Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA
SCIENZE CARDIO-NEFRO-TORACICHE

30° Ciclo

Settore Concorsuale: 06/E1

Settore Scientifico Disciplinare: MED21

TITOLO

EVALUATION OF THE EFFECTIVENESS OF THE USE OF ENDOBRONCHIAL ULTRASOUND GUIDED TRANSBRONCHIAL NEEDLE ASPIRATION (EBUS-TBNA) FOR THE IDENTIFICATION OF CHEMO-RESISTANCE MARKERS THROUGH GENOMIC INVESTIGATION IN PATIENTS WITH NON-SMALL CELL LUNG CANCER (NSCLC)

Presentata da: Dott.ssa JULIANA GUARIZE

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Esame finale anno 2018

Acknowledgements

I would like to thank all the people who, in various ways, have accompanied me in this project.

First, I want to thank to my tutor, Professor Sandro Mattioli for the irrevocable contribution and patience.

I thank Dr. Fabrizio Bianchi, Head of Cancer Biomarkers Unit of the Institute for Stem-cell Biology, Regenerative Medicine and Innovative Therapies - IRCCS - Casa Sollievo della Sofferenza, San Giovanni Rotondo - for the precious and crucial support.

Thanks to the colleagues that supported the clinical activity at the Division of Thoracic Surgery at the European Institute of Oncology, especially Dr. Stefano Donghi and Dr. Monica Casiraghi; the Department of Molecular Medicine, specially Dr. Manuela Vecchi, Elena Belloni, Elena Marino and Giovanni Bertalot for the inestimable contribution in the laboratorial activity; the Department of Pathology, especially Dr. Chiara Casadio, the cytopathologists and technicians and the Biobank for Translational Medicine at IEO for the technical support.

Finally, I want to thank to my husband and my daughter Beatrice, without whom nothing would be possible.

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1. INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths in the developed world, accounting for 156,480 estimated cancer deaths in 2013 in the United States alone (1).

Approximately 85 to 90% of all cases of lung cancer are non–small-cell lung cancer (NSCLC). Despite increasing public awareness of tobacco-associated risk and recent improvements in early detection, about one third of patients present clinically evident metastatic disease that is not curable either by surgery or by combined modality therapy (2).

An additional one third of patients have locally advanced disease, and 80-85% of these patients will relapse with incurable metastatic disease in spite of treatment with aggressive multi-modality therapy (3).

With the advent of third-generation agents (taxanes, gemcitabine, vinorelbine, irinotecan) administered in combination with carboplatin or cisplatin, a significant increase in response rates, better tolerability and quality of life, was observed although without an improvement in overall survival (4-7).

Recent molecular targeted therapies using tyrosine-kinase inhibitors (TKI) or inhibitors of the receptor ALK (anaplastic lymphoma kinase), demonstrated that modern, molecularly targeted drugs can benefit patients with advanced lung cancer (8-10).

However, the complexity of the cancer genome and the relatively propensity of cancer cells to acquire molecular alterations highlights the need to search for cancer biomarkers which could suggest optimal treatment and identify novel drug targets (11).

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 (12).

As known, is worldwide accepted that initial stages NSCLC patients (N0 and N1 disease) should be treated with anatomical surgical resection and complete mediastinal and hilar lymphadenectomy. Advanced stages (stage IIIB and stage IV) should be treated with definitive chemotherapy or chemo/radiotherapy (13,14).

In contrast, the best treatment option for locally advanced disease (Stage IIIA – pN2) is still debated.

According to American College of Chest Physicians guidelines, Stage IIIA (pN2) patients should be treated with definitive chemo/radiotherapy (15) but several series suggests that tree modality treatment (induction chemotherapy, surgery followed by radiotherapy) could be the best treatment option. (16-21)

Analyzing the literature data about N2 NSCLC patients, we found that this group represents a heterogeneous group with different clinical presentation, and both prognosis and treatment strategies are usually based on the extension of the disease to the mediastinum.

As known, patients with stage IIIA (pN2) disease present a very different prognosis according to different grades of mediastinal involvement and according to response to chemotherapy. Thus, these patients could be divided into three different groups according to clinical presentation and prognosis.

The first group, the "occult N2" group, includes those without clinical lymph node involvement (N0) but with unexpected N2 disease discovered after surgery and complete mediastinal lymphadenectomy; these patients are usually treated with adjuvant therapies after complete surgical resection, reaching a 5-year overall survival (OS) up to 35% (16, 22).

The second group is an "unresectable" group of patients with "bulky N2" disease, treated only with definitive chemo-radiotherapy, and with a 5-year OS less than 10% (23).

The third group of patients, "potentially resectable N2" disease, presents a pathological proven mediastinal lymph nodes at the baseline clinical staging, and they have a poor outcome with 5-year OS up to 16% (24), even if resectable.

While surgical resection is still a matter of debate for this last group of patients, the disappointing results reached after either primary surgery alone or radiotherapy alone (25, 26) led induction chemotherapy (IC) to be considered to increase resectability with the final hope of improving long-term survival rates.

After a number of randomized and multi-institutional trials (27-29) testing new drugs and different combinations of surgery, chemotherapy and radiotherapy, there are enough positive results to state that IC followed by surgery was able to further complete resection (R0) and mediastinal downstaging, improving the long-term survival of 5% at 5 years from 40% to 45%, with low morbidity and mortality rates in experienced centers (30).

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 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 (31, 32).

In terms of feasibility in NSCLC specimens and as previous reported, miRNA can be accurately analyzed in formalin-fixed paraffin-embedded (FFPE) and in fresh tissue biopsies (33).

In addition, miRNA are present in different biological fluids (blood, plasma, saliva and urine) and are altered in asymptomatic early stage lung cancer patients (34).

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 (35-37).

Despite the potential clinical value of miRNA signatures, the clinical utility of identified signatures has yet to be demonstrated.

For many years, mediastinoscopy has been considered the gold standard for mediastinal staging with high sensitivity and accuracy, but the procedure was progressively underused due to the high invasiveness, risk of complications and the need to be performed in experienced centers (38).

In the last years, a minimally invasive convex probe endobronchial ultrasound (EBUS) with the ability to perform real-time transbronchial needle aspiration (TBNA) has been described with high accuracy for mediastinal and hilar lymph node staging. (39)

Since then, EBUS-TBNA have gradually changed the way to perform mediastinal staging and in a short period improved its value with new indications in lung cancer management, becoming a standard of care. (40)

Recent studies showed that 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 (41).

2. OBJECTIVES

The aim of this study was to evaluate the feasibility of a high-throughput miRNA expression profile analysis of lung cancer lymph nodal metastasis (pN2) using primary cell lines established from EBUS-TBNA samples. To validate the EBUS-TBNA miRNA profile, the results were compared with those obtained from formalin-fixed paraffin-embedded (FFPE) NSCLC surgical biopsies obtained from mediastinoscopies.

In particular, we want to investigate the genomic profile of tumor specimen from patients with locally advanced disease (stage IIIA - pN2), who are eligible to neoadjuvant chemotherapy. The identification of cancer gene networks involved in mechanisms of chemotherapy resistance will highlight possible novel cancer biomarkers and therapeutic targets to increase survival for lung cancer patients with locally advanced disease.

3. PATIENTS AND METHODS

Research involves two phases: a retrospective study included two different phases (PHASE I and II) and a prospective study.

3.1 RETROSPECTIVE STUDY – PHASE I

3.1.1 Study design

Retrospective analysis of a population of patients with NSCLC - locally advanced - Stage IIIA (pN2), who underwent mediastinoscopy or EBUS-TBNA for the cyto-histological characterization of mediastinal adenopathy and sub sequential induction chemotherapy and surgical resection. Chemotherapy was performed as induction regimens containing cisplatin for a mean of 3 cycles and consecutive surgery.

The main objective was to identify patients with a complete lymph node response after chemotherapy, valuating the ypN2 after the radical surgical resection and identify factors affecting response and survival rates.

3.1.2 Objectives

The aim of this retrospective phase I was to analyze a series of “potentially resectable” stage IIIA-pN2

NSCLC, pathologically confirmed by mediastinoscopy or EBUS-TBNA, undergoing cisplatinum-based- neoadjuvant - induction chemotherapy (IC) followed by surgery, to evaluate short and long-

term outcomes and to identify prognostic factors to improve survival and to investigate future therapies.

3.1.3 Patients and methods

A Retrospective analysis of a cohort of patients with radiologically - computer tomography (CT) and positron emission tomography (PET) suspected of NSCLC with a lymph node involvement (N2) but "potentially resectable" after IC, that underwent mediastinoscopy or EBUS-TBNA for confirmation of pathological N2 (pN2) followed by induction chemotherapy and surgery resection with complete mediastinal and hilar lymphadenectomy with radical intent.

3.1.4 Results

Two hundred and eight seven (287) patients were included into the study and treated with induction chemotherapy.

After a mean of 3 cycles of cisplatin-based chemotherapy regimens, patients were revaluated with CT scan.

One hundred and fourth one (141; 49%) patients from those 287 initially included, were subjected to surgery with radical intent as being considered radiologically "responder" to inductive treatment.

One hundred twenty six (126) patients underwent radical surgery (15 patients underwent exploratory thoracotomies) - after a mean of 27 days from the last chemotherapy cycle and 113 patients had a complete radical surgery without residual disease (R0).

From 113 patients that underwent radical complete resection, 22 patients (17.5%) had a complete "N" response after chemotherapy evaluated by the number of lymph node stations (passing from an initial N2 to N0 disease confirmed by radical lymphadenectomy) and 8 patients (6.3%) had a complete answer also on the T (T0).

