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Clinical Cancer Research

Identification of Circulating MicroRNA Signatures for Breast Cancer Detection

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Abstract

Purpose: There is a quest for novel noninvasive diagnostic markers for the detection of breast cancer. The goal of this study is to identify circulating microRNA (miRNA) signatures using a cohort of Asian Chinese patients with breast cancer, and to compare miRNA profiles between tumor and serum samples.

Experimental Design: miRNA from paired breast cancer tumors, normal tissue, and serum samples derived from 32 patients were comprehensively profiled using microarrays or locked nucleic acid real-time PCR panels. Serum samples from healthy individuals ($n = 22$) were also used as normal controls. Significant serum miRNAs, identified by logistic regression, were validated in an independent set of serum samples from patients ($n = 132$) and healthy controls ($n = 101$).

Results: The 20 most significant miRNAs differentially expressed in breast cancer tumors included miRNA (miR)-21, miR-10b, and miR-145, previously shown to be dysregulated in breast cancer. Only 7 miRNAs were overexpressed in both tumors and serum, suggesting that miRNAs may be released into the serum selectively. Interestingly, 16 of the 20 most significant miRNAs differentially expressed in serum samples were novel. MiR-1, miR-92a, miR-133a, and miR-133b were identified as the most important diagnostic markers, and were successfully validated; receiver operating characteristic curves derived from combinations of these miRNAs exhibited areas under the curves of 0.90 to 0.91.

Conclusion: The clinical use of miRNA signatures as a noninvasive diagnostic strategy is promising, but should be further validated for different subtypes of breast cancers. *Clin Cancer Res; 19(16); 4477–87. ©2013 AACR.*

Introduction

Breast cancer remains the leading cause of mortality in women (1) despite improvements in cancer screening and treatment strategies. Mammography is the current gold standard for breast cancer detection, but can have false negative rates of up to 20% [National Cancer Institute (Bethesda, MD) data; http://www.cancer.gov]. The diagnosis of breast cancer relies on the histologic examination of tissue biopsies or cytology of fine-needle aspirates, which are both invasive procedures. Known serum-based tumor markers, such as CA15.3 or BR27.29, cannot be used for breast cancer detection due to their low sensitivity (2). Thus, there is a need to develop novel markers that are minimally invasive for the improved detection of breast cancer.

microRNAs (miRNA) are approximately 22 nt long noncoding RNAs that can base pair specifically with target mRNAs to induce gene silencing through specific mechanisms involving translational repression or transcript degradation (3). Since their discovery in 1993 (4), miRNA s have been estimated to regulate more than 60% of all human genes (5), with many miRNAs identified as key players in critical cellular functions such as proliferation (6) and apoptosis (7). The current database of MiRNAs, MirBase release 19, has more than 2,000 entries of human MiRNAs constituting a major class of regulatory molecules.

Iorio and colleagues provided the earliest observation that miRNAs are differentially expressed in breast cancer tumors as compared with normal breast tissue (8). Analysis of 76 breast cancer tumors and 10 normal samples (noncancerous breast tissues) using microarrays, which probed for 386 miRNAs, identified 29 dysregulated miRNAs. To identify the dysregulated miRNAs, Persson and colleagues (9) conducted extensive next-generation miRNA sequencing of paired tumor and normal tissue from 5 patients with breast cancer, and detected more than 500 miRNAs, including a novel miRNA (miR-4728) encoded within the *Her2* gene, which was overexpressed in *Her2*-amplified tumors. A plethora of studies have led to the identification of

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Translational Relevance

The high prevalence of breast cancer and the good prognosis for patients with early-stage disease have motivated an active search for diagnostic biomarkers for breast cancer detection. Traditional diagnosis by mammography has its limitations as a screening tool, and efforts to identify serum markers have generally revealed a lack of diagnostic ability of serum proteins. Although there is increasing evidence that microRNAs (miRNA) are linked to malignancy, little is known about the status of circulating miRNAs in breast cancer, or their relationship to miRNAs in the tumor cell. In this study, discriminatory circulating miRNA signatures for breast cancer were identified and validated. Of note, little correlation between tumor and serum expression of miRNAs was observed. The novel miRNA expression signatures identified in this study had sufficient diagnostic efficacy for development into blood-based biomarkers for breast cancer detection.

miRNAs that were differentially expressed depending on breast cancer subtype (10), histologic grade (10), cancer aggressiveness (11, 12), metastasis-free survival (13, 14), as well as estrogen receptor (ER; refs. 10, 12, 15, 16), Her2 (15, 16), or triple-negative status (11, 12, 14, 15).

Circulating miRNAs have been suggested to be able to distinguish breast cancer samples from healthy controls. These studies have usually involved targeted analyses of only 4 to 6 miRNAs by RT-PCR (17–19). However, comparisons between these studies may not be straightforward as they were carried out under diverse experimental conditions. For example, circulatory miRNAs may have been extracted from serum (17, 18), plasma (20, 21), circulating tumor cells (22), or even whole blood (19, 23, 24). Further, although most studies used serum samples collected preoperatively as it has been suggested that miRNA levels may return to baseline within 2 weeks after tumor resection, one other study used postoperative sera (17, 25). Circulating miRNAs may also exhibit racial differences, as the microarray profiling of miRNAs in the plasma of 10 cases each from Caucasian and African patients with breast cancer resulted in only 2 common dysregulated miRNAs between these groups (20). In contrast to targeted studies involving specific miRNAs, there are few comprehensive profiling studies of circulatory miRNAs in breast cancer (20, 26), and a consistent diagnostic signature for circulatory miRNAs is not yet available.

