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miRNA profiling in serum and tissue samples to assess noninvasive biomarkers for NSCLC clinical outcome

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Abstract In NSCLC, the altered expression of some miRNAs in primary tumor tissues has been correlated with diagnosis and prognosis, while the role of circulating miRNAs as cancer biomarkers is currently emerging. MiRNA expression profile through miRNA Affymetrix array was evaluated on a training set formed by the tumor component (n=30 NSCLC serum, n=11/30 tumor tissues) and the control component (n=10 healthy serum and n=11/30 noncancerous counterparts). Statistical analyses highlighted the following: a=55 miRNAs deregulated in tumor serum, b=27 miRNAs deregulated in tumor tissues, and c=2 miRNAs deregulated both in tumor serum and in tumor tissues. MiRwalk tool and enrichment pathway analyses selected some miRNAs whose target genes are correlated with the main pathways involved in NSCLC tumorigenesis. The altered expression of the selected miR-486-5p (*a*), miR-29c* (*b*), and miR-133a (*c*) was confirmed in the validation set (n=40). MiR-486-5p had a higher expression in tumor serum than in tumor tissues (P=0.004),

and miR-29c* showed a lower expression in tumor tissues than in tumor serum (P<0.001). MiR-133a had a not different expression in both tumor serum and tumor tissues (P=0.07). The low level of miR-486-5p expression in the serum of affected patients was associated with a worse time to progression of disease (P=0.010), and serum level of miR-486-5p was a significant prognostic indicator of NSCLC (adjusted hazard ratio=0.179, P=0.019). These data suggest the possibility to monitor affected patients through serum and/or tissue samples, analyzing the altered expression of specific miRNAs, in order to detect prognostic biomarkers in the NSCLC.

Keywords Biomarkers · MicroRNAs · Non small cell lung cancer · Serum · Circulating miRNAs

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Introduction

The diagnosis of non small cell lung cancer (NSCLC) is observed in about 80 % of all lung cancer cases. Its pathogenesis involves exposure to several environmental carcinogens often associated with the onset of different genetic and epigenetic alterations, such as somatic point mutations, microRNA (miRNA) deregulation, and aberrant methylation of gene promoters, which mostly influence the correct expression of some genes [1, 2]. Even though some genetic biomarkers have already been investigated to allow a correct diagnosis of this neoplasia, the early detection of NSCLC is still a challenge; in most patients, NSCLC is diagnosed at an advanced stage, and, despite modern therapeutic improvements, it is characterized by poor prognosis [3]. The 5-year survival rate for stage IV is still about 10 % while 80 % survival is observed when NSCLC is diagnosed at an early stage [4]. The small non coding RNAs of 19–25 oligonucleotides (miRNAs) are

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involved in the regulation of about one third of all human genes, and their aberrant expression in primary neoplastic sites compared to noncancerous tissue counterparts has already been demonstrated to contribute to the development of several solid tumors including NSCLC. They therefore represent valuable diagnostic or prognostic biomarkers [5, 6]. A recent meta-analysis suggested that the detection of a set of miRNAs may be used as a signature of an initial screening test for NSCLC diagnosis [7]. In addition, miRNAs have been proposed as diagnostic biomarkers in NSCLC because of their deregulated expression level in primary resected tissue as compared to the noncancerous tissue counterpart [8]. They seemed to play a role in the classification of lung cancer subtypes, prognosis of lung cancer patients, and response or sensitivity to chemotherapeutic agents [9, 10]. However, in most studies, miRNAs have been analyzed in primary tumor tissues, while biomarkers also need to be detected in the peripheral bloodstream of affected patients due to the minimally invasive nature of sample collection and the widespread vascularization of most tumor tissues [11]. Recently, the role of miRNAs in plasma/serum samples as circulating diagnostic/prognostic biomarkers has been investigated [12]. Some miRNA signatures have already been determined in serum samples of NSCLC patients to distinguish affected patients from healthy subjects [13, 14]. The main diagnostic miRNA signatures have been evaluated in patients affected by NSCLC while only a few studies have focused on high-risk subjects [15, 16]. However, the role of circulating miRNAs in NSCLC in comparison to miRNAs detected in the primary tumor tissue is not yet clear. This uncertain aspect also depends on the source of circulating miRNAs which usually derive from circulating tumor cells (CTSs) or tissue fragments or by apoptotic and lysed tumor cells [17]. Furthermore, miRNAs can be set free in the bloodstream, alone or associated with membrane vesicles released by tumor cells, packaged in exosomes or associated with ribonucleoproteic complexes [18, 19]. Blood-based miRNAs have also been demonstrated to be stable and resistant to RNase digestion, extreme pH, extended storage, and repeated freeze-thaw cycles, and so they could be considered as the right biomarkers for diagnostic purposes [17–20].

The aim of the present study was to determine a specific signature of miRNA expression which could differently characterize serum and primary tumor tissue in order to select novel and noninvasive circulating biomarkers useful both for early diagnosis and for a better definition of prognosis in NSCLC patients.

