: Non-small cell lung cancer; N/A: not applicable.

Table II: *2×2 tables of cluster analysis test result and pathological diagnosis using sputum and BAL samples from cases and sputum from controls*

| Sputum samples | | | |
|---|---|---|---|
| | Pathological diagnosis | | |
| Cluster analysis test result | NSCLC Case (n=21) | Control (n=10) | |
| Positive | 14 | 1 | *PPV=93.3% |
| Negative | 7 | 9 | *NPV=56.3% |
| | Sensitivity= 67.8% | Specificity= 90% | |
| **BAL samples** | | | |
| | Pathological diagnosis | | |
| Cluster analysis test result | NSCLC Case (n=21) | Control (n=10) | |
| Positive | 18 | 0 | PPV=100% |
| Negative | 3 | 10 | NPV=76.9% |
| | Sensitivity= 85.7% | Specificity= 100% | |

*PPV=Positive predictive value; NPV=negative predictive value

periods in both fresh (13) and preserved (14) samples, thus allowing for ease of sample handing and processing.

There has been considerable interest in the use of miRNA-expression profiling of tumor tissue samples (15, 16), serum (17-19), and sputum (13, 20-23) from patients with NSCLC. We present the findings of miRNA-expression profiling of a panel of five miRNAs, which have all been implicated in NSCLC tumorigenesis and growth, namely *miR-21*, *miR-143*, *miR-155*, *miR-210* and *miR-372*, by cluster analysis of BAL fluids and sputum from patients with early-stage NSCLC as a potential means for the detection of early-stage NSCLC.

## Materials and Methods

*Patient selection.* Eligible participants were adults with Zubrod performance status ≤2, with a life expectancy of ≥3 months, who were able to provide a sputum sample. Participants were categorized as NSCLC cases if they had a biopsy confirmed diagnosis of stage I or II NSCLC by the seventh edition of the American Joint Committee on Cancer (24) of any subtype. All NSCLC cases underwent staging investigations as indicated and received standard treatment for their NSCLC in accordance with local guidelines (25, 26). NSCLC cases were excluded if they had any prior or current history of malignancy other than non-melanomatous skin cancer. Participants were categorized as controls if clinical examination and diagnostic imaging [chest X-ray or computed tomography (CT) of the chest] within 12 months prior to study entry were negative for malignancy. Two types of controls were selected for inclusion: i) healthy patients without any active medical conditions, and ii) patients with chronic obstructive pulmonary disease (COPD) and smoking history at significant risk of developing lung cancer.

*Sample collection. Sputum:* Each participant was given an instructional session explaining how to provide a spontaneously expectorated sputum sample. Prior to sputum collection, patients rinsed their mouths thoroughly with water, took a deep inspiration, held their breath, and then coughed. All expectorated sputum was collected into a sterile sample container and was immediately refrigerated at 4˚C. Upon delivery to the laboratory, sputum samples were visually inspected in order to ensure proper sputum sample volume (≥1.0 ml) and consistency. Samples deemed to contain only saliva were discarded and repeat sputum collection was performed.

*BAL:* For NSCLC cases, BAL samples were obtained on the same day as the sputum sample on the day of their surgery for their NSCLC. Sputum samples were obtained first in the preoperative waiting room, 30 to 60 minutes prior to the beginning of the surgical procedure. In the operating room, prior to lung resection, normal saline BAL samples were obtained by flexible bronchoscopy from the lobe in which the tumour was located (based on preoperative imaging). BAL fluids (approximately 40 $cm^3$ per patient) were directly deposited into sterile bottles and were immediately refrigerated at 4˚C.