Few studies have attempted to compare the circulatory miRNA profile to that within the breast cancer tumor, such that the relationship between these 2 profiles of miRNAs is not clear. One study assessed a panel of 7 miRNAs (27), whereas another analyzed 5 miRNAs (28). In a third study, four most discriminating miRNAs, selected from discovery profiling of breast cancer tumors ($n = 84$) and normal tissue samples ($n = 8$), were validated using serum samples from patients with breast cancer ($n = 75$) and healthy volunteers ($n = 20$; ref. 29). Of these 4 miRNAs, which were repressed in breast cancer tumors as compared with normal breast tissues, 3 were also repressed in the sera of patients with breast cancer. A recent study (30) investigated the status of 4 plasma-derived miRNAs in matched tumors, and concluded that miRNAs generally displayed opposite expression patterns in tissue and plasma. However, these comparisons between circulating and tumor miRNA profiles were not comprehensive, as miRNA profiling of the serum or plasma samples were not done.

This study aimed to (i) identify significant miRNAs that are differentially expressed in matched breast cancer tumor tissues and sera samples, (ii) investigate the correlation between miRNAs in the tumor with circulating miRNAs, and (iii) validate the serum-derived miRNA signatures in an independent set of serum samples from patients with breast cancer ($n = 132$) and healthy controls ($n = 101$).

Materials and Methods

Patients

Patients and healthy volunteers were Singaporeans of Chinese ancestry. Written informed consent was obtained from all contributing patients and volunteers, and ethics approval for this study was obtained from the Centralized Institutional Review Board of SingHealth (Singapore). Histopathological records (ER, Her2, and lymph node status) were obtained from SingHealth Tissue Repository.

Tissue and serum samples for the profiling stage

Matched fresh frozen breast cancer tumors, adjacent normal tissues, and preoperative sera from 32 patients with breast cancer were obtained from the SingHealth Tissue Repository. Control serum samples were recruited from 22 healthy female volunteers. The mean age $\pm$ SD for the patients at diagnosis, and healthy volunteers at time of recruitment, were 50 ± 13 years and 47 ± 6 years, respectively. Of the 32 patients with breast cancer used for the profiling stage, 3 (9%), 15 (46%), 9 (28%), or 2 (6%), were diagnosed with stage 1, 2, 3, or 4 cancer, respectively.

All tissue samples were histologically confirmed by a pathologist using hematoxylin and eosin staining of cryosectioned specimens. One tumor sample was rejected because of failure to detect any tumor cells. Except for 2 samples (with 30% and 40% tumor cells), all tumor tissues used had a minimum of 60% tumor cells, as estimated microscopically (Supplementary Table S1). Overall, the breast cancer tumor samples had an average of about 70% tumor cells. The criteria for adjacent normal tissue were absence of tumor cells and presence of epithelial cells. Hence, after histologic confirmation, 31 breast cancer tumors and 23 matched normal tissues were used for miRNA extraction and profiling using microarray.

Blood samples were collected in Becton Dickinson Vacutainer SST tubes. Serum was harvested by centrifugation at 2,200 g after allowing blood to clot for 30 minutes. Thirty-two matched serum samples from patients with breast cancer and 22 samples from healthy controls were obtained for profiling. Sera samples were stored at -80°C.

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Serum samples for the validation stage

Additional serum samples from patients with breast cancer ($n = 132$) were obtained from the SingHealth Tissue Repository (Supplementary Table S1), and additional control serum samples ($n = 101$) were recruited from healthy female volunteers. The mean age ± SD for the patients with breast cancer at diagnosis and healthy volunteers at time of recruitment were 54 ± 11 years and 48 ± 7 years, respectively. Of the 132 patients with breast cancer used for the validation stage, 20 (15%), 52 (39%), 29 (21%), or 11 (8%), were diagnosed with stage 1, 2, 3, or 4 cancer, respectively, and staging information was not available for 20 patients.

microRNA extraction

miRNAs were extracted from tissue or serum samples using the miRVana (Life Technologies) or miRNeasy (Qiagen) kits, respectively, according to manufacturers' instructions. For miRNeasy, the standard protocol was modified on the basis of Exiqon's application note "RNA Purification from Blood Plasma & Serum" (http://www.exiqon.com/ls/Documents/Scientific/serum-plasma-RNA-isolation.pdf), which used MS2 (Roche) as a carrier. miRNA extraction was carried out using 6 to 10 pieces of tissue (~1 × 1 × 1 mm) or 250 μL of serum as the starting material. Quality control of RNA from tissue samples was carried out using the Agilent Bioanalyzer. Quality control of serum samples was carried out using singleplex locked nucleic acid real-time PCR (LNA RT-PCR; Exiqon) and LNA primers for serum markers (miR-16 and miR-20a).

