Lung Cancer 81 (2013) 388–396

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Clinical evaluation of microRNA expression profiling in non small cell lung cancer

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ARTICLE INFO

Article history:
Received 14 December 2012
Received in revised form 8 May 2013
Accepted 13 May 2013

Keywords:
Circulating miRNAs
MicroRNAs
miR-21
Non small cell lung cancer
Tumor markers
Liquid bead array
RT-qPCR

ABSTRACT

Deregulation of miRNAs expression levels has been detected in many human tumor types, and recent studies have demonstrated the critical roles of miRNAs in cancer pathogenesis. Numerous recent studies have shown that miRNAs are rapidly released from tissues into the circulation in many pathological conditions. The high relative stability of miRNAs in biofluids such as plasma and serum, and the ability of miRNA expression profiles to accurately classify discrete tissue types and disease states have positioned miRNAs as promising non-invasive new tumor biomarkers. In this study, we used liquid bead array technology (Luminex) to profile the expression of 320 mature miRNAs in a pilot testing group of 19 matched fresh frozen cancerous and non-cancerous tissues from NSCLC patients. We further validated our results by RT-qPCR for differentially expressed miRNAs in an independent group of 40 matched fresh frozen tissues, 37 plasma samples from NSCLC patients and 28 healthy donors.

We found that eight miRNAs (*miR-21*, *miR-30d*, *miR-451*, *miR-10a*, *miR-30e-5p* and *miR-126**, *miR-126*, *miR-145*) were differentially expressed by three different statistical analysis approaches. Two of them (*miR-10a* and *miR-30e-5p*) are reported here for the first time. Bead-array results were further verified in an independent group of 40 matched fresh frozen tissues by RT-qPCR. According to RT-qPCR *miR-21* was significantly up-regulated ($P = 0.010$), *miR-126** ($P = 0.002$), *miR-30d* ($P = 0.012$), *miR-30e-5p* ($P < 0.001$) and *miR-451* ($P < 0.001$) were down-regulated, while *miR-10a* was not differentiated ($P = 0.732$) in NSCLC tissues. However, in NSCLC plasma samples, only three of these miRNAs (*miR-21*, *miR-10a*, and *miR-30e-5p*) displayed differential expression when compared to plasma of healthy donors. High expression of *miR-21* was associated with DFI and OS both in NSCLC tissues ($P = 0.022$ and $P = 0.037$) and plasma ($P = 0.045$ and $P = 0.065$), respectively. Moreover, we report for the first time that low expression of *miR-10a* in NSCLC plasma samples was associated with worse DFI ($P = 0.050$) and high expression of *miR-30e-5p* was found to be associated with shorter OS ($P = 0.048$). In conclusion, circulating *miR-21*, *miR-10a* and *miR-30e-5p* in plasma should be further evaluated as potential non-invasive biomarkers in NSCLC.

1. Introduction

Worldwide, lung cancer is the most common cause of cancer-related death in men and women while its main types are small cell lung cancer (SCLC) and non small cell lung cancer (NSCLC) accounting about 85% of lung cancers [1,2]. Despite late advancements in NSCLC therapies, the prognosis for patients with advanced NSCLC remains poor, so innovative, non-invasive, sensitive and reliable biomarkers still need to be discovered and exploited.

MicroRNAs (miRNAs) are endogenous short non-coding RNAs that regulate gene expression by targeting mRNAs [3]. Aberrations in miRNAs expression levels have been detected in many human tumor types, and recent studies have demonstrated their critical role in cancer pathogenesis [4,5]. miRNA expression profiles are emerging as potentially useful biomarkers for diagnosis, prognosis, personalized therapy, and disease management [6,7]. In lung cancer, miRNAs have been evaluated as diagnostic and prognostic biomarkers [8–12], and found to be associated with clinical outcome [13]. Recently, miRNA expression profiles that differentiate between primary lung tumors and metastases to lung [14] as well as profiles that discriminate SCLC from NSCLC have been identified [15]. Nowadays, circulating miRNAs have been also identified in serum/plasma in a remarkably stable form [16]. Numerous publications have reported on the dysregulation of circulating miRNAs in blood of tumor patients [17] but to the best of our knowledge, there

Abbreviations: NSCLC, non small cell lung cancer; AD, adenocarcinoma; SCC, squamous cell carcinoma; miRNAs, microRNAs; RT-qPCR, quantitative reverse transcription PCR; LNA, locked nucleic acid; DFI, disease free interval; OS, overall survival.

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http://dx.doi.org/10.1016/j.lungcan.2013.05.007

are only a few studies up to now on circulating miRNAs in serum or plasma of NSCLC patients [18–21]. However, there are still controversial results concerning the relation of miRNA expression levels between tissues and corresponding plasma [16,22].

The primary aim of our study was to evaluate the potential of circulating miRNAs as novel tumor biomarkers in NSCLC.

2. Materials and methods

2.1. Clinical samples

We used 59 cases of surgically resected NSCLC and adjacent normal tissue specimens. All patients gave their informed consent, and the Ethical and Scientific Committees of the participating institutions approved the study. After surgery removal, all tissue samples were immediately flash frozen in liquid nitrogen and stored at −70 °C until use [8]. Data of tumor histology, smoking history, staging and follow-up were obtained from patient files. Blood was also collected in the morning before surgery from 37 NSCLC patients and 28 healthy age-matched volunteers, and plasma was immediately separated and stored at −70 °C. Total RNA from normal lung tissue (Ambion) was used as a quality control in all experiments. We were incompletely blinded to clinical information before sample testing to maintain objectivity. The tumor specimens were sequentially collected and all patients were male. The majority of patients (54/59, 91.5%) were smokers and suffered from mild to moderate chronic obstructive pulmonary disease according to pulmonary function tests that were included as part of the standardized preoperative evaluation of the patients. All patients were treatment naïve when the samples were collected, but after surgery all patients received standard chemotherapy protocols for adjuvant NSCLC, such as gemcitabine plus taxanes (90%) or platinum based chemotherapy (10%). The majority of patients changed stage after the disease relapse to IIIB. We analyzed all samples histologically to assess the amount of tumor component (at least 70% tumor cells) and the quality of material (i.e., absence of necrosis). Normal adjacent tissues were defined histologically confirmed by using the classical pathology approaches. The distance from the primary tumor was >2 cm.

