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microRNA Expression in Prospectively Collected Blood as a Potential Biomarker of Breast Cancer Risk in the BCFR

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Abstract

Background/Aim—Current breast cancer risk assessment models have moderate discriminatory ability. We evaluated whether microRNA (miRNA) expression profiles in peripheral blood mononuclear cells (PBMC) could be a useful biomarker of risk in high-risk women.

Materials and Methods—Next-generation sequencing evaluated miR expression in PBMCs of 20 women who were unaffected at the time of recruitment and later diagnosed with breast cancer and 20 unaffected women.

Results—Out of the 5 miRNAs identified as potential risk markers, miR-144-3p, miR-451a, miR-144-5p and miR-183-5p were up-regulated, while miR-708-5p was down-regulated. We then evaluated these miRs in 28 additional case/control pairs using quantitative reverse transcription polymerase chain reaction (PCR). None of the results in the validation sample were statistically significant possibly due to the much longer interval between blood collection and diagnosis in the validation set.

Conclusions—Differentially expressed miRNAs from PBMCs may be potential non-invasive biomarkers for breast cancer prediction. Larger prospective studies are required to confirm whether our findings with specific miRNA loci were related to timing before diagnosis.

Keywords

microRNA; mononuclear cells; biomarker

To assess individual risk for breast cancer development, risk assessment models have been developed that incorporate epidemiological and clinical risk factors (1). Unfortunately, current models have only moderate discriminatory ability with areas under the curve in the range of 0.55-0.70 for predicting risk for breast cancer (1). For this reason, our group and

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others have been developing molecular biomarkers, such as DNA repair phenotype, as well as white blood cell methylation, that can be added to current models to enhance their predictive value (2, 3) by targeting pathways not described by current risk assessment tools.

microRNAs (miRNAs) are non-coding RNAs of 18-25 nucleotides that are primarily associated with silencing gene expression by binding to the 3' untranslated region of target genes (4) but have also been shown to induce expression of certain genes (5). Based on computational analysis, miRNAs target more than 30% of the human genome (6). In addition, a single miRNA might have more than 1,000 target genes and a single gene can be regulated by multiple miRNAs. miRNAs, therefore, play an important role in various regulatory networks, such as cell proliferation, differentiation and immune cell function. Despite the complexities of miRNA regulatory networks, many studies have made progress toward understanding their roles in signaling pathways in breast cancer with many miRNAs identified as dysregulated in breast tumor tissue (6, 7).

Because of their small size, miRNAs remain intact and are highly resistant to degradation in the extraction process from human tissues or blood samples. Thus, studies have sought to determine whether miRNAs in tumor tissue can be diagnostic of breast cancer or whether miRNAs circulating in plasma or serum can be used for early diagnosis of breast cancer (8, 9). Many miRNAs have been identified as dysregulated in breast cancer suggesting that they may be useful for diagnosis and in treatment. For example, Lu and colleagues provided an early observation that miRNA profiling in tumor tissues, in comparison to adjacent normal tissues, can be used to classify many different cancers (10). Expression of various miRNAs may also impact response to therapy and survival. In terms of studies of plasma miRNAs for early diagnosis of breast cancer, heterogeneity in sample source, study size and method of analysis have likely contributed to the inconsistencies (11). Another major limitation is that most studies used samples collected at the time of diagnosis making it impossible to determine if they will be useful for early diagnosis or are a result of cancer progression. To date, only one study has been carried out prospectively to evaluate serum miRNA expression as an early marker for breast cancer risk; 21 miRNAs were identified as differentially expressed in women who went on to develop cancer compared to women who did not, suggesting the feasibility of this approach (12).

In contrast to the limited work on plasma miRNAs, there have been no studies, to date, to prospectively evaluate miRNAs in peripheral blood mononuclear cells (PBMCs) as a marker of disease risk. Herein, we used next-generation sequencing for miRNA profiling to identify miRNAs differentially expressed in PBMCs of breast cancer cases and controls. Samples were collected prospectively in the New York site of the Breast Cancer Family Registry (BCFR), a multi-center cohort of families at high-risk for breast and ovarian cancer. We then attempted to validate the top candidates from the sequencing studies as biomarkers of risk in additional cases and controls within the same cohort using individual TaqMan miRNA assays.

