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Circulating miRNAs with prognostic value in metastatic breast cancer and for early detection of metastasis

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Abstract

Metastasis is the principal cause of high morbidity and mortality among breast cancer (BC) patients. Identification of markers that can be routinely monitored to predict onset of metastasis in BC patients and prognosis of metastatic breast cancer (MBC) patients would increase their median survival. In this study, plasma miRNAs of 40 MBC patients were profiled by TaqMan low density arrays and miRNAs with prognostic capacity were identified. The candidates were validated initially in the samples of 237 MBC patients and subsequently in 335 samples from an independent study cohort of BC patients. Sixteen miRNAs were established to be significantly associated with overall survival, and were termed as prognostic miRNA panel template (PROMPT). These included miR-141, miR-144, miR-193b, miR-200a, miR-200b, miR-200c, miR-203, miR-210, miR-215, miR-365, miR-375, miR-429, miR-486-5p, miR-801, miR-1260 and miR-1274a. Additionally, 11 of these miRNAs were also associated with progression-free survival. Their prognostic significance was further confirmed in samples from a second study cohort of BC patients. In addition, miR-200a, miR-200b, miR-200c, miR-210, miR-215 and miR-486-5p were found to be significantly associated with onset of metastasis up to 2 years prior to clinical diagnosis in BC patients. We have thus identified panels of miRNAs, which include metastasis promoting miR-200 family and miR-203, as well as oncogenic and tumor-suppressive miRNAs, that can serve as prognostic markers for MBC, and early detection markers of metastasis in BC.

Introduction

Breast cancer (BC) and specifically metastatic breast cancer (MBC) are major health issues worldwide as they account for the highest number of cancer-related deaths among women (1). Due to the early detection of metastasis followed byappropriate intervention, the mortality rate of BC has decreased significantly since 1990s (2), while better stratification of patients into poor and good-prognosis groups would lead to a more personalized medicine approach. Prognosis refers to prediction of progression-free (PFS) and overall survival (OS), both of which are relatively short among MBC patients. Thus, use of blood-based

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Abbreviations

BC	breast cancer
CTC	circulating tumor cell
DDFS	distant disease-free survival
IPE	integrated prediction error
MBC	metastatic breast cancer
OS	overall survival
PROMPT	prognostic miRNA panel template
PFS	progression-free survival

biomarkers that can be routinely monitored for these purposes would be very helpful in improving the overall quality of life for patients. Biomarkers such as receptor status, uPA, PAI-1 and blood-based markers like carbohydrate antigen 15-3 or carcinoembryonic antigen, are confined to specific types of MBC and lack sensitivity and specificity (3–5). Circulating tumor cell (CTC) status has been recommended as an independent prognostic marker for MBC, in general, and has received FDA clearance, although limitations regarding its enrichment and detection methods are cited (6). Currently, prognosis and risk assessment in MBC are largely achieved by clincopathological features such as age of diagnosis, tumor size, number and types of sites of metastasis, receptor status, distant disease-free survival (DDFS), etc (7–9).The most widely used markers for predicting onset of metastasis are the tissue levels of uPA and PAI-1 (10). The recently established Rotterdam signature of 76 genes, which predicts the development of distant metastasis within 5 years, has also gained prominence (11). However, both these markers are applicable only to lymph node negative BC patients; hence it is confined to a subset of BC patients. Thus, there exists a lacuna in the area of biomarkers for predicting prognosis across all types of MBC and early detection of metastasis in BC patients.

Since their discovery, circulating miRNAs, which represent the miRNA population in cell-free portion of blood and body fluids, have attracted tremendous interest in the field of biomarker discovery (12). Features such as high stability, access by minimally invasive methods and possibility of repeated sampling make them ideal candidates for use as biomarkers (13). Various studies have showcased the potential of circulating miRNAs as diagnostic markers in BC (14–18). Particularly, we have previously shown eight miRNAs, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-375, miR-210 and miR-801, to be increased in MBC patients compared to healthy controls, and more importantly, indicative of CTC status while also predicting PFS and OS (19). Other studies have demonstrated differences in levels of miR-10b, miR-34a, miR-155, miR-215, miR-299-5p and miR-411 between MBC patients and healthy individuals (20,21).

The purpose of this study was to identify circulating miRNAs that could predict prognosis in MBC patients by adopting a global profiling approach, followed by validation in two independent cohorts (n = 237 and n = 332). Sixteen miRNA were validated here and miRNA panels were built to predict both PFS and OS, and these panels were found to possess lower prediction errors than the currently recommended CTC status. Most interestingly, by investigating samples from a prospective study cohort we found six miRNAs to indicate onset of metastasis up to 2 years before diagnosis highlighting the importance of these miRNAs as early detection marker of metastasis.

Materials and methods

Samples and study design

Samples used in this study were from two studies. Samples of 237 patients with radiographically confirmed the presence of one or more sites of metastasis at diagnosis, thus diagnosed with MBC at time of blood draw were used from study cohort I (Supplementary Table 1, available at *Carcinogenesis* Online). Tumor progression was routinely monitored approximately every 3 months and response was classified according to the RECIST guidelines (22). Peripheral blood was collected in EDTA tubes (Sarstedt S-Monovette®, Nümbrecht, Germany) after recruitment into the study (MBC patients at base line, MBC_{BL}). An additional blood sample was collected from 117 of the 237 MBC patients after completion of one cycle of therapy (MBC patients after one complete cycle of chemotherapy, MBC_{1C}). Blood was processed within 2 h of phlebotomy by a two-step centrifugation protocol: 1300*g* for 20 min at 10°C, followed by 15 500*g* for 10 min at 10°C of the plasma supernatant obtained from first step. Plasma samples were snap-frozen and stored at –80°C. For each blood draw CTC status was additionally determined by evaluating CTCs using the CellSearch®system (Veridex, Jansen, Raritan, NJ). Depending on the number of CTCs, patients were designated as CTC-positive (≥5 CTCs/7.5 ml blood) or CTC-negative (< 5 CTCs/7.5 ml blood or no detectable CTCs). Samples of study cohort II were drawn from one study region of the population-based case–control MARIE (Mamma Carcinoma Risk factor Investigation) study, in which patients with primary BC diagnosed between 2002 and 2005 were recruited and followed-up until the end of 2009 (Supplementary Table 2, available at *Carcinogenesis* Online). The selected study subjects included all patients with metastasis (M1, n = 67) at diagnosis/blood collection, and patients without metastasis at diagnosis (M0, n = 265). The M0 subjects in turn comprised of all those who developed metastases within 2 years after diagnosis (n = 52) and a subset of those who did not develop metastasis or die during follow-up (n = 196). Here, blood samples were centrifuged at 3300*g* for 10 min at 10°C. Plasma was separated and stored at –80°C. The plasma samples were thawed and a second centrifugation step was applied (12 000*g* for 10 min at 10°C). 200 µl of supernatant from this step was aliquoted into a 2-ml tube and stored at –80°C. PFS, OS and DDFS were calculated as time, in months, from blood draw to progression of disease or last radiologic examination, death or last visit, and development of metastasis or last follow-up time, respectively. All samples were from females and of Caucasian origin. Distribution of clinical characteristics of the samples used from the two study cohorts are given in Supplementary Tables 1 and 2, available at *Carcinogenesis* Online. The study was performed in accordance with the principles embodied in the Declaration of Helsinki and approved by the Ethical Committee of the University of Heidelberg (Heidelberg, Germany). Written informed consent was obtained from all participants.

