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MicroRNA expression profiling identifies decreased expression of miR-205 in inflammatory breast cancer

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Inflammatory breast cancer is the most aggressive form of breast cancer. Identifying new biomarkers to be used as therapeutic targets is in urgent need. Messenger RNA expression profiling studies have indicated that inflammatory breast cancer is a transcriptionally heterogeneous disease, and specific molecular targets for inflammatory breast cancer have not been well established. We performed microRNA expression profiling in inflammatory breast cancer in comparison with locally advanced noninflammatory breast cancer in this study. Although many microRNAs were differentially expressed between normal breast tissue and tumor tissue, most of them did not show differential expression between inflammatory and noninflammatory tumor samples. However, by microarray analysis, quantitative reverse transcription PCR, and *in situ* hybridization, we showed that microRNA-205 expression was decreased not only in tumor compared with normal breast tissue, but also in inflammatory breast cancer compared with noninflammatory breast cancer. Lower expression of microRNA-205 correlated with worse distant metastasis-free survival and overall survival in our cohort. A small-scale immunohistochemistry analysis showed coexistence of decreased microRNA-205 expression and decreased E-cadherin expression in some ductal tumors. MicroRNA-205 may serve as a therapeutic target in advanced breast cancer including inflammatory breast cancer.
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Inflammatory breast cancer is the most aggressive form of breast cancer consisting of 1–5% of newly diagnosed breast cancer in the United States.[1] It is characterized by clinical hallmarks of diffuse erythema and edema (*peau d'orange*) involving one-third or more of the breast skin caused by tumor emboli blocking dermal lymphatics, and rapid progression from the onset of the disease. At the time of presentation, 55 to 85% of patients have regional lymph node metastasis clinically.[2] Despite improved survival in the past three decades because of the introduction of multimodality treatment approaches, the survival outcomes of patients with

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inflammatory breast cancer remain poorer compared with patients with noninflammatory, locally advanced breast cancer,[3,4] underscoring the need for understanding the biology of the disease and exploring new therapeutic targets.

Inflammatory breast cancer is known to be a heterogeneous disease histomorphologically, also reflected at the molecular level. The few published mRNA expression profiling studies to date have indicated that transcriptional heterogeneity exists in inflammatory breast cancer as extensively as in noninflammatory breast cancer, and that the established molecular subtypes such as luminal, HER2-positive, and basal-type can be identified in inflammatory breast cancer.[5–16] Although some of the studies have demonstrated differences in mRNA expression levels between inflammatory breast cancer and noninflammatory breast cancer samples, a specific inflammatory breast cancer signature cannot be deduced from these studies.

One intrinsic limitation in mRNA expression profiling studies of inflammatory breast cancer lies in the combination of the rarity of the disease and poor preservation of mRNA in retrospectively collected formalin-fixed, paraffin-embedded tissue. To this end, recent studies have explored tumor profiling of microRNAs (miRNAs), small (~22 nt), single-stranded nonprotein coding RNA molecules that suppress gene expression by binding to the 3′ untranslated regions of the target mRNAs.[17] Because of their small size, miRNAs are highly stable in fresh frozen as well as formalin-fixed, paraffin-embedded tissue, providing a competitive advantage compared with standard transcriptome analysis with regard to the possibility to explore their potential roles as robust biomarkers in inflammatory breast cancer. More important than its feasibility, recent advances have implicated the role of miRNA as oncogenes or tumor suppressor genes in tumorigenesis, metastasis, and response to treatment in various cancer types including breast cancer.[18–27] Although the number of miRNAs identified is relatively small (~2588 human mature miRNAs from 1881 precusor miRNA genes reported in miRBASEv.21; www.mirbase.org), individual miRNAs can target multiple genes, and it is thought that collectively they can target approximately one-third of the human genes.[28] Thus, they act as global regulators and may be suitable diagnostic markers and therapeutic targets for inflammatory breast cancer.

In this study, the miRNA expression profiles of 23 inflammatory breast cancer, 24 noninflammatory breast cancer, and 12 normal breast tissue fresh frozen samples were generated using a previously validated miRNA microarray assay.[29,30] The differentially expressed miRNA with the highest fold change, miR-205, was further investigated.

Materials and methods

Human Breast Tumor Samples

This study was approved by the institutional review board of MD Anderson Cancer Center. Fifty-three patients treated between 1997 and 2011 at MD Anderson Cancer Center with available fresh frozen breast tissue were identified from the institutional inflammatory breast cancer registry and institutional tissue bank, and included in the microarray study. Among these, 23 patients had the clinical diagnosis of inflammatory breast cancer with biopsy confirmed carcinoma in the underlying breast, and 24 had noninflammatory breast cancer that met the following criteria: clinically and/or pathologically, T4, or tumor $\geq$5 cm, or T2 (tumor $\geq$3 cm) N2. In addition, 12 samples of normal breast tissue, 6 from the above 47 patients and 6 from additional patients, were used in the microarray study as normal controls. Patient age, tumor histologic type, lymph node status, the status of lymphovascular invasion, prognostic/predictive marker status, clinical stage, pathologic stage, and history of neoadjuvant chemotherapy were retrospectively recorded from the medical records. In inflammatory breast cancer patients, because pathologic restaging does not occur after neoadjuvant chemotherapy, the pathologic stage was the same as the clinical stage, except when the ypN was higher than the pretreatment N stage, the ypN was used for pathologic stage. The American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guideline recommendations were used as references with minor modifications for scoring estrogen receptor, progesterone receptor, and HER2[31,32] that were performed as part of the routine pathology evaluation. Estrogen receptor and progesterone receptor status were determined by immunohistochemical staining. Positive staining was defined as nuclear staining in at least 5% of invasive cancer cells. HER2 status was tested by fluorescence *in situ* hybridization or by immunohistochemical staining. Triple-negative (estrogen receptor negative, progesterone receptor negative, and HER2 negative) was defined accordingly.

For *in situ* hybridization analysis, formalin-fixed, paraffin-embedded tissue from 23 of the above patients with available tissue and six additional inflammatory breast cancer patients treated at MD Anderson Cancer Center during the above-mentioned time period was obtained from the surgical pathology files in the Department of Pathology. The total of 29 cases included 5 pretreatment inflammatory breast cancer samples, 9 postchemotherapy inflammatory breast cancer samples, 8 pretreatment noninflammatory breast cancer samples, and 7 postchemotherapy non-inflammatory breast cancer samples. Two pretreatment noninflammatory breast cancer specimens were invasive lobular carcinoma and the remaining 27 specimens were invasive ductal carcinoma.

MicroRNA Microarray Analysis

Total RNA, including miRNA, was extracted from frozen tissues by homogenization with TRI reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. RNA quality and quantity were evaluated by NanoDrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The miRNA expression profiling on miRNA microarray was performed as previously described.[29,30] Briefly, 3 μg of total RNA from each sample was biotin labeled by reverse transcription using 5′-end biotin-labeled random octomer oligo primer. Hybridization of biotin-labeled cDNA was carried out on a miRNA microarray chip (MD Anderson, Version 5.0) that contains 2100 miRNA probes, including 678 human and 472 mouse miRNA genes obtained from Sanger miRBase V12, in duplicate. Hybridization signals were detected by biotin binding of a streptavidin-Alexa647 conjugate by using Axon Scanner 4000B (Axon Instruments, Union City, CA, USA). The images were quantified by GENEPIX 6.0 software (Axon Instruments).

Two-sample *t*-test was applied to identify differentially expressed miRNAs between two groups (normal *vs* tumor; inflammatory breast cancer *vs* noninflammatory breast cancer). The resulting *P*-values, computed from the *t*-test statistics, were then modeled by the Betat-Uniform Modeling algorithm to adjust for multiple hypothesis testing. The significant differentially expressed miRNAs were then identified based on appropriate false discovery rate cutoffs. The results were illustrated using unsupervised Hierarchical Cluster Analysis algorithm based on the identified miRNAs.

Quantitative Reverse Transcription PCR Analysis

The expression levels of miRNAs were detected by quantitative real-time PCR using TaqMan miRNA assays with individual miRNA-specific primers and probes (Life Technologies) to verify the microarray findings. TaqMan mature RNA-specific primers and probes precluded the detection of precursor miRNAs. Briefly, 10 ng of total RNA was reverse transcribed in a volume of 15 μl using TaqMan MicroRNA Reverse Transcription kit (Life Technologies). Quantitative PCR was performed with TaqMan Universal Master Mix (Life Technologies) using standard protocols on ABI Prism 7500 Sequence Detection System (Life Technologies). The mean cycle threshold was determined from triplicate reactions and U48 small nucleolar RNA (SNORD48) was used as endogenous control. Comparative cycle threshold method was used to compare expression levels.

