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Research Article

Downregulation of microRNAs 145-3p and 145-5p Is a Long-term Predictor of Postmenopausal Breast Cancer Risk: The ORDET Prospective Study

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Abstract

Background: miRNAs have been implicated in the regulation of key metabolic, inflammatory, and malignant pathways; hence, they might be considered both predictors and players of cancer development.

Methods: Using a case–control study design nested in the ORDET prospective cohort study, we addressed the possibility that specific mRNAs can serve as early predictors of breast cancer incidence in postmenopausal women. We compared leukocyte miRNA profiles of 133 incident postmenopausal breast cancer cases and profiles of 133 women who remained healthy over a follow-up period of 20 years.

Results: The analysis identified 20 differentially expressed miRNAs, 15 of which were downregulated. Of the 20 miRNAs, miR145-5p and miR145-3p, each derived from another arm of the respective pre-miRNA, were consistently and significantly downregulated in all the databases that we surveyed. For example, analysis of more than 1,500 patients (the UK Metabric cohort) indicated that high abundance of miR145-3p and miR145-5p was associated with longer, and for miR145-3p also statistically significant, survival. The experimental data attributed different roles to the identified miRNAs: Although the 5p isoform was associated with invasion and metastasis, the other isoform seems related to cell proliferation.

Conclusions: These observations and the prospective design of our study lend support to the hypothesis that downregulation of specific miRNAs constitutes an early event in cancer development. This finding might be used for breast cancer prevention.

Impact: The identification of the miRNAs as long-term biomarkers of breast cancer may have an impact on breast cancer prevention and early detection. *Cancer Epidemiol Biomarkers Prev; 23(11); 2471–81. ©2014 AACR.*

Introduction

The identification of molecular biomarkers associated with cancer initiation and progression represents a fundamental step for the risk assessment and development of new prevention strategies. A recently identified class of noncoding small RNAs, miRNA, may provide new insights into cancer prevention and early detection methodology. MiRNAs are small, stable noncoding RNAs whose function is to bind the messenger RNAs (mRNA) of expressed genes and target them for degradation or inhibition of translation, resulting in reduced expressed protein levels (1). It is estimated that miRNAs may actually regulate up to two thirds of the human genome (2). MiRNAs have been shown to be directly involved in many human cancers, including breast, lung, brain, liver, colon, prostate, ovarian cancers, and leukemia (3). Some miRNAs function as tumor suppressors by negatively inhibiting oncogenes that control cell differentiation and apoptosis, whereas others act as oncogenes (oncomirs; refs. 4, 5). There is a growing consensus that miRNA downregulation has a profound impact on the genesis of tumors (6–8). There is evidence that an extensive downregulation of miRNAs is one of the first biologic response of the deregulation in a signaling cascade downstream of specific growth factor receptors implicated in human cancers, including breast cancer. For example, EGF signaling rapidly and simultaneously induces a massive

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

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downregulation of multiple miRNAs, reflecting coordinated regulation at the level of miRNA synthesis, processing, or degradation (9).

In the present study, we aimed to test the hypothesis that miRNAs (as a single entity or as a signature) may represent early indicators of future breast cancer incidence. Previous evidence indicated that miRNAs are deregulated, and, in particular, mainly downregulated in response to environmental/metabolic risk factors for cancer (10–12). To test the working hypothesis, we compared leukocyte miRNA profiles of healthy women who subsequently became affected with breast cancer with women who remained healthy. This was performed using a case–control study design nested in the ORDET [h**OR**mones and **D**iet in the **ET**iology of Breast Cancer) prospective cohort study over a follow-up period of 20 years. The prospective study design also allowed corroborating the evidence that the downregulation of miRNAs represents one of the very early molecular alterations during the development of the disease. Subsequently, we evaluated whether miRNAs differently expressed in the ORDET women candidate to become breast cancer cases versus control subjects were also modulated in breast cancer tissues and had prognostic value using the well-characterized METABRIC cohort of 1,359 breast cancer cases (13). As the final phase of the study, we investigated the functional activity of the identified miRNAs in breast cancer cell lines.

Materials and Methods

Study design and population

The study has been conducted in the context of the ORDET prospective cohort study; the analysis included 133 incident postmenopausal breast cancer cases and 133 matched control subjects.

The ORDET cohort was established in northern Italy between June 1987 and June 1992, in which 10,786 healthy women ages 35 to 69 years were enrolled (14). They were all residents of the Varese province, an area covered by the population-based Lombardy Cancer Registry (15). They had heard about the study through the media, at public meetings, and volunteered to participate. At recruitment, we measured anthropometric variables and collected demographic information and blood samples. Because the study's focus was on endogenous hormones in relation to breast cancer risk, we also applied stringent inclusion criteria and highly standardized conditions on the collection of biologic samples.

Information on cancer outcomes available from the Lombardy Cancer Registry has been linked to the ORDET cohort to identify incident breast cancer cases up to December 31, 2006 (16).

Case subjects were women who developed breast cancer after their recruitment into the ORDET cohort and before the end of the follow-up. We randomly chose one control for each case, from appropriate risk sets consisting of all cohort members who satisfied the matching criteria and were alive and free of cancer at the time of diagnosis of the index case. Matching characteristics were age (±3 years) at enrollment and date of recruitment (±180 days). We applied an incidence density sampling protocol for control selection (17).

After exclusion of women with a history of cancer and women who, immediately after baseline, were lost to follow-up (observed time = 0), 10,633 participants remained to form the base population of ORDET.

