Papers in Press. Published May 19, 2016 as doi:10.1373/clinchem.2015.253716
The latest version is at http://hwmaint.clinchem.org/cgi/doi/10.1373/clinchem.2015.253716

Clinical Chemistry 62:7
000–000 (2016)

Direct Comparison of Metastasis-Related miRNAs Expression Levels in Circulating Tumor Cells, Corresponding Plasma, and Primary Tumors of Breast Cancer Patients

Athina Markou,[1*†] Martha Zavridou,[1†] Ioanna Sourvinou,[1] George Yousef,[2] Sofia Kounelis,[3] Nikos Malamos,[3] Vasilis Georgoulias,[4] and Evi Lianidou[1]

BACKGROUND: Circulating tumor cells (CTCs) and microRNAs (miRNAs) are important in liquid biopsies in which peripheral blood is used to characterize the evolution of solid tumors. We evaluated the expression levels of *miR-21*, *miR-146a*, *miR-200c*, and *miR-210* in CTCs of breast cancer patients with verified metastasis and compared their expression levels in corresponding plasma and primary tumors.

METHODS: Expression levels of the miRNAs were quantified by quantitative reverse transcription PCR (RT-qPCR) in (*a*) 89 primary breast tumors and 30 noncancerous breast tissues and (*b*) CTCs and corresponding plasma of 55 patients with metastatic breast cancer and 20 healthy donors. For 30 of these patients, CTCs, corresponding plasma, and primary tumor tissues were available.

RESULTS: In formalin-fixed, paraffin-embedded tissues, these miRNAs were differentially expressed between primary breast tumors and noncancerous breast tissues. *miR-21* ($P < 0.001$) and *miR-146a* ($P = 0.001$) were overexpressed, whereas *miR-200c* ($P = 0.004$) and *miR-210* ($P = 0.002$) were underexpressed. In multivariate analysis, *miR-146a* overexpression was significantly [hazard ratio 2.969 (1.231–7.157), $P = 0.015$] associated with progression-free survival. In peripheral blood, all miRNAs studied were overexpressed in both CTC and corresponding plasma. There was a significant association between *miR-21* expression levels in CTCs and plasma for 36 of 55 samples ($P = 0.008$). In plasma, ROC curve analysis revealed that *miR-21*, *miR-146a*, and *miR-210* could discriminate patients from healthy individuals.

CONCLUSIONS: Metastasis-related miRNAs are overexpressed in CTCs and corresponding plasma; *miR-21* expression levels highly correlate in CTCs and plasma; and *miR-21, miR-146a*, and *miR-210* are valuable plasma biomarkers for discriminating patients from healthy individuals.
© 2016 American Association for Clinical Chemistry

The liquid biopsy is a minimally invasive blood-based analysis approach that can give information on a solid tumor's evolution in real time. Information is derived mainly from circulating tumor cells (CTCs),[5] circulating tumor DNA, circulating microRNAs (miRNAs), and exosomes, and changes in their concentrations have been associated with tumor burden and malignant progression *(1)*. Numerous studies have demonstrated the feasibility of cancer liquid-biopsy testing, and the clinical utility of CTCs and cell tumor DNA (ctDNA) in many types of cancer *(2)*. Many international consortiums are working on the clinical validation of a variety of liquid-biopsy tests *(3, 4)*. Liquid biopsy–based tests that are specifically designed to predict resistance to specific drugs and select patients for specific molecular-targeted therapies have advantages over classical surgical biopsies. These blood-based targeted molecular assays have a strong potential to be applied as companion diagnostics, in disease monitoring, and even for early cancer detection *(5, 6)*.

[1] Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Athens, Greece; [2] Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada; [3] Oncology Unit and Pathology Department, Helena Venizelou Hospital, Athens, Greece; [4] Department of Medical Oncology, University General Hospital of Heraklion, Heraklion, Greece.
* Address correspondence to this author at: Lab of Analysis Circulating Tumor Cells (ACTC Lab), Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, 15771, Athens, Greece. Fax +0030-210-7274-750; e-mail athina_markoy@yahoo.gr.
† Equal contribution: Athina Markou and Martha Zavridou contributed equally to the work, and both should be considered first authors.

Received December 21, 2015; accepted April 4, 2016.
Previously published online at DOI: 10.1373/clinchem.2015.253716
© 2016 American Association for Clinical Chemistry

[5] Nonstandard abbreviations: CTCs, circulating tumor cells; miRNAs, microRNAs; ctDNA, cell tumor DNA; EpCAM, epithelial cell adhesion molecule; FFPEs, paired formalin-fixed, paraffin-embedded primary tumors; RT-qPCR, quantitative reverse transcription PCR; AUC, area under the curves; DFI, disease-free interval; OS, overall survival; HR, hazard ratio.

Copyright (C) 2016 by The American Association for Clinical Chemistry

It is well known that the presence of tumor cells in the peripheral blood of patients with operable breast cancer is an early indicator of metastasis and may signal tumor spread sooner than clinical symptoms appear and imaging results confirm a poor prognosis *(7–10)*. CTC enumeration with CellSearch has been acquired for use as a prognostic factor when measured in patients with metastatic breast, colorectal, and prostate cancer *(11, 12)*. CellSearch is the only US Food and Drug Administration CTC assay approved for prognostication and disease monitoring in patients with metastatic breast, colorectal, and prostate cancer *(13)*. The clinical utility of CTCs in breast cancer for treatment decisions is currently being evaluated in ongoing clinical studies *(14)*. However, besides detection and enumeration, molecular characterization of CTCs is extremely important because it offers the potential to provide unique information that can be further used to guide individualized targeted treatments *(7, 15, 16)*.

In addition to CTCs, miRNAs seem to be involved in metastasis and might offer the unique potential to better understand the biology of metastasis and resistance mechanisms to established therapies *(17)*. miRNAs are key regulators of gene expression because each miRNA targets, on average, 200 mRNA transcripts *(18)*. The expression of specific miRNAs in primary breast tumors has been associated with the clinical outcome in several studies *(19, 20)*. Because of their small size and remarkable stability *(21)*, miRNAs can also be detected in serum and plasma as cell-free circulating miRNAs *(21–23)*.