MiR205 *In Situ* Hybridization

For each case, one whole-slide unstained tissue section 4 μm thick that had been prepared from a representative paraffin block of primary invasive breast carcinoma was used for *in situ* hybridization by the institutional RNA Center. Briefly, the tissue slides were first digested with 15 μg/ml proteinase K for 10 min at room temperature, and then hybridized with the double-DIG-labeled mercury LNA microRNA probe (Exiqon, Woburn, MA, USA) for 2 h at 50 °C on Ventana Discovery Ultra (Ventana Medical Systems, Tucson, AZ, USA). The digoxigenins were then detected with a polyclonal anti-DIG antibody and alkaline phosphatase-conjugated second antibody (Ventana Medical Systems) using NBT-BCIP as the substrate. Raw images were captured with the same exposure and gain settings from all slides and saved as TIF files, and were analyzed using intensity measurement tools of Image-Pro Plus software (MediaCybernetics, Rockville, MD, USA).

Immunohistochemistry

The polymeric biotin-free horseradish peroxidase method was used for E-cadherin and vimentin immunohistochemical staining on a Leica Microsystems Bond Max stainer (Leica Microsystems, Buffalo Grove, IL, USA). In each case, one whole-slide unstained tissue section 4 μm thick that had been prepared from a representative paraffin block of the invasive breast carcinoma was subjected to heat-induced epitope retrieval with Tris-EDTA buffer for 20 min at 100 °C (E-cadherin) or citrate buffer for 5 min at 100 °C (vimentin). Slides were then incubated with mouse monoclonal antibody to E-cadherin (clone HECD-1, 1:7000, Life Technologies) or vimentin (clone V9, 1:900, Dako North America, Carpinteria, CA, SA). The Refine Polymer Detection kit was used to detect bound antibody, with 3,3-diaminobenzidine serving as the chromogen (Leica Microsystems). Slides were counterstained with Mayer's hematoxylin. Results were evaluated with appropriate positive and negative tissue controls. For E-cadherin, membranous staining was considered positive. For vimentin, positive staining was defined as cytoplasmic staining in >10% of invasive carcinoma cells.

Statistical Analysis

Statistical analysis was carried out using SAS version 9 (SAS Institute, Cary, NC, USA) and SPSS software (IBM, Armonk, NY, USA). Association between tumor type and clinicopathologic factors (Table 1) was assessed using Fisher's exact test. Comparisons of biomarker expression level between tumor and normal, inflammatory breast cancer and

Table 1 Clinicopathologic features of tumor specimens used in microarray analysis

Clinicopathologic features	*IBC* (n = *23*)	*Non-IBC* (n = *24*)	*Total* (n = *47*)	P-*value*
Patient age (year)				0.13
Min	27	37	27	
Median	54	63	57	
Mean	54	62	58	
Max	72	98	98	
Tumor type				0.23
IDC	22 (96%)	21 (87%)	43	
ILC	0 (0%)	3 (13%)	3	
IMC	1 (4%)	0 (0%)	1	
Lymphovascular invasion				>0.99
Present	9 (39%)	11 (46%)	20	
Absent	11 (48%)	13 (54%)	24	
Unknown	3 (13%)	0 (0%)	3	
Axillary lymph node status				0.08
Negative	6 (26%)	4 (17%)	10	
1–3 positive	4 (17%)	10 (42%)	14	
4–9 positive	7 (30%)	9 (38%)	16	
≥10 positive	6 (26%)	1 (4%)	7	
ER status				0.14
Positive (≥5%)	11 (48%)	17(71%)	28	
Negative	12 (52%)	7 (29%)	19	
PR status				0.36
Positive (≥5%)	6 (26%)	10 (42%)	16	
Negative	17 (74%)	14 (58%)	31	
HER2 status				0.41
Positive	4 (17%)	2 (8%)	6	
Negative	18 (78%)	22 (92%)	40	
Unknown	1 (4%)	0 (0%)	1	
Triple-negative status				0.35
Yes	9 (39%)	6 (25%)	15	
No	13 (57%)	18 (75%)	31	
Unknown	1 (4%)	0 (%)	1	
Neoadjuvant chemotherapy				1
Yes	11 (48%)	11 (46%)	22	
No	12 (52%)	13 (54%)	25	
Clinical stage				0.08
IIIB and below (II, IIIA, IIIB)	11 (0, 0, 11)	18 (7, 3, 8)	29	
IIIC and above (IIIC, IV)	12 (6, 6)	6 (6, 0)	18	
Pathologic stage				0.02
IIIB and below (II, IIIA, IIIB)	10 (0, 0, 10)	19 (5, 10, 4)	29	
IIIC and above (IIIC, IV)	13 (7, 6)	5 (5, 0)	18	

Abbreviations: ER, estrogen receptor; IBC, inflammatory breast cancer; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; IMC, invasive mammary carcinoma with mixed ductal and lobular features; PR, progesterone receptor.

noninflammatory breast cancer were carried out using Wilcoxon rank-sum test. Spearman's test was used to assess correlation between biomarker expression measured using different platforms. Overall survival represented duration between the initial breast cancer diagnosis and death from any cause or date of last follow-up. Distant metastasis-free survival represented duration between the initial breast cancer diagnosis and the point of distant metastasis. Survival end points were estimated and plotted using the Kaplan–Meier method. Comparisons of survival between patient groups were carried out using the log rank test. All tests were two sided and *P*-values of ≤ 0.05 were considered statistically significant.

Results

Patient Clinicopathologic Characteristics

Total RNA isolated from fresh frozen tissue of the primary tumors of 23 inflammatory breast cancer, 24 noninflammatory breast cancer, and 12 normal breast tissue samples from 53 patients was subjected to miRNA microarray analysis. The clinicopathologic characteristics of the patients whose tumor samples were used are summarized in Table 1. In all the clinicopathologic factors, except for pathologic stage, the inflammatory breast cancer and noninflammatory breast cancer groups did not show significant difference. The reason for the difference in pathologic stage was most likely because of the fact that inflammatory breast cancer patients were not restaged after neoadjuvant chemotherapy (especially the pT stage) as compared with noninflammatory breast cancer patients who were often downstaged after neoadjuvant treatment.

Distinct MiRNA Expression in Breast Tumors by Microarray Analysis

The heatmaps of miRNA expression comparing tumor with normal tissue samples showed distinct segregation (Figure 1). The 30 miRNAs that were most significantly different between tumor and normal samples based on *P*-value are listed in Table 2. For those that had been reported in previous expression profiling studies, the proposed functions in breast cancer are included in Table 2. However, of these 30 miRNAs, only 6 had a *P*-value of < 0.05 between inflammatory breast cancer and noninflammatory breast cancer, all with very moderate fold changes, suggesting that inflammatory breast cancer shares many common changes in miRNA expression with noninflammatory breast cancer.

Examples of recently identified potential miRNA oncogenes and tumor suppressors and their expression profiles in our microarray analysis are summarized in Tables 3 and 4. Many but not all miRNAs reported in the literature were significantly different between tumor and normal tissue in our analysis. Because our cohort was devoid of early-stage breast cancer, it was not surprising that the miRNA profile obtained from our study did not entirely mirror those reported that were usually derived from breast tumors including all stages.

