SCUOLA DI ALTA FORMAZIONE

DOTTORATO IN SCIENZE E BIOTECNOLOGIE MEDICHE

PHD PROGRAM IN SCIENCES AND MEDICAL BIOTECHNOLOGY

COORDINATOR: PROF. EMANUELE ALBANO

ANNUAL REPORT
Second year

CYCLE: **XXIX** YEAR: **2014-2015**

Title

Analysis of microRNA expression in carcinomas of the lung and their prognostic-predictive significance

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Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide (Siegel et al. 2014). An estimated 1.82 million new cases and 1.59 million lung cancer-related deaths occurred in 2012 (http://globocan.iarc.fr). Lung cancers are classified according to the histologic type and this classification has important implications for the treatment and prognosis of the disease. There are two main categories of lung cancer: small cell lung cancer (SCLC) and non-small cell lung cancers (NSCLC).

SCLC comprises about 15-20% of all lung cancer and it is characterized by aggressive growth and a poor prognosis (the 5-year survival rate is only 2%) (Roberti et al. 2006). Etoposide-based chemotherapeutic regimens and radiotherapy are the main stay of treatment SCLC (Elliottet al. 2002), but most patients relapse rapidly and become resistant to chemotherapeutic agents and no single molecular targeted drug has shown any clinical efficacy over an extended period. Recent papers have tested the possibility to treat SCLC in third line with alchylating agents with conflicting results. The methylation status of MGMT of SCLC has been proposed as biomarker to predict the response to therapy, meaning that only SCLC methylated could respond to alkylating agents (Pietanza et al. 2012). However, the frequency on SCLC methylation and the feasibility of this test in routinely processed samples is not yet known. Our group performed a study of MGMT promoter status evaluation in small biopsies and cytological specimens routinely processed for diagnostic purposes. MGMT promoter was methylated in 35.2% of the cases without any significant difference between histological and cytological samples (37.9% vs. 32%) (Miglio et al. 2015).

NSCLC accounts for approximately 85% of all lung cancers and is divided into three major histological subtypes: adenocarcinoma (AD) (40%), squamous cell carcinoma (SCC) (30%), and large cell carcinoma (15%) (Molina et al. 2008). Moreover, in 2011 the International Association for the Study of Lung Cancer (IASLC), American Thoracic Society (ATS) and European Respiratory Society (ERS) developed a new pathologic classification for adenocarcinoma: acinar, lepidic, micropapillary, papillary or solid (Travis et al. 2011). NSCLC is characterized by a generally poor prognosis, whit a 5-year survival rate for advanced stage (III and IV) of about 5-20% and 60-70% in early stage (I and II). Only around 20% of NSCLC patients are diagnosed at stage I, II, where radical surgical resection of the primary tumour is opted as first line therapy with a favourable efficacy of 60-80% (stage I) and 40-

50% (stage II) (Chee et al, 2008). Locally advanced NSCLC (stage III) is treated with a combination of chemotherapy and radiotherapy, which can be administered sequentially or concurrently. More than 40% of NSCLCs are diagnosed at advanced/metastatic stage IV, where commonly a palliative chemotherapy is applied with a targeted therapy that comprises various biological agents.

Lung cancers develop through a multistep process involving development of multiple genetic and epigenetic alterations, particularly activation of growth promoting pathways and inhibition of tumour suppressor pathways. Specific molecular alterations driving tumour growth and providing targets for therapy can heavily influence the outcomes of therapeutic strategies. Targeted therapies are drugs designed to interfere with specific molecules necessary for tumour growth and progression.

The most frequently genetic abnormalities in SCLC are inactivation mutations in tumour suppressor genes such as TP53 and retinoblastoma gene 1 (RB1) (D'Amico et al. 1992; Yuan et al. 1999); other genetic alterations including overexpression of BCL-2 and only infrequent gain-of-function mutations in oncogenes such as PI3K3 or MYC family members.

In NSCLC, driver genetic alteration occur in multiple oncogenes including, for adenocarcinoma: K-RAS(26,9%), EGFR(9,4%), ALK (4%), MET(4%), PIK3CA(2,6%), RET (1,9%), ROS1(1,7%), BRAF(1,6%), HER2(0,9%). KRAS mutations are mutually exclusive with other driver events including EGFR, HER2 or BRAF mutations and ALK rearrangements (Sequist et al. 2011). Whereas, FGFR1 (20%), PIK3CA (6,5%), PTEN (4-8%), DDR2 (3,8%) are oncogenic drivers for squamous cell lung (Minuti et al. 2013).

The aberrant activation of multiple signal pathways, such as RAS/RAF/MEK, PI3K/AKT/mTOR and STAT leads to the uncontrolled growth of cancer and impaired cell death signalling.
The PI3K/AKT/mTOR signalling pathway is involved in the survival, proliferation, and migration of SCLC and NSCLC(Wojtalla et al. 2013, Tsurutani et al. 2006). The PI3K/AKT/mTOR signalling pathway may be activated in cancer through multiple mechanisms including mutations in PIK3CA, which encodes the catalytic subunit of PI3K, loss or mutation of phosphatase and tensin homolog (PTEN), AKT mutations and deregulation of mammalian target of rapamycin (mTOR) complexes (Samuels et al., 2004).