To the best of our knowledge, the expression of miRNAs has not been studied so far in CTCs, nor have they been compared to their expression in plasma and corresponding primary tumors. For our study, we selected 4 miRNAs that critically regulate various stages of migration and invasion and play critical roles in the multistep metastatic process, namely *miR-21*, *miR-146a*, *miR-200c,* and *miR-210*. In this study, we evaluated the expression levels of 4 metastasis-related miRNAs in the epithelial cell adhesion molecule (EpCAM)-positive CTC fraction of patients with metastatic breast cancer and compared our findings with the expression levels in corresponding plasma and paired formalin-fixed, paraffin-embedded primary tumors (FFPEs). In this study, we aimed to answer the following questions: (*a*) Could we detect metastasis-related miRNAs in CTCs?; (*b*) Was there an association between CTC and plasma in terms of miRNAs expression?; (*c*) Did the expression levels of miRNAs in CTC and plasma reflect those of the primary tumor?; and (*d*) Did the expression levels of miRNAs in CTC and plasma provide any prognostic significance?

Materials and Methods

PATIENTS

All study participants signed an informed consent form to participate in the study, which was approved by the ethics and scientific committees of our institutions. Using quantitative reverse transcription PCR (RT-qPCR), we quantified the expression of *miR-21*, *miR-146a*, *miR-200c, miR-210*, and *miR-191* (used as a normalizer) in (*a*) 55 EpCAM-positive CTC fraction samples isolated from peripheral blood of patients with metastatic breast cancer, (*b*) 55 corresponding plasma samples (same venipuncture), and (*c*) 89 FFPEs. For 30 of these patients, there were available samples of the EpCAM-positive CTC fraction, corresponding plasma, and FFPEs. As a control population, peripheral blood was collected from 20 healthy individuals, and 30 noncancerous breast tissues (mammoplasties) were used as noncancerous tissue controls. In the EpCAM-positive CTC fraction, 1 sample was defined as miRNA overexpressed based on the fold change of miRNA expression in respect to the group of 20 healthy individuals used as a control group. More specifically, a cutoff value was estimated according to the expression of miRNA in the EpCAM-positive fraction of healthy individuals analyzed in exactly the same way as the patient's peripheral blood samples.

EXTRACTION OF MIRNAS FROM FFPES

For miRNAs analysis in FFPE samples, the blocks were cut into 10-mm-thick slices, and each tissue slice was placed into a 1.5 mL nuclease-free microcentrifuge tube. Extraction of miRNAs was performed by using the miRNeasy FFPE kit (Qiagen) according to the manufacturer's instructions.

EXTRACTION OF MIRNAS FROM THE EPCAM-POSITIVE CTC FRACTION

We isolated CTCs from 20 mL peripheral blood by following a positive immunomagnetic selection approach, using anti-EpCAM-coated immunomagnetic beads, as previously described *(24)*. All peripheral blood samples for controls and patients were processed in exactly the same way. Peripheral blood in EDTA-containing tubes was obtained from breast cancer patients with verified metastasis and from female healthy volunteers. To reduce blood contamination by epithelial cells from the skin, the first 5 mL of blood were discarded, and the final collection tube was disconnected before withdrawing the needle. The peripheral blood mononuclear cells were isolated within 2 h of venipuncture by gradient centrifugation with Ficoll, and cell pellets were kept at −70 °C until RNA extraction.

Isolation of total RNA was performed with TRIZOL (Invitrogen). The isolated RNA from each fraction was dissolved in 10 μL RNA storage buffer. RNA con-

centration was determined by using the Nanodrop-1000 spectrophotometer. As a negative control, we followed exactly the same procedure and evaluated miRNAs expression levels in the EpCAM-positive CTC fraction isolated from peripheral blood of 20 healthy blood donors.

EXTRACTION OF CIRCULATING MIRNAS FROM PLASMA

The extraction of miRNAs from plasma (200 μL) was performed as previously described by using the mirVana™ PARIS™ Kit *(25)*. More specifically, whole-blood samples were subjected to centrifugation at 2000*g* for 10 min at room temperature. The upper plasma layer was immediately collected after centrifugation and saved in new RNase-free tubes. Before starting the miRNA extraction procedure, plasma samples were subjected to a second centrifugation at 12 000*g* for 15 min at 4 °C to remove all cellular debris and the vast majority of platelets. Subsequently, 25 fmol of *cel-miR-39* used as an exogenous miRNA spiked-in control was added to all plasma aliquots to allow for normalization of sample-to-sample variations in the RNA-isolation procedure. We evaluated the efficacy and analytical performance of the whole procedure by quantifying the concentrations of the spiked-in exogenous control miRNA *cel-miR-39*. Recovery of *cel-miR-39* in each case was estimated with respect to the concentrations of an equivalent amount of *cel-miR-39* copies that we added to the eluted RNA after the extraction step in each case (representing 100% recovery). In parallel, endogenous tested miRNAs and *miR-191* concentrations were also quantified by RT-qPCR in all samples.

REVERSE TRANSCRIPTION OF MIRNAS

In all cases, cDNA was synthesized using the TaqMan MicroRNA Reverse Transcription Kit and miRNA-specific stem-loop primers in a 15 μL total-volume reaction. Each reaction consisted of (*a*) 3μL of miRNAs eluted from plasma, (*b*) 30 ng total RNA from the EpCAM-positive CTC fraction, and (*c*) 30 ng total RNA from FFPEs tissues and 10μL of Master Mix. The RT reaction mixture was incubated at 16 °C for 30 min, at 42 °C for 30 min, and at 85 °C for 5 min and then held at 4 °C. A no-RT negative control was included in each experiment to ensure that PCR products were not because of contamination by genomic DNA.