Materials and methods

Sample selection

The study was conducted on a subset of eighty subjects. Sixty patients with a first diagnosis of advanced NSCLC and previously untreated were enrolled in the Department of Medical Oncology at the IRCCS Istituto Tumori "Giovanni Paolo II," Bari, from March 2011 to October 2012. Moreover, 20 healthy subjects screened for routine test in the Operative Unit of Clinical and Experimental Pathology at the IRCCS Istituto Tumori "Giovanni Paolo II," Bari, in 2012 were enrolled as control group because of the absence of any cancerous disease and according to sex and age with NSCLC patients.

Thirty NSCLC patients, together with 10 healthy subjects, were included in the training set, while a further 30 NSCLC patients and 10 healthy volunteers were inserted in the validation set. The division of patients in the two sets was carried out to maintain homogeneous clinicopathological features between the two groups. Therefore, each set was formed by the tumor component made up of 30 serum samples collected from NSCLC patients during the baseline visit, and of 11/30 tissue specimens from lung cancer lesions that were evaluated as adequate for molecular analysis by pathologist. The control component was made up of 10 serum samples collected from healthy subjects and of 11 tissue specimens from noncancerous surrounding counterparts of the same patients (Fig. 1).

As patients from the two sets matched for the main features (gender, sex, age, histological classification, etc.), only clinical characteristics of the validation set (Table 1) have been evaluated for correlation analysis with the deregulated miRNAs.

The set of samples was formed by 21 males and 9 females with a diagnosis of NSCLC, with a median age of 65 years. The primary tumor of 19 patients was diagnosed as adenocarcinoma, while the tumor of 12 patients was squamous cell carcinoma (SCC) according to the WHO classification [21]. Moreover, eight of these tumors were classified at stage IIB/IIIA, while the majority of tumors ($n=22$) were classified at stage IIIB/IV according to TNM 7th version of the International Association for the study of lung cancer staging system [22]. The presence of single or multiple metastasis was detected in 20 patients, while 10 patients did not show any metastasis. The study was approved by the Ethics Committee of the IRCCS, Istituto Tumori "Giovanni Paolo II," Bari, as a satellite project of the protocol GOIM 2903_v1.4, EUDRACT code: 2009-015807-19. All patients underwent a chemotherapy regimen based on platinum compounds. Relevant clinical and survival data were available for all patients. All patients signed informed consent. The study was conducted in accordance with the international standards of good clinical practice.

RNA isolation and processing

The process of hemolysis can interfere with the circulating miRNA expression level therefore, particular attention should be paid to the procedure of serum sample collection [23]. Blood samples (5–7 ml) were collected in serum separation

Fig. 1 Design of the study. All patients have been divided into training and validation sets according to homogeneous clinico-pathological features. Microarray analysis of training set has been used to select miRNAs to validate in the second group of patients as reported in M&M
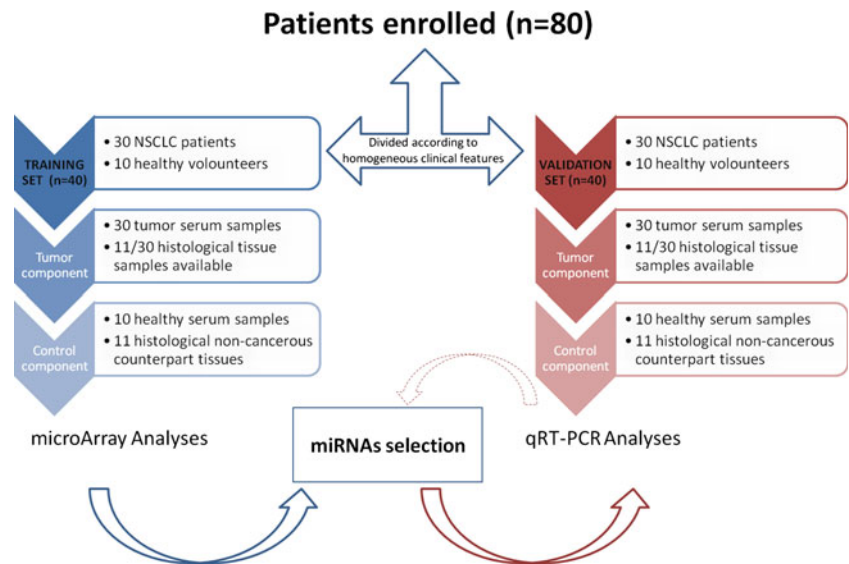
tubes and sent to the laboratory within 2 h of blood collection, at room temperature [24]. The upper serum phases were recovered after 10 min with a centrifugation at 3000 rpm, and immediately stored at −80 °C until further analyses. Circulating RNA was isolated from 250 μl of serum collected from NSCLC patients and control samples by using the TRIzol LS reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol.

Formalin-fixed, paraffin-embedded (FFPE) tissues were cut into 5-μm sections and stained with hematoxylin and eosine, in order to discriminate cancerous regions from noncancerous

Table 1 Clinical characteristics of 30 NSCLC patients in the validation set

Features	Number
Gender	
Male	21
Female	9
Median age (years range)	65 (49–79)
≥65	17
<65	13
Smoking habit	
Current	23
Never	7
Histological classification	
Adenocarcinoma	19
SCC	11
Stage classification	
IIB/IIIA	8
IIIB/IV	22
Metastasis	
Presence	22
Absence	8

NSCLC non small cell lung cancer, *SCC* squamous cell carcinoma

ones. The threshold of 70 % of tumor cells was fixed by the pathologist to make molecular analysis adequate.