The study consisted of three phases: (i) discovery phase, (ii) validation phase which also included the samples from the initial discovery phase and (iii) second independent validation phase. While samples from study cohort I were used for the first two phases, samples from study cohort II were used for the final phase. miRNA was extracted from 400 µl of plasma (cohort I) or 200 µl of plasma (cohort II) using TRIzol® LS (Invitrogen, Life Technologies, Foster City, CA) and Qiagen miRNeasy kit (Qiagen, Hilden, Germany), and spiking in 10 fmol of cel-miR-39, as described previously (19).

Global profiling of circulating miRNAs from MBC_{BL} samples

Circulating miRNA from plasma of MBC_{BL} samples was profiled by TaqMan® Human microRNA cards v3.0 (Applied Biosystems, Life Technologies, Foster City, CA) following the manufacturer's instructions. Briefly, 3 µl of miRNA sample was reverse transcribed by either Megaplex™RT Primers, Human Pool A or Pool B v3.0 (Applied Biosystems). About 5 µl of RT product was preamplified with Megaplex™ PreAmp Primers, Human Pool A or Pool B v3.0 (Applied Biosystems). The final product was used for the quantitative PCR (qPCR) reaction, which was carried out in Applied Biosystems 7900HT machine. Cycle threshold (Ct) value of each miRNA was calculated by the SDS v2.2 software using automatic baseline and threshold setting. 20 samples with poor prognosis (PFS or OS < 3 months) and 20 samples with good prognosis (PFS and OS > 16 months) were profiled, and 754 miRNAs measured (Supplementary Table 3, available at *Carcinogenesis* Online). Of these 40 patients, 12 had died and 28 were still alive. miRNAs not detected or with Ct >35 across all 40 samples were filtered out. The data was then quartile normalized and an additional filtration step to remove invariant miRNAs with interquartile range < 1.5 was applied. Normalized miRNAs remaining after these filtration steps were used for further statistical analysis.

Validation of candidate miRNAs

Candidate miRNAs chosen from the above discovery round were initially validated in an expanded sample set of 237 MBC_{BL} and 117 MBC_{1C} samples from study cohort I by individual TaqMan® assays (Table 1). This was followed by an independent validation in 332 samples from study cohort II. A constant volume input of 2 µl of miRNA was introduced into the reverse transcription reaction, in which a maximum of three miRNAs were multiplexed in a 7.5-µl reaction mixture. About 2.3 µl of reverse transcribed product was subjected to qPCR in a 5-µl reaction mixture containing TaqMan® Universal PCR Master Mix, No AmpEraseUNG (Applied Biosystems), using Roche LightCycler®480 (Roche Applied Sciences, Germany) in triplicates and crossing point was determined. miRNA was normalized to exogenous control, cel-miR-39 and the identified endogenous controls. When a miRNA was undetected in a sample, it was replaced with the maximum crossing point across all samples for that miRNA and used for data analysis.

Statistical analysis

All statistical analysis were performed in R3.0.1 environment (23). For analyzing TLDA data, HTqPCR (24) package from Bioconductor v2.13 (25) was used. Limma analysis was performed to compare miRNA profiles of (i) samples with poor and good prognosis and (ii) samples from patients who died and those who were alive. miRNAs were chosen as candidates for the validation phase if it had $P < 0.05$ and fold change > 2 or <0.5 for one of the above comparisons, and mean Ct < 32 in one of the analyzed groups. Complete-linkage hierarchical clustering with the distance metric defined by the Pearson correlation of samples based on their progression or vital status was accomplished based on the miRNA levels of those which had $P < 0.05$ in the corresponding limma analysis. Endogenous controls were identified from a set of miRNAs with interquartile range < 1 and mean Ct < 30 using NormFinder (26).

In the validation cohort, association between miRNA levels and PFS, OS or DDFS was assessed by log-rank tests and Kaplan–Meier curves. miRNA models with highest prediction accuracy and least redundancy were built using LASSO Cox models, wherein a LASSO penalty term was used for automatic selection of relevant miRNA variables (with penalty parameter tuning done by 10-fold cross-validation), and allowing only miRNAs that were significant in the univariate analysis to enter the model. In addition to the miRNA models, models with CTC status alone or miRNA along with CTC status were also built (allowing for interactions between miRNA and CTC status). The prognostic value of models was assessed by 0.632+ bootstrap estimates of prediction error curves and summarized as the integrated prediction error (IPE) curve, and the IPE of different models were compared, with a lower IPE reflecting a more accurate model. miRNA data was dichotomized as miRNA low levels and miRNA high levels, while CTC status was retained in its binary state (CTC-positive or CTC-negative), and used for the above survival analysis. Comparison of samples of those who developed versus those who did not develop metastasis was done by Wilcoxon rank sum tests with two-sided *P*-value.

