

MicroRNA expression profile in primary lung cancer cells lines obtained by endobronchial ultrasound transbronchial needle aspiration

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Background: Novel cancer biomarkers like microRNA (miRNA) are promising tools to gain a better understanding of lung cancer pathology and yield important information to guide therapy. In recent years, new less invasive methods for the diagnosis and staging of NSCLC have become key tools in thoracic oncology and the worldwide spread of endobronchial ultrasound transbronchial needle aspiration (EBUS-TBNA). However, appropriate specimen handling is mandatory to achieve adequate results and reproducibility. The aim of this single centre prospective study was to evaluate the feasibility of a complete miRNA expression profile in fresh NSCLC cell lines obtained by EBUS-TBNA.

Methods: Patients with proven NSCLC underwent EBUS-TBNA for the diagnosis of suspect lymph node metastasis, and cytological specimens were collected for epithelial cell culture and miRNA expression analysis. To validate the miRNA expression profile, we compared the results from EBUS-TBNA NSCLC specimens with those obtained from formalin-fixed paraffin-embedded (FFPE) mediastinoscopy specimens.

Results: Analysis of the miRNA expression profiles of three independent EBUS-TBNA-derived primary cell lines allowed the screening of 377 different human miRNAs. One hundred and fifty miRNAs were detected in all cell lines. Analysis of the miRNA expression profile in mediastinoscopy specimens showed a strong similarity in the clusters analysed.

Conclusions: The miRNA expression profile is feasible and reliable in EBUS-TBNA specimens. Validation of this protocol in fresh cytological specimens represents an effective and reproducible method to correlate translational and clinical research.

Keywords: Non-small cell lung cancer (NSCLC); bronchoscopy; endobronchial ultrasound; translational research

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Introduction

Lung cancer is the leading cause of death in industrialized nations and is growing in young people and women (1). Despite large studies devised to develop new strategies for early diagnosis to increase chemotherapy response and prognosis, mortality remains high and effective therapies for advanced lung cancer are still lacking (2). A better understanding of lung cancer biology and the discovery of novel cancer biomarkers are paramount to achieve effective therapies especially for patients with advanced non-small cell lung cancer (NSCLC).

Recent studies showed that microRNA (miRNA) could be ideal candidate biomarkers for cancer prognosis since their dysregulation was found to correlate with the onset and progression of several malignancies including lung cancer (3,4). miRNA can be accurately analysed in (formalin-fixed paraffin-embedded) FFPE and in fresh tissue biopsies (5). In addition, miRNA is present in biological fluids (blood, plasma, saliva and urine) and are altered in asymptomatic early stage lung cancer patients (6). Different miRNA expression profiles were found to be associated with different lung cancer subtypes and there is evidence that miRNAs could play a role in targeted therapy in different oncological settings (7-9). Despite the potential clinical value of miRNA signatures, the clinical utility of identified signatures has yet to be demonstrated. Recent studies showed that endobronchial ultrasound transbronchial needle aspiration (EBUS-TBNA) provides adequate cytological specimens for lung cancer diagnosis and staging, including detection of the major genetic alterations identified in lung cancer: epidermal growth factor receptor-tyrosine kinase (EGFR) mutation status, anaplastic lymphoma kinase fusion genes (ALK), and Kirsten ras oncogene homologue (KRAS) mutation status (10).

The present study describes a strategy for high-throughput miRNA expression profile analysis of lung cancer lymph nodal metastasis using primary cell lines established from EBUS-TBNA samples. To validate the EBUS-TBNA miRNA profile, the results were compared with those obtained from mediastinoscopies, the “gold standard” procedure for mediastinal staging in NSCLC patients. FFPE biopsies samples were obtained from mediastinoscopies surgical specimens present in our tissue bank.

Methods

The institutional ethical committee approved this

prospective single centre study (registration number: R65/14-IEO76), and informed consent was obtained. Patients with proven lung cancer underwent routine EBUS-TBNA for the diagnosis of suspect lymph node metastasis, and cytological specimens were collected for epithelial cell culture and subsequent transcriptomic miRNA expression analysis.

EBUS-TBNA procedures

EBUS-TBNA was performed under local anaesthesia (1% lidocaine) and moderate sedation provided by an anaesthesiologist with spontaneous ventilation. All procedures were performed by the same team of interventional pulmonologists using a convex-probe (EBUS Convex Probe BF-UC180F; Olympus) and a dedicated ultrasound processor (EU-ME2; Olympus). EBUS-TBNA specimens were collected with a 22 gauge dedicated needle (Vizishot NA-201SX-4022; Olympus).

A very small amount of the aspirated material was pushed out by the internal stylet and smeared onto glass slides for immediate on-site evaluation [rapid on site evaluation (ROSE)]. The remaining aspirate and other needle passages were put into saline solution for cell block processing and histological evaluation. One dedicated needle passage was put into a culture basal medium for primary cell cultures.

Primary cell culture

The dedicated EBUS-TBNA specimen was processed within 30 minutes after the end of the procedure and placed in a sterile falcon filled with 5 mL of cell culture basal medium Ham's F12/DMEM 1:1 supplemented with 1% foetal bovine serum, 50 ng/mL L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 µg/mL gentamicin, 0.5 µg/mL amphotericin B, 10 µg/mL human transferrin, 1 µg/mL human insulin, 1 µg/mL hydrocortisone, 10 mM Hepes pH 7.5, 50 µM/L-ascorbic acid, 15 nM sodium selenite, 0.1 mM ethanolamine, and 50 ng/mL cholera toxin. EBUS-TBNA samples were spun down for 5 min at 1,000 g at RT, resuspended in 3 mL of complete medium further supplemented with 10 nM epidermal growth factor EGF, 35 µg/mL bovine pituitary extract, and 10 nM triiodothyronine, and cultured on six-well collagen I-coated plates (Collagen Cellware, Biocoat, Corning, USA) in a humidified incubator with 5% CO₂. Primary cells were grown for 6 to 12 days and washed twice with PBS prior to total RNA extraction.