The overall mean survival was 24 months and 5-years survival was 30%.

Multivariate analysis shown that the number of CT cycles performed and the complete lymph node response (down staging) are independent prognostic factors of survival.

Table 1 to 5 shows the demographic data of the population and Figure 1 to 4 shows the survival data.

Table 1: Patients' clinical, surgical and pathological characteristics

	All patients N (%)	Surgical resection N (%)	Exploratory thoracotomy N (%)
All	**141 (100)**	**126 (100)**	**15 (100)**
Sex			
Men	104 (73.8)	92 (73.0)	12 (80.0)
Women	37 (26.2)	34 (27.0)	3 (20.0)
Age (at surgery)			
Median (range)	63 (35-80)	63 (35-80)	62 (36-78)
<50	13 (19.2)	12 (9.5)	1 (6.7)
50-59	35 (24.8)	33 (26.2)	2 (13.3)
60-69	66 (46.8)	55 (43.7)	11 (73.3)
70+	27 (19.1)	26 (20.6)	1 (6.7)
Number of induction CT cycles			
2-3 cycles chemotherapy	102 (72.3)	92 (73.0)	10 (66.7)
4-5 cycles chemotherapy	38 (27.0)	33 (26.2)	5 (33.3)
Unknown	1 (0.7)	1 (0.8)	-
Surgery			
Exploratory	15 (10.6)	-	15 (100)
Pneumonectomy	27 (19.1)	27 (21.4)	-
Lobectomy	96 (68.1)	96 (76.2)	-
Segmentectomy	3 (2.1)	3 (2.4)	-
Extent of resection			
R0	113 (80.1)	113 (89.7)	-
R1	11 (7.8)	11 (8.7)	-
R2	17 (12.1)	2 (1.6)	15 (100)
Histology			
NSCLC	6 (4.3)	-	6 (40.0)
Squamous carcinoma	48 (34.0)	46 (36.5)	2 (13.3)
Adenocarcinoma	79 (56.0)	72 (57.1)	7 (46.7)
Other	8 (5.7)	8 (6.3)	-
Pre-treatment stage			
IIIa	127 (90.1)	115 (91.3)	12 (80.0)
IIIb	14 (9.9)	11 (8.7)	3 (20.0)
Clinical T			
cT0	1 (0.7)	1 (0.8)	-
cT1	18 (12.8)	15 (11.9)	3 (20.0)
cT2	78 (55.3)	74 (58.7)	4 (26.7)
cT3	30 (21.3)	25 (19.8)	5 (33.3)
cT4	14 (9.9)	11 (8.7)	3 (20.0)
cN			
cN0	-	-	-
cN1	-	-	-
cN2	141	126	15
cM			
cM0	141	126	15
Postoperative stage			
Complete response	8 (5.7)	8 (6.3)	-
Ia/Ib	9 (6.4)	9 (7.1)	-
IIa/IIb	16 (11.3)	16 (12.7)	-
IIIa	84 (59.6)	84 (66.7)	-
IIIb	24 (17.0)	9 (7.1)	15 (100.0)
pT			
pT0	13 (9.2)	13 (10.3)	-
pT1	33 (23.4)	33 (26.2)	-
pT2	49 (34.8)	49 (38.9)	-
pT3	19 (13.5)	19 (15.1)	-
pT4	27 (19.1)	12 (9.5)	15 (100)
pN			
pN0	22 (15.6)	22 (17.5)	-
pN1	16 (11.3)	16 (12.7)	-

	pN2	103 (73.0)	88 (69.8)	15 (100)
M				
	M0	140 (99.3)	126 (100)	14 (93.3)
	M1	1 (0.7)	-	1 (6.7)
Adjuvant treatment				
	None	50 (35.5)	47 (37.3)	3 (20.0)
	Chemotherapy	5 (3.5)	5 (4.0)	-
	Radiotherapy	83 (58.9)	71 (56.3)	12 (80.0)
	Chemo-radiotherapy	3 (2.1)	3 (2.4)	-

Table 2: Postoperative complications after radical surgery (126 patients) according to the number of cycles of neoadjuvant chemotherapy*

	ALL N (%)	**2-3 cycles chemotherapy N (%)**	**4-5 cycles chemotherapy N (%)**
All patients	**126 (100)**	**93 (73.8)**	**33 (26.2)**
MAJOR complications	14 (11.1%)	12 (12.9%)	2 (6.1%)
ARDS/ pneumonia/ respiratory failure	6 (4.8%)	5 (5.4%)	1 (3.0%)
Rethoracotomy:	5 (4.0%)	5 (5.4%)	-
- *Hemothorax*	3 (2.4%)	3 (3.2%)	-
- *Cardiac dislocation*	1 (0.8%)	1 (1.1%)	-
- *empyema*	1 (0.8%)	1 (1.1%)	-
Fistula	2 (1.6%)	1 (1.1%)	1 (3.0%)
Renal failure	3 (2.4%)	3 (3.2%)	-
Chylothorax	1 (0.8%)	1 (1.1%)	-
Gastric bleeding	1 (0.8%)	1 (1.1%)	-
MINOR complications	48 (38.1%)	32 (34.4%)	16 (48.5%)
Arrhythmia	21 (16.7%)	14 (15.1%)	7 (21.2%)
Anemia	18 (14.3%)	12 (12.9%)	6 (18.2%)
Atelectasia/ FBS	7 (5.6%)	6 (6.5%)	1 (3.0%)
Prolonged air leak	10 (7.9%)	5 (5.4%)	5 (15.2%)
Dysphonia	1 (0.8%)	1 (1.1%)	-

*several patients reported more than one postoperative complication.
§: re-thoracotomies included hemothorax in 3 cases, cardiac dislocation in 1 case, and empyema in 1.

Table 3: Univariate analysis of the pre and post-surgery variables

	Hazards Ratio (95% CI)	p-value
Number of chemotherapy cycles		
2-3 cycles chemotherapy	1.00	
4-5 cycles chemotherapy	0.47 (0.26-0.85)	0.01
Surgery		
Pneumonectomy	1.00	
Lobectomy	0.68 (0.41-1.13)	0.14
Segmentectomy	1.22 (0.28-5.22)	0.79
Extent of resection		
R0	1.00	
R1/R2	1.39 (0.74-2.63)	0.31
Histology		
Adenocarcinoma	1.00	
Squamous	1.29 (0.83-2.03)	0.26
Other	1.34 (0.53-3.41)	0.54
Pre-treatment stage		
IIIa	1.00	
IIIb	1.42 (0.71-2.84)	0.32
Clinical T		
cT0/cT1*	1.00	
cT2	1.59 (0.78-3.24)	0.20
cT3	1.28 (0.56-2.90)	0.56
cT4	2.00 (0.79-5.05)	0.14
Post-operative stage		
Complete response	1.00	
Ia/Ib	1.84 (0.41-8.28)	0.42
IIa/IIb	3.00 (0.82-11.0)	0.10
IIIa	3.39 (1.05-10.9)	0.04
IIIb	10.6 (2.64-42.2)	0.0009
pT		
pT0	0.36 (0.13-0.98)	0.04
pT1	1.00	
pT2	1.27 (0.72-2.23)	0.42
pT3	1.95 (1.00-3.81)	0.05
pT4	3.76 (1.71-8.28)	0.001
pN		
pN0	1.00	
pN+	2.10 (1.11-4.00)	0.02
pN0	1.00	
pN+	1.77 (0.90-3.49)	0.10
pN+ (multiple N2)	2.72 (1.36-5.44)	0.005
Adjuvant treatment		
None	1.00	
Chemotherapy	1.03 (0.25-4.38)	0.96
Radiotherapy	1.10 (0.69-1.76)	0.68
Chemo-radiotherapy	1.58 (0.37-6.71)	0.53

Hazards ratios and 95% confidence intervals (CIs) obtained from univariate Cox proportional hazards regression model.

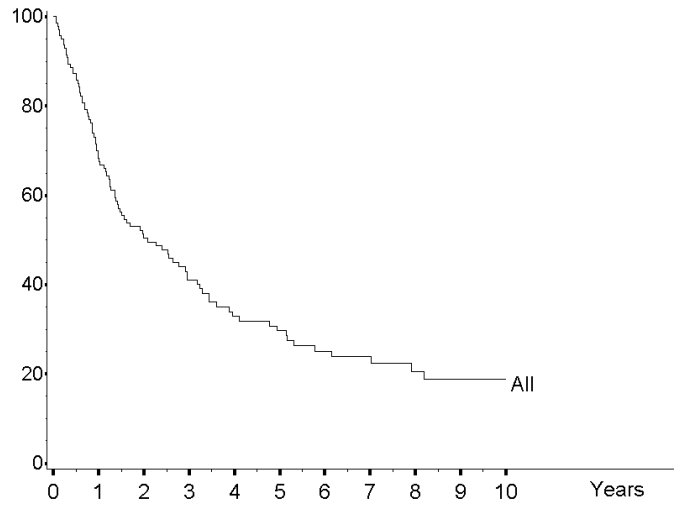
Table 4: Univariate and multivariate analysis

	Univariate		Multivariate	
Variable	**HR (95% CI)**	**P-value**	**HR (95% CI)**	**P-value**
PN+ (multiple N2)	1.00		1.00	
PN+	0.65 (0.41-1.04)	0.07	0.57 (0.35-0.92)	0.02
PN0	0.37 (0.18-0.74)	0.005	0.40 (0.20-0.80)*	0.01
2-3 cycles CT	1.00		1.00	
4-5 cycles CT	0.47 (0.26-0.85)	0.01	0.46 (0.25-0.85)	0.01

*the multivariate risk estimate is HR=0.54 (95%CI 0.25-1.19) for patients pN0 with residual disease and HR=0.23 (95%CI 0.07-0.77) for patients who achieved a complete pathological response (pN0 and pT0).