Two 10-μm FFPE slides were used for RNA extraction after the macrodissection process, necessary to isolate the tumor component from its noncancerous surrounding counterpart. Total RNA was extracted both from the selected tumor tissue area and from the noncancerous counterparts using the FFPE RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The RNA concentration of all samples was measured by the NanoDrop ND-8000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Microarray hybridization and data processing

Five hundred nanograms of RNA of each sample was labeled using the 3DNA Array Detection FlashTag™ RNA Labeling Kit according to the manufacturer's instruction and analyzed by the GeneChipmiRNA v. 1.0 Array (Affymetrix). This contains 46,228 probes comprising 7815 probe sets and covers 71 organisms including 1100 human miRNAs derived from the Sanger miRBase and miRNA database v11 (http://microrna.sanger.ac.uk, accessed April 15, 2008). First, poly (A) tailing was carried out at 37 °C for 15 min in a volume of 15-ml reaction mix, which contained 1× reaction buffer, 1.5 ml MgCl$_2$ (25 mM), 1 ml ATP Mix diluted 1:500, and 1 ml PAP enzyme. Secondly, Flash Tag Ligation was performed at room temperature for 30 min by adding 4 ml of 5X Flash Tag Ligation Mix Biotin and 2 ml T4 DNA Ligase into 15 ml of reaction mix. To stop the reaction, 2.5 ml of Stop Solution was added. Each sample was hybridized on the array, washed and stained with Affymetrix Fluidics Station 450, and scanned with the Affymetrix GeneChip Scanner 3000 7G using the Command Console software (Affymetrix).

Principal component analysis (PCA) was performed as it is an excellent method for visualizing high-dimensional data and underlies outlier samples through Partek Genomics Suite v6.6 (Partek Inc., St. Louis, MO, USA). Raw data (.CEL files) were preprocessed through the Robust Multiarray Average (RMA) method to remove systematic variations. Briefly, RMA corrects raw data for background using a formula which is based on a normal distribution and uses a linear model to estimate values on a log-scale. RMA normalization was performed using the "affy" package of the Bioconductor suite (http://www.bioconductor.org/) for the R statistical language (http://cran.r-project.org/). Normalized values were statistically analyzed with MeV software v.4.8.1 (http://www.tm4.org/mev.html).

qRT-PCR of miRNA expression in validation set samples

Quantification of selected mature miRNA expression levels was performed by RT-PCR using TaqMan MicroRNA Assay Kits (Applied Biosystems, Foster City, CA, USA). Briefly, 10 ng total RNA was reverse-transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. Quantitative PCR was then performed with the ABI 7000 Real-Time PCR system (Applied Biosystems) using the maximum quantity of cDNA indicated in the protocol, following all steps and instructions described by the manufacturer. The threshold cycle (CT) values were calculated with SDS 1.1 software (Applied Biosystems). All RT-PCR reactions were performed in duplicate to test the reproducibility of the detection, and a good correlation (0.920) in each miRNA expression level was found between runs. The average levels of miRNA expression in all tissue and serum samples were normalized relative to the average amounts of U6 small nuclear RNA (U6 snRNA) using the $2-\Delta\Delta Ct$ method [25].

Enrichment analysis

Validated targets of selected miRNAs were analyzed through the miRWalk bioinformatic tool (http://www.ma.uni-heidelberg.de/appsz/zmf/mirwalk/), which is a database including validated gene targets and that allows prediction from eight established miRNA prediction programs [26]. The resulting gene list was submitted to DAVID 6.7 bioinformatic tool in order to identify the targeted pathway, setting the threshold for FDR to 0.01 and considering enrichment in KEGG pathways. P value <0.05 for enriched terms was estimated by David through Fisher's exact test [27].

Statistical and computational analysis

Normalized values by the array were statistically analyzed with MeV software v.4.8.1. t Test was used to compare differences in miRNA expression between tissue and serum samples. Mann-Whitney tests were used to check associations between miRNA expression levels and clinico-pathological features of the patients.

Time to progression of affected patients was estimated by the Kaplan-Meier method and was compared with the log-rank test. Cox regression analysis was performed through R-package "survival" (http://r-forge.r-project.org) to analyze the association between the expression of miRNAs and several risk factors (gender, age, histological type, smoking status, metastases, clinical staging) in the NSCLC outcome. The median fold change of miRNA expression was set as cutoff and was used to classify samples as "high" expressing or "low" expressing.

A P value <0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA).

Results

Difference in microRNA expression levels between serum and tissue samples in NSCLC patients

The miRNA array platform was used to perform a miRNA expression profile in the training set. The differential expression analysis showed 326 miRNAs with a statistically significant difference in mean expression ratio (MER) between tumor serum and tumor tissue samples ($P<0.05$) (supplementary file Fig. S1).

The PCA confirmed this segregation in miRNA expression ratio between the serum collected from affected patients and the tissue representative of the primary tumor (supplementary file Fig. S2).