Table 1 Cohort of EBUS-TBNA primary cells and mediastinoscopies samples with total RNA extracted

Type of sample	Sex	Age	Histology	RNA total ng
EBUS #1	M	72	Squamous cell carcinoma	1,698
EBUS #2	M	64	NSCLC	629
EBUS #3	M	62	Squamous cell carcinoma	4,007
EBUS #4	M	69	Squamous cell carcinoma	3,006
EBUS #5	M	73	Adenocarcinoma	467
MED #1	F	70	Adenocarcinoma	578
MED #2	M	59	Adenocarcinoma	260
MED #3	M	52	Adenocarcinoma	422
MED #4	M	60	Adenocarcinoma	206
MED #5	M	59	Squamous cell carcinoma	203

M, male; F, female; EBUS-TBNA, endobronchial ultrasound transbronchial needle aspiration; EBUS, EBUS-TBNA primary cells; MED, mediastinoscopies FFPE samples.

Immunofluorescence

Immunofluorescence was used to check the expression of lung epithelial (CCA, for bronchiolar epithelium, and SP-C, for alveolar epithelium) and neuroendocrine (chromogranin A) markers on EBUS-derived and plated cells. All the following steps were carried out at room temperature. Permeabilization was achieved with 0.2% BSA, 0.1% Triton, and 1× PBS for 10 min, followed by one wash with 1× PBS. Blocking was carried out with 2% BSA for 30 min. Primary antibodies were added and left for 1 h. Following two washes with 1× PBS, secondary antibodies were added for 30 min (light protected), then another two washes with 1× PBS were performed. Post-fixing was achieved with 2% PFA for 1 min, followed by DAPI staining for 5 min and another two washes with 1× PBS. A final post-fixing was done with 2% PFA followed by one final wash with 1× PBS. Slides were mounted and analysed. The following antibodies were used: CCA, goat polyclonal raised against a peptide mapping near the mouse protein C-terminal (CC10 T-18, Santa Cruz Biotechnologies, sc-9772), dilution 1:400; secondary antibody, donkey anti goat Cy3, dilution 1:400; SP-C, rabbit polyclonal raised against a.a. 1–20 from human protein N-terminal (Anti-Prosurfactant Protein C, pro-SP-C, Millipore, AB3786), dilution 1:1,000; secondary antibody, donkey anti rabbit Alexa 647, dilution 1:100; chromogranin A, rabbit polyclonal raised against the human protein C-terminal (Abcam, Ab15160), dilution 1:100; secondary antibody, donkey anti rabbit Alexa 647, dilution 1:100.

RNA/miRNA extraction and quantitative real-time PCR analysis

Total RNA was extracted from EBUS-TBNA primary cells using the AllPrep DNA/RNA/miRNA Universal Kit and from FFPE tissue samples obtained by mediastinoscopy using the AllPrep DNA/RNA FFPE Kit, both protocols automated on QIAcube, according to the manufacturer's instructions (Qiagen, Hilden, Germany).

Following histological assessment of each FFPE tissue block, RNA was extracted from microdissected areas of one to two tissue sections (5–10 µm thick) on glass slides with adequate tumour cellularity (>60%), selected by a pathologist. The total quantity of RNA extracted in each sample (EBUS-TBNA and mediastinoscopies) is shown in *Table 1*.

Total RNA extracted from EBUS-TBNA-derived primary cells was measured using the NanoDrop® ND-1,000 spectrophotometer and reverse transcribed with the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) in 20 µL of final volume and 5 ng of cDNA/reaction were analysed by PCR. Quantitative PCR was performed using UPL probes (Universal ProbeLibrary; Roche, Switzerland) and primers specific for KRT5 and KRT14 (expressed in epithelial basal cells), KRT18 (expressed in bronchial and alveolar epithelium), or PTPRC and PECAM-1 (expressed in endothelial cells), or PDGFRB (expressed in mesenchymal cells) and LCP2 (expressed in lymphocyte), in a final volume of 15 µL of LightCycler 480 Probe Master mix (Roche). UPL probe and primer combinations specific for each target were designed with the

free web-based ProbeFinder Software (Roche) and reported in *Table S1*. Real-time quantitative PCR analysis (RT-qPCR) reaction was run in a LightCycler 480 real-time PCR instrument (Roche) in 96 wells format, using the following thermal cycling conditions: 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s and 72 °C for 60 s and final cooling at 40 °C for 30 s.

For each mRNA target in each sample, the expression level was measured in triplicate and the average Cq was calculated. Data (average Cq) were normalized to the average Cq value of the two endogenous reference genes (*GAPDH* and *GUSB*) using the $2^{-\Delta Cq}$ method.