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Materials and Methods

Study design/population

Breast cancer cases are participants of the New York site of the BCFR, a six-site international registry (California, New York, Philadelphia and Utah in the USA, Ontario in Canada, and Melbourne and Sydney in Australia) of families with breast and/or ovarian cancer. The BCFR has been described in detail previously (13). At the New York site, we recruited high-risk families from clinical and community settings (2, 14). We collected blood from participants at the time of recruitment for the isolation of mononuclear cells, which were stored in liquid nitrogen until isolation of RNA. At enrollment, each consenting participant was asked to complete several questionnaires, including a family history instrument requesting information on age and site of cancers diagnosed among blood relatives. A questionnaire collected information on demographics, ethnicity, smoking, alcohol consumption, reproductive history, hormone use, weight, height and physical activity; a self-administered food frequency questionnaire collected dietary information. Cases in the current study were selected from family members who were unaffected at the time of recruitment and were later diagnosed with breast cancer (n=48 subjects with available PBMCs). For the discovery phase, we enriched for cases that provided blood closest to diagnosis. Controls were selected from BCFR participants who provided baseline blood and are breast cancer unaffected. Controls were matched to cases on age (±2 years), time of blood draw (within 1 year) and race/ethnicity. The study was approved by the Columbia University's Institutional Review Board and informed consent was obtained from all study participants; strict quality controls and safeguards were used to protect confidentiality. All samples were assayed with the laboratory blinded to case-control status.

The study was carried out in two phases, a discovery phase and a validation phase. For the discovery phase, miRNAs from mononuclear cells of 20 prospectively identified cases and 20 matched controls were profiled by next-generation sequencing as described below. Five miRNAs identified as significantly differentially expressed in the PBMCs of cases and controls by the bioconductor package DESeq in R (15) were then validated using individual TaqMan assays in an additional 28 cases and 28 matched controls.

RNA extraction

Total RNA (including all small non-coding RNAs) was extracted from PBMCs using miRNeasy Mini Kits (Qiagen, Valencia CA, USA) according to the manufacturer's protocol. The RNA pellet was re-suspended in 30 μl nuclease-free water. The quantity and initial quality of total RNA was initially evaluated by 260/280 ratio using an Eon High Performance Microplate Spectrophotometer (BioTek, Winooski, VT, USA).

miRNA profiling by small RNA sequencing

A HiSeq2500 System (Illumina, San Diego, CA, USA) was used to profile small RNAs for the 40 samples in the discovery phase. Equal amounts (1μg) of total RNA were used for the sequencing procedure. Sample quality was ascertained by BioAnalyzer; all had RNA integrity values (RIN) >7. Small RNA libraries were prepared following the manufacturer's

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instruction using TruSeq Small RNA Sample Prep Kits (Illumina). Libraries were sequenced with 1×50 base-pair single-end reads and 10 samples were run per lane.

miRNA mapping and differential expression analysis

The 3' end adaptor sequence was clipped from the reads with FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/commandline.html). Reads were processed with the pipeline miraligner (https://code.google.com/p/seqbuster/wiki/miraligner) to map them to miRBase v.20 sequences (allowing one mismatch). In case of multi-mapping, the count was divided by the number of locations. For example, if a read maps to miR-A and miR-B, 1/2 was counted for miR-A and 1/2 was counted for miR-B.

Real-time quantitative polymerase chain reaction (PCR) analysis

The expression level of the candidate miRNAs was determined by qRT-PCR using TaqMan microRNA assays (Life Technologies, Carlsbad, CA, USA). Each miRNA was reverse transcribed into cDNA from 30 ng of total RNA in a 10 μl reaction according to the manufacturer's instructions. Three μl of the product of the reverse transcription reaction was used as a template for standard two-step real-time quantitative PCR on a 7900HT Fast-Real Time PCR System (Life Technologies). U6 snRNA expression level was used as the endogenous control for normalization purposes. Each sample was run in duplicate. Expression value ΔCt was calculated by subtracting the average Ct value of U6 snRNA from the average Ct value of the miRNAs of interest. The inter-assay coefficient of variation (CV) based on repeat analysis of 10% of samples was used to evaluate the reproducibility of technical replicates across plates.