*Sample preparation.* All sputum and BAL sample containers were labeled with coded identifiers that blinded laboratory personnel of the identity and disease status of the samples. A complete description of our sample handling and analytical methodology used to quantify individual miRNA levels and perform cluster analysis has been previously described in detail (20). In summary, samples were homogenized using a sputolysin solution (Sigma Aldrich, St. Louis, MO, USA) followed by high speed vortexing and incubation

Table III: *Cosine similarity comparison of sputum and BAL samples from the same patient by test result concordance.*

| Patient ID | Sputum result | BAL result | Cosine similarity of matched pairs | Primary tumor location* | Primary tumor diameter (cm) |
|---|---|---|---|---|---|
| | | | Concordant sputum & BAL test results | | |
| MIR089 | Positive | Positive | 1.000 | RUL | 2.1 |
| MIR008 | Positive | Positive | 0.999 | RUL | 1.2 |
| MIR044 | Positive | Positive | 0.997 | RUL | 2.5 |
| MIR016 | Positive | Positive | 0.995 | RLL | 3.5 |
| MIR022 | Positive | Positive | 0.973 | RUL | 1.4 |
| MIR023 | Positive | Positive | 0.919 | LUL | 3.0 |
| MIR013 | Positive | Positive | 0.795 | RUL | 1.3 |
| MIR050 | Positive | Positive | 0.850 | RUL | 4.0 |
| MIR035 | Positive | Positive | 0.848 | RUL | 0.9 |
| MIR072 | Positive | Positive | 0.773 | RLL | 1.0 |
| MIR075 | Positive | Positive | 0.638 | RUL | 2.7 |
| MIR024 | Positive | Positive | 0.616 | LUL | 1.0 |
| MIR003 | False-negative | False-negative | 1.00 | RUL | 4.0 |
| | | | Mean=0.877 | Proportion RUL=69% | Mean=2.2 |
| | | | Discordant sputum & BAL test results | | |
| MIR040 | False-negative | Positive | 0.536 | RUL | 1.4 |
| MIR233 | False-negative | Positive | 0.325 | LUL | 4.5 |
| MIR031 | False-negative | Positive | 0.268 | RLL | 1.5 |
| MIR051 | Positive | False-negative | 0.183 | LUL | 1.8 |
| MIR006 | Positive | False-negative | 0.182 | RML | 1.6 |
| MIR079 | False-negative | Positive | 0.067 | RLL | 0.9 |
| MIR232 | False-negative | Positive | 0.053 | LUL | 4.5 |
| MIR230 | False-negative | Positive | 0.045 | RUL | 1.5 |
| | | | Mean=0.207 | Proportion RUL=25% | Mean=2.2 |

*RUL=Right upper lobe; RLL=right lower lobe; LUL=left upper lobe; RML=right middle lobe.

at 37˚C. RNA was isolated using a TRIzol-based method, and was then quantified using a UV-spectrometer.

*miRNA panel selection*. The selection of the five miRNAs used for expression profiling (*miR-21*, *miR-143*, *miR-155*, *miR-210*, and *miR-372*) was based on an iterative process and prior studies at our institution (20) whereby a panel of 12 miRNAs was evaluated using cluster analysis of a retrospective training set of sputum from NSCLC cases and controls. This panel of 12 miRNAs was then validated and optimized for maximal sensitivity and specificity using a reserve stepwise selection process using receiver-operator curves. The diagnostic characteristics of the miRNA panel were optimal when five miRNAs (*miR-21*, *miR-143*, *miR-155*, *miR-210*, and *miR-372*) were used in this prior study and were thus selected for use in this study.

Each of these five miRNAs have been implicated in various aspects of the tumourigenesis of NSCLC. *miR-21* overexpression in human lung cancer has been shown to inhibit the negative regulators of the rat sarcoma (RAS)/mitogen-activated protein kinase kinase (MEK)/extracellular Signal-regulated Kinases (ERK) pathway, apoptosis (27), and NSCLC growth and metastasis through modulation of the phosphatase and tensin homolog pathway (28). *miR-143* down-regulation in lung cancer has been found to cause to dysregulation of apoptotic pathways (29). *miR-155* has been found to be up-regulated in lung cancer, leading to tumourigenic changes such as promotion of cell survival, proliferation, and replicative immortality (30). *miR-210* modulates hypoxia-inducible factor 1 (HIF-1) activity (31) and promotes a hypoxic phenotype in lung cancer (32). *miR-372* has been found to down-regulate the large tumour suppressor, homolog 2 (LATS2) gene in a post-transcriptional manner (33).