Low MiR-205 Expression in Inflammatory Breast Cancer

Although distinct clustering of miRNA expression was not identified between inflammatory breast

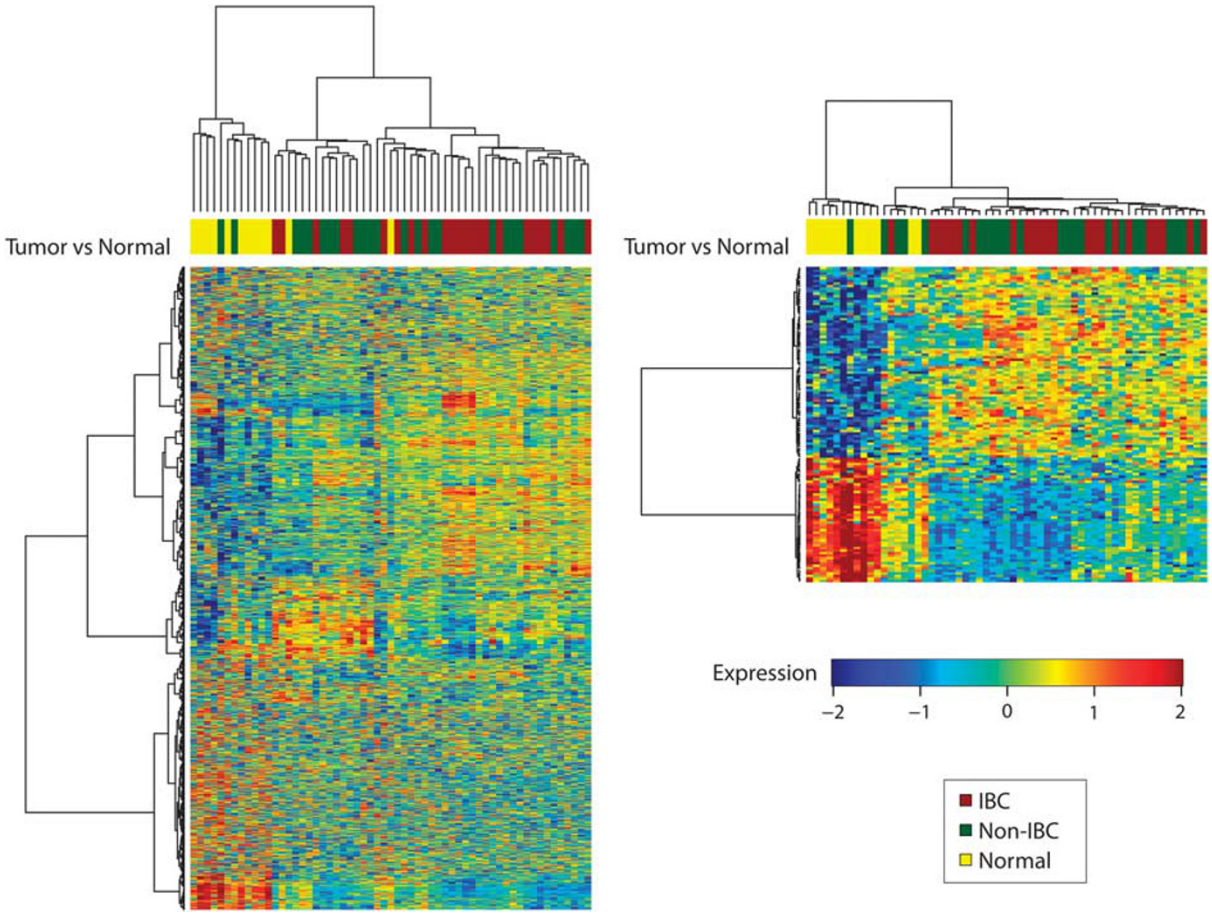


Figure 1 Heatmaps of miRNAs comparing tumor and normal samples. (Left) All 1827 probes; (right) at the false discovery rate level of 5e−05 using two-sample *t*-test. IBC, inflammatory breast cancer.

cancer and noninflammatory breast cancer samples, several miRNAs were shown to be differentially expressed (Figure 2 and Table 5). MiR-205 was the most differentially expressed, with a 3.28-fold lower expression in inflammatory breast cancer ($P=0.001$). MiR-205 was also expressed significantly lower in tumor compared with normal tissue ($P=0.001$, 4.06-fold). Quantitative real-time RT-PCR was performed on eight of the miRNAs in Table 5 selected based on fold change, *P*-value, and expression levels. The results are shown in Table 6. MiR-205 expression was significantly downregulated in tumor tissue compared with normal tissue, as well as in inflammatory breast cancer compared with noninflammatory breast cancer.

Lower Expression of MiR-205 Was Associated with Poor Prognosis

The distant metastasis-free survival and overall survival were assessed in patients according to the miR-205 expression levels in all 47 tumors. Follow-up time ranged from 3 months to 159 months (median follow-up: 39 months). As shown in Figure 3, lower miR-205 expression was associated with shorter distant metastasis-free survival ($P=0.012$) and overall survival ($P=0.025$).

MiR-205 *In Situ* Hybridization Analysis

To further confirm that the difference of miR-205 between inflammatory breast cancer and noninflammatory breast cancer was due to differential expression in the epithelial cells, *in situ* hybridization of miR-205 was performed on formalin-fixed, paraffin-embedded tissue of breast tumors. In all, 15 noninflammatory breast cancer and 8 inflammatory breast cancer samples used for microarray analysis had available formalin-fixed, paraffin-embedded tissue. Formalin-fixed, paraffin-embedded tissue from six additional inflammatory breast cancer patients was also included.

Tumor sections containing nonneoplastic breast epithelium in the same sections were selected for *in situ* hybridization. Staining results of representative cases of inflammatory breast cancer and noninflammatory breast cancer are shown in Figure 4. In the nonneoplastic breast epithelium, strong staining in the basal/myoepithelial cell portion was observed in each case (Figure 4d–f and j–l), consistent with previous reports.[58,59] The invasive carcinoma cells had

Table 2 Top 30 differentially expressed miRNA molecules comparing tumor and normal samples based on *P*-value ($P < 6.0E-08$)

Name	*Trend tumor vs normal*	*Fold change tumor vs normal*	P-*value IBC vs non-IBC*	*Fold change IBC vs non-IBC if* P < *0.05*	*Reported function in breast cancer tissue/cells*	*References*
miR-23b/27b					Pro-metastasis, associated with poor prognosis	[33, 34]
miR-23b*	Up	3.04	**0.025**	1.32		
miR-125a/b					Antiproliferation	[22, 23, 25, 35–45]
miR-125a-5p	Down	−2.15	**0.012**	−1.29		
miR-125a-3p	Down	−2.26	0.807			
miR-185	Up	2.77	0.509		Antiproliferation	[22]
miR-210	Up	3.23	0.234		Anti-apoptosis, pro-angiogenesis	[21–23, 25,35, 38, 44–46]
miR-210MM1G/T	Up	4.02	0.632			
miR-210MM2GA/TC	Up	3.62	0.580			
miR-212	Down	−1.87	**0.043**	−1.21	Not reported	
miR-338-3p	Up	3.21	0.260		(only reported as circulating miRNA)	
miR-448	Down	−1.88	0.195		Associated with chemotherapy-induced EMT	[47]
miR-526b	Up	8.04	0.794		Not reported	
miR-629*	Up	2.00	0.084		Upregulated in multiple cancer types including breast, function unknown	[48]
miR-662	Up	3.24	0.490		Not reported	
miR-891a	Down	−2.46	0.084		Not reported	
miR-922	Up	3.96	0.381		Not reported	
miR-1226	Down	−2.24	**0.017**	−1.28	Proapoptosis	[49]
miR-1226*	Down	−1.88	0.131		Not reported	
miR-1236	Down	−2.18	0.136		Not reported	
miR-1243	Down	−2.29	0.051		Not reported	
miR-1244	Down	−2.12	0.061		Not reported	
miR-1255a	Down	−2.24	**0.030**	−1.24	Not reported	
miR-1255b	Down	−2.26	0.210		Not reported	
miR-1260	Up	2.47	0.473		(only reported as circulating miRNA)	
miR-1260b	Up	2.39	0.101		Not reported	
miR-1262	Down	−2.15	0.067		Not reported	
miR-1263	Down	−2.43	**0.035**	−1.26	Not reported	
miR-1289	Down	−2.94	0.062		Not reported	
miR-1910	Up	1.81	0.056		Not reported	
miR-3140	Down	−2.68	0.263		Not reported	
miR-3197	Up	1.95	0.197		Not reported	

P-values of ≤0.05 in bold.

predominantly cytoplasmic staining. Although staining of the nucleus was inconspicuous, nucleolar staining was readily seen in most of the cases. The staining intensity of the invasive carcinomas varied greatly from case to case. In the majority of the cases, heterogeneity in staining intensity was not detected within each case. In rare cases, scattered strongly stained tumor cells were seen intermixed with lightly stained cells (Figure 4i). The relative staining intensity of the tumors was quantitated using the Image-Pro Plus software (Figure 5). Inflammatory breast cancer tumors (mean ± s.d., 54 ± 20; $n = 14$) had lower staining compared with noninflammatory breast cancer tumors (mean ± s.d., 72 ± 18; $n = 15$) ($P = 0.04$; Figure 4a–c and g–i). No difference was detected between the pretreatment samples (mean ± s.d., 65 ± 21; $n = 13$) and postchemotherapy samples (mean ± s.d., 62 ± 22; $n = 16$) ($P = 0.71$). The two cases of invasive lobular carcinoma had miR-205 expression values of 66 and 78, respectively.