In the ORDET study, as well as in other cohort studies, we found that risk factors differ either in their phenotypic expression or in their distribution by menopausal status (18). Thus, the present report focused on the postmenopausal group of the cohort, defined as those cohort members who had the last menstrual period at least 12 months before their enrollment in the study. In summary, within the postmenopausal members of the cohort and because of the selection criteria, we identified and included in the study 133 incident breast cancer cases and 133 matched control subjects.

miRNA in leukocytes

We evaluated the miRNA expression profile of leukocytes derived from buffy coats collected at recruitment.

Blood collection

Blood samples were drawn after overnight fasting between 7:30 am and 9:00 am from each woman and stored at −80°C.

Samples from each case and related control were handled identically and assayed together in the same laboratory session. Laboratory personnel were blinded to case–control status.

Laboratory methods

RNA extraction, labeling, and microarray hybridization. Leucocytes were lysed in 1 mL of TRI Reagent, a lysis reagent from Ambion, according to the manufacturer's instructions. The concentration and purity of total RNA were assessed using a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies). Total RNA (100 ng) was labeled, hybridized to Human microRNA Microarray V2 (Agilent Technologies), and scanned with Agilent DNA Microarray Scanner (P/N G2565BA) according to the manufacturer's instructions. Feature Extraction Software (Version 10.5) was used for data extraction from raw microarray image files using the microRNA_105_Dec08 FE protocol. This miRNA Agilent expression profile was submitted to the Gene Expression Omnibus (GEO) with the accession number GSE54470. Minimum Information About a Microarray Gene Experiment (MIAME) guidelines were followed as instructions. Furthermore, representative RNA preparations were evaluated for integrity using the 2100 Bioanalyzer RNA 6000 Nano Kit (Agilent Technologies; data not shown).

We also assessed the expression of miR223 in randomly selected samples by Northern blot analysis (data not shown). miR223 is highly specific for hematopoietic cells

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and constitutes a regulator of myelopoiesis (19). The blot was hybridized with a [^{32}P]γATP-radiolabeled LNA oligonucleotide complementary to miR223 sequence. The specificity and strength of hybridization of ORDET RNA samples was as good as that of human promyelocytic HL60 cells treated with retinoic acid (10^{-6} mol/L), a known inducer of miR223.

Microarray data analysis

Data were verified and extracted by the Agilent Extraction 10.7.3.1 software and analyzed using an in-house built routines by Matlab (The MathWorks Inc.). Background-subtracted signal of 851 human miRNA assays was used in the study. All arrays were quantile normalized, assuming that all samples were measured and analyzed under the same condition, enforcing all the arrays to assume the same mean distribution. The Pearson coefficient was calculated to assess the correlation between technical replicates of some randomly chosen samples.

We fitted a linear model to the expression values for each miRNA, to assess the significance of differential expression between case and control. In addition, we used empirical Bayes methods implemented in the LIMMA package to construct moderated t statistics and incorporated the statistical tools to adjust for the multiplicity of the tests. The Benjamini and Hochberg method (1995) was used to control for false discovery.

We considered the linear model including the matched case–control study design, the case–control status, and the error term.

Statistical methods

Data preprocessing and differential expression analysis were done using the Bioconductor AgiMicroRna package (20). The Total Gene Signal (TGS) provided by the Agilent Feature Extraction image analysis software was used as the quantitative measure of miRNA expression. We set all negative TGS values to 0.5 before log transformation, so that the log ratios are shrunk toward zero at lower intensities. The miRNA expression data (i.e., TGS) were quantile normalized before determining differential expression. The data were analyzed using the R software package. For differential expression analysis, the AgiMicroRna package incorporates the linear model with matched pair features from the Bioconductor LIMMA package (21). The LIMMA approach fits a linear model to the expression value for each miRNA to assess the significance of differential expression between different experimental conditions. In addition, the method uses empirical Bayes methods (22) to construct moderated t statistics and incorporates statistical tools to adjust for multiple testing. The Benjamini and Hochberg method (23) was used to control for false discovery rate (FDR), and we ranked the miRNAs according to FDR. We considered the top-ranked 20 miRNAs and investigated the upregulated and downregulated miRNAs identified from postmenopausal samples. We computed agglomerative hierarchical clustering of the dataset. At first, each object is assigned to its own cluster and then the algorithm proceeds iteratively. At each stage, the two most similar clusters are combined to form a larger cluster, continuing until there is just a single cluster. At each stage, distances between clusters are computed by the Lance–Williams dissimilarity update formula. Details about the clustering algorithm are given in the book by Kaufman and Rousseeuw (24). This clustering method partitions the dataset into clusters, in which similar miRNA expression patterns are assigned to the same cluster.

We identified predictive pathways using pathway analysis. Pathway analysis was performed by DIANA miRPath v2.0. The software calculated the union of targeted genes by the selected miRNAs (UNION_SET, all genes targeted by at least one selected miRNA). The

Table 1. Descriptive characteristics of the 133 postmenopausal women candidate to become breast cancer and 133 control women: baseline values