*Sample analysis*. RNA reverse transcription was performed for each miRNA using the TaqMan Reverse Transcription Kit for individual miRNAs (Applied Biosystems, Carlsbad, CA, USA). Quantitative real-time Polymerase Chain Reaction (RT-qPCR) assays (Applied Biosystems) for each miRNA were performed in duplicate using the RT reaction derived from a single sputum sample for each patient using the StepOnePlus™ RT-PCR instrument (Applied Biosystems). Our previous experience using this experimental methodology demonstrated a high degree of analytic reproducibility (20), with an observed standard error range of 0.25 to 0.5 for threshold cycle (CT) (which was defined as the fractional cycle number at which the fluorescence passed the fixed threshold). SDS software (Applied

Biosystems) was used to automatically identify $C_T$ values. The comparative method ($\Delta\Delta C_T$ method) was used to quantify RT-qPCR data for miRNA expression whereby the fold-change in miRNA expression was normalized to that of the endogenous control (U6) and relative to the MRC-5 reference sample. The relative miRNA expression from a sample was expressed as follows: $\Delta\Delta RN=2^{-\Delta\Delta CT}$ where RN is the amount of miRNA required to be tested and $\Delta\Delta CT=(C_{Tm}-C_{Tec})_{sample}-(C_{Tm}-C_{Tec})_{reference}$, where $C_{Tm}$ is the $C_T$ for the measured miRNA, CTec is the CT for the endogenous control miRNA (*U6*) for samples to be tested (BAL fluid or sputum) and the reference sample is the MRC-5 normal lung fibroblast cell line.

*Data collection*. A medical history was obtained as per institutional standard of practice. Epidemiological data were also collected using a self-reported questionnaire which included information such as demographics, functional status, and social/occupational history. Relevant clinical data were obtained for each participant using electronic medical records including: diagnostic imaging scans, pathology reports, pulmonary function data, and previous medical and surgical history.

*Statistical considerations*. Three comparisons were carried-out for this study. Firstly, we assessed the ability of the panel of the five miRNAs to differentiate NSCLC cases, using their BAL samples, from controls, using their sputum samples. Secondly, we assessed the ability of the panel to differentiate cases from controls using sputum samples alone. These two comparisons were performed using the unsupervised hierarchical cluster analysis function of SPSS version 14 (IBM Corp., Armonk, NY, USA) of experimentally normalized miRNA expression profiles using within-group linkage and cosine correlation similarity.

A third analysis was performed in order to assess differences between matched pairs of BAL and sputum samples obtained from the same patient using a cosine similarity test of the $\Delta\Delta Rn^{-1}$ values for each miRNA tested. For cosine similarity, values close to 1 indicate a high degree of similarity, whereas those close to zero indicate a lack of similarity between vectors of an inner product space.

*Ethical considerations*. This study was approved by the Human Research Ethics Board of the University of Alberta (Edmonton, Canada) and the Alberta Cancer Research Ethics Committee (Alberta Health Services, Edmonton, Canada) (study approval number Pro00017473). Study participants provided their written informed consent prior to study entry.