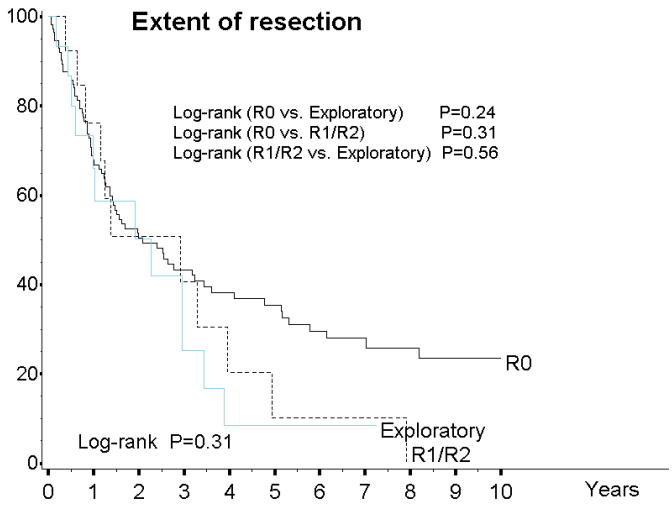
Figure 1: Overall survival of all 141 patients according to extent of resection (A,B). Figure 1C shows the overall survival of 146 patients who were not candidates for surgery but had definitive chemotherapy or chemo/radiotherapy

A)



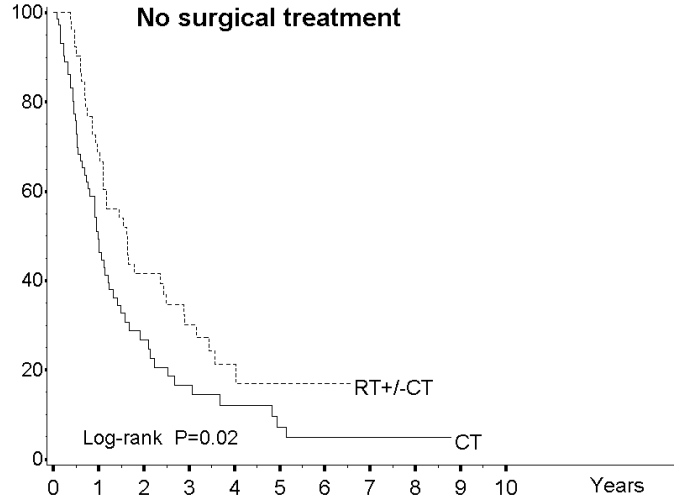
	Baseline	1-year	2-year	3-year	4-year	5-year	10-year
Number at risk							
All	141	86	59	42	31	27	5
Overall survival							
All	100%	68%	50%	41%	33%	30%	19%

B)



	Baseline	1-year	2-year	3-year	4-year	5-year	10-year
Number at risk							
R0	113	68	47	35	28	25	5
R1/R2	13	9	6	4	2	1	0
Exploratory	15	9	6	3	1	1	0
Overall survival							
R0	100%	68%	50%	43%	38%	35%	23%
R1/R2	100%	76%	58%	42%	21%	10%	-
Exploratory	100%	65%	50%	25%	8%	8%	-

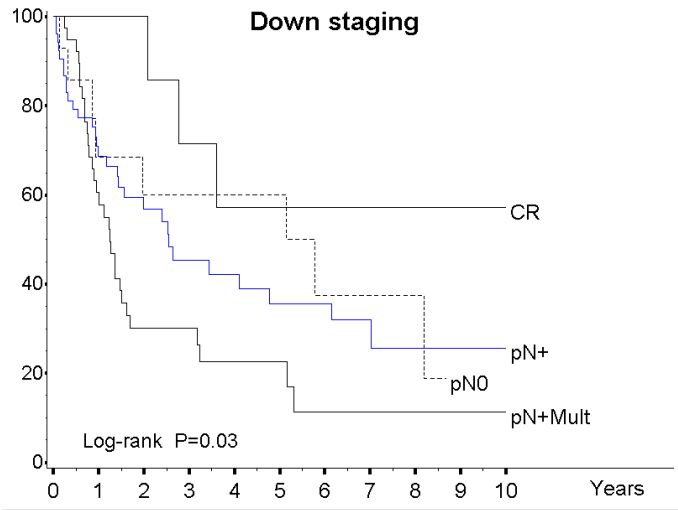
C)



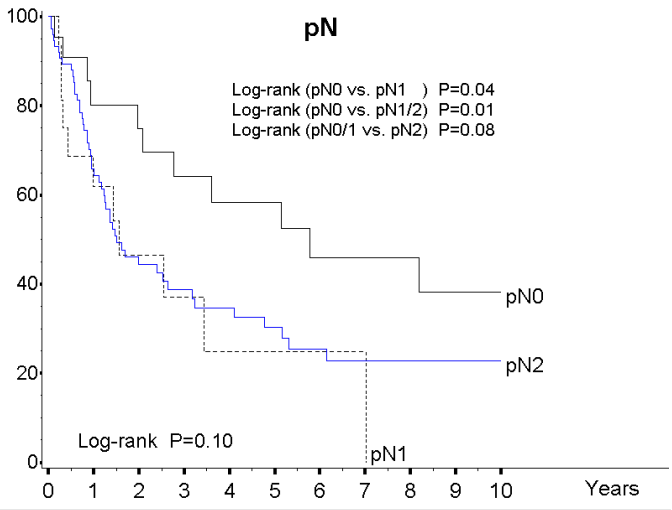
	Baseline	1-year	2-year	3-year	4-year	5-year	10-year
Number at risk							
CT	82	31	13	8	5	3	0
CT+/-RT*	53	33	18	13	5	2	0
Overall survival							
CT	100%	54%	29%	18%	13%	8%	-
CT+/-RT*	100%	69%	41%	29%	21%	16%	-

*6 patients received radiotherapy (RT) without chemotherapy (CT) and 47 received CT and RT

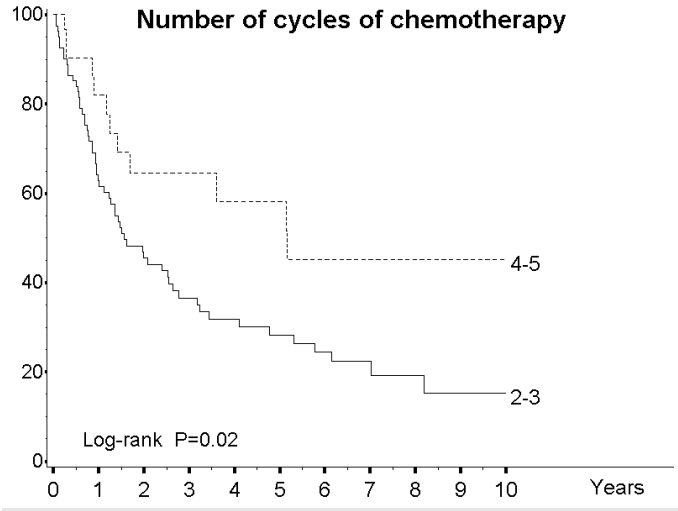
Figure 2: Overall survival of 113 patients after R0 resection according to selected characteristics: downstaging, pN after resection, number of cycles of chemotherapy and number of cycles of chemotherapy and pN



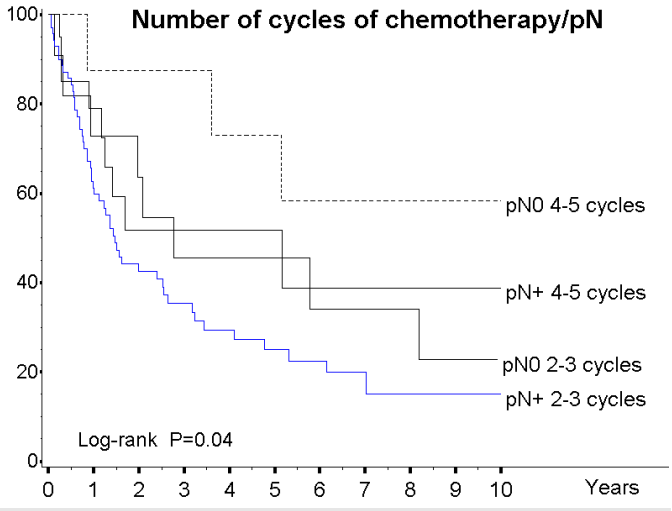
	Baseline	1-year	2-year	3-year	4-year	5-year	10-year
Number at risk							
CR	8	7	7	5	4	4	1
pN0	14	8	7	7	6	6	0
pN+	53	31	23	15	13	11	2
pN+Mult	38	22	10	8	5	4	2
Overall survival							
CR	100%	100%	100%	71%	57%	57%	57%
pN0	100%	69%	61%	61%	61%	61%	39%
pN+	100%	68%	57%	46%	43%	36%	25%
pN+Mult	100%	60%	29%	29%	22%	22%	11%



	Baseline	1-year	2-year	3-year	4-year	5-year	10-year
Number at risk							
pN0	22	15	14	12	10	10	1
pN1	16	9	6	4	2	2	0
pN2	75	44	27	19	16	13	4
Overall survival							
pN0	100%	81%	75%	64%	59%	46%	37%
pN1	100%	61%	47%	38%	27%	27%	27%
pN2	100%	65%	44%	38%	34%	30%	22%



	Baseline	1-year	2-year	3-year	4-year	5-year	10-year
Number at risk							
2-3	81	49	33	23	19	16	1
4-5	31	19	14	12	9	9	4
Overall survival							
2-3	100%	63%	45%	37%	32%	28%	15%
4-5	100%	82%	64%	64%	58%	58%	45%



	Baseline	1-year	2-year	3-year	4-year	5-year	10-year
Number at risk							
pN0 2-3	11	8	7	5	5	5	1
pN0 4-5	11	7	7	7	5	5	1
pN+ 2-3	70	41	26	18	14	11	1
pN+ 4-5	20	12	7	5	4	4	3
Overall survival							
pN0 2-3	100%	73%	64%	46%	46%	46%	24%
pN0 4-5	100%	90%	90%	90%	76%	76%	61%
pN+ 2-3	100%	61%	42%	35%	29%	25%	15%
pN+ 4-5	100%	78%	51%	51%	51%	51%	38%

3.2 RETROSPECTIVE STUDY – PHASE II

Departing from the results of the first retrospective phase study, we decide to evaluate the possibility of predicting the oncological results obtained in patients with full chemotherapy response that showed the best surprising 5-year survival.

