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Prognostic Significance of Metastasis-Related MicroRNAs in Early Breast Cancer Patients with a Long Follow-up

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BACKGROUND: Stability of microRNAs (miRNAs) in formalin-fixed paraffin-embedded (FFPE) tissues enables their reliable analysis in archived FFPE tissue samples, which are an invaluable source for the evaluation of novel biomarkers. Especially in breast cancer, for which late relapses occur in many cases, analysis of miRNAs in FFPE tissues holds great potential, because it can lead to the discovery of novel biomarkers suitable for future routine clinical diagnostics for breast cancer. We investigated the prognostic significance of 6 metastasis-related miRNAs that can critically regulate various stages of migration and invasion and play critical roles in the multistep metastatic process.

METHODS: We quantified the expression of 6 mature miRNAs (namely miR-21, miR-205, miR-10b, miR-210, miR-335, and let-7a) by reverse-transcription quantitative PCR in FFPE tissues of 84 patients with early breast cancer and a long follow-up and 13 cancer-free breast tissue FFPE samples that were used as the control group. We further correlated individual miRNA over- or underexpression with the disease-free interval (DFI) and overall survival (OS).

RESULTS: Univariate analysis revealed that both miR-21 and miR-205 were significantly associated with DFI and only miR-205 with OS. Multivariate analysis demonstrated that miR-205 and miR-21 were independent factors associated with early disease relapse, whereas only miR-205 overexpression was associated with OS.

CONCLUSIONS: Our results clearly indicate that deregulation of metastasis-associated miRNAs in primary tumors is associated with clinical outcome in patients with early breast cancer and can differentiate patients with higher risk in well-characterized subgroups.

The majority of deaths associated with breast cancer are due to the development of metastases. An important focus of current breast cancer research is to increase our understanding of the biology of the metastatic process and to identify panels of biomarkers that may help in early diagnosis and the determination of prognosis and/or the prediction of treatment response, ultimately contributing to more favorable patient outcomes *(1)*.

A variety of clinical and pathological factors are routinely used to classify breast cancer patients to assess their prognosis and to decide on the appropriate therapy. These include patient age, lymph node status, tumor size and histological grade, hormone receptor status [estrogen receptor (ER),[5] progesterone receptor (PR)], and human epidermal growth factor-2 receptor (HER2) amplification/overexpression. Although all of these factors have important clinical value, the prediction of prognosis and metastatic potential of carcinoma at the time of diagnosis is still not possible. Studies on microRNAs (miRNAs) might potentially provide the information needed to overcome this limitation.

The discovery of miRNAs has opened new avenues for breast cancer metastasis research. The deregulation of miRNA in breast cancer was first reported in 2005 by Iorio and colleagues *(2)*. This group first identified a global pattern of miRNA deregulation in breast cancer tissue compared with normal breast tissue, hinting at the importance of miRNA deregulation in the development of breast cancer. Since then, there have been many studies on the expression of various miRNAs and their roles in breast cancer. Increasing data support the value of miRNA expression profiling *(3)* in distinguishing one cancer subtype from another *(4)*, such as luminal vs basal breast cancer *(5)*. Moreover, many recent studies on breast cancer have clearly demonstrated that miRNAs can play an important role in the multistep process of metastasis, functioning as both activators and suppressors of metastasis by crit-

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[5] Nonstandard abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor-2 receptor; miRNA, microRNA; FFPE, formalin fixed paraffin embedded; TNBC, triple negative breast cancer; OS, overall survival; DFI, disease-free interval; Cq, quantification cycle; RQ, relative quantification; IHC, immunohistochemistry; RT-qPCR: reverse-transcription quantitative PCR.

ically regulating various stages of migration and invasion *(6–14)*.

Owing to their small size (22–24 nt), miRNAs are very stable in formalin-fixed paraffin-embedded (FFPE) tissues, in contrast to mRNA *(15, 16)*. This enables reliable miRNA analysis in archived FFPE tissue samples, which are an invaluable source for the evaluation of novel biomarkers. Especially for breast cancer, in which late relapses occur in many cases, analysis of miRNAs in FFPE tissues holds great potential, because it can lead to the discovery of novel biomarkers, suitable for future routine clinical diagnostics for breast cancer.

In this study, we investigated the expression of 6 mature miRNAs, namely miR-21, miR-205, miR-10b, miR-210, miR-335, and let-7a, that can critically regulate various stages of migration and invasion and play critical roles in the multistep metastatic process. In particular, we studied the expression of: (*a*) miR-21, which affects tumor invasion and inhibits tumor cell colonization *(17–20)*; (*b*) miR-205, which is involved in angiogenesis and is downregulated in epithelial–mesenchymal transitions *(21)*; (*c*) miR-10b, which positively regulates cell migration and invasion *(13)*; (*d*) miR-335, which modifies tumor microenvironment *(12)*; (*e*) let-7a, which inhibits the proliferation and self-renewal of breast cancer stem cells *(22)*; and (*f*) miR-210, which has been shown to be inversely associated with cancer aggressiveness and metastatic potential *(23)*. We explored the relationships of the expression profiles for these miRNAs with clinical outcomes in FFPE samples from breast cancer patients, focusing in particular on ER and PR expression, HER2, and lymph node status.