QUANTIFICATION OF MIRNAS EXPRESSION BY RT-QPCR

The expression levels of each miRNA were quantified by RT-qPCR, using the TaqMan microRNA assays (Applied Biosystems), according to the manufacturer's protocols. RT-qPCR was performed in a final volume of 10 μL, containing 2 μL of cDNA template, 2 μL nuclease free water, 1 μL of 20× primer/probe mix from the TaqMan MicroRNA assay, and 5 μL of 2×TaqMan Universal PCR master mix. All reactions were run in triplicate in the Light Cycler 2.0 Real-Time PCR Instrument (Roche). The reaction mixture was incubated at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Expression values were normalized in respect to *miR-191*, which has been shown to be a suitable reference miRNA for breast cancer *(26)*. We evaluated 4 different miRNAs (*miR191*, *RNU44, RNU48*, and *let7a*) as calibrators by measuring their expression levels in 40 primary breast tumors. RNU44 was excluded from the analysis because it was not expressed in the majority of the samples. For the remaining miRNAs, we performed NormFinder algorithm analysis, and according to our results, *miR-191* was ranked as the most-stable RNA (see Supplemental Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue7). Relative quantification was based on the ΔΔCq method, as described by Livak and Schmittgen *(27)*. All RT-qPCR experiments were performed according to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines *(28)*.

STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS statistical package. RT-qPCR data were analyzed by Wilcoxon signed rank tests to statistically evaluate differences in miRNA expression between breast cancer and healthy breast tissues. For the survival analysis, we divided breast cancer patients into 2 different groups, high expression and low expression, using the median ΔCq of noncancerous samples for each miRNA studied at the corresponding cutoffs. Survival time was calculated from the date of endpoint event or last follow-up. The association between survival and miRNA expression was estimated using the Kaplan–Meier method and 2-sided log-rank test. To evaluate the prognostic potential of individual miRNAs and sets of miRNAs, we generated ROC curves and areas under the curves (AUC).

Results

EXPRESSION OF METASTASIS-RELATED MIRNAS IN PRIMARY BREAST TUMORS

The experimental flowchart of our study is outlined in Fig. 1. We initially evaluated the expression levels of *miR-21, miR-146a*, *miR-200c*, and *miR-210* in primary breast tumors. Overexpression and underexpression of these 4 miRNAs were estimated by evaluating the differences in their expression levels between 89 breast cancer tumors (FFPEs) and 30 noncancerous breast tissues (mammoplasties). The expression levels of all 4 miRNAs were significantly different between primary tumors and noncancerous breast tissues. More specifically, *miR-21* ($P < 0.001$) and *miR-146a* ($P = 0.001$) were overexpressed,

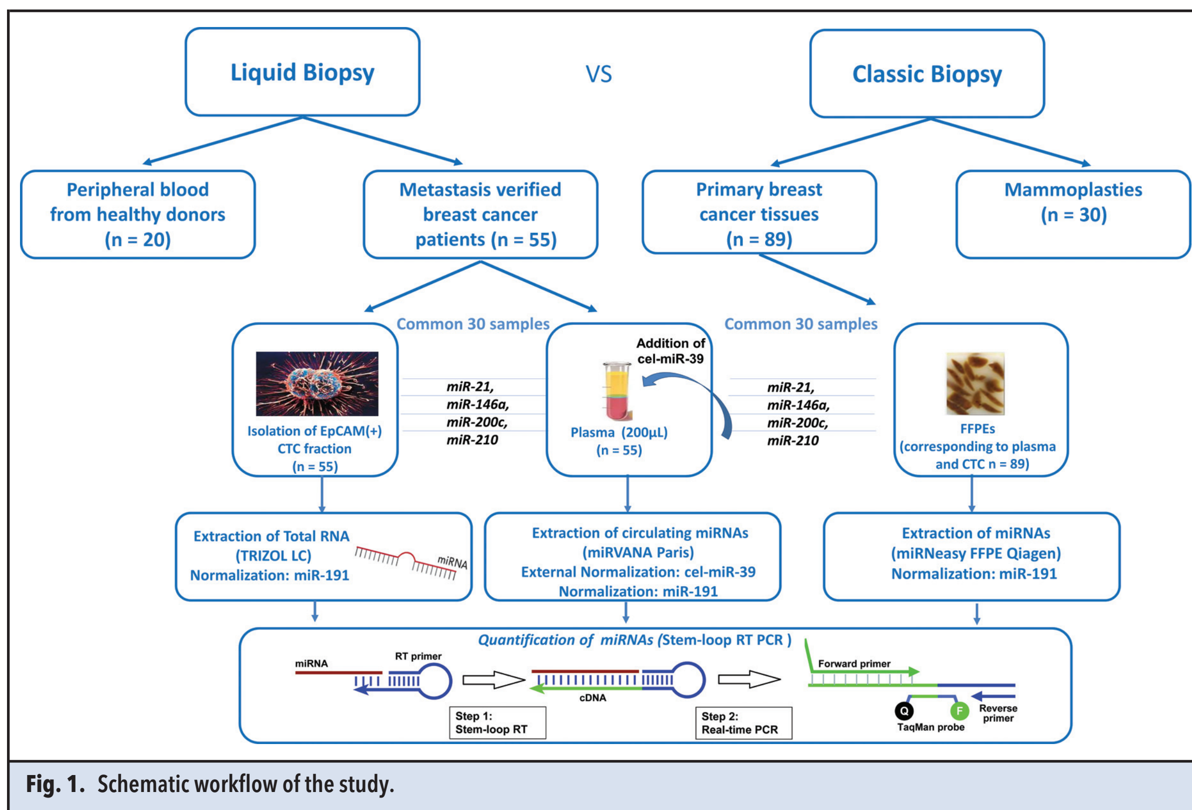

Fig. 1. Schematic workflow of the study.

whereas *miR-200c* (P = 0.004) and *miR-210* (P = 0.002) were found to be underexpressed (Fig. 2A).

The correlation of the expression levels of the 4 miRNAs with patients' clinical outcome revealed that patients with *miR-21* overexpression had significantly shorter disease-free intervals (DFIs) than those with *miR-21* underexpression (43 months vs 84 months, respectively, $P = 0.040$, Fig. 3A). Moreover, patients who had *miR-146a* overexpression had shorter DFIs than those with *miR-146a* underexpression (19 months vs 59 months, respectively; $P = 0.018$, Fig. 3B). The expression levels of *miR-200c* and *miR-210* in FFPEs were not correlated with DFI and overall survival (OS).