The staining intensity in the luminal epithelial cells was light to moderate compared with the myoepithelial cells (Figure 4d–f and j–l). Stromal fibroblasts, blood vessels, and lymphoplasmacytic cells showed light staining in most of the cases and moderate staining in a few cases. Adipose tissue appeared to have light staining in all cases.

To examine whether the significant differences in miR-205 expression by microarray, qPCR, and *in situ* hybridization were coincidental, Spearman's correlation test was performed in order to evaluate correlation among the three platforms. Significant association was seen in the 53 samples used in both the microarray assay and qPCR ($r = -0.8$, $P < 0.0001$; Figure 6a) and the 23 tumors used in qPCR and *in situ* hybridization ($r = -0.5$, $P = 0.02$; Figure 6b). Hence, the differences in miR-205 expression using the three platforms were in good agreement.

Table 3 Oncogene or poor prognostic indicator in breast cancer based on literature review

			Current study		
MiRNA	*References*	*Function/association*	*P-value tumor vs normal*	*Fold change tumor vs normal*	*Trend tumor vs normal if P < 0.05*
miR-9	21, 22, 35, 36, 45, 46, 50	Pro-metastasis	0.998	−1.00	
miR-10b	21–23, 25, 36, 40, 41, 44, 45, 51	Pro-metastasis	**0.001**	−2.75	Down
miR-17-92 cluster (-17,-18a,-19a,-19b-1,-20a,-92a-1)	22, 23, 35, 36, 40–45, 51–54	Pro-metastasis			
miR-17			0.139	1.44	
miR-18a			0.178	−1.60	
miR-19a			**0.0004**	2.00	Up
miR-20a			0.062	1.72	
miR-19b-1			0.106	1.80	
miR-92a-1*			**0.022**	1.72	Up
miR-21	21–23, 25, 35–37, 39, 40–46, 50–52, 54, 55	Anti-apoptosis, pro-metastasis	< **0.0001**	4.50	Up
miR-27	22, 23, 35, 36, 40–45	Pro-proliferation; angiogenesis			
miR-27a			0.083	1.52	
miR-29	21, 25, 41, 42, 45	Pro-metastasis			
miR-29a			**0.002**	−1.72	Down
miR-29b			0.314	1.32	
miR-29c			0.207	−1.44	
miR-103/107	21, 23, 45, 55	Pro-metastasis			
miR-103			0.136	1.49	
miR-107			0.104	1.53	
miR-106b/93/25 cluster	25, 36, 42, 45, 46, 54	Pro-proliferation			
miR-106b			**0.031**	1.60	Up
miR-93			**0.0004**	2.74	Up
miR-25			0.324	−1.31	
miR-128	22,35,40,44	Pro-metastasis	0.225	1.37	
miR-144	35	Pro-proliferation	0.151	−1.70	
miR-155	22, 23, 25, 35–37, 39–45, 50, 54, 56	Pro-metastasis, angiogenesis, anti-apoptosis	**0.010**	1.61	Up
miR-181a	56	Inhibit anoikis	**0.013**	1.93	Up
miR-183/96/182 cluster	40, 54, 56	Pro-proliferation; pro-metastasis			
miR-183			0.294	−1.35	
miR-96			0.777	1.07	
miR-182			**0.003**	3.94	Up
miR-191	22, 25, 45, 56	Pro-proliferation	< **0.0001**	3.15	Up
miR-196a	22, 25, 42, 45	Pro-metastasis	**0.009**	1.81	Up
miR-206	21–23, 35, 36, 40, 42–44, 52, 53, 55	Pro-metastasis; estrogen unresponsiveness	**0.037**	1.31	Up
miR-210	21–23, 25, 35, 38, 44–46	Anti-apoptosis, angiogenesis	< **0.0001**	3.23	Up
miR-221/222	22, 36, 45, 52, 53, 56	Pro-metastasis, pro-proliferation			
miR-221			**0.039**	−1.67	Down
miR-221*MM1C/G			**0.001**	−3.31	Down
miR-222			0.318	1.28	
miR-301	57	Associated with distant metastasis	**0.007**	−2.09	Down
miR-373/520c	21, 35, 36, 40, 42, 44, 50	Pro-metastasis			
miR-373			0.261	−1.30	
miR-520c			0.323	1.38	
miR-766	46	Associated with decreased survival	< **0.0001**	1.46	Up

P-values of ≤0.05 in bold.

Immunohistochemical Staining of E-Cadherin and Vimentin

It has been shown that miR-205 targets the transcription repressors of E-cadherin, ZEB1, and ZEB2 in cultured cells,[60,61] thereby regulating epithelial–mesenchymal transition. Thus, in inflammatory breast cancer, low miR-205 expression is expected to lead to downregulation of E-cadherin. However, it has been reported that inflammatory breast cancers express high levels of E-cadherin compared with noninflammatory breast cancers.[62,63] In light of the high rate of metastasis of inflammatory breast cancer that would correlate with active epithelial–mesenchymal transition, these

Table 4 Tumor suppressor or good prognostic indicator in breast cancer based on literature review

			Current study		
MiRNA	*References*	*Function/association*	P-*value tumor vs normal*	*Fold change tumor vs normal*	*Trend tumor vs normal if* P < *0.05*
Let-7	22, 23, 25, 35–37, 42–46, 50–52, 55	Antiproliferation, anti-metastasis			
Let-7a			0.944	1.01	
Let-7a-2-3p			**0.0004**	−2.94	Down
Let-7b			0.551	−1.10	
Let-7c			0.948	−1.01	
Let-7d			0.719	1.07	
Let-7e			0.625	1.04	
Let-7f			0.309	−1.24	
Let-7g			0.144	−1.19	
Let-7i			0.446	−1.06	
miR-15a/16	22	Proapoptosis			
miR-15a			0.401	−1.17	
miR-16			**0.011**	2.00	Up
miR-26a	35, 56	Proapoptosis, antiproliferation and anti-metastasis	0.104	−1.58	
miR-30 family	21, 36, 42, 45, 46, 55	Antiproliferation			
miR-30a			0.834	−1.05	
miR-30b			0.426	1.25	
miR-30c			0.522	1.22	
mir-30d			0.643	1.13	
miR-30e			0.496	1.19	
miR-30-3p			**0.006**	−2.28	Down
miR-31	21, 22, 25, 35, 36, 40, 42, 44, 45, 55	Proapoptosis, anti-metastasis	0.16	1.43	
miR-34a	23, 36, 37, 42, 44, 46, 52	Proapoptosis, antiproliferation	**0.0006**	2.28	Up
miR-101	22, 42	Anti-metastasis	**0.005**	−2.29	Down
miR-122	22, 37	Antiproliferation	0.095	−1.14	
miR-125a/b	22, 23, 25, 35–45	Antiproliferation			
miR-125a			< **0.0001**	−2.15	Down
miR-125b			**0.001**	−2.27	Down
miR-126	21, 22, 35, 36, 40, 42–46, 54, 56	Anti-metastasis, antiproliferation	**0.002**	−1.90	Down
miR-130	25, 53	Anti-metastasis, antiproliferation			
miR-130a			**0.038**	−1.88	Down
miR-130b			< **0.0001**	−1.83	Down
miR-135a	46	Associated with decreased metastasis	**0.004**	−1.72	Down
miR-145	22, 23, 25, 36, 38–45, 50–54, 56	Proapoptosis, anti-metastasis, suppresses angiogenesis	< **0.0001**	−2.06	Down
miR-146	22, 45	Anti-metastasis			
miR-146a			0.169	1.62	
miR-146b			**0.032**	1.61	Up
miR-185	22	Antiproliferation	< **0.0001**	2.77	Up
miR-193b	21, 44	Antiproliferation	**0.0003**	1.92	Up
miR-200 family	21, 23, 36, 40, 42–46, 51, 53, 54, 57	Antiproliferation			
miR-200a			**0.002**	3.26	Up
miR-200b			< **0.0001**	6.01	Up
miR-200c			**0.023**	2.17	Up
miR-141			< **0.0001**	2.89	Up
miR-429			< **0.0001**	3.34	Up
miR-205	22, 23, 25, 36, 37, 40, 42, 45, 51, 52, 54	Anti-metastasis	**0.001**	−4.06	Down
miR-206	23, 42–45	Antiproliferation, anti-invasion	**0.037**	1.31	Up
miR-302c	44	Antiproliferation	0.136	−1.68	
miR-326	22, 25, 42	Regulates multidrug resistance	< **0.0001**	2.09	Up
miR-335	21, 35, 36, 40, 42–46	Anti-invasion/metastasis	0.100	−1.85	
miR-342-5p	46, 56	Antiproliferation, associated with decreased metastasis	**0.0003**	1.56	Up
miR-497	25, 35, 40, 46, 56	Proapoptosis, antiproliferation	0.352	1.12	
miR-563	46	Associated with increased survival	0.154	−1.77	
miR-1226	22	Proapoptosis	< **0.0001**	−2.24	Down
miR-1539	46	Associated with increased survival	0.065	1.84	

P-values of ≤0.05 in bold.