Statistical analysis

In the discovery phase, the count matrix, generated from small RNA sequencing, was analyzed using the Bioconductor package DESeq (http://bioconductor.org/packages/release/bioc/html/DESeq.html) to compare miRNA expression between cases and controls. DESeq performed a statistical test of differential expression under the hypothesis of a negative binomial distribution of reads and by use of a shrinkage estimator for the distribution's variance (15). Raw read counts were normalized by using the size factors s_j to make counts from different samples *j*, which may have been sequenced to different depths, comparable. The size factor for each sample *j* was computed as the median of the ratios of the *j*-th sample's counts to those of a pseudo-reference sample, the geometric mean across samples for each miRNA i.

$$\hat{s}_j = \mathrm{median}_i \frac{k_{ij}}{\left(\prod_{v=1}^{m} k_{iv}\right)^{1/m}},$$

where indicates the raw counts for the *i*-th miRNA in the *j*-th sample. The normalized counts are calculated as dividing the raw counts k_{ij} by the size factor $\hat{s}_j$. The differential expression of miRNA between cases and controls were then calculated according to the default parameters.

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For the analysis of TaqMan qRT-PCR results in the validation phase, expression value ΔCt for each sample in pairwise comparison was calculated by subtracting the average threshold cycle (Ct) value of U6 snRNA from the average Ct value of the miRNAs of interest. These ΔCt values were then averaged before proceeding with the fold change $2^{-\Delta\Delta Ct}$ calculation to acquire the normalized case miRNA amount relative to control. Differential expression of PBMC miRNAs between breast cancer case and control groups was analyzed using the Mann–Whitney test.

Results

Demographic characteristics of study population

The demographic characteristics of the 20 pairs in the discovery set and 28 pairs in the validation set are summarized in Table I. Cases and controls did not differ in age or ethnicity in either the discovery or validation phase. Mean age at diagnosis in cases in the validation phase is significantly older than in the discovery phase (p=0.03). In the discovery set subjects, four cases are positive for *BRCA1* mutations and one for a *BRCA2* mutation. In the validation subjects, two cases and two controls are positive for *BRCA1* mutations and one case is positive for a *BRCA2* mutation. In the discovery phase, we specifically enriched for subjects who provided blood within two years prior to diagnosis. Thus, the interval between blood draw and diagnosis is larger in the validation phase, which encompassed all remaining prospective cases with available PBMCs with a mean and standard deviation of 78±32 compared to 12±16 in the discovery phase.

Identification of aberrantly-expressed miRNAs as potential risk markers for breast cancer

We used HiSeq 2500 to profile miRNAs in PBMCs collected prospectively from the 20 pairs of breast cancer cases and healthy controls in the discovery phase. In total, more than 184 million read counts from all the samples and on average more than 4.6 million read counts per sample were produced from the small RNA sequencing after the filtering and mapping procedure. There was no statistically significant difference in the number of reads in cases and controls (9.40×10^7 reads (mean=4.70×10^6, SD=2.95×10^6) for cases and 9.03×10^7 (mean=4.51×10^6, SD=2.60×10^6) for controls, p=0.787). miRNAs from the PBMCs matched to 2,087 known miRNAs by mapping to National Center for Biotechnology Information (NCBI) genome, build 37. Among the samples, the number of detected reads ranged from 5.77×10^5 to 1.19×10^7 per sample in cases and from 1.57×10^6 to 1.24×10^7 per sample in controls. Of the 5 miRNAs identified as potential risk markers, miR-144-3p, miR-451a, miR-144-5p and miR-183-5p were significantly up-regulated (miR-144-3p, $p=1.34\times10^{-7}$; miR-451a, $p=8.22\times10^{-6}$; miR-144-5p, $p=8.22\times10^{-6}$; and miR-183-5p, $p=9.42\times10^{-5}$), while miR-708-5p was the only significantly down-regulated miRNA (p=0.005) (Figure 1). The fold changes in miRNA expression of cases compared to controls for miR-144-3p, miR-451a, miR-144-5p, miR-183-5p and miR-708-5p are 3.05, 2.61, 2.77, 2.61 and 0.46, respectively (Table II).