Univariate analysis demonstrated that *miR-21* overexpression [hazard ratio (HR)]: 1.846; 95% CI = 1.016–3.355, $P = 0.044$) and *miR-146α* overexpression (HR: 2.696; 95% CI = 1.143–6.357, $P = 0.025$) were significantly associated with DFI; conversely, there was no association with the ER/PR (estrogen receptor/progesterone receptor) and HER2 (human epidermal growth factor receptor 2) status with DFI in these patients (see online Supplemental Table 1). Multivariate analysis demonstrated that only *miR-146a* overexpression was an independent factor associated with DFI (HR: 2.969; 95% CI = 1.231–7.157, $P = 0.015$) (see online Supplemental Table 1).

EXPRESSION OF METASTASIS-RELATED MIRNAS IN THE CORRESPONDING EPCAM-POSITIVE CTC FRACTION

We further evaluated the expression of these 4 metastasis-related miRNAs in the corresponding EpCAM-positive CTC fraction isolated from peripheral blood. All miRNAs were overexpressed in the EpCAM-positive CTC fraction of the patients in respect to the corresponding healthy controls; *miR-21* ($P < 0.001$, 17.6-fold rise), *miR-146a* ($P < 0.001$, 6.5-fold rise), *miR-200c* ($P < 0.001$, 7.8-fold rise), and *miR-210* ($P < 0.001$, 81.0-fold rise) (Fig. 2B). Univariate analysis revealed that *miR-146a* overexpression (P = 0.068) was not statistically associated with OS in this group of patients. Kaplan–Meier survival analysis and log-rank tests showed that none of the tested miRNAs was correlated with OS (results not shown).

EXPRESSION OF METASTASIS-RELATED CIRCULATING MIRNAS IN CORRESPONDING PLASMA

The expression levels of these 4 metastasis-related miRNAs were further evaluated in corresponding plasma samples isolated from the same peripheral blood samples on the same day and through the same veinpuncture as CTC. All of these 4 metastasis-related miRNAs were significantly overexpressed in patients' plasma compared with healthy donors (*miR-21,* $P < 0.001$; *miR-146a,*

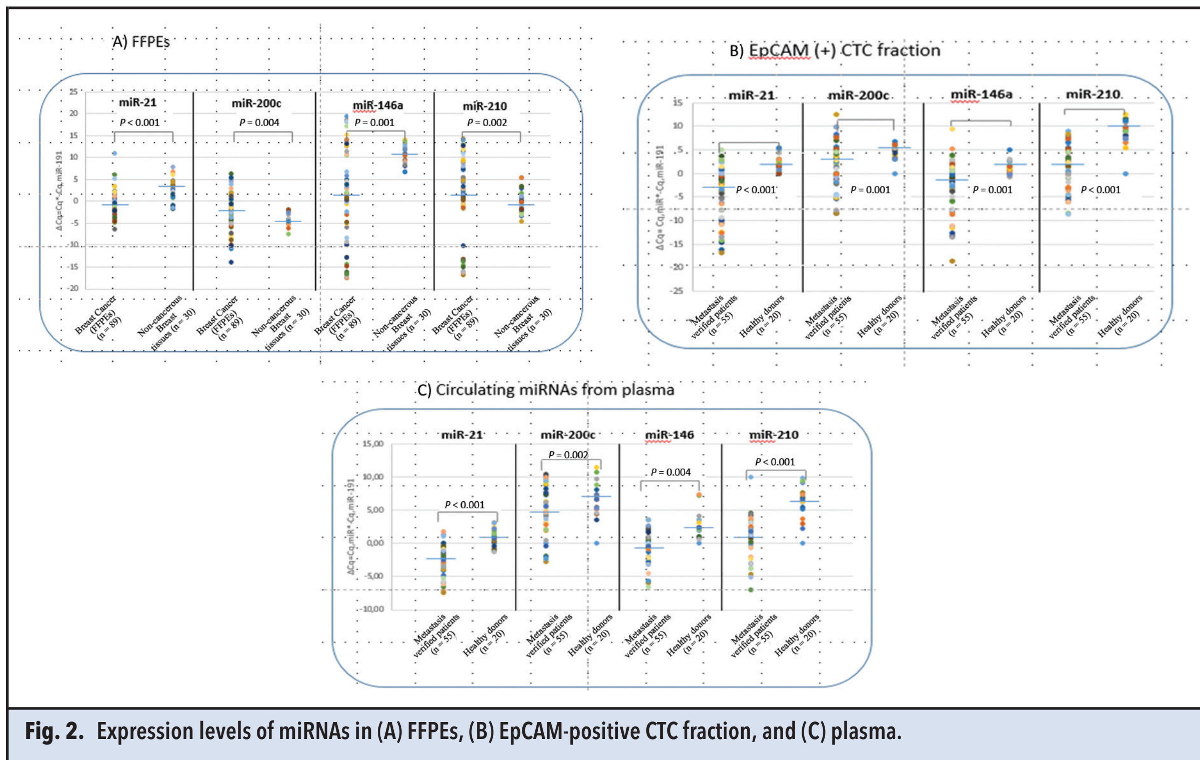

Fig. 2. Expression levels of miRNAs in (A) FFPEs, (B) EpCAM-positive CTC fraction, and (C) plasma.

P = 0.004; *miR-200c*, P = 0.002; and *miR-210*, $P < 0.001$) (Fig. 2C).

The diagnostic utility of these circulating miRNAs in plasma was evaluated by ROC curve analysis. ROC curves showed that *miR-21*, *miR-146a*, and *miR-210* were valuable biomarkers for discriminating patients from healthy individuals, with AUCs of 0.820 (95% CI = 0.691–0.948, $P < 0.0001$), 0.912 (95% CI = 0.847–0.977, $P < 0.0001$), and 0.959 (95% CI = 0.917–1.000, $P < 0.0001$), respectively (Fig. 4). When the plasma concentrations of a combination of *miR-21*, *miR-146a*, and *miR-210* were subjected to combined