For this purpose, in order to evaluate the presence of a predictive genetic different signature in patients responsive to chemotherapy, we performed DNA, RNA and microRNA extraction from a group of mediastinoscopy FFPE specimens in patients that underwent the multimodal treatment described in the phase I.

3.2.1 DNA and RNA extraction

RNA extraction using FFPE specimens was performed with the All-Prep DNA/RNA FFPE kit (Qiagen) to extract both total RNA (including miRNA) and DNA from micro-fiber FFPE tissue sections (Fig 3A).

The extraction was subsequently performed from different amounts of micro-dissected tissue in order to optimize and establish the RNA quantity. The results of RNA extraction according to different quantity of micro-dissected tissue is shown in figure 3B.

Different amounts of total RNA extracted were retro-transcript to determine the minimum quantity of material required to obtain a reliable TLDA (Taqman low-density array) miRNA profile (Figure 3C).

We verified that even at low total RNA amount in the retro-transcription reaction input (5 ng) the miRNA profile obtained was equivalent to other profiles obtained from a panel of 42 controls miRNA (figure 3D) showing that the analysis is reliable also in very small amount of tissue samples.

3.2.2 Evaluation of miRNA expression

We used the optimized protocol to identify the miRNA expression in an initial series of seven patients with NSCLC (Stage IIIA – pN2) treated with neoadjuvant chemotherapy followed by radical surgical resection.

Four patients had a complete lymph node downstage after chemotherapy, with a pN0 after radical surgery – those patients were considered “responders” to chemotherapy.

Three patients had no lymph node downstage after induction chemotherapy and remained pN2 after surgery – those patients were considered “not-responders”.

3.2.3 "Proof-of-principle" experimental results

We profiled 378 miRNAs and 105 of these were detected in at least 6 of the 7 patients.

The hierarchical clustering analysis revealed a clear separation of “responders” (pN0 after surgery) from “non-responders” (pN2 after surgery) patients, based on the expression profile of this miRNA series (Figure 3E).

These results supported the feasibility of identifying a “signature” of miRNA that could be involved in the chemotherapy response in patients with Stage IIIA NSCLC.

Figure 3.

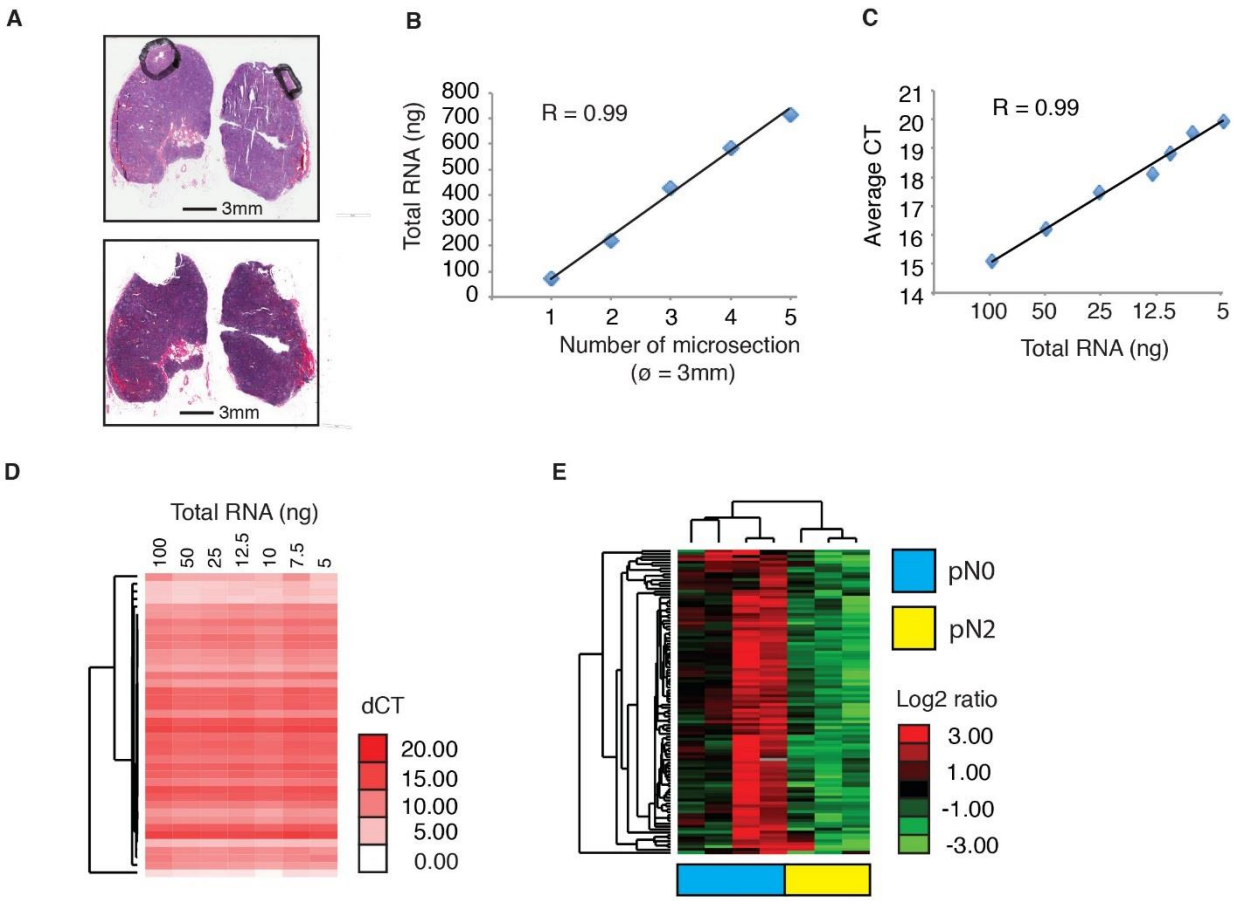


Figure 3: A) Two hematoxylin-eosin-stained microscopic images of the same tissue sample before (mediastinal lymphnodes) and after (below) microdissection of the tissue area containing tumor cells. Scale bars are shown below the pictures (black bar). B) Total RNA extracted from increasing numbers of tissue microsections (3 mm in diameter). C) Quantitative real-time PCR analysis of miRNAs using increasing amounts of total RNA input extracted from microdissected FFPE tissue sections (CT, threshold cycle; average CT, average of 42 miRNAs used as control). D) Hierarchical clustering analysis of miRNA expression profiles obtained using different amounts of total RNA as in (C). E) Hierarchical clustering analysis of 105 miRNA expression profiles of a first set of mediastinoscopies of patients with stage IIIA lung cancer, before induction chemotherapy (color codes indicate chemosensitive patients, pN0 (blue), or chemoresistant patients, pN2 (yellow).

3.3 PROSPECTIVE STUDY

3.3.1 Background

Departing from the results of the retrospective phase of the study and considering that nowadays all patients with suspect NSCLC with mediastinal involvement underwent EBUS-TBNA to confirm the pathological data, the prediction of response to chemotherapy should be ideally predicted in this seating to better identify patients who can benefit from the neoadjuvant treatment followed by surgery.

3.3.2 Objectives

To evaluate the feasibility of culture cell lines obtained from EBUS-TBNA specimens and the DNA and RNA extraction and consecutive high-throughput miRNA expression profile analysis of lung cancer lymph nodal metastasis.

To validate the EBUS-TBNA miRNA profile, the results were compared with those obtained from formalin-fixed paraffin-embedded (FFPE) NSCLC surgical biopsies obtained from a series of mediastinoscopies.

3.3.3 Patients and Methods

3.3.3.1 Patients selection

Patients with suspect Stage IIIA - NSCLC that underwent routine EBUS-TBNA for the diagnosis of suspect lymph node metastasis (pN2) were included into the study.

Informed consent for the procedure was obtained from each patient.

All patients were evaluated with computer tomography (CT) and positron emission tomography (PET) before EBUS-TBNA procedures. Mediastinal lymph nodes were considered suspected when the short axis was larger than 10 mm and there were a PET scan uptake.

Cytological specimens were collected for NSCLC epithelial cell culture and subsequent transcriptomic miRNA expression analysis.

3.3.3.2 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 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) provided by the cytopathologist.

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 separated into a culture basal medium for primary cell cultures.