For miRNA expression analysis, 10 ng of total RNA, measured using the Quant-iT™ RiboGreen® RNA assay kit (Thermo Fisher Scientific), were reverse transcribed with Megaplex™ miRNA-specific stem-loop RT Primers Human Pool A v 2.1 (Thermo Fisher Scientific) and TaqMan® MicroRNA reverse transcription kit (Thermo Fisher Scientific) according to the manufacturer's instructions; 5 µL of reverse transcribed product were pre-amplified for 14 cycles using the TaqMan PreAMP Mastemix and Megaplex PreAMP primers Pool A v 2.1 according to the manufacturer's instructions (Thermo Fisher Scientific). The PCR reaction was performed using the TaqMan Universal Master Mix II, No AmpErase UNG (Thermo Fisher Scientific) by loading 100 µL of the pre-amplified mixture (final dilution 1:200) in each of the eight lanes of the TaqMan® Low Density Array miRNA Panel A v 2.0 (Thermo Fisher Scientific). Real-Time PCR was carried out on the ViiA7 Real-Time PCR System (Thermo Fisher Scientific) using the manufacturer's recommended cycling conditions (50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min) and setting an automatic threshold. Cq data of miRNAs were normalized using the RNU6-1 as housekeeping gene. Normalized Cq (Cqn) were calculated as previously described (9). Hierarchical clustering analysis was performed using Cluster 3.0 for Mac OS X (<http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm>) and Java Treeview (<http://jtreeview.sourceforge.net>). Spearman rank correlation and centroid clustering methods were used on Cqn data.

Results

The immunofluorescence analysis using known markers of alveolar and neuroendocrine cells constituting the airway epithelium confirmed the lung origin of the established EBUS primary cell lines (*Figure 1A*).

RT-qPCR revealed a high expression of epithelial markers in EBUS-derived primary cell lines which was almost absent in the EBUS-TBNA specimen (*Figure 1B*), while the EBUS-TBNA samples were positive to the expression of non-epithelial markers (*Figure 1B*).

As a control, the expression of non-epithelial markers was checked in two commercial cell lines of non-epithelial origin (HL60 and HUVEC) and proved positive (*Figure 1C*). These data confirmed the successful establishment of lung epithelial cells from EBUS-TBNA specimens.

We performed 15 EBUS-TBNA procedures in patients with suspect lymph node NSCLC metastasis for primary cell cultures. Seven out of 15 samples presented an adequate growth on culture and in five patients was possible to extract RNA followed by miRNA profiling analysis. The percentage of adequate growth on culture was about 30% of the total EBUS-TBNA sampled. A flow-chart of the procedure is shown in *Figure 2*.

Analysis of the miRNA expression profile of the five independent EBUS-TBNA-derived primary cell lines using TaqMan® Array Human MicroRNA Card A allowed the screening of 377 different human miRNAs; 130 miRNAs (~35% of the total analyzable) were detected (with Cqn ≤30) in all five cell lines. Relative quantities of miRNAs detected in all five EBUS-primary cell lines ranged from 22 to 26 Cqn (i.e., the 25th and 75th quartile intervals) (*Figure 3A*; *Table S2*).

Analysis of the miRNA expression profile of microdissected formalin-fixed lung cancer tissue ("MED" samples; N=5) compared with the profile of EBUS-primary cells (*Figure 3A*) showed a slight decrease in the average number of miRNA detected (i.e., with Cqn ≤30) in all five FFPE samples (109 vs. 130; *Figure 3A*). This was probably due to a partial degradation of some miRNA species in FFPE samples.

A total of 102 miRNAs (~78% in EBUS-TBNA primary cell lines and ~94% of FFPE mediastinoscopies) were commonly detected in all samples analysed (with Cqn ≤30), confirming a strong similarity of the two miRNA profiles as also shown in hierarchical cluster analysis (*Figure 3B*). A list of miRNAs detected in EBUS-primary cells, mediastinoscopy samples and common miRNAs are shown in *Table S2*.

Discussion

New trends in thoracic oncology are characterized by non-invasive therapies and less-invasive methods for the diagnosis and staging of advanced NSCLC, and targeted