Results

Identification of circulating miRNAs with potential prognostic value

Profiling of plasma samples from patients with two extreme prognostic outcomes resulted in identification of candidate miRNAs for predicting PFS or OS. After the initial filtering steps to eliminate undetected ($n = 271$) and miRNAs whose levels were invariant across all samples ($n = 287$), 199 miRNAs remained which were used for comparisons and clustering. Limma analysis generated eight miRNAs that were significantly different between cases with poor and good prognosis, whereas 21 miRNAs were significant for the deceased-alive comparison. Seven miRNAs namely miR-22, miR-144, miR-149, miR-200a, miR-200b, miR-200c and miR-618 were common hits for both these comparisons. In total 20 miRNAswerechosenforfurthervalidation: miR-22, miR-141, miR-144, miR-146b-3p, miR-149, miR-193b, miR-200a, miR-200b, miR-200c, miR-203, miR-215, miR-365, miR-375, miR-429, miR486-5p, miR-618, miR-758, miR-770-5p, miR-1260 and miR-1274a (Supplementary Table 4, available at *Carcinogenesis* Online). Clustering of samples into poor and good prognosis or into deceased and alive patients based on their respective top hits is shown in Supplementary Figure 1, available at *Carcinogenesis* Online. Apart from identifying circulating miRNAs with prognostic capabilities, combination of miR-29a and miR-139-5p was proposed by NormFinder to be the most stably expressed miRNAs with a stability value of 0.004. Thus, a combination of these miRNAs along with exogenously spiked-in cel-miR-39 was used for normalization in the validation rounds.

Sixteen miRNAs confirmed to be significantly correlated to survival in MBC patients

Candidate miRNAs were first verified in the 40 samples used in the discovery phase by individual TaqMan®assays. miR-146b-3p, miR-149, miR-618, miR-758 and miR-770-5p were found to be present at very low to undetectable levels, and hence not tested in further steps. To the list of remaining 15 miRNAs, miR-210 and miR-801 were added since we had previously demonstrated their association with survival in MBC patients (19). In MBC_{BL} samples, we had a total of 187 patients with progression (83%) and 38 without progression (17%); 85 patients who were deceased (36%) and 149 who were still alive (64%). Log-rank tests after stratification of samples based on their miRNA levels (lower quartile versus rest) revealed 16 miRNAs, namely miR-141, miR-144, miR-193b, miR-200a, miR200b, miR-200c, miR-203, miR-210, miR-215, miR-365, miR-375, miR-429, miR-486-5p miR-801, miR-1260 and miR-1274a to be associated with OS ($P < 0.05$, HR> 2 or < 0.6, Figure 1;Table 1). On the other hand, miR-141, miR-144, miR-193b, miR-200a, miR-200b, miR-200c, miR-203, miR215, miR-375, miR-429, miR-801 and miR-1274a were significantly correlated to PFS ($P< 0.04$, HR > 1.4 or < 0.7, Figure 2). Thus, 16 out of the 17 candidate miRNAs were confirmed to possess prognostic significance with respect to either PFS and/or OS and made up our prognostic miRNA panel template (PROMPT) for MBC. CTC status was also found to be a significant predictor of PFS ($P = 0.006$, HR = 1.5) and OS ($P < 0.0001$, HR = 2.9, Figures 1 and 2) in our tested samples.

Circulating miRNAs of PROMPT correspond to survival after one cycle of therapy

To assess whether the prognostic ability of the miRNAs was valid even after therapy, miRNA levels were measured in MBC_{1C} samples. During the follow-up period, 88 patients had progression (79%) and 23 had no progression (21%), while 35 (30%) had died and 81 were still alive (70%). Thus, distribution of both PFS and OS was similar to those of MBC_{BL} samples analyzed. We found that the majority of miRNAs were still associated with survival and the correlation was found to be, in general, stronger in the MBC_{1C} samples with respect to their *P* values ($P < 0.003$ for OS, $P < 0.045$ for PFS) and HR (HR > 2.8 for OS, HR > 1.6 for PFS, Supplementary Figure 2a and b, available at *Carcinogenesis* Online). However, miR-144 and miR-215were no longer significantly associated with OS and PFS. miR801 was significantly associated with OS only, whereas miR-365, miR-486-5p and miR-1260 lost their correlation to OS after therapy (Table 1).

Panel of miRNAs from PROMPT performs better than CTC status

miRNA models which had the highest predictive power were built with LASSO Cox regression model allowing for automatic

Table 1. Results of log-rank test between miRNA levels or CTC status and survival time, represented by HR, 95% CI and *P* values in MBC_{BL} and MBC_{1C} samples