Materials and Methods

CLINICAL SAMPLES

We retrieved a total of 112 breast tumor FFPE blocks from the early breast cancer tissue biobank archives of the Department of Pathology at the University Hospital of Crete, anonymizing the data according to the guidelines of the local ethics committee. These patients have been followed for up to 12.4 years. Patient characteristics are outlined in Table 1 in the Data Supplement that accompanies the online version of this report at http://www.clinchem.org/content/vol60/issue1. The median age of the patients was 60 years, with 62 patients (55.4%) ≤60 years old. Twenty-three patients (20.5%) had tumor size smaller than 2 cm, and 27 (24.1%) had no lymph node metastasis. When classified into breast cancer subtypes, 31 patients (27.7%) had luminal A, 27 (24.1%) had luminal B, 13 (11.6%) had $HER2^+$, and 29 (25.9%) had triple negative breast cancers (TNBCs). The median (range) overall survival (OS) and disease-free interval (DFI) were 84 months (10–149 months) and 68 months (5–149 months), respectively. The tumor cell content in FFPEs was above 80% in all cases, as verified on a hematoxylin–eosin-stained serial section. All FFPE samples were stored at room temperature until use. Thirteen cancer-free breast tissue FFPE samples (obtained from mammoplasties) were used as the control group.

ISOLATION OF TOTAL RNA FROM FFPE TISSUES

For FFPE samples, the blocks were cut into10-mm–thick slices, and 1 tissue slice each was placed into a 1.5-mL nuclease-free microcentrifuge tube. One milliliter of xylene was added for deparaffinization with vortex mixing for 5 min at room temperature. Samples were left at 60 °C for 3 min and then centrifuged at 18 220 × *g* for 7 min at room temperature. Supernatants were removed, and 1 mL of 100% ethanol was added with vortex mixing for 7 min. This ethanol-washing step was repeated twice and then samples were air-dried. We used the TRIzol LS reagent (Invitrogen) for total RNA isolation. One milliliter of TRIzol LS reagent (Invitrogen) was added, and the mixture was vortex mixed for 5 min at room temperature. Isolated RNA was dissolved in RNA storage buffer (Ambion, Invitrogen) and stored at −70 °C before use. Total RNA concentrations were defined spectrophotometrically in the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

QUANTIFICATION OF miRNA EXPRESSION BY REVERSE TRANSCRIPTION QUANTITATIVE PCR

miRNA expression levels were quantified by using TaqMan microRNA assays (Applied Biosystems, Life Sciences), according to the manufacturer's protocols. The expression levels of miR-10b, miR-21, miR-205, miR-210, miR-335, and let-7a were quantified. Real-time PCR was performed in a final volume of 10 μL, containing 2 μL of the 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 (Applied Biosystems, Life Sciences). All reactions were run in triplicate on the LightCycler 480 system (Roche Diagnostics). 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. The quantification cycles (Cq) were calculated using the LightCycler software (Roche Applied Science). Expression values were normalized to miR-191, which has been shown to be a suitable reference miRNA for solid cancers *(24, 25)*. ΔCq values were calculated by using Cq values for each mature miRNA and the corresponding miR-191 for each sample. We calculated ΔΔCq values using ΔCq values for cancerous tissues and the mean value of ΔCq for normal mammoplasty tissues for each miRNA ($\Delta\Delta Cq = \Delta Cq_{cancer} - \Delta Cq_{normal}$). Relative quantification (RQ) was based on

the ΔΔCq method as described by Schmittgen and Livak *(26)*. Expression data for the miRNAs are presented as fold differences relative to *miR-191* based on the estimation of RQ factor using the following equation: $RQ = 2^{-\Delta\Delta Cq}$ *(26)*.

IMMUNOHISTOCHEMISTRY

HER2 expression in the primary tumors was detected by immunohistochemistry (IHC) with the monoclonal antibody CB11 (Novacastra), using the OPTIMAX automated system (BioGenex Laboratories). Scoring was based on the criteria recommended by Dako A/S for the HercepTest (Dako Corporation). Fluorescence in situ hybridization was performed for tumors with a HER2 score of 2+ by IHC.