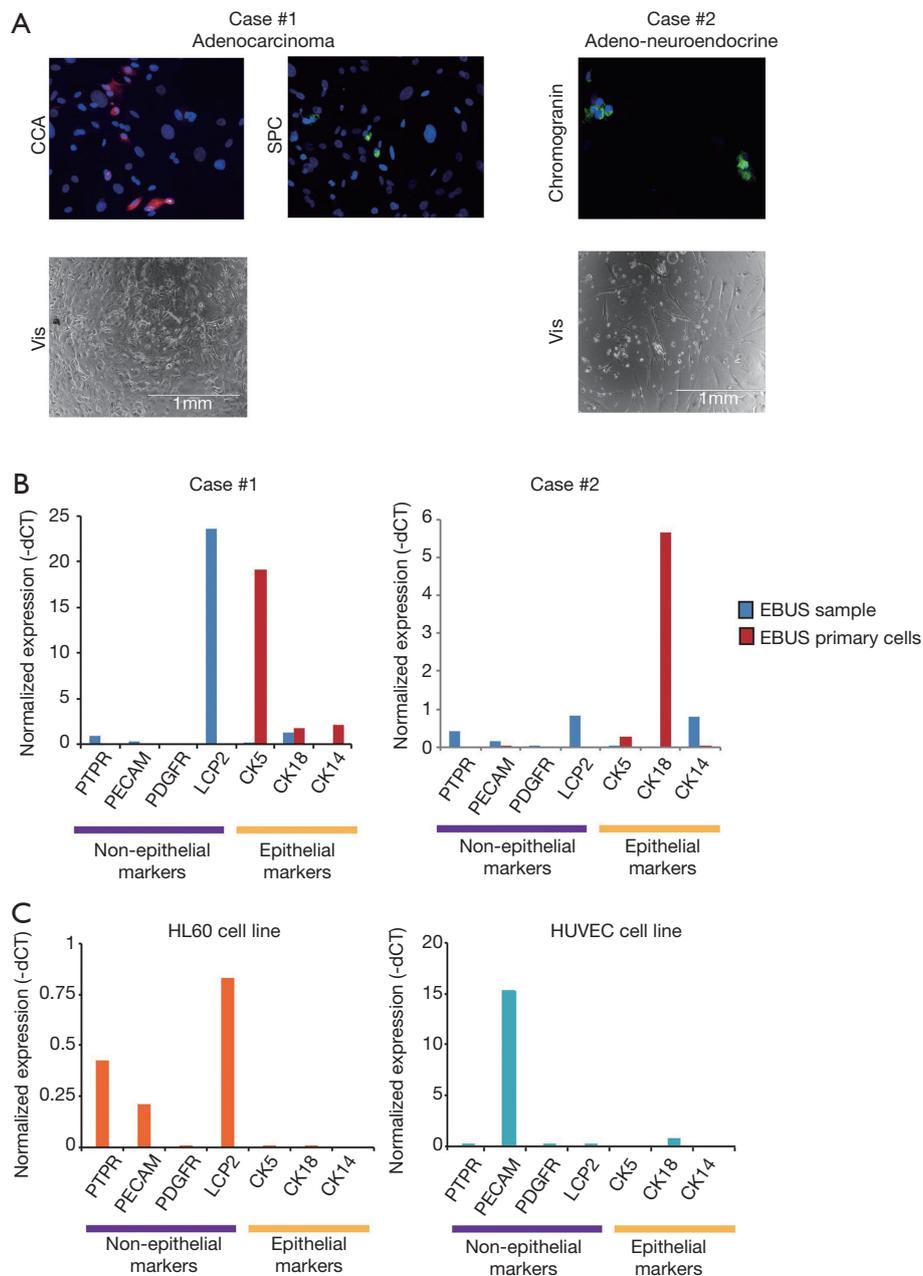


Figure 1 Biological characterization of EBUS derived primary cell lines. (A) Immunofluorescence analysis of primary cell lines obtained from two EBUS-TBNA samples. Left: expression of the Clara cell-specific marker (CCA) and surfactant protein C (SP-C) indicates bronchoalveolar cells in the EBUS specimen of a lung adenocarcinoma. Right: expression of chromogranin indicates neuroendocrine cells in the EBUS specimen of a lung adenocarcinoma with neuroendocrine features. Below, visible microscope analysis representing the morphology of the two cell lines obtained; the neuroendocrine cells appeared with the characteristic spindle-shaped morphology; (B) RT-qPCR analysis of a panel of genes expressed preferentially in non-epithelial cells (i.e., non-epithelial marker) or epithelial cells (i.e., epithelial markers). In blue, gene expression level in all EBUS-TBNA samples. In red, gene expression level in the established primary cell lines; (C) RT-qPCR analysis of two commercial cell lines of non-epithelial origin used as control: HL60 (promyeloblast cell type) and HUVEC (endothelial cell type). Normalized expression refers to the expression of genes normalized to the average of the expression of GUSB and GAPDH (i.e., the -dCT), used as housekeeping genes. EBUS, EBUS-TBNA primary cells; RT-qPCR, real-time quantitative PCR analysis.

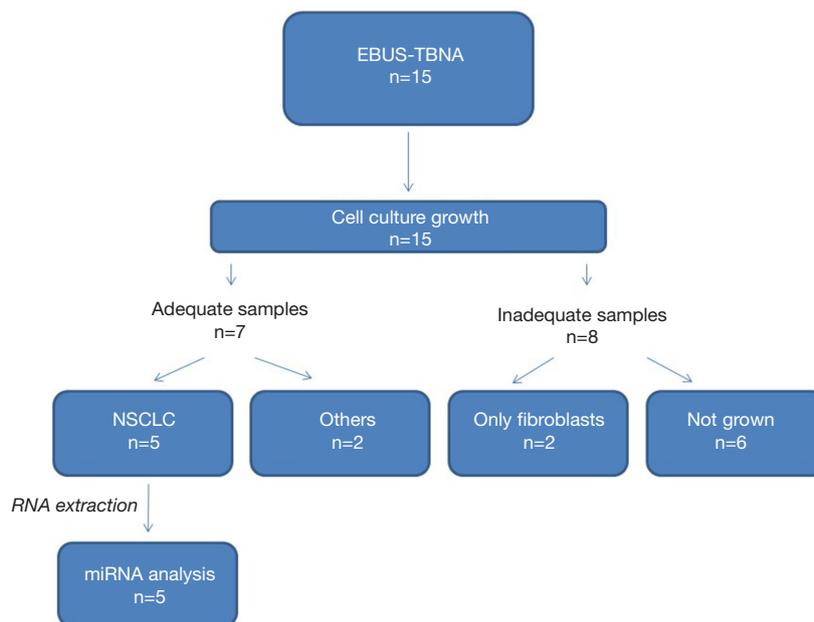


Figure 2 Flow chart of EBUS-TBNA primary cell culture procedure. miRNA, microRNA; EBUS-TBNA, endobronchial ultrasound transbronchial needle aspiration.