## Results

Twenty-seven NSCLC cases and 11 control participants consented to participate. Five NSCLC cases (three with prior malignancies, one withdrew consent, and one with small cell lung cancer) and one control (prior malignancy) were ineligible for participation in the study. Amongst NSCLC cases, the median age was 70 (range=46-84) years and 17 were male and four were female. The majority of cases had adenocarcinoma. With the exception of one case, endobronchial lesions were not detected on inspection by bronchoscopic examination immediately prior to BAL fluid collection. The vast majority of NSCLC cases (20/21) were either previous or current smokers. Amongst the controls, five were healthy without active medical conditions, while five had diagnoses of COPD related to smoking. The median age of the control group 58.5 (range=30-77) years was significantly lower than the cases ($p<0.0001$). Out of the controls, six had prior or current histories of smoking, while four were never smokers. The baseline clinicopathological characteristics of the study cohort are detailed in Table I.

The $\Delta\Delta RN^{-1}$ values for the five miRNAs tested for each sputum sample were analyzed by a cluster analysis which dichotomized the samples into two distinct groups that are depicted with a cluster dendrogram (Figure 1). This cluster analysis correctly classified 14/21 NSCLC cases and 9/10 controls, while producing seven false-negatives and one false-positive (Table II). This corresponded to a sensitivity of 67.8%, specificity of 90%, and a positive likelihood ratio of 6.78.

The cluster analysis was repeated on the BAL fluids and resulted in the dendrogram depicted in Figure 2. This cluster analysis had a higher diagnostic accuracy than the previous one and correctly classified 18/21 NSCLC cases and 10/10 controls, while producing three false-negatives and no false-positives. The diagnostic sensitivity (85.7%) and specificity (100%) were higher than the results using sputum samples from the same patients, with a positive likelihood ratio of >2,000 (Table II).

Given the difference in the diagnostic performance between these cluster analyses, a cosine similarity analysis was performed on matched pairs of BAL and sputum samples obtained from the same individual patient (Table III). The mean cosine similarity between concordant pairs of BAL and sputum samples was high, 0.88, indicating that sputum samples obtained from these patients were of very high quality. By contrast, the mean cosine similarity of the discordant pairs of BAL and sputum samples was low, 0.207, indicating that for these patients, the sputum samples were of lower quality (sampling error), accounting for six out of the seven-false negatives in the sputum cluster analysis. Of note, patients with discordant BAL and sputum cluster analysis results had a smaller proportion of upper right lobe tumors (25% *versus* 69%, $p=0.05$), although tumor sizes were similar between those with concordant and discordant test results ($p=0.49$).

## Discussion

The present study highlights the potential of miRNA-expression profiling as a means to detect NSCLC and potentially improve the ability of diagnostic procedures (such as bronchoscopy) and non-invasive sputum-based tests to detect early stage NSCLC. miRNA Expression profiling of BAL fluids could potentially boost the overall diagnostic yield of a bronchoscopic examination for patients with suspected early-stage NSCLC, with minimal incremental risk to the