	Cases	Controls	Median difference (IC 95%)	*t* Student *P* value
Age, y; median (SD)	57 (5.95)	56 (5.89)	−0.5:2.5	0.85
Age at menarche, y; median (interquartile range)	13 (12–14)	13 (12–14)	−0.36:0.36	0.93
IGF1; median (interquartile range)	115 (95.5–155)	111 (92.2–137.5)	−6.2:14.2	0.16
TTS; median (interquartile range)	0.28 (0.21–0.36)	0.26 (0.2–0.32)	−0.02:0.06	0.21
BMI, kg/m^2; median (interquartile range)	25.6 (23.3–28.3)	25.7 (23.6–28.4)	−1.13:0.78	0.42
Fasting glucose; median (interquartile range)	84 (78–91)	85 (78–90)	−7.8:5.8	0.24
Alcohol intake, g/d; median (interquartile range)	4.8 (0–24)	3.4 (0–18)	−2.3:5.1	0.75
Age at first birth; median (SD)	26 (4.6)	25 (3.6)	−0.09:2.1	0.09
Full-term pregnancies; median (SD)	2 (1)	2 (1)	−0.28:0.28	0.92
Smoking; % of smoker/ex smoker/not smoker	19/12/69	16/12/72	—	0.7[a]

Abbreviations: IGFI, insulin growth factor 1; TTS, total testosterone.
[a]χ^2 *P* value.

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UNION_SET set was used for the statistical analysis. This enrichment analysis identified the pathways significantly enriched with genes belonging to the UNION_SET (25).

To assess the miRNAs' prognostic value, we conducted a survival analysis in the Metabric cohort of breast cancer cases. This analysis was not possible on the ORDET cohort study database for the limited sample size of the breast cancer–specific mortality events. The Metabric cohort is a very well-characterized breast cancer database provided with matching detailed clinical annotation, long-term follow-up, and genomic and miRNA expression data (13). The database includes 1,302 breast tumors, which included a subgroup of 81 breast cancer cases, where each case was provided with samples derived from both the tumoral lesion and the related normal breast tissue. As an initial step of the survival analysis, we tested the consistency of the disregulation (down- versus upregulation) between the observed 20 top-ranked miRNAs in the ORDET cohort and in the tumor tissue versus the normal tissue in the subgroup of 81 breast cancer cases described in the Metabric study. For instance, the downregulated miRNAs in ORDET breast cancer cases versus control subjects were expected to have lower expression levels in breast tumors versus normal tissue. In the subsequent survival analysis, higher expression levels of these miRNAs were expected to be associated with better survival. We expected the opposite effect for the ORDET upregulated miRNAs. For testing the prognostic value, for each miRNA, we computed the P value for its differential expression between tumor tissue and normal tissue (in the subgroup of patients provided with samples of both tumor and normal tissue). Subsequently, for each miRNA, we performed survival analysis and generated Kaplan–Meier plots.

Methods of the experimental study

Cell cultures and transfection. Human breast cancer cell lines MDA-MB-231 and MDA-MB-468 were obtained from the American Type Culture Collection (ATCC; www.atcc.org). ATCC uses morphology, karyotyping, and PCR-based approaches to confirm the identity of human cell lines and to rule out both intra- and interspecies contamination (www.atcc.org). After purchasing, (5 months ago) the cells were routinely tested with PCR approaches. For mature miR145-5p and miR145-3p expression, we used Pre-microRNA Precursor-Negative Control (Ambion), Pre–miR145-5p (Ambion), and Pre–miR145-3p (Ambion) at a final concentration of 5 nmol/L.

The expression levels of miR145-3p and miR145-5p were evaluated by PCR (Supplementary Fig. S1)

Cell proliferation assay. MDA-MB-231 cells were seeded into 6-well dishes and transfected in triplicates as indicated. Cells (6×10^4) were seeded for this assay.

Cells were collected and manually counted at 0, 24, 48, 72 hours after transfection.

Transwell migration assay. Migration assay was performed using a 24-well Boyden chamber with a noncoated 8-mm pore size filter in the insert chamber (BD Falcon). Cells (mimic 145-5p and control-transfected MDA-MB-231 and MDA-MB-468; 5×10^4) were suspended in 0.5 mL DMEM without FBS and seeded into the insert chamber. Cells were allowed to migrate for 48 hours into the bottom chamber containing 0.7 mL of DMEM containing 5% FBS in a humidified incubator at 37°C in 5% CO_2. Migrated cells that had attached to the outside of the filter were visualized by staining with DAPI and counted.

Clonogenic assays. MDA-MB-231 cells were grown to 70% confluence and transfected as indicated. Colony staining and counting were performed as described by Biagioni and colleagues (7).

Table 2. List of the top 20-ranked miRNAs according to FDR values for miRNAs differentially expressed between women candidate to become breast cancer and healthy controls within the ORDET cohort

miRNA	Log FC[a]	P values[b]	FDR[c]
Downregulated miRNAs			
hsa-miR125a-5p	−0.634	0.0021	0.400
hsa-miR141	−0.158	0.0023	0.400
hsa-miR582-5p	−0.496	0.0028	0.400
hsa-miR138	−0.199	0.0034	0.400
hsa-miR199a-5p	−0.581	0.0039	0.400
hsa-miR181c*	−0.321	0.0041	0.400
hsa-miR28-3p	−0.631	0.0042	0.400
hsa-miR224	−0.629	0.0047	0.400
hsa-miR145-3p	−0.261	0.0053	0.408
hsa-miR223	−0.484	0.0079	0.503
hsa-miR145-5p	−0.506	0.0083	0.503
hsa-miR539	−0.364	0.0098	0.504
hsa-miR99b	−0.483	0.0112	0.504
hsa-miR199b-5p	−0.314	0.0117	0.504
hsa-miR920	−0.147	0.0118	0.504
Upregulated miRNAs			
hsa-miR892b	0.460	0.0001	0.102
hsa-miR1288	0.304	0.0045	0.400
hsa-miR520a-3p	0.402	0.0061	0.430
hsa-miR542-5p	0.381	0.0102	0.504
hsa-miR122*	0.393	0.0118	0.504

NOTE: Downregulated and upregulated miRNAs in candidates to become breast cancer cases versus controls.

