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Imaging, Diagnosis, Prognosis

Circulating miRNAs as Surrogate Markers for Circulating Tumor Cells and Prognostic Markers in Metastatic Breast Cancer

Dharanija Madhavan[1,4], Manuela Zucknick[2], Markus Wallwiener[5,6], Katarina Cuk[1,4], Caroline Modugno[5,6], Martina Scharpff[5,6], Sarah Schott[5], Jörg Heil[5], Andrey Turchinovich[1,4], Rongxi Yang[1,4], Axel Benner[2], Sabine Riethdorf[8], Andreas Trumpp[3,7], Christof Sohn[5], Klaus Pantel[8], Andreas Schneeweiss[5,6], and Barbara Burwinkel[1,4]

Abstract

Purpose: The use of circulating tumor cells (CTC) as a prognostic marker in metastatic breast cancer (MBC) has been well established. However, their efficacy and accuracy are still under scrutiny mainly because of methods of their enrichment and identification. We hypothesized that circulating miRNAs can predict the CTC status of patients with MBC, and tested for the same. Furthermore, we aimed at establishing a panel of circulating miRNAs capable of differentiating MBC cases from healthy controls.

Experimental Design: Circulating miRNAs from plasma of CTC-positive and CTC-negative patients with MBC, and healthy controls, were profiled by TaqMan Human MicroRNA arrays. Candidates from the initial screen were validated in an extended cohort of 269 individuals (61 CTC-positive, 72 CTC-negative, 60 CTC-low MBC cases, and 76 controls).

Results: CTC-positive had significantly higher levels of miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-210, miR-375, and miR-801 than CTC-negative MBC and controls ($P < 0.00001$), whereas miR-768-3p was present in lower amounts in MBC cases ($P < 0.05$). miR-200b was singled out as the best marker for distinguishing CTC-positive from CTC-negative patients (AUC 0.88). We identified combinations of miRNAs for differentiating MBC cases from controls (AUC 0.95 for CTC-positive; AUC 0.78 for CTC-negative). Combinations of miRNAs and miR-200b alone were found to be promising prognostic marker for progression-free and overall survival.

Conclusion: This is the first study to document the capacity of circulating miRNAs to indicate CTC status and their potential as prognostic markers in patients with MBC. *Clin Cancer Res; 18(21); 5972–82. ©2012 AACR.*

Introduction

Circulating tumor cells (CTC) are occult tumor cells and purported intermediates of metastasis, through which the primary tumor "seeds" the metastatic site (1). In the past decade, many studies have provided experimental proof for the presence of CTCs in blood of patients with solid carcinomas, and their absence in healthy individuals and those with nonmalignant diseases (2). Subsequently, CTC counts were confirmed to be an independent prognostic marker of progression-free (PFS) and overall survival (OS) in metastatic breast cancer (MBC; ref. 3), metastatic castration-resistant prostate cancer (MCRPC; ref. 4), metastatic colorectal cancer (MCRC; ref. 5), and recently ovarian cancer (6). For MBC, CTC $\geq$5 in 7.5 mL blood has been recommended as an indicator of poor prognosis (3). CTCs have also been proposed as a predictive marker in MBC, MCRPC, and MCRC (3, 7, 8).

Since CTCs are rare, enrichment techniques are required before their detection. Most enrichment and detection methods, including the FDA approved CellSearch system, use positive and/or negative immuno-selection (1). Positive selection depends on the expression of epithelial markers such as epithelial cell adhesion molecule (EpCAM) or cytokeratin on CTCs, which are downregulated in event of epithelial–mesenchymal transition (EMT; refs. 9 and 10).

Authors' Affiliations: [1]Molecular Epidemiology, [2]Division of Biostatistics, and [3]Division of Stem Cells and Cancer, German Cancer Research Center (DKFZ); [4]Molecular Biology of Breast Cancer, [5]Department of Gynecology and Obstetrics, [6]National Center for Tumor Diseases, University of Heidelberg; [7]Hi-STEM-Heidelberg Institute for Stem Cell Technology and Experimental Medicine, GmbH, Heidelberg; and [8]Department of Tumor Biology, University Hospital Hamburg-Eppendorf, Hamburg, Germany

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Corresponding Author: Dharanija Madhavan, Molecular Epidemiology, C080, Im Neuenheimer Feld 581, German Cancer Research Center (DKFZ), Heidelberg 69120, Germany. Phone: 49-6221-568403; Fax: 49-6221-568242; E-mail: d.madhavan@dkfz.de

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Translational Relevance

Metastatic breast cancer (MBC) is a leading cause of morbidity and mortality among females. There is an urgent need for predictive or prognostic biomarkers that can improve the quality of life for these patients. Circulating tumor cells (CTC) have emerged as a promising prognostic biomarker in MBC. Here, for the first time, we have identified circulating miRNAs that can discriminate patients depending on their CTC status. The identified miRNAs seem to have a similar or even better prognostic value than CTCs, and combination of miRNAs and CTCs performs better than CTCs alone. The stability of circulating miRNAs and the relatively cheap methods of their isolation and detection increase their usefulness as a biomarker. Five of the miRNAs identified here are known to play a role in epithelial–mesenchymal and mesenchymal–epithelial transformation. Therefore, these findings might have important implications for other epithelial cancers in which these mechanisms are required for successful metastasis.

On the other hand, negative selection and methods based on principles such as size separation or density centrifugation have lower sensitivity (10, 11). Overall, current methods of CTC identification can miss clinically relevant subpopulations of CTCs and might lead to over or under estimation of CTCs. Thus, despite the enormous benefit of CTCs as a biomarker, they suffer from drawbacks due to the techniques used in their detection.