	MBC_{BL}				MBC_{1C}			
	PFS		OS		PFS		OS	
miRNA	HR (95%CI)	*P*	HR (95%CI)	*P*	HR (95%CI)	*P*	HR (95%CI)	*P*
miR-22	1.10 (0.79–1.55)	0.56	1.57 (0.99–2.51)	0.05	0.74 (0.45–1.23)	0.24	1.05 (0.48–2.32)	0.90
miR-141	1.51 (1.09–2.08)	0.01	3.04 (1.96–4.72)	1.2×10^{-7}	2.37 (1.47–3.81)	2.1×10^{-4}	8.85 (4.47–17.52)	1.2×10^{-14}
miR-144	0.68 (0.49–0.96)	0.02	0.53 (0.31–0.92)	0.02	0.69 (0.43–1.12)	0.13	0.59 (0.26–1.34)	0.20
miR-193b	1.85 (1.34–2.56)	0.00	2.62 (1.69–4.07)	4.1×10^{-6}	1.64 (1.01–2.66)	0.04	2.77 (1.4–5.5)	0.002
miR-200a	1.44 (1.04–2.01)	0.03	3.56 (2.31–5.5)	4.6×10^{-10}	1.89 (1.17–3.07)	0.01	7.67 (3.9–15.1)	9.7×10^{-13}
miR-200b	1.61 (1.15–2.22)	0.00	3.21 (2.07–4.96)	2.1×10^{-8}	2.05 (1.29–3.26)	1.6×10^{-3}	5.92 (3.02–11.61)	2.3×10^{-9}
miR-200c	1.62 (1.17–2.23)	0.00	2.32 (1.5–3.6)	6.6×10^{-5}	2.60 (1.65–4.12)	1.4×10^{-5}	5.62 (2.87–11.02)	7.9×10^{-9}
miR-203	1.41 (1.02–1.94)	0.04	2.17 (1.38–3.42)	4.9×10^{-4}	1.68 (1.05–2.68)	0.03	3.44 (1.74–6.8)	1.4×10^{-4}
miR-210	1.23 (0.88–1.72)	0.22	1.77 (1.12–2.78)	0.01	1.37 (0.85–2.2)	0.20	3.24 (1.62–6.47)	3.6×10^{-4}
miR-215	0.59 (0.41–0.84)	0.00	0.58 (0.33–1.01)	0.05	0.75 (0.45–1.25)	0.27	0.89 (0.39–2.04)	0.78
miR-365	1.12 (0.8–1.57)	0.50	2.01 (1.28–3.17)	2.3×10^{-3}	1.09 (0.68–1.75)	0.73	1.09 (0.5–2.41)	0.82
miR-375	1.42 (1.02–1.93)	0.04	2.42 (1.56–3.76)	4.2×10^{-5}	1.62 (1.02–2.58)	0.04	3.81 (1.94–7.51)	2.3×10^{-5}
miR-429	1.58 (1.15–2.17)	0.00	3.47 (2.25–5.36)	1.2×10^{-9}	2.50 (1.57–3.98)	3.8×10^{-5}	7.37 (3.76–14.45)	2.4×10^{-12}
miR-486-5p	0.76 (0.54–1.07)	0.11	0.50 (0.28–0.91)	0.02	0.82 (0.5–1.34)	0.43	0.69 (0.29–1.67)	0.41
miR-801	1.43 (1.01–1.95)	0.04	2.85 (1.85–4.4)	6.4×10^{-7}	0.98 (0.61–1.58)	0.93	3.04 (1.55–5.99)	6.5×10^{-4}
miR-1260	0.92 (0.65–1.29)	0.61	1.98 (1.26–3.12)	2.1×10^{-3}	0.92 (0.57–1.49)	0.73	1.41 (0.68–2.95)	0.36
miR-1274a	1.49 (1.08–2.06)	0.01	3.04 (1.96–4.71)	1.3×10^{-7}	2.29 (1.43–3.66)	3.4×10^{-4}	5.25 (2.67–10.33)	4.7×10^{-8}
CTC status	1.52 (1.12–2.06)	0.01	2.90 (1.9–4.4)	1.9×10^{-7}	1.71 (1.08–2.68)	0.02	4.65 (2.22–9.78)	6.9×10^{-6}

Samples dichotomized as lower quartile (miRNA high) and upper rest (miRNA low) based on their miRNA Cp values, or as CTC-positive and CTC-negative based on their CTC status. HR calculated as ratio of probability of progression or death of miRNA high/CTC-positive group to that of miRNA low/CTC-negative group. HR greater than 1 denotes that increase in variable decreases probability of survival, while those less than 1 denotes that decrease in variable decreases probability of survival.