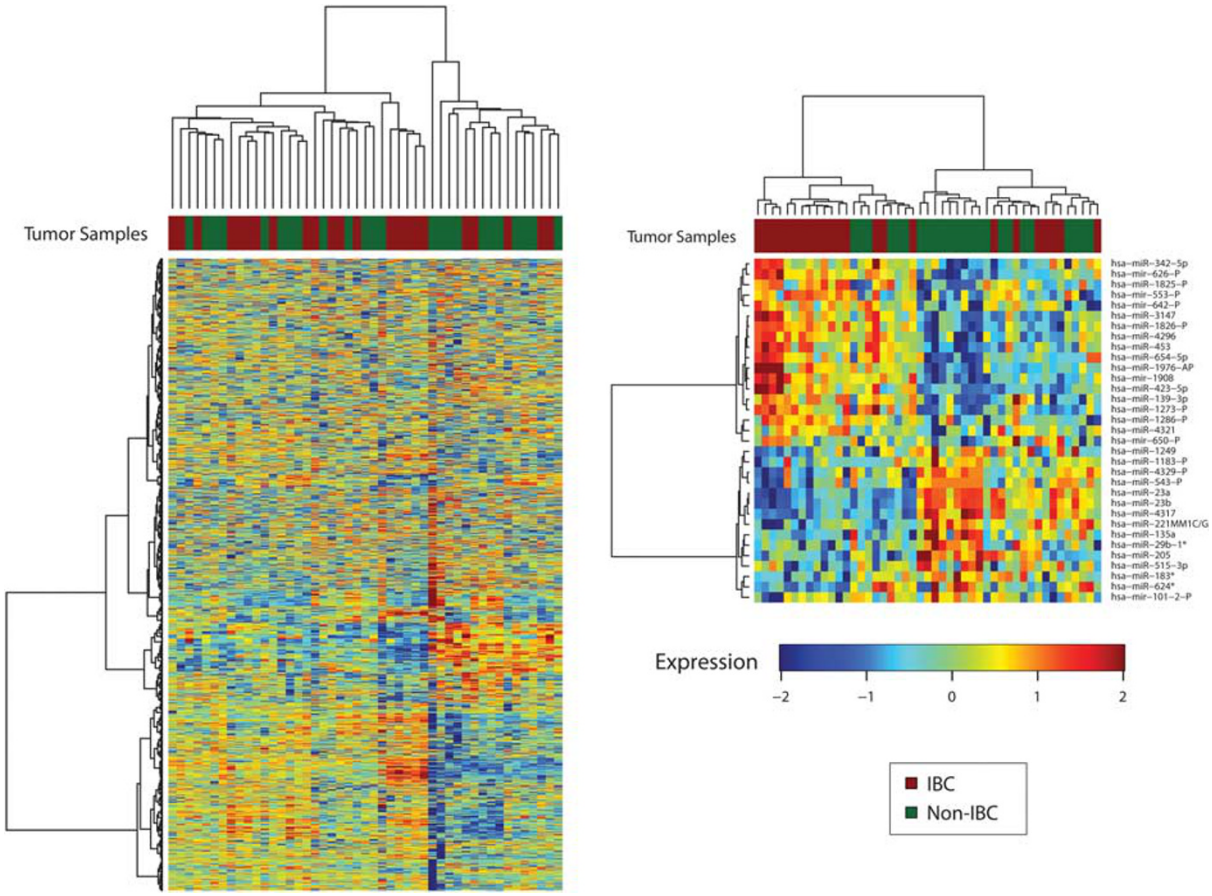


Figure 2 Heatmaps of miRNAs comparing inflammatory breast cancer (IBC) and non-IBC. (Left) All 1827 probes; (right) at the false discovery rate level of 0.1.

Table 5 Top 20 differentially expressed miRNA molecules comparing inflammatory breast cancer and noninflammatory breast cancer at false discovery rate of 0.1 ($P < 0.003427$)

	P-*value IBC vs non-IBC*	*Fold change IBC vs non-IBC*	*Trend IBC vs non-IBC*	P-*value tumor vs normal*	*Fold change tumor vs normal*
miR-23a	0.002	−1.87	Down	0.564	−1.15
miR-23b	0.0005	−1.94	Down	0.484	−1.17
miR-29b-1*	0.002	−1.58	Down	0.160	1.35
miR-135a	0.0003	−1.67	Down	0.004	−1.72
miR-139-3p	0.003	1.26	Up	0.001	1.35
miR-183*	0.0005	−2.28	Down	0.005	2.29
miR-205	0.001	−3.28	Down	0.001	−4.06
miR-221	0.003	−1.89	Down	0.039	−1.67
miR-342-5p	0.0005	1.36	Up	0.0003	1.56
miR-423-5p	0.003	1.39	Up	0.058	1.26
miR-453	0.0007	1.71	Up	< 0.0001	2.35
miR-515-3p	0.003	−1.96	Down	0.024	−1.77
miR-624*	0.001	−2.63	Down	0.056	−2.04
miR-654-5p	0.001	1.51	Up	0.0007	2.31
miR-1249	0.0002	−1.32	Down	0.432	−1.07
mir-1908	0.0001	1.54	Up	0.083	1.32
miR-3147	0.003	1.42	Up	0.040	1.31
miR-4296	0.003	1.53	Up	0.015	1.63
miR-4317	0.0009	−1.83	Down	0.084	−1.45
miR-4321	0.002	1.93	Up	0.0008	2.40

previously reported results appear in conflict. Therefore, it is of interest to examine the association between miR-205 and E-cadherin expression in the inflammatory breast cancer samples. Vimentin is a marker of mesenchymal cells that is infrequently expressed in epithelial tumors, and the expression in the latter suggests mesenchymal features or epithelial–mesenchymal transition. Formalin-fixed, paraffin-embedded tissue of eight tumors representing different miR-205 expression levels by *in situ* hybridization intensities was selected to stain for E-cadherin and vimentin by immunohistochemistry. All tumors except for one case of noninflammatory breast cancer were histologically of ductal differentiation. The single invasive lobular carcinoma was expected to be negative for E-cadherin, whereas others were expected diffusely positive. The staining results are shown in Figure 7. As expected, the case of invasive lobular carcinoma was negative for E-cadherin (Figure 7j). Interestingly, one inflammatory breast cancer and one non-inflammatory breast cancer had markedly decreased E-cadherin staining, with positive staining in small patchy areas intermixed with negatively stained areas (Figure 7d and k). Both tumors had

Table 6 Quantitative PCR results on eight miRNAs comparing inflammatory breast cancer and noninflammatory breast cancer samples

	qPCR normalized Ct value					
	Normal (n = *11*)	*Tumor* (n = *45*)	*IBC* (n = *21*)	*Non-IBC* (n = *24*)	P-*value tumor vs normal*	P-*value IBC vs non-IBC*
miR-23b	3.87	4.61	4.65	4.59	0.09	0.92
miR-135a	5.86	7.79	8.34	7.31	**0.006**	0.08
miR-205	−0.44	2.33	3.22	1.55	**0.0007**	**0.02**
miR-515-3p	—[a]					
miR-139-3p	8.41	11.88	12.31	11.52	< **0.0001**	0.17
miR-342-5p	7.75	7.96	8.37	7.60	0.41	0.06
miR-423-5p	4.17	4.83	4.92	4.76	0.06	0.7
miR-453	—[a]					

[a]Cycle threshold (Ct) values too high for meaningful interpretation.
P-values of ≤0.05 in bold.