Umemura et al. (2014) found in 50 SCLC cases a higher penetrance of activating alterations of the PI3K/AKT/mTOR pathway that act in a mutually exclusive manner. One cause of therapeutic resistance is inactivation of cancer suppressor PTEN, which allows over-activation of the PTEN/PI3K/AKT pathway. Evidence has shown that the poor prognosis of patients with NSCLC of all stages and therapeutic failure are associated with a number of abnormally activated signalling PI3K/AKT pathway, and this is more common in SCC than in AD (Drilon et al. 2013).

The major characteristics of malignant tumour cells are their ability to invade foreign tissues and form metastatic foci at distant locations in the body. Such processes require tumour cell attachment to various matrix proteins and the degradation of the extracellular matrix (ECM), mainly by matrix metalloproteinase (MMP) that may facilitate tumour invasion, metastasis and angiogenesis.

Increased levels of MMPs have been detected in numerous cancers and were correlated with tumour aggressiveness (Shuman et al. 2012). For example, MMP-1, −2, −7, −9, −14, and −15 were overexpressed in NSCLC (Leinonen et al. 2006; Shah et al. 2010; Stenvold et al. 2012), and elevated MMP-1, −9, −11, −13, and −14 levels were also shown in SCLC (Michael et al. 1999; Rintoul et al. 2001). The TIMP3 and RECK has an inhibitory effect on tumour metastasis due to its interaction with MMPs. A clinical study showed that the expression of TIMP-3 was reduced in a number of cancer types and reduced expression of TIMP-3 was significantly associated with pathologic stage, nodal involvement, and poor survival in lung cancer patients. RECK (reversion-inducing cysteine-rich protein with Kazal motifs) is a membrane anchored glycoprotein which regulates MMPs and inhibits angiogenesis. Recent analyses indicate that RECK expression is frequently down-regulated in tumour tissues in comparison with the surrounding non-tumours tissues in several common types of cancer including colon, mammary, pancreatic carcinoma and lung cancer (Noda et al. 2003; Pesta et al. 2009).

DNA damages give rise to mutations and epimutations that, by a process of natural selection, can cause progression to cancer. If DNA damages in proliferating cells are not repaired due to inadequate expression of a DNA repair gene, this increases the risk of cancer. Many known carcinogenic agents cause reduced expression of DNA repair genes or directly inhibit the actions of DNA repair proteins.

The NER system is essential for repairing DNA damage such as pyrimidine dimers, cross-links, and bulky adducts induced by platinum chemotherapy. Excision repair cross-complementation group 1 (ERCC1) is one of polypeptides of the nucleotide excision repair (NER) system. ERCC1 plays a

pivotal role in DNA repair by removal of DNA-platinum adduct, which inhibits DNA synthesis in cancer cells. Some gene expression of markers of sensitivity to specific cytotoxic agents, such as thymidylate synthase (TYMS) and dihydrofolate reductase (DHFR), enzyme that plays an important role in DNA synthesis. In NSCLC, overexpression of TYMS is associated with poor prognosis following lung resection and low TYMS mRNA level is associated with better response to neoadjuvant pemetrexed/gemcitabine treatment (Liu et al. 2015).

Recent evidence suggests significant roles that MicroRNAs (miRNA) play in the prognosis and diagnosis of lung cancer, increasing efforts are dedicated to the development of miRNA-based therapies.

miRNAs are a class of evolutionarily conserved, small non-coding RNAs of 19-24 nucleotides in length that regulate gene expression mostly at the posttranscriptional level (Bartel et al. 2009). Initially, miRNAs were thought to be involved in the regulation of development and cell fate, but more recently it has been discovered that miRNAs participate in a broad range of processes including cell cycling, apoptosis, cell differentiation, tumour development, invasion, metastasis, and angiogenesis (Friedman et al. 2009).

In particular, miRNAs have been observed to be aberrantly expressed in many human cancers (Cortez et al. 2011) and they are promising alternative biomarkers for detecting cancer, establishing prognosis, and monitoring treatment response, as well as crucial players in cancer initiation, development and metastasis (Cortez et al. 2011). From the biological point of view, miRNAs may be better predictive and prognostic markers than DNA or mRNA. A single miRNA, indeed, may regulate hundreds of target mRNAs, frequently grouped in a specific biological pathway. Consequently, a miRNA signature may provide comparable prognostic information several orders of magnitude greater than mRNAs. Besides miRNAs are more stable than other biomarkers during sample processing, thus more suitable for analysis in plasma, urine, stool and tissue (fresh or FFPE - Formalin-fixed, paraffin-embedded) sample and this is a key point in the search for cancer markers.

Recent studies have indicated an emerging role for miRNAs, in addition to genetic and epigenetic changes (methylation/acetylation), in the anticancer-drug-resistant phenotype (Giovannetti et al. 2012), which opens up the possible application of miRNAs in evaluation of outcome and modification of response in known anti-tumour therapies (Hummel et al. 2010).

The possible applications of miRNAs in molecular prognostics, particularly in cancer, are provided by discovery of the role of miRNA in many pathological processes, and for cancer prognosis; miRNA can

be complementary to other genomic and proteomic biomarkers (Cho et al. 2007). Several recent studies have demonstrated how oncogenic miRNAs may interfere with DNA-repair pathways allowing cells to resist drugs that initially were effective against them (Giovannetti et al. 2012). To restore drug sensitivity via miRNAs, potential approaches include activation of tumour suppressor miRNAs or inactivation of oncogenic miRNAs and modulation of miRNA target genes, oncogenes, and tumour suppressor genes, through up- or down-regulation of miRNAs (Sarkar et al. 2010; Giovannetti et al. 2012).