3.3.3.3 EBUS-TBNA 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, 50ng/ml L-glutamine, 100U/mL penicillin, 100µg/mL streptomycin, 10µg/mL gentamicin, 0.5 µg/mL amphotericin B, 10µg/mL human transferrin, 1 µg/mL human insulin, 1µg/mL hydrocortisone, 10mM Hepes pH 7.5, 50µM L-ascorbic acid, 15nM sodium selenite, 0.1mM ethanolamine, and 50 ng/mL cholera toxin.

EBUS-TBNA samples were spun down for 5 min at 1000g at RT, resuspended in 3 ml of complete medium further supplemented with 10nM epidermal growth factor EGF, 35µg/mL bovine pituitary extract, and 10nM triiodothyronine, and cultured on six-well collagen I-coated plates (Collagen Cellware, Biocoat, Corning) in a humidified incubator with 5% CO2.

Primary cells were grown for six to 12 days and washed twice with PBS prior to total RNA extraction.

3.3.3.4 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 1x PBS for 10 min, followed by one wash with 1x PBS.

Blocking was carried out with 2% BSA for 30 min. Primary antibodies were added and left for 1h. Following two washes with 1x PBS, secondary antibodies were added for 30 min (light protected),

then another two washes with 1x 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 1x PBS.

A final post-fixing was done with 2% PFA followed by one final wash with 1x PBS.

Slides were mounted and analyzed.

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 1:1000; 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.

3.3.3.5 RNA/miRNA extraction and quantitative real-time PCR analysis

The total RNA was extracted from EBUS-TBNA primary cells using the All-Prep DNA/RNA/miRNA Universal Kit and from FFPE tissue samples obtained by mediastinoscopy using the All-Prep 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 micro-dissected areas of one to two tissue sections (5-10 μm thick) on glass slides with adequate tumor cellularity (>60%), selected by a pathologist.

Total RNA (200 ng) extracted from EBUS-TBNA-derived primary cells was measured using the NanoDrop® ND-1000 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) 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 Supplemental Table 1.~~

Real-time quantitative PCR analysis (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 seconds and final cooling at 40°C for 30 s.

For each miRNA 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-ΔCq 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 v2.1 (Thermo Fisher Scientific) and TaqMan® MicroRNA reverse transcription kit (Thermo Fisher Scientific) according to the manufacturer's instructions; 5µl of reverse transcribed product were pre-amplified for 14 cycles using the TaqMan PreAMP Mastemix and Megaplex PreAMP primers PoolA 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 (ThermoFisher 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 PanelA v 2.0 (ThermoFisher 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 (42). Hierarchical clustering analysis was performed using Cluster 3.0 for Mac OS X (http://bonsai.hgc.jp/wmdehoon/software/clus- ter/) and Java Treeview (http://jtreeview.sourceforge.net). Spearman rank correlation and centroid clustering methods were used on Cqn data.

4. RESULTS

4.1 Preliminary results I

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 4A). Real-time quantitative PCR analysis (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 4B), while the EBUS-TBNA samples were positive to the expression of non-epithelial markers (Figure 4B).

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 4C).

These data confirmed the successful establishment of lung epithelial cells from EBUS-TBNA specimens and the analysis of the miRNA expression profile of three independent EBUS-TBNA primary cell lines using TaqMan® Array Human MicroRNA Card A, allowed the screening of 377 different human miRNAs; 150 miRNAs (~40% of the total analysable) were detected (with Cqn≤30) in all three cell lines. Relative quantities of miRNAs detected in all three EBUS-primary cell lines ranged from 22 to 27 Cqn (i.e. the 25th and 75th quartile intervals) (Figure 5A).

The analysis of the miRNA expression profile of micro-dissected formalin-fixed lung cancer tissue – FFPE mediastinoscopy samples (MED) evaluated in also 3 cases, compared with the profile of EBUS-primary cells (Figure 5A) showed a slight decrease in the average number of miRNA detected (i.e. with Cqn≤30) in all three FFPE samples (133 vs. 150; Figure 5A). This was probably due to a partial degradation of some miRNA species in FFPE samples.

A total of 117 miRNA (~78% in EBUS-TBNA primary cell lines and ~88% 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 5B).

FIGURE 4.

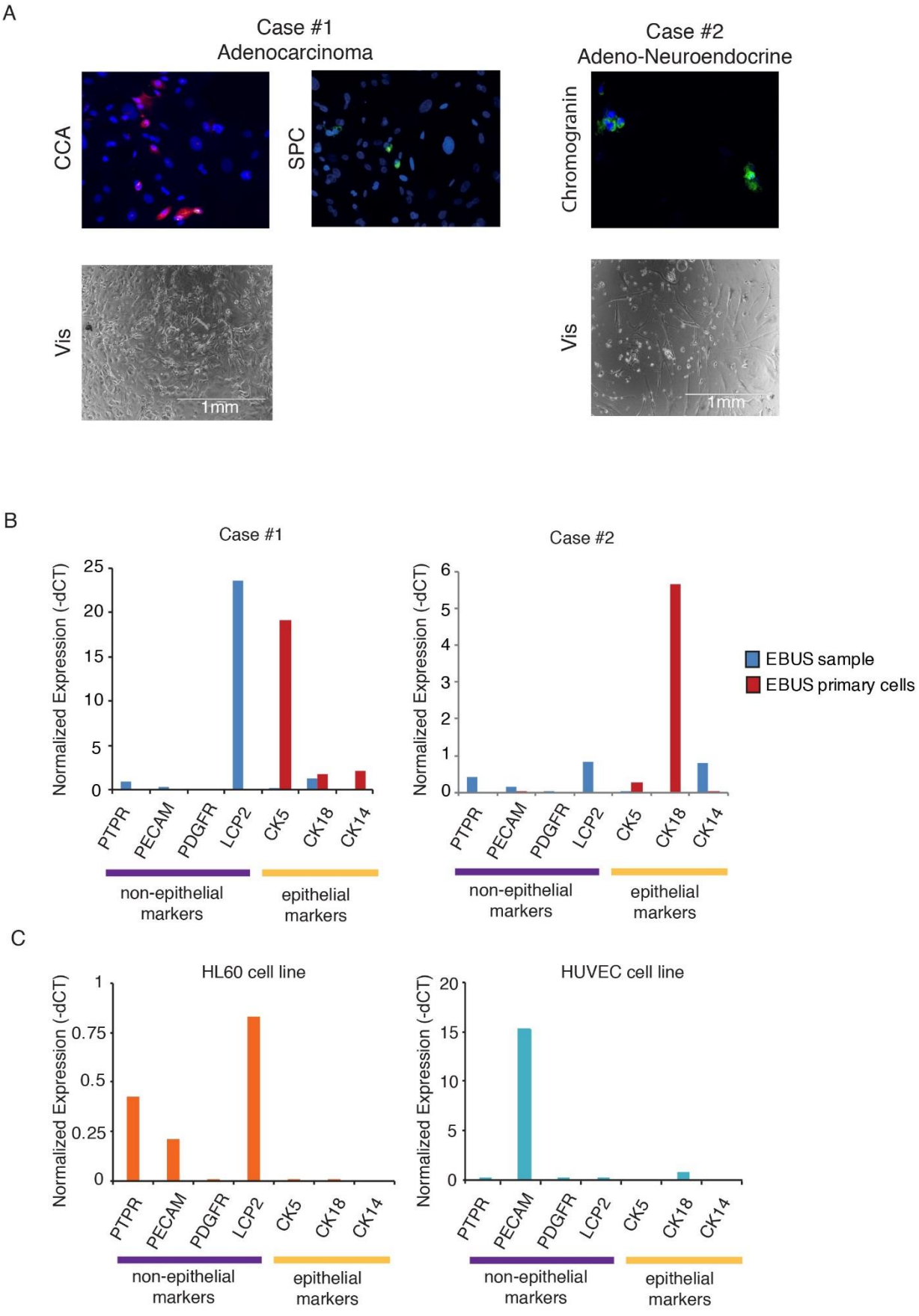

A
Case #1
Adenocarcinoma
Case #2
Adeno-Neuroendocrine
CCA
SPC
Chromogranin
Vis
Vis
1mm
1mm
B
Case #1
Case #2
Normalized Expression (-dCT)
EBUS sample
EBUS primary cells
PTPR
PECAM
PDGFR
LCP2
CK5
CK18
CK14
non-epithelial markers
epithelial markers
C
HL60 cell line
HUVEC cell line
Normalized Expression (-dCT)
PTPR
PECAM
PDGFR
LCP2
CK5
CK18
CK14
non-epithelial markers
epithelial markers

Figure 4) 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**. 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.** 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.

FIGURE 5.