ER and PR expression of the primary tumors was detected by IHC with monoclonal antibodies to ER and PR (DakoCytomation), using the same automated system as described above. All carcinoma cells in 3 hot spots (areas with the highest density of ER-positive or PR-positive carcinoma cell nuclei) per slide were evaluated independently by 2 pathologists, and the mean of the 2 independent counts was considered the final value for each field and hot spot. The final immunoreactivity index (score) was calculated as the mean percentage of ER-positive or PR-positive carcinoma cell nuclei in the 3 hot spots. Staining intensity was graded as negative (0), weak (1+), intermediate (2+), or strong (3+), and reported separately. The triple-negative or basal-like tumors were defined as ER negative/PR negative/HER2 negative (0, 1+ by IHC), the HER2 positive as HER2 (3+ by IHC), and the luminal as ER positive/HER2 negative (0, 1+ by IHC). Luminal A samples were defined as ER+/grade I/II, and luminal B were defined as ER+/grade III tumors.

STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS statistical package (version 21, SPSS). Reverse-transcription quantitative PCR (RT-qPCR) data were analyzed by Wilcoxon signed-rank tests to statistically evaluate differences in miRNA expression between breast cancer and normal breast tissues. Nonparametric tests were used to analyze the relationship between mature miRNA expression levels and various clinicopathological characteristics for each patient (the Mann–Whitney and χ^2 test between 2 groups and the Kruskall Wallis test for 3 or more groups). For the survival analysis we divided breast cancer patients into 2 different groups, high expression and low expression, using the median RQ factors for each miRNA studied at the corresponding cutoffs. Survival time was calculated from the date of end point event or last follow-up (end point is the date of death). The association between survival and miRNA expression was estimated by using the Kaplan–Meier method and 2-sided log-rank test. Clinicopathologic factors known to be associated with prognosis, including tumor size (T2–3 vs T1), nodal infiltration (≥4 vs 0–3), histological grade (III vs I/II), ER status (negative vs positive), PR status (negative vs positive), HER2 status (negative vs positive), and the overexpression of each miRNA separately (yes vs no) were tested with univariate analysis. Variables that were found to be significant in the univariate analysis were then entered in a stepwise multivariate Cox proportional hazard regression model to identify those with independent prognostic information *(27)*.

Results

DIFFERENTIAL EXPRESSION OF METASTASIS-RELATED miRNAs IN PRIMARY TUMORS OF EARLY BREAST CANCER PATIENTS WITH RESPECT TO CANCER-FREE BREAST TISSUES

We selected to study the expression of miR-21, miR-205, miR-10b, miR-210, miR-335 and let-7a, because these miRNAs can critically regulate various stages of migration and invasion and play critical roles in the multistep metastatic process (Fig. 1).

We first evaluated the differences in expression of these 6 metastasis-related miRNAs between breast cancer FFPE tissues and normal breast FFPE tissues. As can be seen in Fig. 2, the expression of all miRNAs except 1 (miR-210) differed significantly in primary tumors with respect to cancer-free breast tissues. More specifically, miR-21 ($P = 0.049$), miR-10b ($P = 0.008$), and miR-335 ($P = 0.008$) were overexpressed and miR-205 ($P = 0.044$) and let-7a ($P = 0.026$) were downregulated, whereas miR-210 expression ($P = 0.708$) was not differentiated.

PROGNOSTIC SIGNIFICANCE OF DIFFERENTIALLY EXPRESSED miRNAS

We further evaluated the correlation between the expression levels of the 6 miRNAs studied and prognosis. Kaplan–Meier survival analysis and log-rank tests were performed by using patients' postoperative survival. Kaplan–Meier survival curves demonstrated that patients with high miR-21 expression (n = 56) had significantly shorter DFI than those with low *miR-21* expression (n = 50) ($P = 0.043$, log-rank test) (Fig. 3). Moreover, patients who had low expression of miR-205 (n = 57) had both shorter DFI and OS times than those who had high expression levels (n = 47) ($P = 0.040$ and $P = 0.047$, respectively; log-rank test) (Fig. 3). However, the expression levels of the other 4 miRNAs did not correlate with the DFI and OS times. Moreover, as can been seen in online Supplemental Table 2, miR-21 overexpression and miR-205 downregulation were not found to be correlated with age, histology grade, tumor size, lymph node status, ER sta-