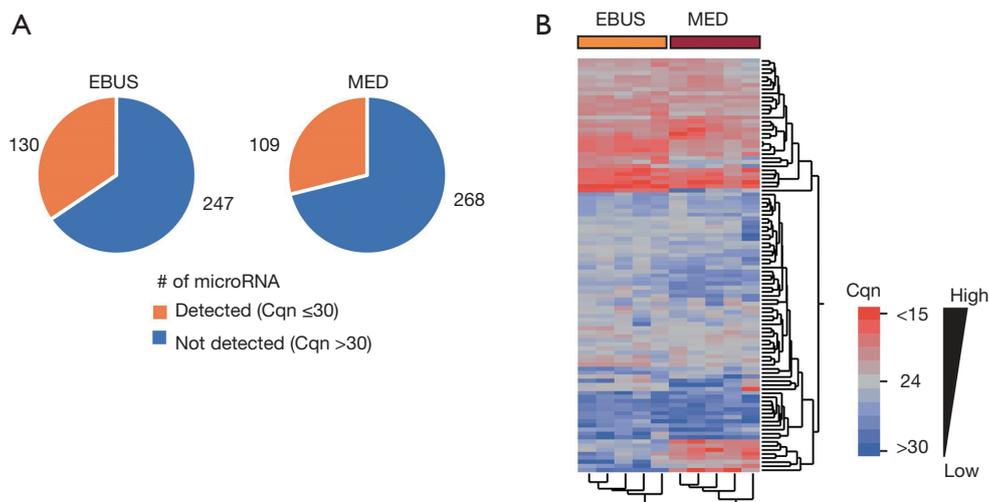


Figure 3 MicroRNA expression profile analysis of EBUS and MED samples. (A) Pie charts of the number of detected ($C_{qn} \leq 30$; in orange), or undetected ($C_{qn} > 30$; in blue) microRNA in all the primary cells obtained from EBUS ($N=5$) or in all mediastinoscopy FFPE samples, i.e., “MED” samples ($N=5$); (B) hierarchical cluster analysis of the expression profile of the 102 commonly detected miRNAs in EBUS ($N=5$) and MED samples ($N=5$). Heatmap indicates the expression level of each individual miRNA analyzed. Normalized Cq values were colour-coded and described by the scale bar. Cqn, normalized Cq; EBUS, EBUS-TBNA primary cells; miRNA, microRNA; FFPE, formalin-fixed paraffin-embedded; MED, mediastinoscopy FFPE samples.

therapies (11) designed to reduce patient discomfort and complications and improve survival rates.

Surgical histological specimens have been supplanted by EBUS-TBNA in different clinical scenarios, especially in lung cancer diagnosis and staging (12). In recent years, several studies have demonstrated that EBUS-TBNA reaches the same percentage of diagnosis as surgical biopsies, and allows molecular analyses and mutation detections for target therapies in advanced lung cancer patients (10). Due to its low invasiveness, EBUS-TBNA can also be repeated on “long survival” patients to re-characterize molecular status for personalized treatment.

Different studies have investigated the association between miRNA alterations and lung cancer onset and progression. miRNAs were shown to be prognostic factors, or early diagnostic markers, or more intriguingly, factors determining chemotherapy or biological drug responses (5).

Developing novel biomarkers in conjunction with less invasive methods of diagnosis and staging of NSCLC patients, especially EBUS-TBNA, is paramount to offer patients optimal care with the new perspectives of targeted therapies. The integration of miRNA screening studies with clinical protocols is mandatory to transfer the proposed miRNA biomarkers to the clinical setting. Standardizing methods for collection of biological samples and optimizing miRNA profile analyses, particularly when starting from limited amounts of specimens, represents a definitive strategy to increase the assessment of proposed biomarkers in clinical studies.

As previously reported, miRNAs are resistant to degradation and can be easily identified in FFPE specimens, but few data are available on the feasibility of miRNA expression analyses in cytological specimens. A recent study investigated mRNA and miRNA expression profiles in EBUS-TBNA stored specimens (13). However, it did not address the purity of the samples to derive miRNA/mRNA expression profiles in terms of lung cancer cells fraction, a major issue since miRNAs have been shown to be tissue-specific (14).

The use of primary cell lines instead of the completely fixed cytological specimens collected from EBUS-TBNA has been never described, but should guarantee a higher specificity of the miRNA expression profile, avoiding “contamination” of the neoplastic cell miRNA profile by other non-epithelial cells (lymphocytes, blood and stromal cells) which are

abundant in lymph node samples.

This study obtained primary cell cultures from EBUS-TBNA specimens removing most of the non-epithelial cells (lymphocytes, blood and stromal cells) that might affect the gene expression profile of NSCLC cellularity and miRNA results. The results demonstrate the feasibility of obtaining pure populations of primary NSCLC cells from EBUS-TBNA specimens and a complete miRNA expression profile analysis, which strongly correlates with those obtained from microdissected lymph nodal metastases from mediastinoscopy specimens. In addition, the establishment of EBUS-TBNA primary NSCLC cell lines also provides experimental models to test the role of these miRNAs in the biology of lung cancer metastases, which may also have therapeutic indications. Our results are promising for the management of advanced NSCLC patients. The possibility to use EBUS-TBNA specimens for a primary cell culture and whole miRNA expression profiles provides an excellent tool in the personalized therapy of advanced lung cancer patients. On the basis of the present results, we have designed a prospective study to collect EBUS-TBNA primary NSCLC cell lines to evaluate the expression of a miRNA signature profile in stage IIIA NSCLC patients. We plan to evaluate a miRNA signature based on response to chemotherapy that could predict a good prognosis and single out the best candidates for therapy, a crucial point in personalized treatment era.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The institutional ethical committee approved this prospective single centre study (registration number: R65/14-IEO76), and informed consent was obtained.