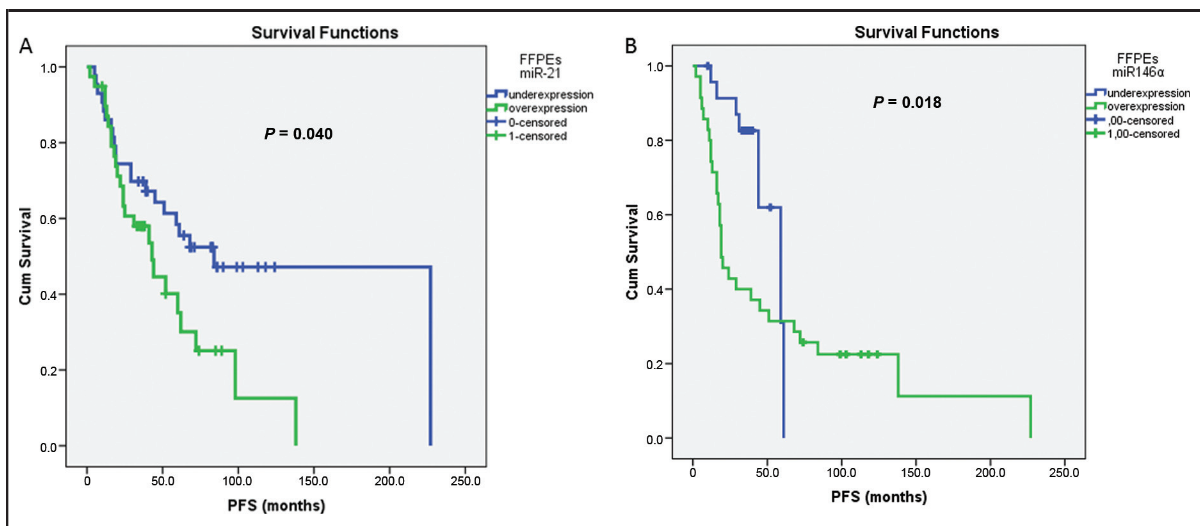

Fig. 3. (A), Kaplan–Meier estimates of DFI or breast cancer patients with verified metastasis in respect to *miR-21* overexpression. (B), Kaplan–Meier estimates of DFI for breast cancer patients with verified metastasis in respect to *miR-146α* overexpression.

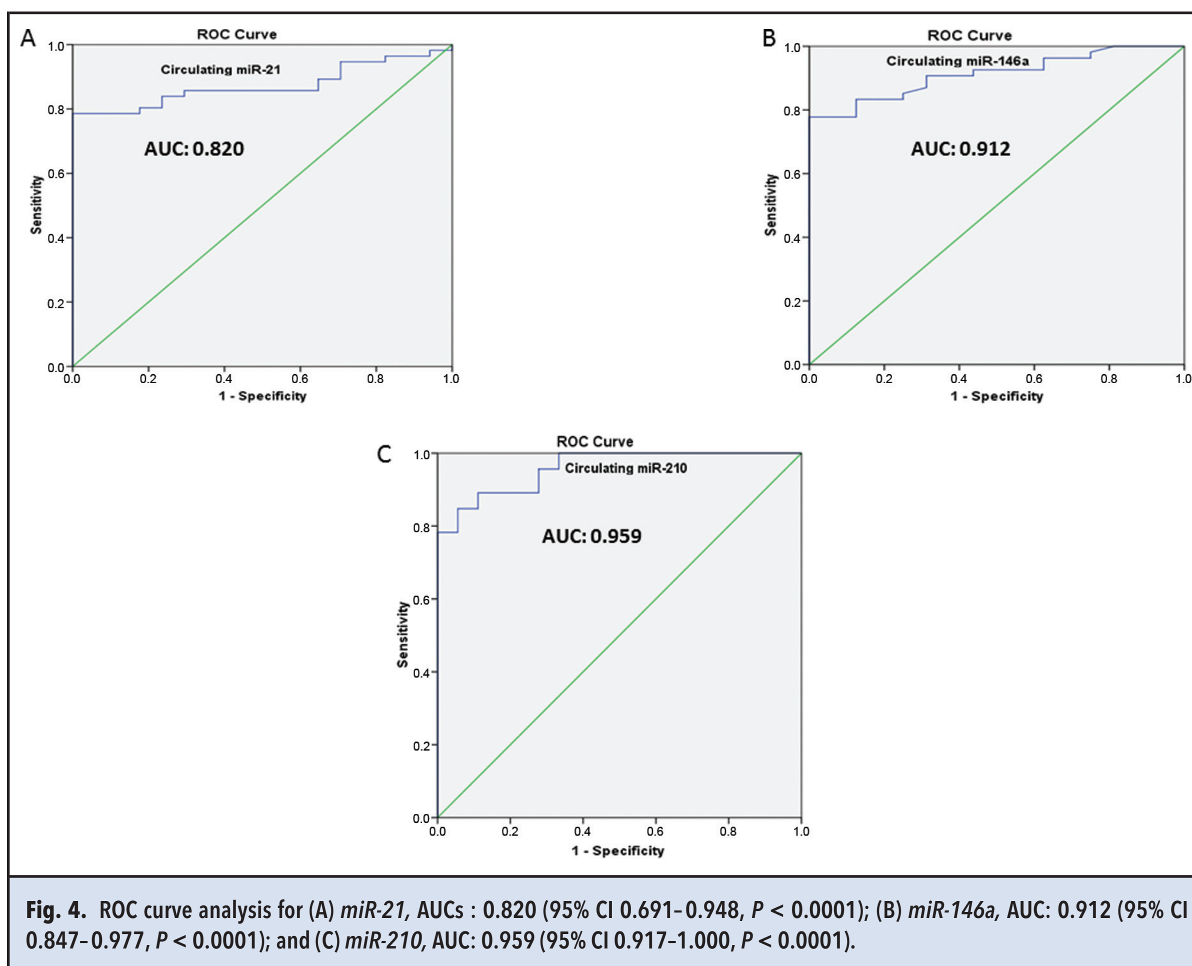

Fig. 4. ROC curve analysis for (A) *miR-21*, AUCs : 0.820 (95% CI 0.691–0.948, $P < 0.0001$); (B) *miR-146a*, AUC: 0.912 (95% CI 0.847–0.977, $P < 0.0001$); and (C) *miR-210*, AUC: 0.959 (95% CI 0.917-1.000, $P < 0.0001$).

analysis by multiple logistic regression, the generated ROC curve reflected a higher ability to differentiate patients from healthy controls (AUC value: 0.997; 95% CI: 0.989 −1.000), demonstrating the diagnostic accuracy of these miRNAs as effective biomarkers in combination.