Independent marker validation

In order to validate the 5 miRNAs identified *via* sequencing in the discovery phase, we conducted qRT-PCR experiments on an independent set of PBMCs miRNA samples from

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28 additional pairs of prospective breast cancer cases and age- and race/ethnicity-matched controls. When expression was normalized to U6snRNA, there were no statistically significant differences in expression of the five miRNAs between cases and controls by the Mann-Whitney test (miR-144-3p, p=0.189; miR-451a, p=0.153; miR-144-5p, p=0.384; miR-183-5p, p=0.308; and miR-708-5p, p=0.499, respectively, Table III and Figure 2). The fold changes ranged from 0.8 to 1.3 and were not consistent in direction to the discovery set. The inter-assay coefficients of variation (CV) of 10% replicated miRNA samples are all <8% (miR-144-3p, CV=4.2%; miR-451a, CV=1.9%; miR-144-5p, CV=1.3%; miR-183-5p, CV=0.6%; miR-708-5p, CV=2.4%; and U6 snRNA, CV=7.4%) suggesting high reproducibility of qRT-PCR results among assays.

Discussion

This is the first prospective study to report on the differential expression of miRNAs in PBMCs of women who went on to develop breast cancer compared to women who did not. In the discovery phase, we used next-generation sequencing to determine expression of miRNAs in 20 cases and 20 ageand ethnicity-matched controls. We found expression of four miRNAs, miR-144-3p, miR-451a, miR-144-5p and miR-183-5p, elevated and expression of one, miR-708-5p, decreased in cases compared to controls. In the validation phase, evaluating 28 case-control pairs, the differences in expression were small, not statistically significant and the direction was not consistent with discovery phase data. In the discovery phase, we specifically selected cases that provided blood primarily within two years of diagnosis. Only one case provided blood six years prior to diagnosis. In contract, in the validation phase, we evaluated all remaining prospective cases. These cases on average provided blood 78 months prior to diagnosis compared to 16 months in the discovery phase. The longer the lag between blood collection and diagnosis, the more difficult it will be to detect differences between cases and controls.

The failure to replicate the discovery phase results may also be partly due to the different methods of analysis in the discovery and validation phases. In the discovery phase that used next-generation sequencing, the number of reads of individual miRNAs was used for quantification. To-date there is no consensus on the application of quantitation methods to small-RNA sequencing results with methods developed to appropriately handle expected variation arising from RNA-Seq experimental (15-17). In previous comparative studies of techniques for RNA-Seq differential expression, DESeq, the analysis method applied in our study, has been shown to be more conservative compared to other packages (18) and is recommended if the number of false-positives is a major concern (19). Differential expression of miRNAs shown in our study would, therefore, be expected to more possibly reflect the biological difference between breast cancer patients and healthy controls. In contrast, for the validation phase that used individual TaqMan assays, it was necessary to standardize data to a control; in our case U6snRNA, which was used in a case-control study of PBMCs in pancreatic cancer (20), as well as in various breast cancer biomarker studies on plasma or tissues (21, 22). However, it is unclear if U6 is a suitable internal control to accurately identify miRNAs differentially expressed in PBMCs of breast cancer patients and healthy controls (23). Thus far, there is little agreement on the selection of internal control genes for normalization. Other endogenous controls, such as miR-16 (24) or external-spiked

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C. elegans miRNA *cel-39* (25), have also been used for the purpose of normalization in qRT-PCR but miR-16 was reported to differ in PBMC (23). Apart from using a single gene as an internal control, researchers have also proposed to use the combination of expression of miR-16 together with miR-425 (26) or miR-1825 and cel-39 (12) as reference genes by using geNorm (27) or NormFinder (28) algorithms. The identification of suitable sample- and disease-specific reference genes for normalization is critical for future studies. Technical variability in qRT-PCR replicates has also been identified as a significant contributor to variability (23); this study found higher variability for the TaqMan assays we used compared to qScript microRNA assays (Quanta Biosciences). The authors suggest that, in addition to more reliable assays, repeated measurements are also needed to reproducibly detect small differences in expression (23).