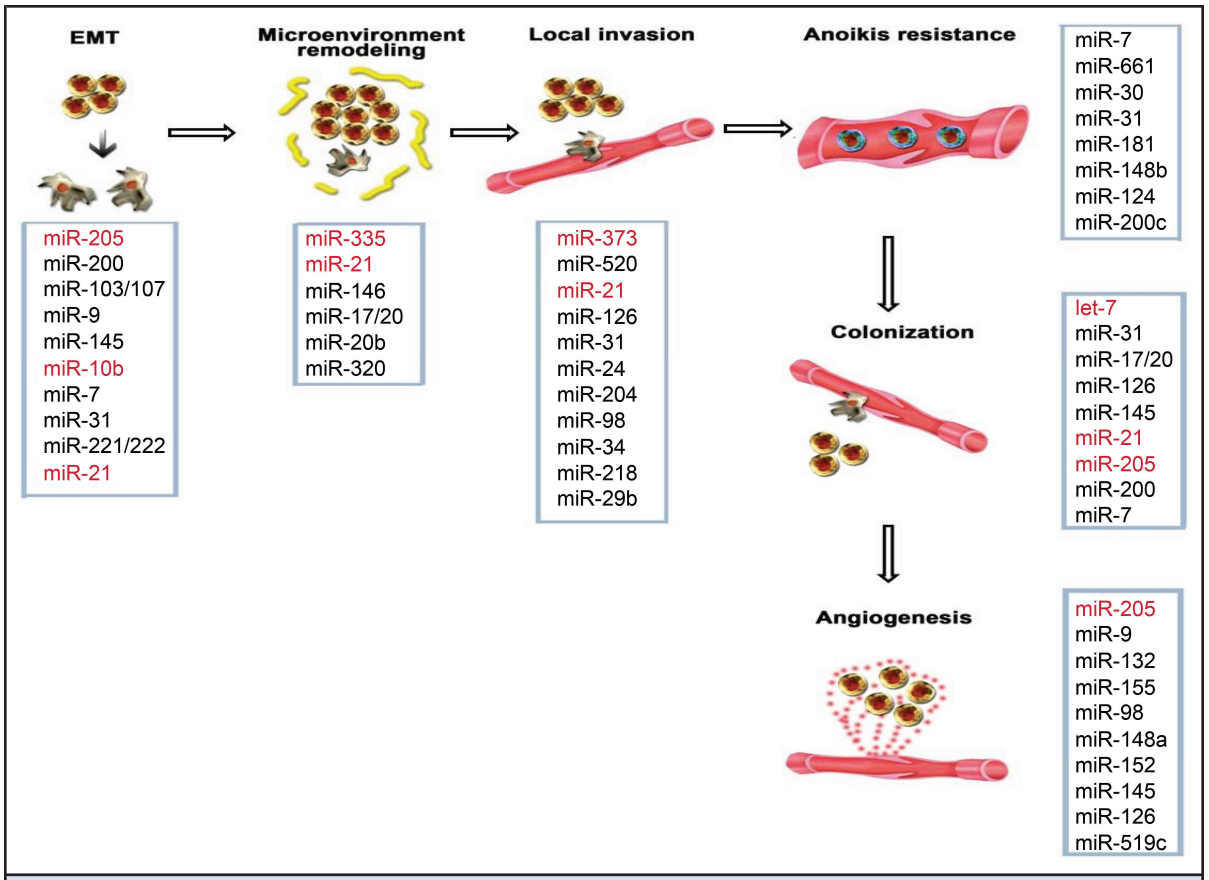

Fig. 1. Metastasis-related miRNAs: miRNAs marked in red are those investigated in this study.

tus, PR status, or HER2 status in the population studied ($P > 0.05$).

miR EXPRESSION ACCORDING TO THE MOLECULAR TUMOR PROFILE

TNBC. In this group (n = 29), relapses were more frequent in patients whose tumors presented miR-21 overexpression (62% vs 47%, $P = 0.035$); consequently, the DFI was significantly shorter compared to the DFI in patients whose tumors did not present miR-21 overexpression ($P = 0.007$) (see online Supplemental Fig. 1A). In the rest of the patients (non–TNBC) (n = 71), DFI was significantly shorter in patients with miR-205 underexpression ($P = 0.026$) (see online Supplemental Fig. 1A). Moreover, in the same group the OS values were significantly different in patients with miR-205 underexpression ($P = 0.036$) (see online Supplemental Fig. 1B).

HER2. In the HER2-negative subgroup (n = 99), patients with miR-21 overexpression had significantly shorter DFI ($P = 0.036$) than patients with miR-21 underexpression, as clearly demonstrated in online Supplemental Fig. 1A. In the HER2–positive subgroup (n = 13), patients with miR-10b overexpression had significantly shorter OS ($P = 0.027$); nevertheless, this finding is limited by the small number of patients.

ER and/or PR positive/HER2 negative. We further defined groups like ER positive/negative and PR positive/negative to analyze the relevance of miRNAs with regard to prognosis in this cohort of patients. For this reason, we analyzed data from all patients that were ER and/or PR positive and HER2 negative with respect to patients with ER negative and/or PR negative.

We found that patients with ER-negative tumors tended to relapse more frequently than patients with ER-positive tumors, but this difference was not statistically significant (58.7% vs 42.8%; $P = 0.082$). Patients with PR-negative tumors relapsed significantly more frequently than patients with PR-positive and HER2-negative tumors (65.9% *v* 35.7%; $P = 0.002$).