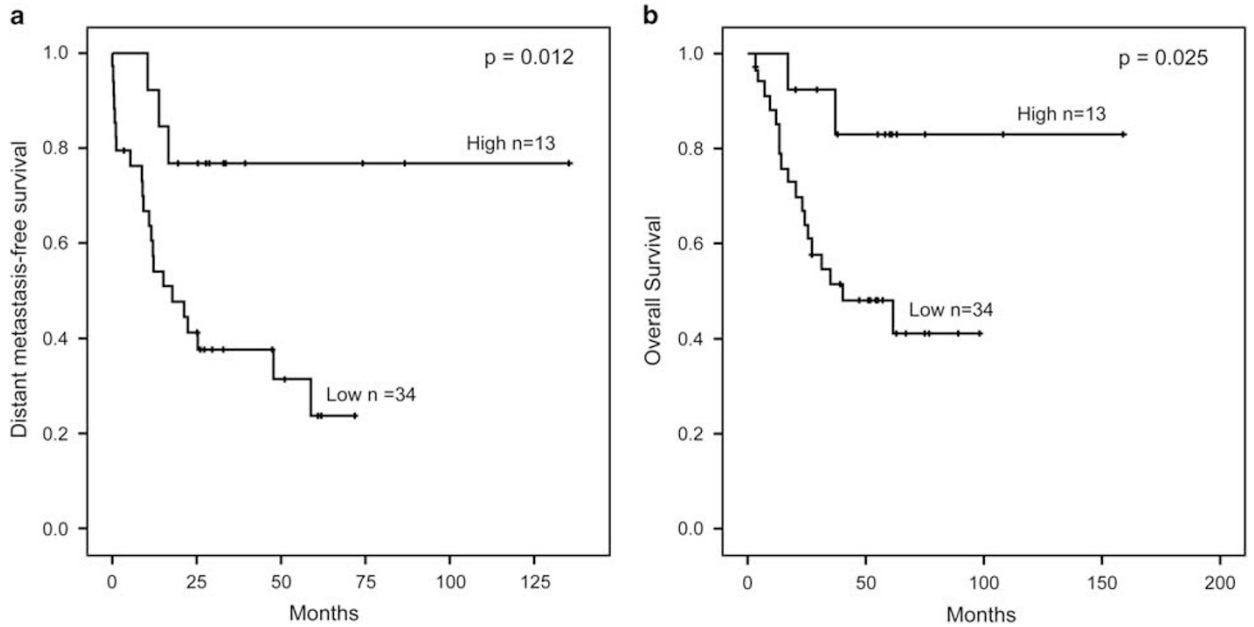


Figure 3 Lower miR-205 expression is associated with shorter distant metastasis-free survival and overall survival in patients with breast cancer. (**a**) Kaplan–Meier plots of distant metastasis-free survival between tumors with high and low miR-205 expression levels. (**b**) Kaplan–Meier plots of overall survival between tumors with high and low miR-205 expression levels.

relatively low miR-205 by *in situ* hybridization. However, not all cases with low miR-205 had decreased staining for E-cadherin. In addition, both cases with decreased E-cadherin staining and one case with diffuse strong E-cadherin staining showed positive staining for vimentin (Figure 7f, h and o). Therefore, some ductal tumors can have decreased expression of miR205 and E-cadherin, regardless of inflammatory breast cancer phenotypes.

Discussion

The current study demonstrated a clear segregation and differential expression of microRNAs between breast tumor and normal samples. The miR-205 expression was significantly lower in inflammatory breast cancer compared with noninflammatory breast cancer tumors by microarray and qPCR analyses of fresh tissue, and by *in situ* hybridization on formalin-fixed, paraffin-embedded tissue. Lower expression of miR-205 was associated with shorter distant metastasis-free survival and overall survival. Further studies are warranted to explore miR-205 as a potential target for inflammatory breast cancer.

Two miRNA profiling studies on inflammatory breast cancer by quantitative RT-PCR have been previously reported.[64,65] In one study, the authors evaluated the expression of 384 miRNAs in 20 inflammatory breast cancer and 50 noninflammatory breast cancer fresh frozen samples, and identified 13 miRNAs whose expression levels were different between inflammatory breast cancer and noninflammatory breast cancer, including 6 with increased expression in inflammatory breast cancer (miR-335, miR-337-5p, miR-451, miR-486-3p, miR-520a-5p,

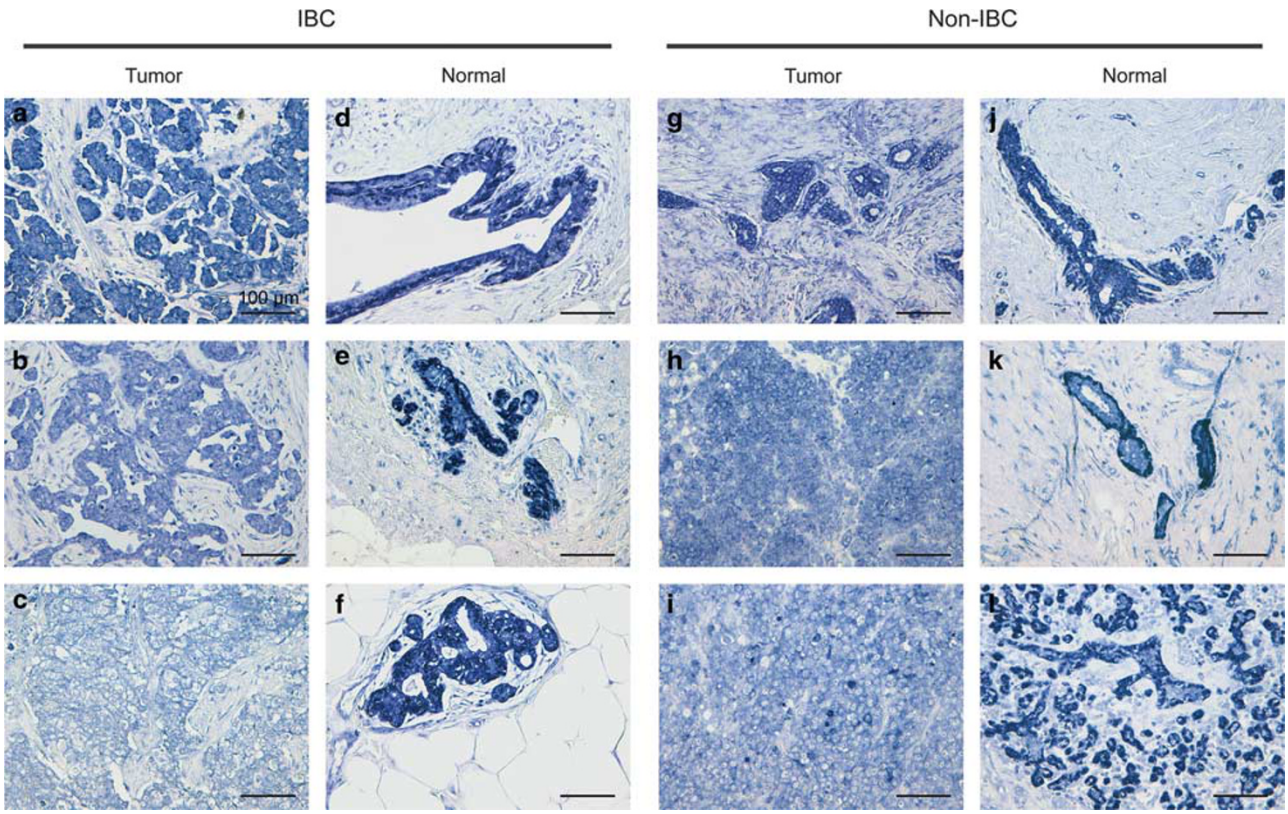


Figure 4 MiR-205 *in situ* hybridization. Blue represents positive staining. (**a–c**) and (**g–i**) are in descending order of staining intensity, respectively. The cases shown in this figure are indicated by arrows in Figure 5. (**a–c**) Representative inflammatory breast cancer (IBC) cases. (**d–f**) Staining of the corresponding normal breast epithelium on the same sections of the IBC tumors to their left. (**g–i**) Representative non-IBC cases. (**j–l**) Staining of the corresponding normal breast epithelium on the same sections of the non-IBC tumors to their left.