2.2. RNA extraction

Total RNA was extracted from tissues using the Trizol reagent (Invitrogen) as previously described [8,23]. The RNA pellet was dissolved in 20 μL RNA storage buffer (Ambion) and stored at −70 °C until use. Total RNA was quantitated using the NanoDrop ND-1000 (NanoDrop Technologies). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Pico Lab Chip Kit, Agilent Technologies). Total RNA was extracted from plasma (200 μL) using the *mir*Vana ™PARIS™ Kit (Ambion). We spiked into each plasma sample 25 fmol of an exogenous synthetic miRNA, *Caenorhabditis elegans miR-39* as an external control for normalization of sample-to-sample variations in RNA isolation procedures [16,24].

2.3. miRNA expression profiling by liquid bead array

FlexmiR miRNA Human Panel array (Luminex, TX, USA) was used to obtain expression profiles for 320 human mature miRNAs in a pilot testing group of 19 NSCLC fresh frozen cancerous and adjacent non-cancerous tissue specimens. This assay sensitively measures the expression of human miRNA sequences by combining xMAP® and locked nucleic acid (LNA™) technologies. The integration of these technologies allows precise detection of miRNAs without prior need for sample RNA size fractionation or amplification. All steps were followed according to the manufacturer's instructions.

2.4. Quantitation of miRNA expression by RT-qPCR

We further evaluated deregulated miRNAs by RT-qPCR using TaqMan MicroRNA assays (Applied Biosystems, CA, USA) in an independent group of 40 fresh frozen NSCLC and adjacent non-cancerous tissue samples. Reverse-transcription using gene-specific primer sets was followed by qPCR amplification for 45 cycles using the LightCycler 2.0 system (Roche Diagnostics, Germany) and miRNA-specific probes (Applied Biosystems, CA, USA). Relative expression was determined using the $\Delta\Delta C_q$ approach [25]. In fresh frozen tissues we used *miR-191* as our reference miRNA for normalization since our RT-qPCR data showed that *miR-191* was stably expressed (Suppl. Fig. 1). In plasma circulating miRNA expression values were normalized in respect to *miR-16* since this miRNA has been reported as a suitable normalizer of miRNA values in plasma [19]. These miRNAs were also ranked as the most stable RNAs when we performed NormFinder algorithm analysis. ΔC_q values were calculated as the difference between C_q values found for each mature miRNA and reference miRNA for each sample. For each target miRNA, $\Delta\Delta C_q$ values were calculated as the differences between ΔC_q values for each cancerous sample and its corresponding adjacent normal tissue ($\Delta\Delta C_q = \Delta C_{q\ \text{cancer}} - \Delta C_{q\ \text{normal}}$). miRNAs expression data are presented as fold change relative to reference miRNA expression based on the formula of $RQ = 2^{-\Delta\Delta C_q}$ [25].

2.5. Statistical analysis

2.5.1. FleximiR miRNA assay

In order to increase reliability and credibility of miRNA profiles liquid bead array data were analyzed using three different statistical analysis approaches:

(a) 2-Tailed paired *t*-test after background correction and normalization (as recommended by Luminex) [26].
(b) Hierarchical clustering.
(c) Bead to bead background subtraction and quantile normalization.

For the multiplex liquid bead array normalization, four normalization microspheres are included in each human pool. These normalization microspheres each contain a unique capture probe designed to specifically target a ubiquitously expressed human small nucleoral RNA (snoRNA). The normalization beads can be used for inter-sample normalization. For each different microsphere type we tested a background control sample. This was water tested in the same manner as an RNA sample throughout the assay. At the completion of the assay the MFI for a given background control was subtracted from its associated microsphere type. Thresholds were defined according to P-value < 0.05, and a fold-change >1.15. We further selected to validate by RT-qPCR all mature miRNAs that were found to be differentially expressed in NSCLC tissues and their corresponding normal samples by all computational approaches.

2.5.2. RT-qPCR

Statistical analysis for RT-qPCR data was performed using the SPSS statistical package (Version 19, SPSS Inc.). RT-qPCR data were analyzed by Wilcoxon signed-rank test to statistically evaluate differences in mature *miR-21*, *miR-30d*, *miR-126**, *miR-30e-5p*, *miR-10a* and *miR-451* expression levels between NSCLC tissues and their corresponding non-cancerous tissues as well as in all NSCLC plasma samples and samples from healthy donors. One-way ANOVA was

used to analyze the association between the expression of miRNAs and clinicopathological features of the patients.

2.5.3. Survival analysis

For the survival analysis we divided NSCLC patients into two different groups: high-expression and low-expression, using the median RQ ratios for *miR-21*, *miR-30d*, *miR-126**, *miR-10a*, *miR-30e-5p* and *miR-451* as the corresponding cut-offs. The association between survival and mature *miR-21*, *miR-30d*, *miR-126**, *miR-10a*, *miR-30e-5p* and *miR-451* expression was estimated using the Kaplan–Meier method and two-sided log-rank test.

3. Results

The experimental flowchart of our study is outlined in Fig. 1.