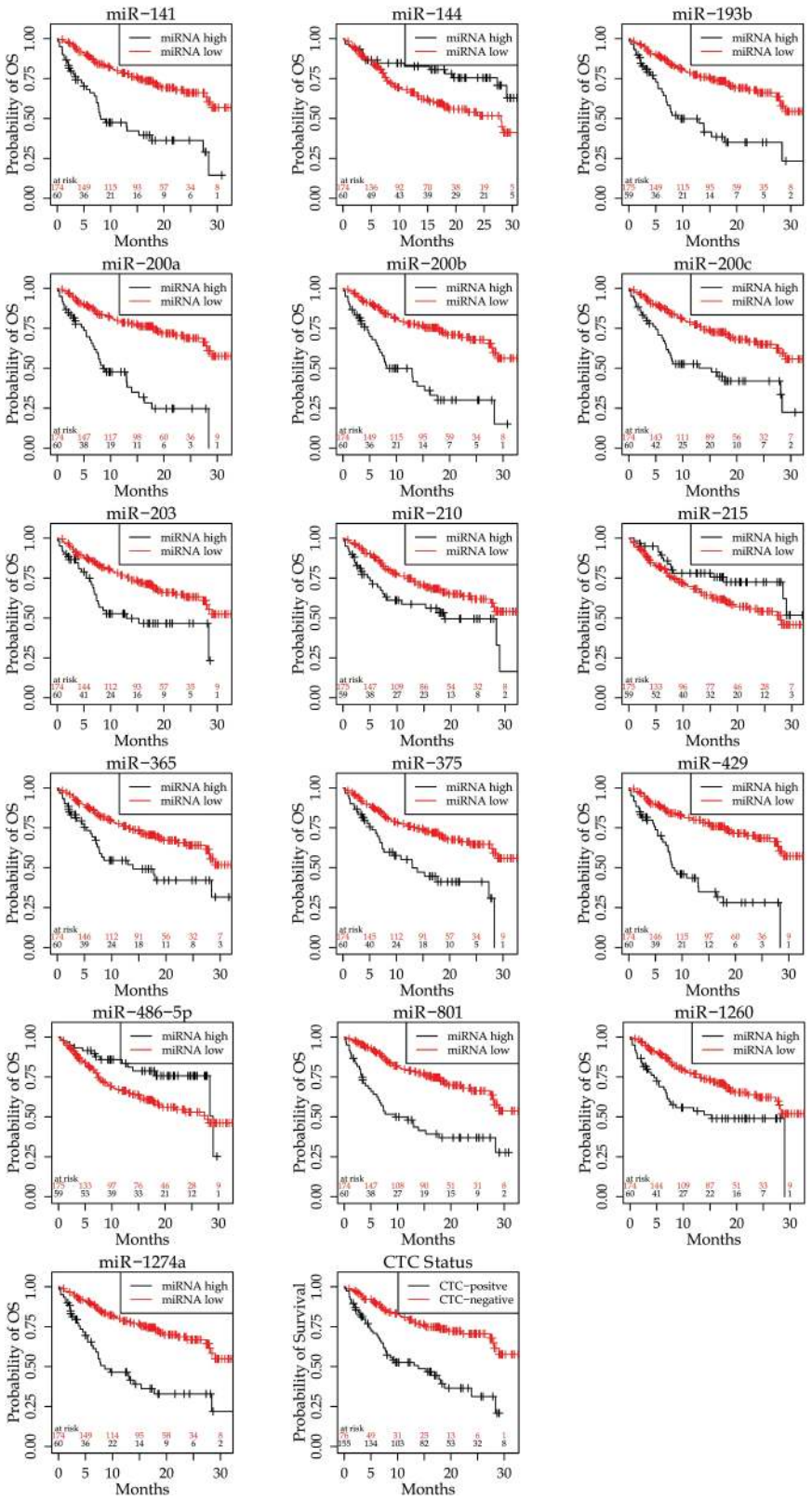

Figure 1. Kaplan–Meier curves of miRNAs significantly correlated to OS and PFS in MBC_{BL} samples and also for CTC status. Samples dichotomized as lower quartile and upper rest based on their miRNA levels or as CTC-positive and CTC-negative based on their CTC status. Number of individuals at risk in each stratum at different time points is indicated along the *x*-axis.

variable selection from PROMPT measured in both MBC_{BL} and MBC_{1C} samples. In MBC_{BL} sample set, themodelcontained 10 miRNAs (miR-141, miR-144, miR-193b, miR200b, miR-200c, miR-203, miR-215, miR-429, miR-801 and miR-1274a) and 11 miRNAs (miR-141, miR-144, miR-193b, miR-200a, miR-200b, miR-215, miR-429, miR-486-5p, miR-801, miR-1260 and miR-1274a) for OS andPFS,

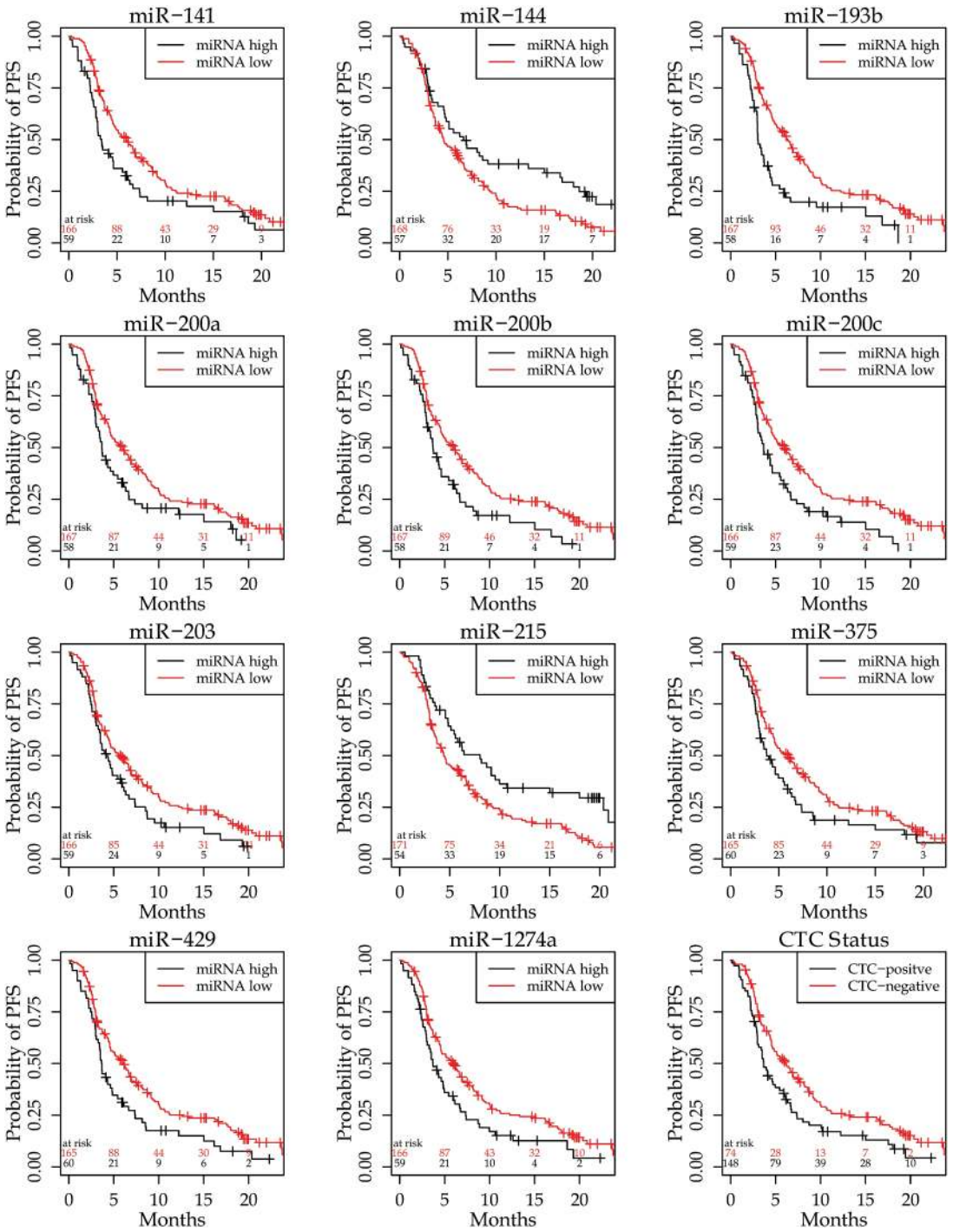

Figure 2. Kaplan–Meier curves of miRNAs significantly correlated to PFS in MBC_{BL} samples and also for CTC status. Samples dichotomized as lower quartile and upper rest based on their miRNA levels or as CTC-positive and CTC-negative based on their CTC status. Number of individuals at risk in each stratum at different time points is indicated along the x-axis.