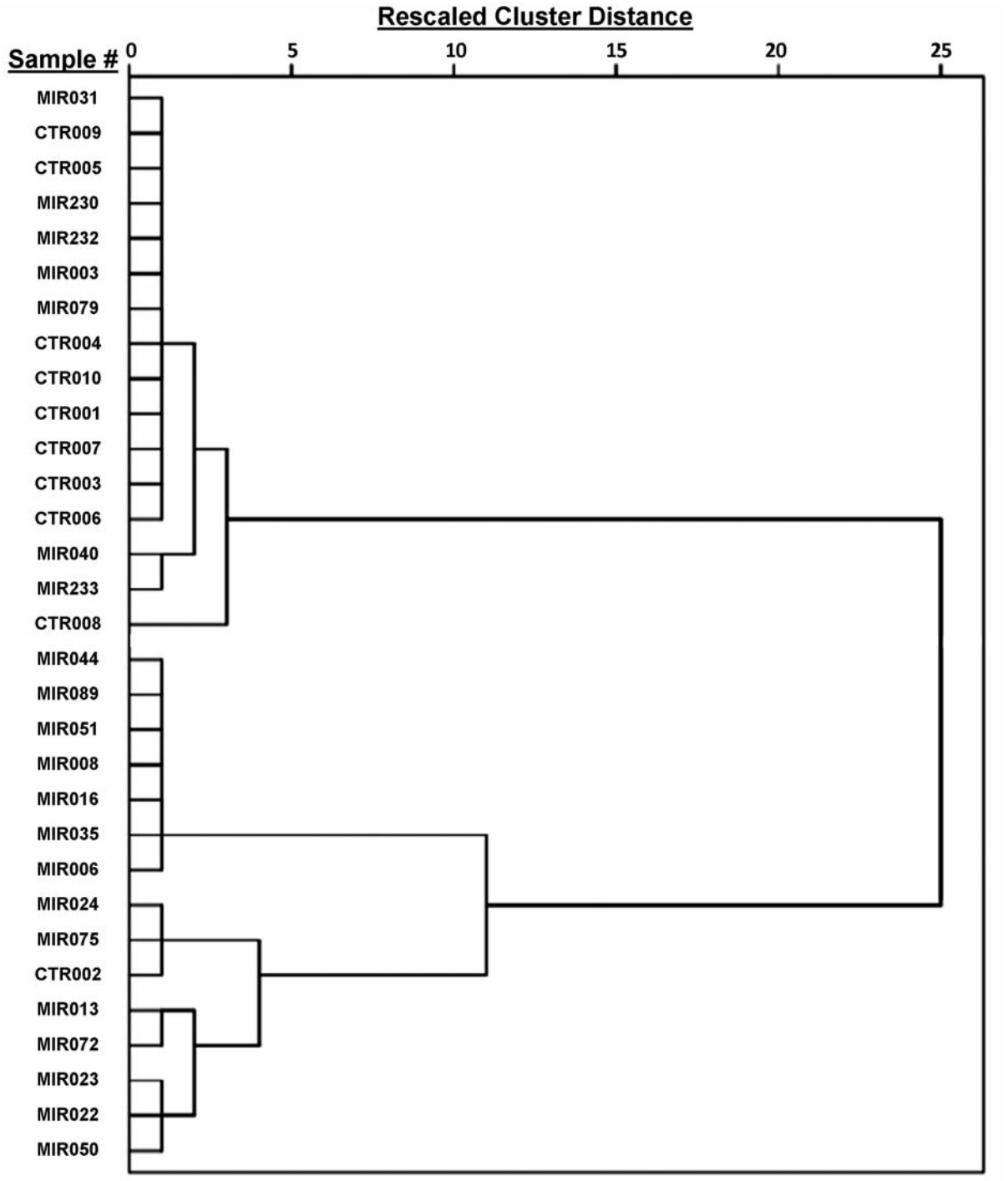


Figure 1. *Dendrogram from the hierarchical cluster analysis using the 21 sputum samples from cases (MIR#) and 10 sputum samples from controls (CTR#) using a five miRNA panel (miR-21, miR-143, miR-155, miR-210, and miR-372). This cluster analysis yielded a sensitivity of 76.2% and specificity of 90% for the diagnosis of non-small cell lung cancer.*

patient. Bronchoscopic evaluations are notably insensitive in the assessment of solitary pulmonary nodules, especially for those that are 2 cm or less for which the diagnostic yield by bronchial brushings and biopsies has been found to be as low as 33% (4). Bronchoscopic assessment of considerably larger solitary pulmonary nodules than those in this cohort by washing, brushing, and biopsy yielded an overall sensitivity of 69% (4). Fluoroscopy and endobronchial ultrasound have