3.1. miRNA expression profiling by liquid bead array

Initially we profiled the expression of 320 mature miRNAs in a pilot testing group of 19 fresh frozen NSCLC tissues and their matched adjacent non-cancerous tissues. Liquid bead array data were analyzed using three different statistical analysis approaches. According to hierarchical clustering 24 miRNAS were found to be differentially expressed, according to 2-tailed paired *t*-test after background correction and normalization (as recommended by Luminex) 23 miRNAs were found to be differentially expressed while according to bead-to-bead background subtraction and Quantile Normalization, 30 miRNAS were found to be differentially expressed. It was surprising to see (Fig. 2A) that each statistical analysis approach resulted in a number of different miRNAs that exhibited differential expression levels. However, by all computational approaches 8 miRNAs were consistently deregulated between cancerous and non-cancerous tissues. More specifically, seven miRNAs, were down-regulated (*miR-30d*, *miR-451*, *miR-30e-5p*, *miR-126*, *miR-145*, *miR-126**, *miR-10a*) while only *miR-21* was up-regulated. Among these, downregulation of *miR-10a* and *miR-30e-5p* is reported here for the first time in NSCLC. Fig. 2B shows a representative heat map of the statistically significant ($P < 0.05$) results.

Using our FlexmiR results a comparison analysis between the miRNAs expression profiling in lung AD versus SCC was also performed. This analysis revealed six miRNAs (miR-520d*, miR-489, miR-181b, miR-513, miR-26b and miR-520e) that were differentially expressed between these two histological types of NSCLC. Our FleximiR data for miR-205 were also confirmed by RT-qPCR. The expression levels of miR-513 and miR-520e were found to be higher in AD than in SCC in contrast to miR-520d, miR-489, miR-189 and miR-26b that were under-expressed.

3.2. Pathways enrichment analysis

In order to understand the potential involvement of miRNAs in the pathogenesis of NSCLC we identified potential targets for these 8 dysregulated miRNAs using the DIANA-mirPath software on the gene targets predicted by microT-4.0, Pic-Tar and TargetScan-5. Our analysis showed that many of the predicted miRNAs targets are involved in critical pathways affected in cancer such as survival, apoptosis, proliferation and epithelial–mesenchymal-transition (Table 1).

3.3. Validation of miRNA expression profiling data in an independent group of NSCLC patients by RT-qPCR (fresh frozen paired tissues)

To validate our liquid bead array findings, we quantified the expression of six out of these eight differentially expressed miRNAs in an independent group of 40 NSCLC fresh frozen paired tissues by RT-qPCR. We selected four miRNAs that have also been found by other studies to be differentiated in NSCLC (*miR-21*, *miR-30d*, *miR-126** and *miR-451*) and two newly reported in this study (*miR-10a* and *miR-30e-5p*). Our RT-qPCR results were in concordance with those obtained by the FlexmiR liquid bead array assay in the pilot testing group for five out of six tested miRNAs, while *miR-10a* was found to be expressed equally in cancerous and non-cancerous tissues (Fig. 3A). This finding is in agreement with a previous publication according to which microarrays expression data vary by as much as 60% when compared with RT-qPCR derived data [27]. The median ΔC_q levels of all these miRNAs – except from miR-10a ($P = 0.732$) – were significantly different between non-cancerous and tumor tissues for the same patients (Fig. 3B).

Table 1
Differentially expressed miRNAs in NSCLC and their predicted target genes.

miRNA	Predicted target genes
miR-10a	*EPHA4, UNC5D, EPHA8, NFAT5, PAK7, PIK3CA, ITGB8, FLT1, FLNA, CRK, CTNNBIP1, BTRC, MAP3K7, ATF2, BDNF, FLNA, CAMK2B, CDK6, SERPINE1*
miR-126	*RGS3, CRK, ITGA6, IRS1*
miR-145	*SEMA6A, SRGAP1, DPYSL2, EFNA3, KRAS, CFL2, MAPK1, PPP3CA, RASA1, SEMA3A, ACTB, TLN2, FLNB, CCND2, ACTG1, FZD7, CTNNBIP1, DVL3, NLK, CCND2, SMAD3, MAP4K4, FLNB, RASA2, MAP3K3, RPS6KA5, NLK, DUSP6, ZFYVE9, ACVR2A, INHBB, BMPR2, SMAD, CDK6, CCND2, BBC33, PTPRF, FOXO1, IRS1*
miR-21	*EPHA4, PPP3CA, RASA1, NFAT5, MAP2K3, NTF3, MAP3K1, FASLG, RPS6KA3, PIK3R1, RPS6KA3, SMAD7, ACVR2A, BMPR2, PITX2*
miR-30e-5p	*SRGAP3, PLXNA2, NCK2, GNAI2, DPYSL2, UNC5C, EFNA3, EPHB2, SEMA6D, KRAS, SEMA6B, CFL2, UNC5D, PPP3CA, RASA1, SEMA3A, NFAT5, PPP3CB, CAMK2D, MAPK8, NRG3, SOS1, ABL2, PIK3R2, CBLB, PLCG1, PIK3CD*
miR-30d	*SRGAP3, SEMA6A, GNAI2, DPYSL2, UNC5C, EFNA3, PLXNA1, EPHB2, SEMA6D, KRAS, SEMA6B, CFL2, NRP1, UNC5D, PPP3CA, RASA1, ABL1, SEMA3A, NFAT5, PPP3CB, KRAS, CACNB2, BDNF, RRAS2, CRKL, TAOK1, IL1A, MAP2K4, RAP1B, PPP3CA, RASA1, MAP3K12, MAP3K5, PDGFRB, CASP3, PPP3CB, CAMK2D, CRKL, PIK3R2, CBLB, PIK3R2, NFAT5, PLCG1, PPP3CB*
miR-451	*TSC1, CAB39, TSC1*

3.4. Validation of selected miRNAs expression profiling in plasma

We further examined whether *miR-21*, *miR-30d*, *miR-126**, *miR-10a*, and *miR-30e-5p* were also differentially expressed in plasma of NSCLC patients. We first measured their expression levels in plasma of 28 healthy volunteers by RT-qPCR ensuring the efficiency of miRNAs isolation by measuring *cel-miR-39* levels. All miRNAs except *miR-126** (which was not at all detectable) had C_q values ≤ 34 in each plasma sample of healthy volunteers indicating that these miRNAs were readily detectable in plasma. We next investigated the expression of *miR-21*, *miR-30d*, *miR-10a*, and *miR-30e-5p* in plasma of 37 NSCLC patients. The median ΔC_q levels of *miR-21*, *miR-10a* and *miR-30e-5p* were significantly different between plasma of healthy donors and plasma of NSCLC patients, while the expression levels of *miR-30d* in plasma samples did not differ between NSCLC patients and healthy donors (Fig. 3C).