The involvement of miRNAs in cancer has been reiterated and irrefutably proven by many studies (12, 13). Circulating miRNAs, defined as miRNAs present in the cell-free component of blood and body fluids, were first reported by Lawrie and colleagues, who observed elevated miR-21 levels in the serum of patients with large B-cell lymphoma (14). Ensuing this, Mitchell and colleagues uncovered the association between circulating miR-141 and prostate cancer, extending the importance of circulating miRNAs to solid cancers (15). Due to their inherent stability (16), ease of sampling by minimally invasive methods, and the proven role of miRNAs in cancer development and progression, circulating miRNAs make attractive candidates for biomarker development. The promise of circulating miRNAs as an early detection/prognostic/predictive marker has been evaluated in different solid carcinomas, including early breast cancer (15, 17–19). In the context of MBC, there has been one study, which linked deregulation of miR-10b, miR-34a, and miR-155 in serum to metastasis (20).

Here, we aimed to identify a panel of circulating miRNAs that could differentiate CTC-positive from CTC-negative MBC cases, and further evaluate its prognostic potential. Such a set of miRNAs could either supplement or complement current CTC detection methods, thereby improving and adding power to existing tests. Simultaneously, we also strived to delineate miRNAs that were specifically deregulated between cases and healthy controls, which might serve as an early detection marker of metastasis. To fulfill these aims, we undertook an array-based approach and screened for circulating miRNAs capable of discriminating CTC-positive from CTC-negative MBC cases, and MBC cases from healthy controls. Candidate miRNAs were subsequently tested in an enlarged sample set of 209 individuals. For correlation to survival, all patients enrolled in this study ($n = 193$) were included irrespective of their CTC status.

Materials and Methods

Sample collection and processing

Patients with MBC as cases and healthy individuals as controls were recruited for the study during 2010 and 2011. Cases and controls were sex (female) and ethnicity (Caucasians) matched. Peripheral blood was collected in EDTA tubes (Sarstedt S-Monovette) and processed within 2 hours. It was centrifuged at 1,300 × *g* for 20 minutes at 10°C, and the plasma was additionally centrifuged at 15,500 × *g* for 10 minutes at 10°C. Samples were snap-frozen and stored at −80°C. CTCs were enumerated in patient's blood by evaluating it in the CellSearch system (Veridex, LLC). On the basis of the CTC numbers, patients were classified as CTC-positive (≥5 intact CTCs/7.5 mL blood), CTC-negative (no intact, apoptotic, or enucleated CTCs), or CTC-low (1–4 intact CTC/7.5 mL blood or no intact CTC but >0 apoptotic or enucleated). For clear definition of phenotypes, CTC-low samples were not included for identification of miRNAs differentially present between CTC-positive and CTC-negative MBC, and their subsequent validation. They were only included for the survival analysis where they were considered as CTC-negative as per clinical definition. Patients had received one or more lines of therapy for their metastatic disease before enrollment into the study (Supplementary Table S1). The study was approved by the Ethical Committee of the University of Heidelberg (Heidelberg, Germany).

miRNAs were extracted from 400 μL of plasma after spiking-in 10 fmol of equimolar mixture of synthetic *C. elegans*-miR-39/238, as previously described (16). Yield of extracted miRNAs was assessed by measuring the levels of miR-16 and miR-24, as they are present in abundance in plasma, and cel-miR-39.

Profiling by TaqMan Human MicroRNA arrays

miRNAs were profiled by the TaqMan Human MicroRNA array Card Set v2.0 (Applied Biosystems) as per the manufacturer's instructions (including preamplification). It quantifies the expression of 667 mature human miRNAs (Sanger's miRBase v10). Three microliters of miRNA was used as input for Megaplex reverse transcription (RT) for all samples, and 2.5 μL of this RT product was taken for the preamplification step. Quantitative PCR (qPCR) was carried out with 9 μL of 1 in 4 diluted preamplification product in Applied Biosystems 7900HT, and cycle threshold (C_t, cycle in which there is the first detectable significant increase in fluorescence) values were retrieved with the SDS software

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v2.2 (automatic baseline and threshold). Eleven CTC-positive cases (here cut-off was increased to ≥20 intact CTCs/7.5 mL blood to select for extreme phenotype), 9 CTC-negative cases, and 10 controls were profiled in this manner. The data were analyzed in HTqPCR package (21) from Bioconductor (v1.2.0) in R 2.14.1 (22). miRNAs undetermined or with C_t less than 15 or more than 35 across all samples, or with interquartile range (IQR) less than 1.5 were removed from subsequent analysis. Data were quantile normalized, duplicates averaged, and limma analysis, with adjustment for multiple testing by controlling for false discovery rate (FDR; Benjamini–Hochberg method), was executed to identify differentially regulated miRNAs between CTC-positive and CTC-negative cases, CTC-positive cases and controls, and CTC-negative cases and controls. With limma, a one-factorial linear model is fitted for each miRNA, after which, the standard errors (SE) are moderated using an empirical Bayes model resulting in two-sided moderated *t* test statistics for each miRNA (23). To select miRNAs for further validation, the following criteria were applied: (i) $\log_2$ fold change $>+2$ or <-2 and FDR < 0.1 for any one of the three comparisons; (ii) mean C_t less than 32 and C_t less than 32 for at least 50% of samples in at least one group.