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Supplementary

Table S1 List of primers and probes (UPL, Roche) used for RT-qPCR analyses of the panel of epithelial and non-epithelial genes

Gene symbol	RefSeq	UPL probe number	Amplicon size	Primer forward	Primer reverse
<i>KRT5</i>	NM_000424.3	63	67	CAGCGTCAAATTTGTCTCCAC	TGCAGCAGGTTCTTAGCTCTT
<i>KRT14</i>	NM_000526.4	19	79	CCTCCTCCCAGTTCTCCTCT	ATGACCTTGGTGCGGATTT
<i>KRT18</i>	NM_000224.2	70	77	AAGCTGGAGGCTGAGATCG	TCCAAGGCATCACCAAGATTA
<i>PTPRC</i>	NM_002838.4	41	73	GAAAAGCTCCCTGAAGCAA	AATGTTCTGGCCCCTCAGT
<i>PECAM-1</i>	NM_000442.4	75	77	TTGGAAATACACAGTGCTGACC	TGGTTTTCCCATTTGTGGAG
<i>PDGFRB</i>	NM_002609.3	61	72	CGGGACCTCTTTCACTACCC	CTCATTTGCCCTCTTTGTCC
<i>LCP2</i>	NM_005565.3	34	63	TTATAGCCGAGCAAATGAACC	CCAAGGCTGATTGATCTCGT
<i>GAPDH</i>	NM_002046.5	45	72	CAAGGAGTAAGACCCCTGGAC	CCCAGCAGTGAGGGTCTCT
<i>GUSB</i>	NM_000181.3	84	61	CCTCCTCCCCGAGTCAG	GAACAGTCCAGGAGGCACTT

UPL, universal probelibrary (roche); RefSeq, NCBI reference sequence database; RT-qPCR, real-time quantitative PCR analysis.

Table S2 The expression data of the 377 miRNA analysed. Target name, the official Assay ID of the TaqMan[®] Low Density Array microRNA Panel v 2.0 (Thermo Fisher Scientific) is shown