3.5. Prognostic significance of differentially expressed miRNAs in NSCLC

We further evaluated the prognostic significance of *miR-21*, *miR-451*, *miR-30d*, *miR-126**, *miR-10a* and *miR-30e-5p* in fresh frozen paired tissues (independent group of 40 NSCLC patients). Kaplan–Meier survival analysis showed that patients with high expression of *miR-21* had significantly lower DFI and OS than those

Table 2
Association between six miRNAs and clinicopathological characteristics in 40 patients with NSCLC.

	N (%)	Tumor *miR-30e-5p* ($2^{-\Delta\Delta C_q}$)		Tumor *miR-10a* ($2^{-\Delta\Delta C_q}$)		Tumor *miR-21* ($2^{-\Delta\Delta C_q}$)		Tumor *miR-30d* ($2^{-\Delta\Delta C_q}$)		Tumor *miR-126** ($2^{-\Delta\Delta C_q}$)		Tumor *miR-451* ($2^{-\Delta\Delta C_q}$)	
		Mean ± SD	*P*	Mean ± SD	*P*	Mean ± SD	*P*	Mean ± SD	*P*	Mean ± SD	*P*	Mean ± SD	*P*
Age													
<60	15 (37.5%)	1.32 ± 3.88	0.542	2.52 ± 7.65	0.429	11.28 ± 23.34	0.347	1.77 ± 4.82	0.450	4.17 ± 9.56	0.276	0.65 ± 1.52	0.131
≥60	25 (62.5%)	0.66 ± 1.75		1.97 ± 3.70		5.67 ± 7.48		0.89 ± 1.57		1.41 ± 4.15		0.10 ± 0.14	
Histological classification													
Adenocarcinoma	16(40%)	0.62 ± 0.65	0.197	1.32 ± 0.68	0.758	13.74 ± 23.25	0.251	2.36 ± 4.93	0.294	3.24 ± 6.79	0.804	0.59 ± 1.46	0.391
SCC	21 (52.5%)	6.08 ± 13.77		1.17 ± 1.38		4.24 ± 5.98		0.56 ± 0.96		2.16 ± 7.29		0.10 ± 0.18	
Other	3 (7.5%)			1.02 ± 0.84		2.35 ± 0.76		0.30 ± 0.33		0.03 ± 0.02		0.18 ± 0.12	
Tumor size													
0-3 cm	11 (27.5%)	0.64 ± 0.88	0.659	1.98 ± 6.33	0.225	4.18 ± 6.29	0.443	0.32 ± 0.41	0.323	0.65 ± 1.12	0.384	0.12 ± 0.12	0.504
>3 cm	29 (72.5%)	1.87 ± 3.08		2.06 ± 4.08		9.27 ± 17.9		1.57 ± 3.67		3.11 ± 7.77		0.38 ± 0.12	
Lymph node													
Negative	23 (57.5%)	2.42 ± 7.17	0.345	1.36 ± 1.01	0.924	6.23 ± 7.76	0.512	6.23 ± 7.76	0.606	1.91 ± 6.89	0.588	0.11 ± 0.13	0.213
Positive	17 (42.5%)	0.53 ± 0.72		1.33 ± 1.63		10.11 ± 22.54		10.11 ± 22.54		3.27 ± 6.78		0.55 ± 1.41	
Stage													
I	11 (27.5%)	0.29 ± 0.23	0.191	2.58 ± 4.91	0.543	6.54 ± 10.27	0.760	6.54 ± 10.27	0.389	3.62 ± 9.70	0.557	0.14 ± 0.10	0.506
II,III,IV	29 (72.5%)	0.55 ± 0.69		4.13 ± 6.93		8.5 ± 17.75		8.5 ± 17.75		2.01 ± 5.36		0.37 ± 1.10	
Smoking status													
Non-smoker	6(15%)	6.47 ± 9.87	0.598	2.87 ± 6.88	0.602	21.46 ± 35.10	0.032*	21.46 ± 35.10	0.050*	4.37 ± 8.07	0.504	1.40 ± 2.54	0.010*
Current smokers	34(85%)	4.42 ± 8.02		3.91 ± 5.41		5.21 ± 6.90		5.21 ± 6.90		2.12 ± 6.61		0.13 ± 0.17	

One-way ANOVA and *t*-test was used to analyze the correlation between the expression of miRNAs and clinicopathological features of the patients.

* Significant difference ($P < 0.05$).