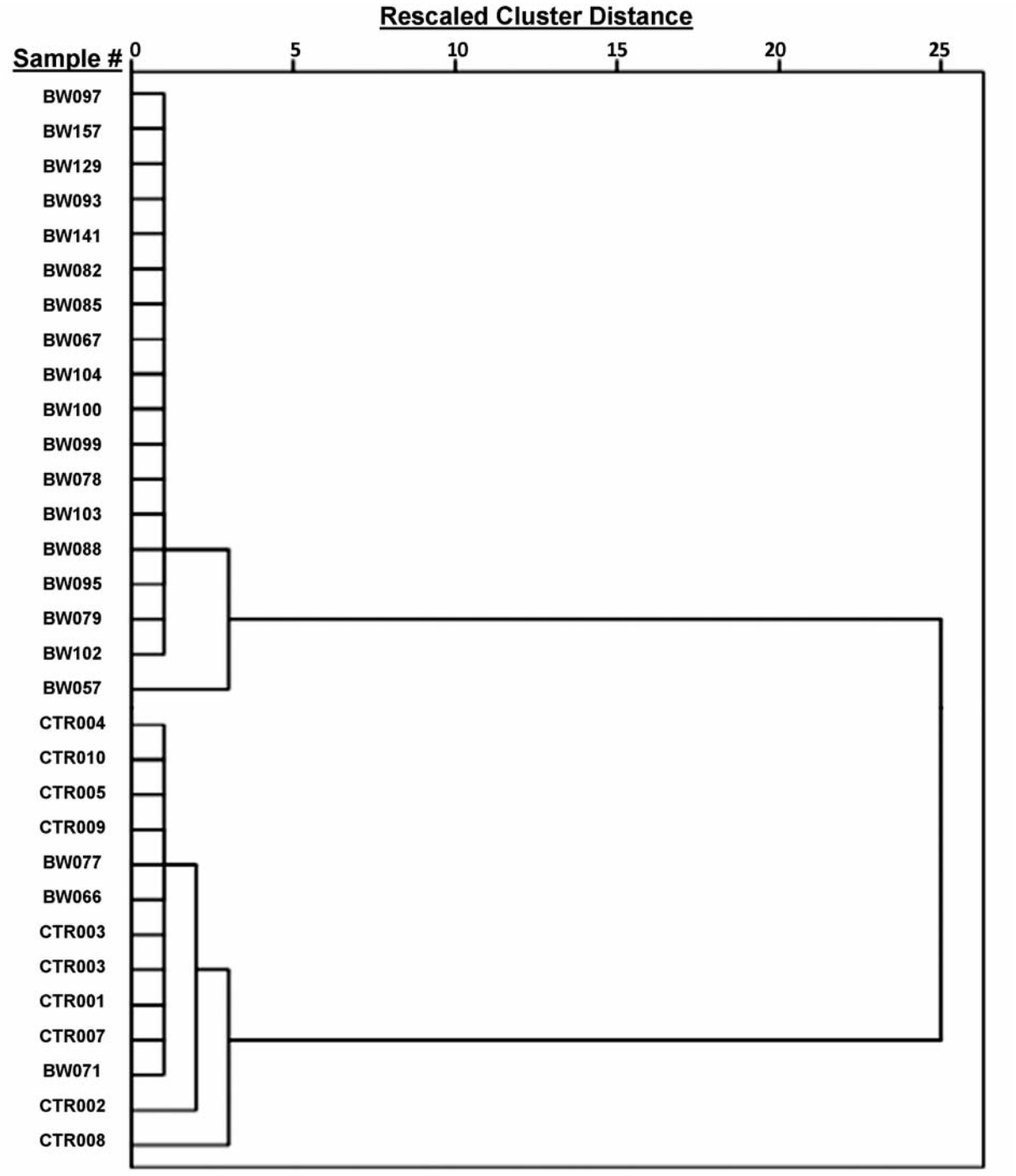


Figure 2. *Dendrogram from the hierarchical cluster analysis using the 21 bronchoalveolar lavage samples from cases (BW#) and 10 sputum samples from controls (CTR#) using a five miRNA panel (miR-21, miR-143, miR-155, miR-210, and miR-372). This cluster analysis yielded a sensitivity of 85.7% and specificity of 100% for the diagnosis of non-small cell lung cancer.*

been shown to improve the diagnostic yield of transbronchial biopsies to approximately 70% (5, 6) in the evaluation of solitary pulmonary nodules. By contrast, in the present study, miRNA-expression profiling of BAL fluids alone yielded a diagnostic sensitivity of 85.7% and positive likelihood ratio greater than 2,000, despite the lack of visualized endobronchial lesions to guide BAL sampling and the diminutive size of the primary tumours in the cohort. miRNA.