respectively. In MBC_{1C} sample set, the final model consisted of only a small subset of these miRNAs for predicting OS (miR-141, miR-200a, miR-200b, miR-429 and miR-1274a) and PFS (miR-141, miR-200c, miR-429 and miR-1274a) (Supplementary Table 5, available at *Carcinogenesis* Online). While for PFS, miRNA model (IPE = 2.05) had a marginally lower IPE than CTC status (IPE = 2.058, Figure 3a), for OS, Cox model with miRNA variables (IPE = 1.347) performed significantly better than the model with CTC status (IPE = 1.457, Figure 3b). The superiority of the miRNA model to CTC status with reference to IPE was much more profound in MBC_{1C} (blood taken after one cycle of therapy) sample set (Figure 3c and d). Adding CTC status to the miRNA variables did not improve the accuracy of the miRNA models, with the exception of PFS in MBC_{BL} data set, in which the combination of miRNAs, and CTC introduced as an unpenalized variable was proposed as the best model (Figure 3). In our data set, we found lung metastasis, visceral metastasis, number of sites of metastasis and progesterone receptor (PR) status of primary tumor to be significantly associated with both

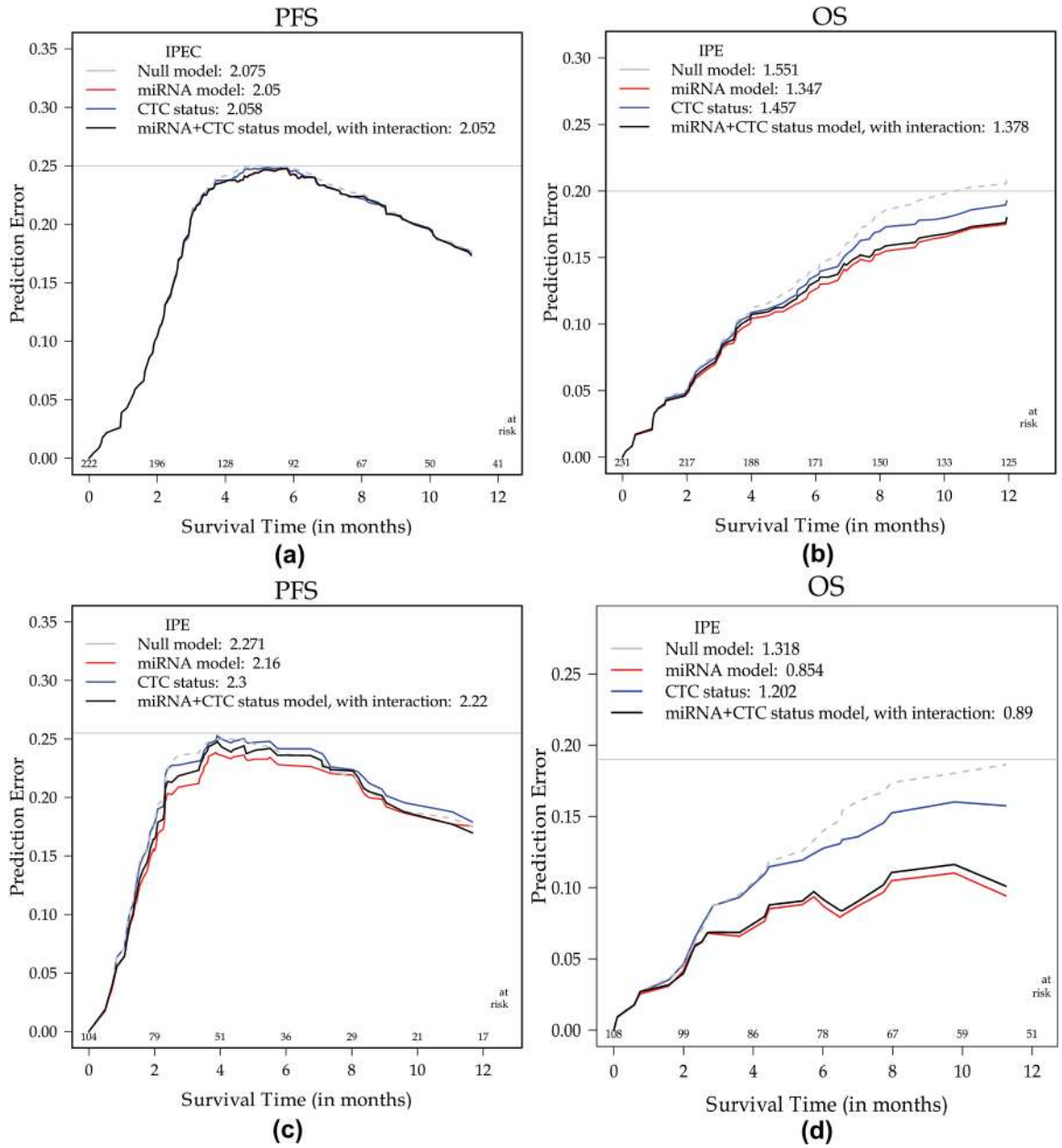

Figure 3. Integrated prediction error (IPE) curves shown for null model without co-variates, miRNA model, CTC model and miRNA + CTC model in MBC_{BL} samples, (**a**) and (**b**), and MBC_{1C} samples, (**c**) and (**d**).

OS and PFS (data not shown). Comparison of multivariate models containing these established clinical prognostic variables with and without the addition of miRNAs demonstrated that the addition of miRNAs decreased the prediction error for OS from 1.47 to 1.30, and PFS from 1.97 to 1.92.

Correlation of PROMPT to OS confirmed in an independent cohort

The 16 miRNAs significantly predicting OS, were interrogated in a second sample set consisting of 332 patients (both M0 and M1) of study cohort II. Of the 332 patients, 225 (62%) were still alive with a 5-year follow-up and 107 (38%) had died. Seven miRNAs, miR-144, miR-200a, miR-200b, miR-200c, miR-210, miR-215 and miR-486-5p were confirmed to predict OS in these samples ($P < 0.02$ for all, HR > 1.7 or < 0.65, Table 2; Figure 4).