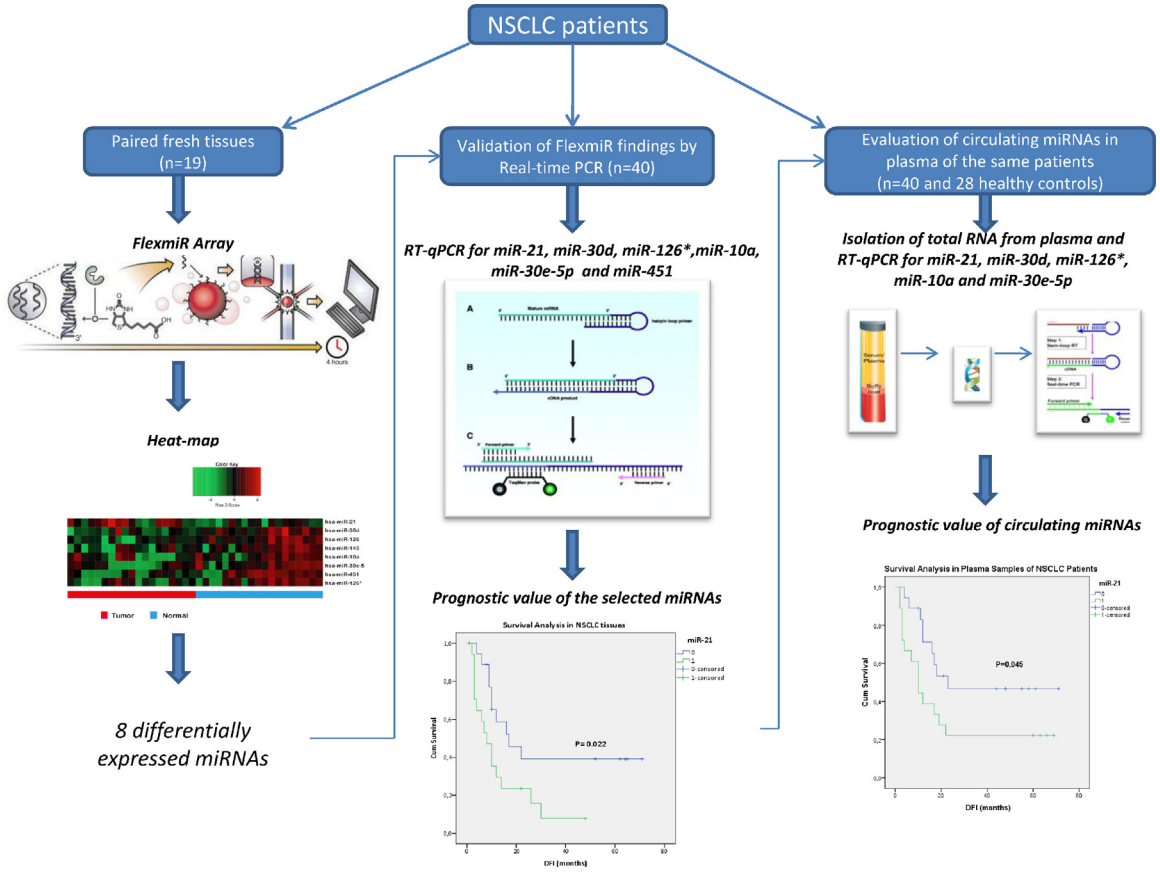


Fig. 1. Exprerimental flowchart of the present study.

with low expression (Fig. 4). However, the expression of *miR-451*, *miR-30d*, *miR-126**, *miR-10a* and *miR-30e-5p* did not correlate with DFI and OS in this group. We further evaluated the prognostic significance of these miRNAs (*miR-21*, *miR-30d*, *miR-10a* and *miR-30e-5p*) circulating in plasma of 37 NSCLC patients. *miR-21* overexpression both in NSCLC tissues and plasma was found to be of prognostic value both for DFI and OS, while circulating *miR-30e-5p* and *miR-10a* were associated with OS and DFI, respectively (Fig. 4). There was no correlation between these miRNAs and the clinico-pathological features of NSCLC patients; only the smoking status of these patients correlated with *miR-21*, *miR-451*, and *miR-30d* expression levels. However, the fact that the number of non-smokers was significantly lower than the number of smokers poses a question concerning this finding (Table 2).

4. Discussion

Recently miRNAs that either characterize histologically the lung tissues [14,28] or mark patients with poor prognosis were identified through studies that profiled miRNA expression in lung cancer [8–12,16,20,21,29,30].

We used liquid bead array technology to discover which miRNAs were differentially expressed among 320 mature miRNAs in a pilot group of NSCLC fresh frozen paired tissues. By using three different statistical analysis approaches we found that eight miRNAs were differentially expressed (7 down-regulated and *miR-21* up-regulated when compared to adjacent normal tissues). We report for the first time that *miR-10a* and *miR-30e-5p* are down-regulated in NSCLC in respect to their non-cancerous adjacent tissues. Six out of these eight differentially expressed miRNAs found in our study were consistent with those found by other studies. The discrepancy of our results in respect to other similar miRNA profiling studies might be attributed to the different miRNA microarray probes, and different technologies used.

We further studied the expression of six out of these 8 differentially expressed miRNAs in an independent group of NSCLC patients by RT-qPCR. We selected *miR-451*, *miR-126** and *miR-30d*, that were found to be down-regulated and *miR-21* that was highly over-expressed in our bead-array experiments and which according to our previous study was of prognostic significance in NSCLC [8]. Moreover, we tested for the first time the expression of *miR-10a* and *miR-30e-5p*. First of all our experiments have shown a good correlation between bead-array profiling and RT-qPCR results concerning fold change for five out of six tested miRNAs in tissues samples (except of *miR-10a*).

Wang et al. have very recently shown that *miR-451* was the most down-regulated miRNA in NSCLC tissues and that low *miR-451* expression levels were correlated with shorter overall survival in NSCLC patients [31]. Based on this data, this group proposed that *miR-451* functions as a tumor suppressor in NSCLC by targeting ras-related protein 14 (RAB14) [31]. Moreover, according to Bian et al., upregulation of *miR-451* increases cisplatin sensitivity of NSCLC cell line A549 [32]. We also verified both by our microarray experiments and by RT-qPCR that *miR-451* was significantly under-expressed in NSCLC tissues compared to their corresponding adjacent tissues. We also studied for the first time *miR-451* expression levels in plasma of NSCLC patients. Our data show that *miR-451* expression is significantly different between plasma samples of NSCLC patients and healthy volunteers; however, there was no correlation between *miR-451* under-expression and survival of NSCLC patients.