Finally, there are very few studies dealing with the feasibility of the expression of miRNA on cytological samples, following the report of two examples. Fassina et al. develop a miRNA expression method for differentiating AD from SCC in cytologic specimens obtained by means of such a minimally invasive and safe technique as CT-guided TTNA (Fassina et al. 2011). Gilad et al. develop an assay based only of the expression of a small set of miRNAs that may help to differentiate the four main types of lung cancer. The assay displays high levels of accuracy in pathologic and cytologic samples. For the latter, fine-needle aspiration (FNA) and bronchial brushing and washing samples were tested, demonstrating the versatility of the assay (Gilad et al 2012).

In light of these results, we selected three miRNA that were previously reported to be involved in processes or pathways that have been associated with a prognostic and predictive role, namely PI3K/AKT/mTOR pathway (miR-192; miR-200c, miR-205), regulation of MMPs expression (miR-200c, miR-205) and DNA damages/repair (miR-192).

	Target			
	PI3K/Akt/mTOR pathway	MMPs regulation	DNA repair/damage/ biosynthesis	Other target
miR-192			TYMS; DHFR; ERCC3-4; damage DNA	RB1; BIM; ZEB2; MDM2
miR-200c	PTEN			ZEB1-2, KRAS
miR-205	PTEN	TIMP3; RECK		ERBB3, E2F1, ZEB1

Table 1. Panel of MicroRNA, targets and the presence in the tissues examined.

Aims

To evaluate the feasibility of molecular analysis using cytological sample and small biopsies from patients with NSCLC and the possible influence of the different types of fixative solution on miRNA expression in such samples. Moreover, we tried to identify a panel of miRNAs related to prognosis, that could be useful in clinical practice.

Methodologies.

1) Patients and Samples (done).

Cytologic samples from 50 patients with SCLC were collected from the archives of Pathology Unit of University Hospital of Novara between January 2006 and December 2014.

Twenty-five samples (50%) were from primary tumours and 25 from metastatic regional lymph nodes. Forty-four samples (88%) were obtained by fine needle aspirations (FNAs), and six (12%) by bronchial washing (BWs).

FNAs were performed using 21 or 22-gauge disposable needles, immediately smeared onto a glass slide and spray-fixed using ethanol (Bio-Fix, Bio Optica, Milan, Italy). Part of the needle content was also washed in lysing solution and centrifuged at 1,800 g to obtain cell blocks. They were prepared using agarose gel and fixed in ethanol.

Specimens from bronchial washing were centrifuged to obtain a cell pellet, then the pellet was partially smeared onto a glass slide and spray-fixed, whereas the remaining part was fixed with Duboscq-Brazil fluid for 24 hours; afterward, paraffin-embedded cell blocks were prepared and sectioned.

Specimens from slide obtained by transferring smear to glass slide were stained with the Papanicolaou stain and permanently mounted. The sections obtained by cutting paraffin-embedded cell blocks, were stained by haematoxylin-eosin and by immunohistochemistry to define the diagnosis of SCLC. A panel of immunohistochemistry was used in all the cases and included the following antibodies: synaptophysin, CD56, chromogranin, and CK AE1/AE3 (Ventana Medical System, Tucson, AZ). The reactions were performed by means of Benchmark instruments (Ventana Medical System).

Ten normal lung tissue samples obtained from surgical resection of lung carcinoma were selected after light microscopy examination and used as positive controls.

2) Patients and samples (in progress).

Seventy cytological samples obtained from patients with a diagnosis of advanced NSCLC (stage III and IV) and with a minimum of 2-years follow-up will be retrospectively collected.

The samples have been obtained by sputum, bronchial brushing or washing and by fine-needle aspiration biopsy during routine procedures for diagnostic purposes. The samples are generally composed by cellular smears an/or by cell block and in all the cases the morphologic diagnosis was confirmed by a specific panel of immunohistochemistry. Before the inclusion in the present project, all the cases will be reviewed by an experienced pathologist. We hypothesise that al least 40 cases of AD and 30 cases of SCC will be included in the study. In all the cases, the neoplastic cellularity and the percentage of the tumour cells versus normal cells will be evaluated in order to establish a cut-off of expression. In analogy with our previous work on the analysis of EGFR in routine cytologic specimens of lung adenocarcinoma (Allegrini et al. 2012), we will also investigate the influence of the different types of fixative solution on the feasibility of miRNA expression in such samples.

Whereas, tissue samples of early NSCLC, obtained by surgery from patients with stage I and II tumours will be evaluated in order to investigate miRNA expression from at least 50 cases, matched for grading and histological subtype. Also in these patients a minimum of 2-years of follow-up will be required and the disease-free survival will be compared with miRNA expression.