Identified circulating miRNAs may also serve as early indicators of metastasis

The successfully validated 16 miRNAs were also tested for their ability to prospectively detect onset of metastasis in M0 samples from study cohort II. Of the 248 subjects, 52 (20%) developed metastasis within 2 years and 196 (80%) did not develop metastasis for at least 50 months. The analysis showcased the potential of miR-200a, miR-200b, miR-200c, miR-210, miR-215 and miR-486-5p to detect the onset of metastasis as early as 2 years prior to clinical diagnosis ($P < 0.02$). These miRNAs were significantly increased in patients who developed metastasis within 2 years in comparison to those who did not, with the exception of miR-215, which was decreased in the former subtype (Supplementary Figure 3, available at *Carcinogenesis* Online). A combination of these six miRNAs could discriminate the two

Table 2. Results of independent validation

	Overall survival		Metastasis onset	
miRNA	HR	*P*	Fold change	*P*
miR-141	0.99 (0.68–1.45)	0.96	0.96	0.50
miR-144	0.64 (0.44–0.94)	0.02	0.78	0.07
miR-193b	1.41 (0.96–2.08)	0.08	1.20	0.40
miR-200a	2.77 (1.82–4.20)	5.9×10^{-7}	1.50	1.4×10^{-8}
miR-200b	1.92 (1.29–2.86)	0.001	1.61	0.006
miR-200c	1.72 (1.16–2.55)	0.006	1.15	0.02
miR-203	0.79 (0.54–1.16)	0.23	0.63	0.07
miR-210	1.78 (1.21–2.66)	0.003	1.13	0.05
miR-215	0.53 (0.35–0.78)	0.001	0.72	0.01
miR-365	0.74 (0.53–1.08)	0.12	0.99	0.58
miR-375	1.22 (0.82–1.76)	0.34	1.25	0.73
miR-429	1.13 (0.77–1.66)	0.52	0.96	0.93
miR-486-5p	2.65 (1.75–4.00)	1.4×10^{-6}	1.77	1.1×10^{-5}
miR-801	1.33 (0.91–1.95)	0.14	1.16	0.79
miR-1260	1.18 (0.81–1.73)	0.39	1.15	0.07
miR-1274a	0.84 (0.57–1.23)	0.36	0.89	0.81

HR with 95% CI and *P* values from log-rank test representing the correlation of miRNAs to OS are given. HR calculated as ratio of probability of progression or death of miRNA high group to that of miRNA low group. Fold change between M0 patients who developed metastasis to those M0 patients who did not develop metastasis along with their *P* values is represented under 'Metastatic onset.'

subtypes of M0 samples, i.e. those who developed metastasis and those who did not develop metastasis, with an area under the curve of 0.82 (sensitivity = 77%, specificity = 75%). miR-200a (HR = 1.6, $P < 10^{-7}$), miR-200b (HR = 1.2, $P = 0.006$), miR-200c (HR = 1.2, $P = 0.02$), miR-210 (HR = 1.1, $P = 0.049$) and miR-486-5p (HR = 1.1, $P < 10^{-4}$) could not only detect the development of metastasis prospectively, but were also correlated with DDFS time in the subset of M0 patients who developed metastasis. Additionally, we found no specificity to any particular site of metastasis (data not shown).

Discussion

Prognostic biomarkers that divulge information regarding the spread of disease to distant sites, progression of disease and survival of patients have important clinical applications. They help oncologists in decision-making processes and for adoption of appropriate treatment regime for the patients (8). Blood-based biomarkers have advantages over tissue markers as they are easily accessible and can also be routinely monitored. We have explored the use of circulating miRNAs as prognostic markers for MBC, and have successfully identified miRNA panels by a systematic approach, consisting of a discovery phase and two independent validation phases (Supplementary Figure 4, available at *Carcinogenesis* Online).

Through global profiling of plasma miRNAs of MBC patients, we could identify 20 miRNAs which were selected for further validation. Since OS and PFS are closely related, we hypothesized that miRNAs which were predictive of OS would be capable of predicting PFS, and vice versa. Hence, we used two approaches to identify miRNAs of prognostic value, those that can predict OS and PFS. Of note six miRNAs, miR-200 family (miR-141, miR-200a, miR-200b, miR-200c), miR-203 and miR-375, that we previously identified as prognostic miRNAs with a different approach utilizing CTC status as a surrogate end point for prognosis, was once again significantly associated with survival in the discovery phase in the here presented study. On the other hand, miR-210 and miR-801 were not in the list of candidate miRNAs generated from analyzing the TLDA array data, however, we included them in the validation phase of this study based on previous results (19).

Our present work has identified plasma levels of miR-141, miR-144, miR-193b, miR-200a, miR200b, miR-200c, miR-203, miR-215, miR-375, miR-429, miR-801 and miR-1274a to be significantly associated to PFS, while miR-210, miR-365, miR-486-5p and miR-1260, in addition to the above 12 miRNAs, to correlate to OS in MBC patients (Table 1). These 16 miRNAs together make up the PROMPT. Interestingly, majority of the miRNAs remained significantly correlated to OS and PFS even after one cycle of therapy (Table 1 and summarized in Supplementary Figure 5, available at *Carcinogenesis* Online). This is important, since prognosis of a patient is dynamic and may change depending on their response to therapy, and the biomarker one has identified should accurately reflect the current prognostic status of the patient.

Independent validation of 7 out of 16 miRNAs, miR-144, miR-200a, miR-200b, miR-200c, miR-210, miR-215 and miR-486-5p in plasma samples from BC patients, which included those with and without metastasis, further strengthened our results. miR-141, miR-203 and miR-429 were present in very low amounts in these samples, which might contribute to their loss in significance. Possibly, the remaining miRNAs, miR-193b, miR-365, miR-375, miR-1260 and miR-1274a are specific for only MBC patients. Lastly, the ability of miR-200a, miR-200b, miR-200c, miR-210, miR-215 and miR-486-5p to predict onset of metastasis demonstrates their huge potential as early detection markers of metastasis, which can indicate disease spread even up to 2 years before clinical diagnosis of metastasis, thus further increasing their diagnostic value.