Validation of candidate miRNAs

The identified miRNAs were validated using TaqMan Human MicroRNA assays for mature human miRNAs (Applied Biosystems), as described previously (24). A constant volume of 1 μL of miRNA input was used for the RT reaction. The qPCR was done in Roche LightCycler 480 (Roche Applied Sciences) in triplicates. Crossing point (Cp), the point at which the maximal increase of fluorescence within the log-linear phase takes place as calculated by determining the second derivative maxima of the amplification curves, is given as output. Samples were randomized and blinded to the person carrying out the experiment. Run controls were included in each batch of samples to rule out

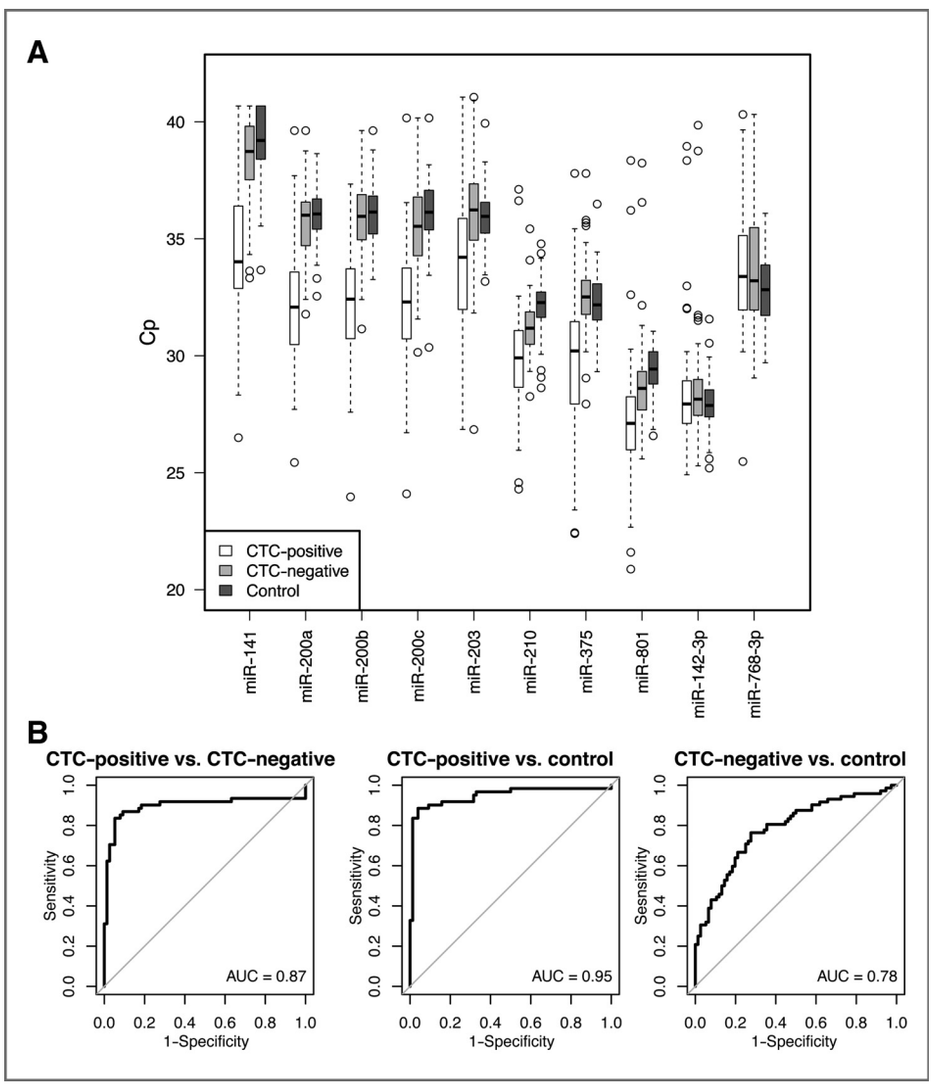

Figure 1. A, box and whisker plots of the 10 candidate miRNAs, represented as Cp values, across 61 CTC-positive, 72 CTC-negative MBC cases and 76 controls. B, multiparametric panel based on penalized LASSO logistic regression model. CTC-positive versus CTC-negative: miR-141, miR-200b (80% sensitivity, 83% specificity); CTC-positive versus control: miR-141, miR-200b, miR-200c, miR-210, miR-768-3p (90% sensitivity, 91% specificity); CTC-negative versus control: miR-200c, miR-210, miR-768-3p (80% sensitivity, 65% specificity). AUC, area under the curve.

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Table 1. Validation of candidate miRNAs

	CTC-positive vs. CTC-negative			CTC-positive vs. control			CTC-negative vs. control		
	FC	*P*	AUC	FC	*P*	AUC	FC	*P*	AUC
miR-141	26.17	**8.27E−13**	0.85	36.25	**1.69E−16**	0.90	1.39	**6.75E−03**	0.59
miR-200a	15.24	**6.85E−13**	0.85	15.78	**7.34E−15**	0.88	1.04	3.05E−01	0.47
miR-200b	11.63	**9.53E−15**	0.88	13.18	**9.65E−17**	0.91	1.13	8.52E−01	0.03
miR-200c	9.38	**5.73E−13**	0.86	14.22	**1.54E−17**	0.92	1.52	**1.22E−02**	0.59
miR-203	4.06	**6.37E−06**	0.71	3.36	**5.89E−06**	0.71	0.83	2.47E−01	0.49
miR-210	2.41	**2.77E−07**	0.74	5.17	**2.29E−14**	0.87	2.14	**2.57E−07**	0.73
miR-375	4.96	**5.98E−10**	0.80	3.89	**2.22E−09**	0.79	0.78	1.27E−01	0.52
miR-801	2.83	**2.54E−06**	0.72	4.99	**5.91E−13**	0.85	1.77	**2.87E−05**	0.67
miR-142-3p	1.16	4.44E−01	0.17	0.96	6.73E−01	0.45	0.83	1.72E−01	0.52
miR-768-3p	0.88	6.76E−01	0.35	0.68	**6.12E−03**	0.61	0.77	**2.96E−02**	0.58

NOTE: Results of Wilcoxon rank sum tests with median fold change (FC $= 2^{-\Delta Cp}$), corresponding two-sided *P* value, and leave-one-out cross-validated area under the curve (AUC) estimates for the 10 candidate miRNAs. Significant results are in bold.

interrun variation (Supplementary Table S2). Validation was conducted in an extended cohort of 209 individuals: 61 CTC-positive and 72 CTC-negative cases, and 76 controls. The miRNAs were also measured in additional 60 CTC-low samples, which were used for survival analysis. Cp value of each miRNA was normalized to cel-miR-39 (24). For statistical analysis, when a miRNA was undetected in a sample, its Cp value was set to the maximum Cp across all samples for that miRNA.