MeV software analyses revealed miRNAs with a deregulated expression between tumor serum and control serum ($n=57$) and between tumor tissue and noncancerous tissue samples ($n=29$) ($P<0.05$). These data were used to identify, through a Venn diagram, three categories of miRNAs: miRNAs with a statistically significant deregulation in tumor serum samples ($a=55$), miRNAs significantly deregulated in tumor tissue samples ($b=27$), and a third category (c), formed by two miRNAs, deregulated both in tumor serum and in tumor tissue samples (Fig. 2 and supplementary file Table S1).

Considering a, b, and c categories, we used the MiRwalk tool to select only those miRNAs whose validated target genes were simultaneously involved in NSCLC, EGFR, PIK3CA/AKT/mTOR, and MAPK/ERK pathways. MiRNAs that met this criteria in the a category were miR-486-5p and let-7b, whereas in the b category, these were miR-200c* and miR-29c*. In the c category, only miR-133a was eligible. The involvement of these selected miRNAs in NSCLC tumorigenesis was also confirmed by enrichment pathway analysis. The
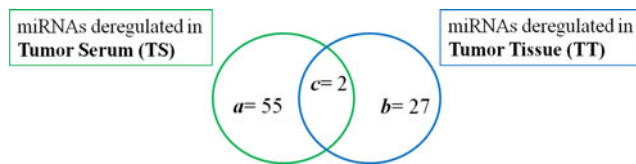
Fig. 2 Venn diagram allows to divide miRNAs according to the deregulated expression between tumor serum and tissue samples. We can identify three different categories of miRNAs with deregulated expression levels: (**a**) 55 serum-specific miRNAs; (**b**) 27 tissue-specific miRNAs; (**c**) two miRNAs commonly deregulated in the two tumor sites

validated target genes of the five selected miRNAs resulted significantly associated with specific pathways correlated with cancer development (Table 2).

In the *a* category, miR-486-5p and let-7b were both significantly downregulated in the tumor serum compared to control serum ($P=0.01$ and $P=0.03$, respectively); unlike let-7b, miR-486-5p had a lower expression in the tumor tissues compared to noncancerous counterparts, even if these difference were not statistically significant. In the *b* category, miR-200c* and miR-29c* were both significantly downregulated in the tumor tissues compared to noncancerous counterparts ($P=0.04$ and $P=0.02$, respectively) while their higher expression level in tumor serum than in control serum was not statistically significant. The miR133a (*c* category) showed a significant upregulation of its expression level both in tumor tissue compared to noncancerous counterparts and in tumor serum compared to control serum samples ($P=0.04$ and $P=0.02$, respectively).

When comparing the mean expression ratio of miRNAs in tumor tissue versus tumor serum, the levels of miR-486-5p were higher in the serum of the affected patients than in tissue samples ($P<0.00$), while the let-7b expression level resulted lower in the serum of affected patients than in tissue samples ($P<0.00$). MiR-200c* and miR-29c* evidenced a lower mean expression ratio in tumor tissue than in serum samples ($P=0.03$ and $P=0.00$, respectively). Moreover, for miR-133a, the difference in mean expression ratio was not statistically significant between the two tumor components (Fig. 3).

The data of the five selected miRNAs were only confirmed in a further validation set of samples through qRT-PCR analyses. The median fold change of miRNA expression levels both in tumor serum and in tumor tissue samples was evaluated for each selected miRNA belonging to the three categories (*a*, *b*, *c*) identified in the training set.

In the *a* category, miR-486-5p was downregulated in tumor serum compared to control serum and the median fold change of miR-486-5p expression resulted higher in tumor serum than in tumor tissue ($P=0.004$). Let-7b also resulted downregulated in tumor serum compared to control serum, but unlike in the training set, the median fold change expression of let-7b was not significantly higher in tumor tissue that in tumor serum. In the *b* category, the expression level of miR-200c* was not detectable in tumor serum as it was too low, while it was

upregulated in tumor tissues compared to noncancerous counterparts; therefore, its fold change expression in NSCLC serum samples could not be compared to that of tumor tissues. In the same category, miR-29c* was downregulated in tumor tissue compared to noncancerous counterparts and the median fold change of miR-29c* expression was lower in tumor tissues than in tumor serum samples ($P<0.001$).

In the third *c* category, miR-133a was upregulated both in tumor tissue and in tumor serum compared to control counterparts, but the difference in the median fold change of miR-133a expression both in tumor serum and in tumor tissues was not significantly different, with a trend for lower expression in tumor tissues (Fig. 4).

The deregulation in miRNA expression level analyzed in the training set was confirmed in the validation set only for three miRNAs, whose expression was specific for each of the three categories.

Association between miRNA expression levels and clinico-pathological features of NSCLC patients

The three miRNAs specific of each category: miR-486-5p, miR-29c*, and miR-133a were correlated with clinicopathological characteristics. Analyzing the serum of affected patients, a higher miR-133a expression level resulted significantly associated with the clinical stage IIB/IIIA (Table 3), while in tumor tissues, miRNA expression levels did not prove to be associated with any features (Table 4).

miRNA expression levels and clinical outcome of NSCLC patients

Investigating miRNA expression levels and clinical outcome, the univariate Cox hazard regression analysis demonstrated that the serum level of miR-486-5p was a significant prognostic indicator of NSCLC (hazard ratio=0.179, 95 % CI 0.043–0.759, $P=0.019$), while no prognostic role was observed for expression levels of miR-29c* and miR-133a both in serum and in tissue samples (Table 5).