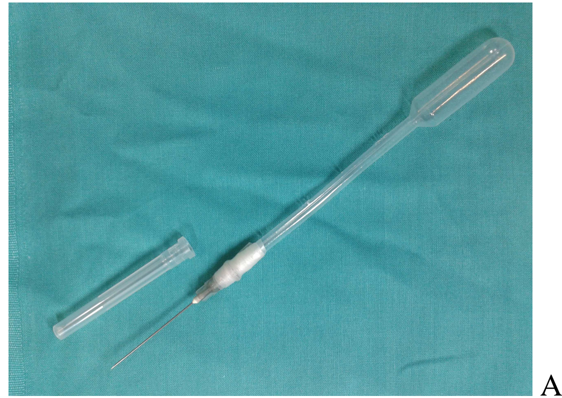
All the cases will be evaluated by an experienced pathologist and the area of interest will be selected, including a maximum number of cancer cells, avoiding necrotic areas.

RNA Isolation

Total RNA from cell blocks and papanicolaou-stained smear slide was extracted using RecoverAll^{TM} Total Nucleic Acid Isolation Kit (Life Technologies^{TM}).

An alternative method of microdissection was set-up with a higher yield and it turned up to be more accurate to extract RNA from neoplastic areas, discriminating between normal cells and necrotic areas. A different method consists in this: 3-µm-thick sections were placed on glass slides, ten slides for each cellblock. Then, each slide was stained with H&E staining and uncovered. H&E staining was performed with Leica ST5020 Multistainer, and the program was set as follows: 4 times in ovens/slide drying stations for 5 minute at 62°C, xylene for 3 minute x 3, absolute (100%) ethanol for 2 minute x 2, ethanol 95% for 1 minute, water for 4 minute, Mayer's hemalum for 10 minute, water for 8 minute, eosin for 1 minute, water for 10 second, ethanol 95% for 40 second, absolute ethanol for 2 minute x 2, xylene for 4 minute.

Throughout microdissection, it was necessary to use absolute ethanol with the aid of a modified pipette-needle, to prevent the loss of material (due to electrostatic phenomena during sample collection). This device consists of a 22-gauge-needle inserted in a Pasteur pipette and wrapped with parafilm in order to maintain an airtight seal between the two devices (figure 1 A). All glass slides samples were observed with a microscope Eclipse 80i (Nikon) with magnification of 10X. RNA detection was performed from uncovered stained slides by collecting tumour cells with modified pipette-needle and absolute ethanol (figure 1 B). The specimen soaking in absolute ethanol was transferred into a 1.5 mL tube, made up to 1 mL with ethanol and centrifuged at 14.000 rpm for 7 min. Then, supernatant was carefully discharged and the pellet was retained. The pellet was dried in Concentrator 5301 (Eppendorf) at 30°C for 45 minute. Thereafter, manufacturer's instructions of RecoverAllTM Total Nucleic Acid Isolation Kit (Life TechnologiesTM) were followed by Protease Digestion to the Final Nucleic Acid Purification. RNA purity and concentration were determined by NanoDrop.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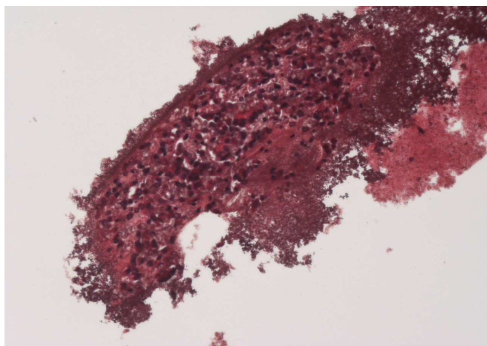
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B2

Figure 1. A) Modified pipette-needle: 22-gauge-needle inserted in a Pasteur pipette and wrapped with parafilm; B) slide stained with H&E staining and uncovered B1) before collecting tumour cells and B2) after collecting tumour cells.

cDNA synthesis and miRNA expression analysis by quantitative real-time PCR

Complementary DNA (cDNA) was synthesised using the TaqMan® MicroRNA Reverse Trancription Kit and TaqMan® MicroRNA Assays.

For each reverse transcription reaction 50 ng of RNA and 3 µL of 5X RT microRNA-specific primers were used in a reaction volume of 15 µL. cDNA synthesis was conducted by incubation at 16 °C for 30 minute, 42 °C for 30 minute, 85°C for 5 minute and hold at 4°C.

The Real-Time PCR reaction was performed using 1,40 µL of cDNA, 10 µL TaqMan® Fast Universal PCR Master Mix without AmpErase® UNG and 1 µL of primer/probe mixture in a total reaction volume of 20 µL. Real-time PCR was conducted in the 7500 Fast Real Time PCR System (Applied Biosystems) with PCR initial enzyme activation step at 95°C for 10 min and then 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min.

For this study was analysed the expression of hsa-miR-192-5p (Assay ID:000491), hsa-miR-200c-3p (Assay ID:002300), hsa-miR-205-5p (Assay ID:000509), and the internal control RNU6B (U6 snRNA; Assay ID:001093). The relative miRNA level was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak et al, 2001) and normalized to RNU6B.