Statistical analyses of validation data

All statistical analyses were conducted in R 2.14.1 with the following R packages: coin v1.0-21, ROCR v1.0-4, penalized v0.9-39, survival v2.36-14, peperr v1.1-6 (22). Power simulations for two-group comparisons were done to assess if our sample sizes were sufficient to find a true 2-fold change with at least 90% statistical power. It was estimated on the basis of observed standard deviations (SD) in preliminary small-scale validation experiments. Wilcoxon rank sum tests were applied to assess the significance of differences between the groups. Leave-one-out cross-validated receiver operating characteristic (ROC) curves were built for logistic regression models based on individual miRNAs. Penalized LASSO logistic regression model (with penalty parameter tuning conducted by 10-fold cross-validation) was used to compute the least redundant and most informative panel of miRNAs that can discriminate two groups. Corresponding area under the curve (AUC) was calculated for each model. Specificity at a predefined sensitivity (80% or 90%) was determined for the multivariable models. Interrelationships between miRNAs were analyzed by partial correlations, that is Spearman correlation between two variables conditioned on the remaining variables. The approach of Smith and Thompson (ref. 25; to control for confounding effects) was used to deduce the interaction of miRNA expression with age. Age-adjusted *P* values were computed from Wald tests in logistic regression models including age as a covariable.

PFS and OS were calculated as time (in months) from blood take to progression of disease or last radiologic examination and death or last visit, respectively. Kaplan–Meier method was used to estimate the distribution of OS and PFS. Log-rank tests were used to compare survival curves between groups. Cox models were used to identify prognostic variables, build multivariable models, and to assess and compare the prognostic value of resulting models (26). A LASSO penalty term was used for automatic selection of relevant miRNA variables (with penalty parameter tuning done by 10-fold cross-validation). The prognostic value of models was assessed by 0.632+ bootstrap estimates of prediction error curves and summarized as the integrated prediction error curve (IPEC) up to 15 months (PFS) or 7.5 months (OS; ref. 27). miRNA and CTC data entered the survival models as dichotomized variables (lower quartile vs. the rest for miRNA, CTC-positive vs. CTC-negative for CTC).

Table 2. Correlation of miRNA and CTC counts

	ρ	*P*
miR-141	−0.66	<0.00001
miR-200a	−0.65	<0.00001
miR-200b	−0.70	<0.00001
miR-200c	−0.67	<0.00001
miR-210	−0.48	<0.00001
miR-375	−0.50	<0.00001
miR-203	−0.60	<0.00001
miR-801	−0.47	<0.00001
miR-142-3p	−0.12	0.16
miR-768-3p	−0.01	0.88
miR-16	−0.12	0.17

NOTE: Spearman rank correlation of miRNA amount (Cp value) and number of CTCs.

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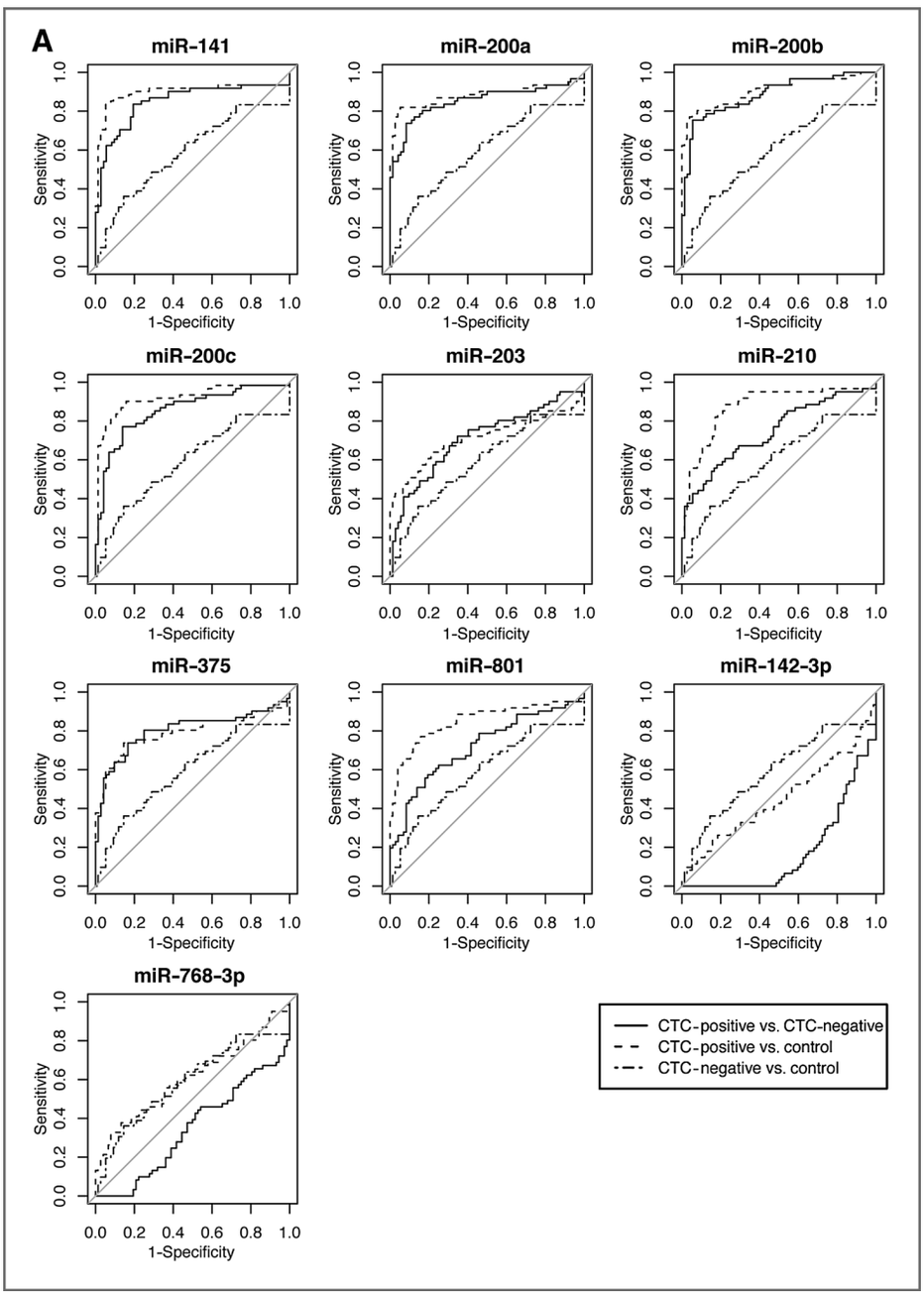