[a]The Log FC column gives $\log_2$ fold change between cases' and controls' expression.

[b]P values for moderated t statistics.

[c]FDR gives the P value adjusted with the Benjamini and Hochberg method to control the FDR.

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Results

Most of the baseline characteristics did not differ between the 133 breast cancer cases and 133 controls (Table 1), in particular for age, reproductive, hormonal, and life-style risk factors that could have represented confounders of the studied association.

When we conducted class comparisons to identify differentially expressed miRNAs, we first performed a moderated *t* test (22) for each miRNA on all 266 postmenopausal women. Most of the difference in miRNA expression between cases and controls was toward the downregulation in women who were later affected with breast cancer.

Table 2 reports all the top-ranked 20 miRNAs (ranked according to FDR values). Of these 20 miRNAs, 15 (75%) were downregulated. Among the upregulated miRNAs, miRNA892b was characterized by the lowest FDR (close to 10%), resulting in the most statistically significant differentially expressed miRNA.

An examination of miRNA expression revealed that the 20 top-ranked miRNAs were grouped in three different clusters. Figure 1 shows the heatmap of the distinct miRNA profiles following an unsupervised hierarchical clustering analysis. None of the reported 20 top-ranked miRNAs correlate to age (in either cases or controls), body mass index (BMI), serum fasting glucose, and serum

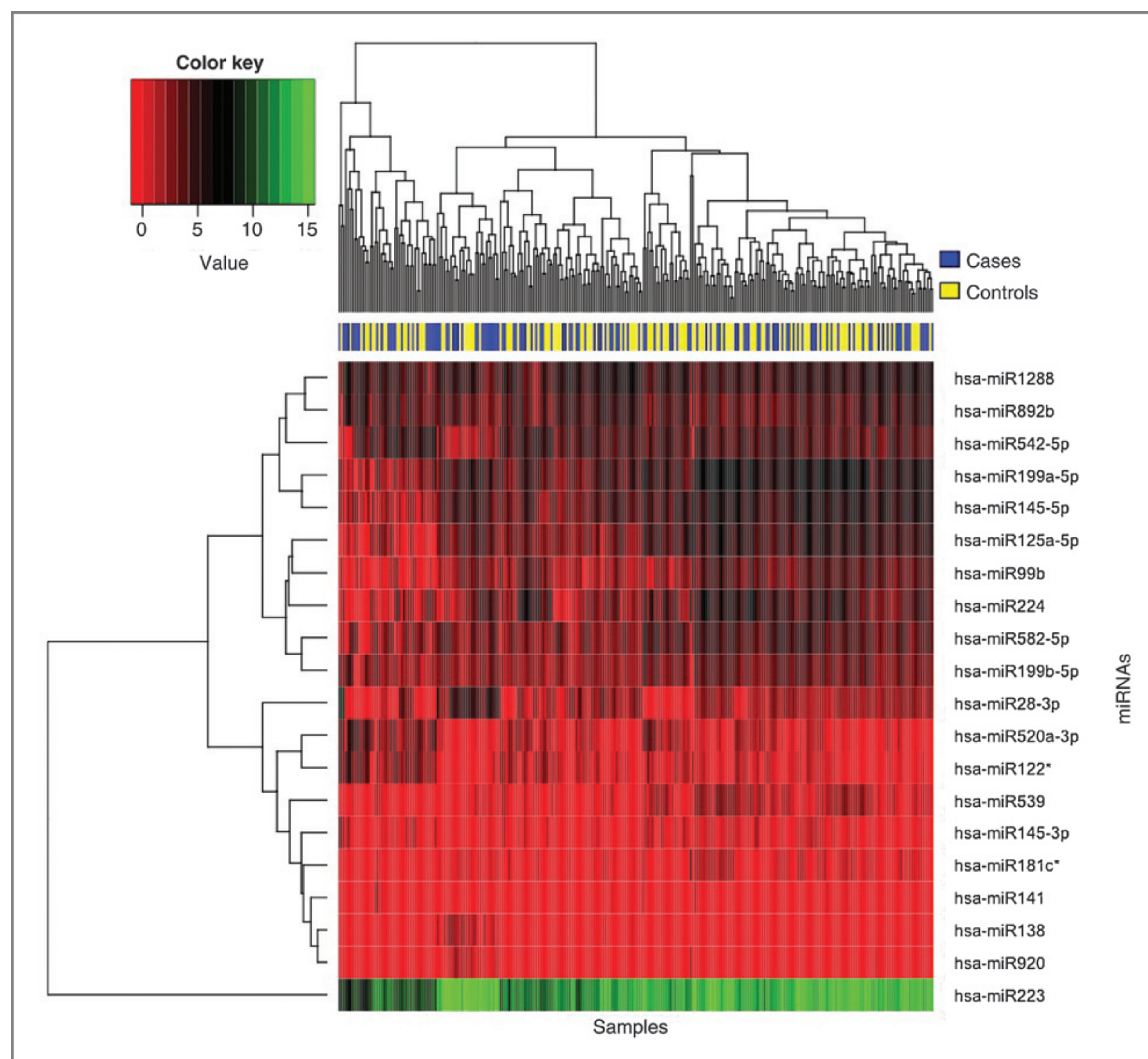

Figure 1. Heatmap of top-ranked 20 miRNAs from postmenopausal samples. The heatmap provided insight into the data structure for each miRNA and sample. We used red and green colors for defining low and high expression values. Blue, cases; yellow, controls. The clustering methods partitioned the dataset into three clusters, identified on the left of the figure. Each miRNA is listed on the right.