Moreover, a higher level of miR-486-5p in the serum of affected patients was associated with a longer time to progression of the disease after platinum-based chemotherapy ($P=0.010$; using a log-rank test, Fig. 5).

A multivariate Cox proportional hazard regression analysis taking into account the expression levels of miR-486-5p, miR-29c*, miR-133a, and some clinical features (gender, age, histological type, smoking habit, metastasis, clinical staging) showed that only the serum level of miR-486-5p was an independent indicator of NSCLC prognosis (Table 6).

Otherwise, levels of expression of miR-29c* and miR-133a both in tumor serum and in tumor tissue were not

Table 2 Pathways involved in NSCLC tumorigenesis associated with the five selected miRNAs

miRNA categories	miRNAs	Pathways	*P* value
a) Tumor serum	miR-486-5p	Pathways in cancer	3.33E−19
	Let-7a	Non small cell lung cancer	2.94E−09
		p53 signaling pathway	2.04E−05
		Cell cycle	1.99E−05
		MAPK signaling pathway	0.001
b) Tumor tissue	miR-200c*	Pathways in cancer	1.21E−28
	miR-29c*	Non small cell lung cancer	1.56E−13
		Cell cycle	8.25E−10
		MAPK signaling pathway	0.003
		mTOR signaling pathway	0.021
c) Tumor samples	miR-133a	Pathways in cancer	9.65E−10
		MAPK signaling pathway	6.48E−04
		p53 signaling pathway	0.007
		Non small cell lung cancer	0.020
		Jak-STAT signaling pathway	0.030

KEGG pathways for selected miRNA with *P* values <0.05

associated with disease progression in NSCLC patients (data not shown).

Discussion

NSCLC is a solid tumor often diagnosed at an advanced stage because of the lack of specific early symptoms, thus making surgery an impossible treatment option. In fact, clinical investigation is mostly possible on small biopsies or cytological samples from bronchoscopy, which provide few tumor cells often of poor quality.

Furthermore, these tumor samples might not always be adequate for molecular analysis because of the heterogeneity of tumor content, changes in genetic alterations during the metastatic process, or chemotherapy treatments.

Therefore, a noninvasive and easy strategy of sampling that could give reliable information on the growth and biology of NSCLC is being studied. Blood-based biomarkers might greatly improve the early detection and prognosis of NSCLC
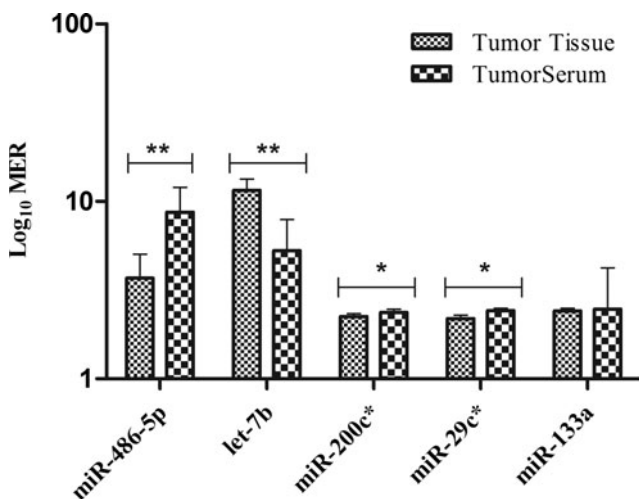
Fig. 3 miRNA expression level in serum and tissue samples of the training set. Expression level, through microarray analysis, of the five selected miRNAs: $\log_{10}$ of the mean expression ratio of miR-486-5p and let-7b (deregulated in tumor serum samples—*a*), miR-200c* and miR-29c* (deregulated in tumor tissue samples—*b*), and miR-133a (deregulated both in tumor serum and in tumor tissue—*c*) was shown (*$P<0.05$; **$P<0.01$)
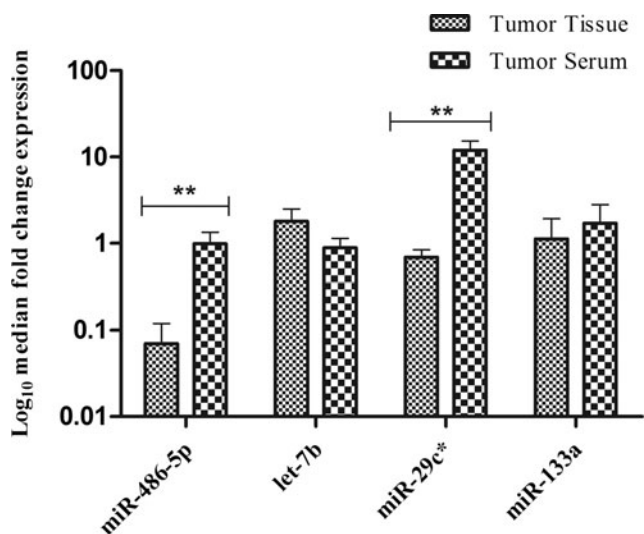
Fig. 4 qRT-PCR data on miRNA expression levels of selected miRNAs in the validation set. $\log_{10}$ of the median fold change of expression level for each miRNA belonging to the three categories (*a*, *b*, *c*) identified in the training set (*$P<0.05$; **$P<0.01$)