Since multiple marker panels are more informative than single miRNAs, we constructed panels of miRNAs possessing highest accuracy and least redundancy. The performance of miRNA panels was compared to the only currently available and FDA-cleared prognostic marker for MBC, the CTC status. This proved that compared to CTC status the miRNA panels have higher accuracy with respect to predicting PFS and OS, which further

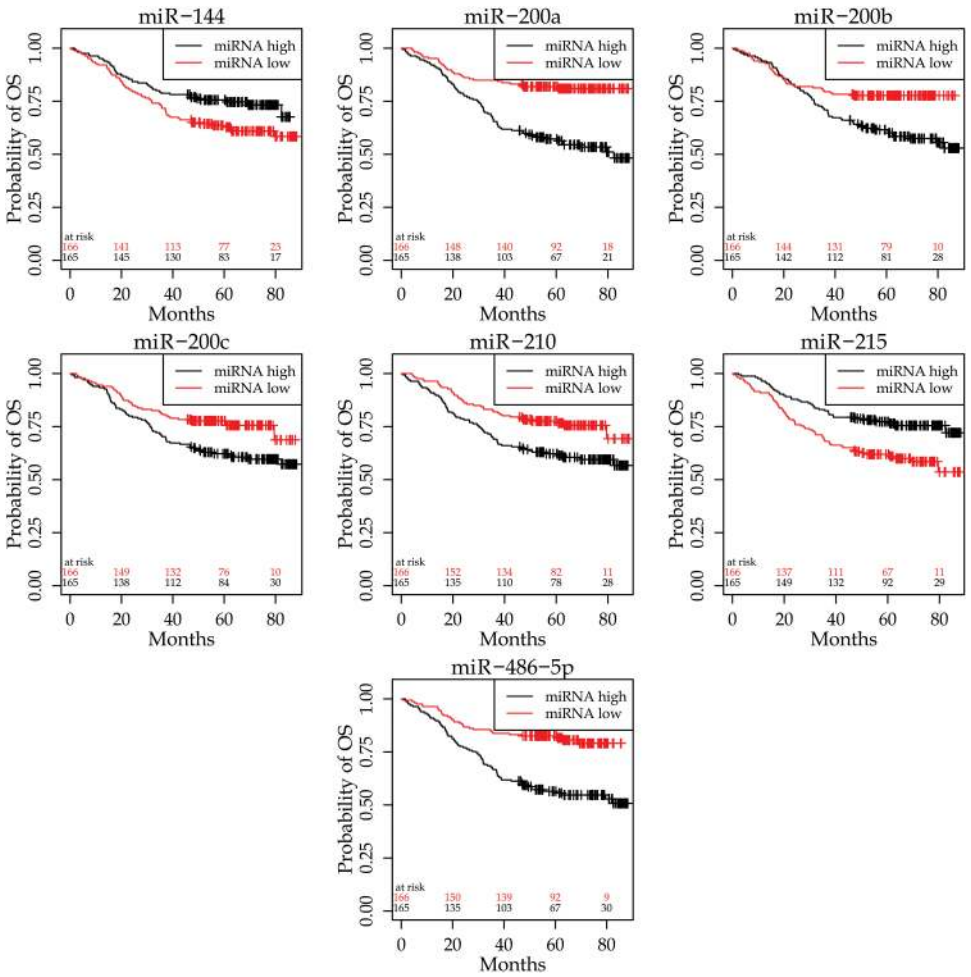

Figure 4. Kaplan–Meier curves of miRNAs significantly correlated to OS in all samples of cohort II. Samples dichotomized as less than median and greater than median based on their miRNA levels. Number of individuals at risk in each stratum at different time points is indicated along the x-axis.

improved when measured after therapy. This indicates the importance of these miRNA markers as independent prognostic markers. In clinical practice, prognosis of patients is estimated mainly based on clinical characteristics of the disease presented, however a portion of cases cannot be explained by clinical features alone. Hence, we further examined the ability of our described panel of miRNAs to improve the prognostic accuracy when added to clinical features such as lung metastasis, number of sites of metastasis, DDFS and receptor status. By combining clinical features and miRNA levels we were further able to decrease the prediction error, thus enhancing their utility in a multimarker assay. Thus, we can safely surmise, that our panel of miRNAs can be used in combination with clinical features to better predict prognosis in MBC patients.

Increased level of miR-200 family miRNAs (miR-141, miR-200a, miR-200b, miR-200c, miR-429, miR-203 and miR-375) are associated with decreased survival and increased metastasis onset in the present study. miR-200 family members are well known negative regulators of epithelial–mesenchymal transition, and positively regulate mesenchymal–epithelial transition in BC (27,28). Hence, they are important for successful colonization of metastasis, which would explain why we find increased levels of these miRNAs to be correlated to decreased survival as well as increased onset of metastasis (27–30).With respect to BC, miR-365 has been shown to be increased in cancer cells compared to normal cells (31,32), although tumor suppressive roles have been attributed to it in other cancers (33,34). miR-215 on the other hand has been previously reported as increased in serum of MBC patients, however in this study, unlike our results, they found higher levels of miR-215 to be present in the group with progressive disease. The difference could be due to different sample types as it has already been shown that plasma and serum have different circulating miRNA profile (35). miR-193b and miR-486-5p have been shown to be decreased in malignant cells compared to normal cells in BC, thus having tumor suppressive properties (31). While, this is in concordance with our observed trend in circulation for miR-486-5p, our results point to an oncogenic role for miR-193b in circulation. This conflict could be due to the complex origin of circulating miRNAs which might not be necessarily from tumor cells only (36). There have been no reports regarding the functional role of miR-144, miR-801, miR-1260 and miR-127a in BC so far.

To summarize, in the study presented here we have identified individual and panels of circulating miRNAs which were validated to predict PFS and OS in plasma of BC patients, and specifically MBC patients. Additionally, we have also identified circulating miRNAs with ability to detect metastasis up to 2 years prior diagnosis. Therefore, the circulating miRNAs described here have wide application as prognostic markers and early detection markers of metastasis for BC.

Supplementary material

Supplementary Tables 1–5 and Figures 1–5 can be found at http://carcin.oxfordjournals.org/

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Conflict of Interest Statement: B.B. and D.M. are inventors of a provisional patent application relating to the subject matter of this manuscript and therefore declare a potential conflict of interests.

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