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Table 3. Cancer predicted pathways targeted by the three clusters identified by the cluster analysis

miRNA cluster	Most significant predicted pathways
Cluster 1	Wnt signaling pathway
miR145-5p miR199a-5p miR542-5p miR892b miR1288	Steroid biosynthesis
	Glycosylphosphatidylinositol(GPI)-anchor biosynthesis
	Hedgehog signaling pathway
	Adherens junction
	Transcriptional misregulation in cancer
	Pathways in cancer
	TGFβ signaling pathway
	MAPK signaling pathway
	Cell cycle
Cluster 2	MAPK signaling pathway
miR28-3p miR122* miR138 miR141 miR145-3p miR181c* miR520-3p miR539 miR920	ErbB signaling pathway
	mTOR signaling pathway
	Insulin signaling pathway
	PI3K-Akt signaling pathway
	Transcriptional misregulation in cancer
	Chemokine signaling pathway
Cluster 3	MAPK signaling pathway
miR99b miR582-5p miR199b-5p	Transcriptional misregulation in cancer
	Pathways in cancer

fasting insulin. When we looked at the hormone receptor status of the ORDET incident breast cancer cases (e.g., estrogen and progesterone receptor status in breast cancer cases), in the subgroup of progesterone receptor–positive cases, we found that low expression levels of miR99b were statistically associated with a higher probability to develop progesterone receptor–positive breast cancer.

In Table 3, we describe the most significant cancer predicted pathways targeted by the three clusters. It is worth noting that most of the pathways identified by the clusters of differentially expressed miRNAs were related to both breast cancer and more general cancer development (e.g., ErbB, mTOR, TGFβ, Hedgehog, and Wnt pathways; refs. 26–30). We then observed that the three miRNA clusters targeted pathways also related to metabolic and endocrine systems, such as cholesterol synthesis, steroid biosynthesis, and the insulin pathway recognized in the ORDET cohort, as well as in other studies as pathways involved in breast cancer development (27, 31, 32). Finally, the mitogen-activated protein kinase (MAPK) pathway was targeted at a very high level of statistical significance by all the three clusters.

We also noticed that among the top 20 differentially regulated miRNAs, a pair of downregulated miRNAs share the same precursor: the miR145-3p and miR145-5p located on chromosome 5.

To further investigate the consistency of the observed downregulation of miR145-3p and miR145-5p in breast cancer, we analyzed three different publicly available databases of miRNA expression in breast cancer [GSE28884 (33) and GSE19536 (34) and The Cancer Genome Atlas Network (35)] and Biagioni and colleagues article database (7). As shown in Tables 4 and 5, miR145-3p and miR145-5p were consistently downregulated across all databases in tumor tissue versus peritumoral or normal tissues.

In Table 6, we also included, as the fifth database, the comparison between the tumor tissue and the normal tissue observed in the Metabric cohort study: Again, both miRNAs were downregulated in the tumor tissue versus normal tissue at a very high level of statistical significance. We conducted the same analysis for all the remaining 18 miRNAs; however, we did not notice a similar level of consistency for any of them (Table 6 and Supplementary Tables S1–S18). For instance, the downregulated miRNA-199a-5p allocated in the first cluster was equally downregulated in both tumor tissue and normal tissue in the Metabric study (13) and upregulated in the Biagioni and colleagues study (ref. 7; Supplementary Table S1).

To evaluate whether miR145-3p and miR145-5p played a role in the progression of breast cancer, we analyzed the disease-specific survival rate as a function of their low and high expression. In the Metabric breast cancer cohort, we saw that the high expression of both miR145-3p and miR145-5p was associated with longer survival: For miR145-3p, the difference with its low expression reached statistical significance (Fig. 2).

As the final step of our study, we investigated the functional activity of miR145-3p and miR145-5p in MDA-MB-231 and MDA-MB-468 breast cancer cell lines. We used these breast cancer cell lines, which are both

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Table 4. miR145-3p modulation in breast cancer cases and in a variety of breast cancer tissues and peritumoral tissues by different databases

miR145-3p	ORDET	Biagioni et al. (7)	CGAN	Farazi et al. (33)	Enerly et al. (34)
Tumor	133 (cases)	63	694	168	101
Peritumor	—	59	83	—	—
Normal	133 (controls)	—	—	11	—
Platform	Agilent	Agilent	Illumina	Solexa	Agilent
Subsets	case vs. control	T vs. PT	Lum A-B vs. basal	—	Basal vs. Lum A mut p53 vs. wt p53 ER-p53 mut vs. ER-p53 wt proliferative samples
Modulation	Down	Down	Down	—	Down

Abbreviations: basal, basal breast cancer; DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; IDC, invasive ductal carcinoma; lum, luminal breast cancer; mut p53, mutated p53; PT, peritumoral tissue; T, tumor tissue; wt, wild-type.

representative of a basal-like phenotype, because they were previously used by Sachdeva and Mo, to demonstrate the involvement of miR145-5p in breast cancer invasion and metastatization (36). Because this breast cancer histotype is known to be the most aggressive one, it is also the best model to perform a migration assay. As reported in Fig. 3A–E, we observed that the two miRNAs had different and complementary activities in controlling breast cancer cells. Ectopic expression of miR145-5p inhibited cell migration but did not affect proliferation of the analyzed breast cancer cell lines (Fig. 3B). On the contrary, overexpression of miR145-3p impinged on cell proliferation and colony formation, but had no effect on cell migration (Fig. 3C and D); we did not observe any effect of miR145-3p on the migration of breast cancer cells (Fig. 3E).