Reverse transcription and RT-PCR

For quality control and individual LNA RT-PCR assays, reverse transcription was carried out using the Universal cDNA Synthesis kit (Exiqon), using 4 μL of miRNA containing total RNA, 2 μL of enzyme mix, and 4 μL of 5× reaction buffer, made up to a 20 μL reaction volume using nuclease-free water. Reverse transcription was carried out at 42°C for 60 minutes, followed by inactivation at 95°C for 5 minutes. Every RT-PCR experiment included no reverse transcription controls. For RT-PCR, 10 μL reactions were prepared in the following proportions: 5 μL of SYBR Green master mix, 1 μL of LNA primer mix, and 4 μL of cDNA template (55× dilution). RT-PCR was conducted at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds/60°C for 1 minute using an Applied Biosystems 7500 Real-Time PCR System (Life Technologies).

microRNA microarray and LNA RT-PCR panels

The Agilent human miRNA microarray was based on miRBase Release 16.0, with probes for about 1,300 miRNAs. The microarray is based on a direct labeling (Cy3) chemistry and was carried out according to the manufacturer's standard protocol. Each microarray experiment used 200 ng of miRNA containing total RNA.

The LNA RT-PCR human miRNA panels (Exiqon) comprised two 384-well plates for the detection of 742 miRNAs. Reverse transcription was conducted using the Universal cDNA Synthesis kit (Exiqon) in 40 μL reactions per panel, using 8 μL of miRNA containing total RNA, 4 μL of enzyme mix, and 8 μL of 5× reaction buffer, made up to 40 μL using nuclease-free water. For each 384-well plate, the cDNA was diluted 55× (using 2160 μL of nuclease-free water). Two milliliter of the diluted cDNA was combined with an equal volume of 2× SYBR Green Master Mix (Exiqon) and dispensed at 10 μL per well. The RT-PCR was executed according to Exiqon's protocol for serum and plasma on an Applied Biosystems 7900HT Real-Time PCR System (Life Technologies) which was set using run templates (SDS files) downloaded from Exiqon's website.

The Gene Expression Omnibus accession number for the miRNA expression profiles from the microarray and RT-PCR panels reported in this study is GSE42128.

Biocomputational analysis

Microarray expression data was imported into the GeneSpring software (Agilent). Global normalization was carried out based on 90 percentile shift followed by $\log_2$ transformation. Principal component analysis (PCA), paired and unpaired *t* test, and cluster analysis were computed using the GeneSpring software.

C_t values from RT-PCR were imported into the GenEx software (Exiqon). The analysis workflow included (i) quality control using no reverse transcription controls, (ii) interplate calibration, (iii) selection of reference genes using NormFinder and GeNorm, and (iv) normalization and $\log_2$ transformation. PCA, cluster analysis, *t* test (unpaired, two-tailed), Mann–Whitney test (two-sided), and Kolmogorov–Smirnov test (for normal distribution) were done using the GenEx software where appropriate.

To derive the most important serum miRNA species for the validation stage, breast cancer-associated serum miRNAs that remained significant after Bonferroni correction ($n = 21$) were used for analysis by collinearity statistics so as to obtain sets of noncollinear miRNA markers suitable for logistic regression (31). miRNAs showing evidence of collinearity are not desirable as diagnostic markers because collinearity will amplify errors in the subsequent regression analysis. Variance inflation factor (VIF) scores of ≥ 5 was taken as indicative of collinearity, and thus only miRNAs with VIF <5 were used for logistic regression. Sets of miRNAs derived with VIF < 5 were subjected to binary logistic regression (32, 33). Binary logistic regression was carried out using the PASW software (IBM Corporation; version 18) using the Forward: likelihood ratio method. Receiver operating characteristic (ROC) curves were plotted using PASW.

Data reproducibility

To verify the reproducibility of the microarray platform, technical replicates were conducted for 4 samples. The R^2 values obtained from the correlation plots between replicates ranged from 0.96 to 0.99 (data not shown), confirming the technical reproducibility of the platform. Three other tissue samples were extracted twice using the miRVana kit and subjected to microarray analysis. The R^2 values ranged from 0.89 to 0.96 for the correlation plots between

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the duplicate samples (data not shown), validating the consistency of the miRVana extraction method.

Similarly, to validate the consistency and reliability of the LNA RT-PCR platform, one sample was reversed transcribed twice and run on the LNA RT-PCR panels, with $R^2 = 0.97$ obtained on the correlation plot, confirming the reproducibility of this platform (data not shown). A no reverse transcription control was also run on a complete set of the LNA RT-PCR panels. Input of the resultant background C_t values into the quality control workflow in the GenEx program did not identify any problematic miRNA with C_t values considered too close (within 3 cycles) to background values.

Results

microRNA profiling of tumor and adjacent normal tissue samples

Significant differentially expressed miRNAs were identified by applying the paired *t* test (23 pairs of breast cancer tumors vs. adjacent normal tissues) or the unpaired *t* test (31 breast cancer tumors vs. 23 adjacent normal tissues). This resulted in 73 miRNAs that were significant ($P \leq 0.05$) after correction for multiple testing by Benjamini–Hochberg false discovery rate (FDR) in both paired as well as unpaired *t*tests. The 20 most significant miRNAs with corrected *P* values ranging from 1.6E-06 to 8.0E-09 are shown in Table 1. A complete list of significant miRNAs is provided in Supplementary Table S2. Seven out of 20 dysregulated miRNAs were overexpressed.