Figure 2. A, leave-one-out cross-validated ROC curves for logistic regression models based on individual miRNAs for all three comparisons. (*Continued on the following page*.)

Results

Circulating miRNA profiles of CTC-positive and CTC-negative MBC are significantly different

Thirty plasma samples consisting of 11 CTC-positive (CTC $\geq$ 20/7.5 mL blood), 9 CTC-negative cases, and 10 controls were profiled by low-density TaqMan arrays. After filtering and averaging of duplicates, 216 unique and variably expressed miRNAs remained, which were used for clustering and limma analysis. Surprisingly, we observed that the differences in profiles between CTC-positive and CTC-negative patients with MBC were larger than those between CTC-negative and healthy controls. Clustering of samples revealed that CTC-positive cases formed one cluster, whereas CTC-negative cases and controls formed two subclusters of another branch (Supplementary Fig. S1). Concomitantly, limma analysis returned more miRNAs significant for the comparison of CTC-positive (19 miRNAs) than for CTC-negative cases (4 miRNAs) with controls. Analysis of CTC-positive against CTC-negative cases engendered 12 and 3 miRNAs in the CTC-positive group

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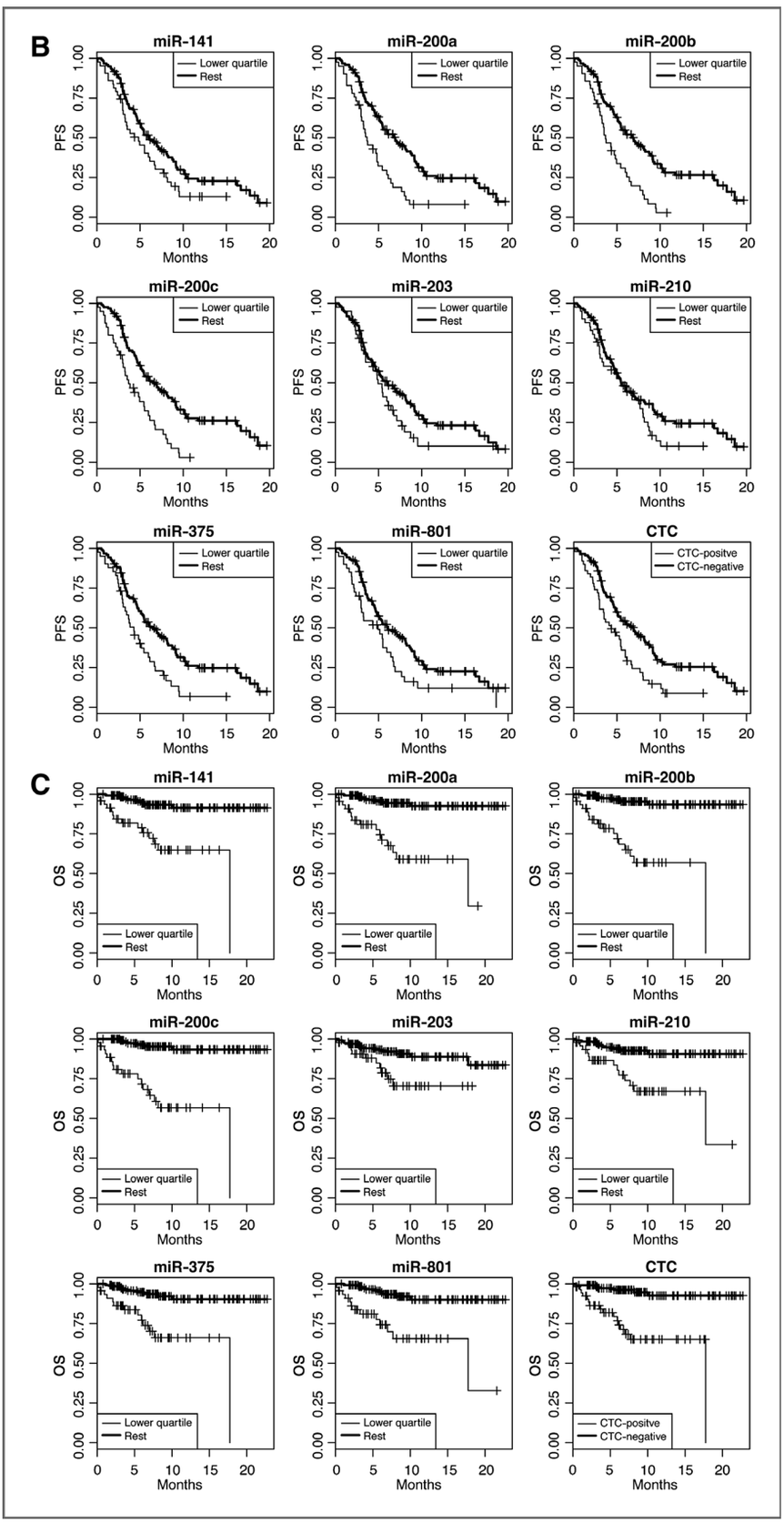

Figure 2. (*Continued*.) Kaplan–Meier curves of miRNA amounts stratified based on the Cp values as lower quartile (or 25th percentile) and rest, and of CTC stratified as CTC-positive and CTC-negative for progression-free survival (B) and overall survival (C).