and miR-548d-5p) and 7 with decreased expression in inflammatory breast cancer (miR-15a, miR-24, miR-29a, miR-30b, miR-320, miR-342-5p, and miR-342-3p).[64] In the other study, the expression of 804 miRNAs in 12 inflammatory breast cancer and 31 noninflammatory breast cancer human samples was examined, and the differentially expressed miRNAs were then validated in 65 inflammatory breast cancer and 95 noninflammatory breast cancer human samples. Thirteen miRNAs were validated to be differentially expressed between inflammatory breast cancer and noninflammatory breast cancer, including 12 with increased expression in inflammatory breast cancer (miR-7, miR-21, miR-301b, miR-324-5p, miR-421, miR-486, miR-503, miR-720, miR-1234, miR-1274a, miR-1308, and miR-1825) and 1 with decreased expression in inflammatory breast cancer (miR-1303). In addition, a 5-miRNA signature composed of miR-421, miR-486, MiR-503, miR-720, and miR-1303 was predictive for inflammatory breast cancer.[65] Apparently, the two published miRNA profiles in inflammatory breast cancer and our study results do not overlap. Small sample size may partly contribute to the lack of common profile. In addition, the selection of the noninflammatory breast cancer group varied among the three studies. The second published study selected a group of noninflammatory breast cancer cases that were significantly different from the inflammatory breast cancer ones in age, tumor histologic grade, stage, estrogen receptor status, progesterone receptor status, HER2 status, and distant metastasis, in contrast to the other two studies where the inflammatory breast cancer group and noninflammatory breast cancer group were only significantly different in tumor stage. Whereas the first published study included a considerable proportion of early-stage patients in the noninflammatory breast cancer group (42% of the noninflammatory breast cancer cases were stage I), our study included only clinically and/or pathologically T4, or tumor ≥ 5 cm, or T2 (tumor ≥ 3 cm) N2 patients in the noninflammatory breast cancer group, thus eliminating any early-stage patients. In our study, the inflammatory breast cancer and noninflammatory breast cancer groups were only different in pathologic stage but not clinical stage, most likely because of the fact that inflammatory breast cancer patients were not clinically restaged after neoadjuvant chemotherapy based on residual tumor size (T stage) as noninflammatory breast cancer patients, and hence the inflammatory breast cancer patients in our cohort appeared to have a higher

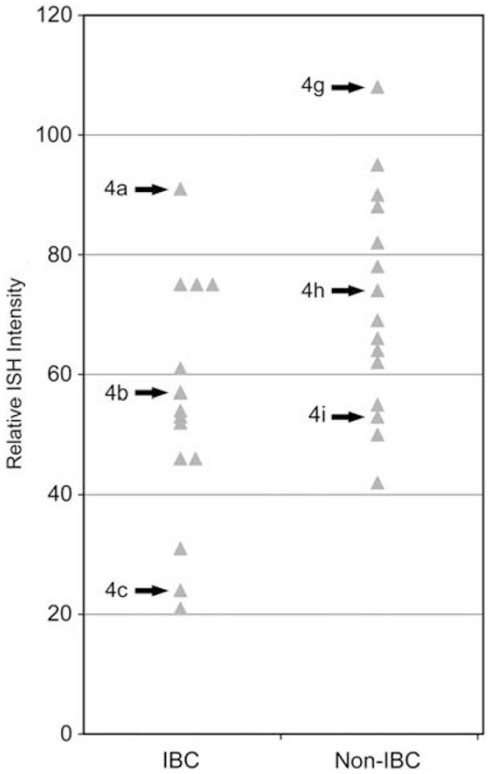


Figure 5 The miR-205 *in situ* hybridization results showing relative intensities. Each triangle represents one tumor. Arrows on the left sides of the triangles mark the tumors shown in Figure 4. IBC, inflammatory breast cancer.

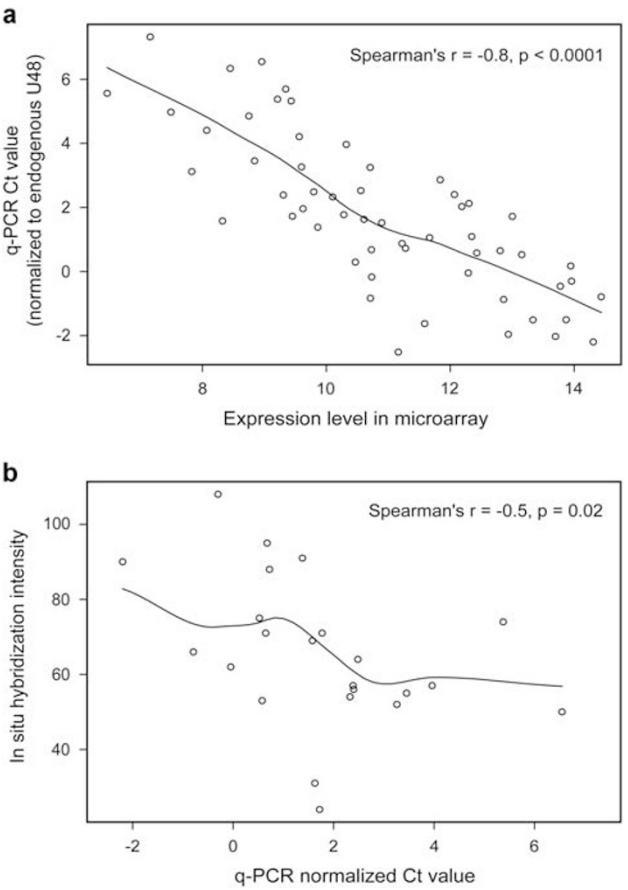
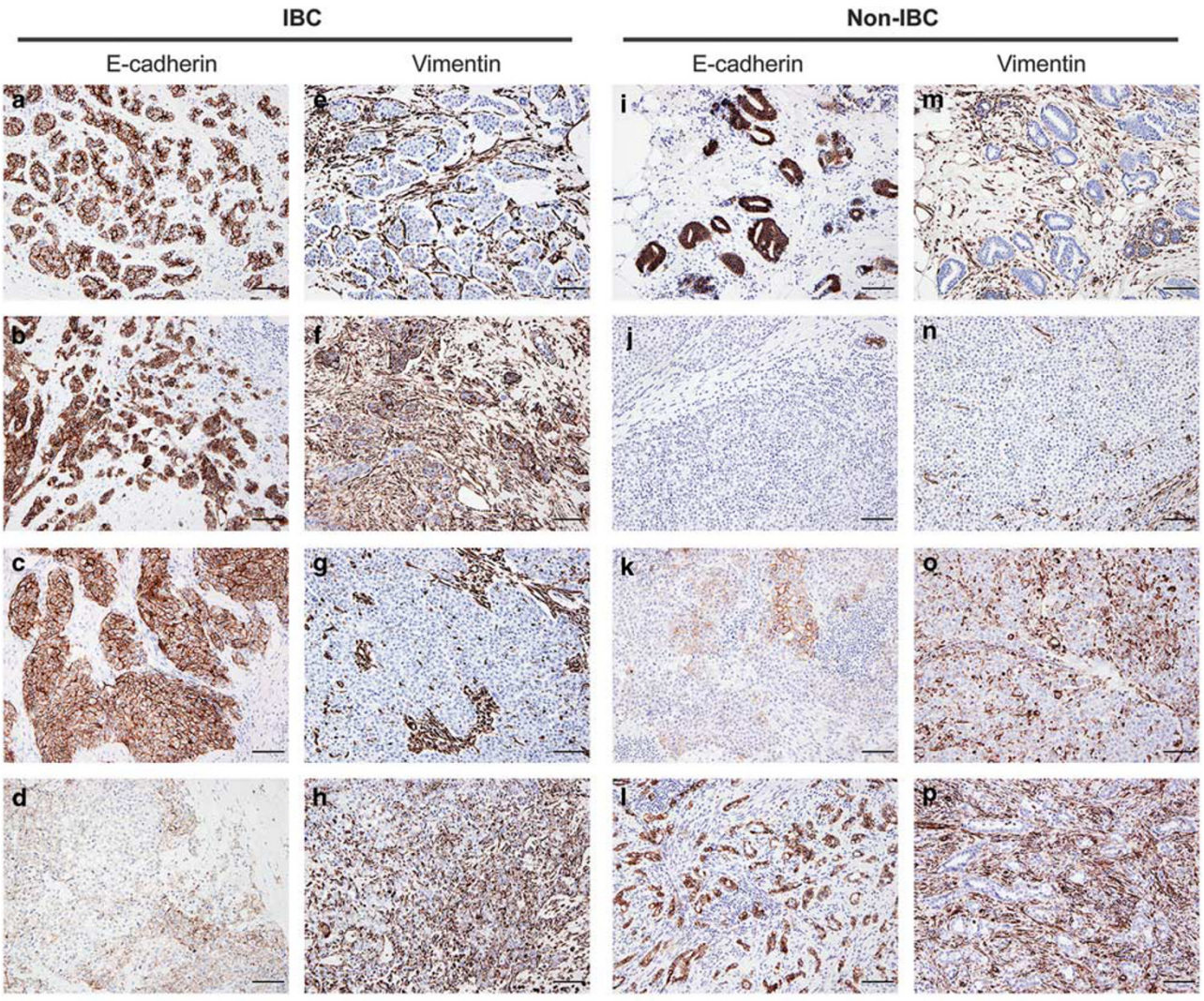


Figure 6 Correlation between different miR-205 analyses. (**a**) Correlation between microarray analysis and quantitative PCR. (**b**) Correlation between quantitative PCR and *in situ* hybridization. Ct, cycle threshold.

pathologic stage. Overall, our study results represented a miRNA profile of advanced breast cancers, and the differential expression of miRNA was obtained between inflammatory breast cancer and more advanced noninflammatory breast cancer in comparison with the other two studies. Nevertheless, the significant miRNAs from the three studies may serve as candidate markers for inflammatory breast cancer and can be further investigated.