Table 3 Association of expression levels of selected miRNAs in tumor serum of 30 NSCLC patients with clinico-pathological characteristics. In *italics* significant value (p<0.05 by Mann-Whitney test); *SCC* squamous cell carcinoma

Characteristics	N	miR-486-5p		miR-29c*		miR-133a	
		Median (IR)	P value	Median (IR)	P value	Median (IR)	P value
Gender							
Male	21	0.99 (0.84—8.73)	0.29	11.77 (7.66–19.09)	0.53	1.63 (1.17–1.88)	0.39
Female	9	3.26 (0.96–21.59)		16.29 (8.51–17.13)		1.75 (1.44–2.03)	
Median age							
>65	17	0.99 (0.84–4.29)	0.19	11.94 (9.38–16.71)	0.80	1.7 (1.36–1.93)	0.83
<65	13	1.94 (0.95–27.54)		12.08 (4.09–19.63)		1.74 (1.14–1.93)	
Histological classification							
Adenocarcinoma	19	2.43 (0.94–13.89)	0.33	14.74 (9.00–17.40)	0.30	1.72 (1.51–1.96)	0.39
SCC	11	0.99 (0.81–8.94)		9.77 (6.83–16.68)		1.45 (1.16–1.86)	
Smoking habit							
Current	23	0.99 (0.90–10.85)	0.89	12.08 (7.07–21.50)	0.92	1.63 (1.19–1.90)	0.49
Never	7	3.26 (0.96–22.56)		11.77 (8.50–16.73)		1.75 (1.51–2.05)	
Stage classification							
IIB/IIIA	8	1.35 (0.98–8.94)	0.78	8.62 (6.33–24.34)	0.62	1.88 (1.71–2.26)	*0.03*
IIIB/IV	22	0.99 (0.89–10.84)		12.67 (9.45–16.76)		1.56 (1.14–1.76)	
Metastasis							
Presence	22	0.99 (0.94–8.11)	0.91	11.94 (7.07–16.68)	0.34	1.58 (1.16–1.78)	0.17
Absence	8	1.17 (0.67–11.28)		12.08 (8.25–24.70)		1.76 (1.37–2.19)	

Table 4 Association of expression levels of selected miRNAs in tumor tissue of 11/30 NSCLC patients with clinico-pathological characteristics

Characteristics	N	miR-486-5p		miR-29c*		miR-133a	
		Median (IR)	P value	Median (IR)	P value	Median (IR)	P value
Gender							
Male	8	0.07 (0.01–7.09)	0.84	0.67 (0.00–8.59)	0.77	1.14 (0.02–3.24)	0.26
Female	3	0.51 (0.01–7.04)		0.46 (0.05–11.84)		0.68 (0.10–1.15)	
Mean age (year)							
>65	8	0.24 (0.01–7.09)	0.54	4.31 (0.02–11.84)	0.08	1.15 (0.02–3.24)	0.28
<65	3	0.02 (0.01–7.04)		0.05 (0.00–0.43)		1.05 (0.68–1.05)	
Histological classification							
Adenocarcinoma	9	0.41 (0.04–5.66)	0.29	0.41 (0.04–5.66)	0.72	1.13 (0.39–1.40)	0.64
SCC	2	0.03 (0.02–0.04)		0.22 (0.02–0.43)		1.87 (1.05–2.69)	
Smoking habit							
Current	8	0.07 (0.01–7.04)	0.68	0.27 (0.03–6.00)	0.08	1.14 (1.05–2.43)	0.12
Never	3	0.51 (0.01–7.09)		8.59 (0.46–11.84)		0.10 (0.02–1.15)	
Stage classification							
IIB/IIIA	3	0.07 (0.02–0.07)	0.47	0.92 (0.43–8.57)	0.63	1.16 (1.05–3.24)	0.31
IIIB/IV	8	0.46 (0.02–6.35)		0.29 (0.03–8.37)		1.09 (0.24–1.52)	
Metastasis							
Presence	8	0.46 (0.02–6.35)	0.47	0.29 (0.03–8.37)	0.63	1.09 (0.24–1.52)	0.31
Absence	3	0.07 (0.02–0.07)		0.92 (0.43–8.57)		1.16 (1.05–3.24)	

SCC squamous cell carcinoma

P<0.05 between groups using Mann-Whitney test

Table 5 Univariate Cox hazard regression analysis. In *italics* significant value

	HR (95 % CI)	*P* value
Serum miR-486-5p	0.179 (0.043~0.759)	*0.019*
Serum miR-29c*	0.491 (0.137~1.755)	0.274
Serum miR-133a	1.043 (0.301~3.611)	0.947
Tissue miR-486-5p	2.000 (0.181~2.06)	0.571
Tissue miR-29c*	0.500 (0.045~5.514)	0.591
Tissue miR-133a	0.537 (0.049~5.846)	0.613

HR hazard ratio, *CI* confidence interval

Table 6 Multivariate Cox hazard regression analysis. In *italics* significant value.