Three component PCA (Supplementary Fig. S1A) was able to cluster 84.4% of the samples into tumor and normal tissue groups. Nonsupervised hierarchical clustering of the expression profiles of breast cancer tumors and adjacent normal tissues based on Euclidean distance using the 20 most significant miRNAs in a self-organizing map was able to cluster the majority of breast cancer tumors from the adjacent normal tissues (Fig. 1A).

Table 2 lists the miRNAs that were significantly associated with ER, Her2, and lymph node positivity, as determined using the unpaired Student *t* test, without correction for FDR. Interestingly, almost all of the differentially expressed miRNAs were novel, with the majority being unique from those identified in other studies (11–13, 15, 16). Notably, these previous studies (11–13, 15, 16) did not share common significant miRNAs between each other. A complete list of miRNAs that are associated with ER, Her2, and lymph node positivity is provided in Supplementary Table S3.

microRNA profiling of serum samples

Among the 6 suggested reference gene candidates provided in the LNA RT-PCR panels, both the geNorm and NormFinder algorithms identified miR-103 and miR-191 as the most stably expressed, best gene combination for use as reference genes for normalizing the RT-PCR data. Statistical analysis of the serum miRNA profiles led to the identification of 85 miRNAs that were significant ($P \leq 0.05$) after FDR correction for multiple testing. The most significant 20 miRNAs are shown in Table 1, and 18 of these were upregulated in breast cancer. Most of these miRNAs seemed to be novel and have not been reported in the context of circulating miRNA in breast cancer. A complete list of significant miRNAs identified from serum is provided in Supplementary Table S2.

PCA (Supplementary Fig. S1B) and cluster analysis using the 20 most significant miRNAs (Fig. 1B) were able to cluster the breast cancer sera from those belonging to healthy controls.

Furthermore, serum miRNAs differentially expressed according to ER, Her2, and lymph node status could also be identified (Table 2) using the unpaired *t* test without correction for FDR. A complete list of serum miRNAs differentially expressed according to ER, Her2, and lymph node status is shown in Supplementary Table S4.

Interplatform comparison

Because the serum and tissue samples were extracted and profiled using different kits and platforms, we sought to ascertain that the breast cancer serum and tumor datasets are comparable. Hence, the correlation between the miRVana and miRNeasy extraction methods, and that between the Agilent miRNA microarray and LNA RT-PCR panels, were examined. All the 742 miRNA detected by LNA RT-PCR panels were also included in the miRNA microarray ($n = 1300$). Profiling of the same breast cancer tumor, extracted by mirVana or miRNeasy on the LNA RT-PCR panels showed a high degree of correlation between these extraction methods ($R^2 = 0.96$; Supplementary Fig. S2A). Profiling of the same breast cancer tumor sample on microarray and RT-PCR showed appreciable correlation for the 742 miRNAs common between these platforms ($R^2 = 0.61$; Supplementary Fig. S2B), suggesting that they have comparable dynamic ranges, and that the microarray and RT-PCR datasets are comparable.

Comparison between the breast cancer serum and breast cancer tumor profiles

Interestingly, there were only 7 common significant miRNAs that were overexpressed in both breast cancer tumors and sera from patients with breast cancer, and one miRNA that was downregulated in both sample types (Table 3). Another 13 miRNAs were dysregulated in breast cancer sera and tumors, but in opposite directions. Hence, circulating miRNAs are not highly similar to those within breast cancer cells, suggesting that some miRNAs are released into the circulation selectively.

Validation of miR-1, miR-92a, miR-133a, and miR-133b

Twenty-three breast cancer-associated serum miRNAs, with *P* values that remained significant after Bonferroni correction ($P \leq 1.3E-04$), were selected for analysis by collinearity statistics. As a result, 3 sets of miRNAs were derived, in which each set comprised 10 miRNAs with VIF < 5 and were hence not impeded by collinearity (Fig. 2). Logistic regression was carried out to identify miRNA signatures with the highest diagnostic efficacy for further validation. As a result, 3 models were identified (Fig. 2), which comprise miR-1, miR-92a, miR-133a, and miR-133b as the most important diagnostic miRNA markers.

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Table 1. Twenty most significant miRNAs differentially expressed in breast cancer tumors versus adjacent normal tissues, and in breast cancer sera versus sera from healthy individuals. Other studies which have also reported the miRNAs in relation to breast cancer are referenced