Yanaihara et al. have shown that *miR-126** was down-regulated in NSCLC [29]. According to Meister et al., *miR-126* and *miR-126**

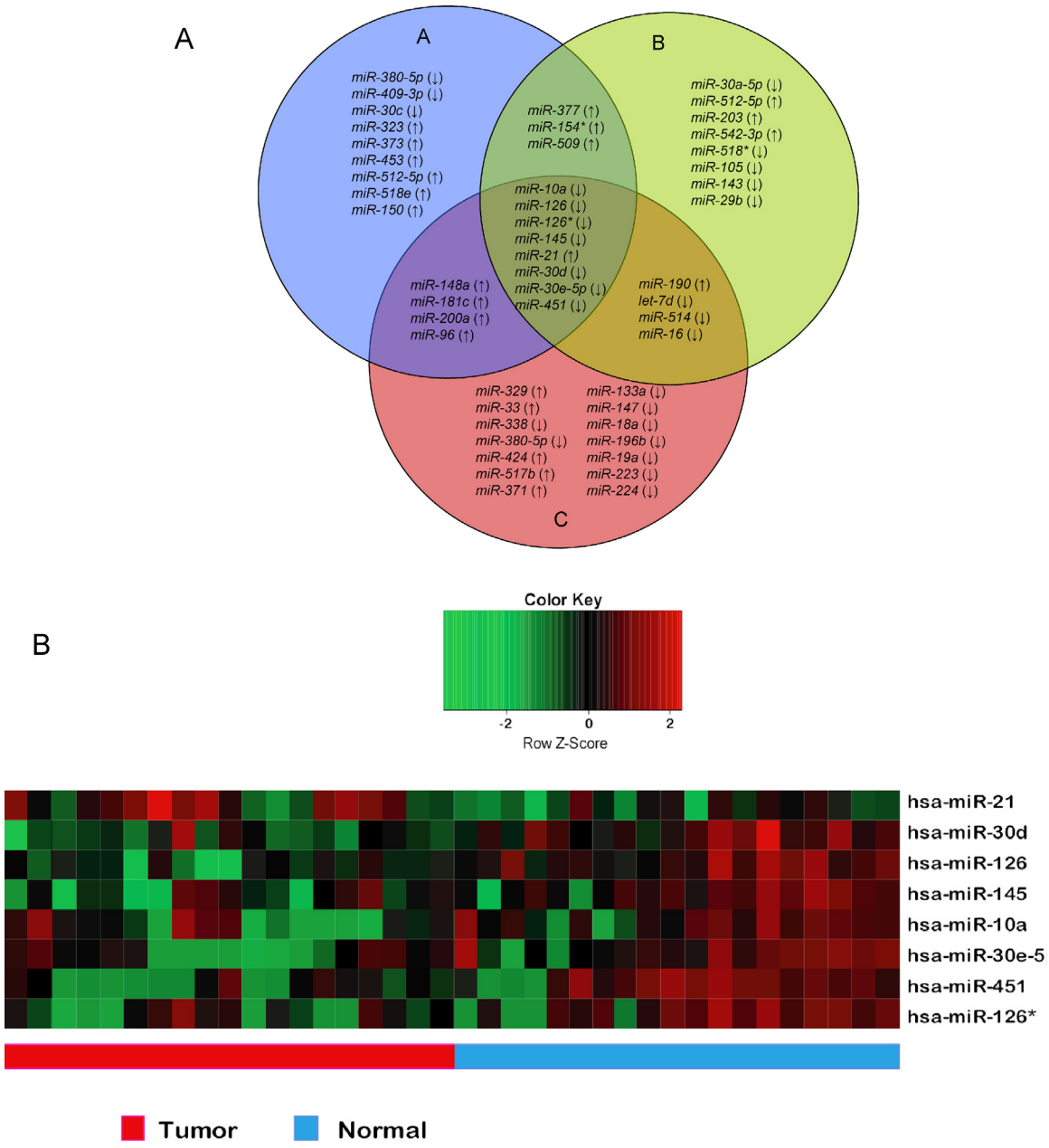
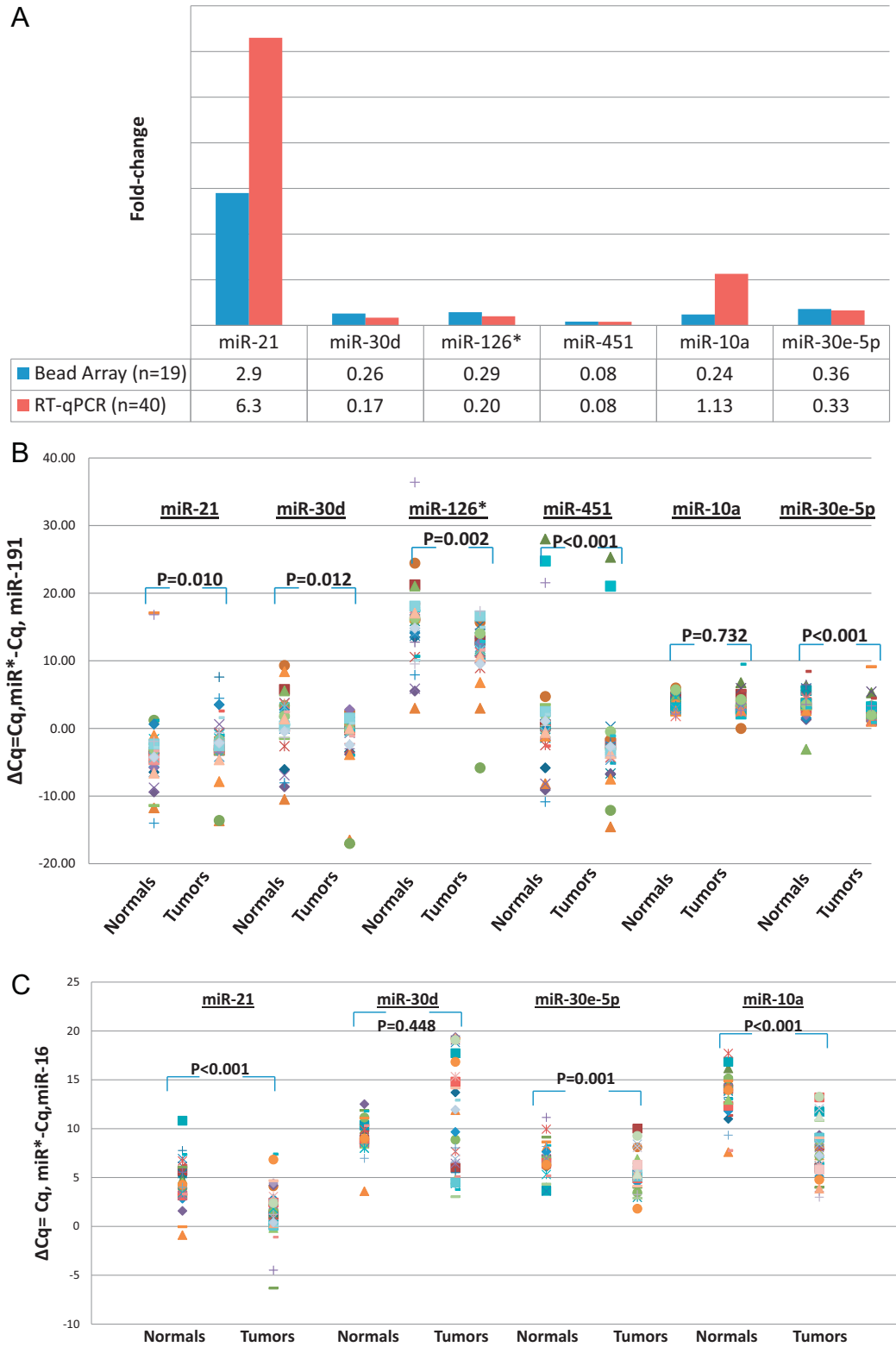