Discussion

In this study, we provide evidence that miRNAs might serve as early indicators of breast cancer occurrence. We observed that in leukocytes collected from healthy women who later became affected with breast cancer, differences were found in miRNA expression profiles, in comparison with leukocytes of women who did not develop the disease during the same follow-up period. The observation that among the microRNAs listed in the top 20 most differentially expressed microRNAs, 15 were down-regulated, supports our working hypothesis that downregulation of regulatory miRNAs might herald breast cancer initiation. This concept has also been experimentally validated by the progressive downregulation of miRNAs observed when passing from healthy breast tissue to breast cancer with high cell-proliferation rates (37).

The high blood stability of miRNAs, their resistance to RNA degradation, and their reproducible detection make miRNAs suitable biomarker candidates (38). A number of lifestyle factors and conditions, often related to inflammation status, have been reflected into specific deregulation of miRNA in peripheral leukocytes (39). Lifestyle and dietary factors are both related to inflammation status and

Table 5. miR145-5p modulation in breast cancer cases and in a variety of breast cancer tissues and peritumoral tissues by different databases

miR145-5p	ORDET	Biagioni et al. (7)	CGAN	Farazi et al. (33)	Enerly et al. (34)
Tumor	133 (cases)	63	694	168	101
Peritumor	—	59	83	—	—
Normal	133 (controls)	—	—	11	—
Platform	Agilent	Agilent	Illumina	Solexa	Agilent
Subsets	Case vs. control	T vs. PT	Lum. B vs. Basal	DCIS vs. normal; IDC HER2$^+$ ER$^-$ vs. normal	Basal vs, Lum A; mut p53 vs. wt p53; ER-p53 mut vs. ER-p53 wt proliferative samples
Modulation	Down	Down	Down	Down	Down

Abbreviations: basal, basal breast cancer; DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; IDC, invasive ductal carcinoma; lum, luminal breast cancer; mut p53, mutated p53; PT, peritumoral tissue; T, tumor tissue; wt, wild-type.

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Table 6. miRNAs modulation in breast cancer tissues versus normal tissues in the 1,359 patients with breast cancer from the METABRIC study and in subtypes of breast cancer tissues versus normal tissues derived from the highly characterized subgroup of 81 participants

		P value for all subtyped together			*P* value for matched samples by subtype					
	Expression in Caldas dataset (METABRIC)	Direction of change	N vs. T (81 vs. 1,278)	Matched N vs. T (81)	Normal-like (15)	Her2 (5)	Basal-like (15)	Lum A (27)	Lum B (19)	KM *P* value
Downregulated miRNAs										
hsa-miR125a-5p	Expressed	Upregulated in T	<0.001	<0.001	0.814	0.110	0.724	<0.001	<0.001	0.791
hsa-miR141	Expressed	Upregulated in T	<0.001	<0.001	0.198	0.056	0.445	<0.001	<0.001	0.0637
hsa-miR582-5p	Expressed	Upregulated in T	<0.001	<0.001	0.421	0.503	0.483	0.004	0.130	0.144
hsa-miR138	Not Expressed	—	—	—	—	—	—	—	—	—
hsa-miR199a-5p	Expressed	No difference	0.109	0.304	0.743	0.079	0.615	0.094	0.018	0.0043
hsa-miR181c*	Expressed	No difference	0.063	0.489	0.470	0.008	0.353	0.018	0.987	0.00857
hsa-miR28-3p	Not Expressed	—	—	—	—	—	—	—	—	—
hsa-miR224	Expressed	Downregulated in T	<0.001	<0.001	<0.001	0.598	0.511	<0.001	<0.001	0.202
hsa-miR145-3p	Expressed	Downregulated in T	<0.001	<0.001	<0.001	0.061	0.012	0.002	<0.001	0.00121
hsa-miR145-5p	Expressed	Downregulated in T	<0.001	<0.001	0.005	0.007	<0.001	<0.001	<0.001	0.112
hsa-miR223	Expressed	No difference	0.169	0.364	0.372	0.427	0.101	0.870	0.498	0.0086
hsa-miR539	Expressed	Downregulated in T	<0.001	0.081	0.263	0.570	0.974	0.747	0.008	0.0113
hsa-miR99b	Expressed	Downregulated in T	0.003	0.439	0.315	0.114	0.673	0.392	0.160	0.0197
hsa-miR199b-5p	Expressed	Downregulated in T	<0.001	0.003	0.008	0.184	0.125	0.476	0.012	<0.001
hsa-miR920	Not expressed	—	—	—	—	—	—	—	—	—
Upregulated miRNAs										
hsa-miR892b	Not expressed	—	—	—	—	—	—	—	—	—
hsa-miR1288	Not expressed	—	—	—	—	—	—	—	—	—
hsa-miR520a-3p	Not expressed	—	—	—	—	—	—	—	—	—
hsa-miR542-5p	Expressed	No difference	0.122	0.249	0.565	0.256	0.060	0.095	0.103	0.0188
hsa-miR122*	Not expressed	—	—	—	—	—	—	—	—	—

NOTE: For each miRNA, it is indicated whether it is expressed in the METABRIC dataset (column 2); for the expressed miRNAs, it is also indicated whether they are over- or underexpressed in the tumor tissue relative to normal samples (column 3), and corresponding *P* values for all samples together (column 4), only in matched tumor and normal samples from the same patient across all subtypes (column 5), and for each subtype alone (columns 6–10). The number of patients in each comparison is indicated in parentheses in the column heads. The last column contains *P* values for Kaplan–Meier analysis, comparing the difference in survival between the third of patients with the highest expression of the miRNA to the third of patients with lowest expression.