Expression profiling of BAL fluids is also superior to other adjunctive molecular testing methods, such as PCR for tumor-specific oncogene mutations on BAL samples (34), as it does not require the discrete presence of intact cancer cells since miRNAs secreted in the extracellular milieu are sufficient for diagnostic purposes.

In our study, miRNA expression profiling of a single, spontaneously expectorated sputum sample yielded a diagnostic sensitivity of 67.8%. This diagnostic sensitivity achieved using our experimental method was 8.5-times the sensitivity of conventional cytological analysis of spontaneously expectorated sputum amongst patients with similar-sized tumours to those in the present study (8). A cosine similarity assessment using matched pairs of concordant and discordant BAL and sputum samples found that the difference in sensitivity between the cluster analysis of BAL fluids and the cluster analysis using the sputum samples is largely attributable to sampling error. In other words, the six false-negatives from the sputum analysis were very likely due to an inability of those patients to properly expectorate the miRNAs that were captured at the time of BAL and this may have been due to the location of these primary tumours in lobes other than the upper right lobe.

We have identified two potential means for boosting the accuracy of our miRNA expression profiling methodology for expectorated sputum samples. Firstly, sampling errors for diagnostic tests have traditionally been overcome through the use of repeated sample collection. For example, the sensitivity of a single faecal occult blood test (FOBT) is estimated to be 30% (35); however, when FOBT tests are performed in triplicate, the sensitivity of the FOBT test reaches up to 80% (36). Likewise, the sensitivity of the conventional cytological analysis of sputum has been shown to increase from 8% to 40% when the number of sputum samples examined from each patient was increased from one to six (8). Secondly, induced sputum collection using inhaled hypertonic saline has been shown to increase the diagnostic sensitivity of conventional cytological assessment of sputum for NSCLC by as much as 12% (8) when compared to spontaneously expectorated samples. These additional measures, aimed at improving the sputum sample collection process, could potentially improve the overall diagnostic yield to a level that is suitable for use as a non-invasive test for the detection of early-stage NSCLC.

Due to the sample size of our study cohort, we cannot exclude the possibility of bias arising from the presence of unmatched confounding factors, especially age and smoking status. In our opinion, the ideal control patients are smokers with COPD since these patients constitute the most comparable population from which incident cases of NSCLC arise. Since this population does not routinely undergo bronchoscopy without an imaging indication (such as a solitary pulmonary nodule), obtaining BAL fluids from these patients would expose them to the undue risk of complications from broncoscopy, and to do so would be unethical. As such, our control participants were only able to provide sputum samples during this study. A method to overcome this potential control selection bias would be to accrue a larger cohort and employ multivariable adjustment for confounding factors.

In summary, miRNA expression profiling of sputum and BAL fluids distinguished cases from controls with high accuracy in the setting of this study. Application of this approach in the screening setting would require: i) validation studies which feature considerably larger populations with lower NSCLC prevalence (similar to studies which assessed low-dose CT-based screening); ii) assessment for variations in expression profiles in NSCLC by phenotypic and genetic subtypes (such as epidermal growth factor receptor mutation-positive disease); and iii) multivariable assessment of the impact of potentially confounding patient and disease factors.

## Conclusion

miRNA Expression profiling of sputum and BAL fluids represents a potential means to detect NSCLC in those with suspected early-stage NSCLC. Further studies are required to validate this promising approach.

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