	HR (95 % CI)	*P* value
Serum miR-486-5p	0.148 (0.030~0.719)	*0.018*
Serum miR-29c*	0.414 (0.086~1.981)	0.269
Serum miR-133a	1.642 (0.332~8.124)	0.544

HR hazard ratio, *CI* confidence interval

patients, and recently, it has been suggested that this role could be played by circulating miRNAs. In fact, miRNA profiles in blood could arise from the primary tumor and might already be detectable at early stages in the development of tumors; thus, it could be useful to understand real-time information on the tumor status of affected patients as compared to that observed in the primary tumor tissue [28]. Comparison of miRNA signature between primary tissue and the bloodstream remains a field of great interest in order to understand lung tumorigenesis and to detect specific prognostic miRNAs through a minimally invasive approach.

We have already shown the possibility to use fine needle aspirate (FNA) samples collected from the primary tumor site of affected patients as less invasive sources of tumor cells helpful to analyze genetic (EGFR and KRAS gene mutations) and epigenetic (miRNA) changes in lung cancer tumorigenesis showing a strict correlation of miRNA levels in the two cell sites [29]. The present paper aims to study the expression of miRNAs in serum and in primary tumor tissue in order to improve the early prognosis of NSCLC patients.
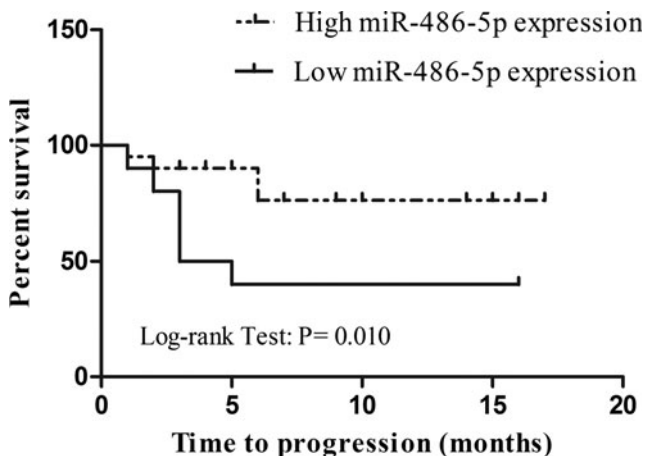

Fig. 5 Kaplan-Meier curves for NSCLC patients according to serum level of miR-486-5p. *P* value for time to progression of disease for patients with high and low levels of miR-486-5p expression was calculated using the log-rank test (log-rank test, *P*=0.010)

It is already known that both plasma and serum samples are a useful blood-based source to perform genetic and epigenetic analysis. MiRNA expression levels in serum might not always correlate with that in plasma due to the difference in their biological components associated with the hemolytic process. In fact, a particular approach has been proposed to discriminate hemolysis-related miRNAs by circulating miRNAs useful as biomarkers [30]. We decided to overcome this aspect and perform our miRNA analysis in serum samples of NSCLC patients.

Firstly, through the comparison of microarray profile between tumor component and control counterparts, we detected two classes of miRNAs: miRNAs deregulated in tumor serum and miRNAs deregulated in tumor tissue. Then, the merge of these classes was used to select three categories of miRNAs with differentially deregulation in tumor serum (*a*), in tumor tissue (*b*), and in both tumor serum and tissue (*c*). Among the cluster of miRNAs belonging to each category, our attention was focused on those miRNAs whose validated target genes are simultaneously involved in EGFR, PIK3CA/AKT/ mTOR, and MAPK/ERK pathways. These target genes often resulted deregulated in NSCLC, so the regulation of their expression by miRNAs could be useful for clinical outcomes of affected patients.

We confirmed miR-486-5p in the *a* category, miR-29c* in the *b* category, and miR-133a in the *c* category through qRT-PCR in the validation set.

Previous studies on miRNA expression pattern in tumor plasma/serum and primary tumor tissue reported discordant results and always compared miRNA level in the two sites with respect to the normal counterpart more than the two districts with respect to each other.

Our approach previously compared miRNA expression level in tumor and control component both in tissue and in serum samples and then compared the deregulated miRNA pattern evidenced in both groups (tissue vs serum).

In some studies on lung cancer, the expression of specific miRNA appeared to be increased both in serum and in tumor tissue samples [31, 32]. A set of eight miRNAs showed deregulated expression in the tumor tissues of NSCLC patients compared to noncancerous tissues, and some of them

displayed also a differential expression in the plasma of affected patients when compared to that of healthy donors [33].

Circulating miRNAs in plasma samples of NSCLC patients were considered more tissue specific than tumor specific, because their deregulated expression in tissues was not detected in the corresponding plasma samples [34, 35]. This was confirmed by the present data which highlighted that miRNAs deregulated in tissue are different from those deregulated in serum. Our work highlighted miRNAs clearly deregulated in tumor serum samples, such as miR-486-5p, or in tumor tissue, such as miR-29c*, that could reflect the tumor status of the patient investigating different body districts. Furthermore, the simultaneous presence of a deregulated miRNA (miR-133a) in primary tumor tissue and in serum collected from NSCLC patients stressed the possibility that the expression of a few miRNAs in serum/plasma samples could reflect the miRNA expression in tumor tissue. This result could allow clinicians to monitor tumor status through a noninvasive approach, while the bloodstream-specific miRNAs could be used as prognostic biomarkers in all stages of disease. The reduction of miR-486-5p expression level that we observed in tumor serum with respect to the control counterpart has also been observed in different studies showing that reduced miR-486-5p expression is one of the most frequent molecular events in NSCLC both in serum, sputum samples [36, 37], and in tumor tissues [34, 38].