Abbreviations: N, normal tissue; T, tumoral tissue.

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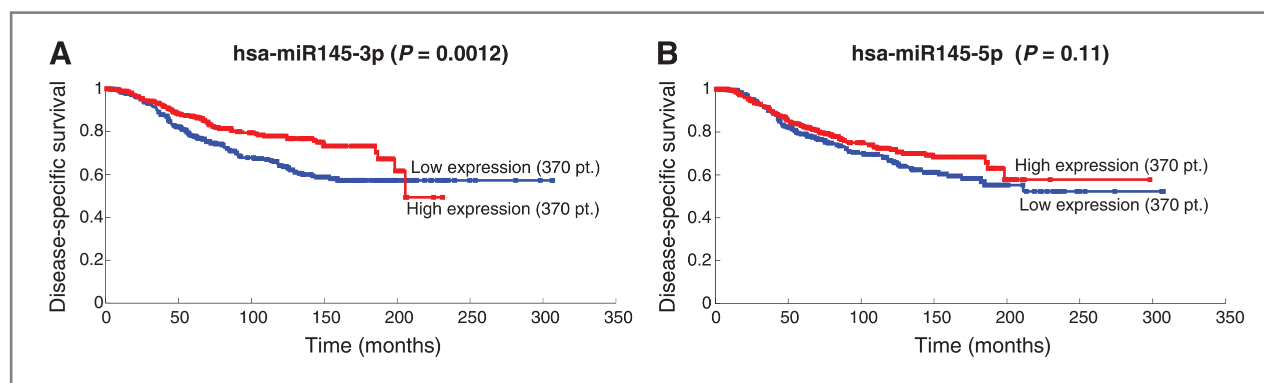

Figure 2. Breast cancer disease-specific survival by miRNA expression. Kaplan–Meier analysis for the association between (A) miR145-3p and (B) miR145-5p expression levels with survival of patients with breast cancer (based on the METABRIC dataset). For each miRNA, we compared the third of patients with the highest expression levels of the corresponded miRNA (red) to the third of patients with the lowest expression (blue). The miRNA name and *P* value are indicated in the title.

breast cancer risk. Macronutrient intake, characteristic of the Western diet, and obesity may activate inflammatory signaling pathways (40). Elevated levels of the proinflammatory cytokines promote angiogenesis, tumor progression, and metastasis (41).

The role of miRNAs isolated from leukocytes as biomarkers of cancer occurrence has been underlined in this study by the predicted pathway analysis conducted on the basis of the three miRNA clusters. All clusters seem to target, with the highest level of statistical significance, pathways related to both breast and other cancer development. At the same time, the clusters targeted genes involved in metabolic and hormone risk factors for breast cancer.

When considering miR145-3p and miR145-5p downregulation, we found that it was consistent across five unrelated databases, with their high expression associated with better survival. This association was statistically significant for miR145-3p. This was further corroborated by our findings that miR145-3p apparently controls cell proliferation, whereas miR145-5p's activity seems to modulate cell migration and invasion. Thus, we propose that biologic functions of these two miRNAs might differ according to the different phases of cancer development. The two miRNAs derive from each arm of their pre-miR hairpin. In a previous publication of our group, Biagioni and colleagues (7) observed a similar opposite and complementary behavior of two miRNAs that, similar to miR145-3p and miR145-5p, shared the same precursor: miR10b-5p and miR10b-3p. The overexpression of miR10b-5p was associated with tumor invasion and metastasis (42), whereas the overexpression of miR10b-3p was related to cell-cycle progression and proliferation (7). Following this model, we may consider miR145-3p downregulation as a very early signal of breast cancer development for its specific control of the first step of carcinogenesis: cell proliferation. miR145-5p has been shown to act as a tumor suppressor in different tumor types (36). Its downregulation was associated with neoplastic cell growth, invasion, and metastasis and is paired with increased expression of different oncogenic mRNA targets expression, such as EGFR, MYC, MUC1, OCT4, and RTKN (8, 36, 43).

There is very scarce evidence on the involvement of miR145-3p in cancer progression. Indeed, Camps and colleagues (44) observed downregulation of miR145-3p expression in MCF-7 cells exposed to hypoxia.

The evidence showing that only two miRNAs out of 20 were corroborated in all different databases was expected. Differences in phase of the natural history of the disease at sample collection (ORDET was based on samples derived from women still healthy, whereas all the other samples were derived from breast cancer lesions) and differences in study design reflected in differences in study populations by age and/or menopausal status may explain the results. In particular, a comparison of miRNA profiling between leukocyte samples (ORDET) and tissue samples (Metabric and the other database) represents a limitation for our direct study inference because miRNA profiling was performed in different tissues. However, we hypothesized that leukocyte miRNA profile (ORDET samples) did reflect, in still healthy women, exposure to nutritional and metabolic determinants of breast cancer. Then, differences in miRNA profiling between women who later developed breast cancer and women who remained healthy had potential etiologic meaning. Subsequently, in the Metabric study, as well as in other databases, we investigated whether tissue from breast cancer cases, as a later event, reflected similar miRNA profiling, effect of exposure to risk factors, we observed in leukocytes collected from those ORDET women candidate to develop breast cancer.