Systematic name	FDR Corrected *P*	Regulation	Fold change	References
Breast cancer tumors versus adjacent normal tissues				
miR-145	8.04E-09	Down	2.48	(8, 9)
miR-21	1.23E-07	Up	1.95	(8, 9)
miR-497	1.76E-07	Down	2.02	(46)
miR-720	2.16E-07	Up	2.05	
miR-1274b	2.16E-07	Up	1.92	
miR-99a	2.47E-07	Down	2.33	(9)
miR-195	3.16E-07	Down	1.69	(47)
miR-143	3.42E-07	Down	1.61	(8, 9)
miR-1260	4.15E-07	Up	1.84	
miR-30c	4.43E-07	Down	1.73	
miR-125b	6.88E-07	Down	1.66	(8)
miR-140-5p	6.96E-07	Down	1.29	(8, 9)
miR-10b	6.96E-07	Down	1.45	(8, 9)
miR-376c	1.02E-06	Down	2.08	
miR-103	1.02E-06	Up	0.78	(48)
miR-100	1.04E-06	Down	1.67	(9)
miR-4324	1.19E-06	Down	2.19	
miR-93	1.19E-06	Up	1.32	(49)
miR-140-3p	1.30E-06	Down	1.34	(8, 9)
miR-107	1.63E-06	Up	0.72	(9)
Breast cancer sera versus sera from healthy female controls				
miR-1	1.77E-07	Up	3.59	
miR-133b	4.04E-07	Up	3.41	
miR-133a	8.61E-07	Up	3.29	
miR-92a	8.97E-07	Up	1.34	
miR-10b	1.67E-06	Up	1.77	(23)
miR-486-5p	3.36E-06	Up	1.65	
miR-423-5p	6.38E-06	Up	1.10	
miR-7	6.38E-06	Up	1.64	
miR-223	1.80E-05	Down	0.98	
miR-20a	1.80E-05	Up	0.92	(50)
miR-185	2.06E-05	Up	1.04	
miR-338-3p	2.06E-05	Down	1.02	
let-7i	2.06E-05	Up	0.64	
miR-16	2.68E-05	Up	1.30	
miR-214	5.27E-05	Up	1.80	(50)
let-7b	8.04E-05	Up	0.85	(39)
miR-144*	1.18E-04	Up	1.00	
miR-16-2*	1.53E-04	Up	1.08	
miR-320a	1.97E-04	Up	0.98	
miR-93	5.45E-04	Up	0.74	

The 4 significant miRNAs identified were then subjected to validation by LNA RT-PCR using additional breast cancer sera ($n = 132$) and healthy control sera ($n = 101$). MiR-103 and miR-191, identified earlier by GenEx software as the best reference genes, were used for data normalization. Validation results were consistent with data from the sera profiling experiments. As expected, all the 4 miRNAs were overexpressed in breast cancer sera (Fig. 3A). The $\log_2$ fold changes for miR-1, miR-92a, miR-133a, and miR-133b were 2.67, 1.32, 2.52, and 2.41, respectively, comparable with those from the sera profiling experiments (3.59, 1.34, 3.29, and 3.41, respectively). The *P* values were highly significant ($P < 1E-8$) for all the 4 miRNAs (the Mann–Whitney test was used for calculating statistical significance as the C_t values

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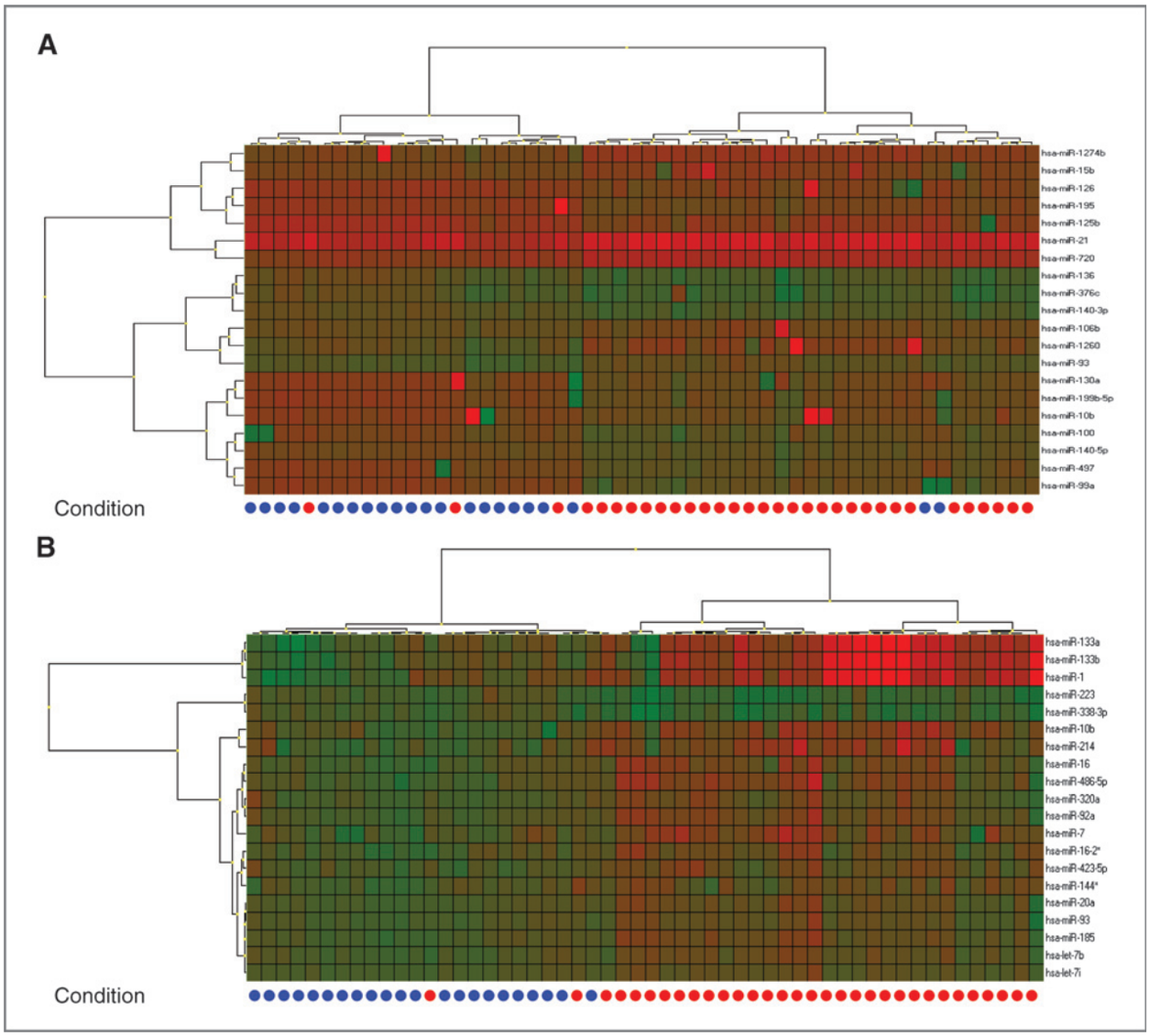