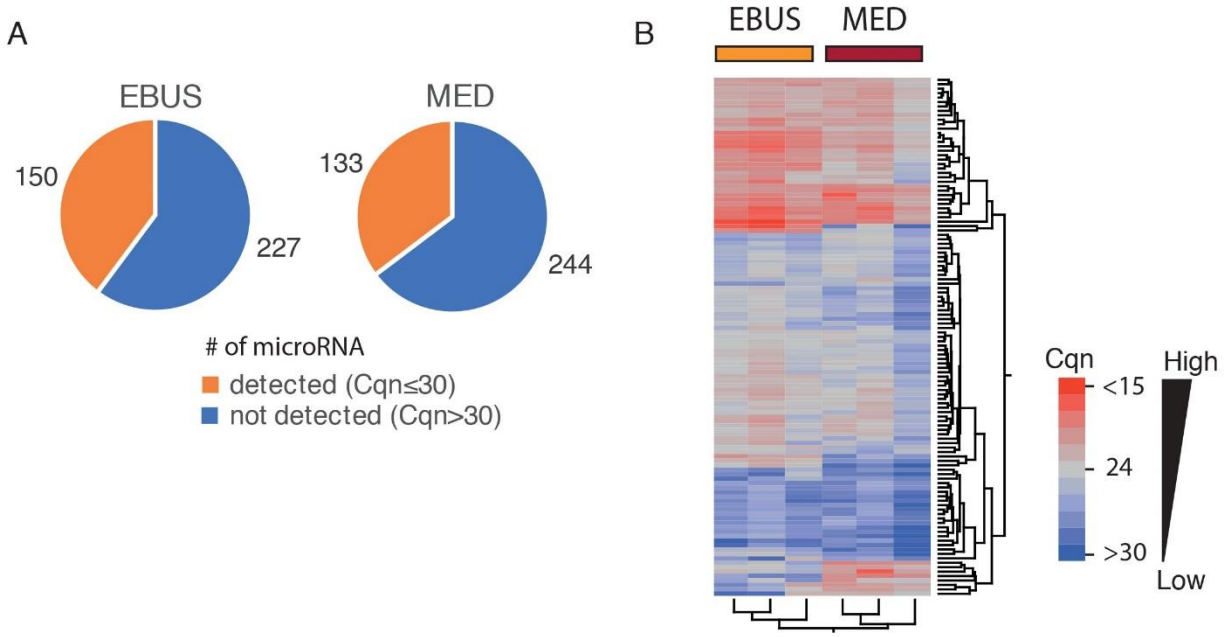


Figure 5) A. Pie charts of the number of detected (Cqn≤30; in orange), or undetected (Cqn>30; in blue) microRNA in all the primary cells obtained from EBUS (N=3) or in all mediastinoscopy FFPE samples, i.e. "MED" samples (N=3). **B.** Hierarchical cluster analysis of the expression profile of the 117 commonly detected miRNAs in EBUS (N=3) and MED samples (N=3). Heat map indicates the expression level of each individual miRNA analyzed. Normalized Cq values were colour-coded and described by the scale bar.

4.2 Preliminary results II

The first analysis of microRNA extraction in an initial cohort of seven patients, showed a strong different miRNA profile between patients responders to chemotherapy and patients not-responders.

Figure 6.

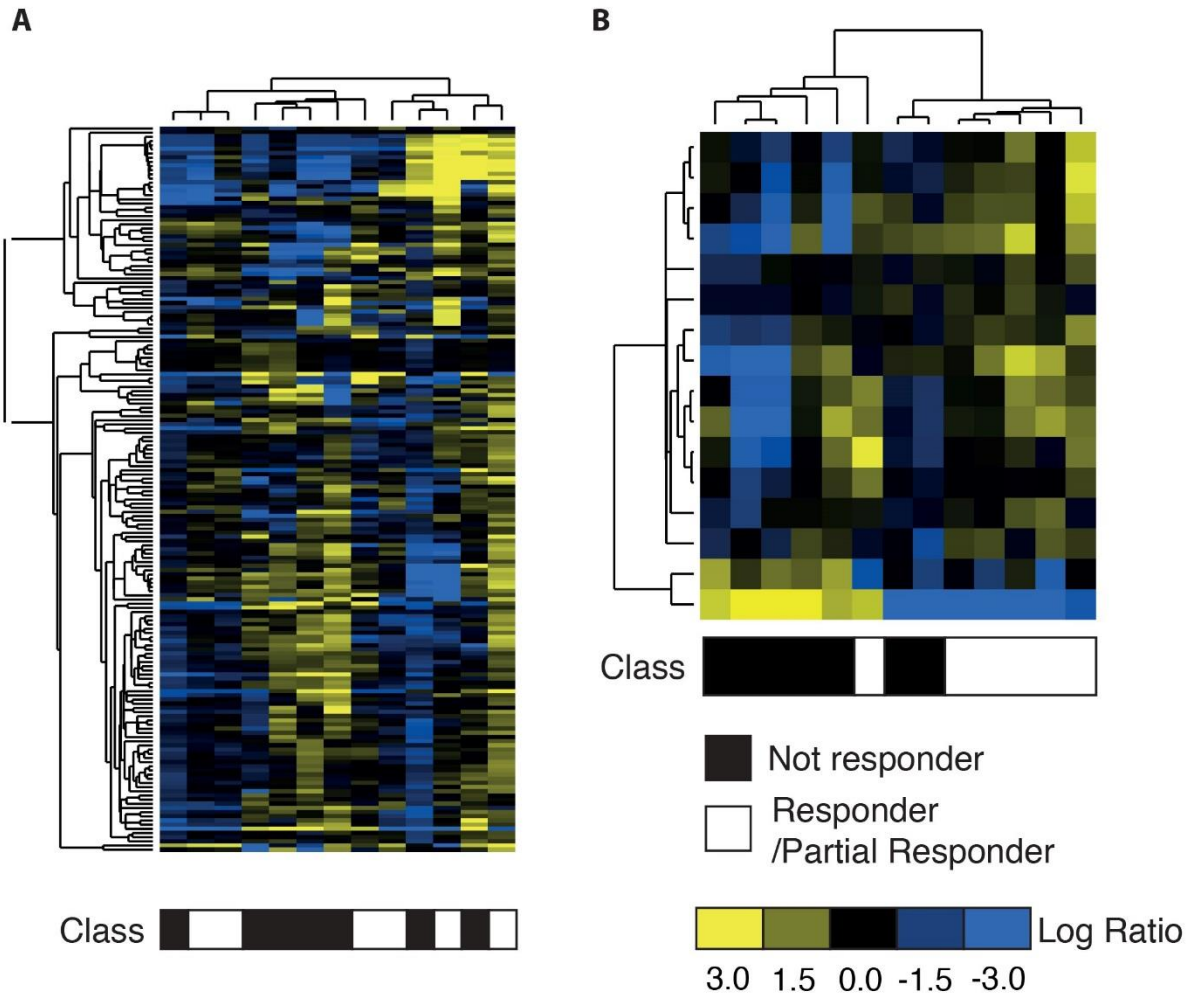


Figure 6. A. Cluster analysis of all the microRNAs expressed (N=174) in the EBUS samples. B. Subset of microRNAs most discriminating between responder/partial responder vs. not responder patients to neoadjuvant chemotherapy.

In yellow, more expressed microRNAs. In blue, less expressed microRNA. Value of microRNAs expression were log2 transformed before clustering; color codes indicate relative microRNAs expression.

4.3 Final results

A total of 82 patients underwent EBUS-TBNA for the confirmation of pathological lymph node involvement (Stage IIIA - pN2 NSCLC).

In 38 (46.3%) was possible to obtain primary lung cancer cell cultures and in 19 (23.2%) patients was possible to proceed with consecutive DNA, RNA extraction and microRNA profiling.

The percentage of success of RNA extraction from primary EBUS-TBNA epithelial cell cultures was about 50%.

Table 5 shows patients' characteristics and pathological EBUS-TBNA results.

Ten (10) out of 19 (53%) patients completed the treatment with neoadjuvant chemotherapy and surgery being possible to evaluate the lymph node down staging after surgery.

Table 6 shows the type and number of chemotherapy cycles and the final follow-up with the lymph node down staging after surgery.

Table 7 shows the quantity of DNA and RNA extracted from each EBUS-TBNA primary culture cells sample.

MicroRNA expression profile results:

Analysis of the miRNA expression profile of three independent EBUS-TBNA-derived primary cell lines using TaqMan® Array Human MicroRNA Card A allowed the screening of 377 different human miRNAs; 150 miRNAs (~40% of the total analyzable) were detected (with Cqn≤30) in all three cell lines. Relative quantities of miRNAs detected in all three EBUS-primary cell lines ranged from 22 to 27 Cqn (i.e. the 25th and 75th quartile intervals).

To better homogenise the casuistic, we compared the results of miRNA profile founded in EBUS-TBNA primary cell cultures with 10 cases of FFPE from mediastinoscopies (5 responders and 5 not-responders). The results of the analysis showed a strong similarity between the two profiles.

A total of 117 miRNA (~78% in EBUS-TBNA primary cell lines and ~88% 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.

A series of 13 most promising differentially expressed miRNA were identified and highlighted in a comparison table that is shown in figure 7.

Figure 8 shows the hierarchical clustering of microRNA expression profile of 117 microRNA commonly expressed in EBUS-TBNA biopsy specimens (N = 20) – on the left - or mediastinoscopy (MED) – on the right. The heat map indicates the level of expression (log2 Ratio) of each single microRNA (green, less expressed, red, more expressed) in the analyzed samples. Bottom, yellow samples of patients who responded to neoadjuvant therapy and in black those who did not respond.

Figure 9 shows the hierarchical clustering expression profile of a 13 differentially expressed microRNA ($p < 0.05$) set in EBUS-TBNA samples of patients responders to neoajuvant chemotherapy vs. not-responders. On the left, the expression level logic (log2 Ratio) of each single microRNA (green, less expressed, red, more expressed) in the EBUS-TBNA samples. On the right, heatmap of the expression profile in the mediastinoscopy samples (MED). Bottom, yellow samples of patients who responded to neoadjuvant chemotherapy, while in black those who did not respond.