The prospective nature of the observation that specific miRNAs were deregulated in leukocytes collected from healthy women to develop breast cancer 20 years before disease onset makes our study unique when compared with previous studies. The prospective design of our study assigns to both miR145-3p and miR145-5p a role

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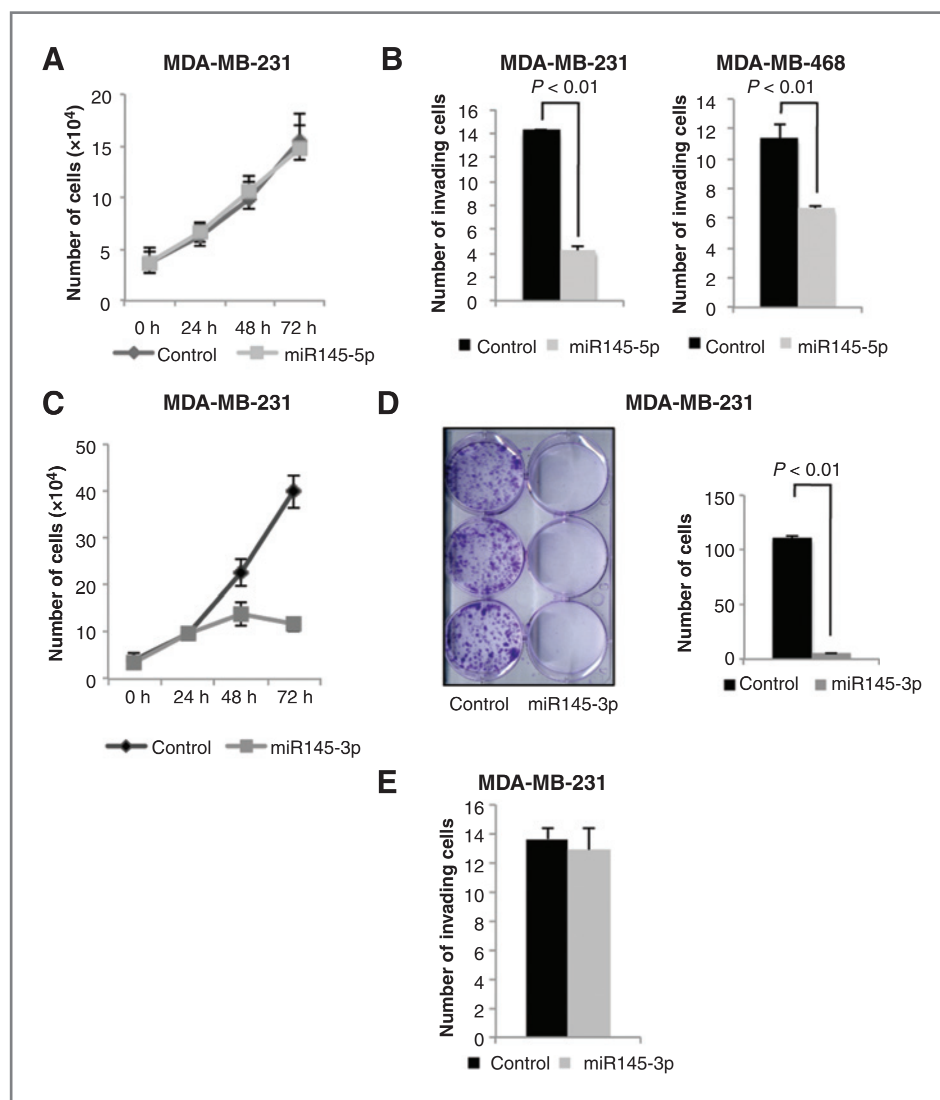

Figure 3. *In vitro* effects of miR145-5p and miR145-3p. A, miR145-5p does not affect breast cancer cell proliferation. Proliferation assay was performed in MDA-MB-231 cells upon miR145-5p exogenous expression. Cells were collected and manually counted at the indicated time points. B, miR145-5p inhibits breast cancer cell migration. Transwell migration assay was performed in MDA-MB-231 and MDA-MB-468 cells upon miR145-5p exogenous expression. C, miR145-3p impinges breast cancer cell proliferation. Proliferation assay was performed in MDA-MB-231 cells upon miR145-3p exogenous expression. Cells were collected and manually counted at the indicated time points after transfection. D, miR145-3p impinges breast cancer cell colony formation ability. Clonogenic assay was performed in MDA-MB-231 cells upon miR145-3p exogenous expression. E, miR145-3p does not affect breast cancer cell migration. Transwell migration assay was performed in MDA-MB-231 cells upon miR145-3p exogenous expression. B, C, and E, histogram bars represent mean $\pm$ standard deviation of at least three independent replicates.

as long-term breast cancer predictors and it also opens a new avenue for prevention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Conception and design: P. Muti, F. Berrino, S. Strano, G. Blandino
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Muti, A. Sacconi, A. Hossain, N.B.B. Moshe, V. Krogh, J. Beyene
Writing, review, and/or revision of the manuscript: P. Muti, A. Hossain, S. Sieri, V. Krogh, F. Biagioni, S. Strano, J. Beyene, G. Blandino
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Muti, Y. Yarden
Study supervision: P. Muti, G. Blandino
Other (provided microarray data): F. Biagioni

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