Figure 1. Hierarchical clustering of (A) tissues and (B) serum samples. A, breast cancer tumor and adjacent normal tissues are indicated with red (●) and blue (●) circles, respectively. B, serum from patients with breast cancer and healthy individuals are indicated with red (●) and blue (●) circles, respectively.

did not follow normal distribution). The resultant ROC curves plotted using the miRNA combinations derived by logistic regression showed areas under the curves (AUC) of 0.90 to 0.91 (Fig. 3B), confirming the diagnostic efficacies of the miRNA models.

Discussion

Among the 20 most significant miRNAs that are differentially expressed in breast cancer tumors identified in this study, several have also been reported to be similarly dysregulated in other studies (Table 1), attesting to the ability of our approach to isolate known differentially expressed miRNAs associated with breast cancer. Among known tumor-derived miRNAs, mir-145 and miR-21 are amongst the most consistently detected (8, 9) and are hence very attractive candidates for clinical application. Furthermore, the observation from this study that among the 20 most significant differentially expressed miRNAs in breast cancer tumors, 13 were downregulated, whereas only 7 were upregulated, is consistent with the notion that tumorigenesis is apparently more associated with downregulation of tumor-derived miRNAs (8, 34).

Six out of the 20 most significant tumor-derived miRNAs have not been previously reported in literature in association with breast cancer, suggesting that novel miRNAs can still be identified. The *in vitro* functionality of these novel miRNAs should be investigated. For example, miRNAs that

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Table 2. Ten most significant breast cancer tumor miRNAs and serum miRNAs associated with clinicopathological features

ER Positivity				Her2 Positivity				Node positivity			
Systematic name	*P*	Regulation	Fold change	Systematic name	*P*	Regulation	Fold change	Systematic name	*P*	Regulation	Fold change
Breast cancer tumor miRNAs				Breast cancer tumor miRNAs				Breast cancer tumor miRNAs			
miR-622	3.3E-04	Up	1.26	miR-361-5p	4.15E-04	Down	0.86	miR-181d	0.025	Up	0.77
miR-342-3p	0.0013	Up	1.44	miR-503	0.0010	Up	1.96	miR-24	0.025	Up	0.39
miR-25	0.0022	Down	0.61	miR-143*	0.0011	Up	1.35	miR-452	0.001	Up	2.25
miR-29a*	0.0036	Down	1.01	miR-1260b	0.0061	Up	1.09	miR-582-5p	0.015	Up	1.51
miR-1274a	0.0096	Down	1.14	miR-3613-3p	0.008	Up	0.91	miR-605	0.039	Up	0.93
miR-590-5p	0.011	Down	0.47	miR-221	0.009	Down	0.84	miR-625	0.032	Down	0.83
miR-1290	0.012	Down	2.06	miR-4284	0.013	Up	1.20	miR-636	0.006	Up	0.75
miR-106b	0.012	Down	0.50	miR-1181	0.017	Up	1.47	miR-877*	0.010	Down	1.80
miR-1181	0.013	Down	1.35	miR-335*	0.018	Down	1.62	miR-95	0.018	Down	1.03
miR-93	0.015	Down	0.68	miR-15b*	0.018	Up	0.90	miR-514b-5p	0.045	Up	1.42
Breast cancer serum miRNAs				Breast cancer serum miRNAs				Breast cancer serum miRNAs			
miR-134	0.0037	Up	1.11	miR-1908	0.0049	Down	1.01	miR-30d	0.00041	Down	0.90
miR-555	0.0047	Up	1.05	miR-450a	0.010	Down	0.89	miR-29b-1*	0.0013	Down	1.09
miR-596	0.0048	Up	1.17	miR-340	0.026	Down	1.06	let-7c	0.0014	Down	0.71
let-7g*	0.0056	Up	0.97	miR-206	0.029	Down	1.01	miR-551b	0.0019	Down	1.53
miR-671-5p	0.0079	Up	1.82	miR-28-3p	0.031	Dwn	0.70	miR-505	0.0020	Down	1.42
miR-602	0.0086	Up	1.10	miR-551a	0.038	Down	0.98	miR-379	0.0024	Down	1.25
miR-579	0.0088	Up	1.09	miR-223*	0.042	Down	0.82	miR-98	0.0027	Down	0.77
miR-181a*	0.0091	Up	0.99					miR-671-5p	0.0031	Down	1.89
miR-124	0.0094	Up	1.34					miR-485-3p	0.0043	Down	1.06
miR-23a*	0.011	Up	0.89					miR-15b	0.0068	Down	0.61