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present in higher and lower amounts, respectively (data not shown). Stringent cut-offs were applied to ensure reduction in false positives and feasibility of testing. Consequently, 17 miRNAs were selected for the validation study: miR-99a, miR-133b, miR-139-3p, miR-141, miR-142-3p, miR-193b*, miR-200a, miR-200b, miR-200c, miR-203, miR-206, miR-210, miR-375, miR-571, miR-630, miR-768-3p, and miR-801 (Supplementary Table S3).

Eight circulating miRNAs showed significantly higher abundance in CTC-positive MBC compared with CTC-negative MBC or controls

After preliminary testing, 10 miRNAs, including 4 members of the miR-200 family (miR-141, miR-200a, miR-200b, miR-200c), along with miR-142-3p, miR-203, miR-210, miR-375, miR-768-3p, and miR-801, were analyzed in an expanded sample set of 133 MBC cases (Supplementary Table S1) and 76 controls. The remaining 5 candidates (miR-133b, miR-139-3p, miR-193b*, miR-206, miR-99a) could not be analyzed because of low expression, whereas miR-571 and miR-630 could not be detected by TaqMan miRNA assays (data not shown). Power simulations showed that in the tested scenarios with our sample sizes sufficient statistical power of 90% or higher were achievable, as long as SDs of miRNA expression were below 3.5.

Wilcoxon rank sum tests confirmed that miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-210, miR-375, and miR-801 were significantly increased in CTC-positive in comparison to CTC-negative cases (fold change [FC] of 2.41–26.17, $P < 0.00001$ for all miRNAs). On the basis of the trend of our array results, the differences in circulating miRNAs between these subgroups and controls were additionally explored. These 8 miRNAs were also found to have significantly increased levels in CTC-positive cases than controls (FC of 3.36 to 36.25, $P < 0.00001$ for all miRNAs). However, only 4 of these 8 miRNAs had significantly higher levels in CTC-negative cases than controls (miR-141, miR-200c, miR-210, miR-801; FC of 1.39–2.14, $P < 0.05$ for all miRNAs). Although miR-768-3p had only a negligible decrease when comparing CTC-positive and CTC-negative cases, it was found to be present in significantly lower quantities in CTC-positive ($P = 0.006$, FC = 0.68), and CTC-negative cases ($P = 0.003$, FC = 0.77) in comparison to controls. No significant changes in levels in miR-142-3p were found in any of the comparisons. These results are represented in Fig. 1A and Table 1. Analysis of the relationship between these 10 miRNAs discerned a high correlation among the members of the miR-200 family ($\rho > 0.3$, $P < 0.00001$), between miR-210 and miR-801 ($\rho = 0.53$, $P < 0.00001$), and between miR-142-3p and miR-768-3p ($\rho = 0.41$, $P < 0.00001$; Supplementary Fig. S2).

Circulating miRNAs differentiate CTC-positive from CTC-negative MBC

Leave-one-out cross-validated ROC analysis predicted the ability of the investigated miRNAs to differentiate CTC-positive from CTC-negative cases, and CTC-positive cases from controls with high AUCs (Fig. 2A; Table 1). For CTC-positive versus CTC-negative cases, although a multivariable model comprising miR-141 and miR-200b was predicted (0.87), the AUC of miR-200b alone (0.88) was found to be marginally greater than that of the model. Combination of miR-141, miR-200b, and miR-375 performed with equal accuracy (AUC 0.88, data not shown). With an equal sensitivity and specificity as the models (80% and 83% respectively), we reckon miR-200b alone might be sufficient for distinguishing CTC-positive from CTC-negative cases. For CTC-positive cases versus controls, the predicted multivariable model with miR-141, miR-200b, miR-200c, miR-210, and miR-768-3p had a very high AUC of 0.95 (90% sensitivity and 91% specificity). Even though, individually the miRNAs could not differentiate CTC-negative cases from controls with high certainty, the model predicted combination of miR-200c, miR-210, and miR-768-3p, had an appreciable AUC of 0.78 (80% sensitivity and 65% specificity; Fig. 1B).

Circulating miRNAs correlate with CTC counts

The 8 miRNAs that were significantly elevated in the CTC-positive and CTC-negative comparison also evinced a strong correlation to CTC counts. Spearman correlation analysis showed lower Cp values, and thus higher miRNA amounts, correlated with higher number of CTCs ($P < 0.00001$). In contrast, miR-142-3p and miR-768-3p had very poor correlation to CTC numbers ($\rho = -0.12$ and -0.01, respectively). miR-16, which is considered as an endogenous control for breast cancer tissue (28), also had poor and nonsignificant correlation to CTC ($\rho = -0.12$, $P = 0.17$; Table 2). This repudiates a nonspecific increase of miRNAs in CTC-positive samples, and underscores the specificity of the miRNAs present in circulation.