In the ER-negative subgroup (n = 46), disease relapse was more common in patients with miR-10b overexpression ($P = 0.019$)(see online Supplemental Fig. 1A), whereas in the PR-negative subgroup (n =

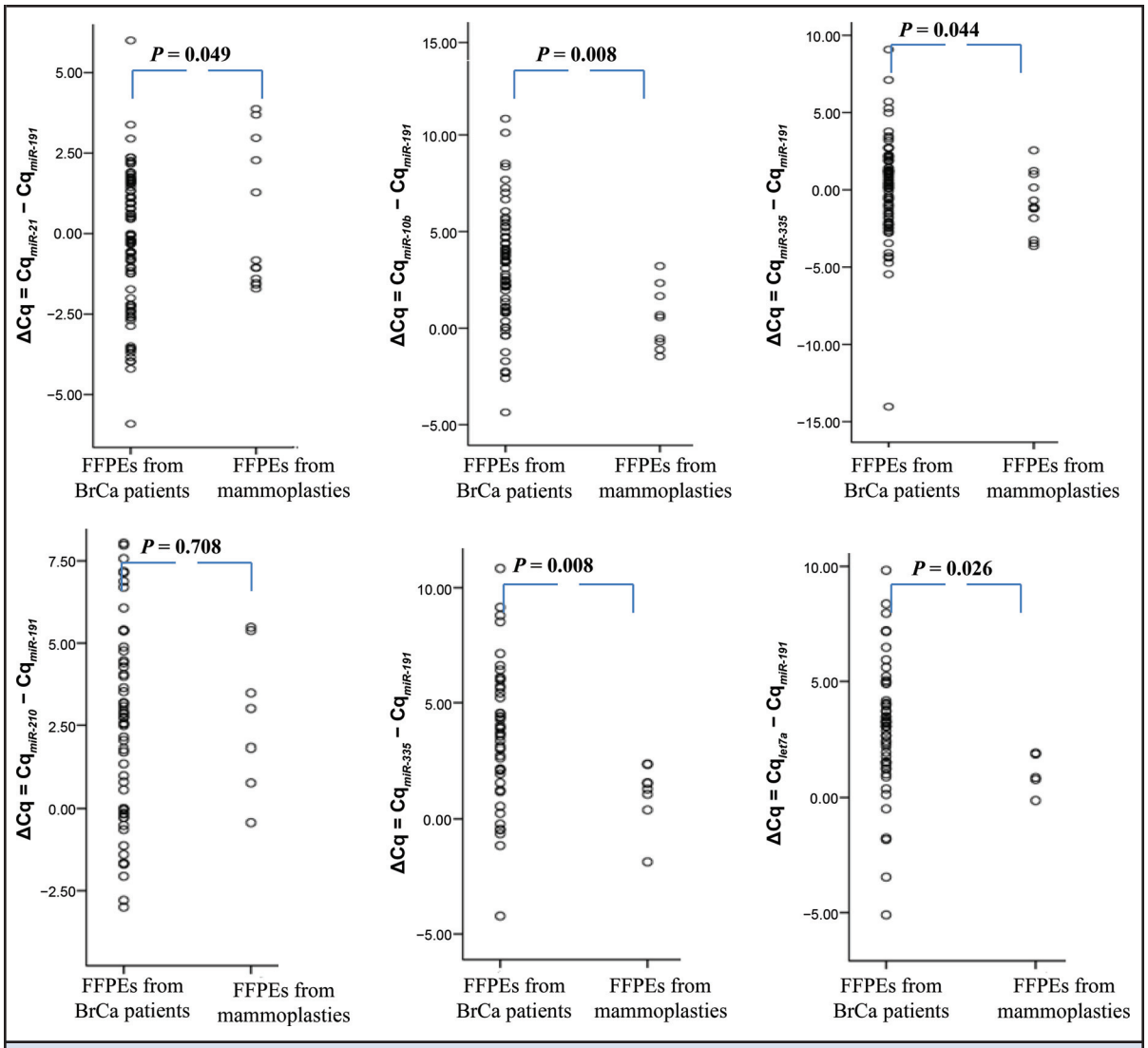

Fig. 2. Quantification of metastasis-related miRNAs in FFPEs of patients with early breast cancer (n = 84) and noncancerous breast tissues (n = 13). ΔCq values for each miRNA studied are shown referenced to the expression of the endogenous control, miR-191.

47), disease relapse was more common in patients with miR-21 ($P < 0.001$) or miR-10b ($P = 0.016$) overexpression (see online Supplemental Fig. 1A). Moreover, in the PR-negative subgroup, patients who overexpressed miR-21 had shorter OS ($P = 0.021$) (see online Supplemental Fig. 1B).