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Table 3. miRNAs differentially expressed in both breast cancer sera and breast cancer tumor tissue

miRNA	Regulation	
	Serum	Tumor
Dysregulated in the same direction		
miR-15b	Up	Up
miR-16	Up	Up
miR-17	Up	Up
miR-25	Up	Up
miR-93	Up	Up
miR-107	Up	Up
miR-185	Up	Up
miR-199a-5p	Down	Down
Dysregulated in opposite directions		
let-7a	Up	Down
let-7b	Up	Down
let-7c	Up	Down
let-7i	Up	Down
miR-10b	Up	Down
miR-130a	Up	Down
miR-143	Up	Down
miR-195	Up	Down
miR-214	Up	Down
miR-30a	Up	Down
miR-451	Up	Down
miR-142-3p	Down	Up
miR-181a	Down	Up

were upregulated (miR-720, miR-1274b, and miR-1260) or downregulated (miR-30c, miR-376c and miR-4324) in breast cancer tumors will be likely candidates for novel oncomirs or tumor suppressors, respectively.

Published studies on circulating miRNAs have identified a wide diversity of miRNAs between studies. This is not surprising, considering the wide variation of sample types (plasma, serum, or whole blood; refs. 18, 24, 30) and experimental approaches (next generation miRNA sequencing, RT-PCR profiling, or targeted analysis of specific miRNAs; refs. 17, 28, 30) used in these studies. The use of whole blood will lead to the isolation of miRNAs from many cell types including those within the blood cells (35), and not just circulating miRNAs, warranting caution when comparing miRNA profiles derived from blood with those from sera or plasma. Serum and plasma are considered equivalent, although miRNA concentration seemed to be higher in serum (36).

To our knowledge this study represents the largest serum and tumor cohort in terms of extensive profiling of miRNAs. In this study, a total number of 108 samples, including 54 sera samples, were profiled. By comparison, 2 other studies (21, 30) profiled 20 samples in their marker discovery stage. In addition, the use of appropriate normalization controls is a well-known crucial issue for RT-PCR experiments. The use of a larger profiling cohort in this study facilitated the selection of reference miRNAs empirically. Conversely, the use of a spike-in or a small RNA for data normalization in similar studies (21, 30) have sometimes been considered to be problematic due to their suspected instability (37).

Because the histopathological records for the samples used in this study were available, we were also able to identify miRNA signatures that were associated with ER, Her2, or lymph node metastasis. Such signatures may have the potential to be developed as tools to substantiate histologic tests in breast cancer. Interestingly, we also identified significant serum miRNAs that were indicative of the tumor's ER, Her2, or lymph node status. Circulating miRNAs associated with ER, progesterone receptor, and Her2 status have been reported in one other study (26). The possibility of a serologic test that can augment histologic information of a tumor without the need for biopsy is an exciting avenue for further research.

In this study, the miRNA profiles between sera and the corresponding matched tumor were largely dissimilar. Similarly, Wu and colleagues, observed that out of 19 miRNAs

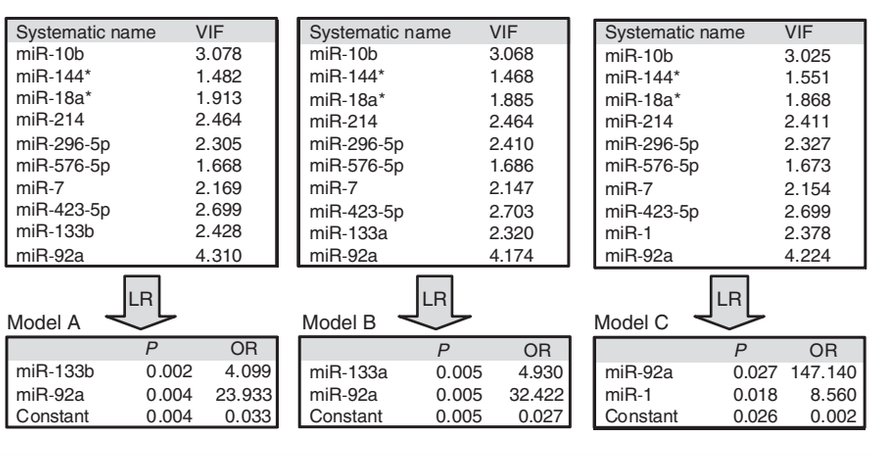

Figure 2. Test for collinearity by VIF computation and derivation of diagnostic models and significant markers by logistic regression (LR). OR, odds ratio (which is also the exponentiation of the B coefficient). Statistical significance is represented by the *P* value.