Fig. 2. Liquid bead array: (A) differentially expressed miRNAs according to three different statistical approaches. (a) Hierarchical clustering, (b) paired *t*-test after background correction and normalization (as recommended by Luminex), (c) bead to bead background and quantile normalization. (B) Bead-array results showing the 8 statistically significant ($P < 0.05$) dysregulated miRNAs in 19 NSCLC tissues and their corresponding non-cancerous adjacent tissues. Red represents over-expressed miRNAs while green indicates low expressed miRNAs in lung cancer compared to adjacent tissues. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

may have a supportive role in the progression of cancer that might be mediated by the promotion of blood vessel growth and inflammation [33]. We also verified both by our microarray experiments and by RT-qPCR that *miR-126** was significantly down regulated in NSCLC. Nevertheless, in our study the expression levels of *miR-126** were not detectable in plasma samples.

Concerning *miR-30d*, our results indicate that low expression levels of this miRNA were significantly lower in NSCLC tissues than in adjacent non-cancerous tissues. Hu et al. have recently reported that *miR-30d* was among eleven serum miRNAs that were found to be altered more than five-fold between longer-survival and shorter-survival groups in NSCLC, and that its levels were significantly associated with overall survival [34]. Moreover, Xie et al. have recently shown that cell-free *miR-30d* is a potential diagnostic biomarker for malignant and benign effusions [35]. It is important to note that the *miR-30* family microRNAs target mesenchymal gene trancripts and maintain them in a translational inactive state [36]. More especially, *miR-30d* and *miR-30e-5p* contain the same seed sequence (–GUAAACA–) and have been shown to bind to the 3′UTR of mesenchymal genes such as *Snail*, *Slug*, and *Vimentin* [36]. This finding has significant implications in several areas of cancer development and disease, since these genes play a critical role in epithelial mesenchymal transition (EMT) [37]

One of the most studied miRNAs that is over-expressed in several solid malignancies including breast and lung cancer is *miR-21* [8,9,38,39]. According to our data there was a concordance between liquid bead array and RT-qPCR concerning *miR-21* expression in NSCLC paired fresh frozen tissues. Moreover our data clearly show



	miR-21	miR-30d	miR-126*	miR-451	miR-10a	miR-30e-5p
Bead Array (n=19)	2.9	0.26	0.29	0.08	0.24	0.36
RT-qPCR (n=40)	6.3	0.17	0.20	0.08	1.13	0.33

Fig. 3. RT-qPCR: expression levels of *miR-21*, *miR-30d*, *miR-126**, *miR-10a*, *miR-30e-5p* and *miR-451* (A) Concordance of bead array results with RT-qPCR, (B) in NSCLC and corresponding non-cancerous tissues ($n = 40$ pairs), (C) in plasma of NSCLC patients ($n = 37$), and healthy volunteers ($n = 28$).

that NSCLC patients with over-expression of *miR-21* both in their primary tumors and plasma had a shorter disease free survival and overall survival. We and others have previously already shown that over-expression of mature *miR-21* is an independent negative prognostic factor for overall survival in NSCLC patients based on its over expression in primary tumors [8,38]. Recently, Saito et al. showed that increased *miR-21* expression is associated with disease progression and survival in stage I lung cancer [9]. In addition to these studies, Wang et al. have shown that high levels of circulating *miR-21* in serum of NSCLC patients are significantly correlated with overall survival and suggested that expression of *miR-21* might be useful as a prognostic marker [39]. Consistent to these results,