In the PR-positive and HER2-negative subgroups (n = 56), disease relapses and deaths were significantly different in patients with miR-205 underexpression ($P = 0.006$ and $P = 0.007$) (see online Supplemental Fig. 1, A and B, respectively). Moreover, in the ER-positive and HER2-negative subgroup, patients whose tumors presented miR-210 underexpression had significantly shorter OS ($P = 0.041$) (see online Supplemental Fig. 1B). However, in the ER-positive and HER2-negative subgroup (n = 56), relapses and deaths were not found to differ significantly for patients with miR-10b overexpression and those without ($P = 0.361$ and $P = 0.212$, respectively).

In the ER- and/or PR-positive /HER2-negative subgroup (n = 58), OS was significantly different in patients with miR-205 underexpression ($P = 0.030$) (see online Supplemental Fig. 1B).

LYMPH NODES

In the lymph node–negative subgroup (n = 27), disease relapses were significantly different in patients

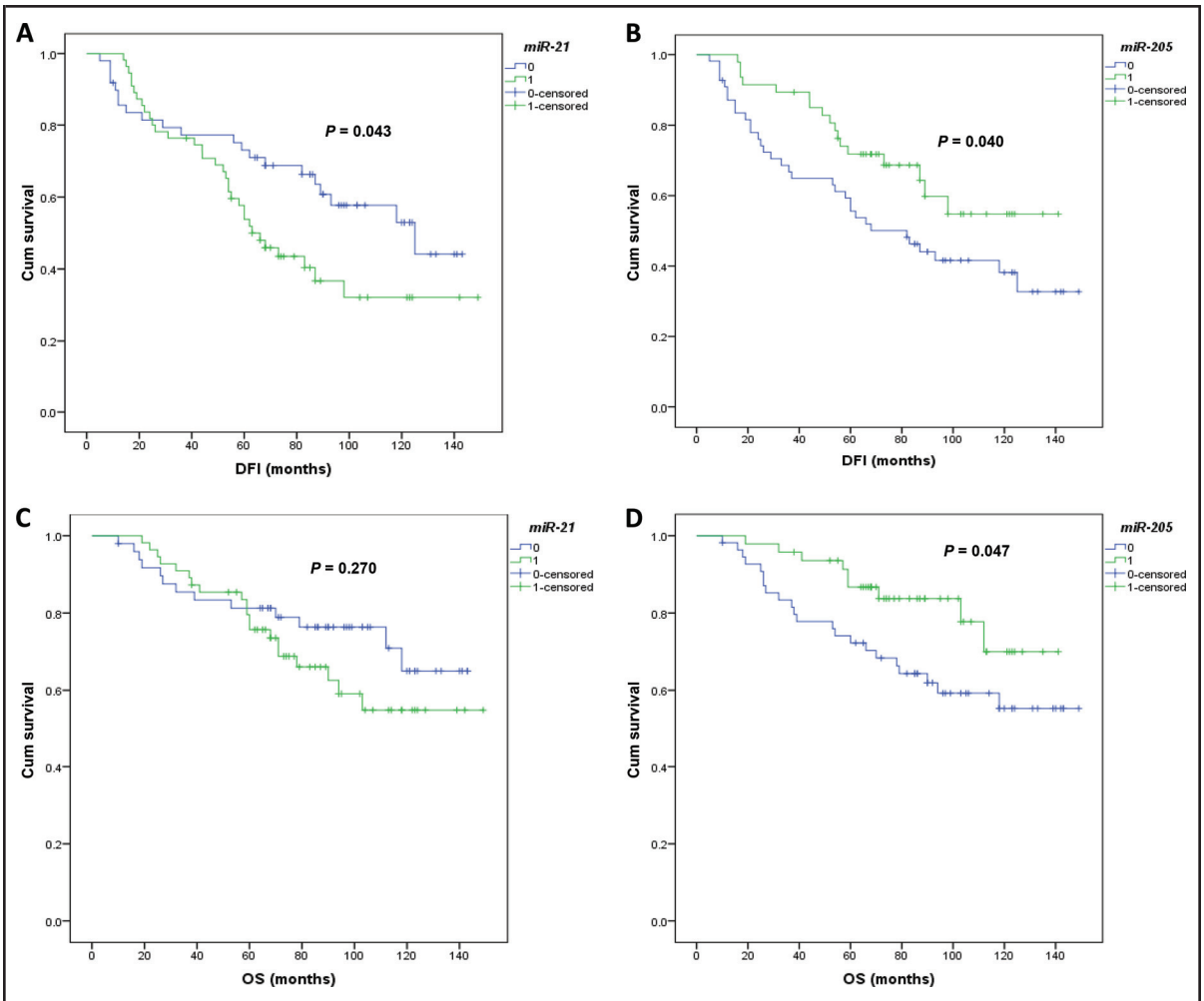

Fig. 3. (A), Kaplan–Meier estimates of DFI for early breast cancer patients with or without miR-21 overexpression. (B), Kaplan–Meier estimates of DFI for early breast cancer patients with or without *miR-205* downregulation. (C), Kaplan–Meier estimates of OS for early breast cancer patients with or without miR-21 overexpression. (D), Kaplan–Meier estimates of OS for early breast cancer patients with or without miR-205 downregulation. Cum, cumulative.

with miR-21 overexpression ($P = 0.014$) (see online Supplemental Fig. 1A), whereas in the lymph node–positive subgroup (n = 84) disease relapses and deaths were significantly different in patients with miR-205 underexpression ($P = 0.035$ and $P = 0.028$, respectively) (see online Supplemental Fig. 1, A and B).