DIRECT COMPARISON BETWEEN MIRNAS EXPRESSION LEVELS IN THE EPCAM-POSITIVE CTC FRACTION, IN PLASMA, AND IN CORRESPONDING PRIMARY TUMORS

We further investigated whether there was a direct association between the expression of these miRNAs in the EpCAM-positive CTC fraction, in corresponding plasma, and paired FFPEs. A heat map summarizing our results is shown in Fig. 5.

For a direct comparison of miRNAs expression between the EpCAM-positive CTC fraction and corresponding plasma, material for matched EpCAM-positive CTC fraction and plasma was available for 55 patients. The results for the direct comparison with respect to *CK-19* expression and death are shown in Fig. 5A. The higher percentage agreement between circulating miRNAs in plasma and miRNAs in the EpCAM-positive CTC fraction was observed for *miR-21* (67.2%, $\kappa = 0.187$; Table 1). Contingency tables for these statistics are presented in Table 1.

For the direct comparison of miRNAs expression between the EpCAM-positive CTC fraction, plasma, and corresponding FFPE, material for matched EpCAM-positive CTC fraction, corresponding FFPEs, and plasma was available for 30 patients. *miR-21* expression levels were concordant between the EpCAM-positive CTC fraction and FFPEs in 18 of 30 (60%) patients (k = 0.143, Fig. 5B). *miR-21* expression levels between plasma and corresponding FFPEs showed a concordance for 16 of 30 (53.3%) cases (k = 0.16, Fig. 5B). Among the remaining 12 discordant cases between CTC and plasma, the expression of *miR-21* in CTC matched the corresponding FFPEs in 6 cases, and the expression of *miR-21* in plasma matched the FFPEs in the other 6 cases. However, the expression levels of all other miRNAs studied were not correlated between the EpCAM-positive CTC fraction and paired FFPEs (Fig. 5B). The concordance of miRNAs expression levels between plasma and corresponding FFPEs varied between 46.6% and 66.6%.

Fig. 5. Heat maps of 4 miRNAs in FFPEs, EpCAM-positive fraction, and plasma samples as quantified by RT-qPCR.
Red and green indicate overexpression and underexpression, respectively.

Discussion

The liquid biopsy is a very promising approach for cancer diagnosis, prognosis, and evaluation of therapy response. However, there is a constantly increasing number of biomarkers for evaluation in CTC and plasma, and well-designed comparison studies between different liquid-biopsy information sources, such as CTC, ctDNA, and

Table 1. Direct comparison of miRNAs expression levels in the EpCAM-positive CTC fraction and corresponding paired plasma samples in metastatic breast cancer patients (n = 55).

miRNA	Plasma	EpCAM-positive CTC fraction U[a]	EpCAM-positive CTC fraction O[b]	Total	*P*	Concordance
miR-21	U	6	6	12	0.008	37/55 (67.2%) k = 0.187
	O	12	31	43		
	Total	18	37	55		
miR-146a	U	8	19	27	0.010	18/55 (32.7%) k = −0.347
	O	18	10	28		
	Total	26	29	55		
miR-200c	U	15	16	31	NS[c]	28/55 (50.9%) k = 0.025
	O	11	13	24		
	Total	26	29	55		
miR-210	U	18	10	28	NS[c]	32/55 (59.2%) k = 0.166
	O	14	13	27		
	Total	32	23	55		

[a] Underexpression.
[b] Overexpression.
[c] Nonsignificant.

circulating miRNAs and conventional-biopsy samples are still missing.

There have been only a few studies that correlated circulating miRNAs with CTC enumeration. Sieuwerts et al., using CellSearch, reported that 55 mRNAs and 10 miRNAs were more abundantly expressed in blood of patients with at least 5 CTC/7.5 mL of blood compared with samples from patients without detectable CTC and healthy blood donors *(29)*. Recently, Gasch et al., using in-situ-hybridization protocols combined with antibody-based immunofluorescence, in combination with CellSearch, demonstrated a high heterogeneity in *miR-10b* expression in CTCs isolated from blood of metastatic-cancer patients *(30)*.

In the present study, all peripheral blood samples were collected in a way that CTC and plasma were isolated from the same blood draw, and all samples were analyzed in parallel with the same methodologies. The expression levels of 4 metastasis-related miRNAs in the EpCAM-positive CTC fraction of patients with metastatic breast cancer was investigated; moreover, the expression levels of the same miRNAs in corresponding plasma and paired primary tumors was evaluated. The selection of these miRNAs was based on their critical role in the multistep metastatic process. Indeed, *miR-21* is involved in invasion *(31)*, *miR-146a* in invasion and migration *(32)*, *miR-210* in tumor proliferation and hypoxia *(33, 34)*, and *miR-200c* in mesenchymal-to-epithelial transition *(35)*.

Our results demonstrate that all of these miRNAs were significantly differentially expressed in primary breast tumors when compared with noncancerous breast tissues. *miR-21* overexpression in FFPEs was associated with reduced progression-free survival, but not with OS. This finding is in concordance with our previous results concerning the prognostic significance of *miR-21* in breast cancer FFPEs *(19)* as well as with other studies *(36)*. Furthermore, *miR-146a* overexpression was found to be an independent factor associated with progression-free survival.

We report for the first time that, in the EpCAM-positive CTC fraction, the expression levels of all 4 of these miRNAs were significantly different between cancerous and noncancerous breast samples. A striking finding of our study is the upregulation of *miR-21* both in the EpCAM-positive CTC fraction and in corresponding plasma. *miR-21* expression levels were concordant between CTC and paired plasma in the majority of the patients. This is the first time that a high correlation for *miR-21* expression is reported between plasma and CTC. It is important to note that *miR-21* is a well-studied miRNA and the only miRNA known to be upregulated in all types of human malignancies *(37, 38)*. Our group has reported that *miR-21* is an independent negative prognostic factor for OS in non-small-cell lung cancer patients *(37)*. Very recently, Ortega et al. have developed a protocol for the detection of miRNAs in CTC using in situ hybridization combined with immunomagnetic selection based on cytokeratin expression and immunocytochemistry. According to their data, all CTC-positive samples were expressing both cytokeratin and *miR-21* *(39). It is also important to note that miR-21* plays an important role in the regulation of anticancer drug sensitivity and resistance *(40)* because aberrant *miR-21* expression can reduce the sensitivity of cancer cells to anticancer agents such as tamoxifen, gemcitabine, docetaxel, and 5-fluorouracil *(41–42)*.