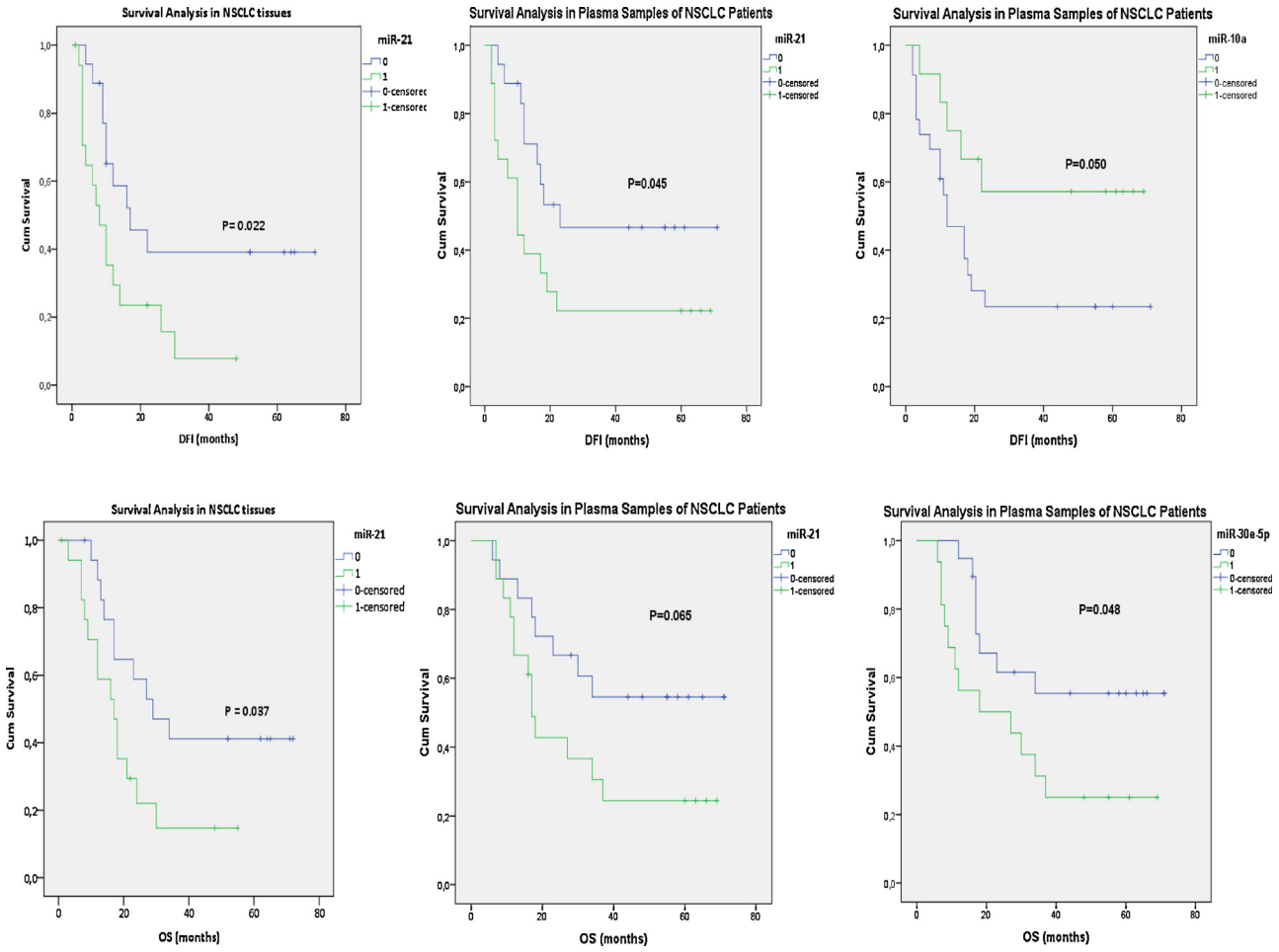


Fig. 4. Kaplan–Meier analysis for *miR-21* (tissues and plasma), *miR-10a* and *miR-30e-5p* (plasma) in NSCLC patients.

our data indicate that high levels of circulating *miR-21* in plasma of NSCLC patients are indicative of shorter disease free survival and overall survival. There is thus accumulating evidence that *miR-21* is a very promising prognostic biomarker in NSCLC. Moreover for normalization of miRNA expression levels by RT-qPCR we have used *miR-191* as an internal endogenous control for tissues samples, since its expression was found to be stable in tissues and *miR-16* as an internal endogenous miRNA control for plasma [40,41]. Especially for the normalization of miRNA levels in plasma, we have also included in our experiments an external synthetic non-human miRNA, *cel-miR-39*, that we have spiked in all our plasma samples prior to miRNA isolation in order to correct for differences in RNA extraction recoveries.

We report for the first time that circulating *miR-10a* levels in plasma are differentiated between healthy donors and NSCLC patients. We also report for the first time that *miR-30e-5p* is deregulated in NSCLC tissues and its expression levels in plasma were significantly different between NSCLC patients and healthy volunteers. It is important to note that high levels of circulating *miR-30e-5p* in plasma were found to be correlated with OS in NSCLC patients while low expression levels of miR-10a were found to be associated with shorter DFI of NSCLC patients. Moreover, according to our findings, over-expression of *miR-21* both in tissues and plasma was associated both with DFI and OS.

Our findings add a new dimension to the potential of miRNAs as promising biomarkers in the prognosis of NSCLC. Whether or not over- or down-regulation of these differentially expressed miRNAs is of prognostic, predictive or therapeutic importance has yet to be determined in a larger number of samples. We will further explore our findings in a prospective study, in respect to the clinical outcome of NSCLC patients.

Conflict of interest statement

None declared.

Acknowledgements

We would like to thank Dr. A. Tsaroucha ("Sotiria" General Hospital for Chest Diseases) for providing the clinical samples used in our study and Dr. L. Kaklamanis (Onassis Center for Cardiovascular Diseases) for the histological examination of all NSCLC tissue samples. This work was supported by a research grant to Dr. E.S. Lianidou, from the Special Research Account of the University of Athens. Especially we would like to thank Luminex Corporation for their generous supply of FlexmiR kits to perform the bead array experiments and Dr. Ch. Moschoyianni for technical support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.lungcan.2013.05.007.

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