UNIVARIATE AND MULTIVARIATE ANALYSES

Univariate analysis revealed that histology grade, lymph nodes, ER/PR receptor status, and HER2 status were significantly associated with DFI in these patients, whereas lymph nodes, ER/PR receptor status, HER2 status, and triple-negative tumors were significantly associated with OS (Table 1). Concerning miRNAs, univariate analysis revealed that both miR-21 and miR-205 were significantly associated with DFI and only miR-205 with OS (Table 1). Multivariate analysis demonstrated that the number of lymph nodes, ER/PR receptor status, histology grade, and the expression of miR-205 and miR-21 were independent factors associated with early disease relapse, whereas only lymph nodes, ER/PR receptor status, and miR-205 overexpression were independent factors associated with OS (Table 2).

Discussion

In this study we performed a systematic evaluation of the prognostic significance of metastasis-related miRNAs in early breast cancer. We explored the expression levels of miR-10b, miR-21, miR-205, miR-

Table 1. Univariate analysis for DFS and OS of patients with early-stage breast cancer.

	DFI		OS	
Factor	Hazard ratio, 95% CI	*P*	Hazard ratio, 95% CI	*P*
Tumor size, T2–3 vs T1	1.215 (0.653–2.260)	0.540	1.576 (0.613–4.055)	0.345
Histology grade, III vs I/II	1.706 (1.004–2.897)	0.048	1.548 (0.797–3.006)	0.197
Lymph nodes, 4 vs 0–3	1.714 (1.011–2.905)	0.045	2.699 (1.376–5.295)	0.004
ER, negative vs positive	1.696 (0.991–2.903)	0.054	2.306 (1.145–4.644)	0.019
PR, negative vs positive	2.289 (1.330–3.941)	0.003	1.949 (0.974–3.898)	0.059
Receptors, negative vs at least 1 positive	2.080 (1.213–3.567)	0.008	2.292 (1.157–4.541)	0.017
HER2, positive vs negative	2.656 (1.294–5.453)	0.008	2.293 (1.003–5.242)	0.049
Triple negative, yes vs no	1.549 (0.904–2.655)	0.111	1.938 (1.004–3.741)	0.049
miR-21, up vs down	1.762 (1.010–3.074)	0.046	1.478 (0.734–2.979)	0.274
miR-10b, up vs down	1.098 (0.605–1.993)	0.759	1.309 (0.592–2.898)	0.506
miR-205, up vs down	1.835 (1.019–3.303)	0.043	2.156 (0.990–4.693)	0.053
miR-335, up vs down	1.092 (0.586–2.034)	0.781	1.058 (0.495–2.262)	0.884
miR-210, up vs down	1.049 (0.581–1.895)	0.873	1.028 (0.486–2.174)	0.943
let-7a, up vs down	1.350 (0.752–2.423)	0.315	1.405 (0.681–2.897)	0.358

210, miR-335, and let-7a in early breast cancer patients and investigated the relationship of the relative expression levels of these biomarkers with clinical outcomes in all patients and in specific subgroups that were defined on the basis of ER/PR receptor expression, HER2, and lymph node status.

We found that miR-21, miR-10b, and miR-335 were overexpressed in primary breast tumors compared with noncancerous breast tissues. However, we found that miR-21 overexpression was associated only with reduced DFI, but not with OS. Our results are in agreement with those reported recently by Yu and coworkers, who evaluated the effect of miR-21 on disease progression and its association with transforming growth factor-β by analyzing miR-21 expression in breast cancer. Yu and coworkers also reported that high miR-21 expression was associated with poor DFI in patients with early stage disease, but they did not find any associations between patient survival and miR-21 expression among all patients *(28)*. Another study revealed that miR-21, among other miRNAs, was consistently upregulated in atypical ductal hyperplasia, ductal carcinoma in situ, and invasive ductal carcinoma *(29)*. This deregulation of miR-21 expression during breast tumorigenesis might be an early event, because it occurred significantly during the transition from normal tissue to atypical ductal hyperplasia *(29)*. Our results are in agreement with numerous reported studies that demonstrated upregulation of miR-21 in breast cancer *(2, 4, 16–20)*, as well as with our previous findings that miR-21 upregulation is of prognostic significance in non–small cell lung cancer *(30, 31)*.