The miRNAs we identified were previously reported as differentially expressed in breast cancer. miR-183 expression in breast tumors has been evaluated in four studies and found up-regulation in three of them (29). In early breast tumorigenesis, miR-183 expression begins to increase during the normal-atypical ductal hyperplasia transition (30) and is greatly increased in ductal carcinoma in situ compared to normal tissues prior to invasive breast cancer (31). miR-183 up-regulation was detected with increased histological severity of lesions in lobular carcinoma in situ and correlated with invasive lobular carcinoma progression (32). Moreover, elevated expression of miR183 has been detected in tumor tissue (33) and circulating tumor cells (CTCs) (34) from women with metastatic breast cancer. However, miR-183 also was found to inhibit cell migration in breast cancer cell lines (35) and was down-regulated in invasive, compared to less-invasive, cell lines (36). Further functional assays are required to resolve these discrepancies in human samples and cell lines and elucidate the underlying molecular mechanism of miR-183. Nevertheless, according to previous studies on the correlation of miR-183 and tumor progression, it is possible that the aberrantly increased expression of miR-183 occurs early in breast cancer development.

mir-144 and mir-451 are in the same miRNA cluster and usually transcribed together (37). Down-regulation of miR-144 has been identified in several cancers including liver, lung and prostate (38). However, in a study of nasopharyngeal cancer, miR-144 was found to be up-regulated more than 2-fold; repression of miR-144 significantly decreased cell proliferation, clonogenicity, migration and tumor formation in nude mice (39). miR-144 was also shown to increase HeLa cell growth (40). In breast cancer cell lines, miR-144 was found to target and repress expression of the tumor suppressors PTEN and Rb1 (41). miR-144 expression in PBMCs has been reported in a study of tuberculosis patients and controls; an almost 6-fold increase in miR-144 was observed in patients with expression primarily in T cells (42). Conflicting data have also been observed for miR-451 with decreased levels in gastric and colorectal tumors but over-expression in ovarian with a correlation to poor prognosis (reviewed in (43)). One study on triple-negative tumors found miR-451 expression decreased compared to adjacent tissue (44). PAX4 was shown to be responsible for the down-regulation of miR-144/451 in breast cancer tissues resulting in increased cell migration and invasion in breast cancer cell lines (45). Extensive work has been carried out on miR-451 in glioblastoma and demonstrated increased but variable levels in tumor compared to adjacent tissue and poorer survival in those with higher expression (46).

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miR-451 plays a complicated role in glioblastoma regulating the balance between cell proliferation and migration; its expression is regulated by levels of glucose with glucose deprivation resulting in decreased levels of miR-451 (46). Studies of plasma miRs for early diagnosis identified over-expression of miR-451 as one of the best predictors of breast cancer; levels decreased in postoperative plasma samples (21). Taken together, these data suggest we are far from a definitive understanding over the role of these miRs in cancer and that their role might differ by cancer type and tumor microenvironment.

The effect of miR-708 down-regulation has been studied in breast cancer cell lines, as well as a mouse model, and decreased expression has been observed in cases compared to controls by in situ hybridization analysis of lymph nodes (47). In a well-designed functional study, miR-708 was found to have a metastasis-suppressive role in breast cancer by targeting the endoplasmic reticulum protein neuronatin, which regulates cell migration through adjustment of intracellular calcium levels. miR-708-mediated inhibition of cell migration may lead to a reduced number of disseminated CTCs. On the other hand, B7-H3, an immunoregulatory protein, which is over-expressed in different cancers and often associated with metastasis and poor prognosis (48), is targeted directly by miR-708 (49). As the composition of PBMCs is heterogeneous, ranging from CTCs to critical components of the immune system, such as lymphocytes, monocytes and macrophages, the source of miR-708 down-regulation observed in our study remains unclear. However, CTCs are extremely rare (50) and the level of CTC is likely too low to impact miR-708 levels. The reduced expression of miR-708 in PBMCs may be due to distinct or synergistic effects from the variety of immune cells.

Strengths of our study are the use of prospective samples, which allowed us to evaluate whether the miRNAs were predictive of risk, and the use of sequencing in the discovery phase to comprehensively identify aberrantly expressed miRs. However, a major limitation is the relatively small sample size, which limited the power to acquire statistically significant results at the validation phase by time between blood draw and diagnosis.