Prognostic markers of PFS and OS

Strength of the 8 miRNAs, increased in CTC-positive MBC to predict PFS and OS, was interrogated across 176 MBC cases with survival data (data on progression status available for only 164 of these 176 patients). Log-rank tests showed miR-141, miR-200a, miR-200b, miR-200c, miR-375, and miR-801 to be significantly correlated to PFS, with higher levels of these miRNAs associated with lower probability of PFS ($P < 0.05$). All the 8 miRNAs elevated in CTC-positive cases were found to be promising markers of OS ($P < 0.008$; Table 3 and Fig. 2B and C). Lasso Cox model predicted combinations of miR-200a, miR-200b, and miR-200c for PFS (IPEC = 2.041 compared with $IPEC_0 = 2.097$ for the null model without covariate information), and miR-200a, miR-200b, miR-200c, and miR-801 for OS (IPEC = 0.328 compared with $IPEC_0 = 0.369$ for the null model) to be the best-fitting multivariable models. When CTCs were introduced into these multivariable miRNA models, the IPEC was essentially unchanged (PFS: 2.043, OS: 0.330; Supplementary Fig. S3A and S3B). For PFS, miR-200b outperformed the multivariable models (IPEC = 2.011), while performing equally well for OS (IPEC = 0.331). In comparison to CTC (IPEC of 2.074 for PFS and 0.338 for OS), the multivariable models and miR-200b performed

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Table 3. Association between miRNA and prognosis

	PFS	OS
miR-141	**4.58E−02**	**6.77E−06**
miR-200a	**6.69E−05**	**1.24E−07**
miR-200b	**1.74E−05**	**3.72E−09**
miR-200c	**3.06E−05**	**1.06E−09**
miR-203	9.20E−02	**7.28E−03**
miR-210	1.07E−01	**2.30E−04**
miR-375	**1.45E−03**	**3.96E−05**
miR-801	**1.51E−02**	**2.45E−05**
CTC	**1.70E−03**	**4.49E−07**

NOTE: Log-rank model test for assessing significance of miRNA in plasma or CTC counts and progression-free survival and overall survival. Significant results are in bold.

perceptibly better with respect to PFS and slightly better for OS. Combining miR-200b and CTCs did not improve the prediction accuracy drastically; nevertheless, it gave the lowest IPEC of 2.009 for PFS (Fig. 3A and B). However, an additional evaluation of the model fit of Cox regression model with miR-200b and CTC compared with that of CTC alone showed that inclusion of miR-200b into the CTC model clearly improves model fit to the data (likelihood-ratio test, $P = 0.002$ for PFS and $P < 0.001$ for OS).

Discussion

Because 40% of patients with primary breast cancer are estimated to succumb to metastatic relapse and death, MBC is a major health issue worldwide (29). In 2004, the U.S. Food and Drug Administration (FDA) approved the use of CTC numbers estimated by the CellSearch system as an index of PFS and OS. However, many studies have highlighted its disadvantages, such as identification of only EpCAM positive circulating epithelial cells and interreader variability (30). Reported in only 60% of patients with MBC, there is also an apparent discrepancy in its detection between breast cancer subtypes, and hence its prognostic value (10). We hypothesize circulating miRNAs present in plasma can predict the presence of CTCs, and could thus be developed into a prognostic marker in MBC.

The strengths of this study are (i) the carefully standardized and uniform processing of blood samples within a limited window of 2 hours from blood collection, (ii) application of a two-step centrifugation protocol before snap freezing, (iii) processing of validation samples in a blinded manner, and (iv) the large sample size. Standardized sample processing is very important when investigating circulating miRNAs (31). On the basis of previous reports and our own observations, a second high-speed centrifugation step of plasma before snap freezing is critical for avoiding miRNA contamination from cells or cell debris (31, 32). Hence, precautions were taken to ensure that the circulating miRNAs originated exclusively from the cell-free portion of the blood.

Eight miRNAs, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-210, miR-375, and miR-801, had significantly higher expression in CTC-positive compared with CTC-negative MBC cases. miR-200b emerged as the best parameter for differentiating CTC-positive from CTC-negative MBC, with an AUC of 0.88 (80% sensitivity, 83% specificity). It was also the most accurate miRNA individually for predicting PFS and OS, and its prediction accuracy increased by a small margin when used in combination with CTC. These results could be indicative of a very specific role for miR-200b in determining the CTC status and prognosis in MBC. Even with this relatively short follow-up time and few events in our patient population, we were able to show the correlation of these miRNAs, especially miR-200b, to PFS and OS. We found the prognostic value of miRNA to be at least equal to or even better than that of CTC alone. Since both the multivariable miRNA model as well as miR-200b alone had lower prediction errors than CTC, they have the potential for prognostication and prediction in MBC either alone or in combination with CTC.

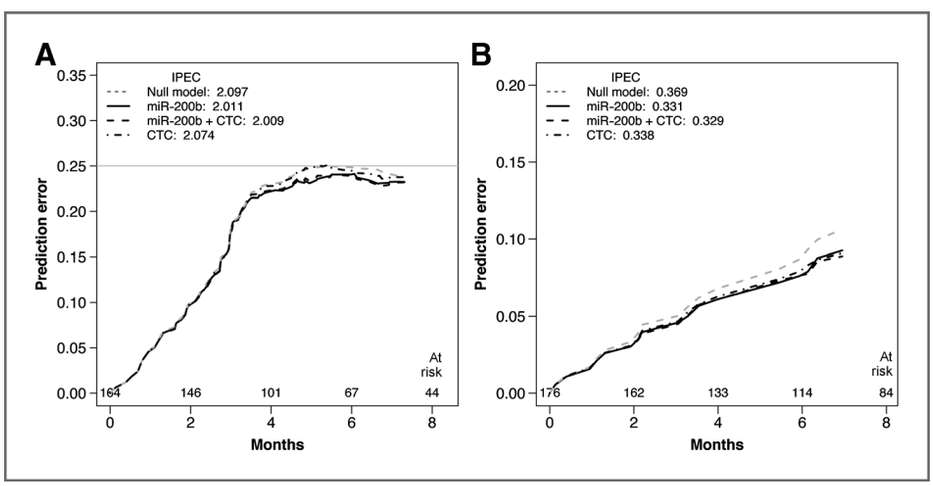

Figure 3. Prediction error curves up to 15 months (PFS) or 7.5 months (OS) for the null model (Kaplan–Meier model without any covariate information), CTC, miR-200b, miR-200b + CTC for PFS (A) and OS (B).