Statistical analysis

Stata Statistical Software (release 13.0 College Station, Stata Corporation, TX, USA) was used in all the statistical analyses. Survival data were analyzed by the Kaplan-Meier method and the survival curves were compared by the log-rank test. The variables putatively associated with the survival were analyzed by use of univariate Cox proportional hazards regression model. The variables selected by the univariate analysis were entered into a multivariate Cox model using a forward stepwise elimination algorithm (terms with $p > 0.05$ were eligible for removal)

Results of SCLC

The diagnosis of SCLC was performed on the base of the cytologic findings, the positivity for cytokeratin and at least one of the neural markers (*i.e.,* synaptophysin, CD56 or chromogranin) (18)

Main demographics and clinicopathologic findings are summarised in table 1. The average age at diagnosis was 65,8 years (range 34-82), 32 patients were male and 18 were female. At the end of the study 43 patients (86%) had died. The average overall survival (OS) was 363,4 days (range 9 to 2482 days). Eighteen patients (36%) had limited-stage disease (III A or III B), whereas 32 (64%) had extensive-stage disease. Twenty four patients (48%) received cisplatin-containing chemotherapy regimens, 13 (26%) were treated with carboplatin-containing chemotherapy, whereas 13 patients received only best supportive care.

Clinical characteristics of SCLC patients in this study	
Variables	Values¶
Age (mean±SD)	65,8 ±11,9
	N°(%)
Sex	
Male	32 (64)
Female	18 (36)
Disease Stage	
Limited-stage disease (III A or B)	18 (36)
Extensive-stage disease	32 (64)
Chemotherapy	
Etoposide	27 (54)
Irinotecan	10 (20)
Other	13 (26)
Platinum-Based Chemotherapy	
Cisplatin	24 (48)
Carboplatin	13 (26)
Other	13 (26)

Table 2. Clinical characteristics of SCLC patients in this study

Association between single miRNA, selected panel of miRNAs and OS

All 50 samples had sufficient RNA to perform miRNA expression profiling of the three miRNA panel selected in this study (miR-192, miR-200c, miR-205), independently to the source, the type of the specimen and the fixative medium. The medians of relative expression levels of each miRNA (miR-192, median $2^{-\Delta\Delta Ct}$ = 13,59; miR-200c, median $2^{-\Delta\Delta Ct}$ = 1,81, and miR-205, median $2^{-\Delta\Delta Ct}$ = 2,52) were used as the cut-off point in order to distinguish the patients into high (score 1) and low (score 0) expression groups. The association between the two groups of miRNA expression and the overall survival (OS) was investigated by Kaplan–Meier analysis and log-rank test. The OS between the high and low expression group of each single miRNA was not statistically significant (median miR-192 log-rank p-value = 0,3621; median miR200c log-rank p-value = 0,2191; median miR205 log-rank p-value = 0,2237).

Then, for every patient the sum of the expression levels of the three miRNAs was calculated, obtaining a score ranging from 0 (level of expression of each miRNA lower than the median value) to 3 (level of expression of each miRNA higher than the median value). Afterwards, the patients were divided in two groups: group A (n#25), with a score 0 or 1 (low expression miRNA panel), and group B (n#25), with a score 2 or 3 (high expression miRNA panel) (Table 3).

Score distribution in Group A and B

	miRNA	N° (%)
Group A		25 (100%)
Score 0	No miRNA ↑	9 (36%)
Score 1	1 miRNA ↑	16 (64%)
	miR-192	5 (20%)
	miR-200c	8 (32%)
	miR-205	3 (12 %)
Group B		25 (100%)
Score 2	2 miRNAs ↑	16 (64%)
	miR-192 and miR-200c	3 (12%)
	miR-192 and miR-205	8 (32%)
	miR-200c and miR-205	5 (20%)
Score 3	3 miRNAs ↑	9 (36%)

Table 3. group A with a score 0 or 1 (low expression miRNA panel), and group B with a score 2 or 3 (high expression miRNA panel).

Next, we compared the OS between the two groups of patients: as shown in figure 2 the log-rank test indicated the group A had a statistically significant advantage of survival (p=0,0291)

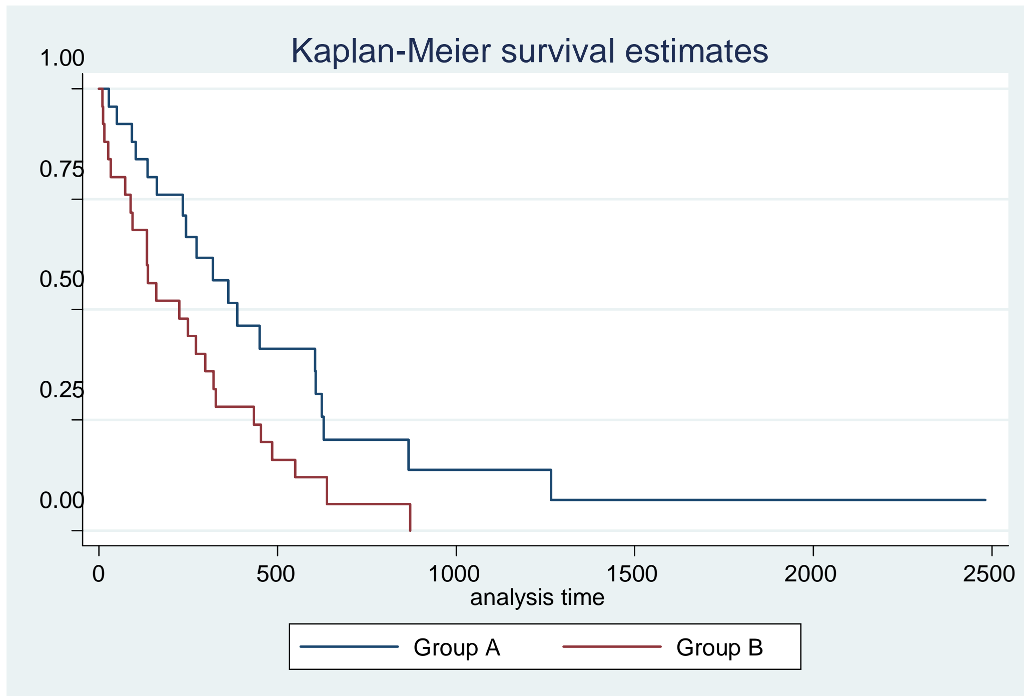
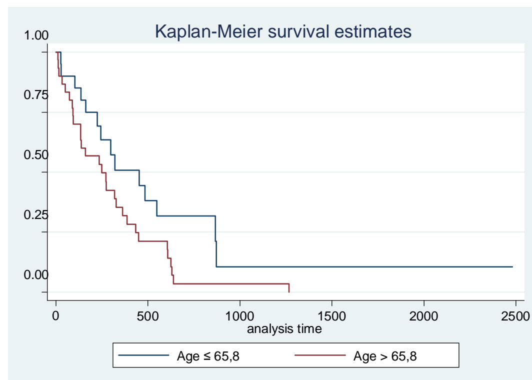