Several recent studies have evaluated PBMC miRNA expression in other diseases. For example, down-regulation of miR-548 was found in chronic heart failure patients (51), while several miRNAs were dysregulated in sepsis (52) and in depression (53). Our study sheds light on the application of differentially expressed miRNAs from PBMCs, identified by small RNA sequencing, as potential non-invasive biomarkers for breast cancer prediction but also highlights some of the difficulties in these types of studies. Future prospective studies using a large cohort of women across the spectrum of breast cancer risk and a greater range of blood collection prior to cancer diagnosis will be needed to confirm whether our findings support that selected miRNAs markers close to the time of diagnosis can be used clinically to predict risk.

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views or policies of the National Cancer Institute or any of the collaborating centers in the BCFR, nor does mention of trade names, commercial products or organizations imply endorsement by the USA Government or the BCFR.

Abbreviations

BCFR	breast cancer family registry
miRNAs	
PBMC	peripheral blood mononuclear cells

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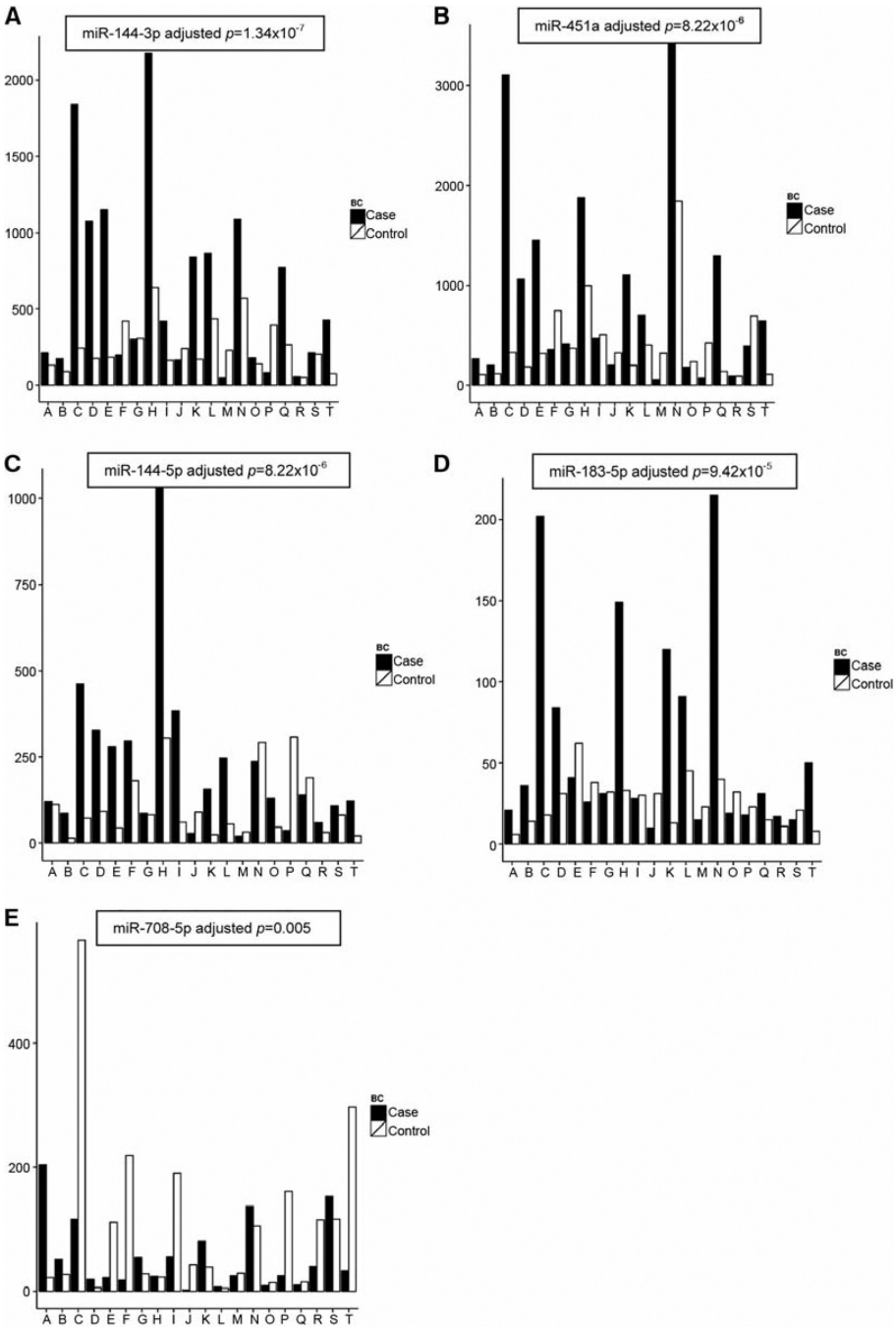