Table 5. Patient's characteristics and EBUS-TBNA pathological results

PATIENTS	AGE	SEX	PATHOLOGICAL EBUS-TBNA RESULTS
1	72	Male	squamous cell carcinoma
2	64	Male	NSCLC
3	62	Male	squamous cell carcinoma
4	69	Male	squamous cell carcinoma
5	73	Male	adenocarcinoma
6	66	Male	squamous cell carcinoma
7	72	Male	adenocarcinoma
8	55	Male	adenocarcinoma
9	64	Male	adenocarcinoma
10	61	Male	adenocarcinoma
11	67	Male	squamous cell carcinoma
12	53	Female	adenocarcinoma
13	66	Female	adenocarcinoma
14	76	Male	squamous cell carcinoma
15	75	Male	poorly differentiated adenocarcinoma
16	57	Female	Poorly differentiated adenocarcinoma
17	69	Male	Poorly differentiated adenocarcinoma
18	74	Female	Poorly differentiated adenocarcinoma
19	74	Male	Adenocarcinoma

Table 6. Type and number of chemotherapy cycles and follow-up after surgery

Patient	Type of chemotherapy	Number of cycles	Type of surgery	Surgical results	Lymph node down staging
1	cisplatin + gemcitabine	3	Pneumonectomy	ypT3 ypN2	NO
2	No	0	ND	ND	ND
3	cisplatin + gemcitabine	3	Explorative Thoracotomy	ND	ND
4	cisplatin + gemcitabine	4	Lobectomy	ypT1a ypN0	YES
5	cisplatin + gemcitabine	3	ND	ND	ND
6	cisplatin + gemcitabine	3	ND	ND	ND
7	cisplatin + gemcitabine	3	Lobectomy	ypT2a ypN2	NO
8	cisplatin + gemcitabine	3	Pneumonectomy	ypT4 ypN2	NO
9	cisplatin + gemcitabine	4	Lobectomy	ypT2a ypN0	YES
10	cisplatin + pemetrexed	3	ND	ND	ND
11	cisplatin + gemcitabine	1	ND	ND	ND
12	cisplatin + gemcitabine	3	Lobectomy	ypT3 ypN2	NO
13	cisplatin + gemcitabine	3	Pneumonectomy	ypT0 ypN2	NO
14	cisplatin + gemcitabine	3	Lobectomy	ypT0 ypN0	YES
15	cisplatin + gemcitabine	3	ND	ND	ND
16	cisplatin + gemcitabine	3	Bilobectomy	ypT2a ypN2	NO
17	cisplatin + gemcitabine	3	ND	ND	ND
18	cisplatin + gemcitabine	4	Lobectomy	ypT3 ypN1	YES
19	cisplatin + gemcitabine	3	ND	ND	ND

Table 7. Total quantity of DNA and RNA extracted from each EBUS-TBNA cell cultures misured with Ribogreen (for RNA) and Picogreen (for DNA).

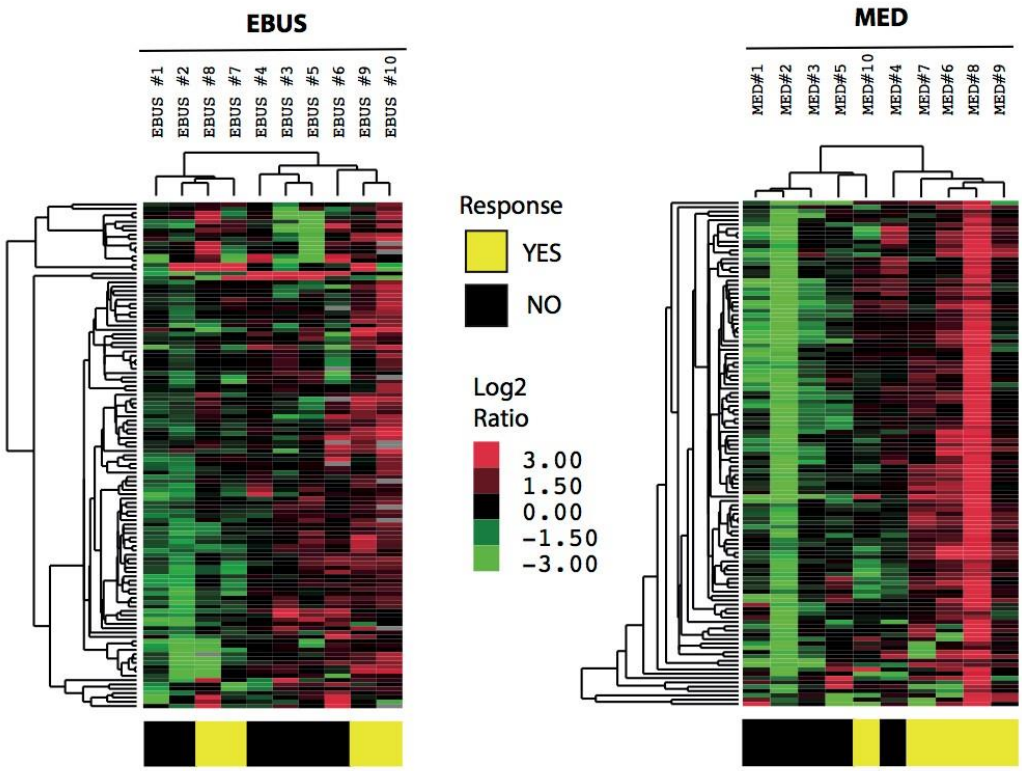
Patients	**[RNA](ng/ul)**	**TOT RNA(ng)**	**[DNA] (ng/ul)**	**TOT DNA(ng)**
1	30,87	1698	2,98	298
2	6,23	62,9	0,4	40
3	72,86	4007	6,92	692
4	54,67	3006	5,92	592
5	9,50	95,05	0,338	33,8
6	38	2090	3,66	366
7	6,05	60,51	0,106	10,6
8	175,67	9661	23,2	2320
9	15,64	859,95	2,28	228
10	9,84	541,03	1,87	187
11	142,91	8574,6	6,94	694
12	3,16	31,63	0,29	29
13	209,75	11536,28	19,7	1970
14	3,62	199,33	0,174	17,4
15	18,63	1024,82	1,71	171
16	161,14	8862,75	100	10000
17	165,12	9081,77	30,2	3020
18	328	19680	47,4	4740
19	38,28206837	2296,924102	4,86	486

Figure 7. Normalized expression data (small-nuclear RNA - U6 - was used as housekeeping) of the 13 microRNAs in EBUS (above) and MED (below) samples. Data is shown in CQ (quantification - real-time qPCR- cycle value) form. The statistical significance of the expression difference in "risponders" compared to "not-risponders" patients is reported on the right (p-value; Student's "t" test), along with the mean difference in logarithmic expression (Log2 Ratio).

RESPONSE	NO	NO	NO	NO	NO	NO	YES	YES	YES	YES		
ID	**EBUS #1**	**EBUS #2**	**EBUS #3**	**EBUS #4**	**EBUS #5**	**EBUS #6**	**EBUS #7**	**EBUS #8**	**EBUS #9**	**EBUS #10**	**p-value**	**EBUS Log Ratio**
MIR_#89	24,2580358	24,8715358	24,2430358	22,8735358	23,8032858	24,1566978	22,1710358	23,4685358	22,7895358	21,6660308	**0,01092948**	-1,72
MIR_#60	22,4240358	22,8415358	23,4910358	21,6895358	23,6232858	24,8637988	21,9290358	21,6835358	20,6565358	19,6132118	**0,01417213**	-2,00
MIR_#99	23,7460358	23,2215358	23,3110358	22,5815358	23,9952858	21,7095478	22,7040358	21,3115358	20,4155358	20,9995048	**0,01633716**	-2,11
MIR_#2	25,9980358	26,0855358	25,5170358	25,2935358	28,1252858	23,5285078	23,5120358	22,4545358	24,0185358	23,6465068	**0,01903621**	-2,18
MIR_#70	19,7480358	20,0815358	19,1050358	18,9865358	21,0762858		19,1270358	18,3635358	16,4985358	16,7091228	**0,01967592**	-2,21
MIR_#88	26,9880358	27,5795358	26,2350358	25,6775358	27,1012858	25,9000378	25,9460358	25,7455358	25,3195358	24,1893608	**0,03200948**	-1,08
MIR_#105	25,9640358	27,1545358	27,6600358	26,2345358	29,0932858	26,6766578	26,6790358	25,6635358	24,3795358	24,1522848	**0,03343142**	-1,89
MIR_#49	24,7830358	25,0785358	26,0000358	24,4485358	25,3142858	24,6207578	24,4220358	24,6945358	23,6805358	22,6417148	**0,03416368**	-0,88
MIR_#36	22,7730358	22,9915358	22,0270358	22,1275358	22,5462858	23,2413758	21,5990358	22,5155358	21,5225358	19,9834968	**0,03591171**	-1,10
MIR_#10	22,5550358	22,3005358	23,3590358	21,6815358	21,6822858	21,9105178	20,6940358	20,9575358	22,3525358	20,0857578	**0,04056355**	-1,28
MIR_#113	24,4450358	24,8175358	25,6780358	23,7465358	23,2742858	25,0059248	23,9080358	24,0115358	23,3685358	21,6185128	**0,07708431**	-0,99
MIR_#1	21,0390358	20,7665358	20,3810358	19,5395358	20,6472858	19,4752228	20,8420358	18,8425358	18,3045358	18,9817098	**0,08891307**	-1,60
MIR_#95	20,7570358	20,3485358	20,1150358	19,2435358	21,2072858	18,5788928	20,3000358	18,9045358	17,6605358	18,3090608	**0,09828997**	-1,62