Target name	EBUS #1	EBUS #2	EBUS #3	EBUS #4	EBUS #5	MED #1	MED #2	MED #3	MED #4	MED #5	DETECTED	
hsa-let-7a-4373169	21.15	20.48	20.88	20.84	20.53	21.02	22.92	21.80	21.17	21.37	Commonly detected in all	
hsa-let-7c-4393167	26.11	25.19	26.20	23.51	24.96	23.45	25.30	23.64	23.02	22.87	Commonly detected in all	
hsa-let-7d-4395394	23.74	23.17	23.33	22.97	24.66	23.06	25.32	23.78	22.46	23.24	Commonly detected in all	
hsa-let-7e-4395517	20.63	20.44	20.08	20.49	19.91	19.25	21.19	20.14	19.56	20.10	Commonly detected in all	
hsa-let-7f-4373164	23.75	23.29	23.84	23.11	22.77	24.63	26.25	24.01	24.46	22.11	Commonly detected in all	
hsa-let-7g-4395393	24.02	23.68	24.08	23.74	22.74	23.42	25.21	24.37	23.26	24.60	Commonly detected in all	
hsa-miR-424-373285	27.16	28.05	25.28	21.98	20.13	28.12	27.98	27.06	19.06	25.07	Commonly detected in all	
hsa-miR-10a-4373153	27.04	27.03	24.94	25.54	23.81	25.04	26.23	23.33	21.08	25.22	Commonly detected in all	
hsa-miR-15a-4373123	26.31	26.93	27.89	28.75	24.38	28.00	27.32	25.65	21.43	24.93	Commonly detected in all	
hsa-miR-15b-4373121	22.67	22.60	22.41	20.69	22.66	24.36	25.04	23.67	19.13	22.81	Commonly detected in all	
hsa-miR-16-4373122	22.04	21.99	22.29	20.38	19.64	21.51	22.37	20.82	19.27	21.69	Commonly detected in all	
hsa-miR-17-4395419	18.41	18.12	18.98	18.45	17.81	21.12	22.74	20.67	17.49	21.06	Commonly detected in all	
hsa-miR-19a-4373099	22.60	21.89	23.36	22.41	21.05	25.92	27.33	24.04	14.52	25.32	Commonly detected in all	
hsa-miR-19b-4373098	17.81	17.28	18.90	18.13	16.25	20.52	22.21	19.36	14.86	19.99	Commonly detected in all	
hsa-miR-20a-4373283	18.35	17.96	19.10	18.24	17.02	21.74	23.22	21.11	19.24	21.59	Commonly detected in all	
hsa-miR-20b-4373063	23.57	23.06	24.28	23.02	22.76	26.78	28.21	25.82	22.20	25.25	Commonly detected in all	
hsa-miR-21-4373090	18.54	16.82	19.47	17.77	15.82	19.09	20.13	17.50	14.28	21.26	Commonly detected in all	
hsa-miR-22-4373079	25.35	22.41	25.73	21.91	21.38	26.00	28.12	23.72	20.50	28.96	Commonly detected in all	
hsa-miR-24-4373072	16.28	15.20	17.58	16.79	16.66	18.71	20.82	19.12	15.45	18.53	Commonly detected in all	
hsa-miR-25-4373071	24.08	23.52	23.91	22.04	20.96	25.13	26.32	22.39	21.16	23.13	Commonly detected in all	
hsa-miR-26a-4373061	22.03	20.99	22.25	21.57	20.97	20.36	23.34	21.14	19.94	21.80	Commonly detected in all	
hsa-miR-26b-4395167	23.64	22.82	24.60	22.07	22.04	23.43	26.16	23.35	24.08	23.64	Commonly detected in all	
hsa-miR-27a-4373287	21.84	20.25	22.72	24.73	22.24	23.55	25.29	23.10	19.92	21.83	Commonly detected in all	
hsa-miR-27b-4373068	23.16	22.68	24.25	23.06	25.17	24.13	26.91	24.23	20.76	23.23	Commonly detected in all	
hsa-miR-28-3p-4395557	24.54	23.37	24.02	23.59	22.28	23.20	25.12	22.04	19.94	22.24	Commonly detected in all	
hsa-miR-28b-3p-4393067	24.14	23.06	24.22	23.37	22.34	25.64	26.88	24.26	20.41	23.05	Commonly detected in all	
hsa-miR-29a-4395223	20.63	18.71	21.41	21.61	19.63	20.97	22.75	20.93	17.78	19.64	Commonly detected in all	
hsa-miR-29b-4373288	23.92	21.61	25.14	24.73	22.06	24.92	27.25	24.52	23.30	23.45	Commonly detected in all	
hsa-miR-30b-4373290	19.63	19.11	20.98	18.92	19.79	16.87	21.00	20.41	18.20	19.02	Commonly detected in all	
hsa-miR-30c-4373060	18.91	18.32	20.34	18.17	19.33	18.37	21.69	20.42	18.31	18.79	Commonly detected in all	
hsa-miR-31-4395390	17.00	16.20	19.24	18.74	17.99	28.10	29.12	23.93	19.93	21.14	Commonly detected in all	
hsa-miR-34a-4395168	22.01	21.23	20.41	22.78	20.22	22.94	24.28	22.99	19.07	21.27	Commonly detected in all	
hsa-miR-92a-4395169	20.23	19.79	20.11	20.06	20.68	22.55	24.16	21.56	18.64	20.17	Commonly detected in all	
hsa-miR-93a-4373302	22.89	22.58	23.10	21.60	23.48	25.11	25.96	22.63	19.29	24.31	Commonly detected in all	
hsa-miR-99a-4373008	19.41	19.64	18.94	20.65	19.48	23.50	25.32	21.71	22.07	21.37	Commonly detected in all	
hsa-miR-99b-4373007	20.08	18.99	20.38	19.87	19.98	21.99	24.14	20.97	19.23	21.27	Commonly detected in all	
hsa-miR-100-4373158	19.83	19.81	19.56	20.67	19.55	23.12	24.93	21.16	19.62	21.53	Commonly detected in all	
hsa-miR-103-4373150	23.01	22.23	24.34	22.75	22.14	24.59	25.94	24.14	21.20	23.17	Commonly detected in all	
hsa-miR-106a-4395280	18.56	18.15	19.26	18.52	17.73	21.13	22.79	20.71	17.52	21.14	Commonly detected in all	
hsa-miR-106b-4373155	22.26	21.53	23.06	20.99	21.19	23.82	24.86	21.78	19.04	21.64	Commonly detected in all	
hsa-miR-125a-5p-4393223	23.27	22.29	23.13	23.11	22.72	24.86	25.83	23.67	22.20	24.74	Commonly detected in all	
hsa-miR-125b-4373148	21.30	21.25	19.92	20.82	20.89	21.80	23.20	19.94	20.12	21.31	Commonly detected in all	
hsa-miR-126-4395393	25.02	23.10	26.28	23.91	22.10	20.44	20.44	19.84	17.30	28.66	Commonly detected in all	
hsa-miR-127-3p-4373147	26.90	29.08	22.60	25.56	27.16	26.45	28.00	25.89	22.17	25.49	Commonly detected in all	