According to our findings, although *miR-200c* and *miR-210* were down-regulated in primary tissue samples, we found them up-regulated in corresponding plasma of patients at the time they developed metastasis. We believe that these findings can be explained by the fact that we are analyzing liquid-biopsy material from metastatic breast cancer patients. It is now known that tumors are continuously evolving during time and that the metastatic sites are not identical to the primary tumors.

It is known that *miR-200c* possesses regulatory functions in the EMT pathway and tumor-suppressive features *(35)*, and in vivo studies suggest that overexpression of the *miR-200* family increases the metastatic potential in breast cancer by inducing mesenchymal-to-epithelial transition (MET). MET is required for successful colonization and establishment of metastasis *(43)*. Alternatively, low expression of *miR-200c* in the primary tissue might reflect changes taking place in the primary tumor associated with EMT that facilitate exit from the primary tumor, a process that is later reversed in the metastatic deposit.

Based on our findings, we suggest that plasma *miR-200c* and *miR-210* from patients with metastasis might originate from the metastatic site, from which the cancer cells are secreted abundantly into the systemic circulation in patients. Our results are in full agreement with the findings of Madhavan et al. that have also shown that CTC-positive metastatic breast cancer patients had significantly higher levels of *miR-200c* and *miR-210* than CTC-negative metastatic breast cancer patients *(23)*.

The choice of a reference gene remains problematic and can have a serious impact on the actual available transcript levels and, consequently, on the biological interpretation of data *(44)*. Although several groups have been focused on studying the expression levels of circulating miRNAs in plasma, it is remarkable that limited overlap has been observed between the findings of even very similar studies of the same disease. Regarding breast cancer, as Thompson et al. mentioned, there is a dampening enthusiasm for circulating miRNAs because after the analysis of 15 reports on circulating miRNAs in breast cancer patients revealed a very low reproducibility between the datasets published previously *(45)*. The lack

of reproducibility may have several reasons: (*a*) sample type (plasma, serum, whole blood), (*b*) differences in blood processing protocols, (*c*) differences in study populations, (*d*) differences in time points of sample collections *(46)*. General pitfalls concerning circulating miRNAs as biomarkers, not the least of which is the need for more validation and the contribution to miRNA secretion by blood cells, together with possible solutions, have been extensively reviewed elsewhere *(46)*.

In conclusion, this first direct-comparison study of miRNAs expression in CTCs, corresponding plasma, and paired primary tissues demonstrates that: (*a*) metastasis-related miRNAs are overexpressed in CTCs and corresponding plasma, (*b*) *miR-21* expression levels are highly correlated in CTCs and plasma, and (*c*) *miR-21, miR-146a*, and *miR-210* are valuable plasma biomarkers for discriminating patients from healthy individuals.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: A.N. Markou, CANCER-ID project; E. Lianidou, CANCER-ID project.
Expert Testimony: None declared.
Patents: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, and final approval of manuscript.

Acknowledgments: We thank Dr. Loukas Kaklamanis (Onassis CardioSurgery Center, Athens, Greece) for providing the healthy breast tissue samples (mammoplasties).