The role of miR-486-5p as a tumor suppressor involved in cellular proliferation, migratory capacity, and tumor growth both in vitro and in vivo is already known. Small et al. recently demonstrated that miR-486-5p targeted both PTEN and FOXO1A inducing an increase in PI3K/AKT signaling, thus explaining the role of its downregulation on cell proliferation [39]. PTEN is considered a tumor suppressor gene with a negative regulator role in the PIK3CA pathway, inducing control on tumor growth, survival, and proliferation. The involvement of miR-486-5p in the PIK3CA pathway was also confirmed by the observation that this miRNA directly targets PIK3R1, a member of the pathway in cancer whose expression can inhibit the activation of the PI3K-AKT pathway [40].

The tumor suppressor role of miR-486-5p was also evidenced by the reduction in downstream pAKT and pFoxo3a, belonging to the validated targeted components of IGF signaling including IGF1, IGF1R, and p85α [41].

In our set of samples, the downregulation of miR-486-5p in tumor serum could confirm the already demonstrated tumor suppressor role of this miRNA in tissue. Moreover, we observed that patients with lower serum level of miR-486-5p expression showed a worse time to progression of disease after starting a platinum-based chemotherapy, compared to patients with a high expression level of this miRNA, confirming that the reduced expression of miR-486-5p could contribute to progression and metastasis of NSCLC, as observed by its behavior on cancer cell migration and invasion in vitro and on metastasis development in vivo [42].

Furthermore, we observed that a low serum level of miR-486-5p was a statistically significant indicator of disease prognosis in NSCLC patients, irrespective of the other clinical features.

This underlined that patients with a low serum level of miR-486-5p had a more aggressive disease than patients with a high miRNA expression level, as reported elsewhere [43]. The Cox proportional hazard regression analysis showed that the low level of miR-486-5p expression in tumor serum was also a good predictor of disease progression after administration of platinum based-chemotherapy. In fact, downregulation of miR-486-5p in the serum of affected patients was significantly correlated with worse response to all chemotherapeutic combination with platinum: platinum-gemcitabine ($P=0.038$), platinum-pemetrexed ($P=0.048$), and platinum-taxanes ($P=0.032$) (data not shown).

Interestingly, a role of miR486-5p as a therapeutic target has been hypothesized due to its regulatory power on PIM1 that improved the efficacy of epidermal growth factor receptor (EGFR) targeted therapies [44].

In the category **b**, miR-29c* was downregulated in tumor tissue with a lower expression in tissue when compared to serum. MiR-29c* is a member of miR-29 family and is implicated in the pathogenesis of cancer and its expression is reduced in solid tumors, such as lung cancer, because it targets several genes involved in the cell cycle (MCL-1, CDK6).

Supporting our data on the higher expression level of miR-29c* in tumor serum compared to tumor tissue, a study showed a significant upregulation of miR-29c* in the plasma of patients affected by malignant pleural mesotelioma [45]. Furthermore, miR-29c was upregulated in serum samples of early stage NSCLC patients [46]. In our set of samples, no prognostic role was observed for expression levels of miR-29c* both in serum and in tissue samples.

MiR-133a belongs to a category **c** of miRNAs deregulated both in tumor serum and in tumor tissue. It was upregulated in both tumor sites when compared to their control counterparts, but its expression level resulted similar in tissue when compared to serum confirming the hypothesis that some miRNAs are released by the primary tumor into the bloodstream and could be representative of the tumor status of patients [17, 37]. This class of circulating miRNAs could mirror the miRNA signature of primitive tumor tissue and could be useful to obtain real-time information on the biology of lung cancer.

MiR-133a was considered a potent inhibitor of cell survival because of its cytotoxic power observed in three NSCLC cell lines [47]. In fact, this miRNA was able to activate apoptotic pathways and promote cell cycle arrest, regulating S phase progression. Some target prediction analysis showed that miR-133a has both oncogenes and tumor suppressors as target genes.

In particular, in lung SCC, miR133a functions as a tumor suppressor and its restoration in SCC cell lines inhibited cell proliferation, invasion, and migration [48]. The potential role played by this miRNA in lung tumor progression could be demonstrated in our set of samples because the high expression of miR-133a was statistically associated with advanced stage of disease (IIIB/IV).

In conclusion, we can underline the role of serum miR-486-5p expression as a prognostic marker and the role of serum miR-133a expression as mirror of tissue miRNA level. Even though our data are preliminary and limited by the small cohort of patients, they are supported by the fact that the enrolled series of samples came from a single institution and was homogeneous.

Therefore, further prospective studies using a larger cohort of samples have been designed in order to verify the clinical value of the selected circulating miRNAs in the diagnosis, prognosis, and outcome of NSCLC patients. This could overcome the use of tumor tissue for clinical analysis. Finally, this can be useful to monitor miRNA expression deregulation during tumor growth to follow up the response to antineoplastic treatments, thus improving the clinical outcome of NSCLC patients

Compliance with ethical standards

Conflicts of interest None

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