Figure 2. Survival by miRNA panel. The red line depicts survival curve for Group B, and the blue line depicts survival curve for Group A. The survival curves were found to be significantly different with a log-rank p value of 0,0291.

Association between miRNA panel, clinical variables and OS.

In our cohort, the average age at diagnosis was 65.8 years: the group of patients older than the average age (n#30) showed a lower OS than the younger patients (Figure 3 A), although the result was not statistically significant (p=0,0516). As expected, also in our cohort, the patients with limited-stage disease (LD) showed significantly longer OS than patients with extensive-stage disease (ED) (p= 0.049) (Figure 3 B).



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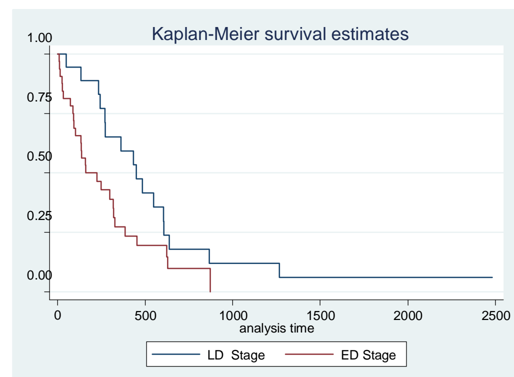

B

Figure 3. A) Survival by mean Age. The red line depicts survival curve for old patients (Age > 65,8), and the blue line depicts survival curve for young patients (Age ≤ 65,8); The log-rank p value of 0,0516.
B) Survival by Stage. The red line depicts survival curve for patients with extensive-stage disease (ED), and the blue line depicts survival curve for limited-stage disease (LD)(Age ≤ 65,8); The log-rank p value of 0,049.

Also, we analysed the association of miRNA panel, age at diagnosis and OS. In the older patients, the group A showed a statistically significant longer OS than the group B (p = 0.0129, figure 4); conversely, in the younger patients the levels of expression of miRNA panel did not change the OS (p = 0.407)

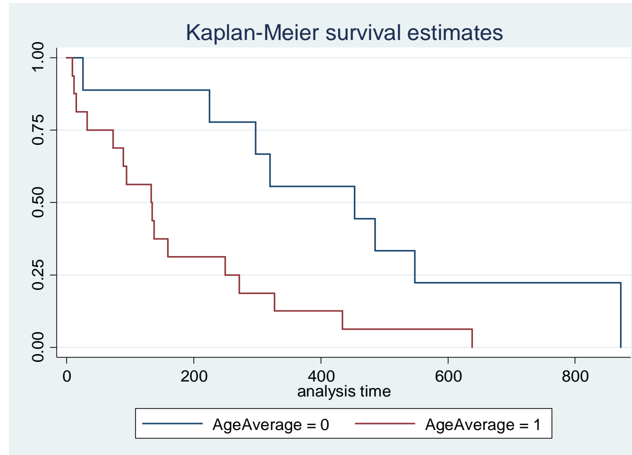

Figure 4. Survival by only mean Age > 65,8) and miRNA panel. The red line depicts survival curve for old patients Group B, and the blue line depicts survival curve for old patients Group A; The log-rank p value of 0,0129.

Finally, we did not find any association between the expression of miRNA panel and OS in each group of patients with limited and extensive-stage disease (p = 0,515 and p=0,40) (not shown).

Survival data were analyzed by the Kaplan-Meier method and the survival curves were compared by the log-rank test. The variables putatively associated with the survival were individually analyzed by use of univariate Cox proportional hazards regression model (Table 4)

The variables selected by the univariate analysis (Age Mean , miR-200c, miR-205, panel of miRNA and stage) were entered into a multivariate Cox model using a backward elimination algorithm (terms with $p > 0.05$ were eligible for removal).

Parameters that were independent predictive variables were: Mean Age (≤ 65,8 years vs > 65,8 years), Stage (limited vs extended) and Panel miRNA (low vs high expression).