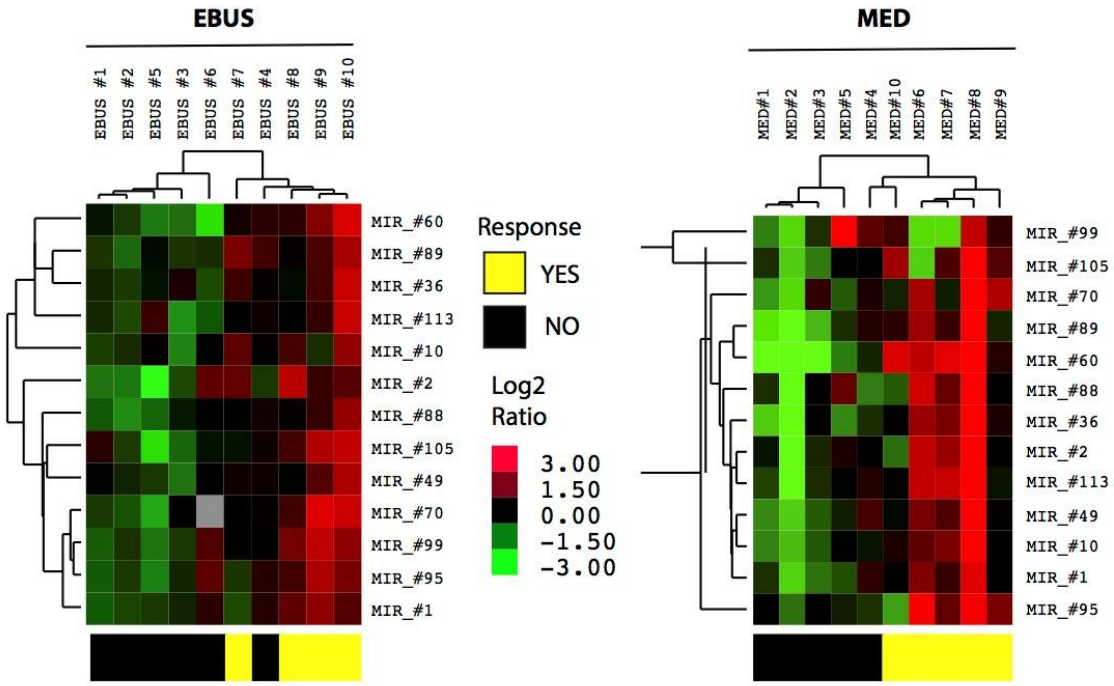
RESPONSE	NO	NO	NO	NO	NO	YES	YES	YES	YES	YES		
ID	**MED#1**	**MED#2**	**MED#3**	**MED#4**	**MED#5**	**MED#6**	**MED#7**	**MED#8**	**MED#9**	**MED#10**	**p-value**	**MED Log Ratio**
MIR_#89	26,1429991	27,9314991	25,5682497	22,9697499	23,9257497	21,5402504	22,7422495	20,3365005	23,8287499	22,9022508	**0,02071496**	-2,83
MIR_#60	27,1230005	28,1394993	26,6832495	23,3427491	24,3837499	20,7122493	20,2242498	19,6035007	22,4837506	20,3232503	**0,00084831**	-6,36
MIR_#99	28,8870005	29,945499	27,9552507	26,2897496	24,2657499	30,01	30,01	25,1045002	26,8147499	26,6212507	0,86658374	-1,14
MIR_#2	23,4539999	26,3005005	23,6412496	23,1687493	22,8657503	20,9552494	21,4302492	20,2204994	23,2457497	24,51825	0,10815284	-2,02
MIR_#70	22,3560003	23,1685005	20,0362501	20,2727494	21,6627511	18,6472507	20,9292498	17,5844997	18,5737488	20,9942499	**0,04570609**	-3,02
MIR_#88	26,9320006	29,6084991	26,30025	27,8327508	25,1937513	23,9182506	25,2002497	22,9295009	26,4557507	27,4642492	0,1120981	-1,73
MIR_#105	28,0899996	29,9715	28,99825	27,4777493	27,6357507	30,01	26,6602507	24,3195003	26,5707504	25,6812501	0,12707552	-1,52
MIR_#49	27,1359991	27,9505001	26,6422491	24,7397504	25,8897504	24,1552501	24,6332502	20,040501	25,3887493	25,70225	**0,06436649**	-2,01
MIR_#36	25,1109995	25,9584995	22,6342497	23,2477498	24,3127508	20,8862491	21,2732496	19,1684994	22,3697502	22,7832494	**0,00969718**	-3,04
MIR_#10	24,3649992	25,0384994	23,8652506	23,07375	22,8097505	21,7222496	21,3882494	19,4904999	22,8237488	22,4972501	**0,01361007**	-2,14
MIR_#113	25,6319994	27,9344994	25,3762502	24,46275	24,9817514	22,5932499	22,5092497	21,2785	25,1227505	24,7362504	**0,03259979**	-2,78
MIR_#1	21,0209994	22,9234997	21,7992506	19,9007497	21,3707513	18,9532505	19,7922506	17,783501	20,4107506	20,4702497	**0,02622825**	-1,58
MIR_#95	18,8579992	20,2464996	18,9622507	19,488749	19,2507505	15,9552494	17,7642508	16,0184997	17,4807503	20,7842508	**0,0894594**	-1,77

Figure 8.



Hierarchical clustering of microRNA expression profile of 117 microRNA commonly expressed in EBUS-TBNA biopsy specimens (N = 20) – on the left - or mediastinoscopy (MED) – on the right. The heat map indicates the level of expression (log2 Ratio) of each single microRNA (green, less expressed, red, more expressed) in the analyzed samples. Bottom, yellow samples of patients who responded to neoadjuvant therapy and in black those who did not respond.

Figure 9.



Hierarchical clustering expression profile of a 13 differentially expressed microRNA ($p < 0.05$) set in EBUS-TBNA samples of patients responders to neoajuvant chemotherapy vs. not-responders. On the left, the expression level logic (log2 Ratio) of each single microRNA (green, less expressed, red, more expressed) in the EBUS-TBNA samples. On the right, heatmap of the expression profile in the mediastinoscopy samples (MED). Bottom, yellow samples of patients who responded to neoadjuvant chemotherapy, while in black those who did not respond.

5. 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 therapies (43) 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 (44). 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 (41). 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. (33)

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 (45). 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 (46).

In the first phase of our study, we retrospective analysed a consecutive intent-to-treat series of selected patients with "potentially resectable" N2 NSCLC, pathologically proven by mediastinoscopy or EBUS-TBNA after induction chemotherapy, to obtain the most homogeneous group possible to identify prognostic factors to improve survival but also to identify the best group of survivors to investigate future therapies.

Although survival was significantly compromised in most studies when residual nodal disease was present after induction therapy, and these patients might do as well overall without surgery, for those showing a response to induction, multiple studies have already demonstrated that a more important determinant of survival in patients with stage III-N2 disease is downstaging the mediastinal lymph nodes involved (47-50). Patients with pathologic downstaging to N0 status demonstrated a survival benefit compared with those with persistent nodal involvement (N1–N3 disease, or nodal involvement ''unknown'') at the time of surgery, and with patients who did not undergo surgical resection (5-year survival 41% versus 24% versus 8%, respectively) (25).

Our study observed mediastinal downstaging in 30% of the patients, with a complete pathological response in only 8 patients (6%). Even if we had a moderate mediastinal response after IC, complete lymph node downstaging (pN0) was found to be one of the most important predictors of survival (46% for pN0 vs. 26% for pN+), above all considering that most of the patients with N1 or persistent N2 disease underwent adjuvant radiotherapy.

In multivariate analysis, lymph nodal downstaging and the number of chemotherapy cycles were both independent prognostic factors, with the benefit of downstaging mostly due to complete pathological response. In our patients, 5-year OS was 35% for all patients undergoing radical resection but when the lymph nodal downstaging was associated with 4 or more cycles of IC, the 5-year OS rose to 76%. This excellent result was related to the fact that those patients were already good responders after 2-

3 cycles of chemotherapy, and that is why we decided to continue with subsequent cycles of chemotherapy.

Another important prognostic factor was the number of nodal stations involved. Daly et al. (48) found a significant difference in 5-year OS between persistent single versus multilevel N2 at resection (37% vs. 7.1%; $p<0.005$), as did our study (33% and 18%, $p=0.005$).

Since persistent lymph node disease after chemotherapy has a negative prognostic significance, the appropriate selection of patients for surgery following IC becomes essential not only for a correct analysis of the data but also to reduce the operative risk of surgery in patients who may not benefit.

The preliminary retrospective analysis of a miRNA expression profile in mediastinoscopies specimens derived from the first retrospective cohort of patients showed a specific pattern of expression highly different between "responders" and "not-responders" patients.

Departing from these results, we tested the feasibility of the use of EBUS-TBNA specimens to profile the whole genome and miRNA expression that has never been described.

The use of primary cell lines instead of the completely fixed cytological specimens collected from EBUS-TBNA 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.

Our 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 micro-dissected lymph nodal metastases from mediastinoscopy specimens.

In addition, the evaluation of miRNA profile in NSCLC cell cultures derived from EBUS-TBNA specimens also provided a specific pattern of expression completely different from "responders" and

"not responders" patients. This pattern was also verified in a series of FFPE samples derived from mediastinoscopies confirming the results.

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.

Based on our results the evaluation of a miRNA signature based on response to chemotherapy that could predict a good prognosis and single out the best candidates for therapy represents a step forward in the personalized treatment era.

6. CONCLUSIONS

Response to chemotherapy is the most important prognostic factor affecting survival in Stage IIIA – pN2 NSCLC patients.

The ability to predicted response to chemotherapy is crucial and the genomic approach represents the best tool.

The identification of chemo-resistance markers through miRNA expression profile is feasible and reproducible in EBUS-TBNA specimens from patients with NSCLC.

Our results are very promising in a new future the management of NSCLC patients in the personalized therapy era.

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