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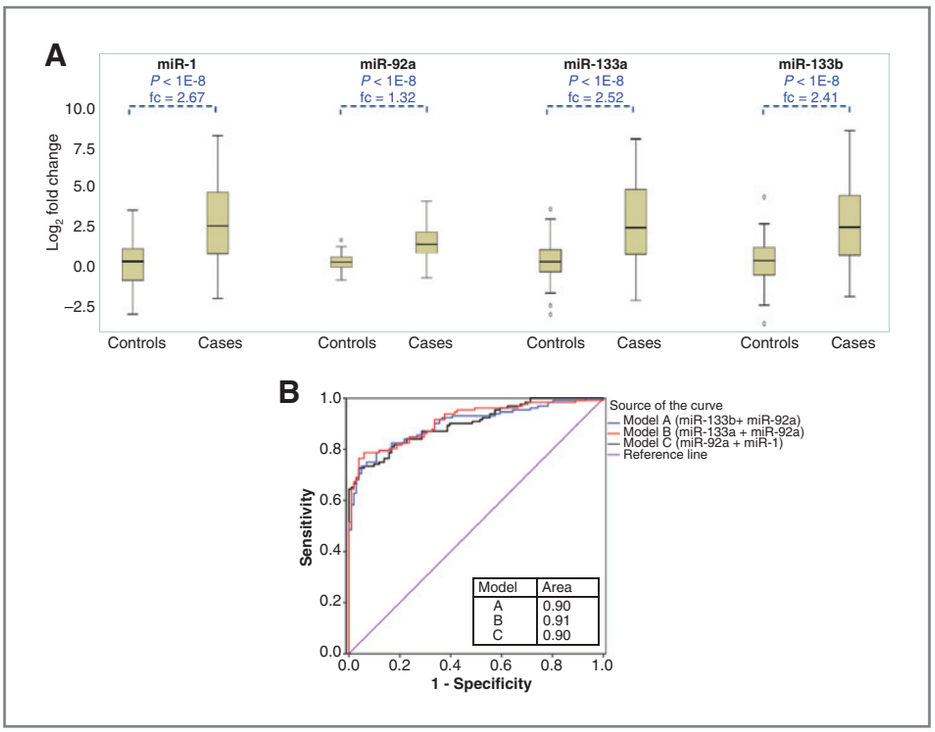

Figure 3. Validation of significant miRNAs by RT-PCR using 132 cases and 101 controls. A, box and whisker plots (generated by PASW) representing RT-PCR results for miR-1, miR-92a, miR-133a, and miR-133b, respectively. The *y*-axis depicts $\log_2$ fold change. The lines inside the boxes denote the medians. The boxes mark the interval between the 25th and 75th percentiles. The whiskers denote the interval between the maximum and minimum values. Open circles indicate outliers, defined as values beyond one and a half box lengths from either end of the box. Statistical significance was determined using the Mann–Whitney test. B, ROC curves plotted using the miRNA combinations derived by logistic regression.

that were upregulated in breast cancers, only 2 were also upregulated in sera (28). Studies on breast cancer cell lines have shown that the extracellular and cellular miRNA profiles differ, thus suggesting that circulating miRNAs do not reflect their abundance in the malignant cells (38). Furthermore, Cookson and colleagues (39), upon investigating miRNA changes in plasma after tumor resection, concluded that circulating miRNA profiles reflected the presence of breast cancers but not the profiles of miRNAs within the tumors.

In this study, we used ROC curve analysis to show the diagnostic use of 3 diagnostic models, which were derived from two-marker combinations of miR-1, miR-92a, miR-133a, and miR-133b. In a study by Cuk and colleagues, the diagnostic efficacy of 4 miRNAs (miR-148b, miR-376c, miR-409-3p, and miR-801), and that of a three-marker combination (miR-148b, miR-409-3p, and miR-801) were evaluated (30). Individually, the miRNAs had AUCs of 0.64 to 0.66, whereas the three-marker combination had an AUC of 0.69. Relatively higher AUCs of 0.90 to 0.91 were obtained for the 3 diagnostic models evaluated in this study, as well as for each of the 4 miRNAs individually (AUCs of 0.78–0.87; ROC curves not shown).

Intracellularly, miR-1, miR-92a, miR-133a, and miR-133b seem to play tumor suppressor roles in cancer cells (40–43). It is not known whether these miRNAs have antitumorigenic properties in their circulating forms. The presence of circulating miRNAs has only been recognized over the last few years (44), and the understanding of their biologic roles is just emerging. Circulating miRNAs have been proposed to play either oncogenic or tumor-suppressive roles (37). For example, exosomes containing miRNAs derived from human melanomas and colorectal carcinomas were able to promote tumor growth and immune escape (45). Alternatively, immunocytes may secrete tumor-suppressive miRNAs so as to block tumor proliferation or promote apoptosis (37).

In conclusion, serum-based miRNA signatures associated with breast cancer were successfully derived and validated. The clinical deployment of these signatures as a noninvasive diagnostic strategy is promising, and could be validated further for clinically important subtypes of breast cancer such as triple-negative or metastatic breast cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Chan, A.S. Lee
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Chan, C.S. Liaw, S.M. Ji, H.H. Tan, C.-Y. Wong, A.A. Thike, P.H. Tan, G.H. Ho
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Chan
Writing, review, and/or revision of the manuscript: M. Chan, H.H. Tan, C.-Y. Wong, P.H. Tan, G.H. Ho, A.S. Lee
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.S. Liaw, G.H. Ho
Study supervision: A.S. Lee

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