Univariate Cox regression	HR (95% CI)	P-value
N=50		
Age	1,02 (0,99 - 1,05)	0,181
Sex	1,06 (0,56 - 1,97)	0,861
miR-192	1,00 (0,997 - 1,01)	0,308
miR-200c	1,04 (1,00- 1,1)	0,018*
miR-205	1,00 (1,00 - 1,005)	0,039*
Mean Age (1,0)	1,88 (0,98 - 3,58)	0,05*
Median miR-192 (1,0)	1,33 (0,72 - 2,45)	0,364
Median mirR-200c (1,0)	1,46 (0,8 - 2,67)	0,222
Median miR-205 (1,0)	1,46 (0,79 - 2,69)	0,226
Stage	1,89 (0,99 -3,58)	0,05*
Multivariate Cox Regression		
Age Mean	2,63 (1,32 – 5,25)	0,006
Stage	2,09 (1,07 – 4,08)	0,030
Panel-MiR	2,1 (1,11 – 4,04)	0,022

Table 4. Univariate and Multivariate Cox regression; * these variables were entered into the multivariate Cox Model

CONCLUSIONS

The diagnosis of SCLC could be suspected on the basis of clinical presentation and imaging studies but pathologic confirm is required. Cytology from bronchoscopy or from fine-needle aspiration biopsies (FNAB) are often the preferred approaches in order to achieve the diagnosis, either from primary tumour or from metastasis because the minimal trauma to the patient and the ease of sample acquisition. The prognosis of SCLC is poor; best prognostic factors include limited stage disease (TNM stages I-III), age < 70 years, female sex, performance status <1 and baseline laboratory test (LDH, alkaline phosphatise and sodium) (van Meerbeeck et al 2011). Unlike other lung cancers, molecular markers of SCLC able to predict the prognosis or driving the choice to therapy are still lacking.

In the last few years, miRNAs have been considered promising biomarkers in order to discriminate different subtypes of cancer, and to foresee prognosis and response to the conventional chemotherapy in several types of human cancers. Moreover, the higher stability of miRNAs compared with mRNAs, allowed to obtain high-quality miRNA from a wide range of tissue and cell sources taken during the *routine* diagnostic procedures. However, to the best of our knowledge, no authors have investigated the feasibility of miRNA analysis expression in different cytology samples, fixed in several ways, and the lower cut off of cellularity below which the results are not reliable.

In our study, we were able to isolate sufficient quantity of miRNAs from SCLC cells obtained by FNABs of lung or metastatic lymph nodes either from cell blocks or by scraping cells from slides, fixed in ethanol, and by BWs fixed in Dubosq-Brazil. miRNA analysis was reliable also in low-cellular samples with a minimum of 80 cancer cells.

In our cohort of patients, the average age at diagnosis was 65.8 years: the group of patients older than the average age showed a strong tendency with a poor prognosis. Moreover, we compared the OS between patients with limited and extensive stage disease. As expected, also in our cohort, the patients with limited-stage disease showed better survival than patients with extensive-stage disease. Both results are in line with the findings of literature (van Meerbeeck et al 2011; Amini et al. 2014).

The comparison between miRNA panel expression, stage at diagnosis and age, indicated that high expression of miRNA was associated with poor OS in the group of older patients with extensive-stage disease. Moreover, we found that high expression of the selected miRNA panel (miR-192, miR-200c and miR-205) is *independently* associated with short overall survival in SCLC.

Different molecular mechanisms are involved in the effect of these miRNAs: miR-192 target genes involved in the repair, biogenesis and damage DNA processes, miR-205 seems to act through

angiogenesis by inhibiting TIMP-3 and RECK and increasing MMPs levels, miR-200c plays a role in the regulation of the PI3K/Akt/mTOR pathway.

Several studies have shown that miR-192 is involved in the processes of chemo-resistance, progression, metastasis and cell proliferation. Zhang et al. show that an over-expression of mir-192 confers drug resistance to cisplatin and inhibits apoptosis in cells of lung adenocarcinoma, through the negative regulation of BIM (Bcl-2 family member) (Zhang et al. 2014). Another target gene regulated by miR-192 for cell proliferation of lung cancer is the RB1(Feng et al. 2011). Moreover, miR-192 target genes involved in DNA biogenesis as dihydrofolate reductase (Song et al., 2008), TYMS (Khella et al. 2013) and DNA repair ERCC 3-4 (Xie et al. 2011). ERCC3 and ERCC4, two proteins involved in NER pathway, were down-regulated by miR-192, with consequent impairing of NER machinery (Xie et al. 2011).

Many studies examined the prognostic value of miR-200c expression in human cancers. Liu et al. (2012) showed a miR-200c overexpression as a marker of poor prognosis in NSCLC patients. Overexpression of miR-200c influences tumour growth and survival by regulating the expression of tumour suppressive PTEN and subsequent activation of the Akt signaling pathway in many carcinomas. A decrease in functional PTEN causes activation of Akt and FAK and the expression of the MMPs (Kwiatkowska et al. 2011). As MMPs appear to be essential for tumour invasion and metastatic spread, characterizing their expression may help to determine a treatment or prognosis in patients whit cancer, included NSCLC. A study shows miR-205 directly target two key upstream inhibitors of MMPs, tissue inhibitor of metalloproteinases 3 (TIMP3) and reversion inducing cysteine-rich protein with kazal motifs (RECK), that in turn led to ECM degradation. Overexpression of miR-205 was detected in tissues from multiple subtypes of NSCLC that led to increased proliferation and angiogenesis (Cai et al., 2013). Oncogenic miR-205, overexpressed in NSCLC cell lines and tissues, was shown to enhance cell growth, metastasis, and chemoresistance to Cisplatin of A549 cells by targeting PTEN (Liu et al., 2013).