hsa-miR-130a-4373145	24.05	22.42	24.62	23.69	22.92	23.75	26.31	25.25	22.22	24.91	Commonly detected in all	
hsa-miR-130b-4373144	24.90	24.35	25.19	24.42	24.56	27.14	27.95	26.84	21.76	25.89	Commonly detected in all	
hsa-miR-132-4373143	22.00	22.56	23.18	22.67	20.98	24.01	25.08	22.41	20.44	22.28	Commonly detected in all	
hsa-miR-135b-4395372	24.65	21.31	26.22	25.86	21.04	23.07	25.90	25.07	22.96	27.79	Commonly detected in all	
hsa-miR-139-5p-4395400	27.66	26.02	27.90	22.92	23.39	24.07	26.87	28.25	27.06	24.39	24.62	Commonly detected in all
hsa-miR-140-5p-4373274	25.85	24.33	25.78	26.21	26.01	24.35	25.49	24.64	19.35	23.92	Commonly detected in all	
hsa-miR-141-5p-4373137	22.28	20.84	25.33	21.94	19.84	22.67	25.02	22.04	21.14	21.72	Commonly detected in all	
hsa-miR-145-4395389	27.76	25.83	21.21	22.09	23.60	20.31	21.79	21.20	15.08	19.43	Commonly detected in all	
hsa-miR-146a-4373132	22.65	21.90	20.55	22.74	21.13	21.83	22.12	20.01	17.45	18.84	Commonly detected in all	
hsa-miR-146b-5p-4373178	26.69	25.51	23.53	23.89	23.90	21.02	22.28	20.79	16.30	19.88	Commonly detected in all	
hsa-miR-148a-4373130	25.29	24.15	25.40	24.10	25.02	24.45	26.92	24.01	22.56	24.62	Commonly detected in all	
hsa-miR-149-4395366	22.54	21.49	22.95	21.93	27.76	27.12	28.14	26.68	20.30	24.38	Commonly detected in all	
hsa-miR-150-4373127	25.99	27.89	27.69	27.27	26.46	20.34	19.89	18.86	19.17	15.70	Commonly detected in all	
hsa-miR-151a-4395170	23.43	23.59	24.61	23.30	24.07	23.90	26.13	24.39	21.76	23.05	Commonly detected in all	
hsa-miR-181a-4373117	25.10	21.36	23.45	23.25	21.17	22.01	25.26	23.00	21.27	22.56	Commonly detected in all	
hsa-miR-181b-4395382	27.86	26.33	28.25	27.06	26.61	27.89	28.80	26.67	24.14	25.10	Commonly detected in all	
hsa-miR-181c-4395396	24.00	22.41	24.78	24.01	23.47	22.10	25.95	22.95	20.56	22.75	Commonly detected in all	
hsa-miR-191-4395410	19.86	18.83	20.67	20.09	19.65	19.29	22.04	19.16	18.73	19.74	Commonly detected in all	
hsa-miR-193a-5p-4395392	28.76	26.48	25.07	25.45	24.01	26.90	27.97	26.81	22.11	25.31	Commonly detected in all	
hsa-miR-193b-4395478	19.86	17.98	20.19	19.13	18.84	22.36	23.17	20.04	16.65	21.66	Commonly detected in all	
hsa-miR-194-4373106	27.72	26.09	29.03	25.21	23.22	28.87	28.57	27.51	24.55	27.01	Commonly detected in all	
hsa-miR-197-4373022	22.89	21.41	22.72	22.18	23.63	25.10	22.64	20.90	20.71	21.91	Commonly detected in all	
hsa-miR-199a-3p-4395415	29.19	29.02	22.43	24.17	26.97	22.59	24.10	22.20	19.00	22.04	Commonly detected in all	
hsa-miR-200a-4378069	21.93	20.60	24.64	21.95	20.90	22.38	25.45	23.01	18.96	22.73	Commonly detected in all	
hsa-miR-200b-4395362	20.96	19.88	23.25	20.85	19.94	22.34	25.29	21.90	20.07	23.50	Commonly detected in all	
hsa-miR-200c-4395411	18.25	17.40	20.56	17.35	16.73	19.30	21.86	18.09	17.32	20.46	Commonly detected in all	
hsa-miR-203-4395365	20.16	18.18	22.91	19.44	17.73	22.65	24.68	23.39	22.20	21.12	Commonly detected in all	
hsa-miR-210-4373089	21.17	20.39	22.60	20.81	21.74	20.94	23.19	20.57	19.55	20.11	Commonly detected in all	
hsa-miR-218-4373081	23.82	23.10	23.79	22.23	26.67	22.92	24.99	24.97	20.18	21.58	Commonly detected in all	
hsa-miR-219-4373077	20.00	19.07	19.49	20.58	22.31	24.72	26.24	24.16	20.28	23.00	Commonly detected in all	
hsa-miR-223-4395387	18.13	17.06	18.75	18.10	18.09	19.28	21.26	18.42	16.48	18.73	Commonly detected in all	
hsa-miR-224-4395406	22.66	24.26	25.24	23.65	23.36	20.92	21.35	16.93	16.12	26.20	Commonly detected in all	
hsa-miR-224-4395102	24.01	23.25	24.74	21.53	22.21	27.20	28.07	24.44	21.15	22.95	Commonly detected in all	
hsa-miR-301a-4373064	24.04	23.22	26.26	22.87	23.79	24.84	27.59	26.10	22.47	26.05	Commonly detected in all	
hsa-miR-302a-4395388	21.66	21.13	22.14	20.60	20.04	21.74	22.56	20.32	20.07	21.00	Commonly detected in all	
hsa-miR-324-5p-4395272	27.10	26.22	24.98	25.95	26.10	26.93	29.61	26.30	22.61	25.19	Commonly detected in all	
hsa-miR-324a-5p-4393205	24.37	23.75	26.86	22.17	24.05	26.14	27.93	25.57	21.26	23.93	Commonly detected in all	
hsa-miR-331-3p-4373046	20.66	19.23	21.93	19.90	19.14	20.79	23.29	21.11	18.89	21.06	Commonly detected in all	
hsa-miR-339-5p-4395368	24.47	23.55	25.19	24.26	22.51	25.64	27.37	24.87	23.73	23.62	Commonly detected in all	
hsa-miR-355-4395459	24.85	25.17	24.53	26.27	27.70	24.72	24.83	22.08	16.56	20.23	Commonly detected in all	
hsa-let-7b-4395446	20.87	20.41	20.46	20.30	20.09	18.86	20.25	18.96	17.52	19.25	Commonly detected in all	
hsa-miR-342-3p-4395371	23.87	23.63	25.23	24.08	24.07	21.13	22.79	20.59	16.96	19.01	Commonly detected in all	
hsa-miR-345-4395297	24.95	24.97	26.48	25.37	26.14	29.03	29.71	27.59	20.72	24.33	Commonly detected in all	
hsa-miR-365-4373194	23.61	21.89	23.33	22.70	22.38	28.89	29.95	27.96	18.87	24.27	Commonly detected in all	
hsa-miR-374a-4												