Figure 1.

miRNA expression in mononuclear cells of prospective breast cancer cases and age- and ethnicity-matched controls from the New York site of the BCFR from the discovery phase. (A) miR144-3p; (B) miR-451a; (C) miR-144-5p; (D) miR-183-5p; (E) miR-708-5p. The horizontal axis represents each of the 20 paired cases and controls (from pair A to pair T) in the discovery phase. The vertical axis indicates the read counts of each miRNA in each sample. Black bars show miRNA expression in breast cancer cases, while white bars

indicate the level of expression in healthy controls. The p value of differential expression comparison is shown at the top of each miRNA bar chart.

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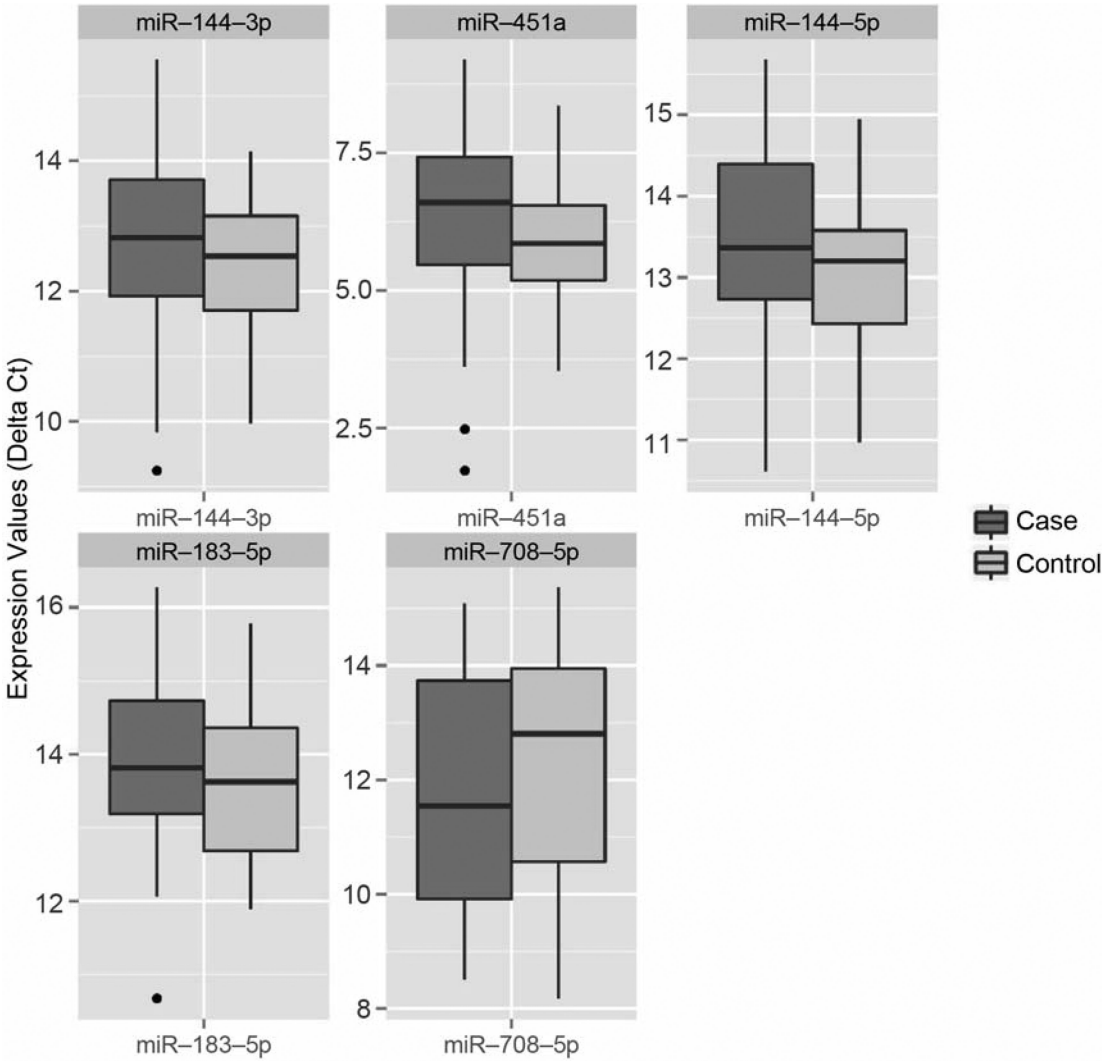