Table 2. Independent predictive and prognostic factors by multivariate analysis for DFS and OS of patients with early-stage breast cancer.

	DFI		OS	
Factor	Hazard, 95% CI	*P* value	Hazard, 95% CI	*P* value
miR-21, up vs down	2.494 (1.295–4.802)	0.006		
miR-205, down vs up	2.018 (1.069–3.811)	0.030	2.453 (1.061–5.671)	0.036
Histology grade, III vs I/II	1.916 (1.017–3.611)	0.044		
Lymph nodes, 4 vs 0–3	1.989 (1.082–3.657)	0.027	4.120 (1.909–8.888)	0.0001
ER/PR receptors, negative vs at least 1 positive	2.397 (1.296–4.435)	0.005	2.467 (1.161–5.244)	0.019
HER2, positive vs negative	1.476 (0.613–3.551)	0.385	1.645 (0.603–4.484)	0.331

We found that miR-10b was deregulated in breast cancer but was not associated with reduced DFI or with OS. Overexpression of miR-10b in otherwise nonmetastatic breast tumors has been shown to initiate robust invasion and metastasis *(13, 32)*. Another reported study showed miR-10b expression levels to be positively correlated with tumor size, pathological grading, clinical staging, lymph node metastasis, HER2 positivity, and tumor proliferation but negatively associated with ER positivity, PR positivity, and E-cadherin mRNA and protein levels *(33)*. However, in agreement with our results, miR-10b overexpression in breast cancer up to now has not been shown to correlate with prognosis and survival.

miR-205 and let-7a in our study were downregulated in early breast cancer, as has already been reported by other groups *(21, 22)*. A systematic evaluation of functional miRNA–mRNA interactions associated with the invasiveness of breast cancer cells has revealed that miR-205 was included in a group of 7 downregulated miRNAs in invasive cell lines compared to normal and less invasive cell lines *(34)*. It has recently been reported that miR-205 is a tumor suppressor in breast cancer, because breast cancer cell lines express a lower level of miR-205 than nonmalignant cells, and that ectopic expression of miR-205 significantly inhibits cell proliferation and anchorage-independent growth, as well as cell invasion *(35)*. We report for the first time that downregulation of *miR-205* is associated with reduced DFI and OS in early breast cancer. These results were verified by both univariate and multivariate analysis.

When we evaluated miRNA expression according to different subgroups defined by the expression of steroid receptors, HER2, histology, and tumor grade, we were surprised to see that deregulation of miRNAs could be of prognostic significance in different subgroups. miR-10b overexpression was associated with disease relapse in the ER-negative subgroup. In the PR-negative subgroup, disease relapse was more common in patients with miR-21 and miR-10b overexpression, whereas patients who overexpressed miR-21 had shorter OS. In the same subgroup, miR-205 underexpression could differentiate patients in respect to disease relapse and death.

Our current study is the first to demonstrate that miR-21 overexpression was associated with a shorter DFI in the group of patients with TNBC and in the HER2-negative subgroup of patients as well. Very recently, Cascione et al. determined miRNA expression profiles to stratify TNBCs and identified miRNA signatures that correlated with prognosis and were independent predictors for OS and DFI *(36)*. Radojicic et al. recently explored the expression profile of miRNAs in TNBC and reported that there was a nonsignificant trend for high expression levels of the miRNAs miR-21, miR-210, miR-221, and miR-222 to be associated with worse patient DFI and OS *(37)*.

A metaanalysis recently summarized the recent studies regarding miR-210 involvement in human breast cancer and analyzed the predictive role of miR-210 for survival. The authors of this metaanalysis concluded that higher miR-210 expression in breast cancer might predict poor survival in patients with breast cancer *(38)*. However, according to our findings, miR-210 expression levels did not differentiate between primary tumors and noncancerous breast tissues and were not correlated with reduced DFI or OS. It was only in the ER-positive/HER2-negative subgroup that miR-210 overexpression was significantly associated with shorter OS.

Finally, Dving et al. have recently profiled miRNA expression across a cohort of 1302 patients with breast tumors for whom clinical follow-up information and matching genomic and messenger RNA expression data were available. The results reveal context-dependent interactions and demonstrate an important role for miRNAs in the biology and outcome of breast tumors devoid of somatic copy-number aberrations, suggesting an important modulatory role for miRNAs in this common subtype of the disease *(39)*.

Conclusions

Our results clearly indicate that deregulation of metastasis-associated miRNAs in primary tumors is associated with clinical outcomes in patients with early breast cancer with a long follow-up. Overexpression of miR-21 and underexpression of miR-205 are clearly associated with shorter DFI in all early breast cancer patients, whereas miR-205 underexpression is associated with OS. We report for the first time that deregulation of specific expression of miRNAs is associated with clinical outcomes in well-characterized breast cancer subgroups, and this deregulation may be an indicator of patients with higher risk.

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