References

1. Lianidou ES, Markou A. Circulating tumor cells in breast cancer: detection systems, molecular characterization, and future challenges. Clin Chem 2011;57:1242–55.
2. Pantel K, Alix-Panabières C. Real-time liquid biopsy in cancer patients: fact or fiction? Cancer Res 2013;73:6384–8.
3. Bidard FC, Peeters DJ, Fehm T, Nolé F, Gisbert-Criado R, Mavroudis D, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. Lancet Oncol 2014;15:406–14.
4. Ignatiadis M, Lee M, Jeffrey SS. Circulating tumor cells and circulating tumor DNA: challenges and opportunities on the path to clinical utility. Clin Cancer Res 2015;21:4786–800.
5. Heitzer E, Auer M, Ulz P, Geigl JB, Speicher MR. Circulating tumor cells and DNA as liquid biopsies. Genome Med 2013;5:73.
6. Lianidou ES. Molecular characterization of circulating tumor cells: Holy Grail for personalized cancer treatment? Clin Chem 2014;60:1249–51.
7. Lianidou ES, Markou A, Strati A. Molecular characterization of circulating tumor cells in breast cancer: challenges and promises for individualized cancer treatment. Cancer Metastasis Rev 2012;:663–71.
8. Stathopoulou A, Ntoulia M, Perraki M, Apostolaki S, Mavroudis D, Malamos N, et al. A highly specific real-time RT-PCR method for the quantitative determination of CK-19 mRNA positive cells in peripheral blood of patients with operable breast cancer. Int J Cancer 2006;119:1654–9.
9. Xenidis N, Ignatiadis M, Apostolaki S, Perraki M, Kalbakis K, Agelaki S, et al. Cytokeratin-19 mRNA-positive circulating tumor cells after adjuvant chemotherapy in patients with early breast cancer. J Clin Oncol 2009;27:2177–84.
10. Lucci A, Hall CS, Lodhi AK, Bhattacharyya A, Anderson AE, Xiao L, et al. Circulating tumour cells in non-metastatic breast cancer: a prospective study. Lancet Oncol 2012:688–95.
11. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 2004;351:781–91.
12. Hayes DF, Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Miller MC, et al. Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. Clin Cancer Res 2006;12:4218–24.
13. Miller MC, Doyle GV, Terstappen LW. Significance of circulating tumor cells detected by the CellSearch system in patients with metastatic breast colorectal and prostate cancer. J Oncol 2010;2010:617421.
14. Bidard FC, Fehm T, Ignatiadis M, Smerage JB, Alix-Panabières C, Janni W, et al. Clinical application of circulating tumor cells in breast cancer: overview of the current interventional trials. Cancer Metastasis Rev 2013;32:179–88.
15. Lianidou ES, Mavroudis D, Georgoulias V. Clinical challenges in the molecular characterization of circulating tumour cells in breast cancer. Br J Cancer 2013;108:2426–32.
16. Plaks V, Koopman CD, Werb Z. Cancer. Circulating tumor cells. Science 2013;341:1186–8.
17. Ohtsuka M, Ling H, Doki Y, Mori M, Calin GA. MicroRNA processing and human cancer. J Clin Med 2015;4:1651–67.
18. Griffiths-Jones S, Moxon S, Marshall M, Khanna A, Eddy SR, Bateman A. Rfam: annotating non-coding RNAs in complete genomes. Nucl Acid Res 2005;33:D121–4.
19. Markou A, Yousef GM, Stathopoulos E, Georgoulias V, Lianidou E. Prognostic significance of metastasis-related microRNAs in early breast cancer patients with a long follow-up. Clin Chem 2014;60:197–205.
20. Pecot CV, Rupaimoole R, Yang D, Akbani R, Ivan C, Lu C, et al. Tumour angiogenesis regulation by the miR-200 family. Nat Commun 2013;4:2427.
21. Schwarzenbach H, Nishida N, Calin GA, Pantel K. Clinical relevance of circulating cell-free microRNAs in cancer. Nat Rev Clin Oncol 2014;11:145–56.
22. Müller V, Gade S, Steinbach B, Loibl S, von Minckwitz G, Untch M, et al. Changes in serum levels of miR-21, miR-210, and miR-373 in HER2-positive breast cancer patients undergoing neoadjuvant therapy: a translational research project within the Geparquinto trial. Breast Cancer Res Treat 2014;147:61–8.
23. Madhavan D, Zucknick M, Wallwiener M, Cuk K, Modugno C, Scharpff M, et al. Circulating miRNAs as surrogate markers for circulating tumor cells and prognostic markers in metastatic breast cancer. Clin Cancer Res 2012;18:5972–82.
24. Strati A, Markou A, Parisi C, Politaki E, Mavroudis D, Georgoulias V, Lianidou E. Gene expression profile of circulating tumor cells in breast cancer by RT-qPCR. BMC Cancer 2011;11:422.
25. Sourvinou IS, Markou A, Lianidou ES. Quantification of circulating miRNAs in plasma: effect of preanalytical and analytical parameters on their isolation and stability. J Mol Diagn 2013;15:827–34.
26. Peltier HJ, Latham GJ. Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. RNA 2008;14:844–52.
27. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001;25:402–8.
28. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009;55:611–22.
29. Sieuwerts AM, Mostert B, Bolt-de Vries J, Peeters D, de Jongh FE, Stouthard JM, et al. mRNA and microRNA expression profiles in circulating tumor cells and primary tumors of metastatic breast cancer patients. Clin Cancer Res 2011;17:3600–18.
30. Gasch C, Plummer PN, Jovanovic L, McInnes LM, Wescott D, Saunders CM, et al. Heterogeneity of miR-10b expression in circulating tumor cells. Sci Rep 2015;5:15980.
31. Song B, Wang C, Liu J, Wang X, Lv L, Wei L, et al.

MicroRNA-21 regulates breast cancer invasion partly by targeting tissue inhibitor of metalloproteinase 3 expression. J Exp Clin Cancer Res 2010;29:29.

32. Hurst DR, Edmonds MD, Scott GK, Benz CC, Vaidya KS, Welch DR. Breast cancer metastasis suppressor 1 upregulates miR-146, which suppresses breast cancer metastasis. Cancer Res 2009;69:1279–83.

33. Rothé F, Ignatiadis M, Chaboteaux C, Haibe-Kains B, Kheddoumi N, Majjaj S, et al. Global microRNA expression profiling identifies MiR-210 associated with tumor proliferation, invasion and poor clinical outcome in breast cancer. PLoS One 2011;6:e20980.

34. Camps C, Buffa FM, Colella S, Moore J, Sotiriou C, Sheldon H, et al. hsa-miR-210 Is induced by hypoxia and is an independent prognostic factor in breast cancer. Clin Cancer Res 2008;14:1340–8.

35. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol 2008;10: 593–601.

36. Chen J, Wang X. MicroRNA-21 in breast cancer: diagnostic and prognostic potential. Clin Transl Oncol 2014;16:225–33.

37. Markou A, Tsaroucha EG, Kaklamanis L, Fotinou M, Georgoulias V, Lianidou ES. Prognostic value of mature microRNA-21 and microRNA-205 overexpression in non-small cell lung cancer by quantitative real-time RT-PCR. Clin Chem 2008;54:1696–704.

38. Markou A, Sourvinou I, Vorkas P, Yousef G, Lianidou E. Clinical evaluation of microRNA expression profiling in non small cell lung cancer. Lung Cancer 2013; 81:388–96.

39. Ortega FG, Lorente JA, Garcia Puche JL, Ruiz MP, Sanchez-Martin RM, de Miguel-Pérez D, et al. miRNA in situ hybridization in circulating tumor cells–MishCTC. Sci Rep 2015;5:9207.

40. Ling H, Fabbri M, Calin GA. MicroRNAs and other noncoding RNAs as targets for anticancer drug development. Nat Rev Drug Discov 2013;12:847–65.

41. Zhang S, Han L, Wei J, Shi Z, Pu P, Zhang J, Yuan X, Kang C. Combination treatment with doxorubicin and microRNA-21 inhibitor synergistically augments anticancer activity through upregulation of tumor suppressing genes. Int J Oncol 2015;46:1589–600.

42. Fabbri M. MicroRNAs and cancer: towards a personalized medicine. Curr Mol Med 2013;13:751–6.

43. Dykxhoorn DM, Wu Y, Xie H, Yu F, Lal A, et al. miR-200 enhances mouse breast cancer cell colonization to form distant metastases. PLoS One 2009;4:e7181.

44. Schwarzenbach H, da Silva AM, Calin G, Pantel K. Data normalization strategies for microRNA quantification. Clin Chem 2015;61:1333–42.

45. Leidner RS, Li L, Thompson CL. Dampening enthusiasm for circulating microRNA in breast cancer. PLoS One 2013;8:e57841.

46. Witwer KW. Circulating microRNA biomarker studies: pitfalls and potential solutions. Clin Chem 2015;61: 56–63.