In conclusion, we have identified panel of miRNA as potential prognostic markers in SCLC. Although further studies in larger cohorts are needed, our findings have revealed the usefulness of microRNAs as potential prognostic markers in SCLC.

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Seminars 2014

Department of translational medicine.

PhD program in Sciences & Medical Biotechnology

28/07/2015 Dott. Vincenzo Cantaluppi "Le cellule staminali nel danno renale acuto e nel trapianto di rene"

21/04/2015 Prof. Percipalle "Actin-based mechanisms in the control of gene expression and cell fate"

27/01/2015 Prof. Antonio Sica "Myeloid cells as therapeutic target in cancer"

21/01/2015 Prof. Valeria Poli "Targeting the liver to cure myocarditis: a lesson from a model of STAT3-dependent auto-immune myocarditis "

20/01/2015 Dr Tonino Alonzi "Regulation of hepatocytes differentiation during the transitions between epithelial and mesenchymal states"

19/01/2015 Prof. Dr Yong-Sang Song "Anticancer strategy Targeting cancer cell metabolism in ovarian cancer"

6/12/2014 Dr. A. Snow "Dysregulated antigen receptor signaling: molecular lessons from two congenital lymphoproliferative disorders."

05/12/2014 Prof. Rifaat Safadi "Focus on the liver: from basics of NAFLD to hot topics in HBV & HCV infections"

4/12/2014 Girish Patel"Uncovering the role of β-HPV in field cancerization: a collaboration in progress"

25/11/2014 Roberta Arcidiacono e Marta Ruspa"La scoperta del bosone di Higgs"

14/11/2014 Dott.Boccafoschi “Tissue engineering: the state of the art”

The Borghese Sessions Steven R Ellis

08/09/2014
10:00 Clinical case - Skin as an organ
11:00 Layers of skin, cell types, developmental origins

09/09/2014
10:00 Cell-Cell Interactions - anchoring junctions
11:00 Cell-Cell Interactions - occluding junctions, tight junctions

10/09/2014
10:00 Cell Matrix Interactions - basal lamina
11:00 Epithelial-mesenchymal transition

11/09/2014
10:00 Angiogenesis
11:00 Innervation

15/09/2014
10:00 Basal layer stem cells, symmetric versus asymmetric divisions, transient amplifying cells
11:00 Solar radiation, nucleotide excision repair

16/09/2014
10:00 Basal and squamous cell carcinomas
11:00 Melanoma - biology

17/09/2014
10:00 Melanoma - treatment
11:00 Contact dermatitis

22/09/2014
10:00 Other skin disorders
11:00 Other components of skin

21/07/14 Dr Maria Giuseppina Miano "a functional link between arx and kdm5c genes linked to neuronal diseases defines a crucial epigenetic path"

16/07/2014 at 14.30 Prof. John F. McDonald "The potential of small regulatory RNAs for the treatment of ovarian cancer "

15/07/2014 ore 14.30-16 Prof.ssa Follenzi "applicazioni terapia genica"

30/06/2014 at 14-16 Dott. Cotella "the C-value paradox, junk DNA and ENCODE"

27/06/2014 at 14 Manuela Sironi "Has nature done the experiment for us? Evolutionary insights into infection susceptibility and autoimmunity"

26/06/2014 at 14 Prof Gianni Del Sal "Disarming mutant P53 in cancer"

19/06/2014 at 12-13.30 Prof.ssa Follenzi "terapia genica"

12/06/2014 at 14 Gianni Cesareni "Metformin rewires the signaling network of breast cancer cells and changes their sensitivity to growth and apoptotic stimuli"

11/06/14 at 14 Prof. Fabrizio Loreni "Ribosome alteration in cancer: effect or cause?"

9/06/2014 at 14 Dott Iacopo Baussano "Assessment of cervical cancer control in Rwanda and Bhutan"

5/05/2014 ore 12 Prof. Vittorio Colombo e Dr. Matteo Gherardi, "atmospheric pressure plasma sources ad processes for biomedical and surface treatment applications"

19/03/2014 14.30 Prof Emilio Hirsch “role of phosphoinositides-3-kinase C2-alph, a Class II PI 3-kinase, in development and cancer”

List of publications.

Tarallo S, Pardini B, **Mancuso G**, Rosa F, Di Gaetano C, Rosina F, Vineis P, Naccarati A. MicroRNA expression in relation to different dietary habits: a comparison in stool and plasma samples. Mutagenesis. 2014; 29(5):385-91.

Miglio U, Mezzapelle R, Paganotti A, Veggiani C, Mercalli F, **Mancuso G**, Gaudino E, Rena O, Buosi R, Boldorini R. Frequency of O6 -methylguanine-DNA methyltransferase promoter methylation in cytological samples from small cell lung cancer. Diagn Cytopathol. 2015 Jul 7. doi: 10.1002/dc.23319.