Figure 2.

Validation phase for expression of the top five candidate miRNAs in PBMCs of an independent set of 28 prospective breast cancer cases and matched controls from the New York site of the BCFR by qRT-PCR analysis. Box plots of data for (from left to right) miR-144-3p, miR-451a, miR-144-5p, miR-183-5p and miR-708-5p. The change of each miRNA (Delta Ct) is the result of normalization to U6 expression. The lines inside the boxes denote the medians. The boxes mark the interval between the 25th (Q1) and 75th (Q3) percentiles. IQR (Inter-Quartile Range)=Q3-Q1. Filled circles indicate data points that are less than Q1-1.5*IQR or greater than Q3+1.5*IQR.

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Table I

Characteristics of study participants from the New York site of the BCFR in the discovery and validation phases.

	Discovery Set		Validation Set	
Characteristic	**Cases n=20**	**Controls n=20**	**Cases n=28**	**Controls n=28**
Age at blood collection years, mean±SD	45.1±9.3	44.9±9.1	45.8±9.4	45.8±9.7
Age at diagnosis years, mean±SD	46.2±9.3		52.3±9.9	
Race				
White	14	14	26	26
Hispanic	6	6	2	2
BRCA mutation status				
BRCA1 mutation carrier	4		2	2
BRCA2 mutation carrier	1		1	
Interval between blood collection and diagnosis				
Months, mean±SD	16±22		78±36	
Range	0*-108		9-138	

*One case provided blood three days before diagnosis.

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Table II

Distribution of read counts for candidate miRNA expression by sequencing in cases and controls from the New York site of the BCFR in the discovery phase.

miRNA read count Mean (SD) Median (Max-Min)	Cases (n=20)	Controls (n=20)	Fold change	*p*-Value
hsa-miR-144-3p	789 (1266)	257 (232)	3.05	1.34×10^{-7}
	549 (5842-50)	201 (1135-47)		
hsa-miR-451a	10238 (13013)	3926 (2932)	2.61	8.22×10^{-6}
	6346 (47511-628)	3020 (10493-785)		
hsa-miR-144-5p	230 (349)	83 (43)	2.77	8.22×10^{-6}
	151 (1663-31)	90 (150-18)		
hsa-miR-183-5p	82 (138)	32 (34)	2.61	9.42×10^{-5}
	34 (615-9)	20 (135-5)		
hsa-miR-708-5p	50 (48)	108 (148)	0.46	0.005
	36 (157-7)	47 (608-6)		

Fold change=ratio of read count of cases and read count of controls.

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Table III

Distribution of candidate miRNA expression by real time PCR in cases and controls from the New York site of the BCFR in the validation phase.

ΔCt (Mean, SD)	Cases (n=28)	Controls (n=28)	Fold Change
has-miR-144-3p	12.7 (1.5)	12.3 (1.0)	0.78
has-miR-451a	6.3 (1.8)	5.9 (1.2)	0.78
has-miR-144-5p	13.3 (1.3)	13.1 (1.0)	0.86
has-miR-183-5P	13.9 (1.3)	13.6 (1.0)	0.81
hsa-miR-708-5p	11.9 (2.2)	12.2 (2.2)	1.3

Fold change=2^(–ΔΔCt).