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We also propose panels of miRNAs capable of discriminating cases from controls with AUCs of 0.95 (90% sensitivity, 91% specificity) for CTC-positive, and 0.78 (80% sensitivity, 65% specificity) for CTC-negative. Analysis of miRNA levels and its dependence on age predicted that miR-141, miR-200a, miR-200b, miR-210, and miR-375 interacted with age in the case–control comparison (Supplementary Table S4). This could marginally compromise their power to differentiate cases and controls, as there is a significant difference in age distribution between them ($P < 0.00001$). Due to this caveat, our results of MBC cases and control comparisons have to be treated with caution. Since there was a borderline significance in age distribution between CTC-positive and CTC-negative ($P = 0.041$), we additionally analyzed the miRNA expression differences adjusting for age. No significant effect on the results was observed (Supplementary Table S5).

The miR-200 family and miR-203 possess regulatory functions in the EMT pathway and tumor suppressive features (33–35). The miR-200 family are inhibitors of EMT via the ZEB1/2-E-cadherin axis (33, 36). However, *in vivo* studies suggest that overexpression of miR-200 family increases the metastatic potential in breast cancer by inducing mesenchymal-to-epithelial transition (MET), which is required for successful colonization and establishment of metastasis (37). These miRNAs, which are upregulated in MET are found in higher levels in cases and are negatively correlated with prognosis in our study. This might point to a critical role for MET in the unfavorable outcome of metastasis. Furthermore, miR-200a/b and miR-141 were shown to be upregulated in serum of pancreatic cancer (38), and metastatic prostate cancer (15, 19) patients, respectively. Thus, our results are in agreement with the findings of these studies probing circulating miR-200 family. More importantly, we revealed for the first time that expression of the miR-200 family is an excellent indicator of the CTC status and prognosis. miR-203 is another EMT repressor by targeting 3′UTR of *ZEB2* and *SMAD4* (34, 35). Despite its tumor suppressive functions, an increased expression of miR-203 in tumor tissues has been discerned in ovarian (39), bladder (40), colon (41), and breast cancer (42). This and our results hint at paradoxical roles for miR-203, especially in breast cancer, and needs further clarification.

miR-210, whose expression is regulated by HIF1α, is proclaimed to be intimately involved in orchestrating cell response to hypoxia (43). In line with our findings, elevated miR-210 levels were associated with decreased overall survival (43), increased aggressiveness and metastatic capacity (44) in breast cancer. In contrast, miR-375 exhibits tumor and metastatic suppressive properties in other cancer models (45). However, higher miR-375 expression has been indicted in progression of invasive lobular breast carcinoma (46). Recently, it has been linked to EMT, where reexpression of miR-375 was found to partly reverse EMT-like properties in MCF-7 cells (47). The upregulation of miR-375 noted by us in MBC coincides with prior reports in metastatic prostate cancer (19), and points to a prometastatic role for it in these cancers. miR-801 and miR-768-3p were described as overexpressed in plasma of patients with hepatocellular carcinoma (48) and downregulated in gastric and thyroid tumors (49, 50), respectively. Functional elucidation of these miRNAs would shed light on the specific roles of these miRNAs in the metastatic processes in breast cancer and maybe in other epithelial tumors too.

Our study is the first to explore the differences in circulating miRNA profiles of plasma between CTC-positive and CTC-negative individuals with MBC. Through an initial array-based screening round, followed by a validation step on a large cohort, circulating miRNAs that can indicate the CTC status of patients with MBC were identified. However, the ability of these miRNAs to detect CTCs that have undergone EMT has to be further tested. Nevertheless, we were able to verify that these miRNAs are promising biomarkers of PFS and OS, both independent of and in combination with CTC. These results will have to be further verified in large study cohorts with longer follow-up. Furthermore, these findings might have important implications for other epithelial cancers where the CTC status is used as a prognostic marker. Finally, based on the differences between cases and healthy controls, the detected miRNAs hold promise as an early detection marker of metastasis in breast cancer.

Disclosure of Potential Conflicts of Interest

D. Madhavan, M. Zucknick, K. Cuk, and B. Burwinkel have ownership interest in patent application for the signature of miRNAs described in this work. K. Pantel has honoraria from speakers bureau from VERIDEX. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: D. Madhavan, M. Wallwiener, S. Schott, J. Heil, A. Turchinovich, A. Trumpp, A. Schneeweiss, B. Burwinkel

Development of methodology: D. Madhavan, A. Turchinovich, K. Pantel, B. Burwinkel

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Madhavan, M. Wallwiener, K. Cuk, M. Scharpff, S. Schott, J. Heil, S. Riethdorf, C. Sohn, K. Pantel, A. Schneeweiss, B. Burwinkel

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Madhavan, M. Zucknick, S. Schott, A. Benner, A. Trumpp, K. Pantel, A. Schneeweiss, B. Burwinkel

Writing, review, and/or revision of the manuscript: D. Madhavan, M. Zucknick, K. Cuk, S. Schott, J. Heil, A. Turchinovich, R. Yang, A. Benner, S. Riethdorf, K. Pantel, A. Schneeweiss, B. Burwinkel

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Modugno, M. Scharpff, A. Trumpp, B. Burwinkel

Study supervision: S. Schott, B. Burwinkel

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