Human miR-205 is located at the junction of the second intron and third exon of LOC642587 locus in chromosome 1.[66] It has been found that miR-205 is a highly conserved miRNA with homologs in different species. Recent studies on the functions of miR-205 have implicated its role in normal development and cancer. In mouse mammary gland development, miR-205 is strongly expressed in the basal epithelial cells, including both myoepithelial cells and basal stem cells, until the mature virgin stage, and has increased expression in both the luminal and basal epithelium during pregnancy and in late involution.[67] In addition, high expression of miR-205 has been observed in stem cell-enriched populations of normal mouse mammary epithelial cells isolated by FACS purification.[68] Thus, miR-205 may play a role in mammary epithelial stem cell proliferation and differentiation. Its functions in cancer appear to be tissue type specific. MiR-205 expression level is reportedly upregulated in human samples of lung cancer,[69] bladder cancer,[70] and endometrioid adenocarcinoma,[71] and downregulated in breast cancer,[72–74] prostate cancer,[75] and melanoma.[76] It has been reported that in a cohort of early breast cancer patients, decreased miR-205 is associated with worse disease-free interval and overall survival.[77] Similarly, in our cohort of advanced breast cancer patients, lower miR-205 expression is also associated with worse survival.

In cultured breast cancer cell lines, miR-205 is shown to suppress proliferation, clonogenic survival, anchorage-independent growth, and invasiveness, indicating its role in tumor cell growth, invasion, and metastasis.[72] Furthermore, miR-205 has been found to negatively regulate epithelial–mesenchymal transition, an essential early step in tumor metastasis. In cell culture models, miR-205, along with the miR-200 family, suppresses the expression of ZEB1 and ZEB2, repressors of E-cadherin transcription that have been implicated in epithelial–mesenchymal transition.[60] Downregulation of miR-205 is also seen in epithelial–mesenchymal transition induced by mammosphere culture of breast cancer cell line MCF-7.[78] Interestingly,



IBC			Non-IBC		
ISH	E-cadherin	Vimentin	E-cadherin	Vimentin	ISH
91	Positive	Negative	Positive	Negative	108
75	Positive	Positive	Negative	Negative	78
31	Positive	Negative	Marked decrease/patchy	Positive	53
24	Marked decrease/patchy	Positive	Positive	Negative	42

Figure 7 Immunohistochemical staining for E-cadherin and vimentin. The inflammatory breast cancer (IBC) cases are shown on the left (**a–d**, E-cadherin; **e–h**, vimentin in corresponding cases) and the noninflammatory breast cancer cases on the right (**i–l**, E-cadherin; **m–p**, vimentin in corresponding cases). The table summarizes the results in the same order as shown in the photomicrographs, with the relative miR-205 *in situ* hybridization intensities on the left side for the IBC tumors and on the right side for the non-IBC tumors.

expression of miR-205 is downregulated in breast cancer cell lines that belong to claudin-low subtype, which is known to be associated with high epithelial–mesenchymal transition,[79] as well as in triple-negative breast cancer human samples.[74,80] Of note, the commonly used triple-negative inflammatory breast cancer cell line SUM149, unlike the claudin-low subtype of breast cancer cell lines, does not contain a detectable mesenchymal subpopulation[81,82] and demonstrates a much higher expression level of miR-205 than the triple-negative breast cancer cell lines with a mesenchymal phenotype.[79] Consistent with its potential role in tumor metastasis demonstrated *in vitro*, a recent miRNA expression profiling study of human metastatic cancers demonstrated miR-205 downregulation in metastatic breast cancer in lymph nodes compared with primary breast cancers, but not

in metastatic cancers from the colon, bladder, or lung.[26] Identified direct targets of miR-205 in breast cancer include VEGF-A, a key regulator of angiogenesis and tumor metastasis,[72] E2F1, LAMC1,[79] and HER3.[72,73] Heterodimerization of HER2–HER3 leads to the activation of PI3K/Akt survival pathway, critical for HER2-mediated tumorigenesis. It was found that enforced expression of miR-205 in cultured breast cancer cells increases the responsiveness to EGFR inhibitor Gefitinib and EGFR/HER2 inhibitor Lapatinib.[73] It is conceivable that miR-205 may affect tumor growth and metastasis by simultaneously targeting multiple genes. Additional targets of miR-205 identified in other tissue types have not been validated in breast cancer.[83–86] Likely, miR-205 plays a role in specific pathways in tumorigenesis and tumor progression in a cell type-specific manner.

It has been reported that miR-205 is restricted to myoepithelial cells in normal epithelial structures,[58,59] and its expression is reduced or completely eliminated in matching tumor specimens.[58] However, our *in situ* hybridization analysis showed expression in luminal cells in normal ducts and lobules in some cases. This observation suggests that miR-205 expression may be regulated by physiologic changes in the mammary tissue, similar to the shift of expression from the basal epithelium to both the basal and luminal epithelium in mouse mammary gland development.[67] We did observe a stronger expression in the basal cell layer in the majority of cases, and it is possible that the less frequent expression in luminal cells was not observed in previous studies because of the use of tissue microarrays.

It has been reported that in early invasive breast cancer, miR-205 expression detected by *in situ* hybridization is positively related to ductal morphology, presumably because miR-205 regulates E-cadherin expression, and E-cadherin is known to be lost in lobular carcinoma.[59] However, in the cohort of advanced breast cancers used for the current study, the two invasive lobular carcinomas (noninflammatory breast cancer) appeared to have intermediate staining of miR-205 compared with other ductal carcinomas, suggesting that the regulation of E-cadherin many have different mechanisms. Furthermore, it has been shown that E-cadherin is expressed at higher levels in inflammatory breast cancer compared with noninflammatory breast cancer, regardless of ductal *vs* lobular phenotype.[62,63] However, from the role of E-cadherin in epithelial–mesenchymal transition, it is expected that inflammatory breast cancer would have decreased expression of E-cadherin, although it is possible that its expression is temporally controlled. Consistent with the latter hypothesis, one study showed that the xenografts of MDA-IBC-3 cells (derived from inflammatory breast cancer patients) had faster growth rate when coinjected with human bone marrow-derived mesenchymal stem cells, and had decreased expression of E-cadherin.[87] The mesenchymal stem cells used in that study were known to promote epithelial–mesenchymal transition and breast cancer metastasis. Although our results on E-cadherin and vimentin expression raise the possibility that a subset of tumors with low miR-205, including but not restricted to inflammatory breast cancer, may have decreased E-cadherin expression and expression of vimentin even though they have ductal differentiation, the data are very limited. Additional large studies are necessary to establish the correlation between E-cadherin, vimentin, and miR-205 expression.

In summary, by microarray analysis and quantitative RT-PCR performed on fresh frozen tissue and *in situ* hybridization on formalin-fixed, paraffin-embedded samples, our study showed that miR-205 was downregulated in advanced breast cancer, especially in inflammatory breast cancer. Recent studies have shown that in mouse models, delivery of miR-205 through nanoliposomes can sensitize breast tumors to radiation,[88] and induction of miR-205 expression by an antioxidant negatively modulates epithelial–mesenchymal transition and inhibits triple-negative breast cancer metastasis.[89] Thus, miR-205 may be a potential therapeutic target for advanced breast cancer including inflammatory breast cancer.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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