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Deregulation of cancer-related miRNAs is a common event in both benign and malignant human breast tumors

Andliena Tahiri[1,2], Suvi-Katri Leivonen[2–4], Torben Lüders[1,2,†], Israel Steinfeld[5,†], Miriam Ragle Aure[2,3], Jürgen Geisler[2,6], Rami Mäkelä[4], Silje Nord[3], Margit L.H.Riis[2,7], Zohar Yakhini[5,8], Kristine Kleivi Sahlberg[3,9], Anne-Lise Børresen-Dale[2,3], Merja Perälä[4], Ida R.K.Bukholm[2,7,10] and Vessela N.Kristensen[1–3,*]

[1]Department of Clinical Molecular Biology (EpiGen), Division of Medicine and Laboratory Sciences, Akershus University Hospital and [2]Institute for Clinical Medicine, Faculty of Medicine, University of Oslo, Oslo, Norway, [3]The K.G. Jebsen Center for Breast Cancer Research, Department of Genetics, Institute for Cancer Research, Oslo University Hospital Radiumhospitalet, Oslo, Norway, [4]Medical Biotechnology, VTT Technical Research Centre of Finland, Turku, Finland, [5]Department of Computer Science, Technion, Haifa, Israel, [6]Department of Oncology and [7]Department of Surgery, Akershus University Hospital, Lørenskog, Norway, [8]Agilent Laboratories, Tel Aviv, Israel, [9]Department of Research, Vestre Viken Hospital Trust, Drammen, Norway and [10]Institute of Health Promotion, Akershus University Hospital, Lørenskog, Norway

*To whom correspondence should be addressed. Akershus University Hospital, Institute of Clinical Molecular Biology, PO Box 28, N–1478 Lørenskog, Norway. Tel: +47 02900 (central board); Fax: +47 67963820; Email: v.n.kristensen@medisin.uio.no

MicroRNAs (miRNAs) are endogenous non-coding RNAs, which play an essential role in the regulation of gene expression during carcinogenesis. The role of miRNAs in breast cancer has been thoroughly investigated, and although many miRNAs are identified as cancer related, little is known about their involvement in benign tumors. In this study, we investigated miRNA expression profiles in the two most common types of human benign tumors (fibroadenoma/fibroadenomatosis) and in malignant breast tumors and explored their role as oncomirs and tumor suppressor miRNAs. Here, we identified 33 miRNAs with similar deregulated expression in both benign and malignant tumors compared with the expression levels of those in normal tissue, including breast cancer-related miRNAs such as let-7, miR-21 and miR-155. Additionally, messenger RNA (mRNA) expression profiles were obtained for some of the same samples. Using integrated mRNA/miRNA expression analysis, we observed that overexpression of certain miRNAs co-occurred with a significant downregulation of their candidate target mRNAs in both benign and malignant tumors. In support of these findings, *in vitro* functional screening of the downregulated miRNAs in non-malignant and breast cancer cell lines identified several possible tumor suppressor miRNAs, including miR-193b, miR-193a-3p, miR-126, miR-134, miR-132, miR-486-5p, miR-886-3p, miR-195 and miR-497, showing reduced growth when re-expressed in cancer cells. The finding of deregulated expression of oncomirs and tumor suppressor miRNAs in benign breast tumors is intriguing, indicating that they may play a role in proliferation. A role of cancer-related miRNAs in the early phases of carcinogenesis and malignant transformation can, therefore, not be ruled out.

Introduction

Global gene expression analysis based on microarray technology has facilitated a molecular taxonomy of breast cancer using pervasive differences in the gene expression patterns (1–4). Several breast cancer gene signatures have been identified, allowing stratification of patients into subgroups with either good or poor prognosis (5). Although global gene expression analysis has been extensively used for taxonomy or prognostication, it has revealed little of the underlying deregulation leading from benign tissue to malignant transformation and growth, as the studied specimens only provide a molecular snapshot at the time of diagnosis in an already established infiltrating breast tumor.

MicroRNAs (miRNAs) are small nucleic acid molecules (~18–24 nucleotides), proven to be suitable to classify breast cancer into prognostic molecular subtypes (6). Typically, miRNAs negatively regulate messenger RNAs (mRNAs) at the posttranscriptional level by binding primarily to the 3′-untranslated region of their target genes (7). As of today, >2000 mature miRNAs have been identified in humans (http://www.mirbase.org/), and many of them have been identified to have an important role in disease progression, including cancer. miRNA expression profiling is considered as highly informative as miRNAs reflect the developmental lineage and differentiation state of tumors (8). In cancer, miRNAs are either down- or upregulated and are thought to behave as either tumor suppressors and/or oncogenes (oncomirs), respectively (9). The first study identifying deregulated miRNAs in breast cancer tissue was performed by Iorio *et al*. in 2005 (10). Thereafter, many studies have identified miRNAs with differential expression patterns in multiple human cancer types (11), including breast cancer (12–16). Deregulated expression of miRNAs in breast cancer has been linked to different clinicopathological features such as tumor stage, receptor status and survival (12), and miRNA expression profiles have proven to be more reliable in classifying tumors than mRNA expression profiles (8).

Although extensive research has been conducted on malignant tumors, there are far more benign lesions occurring in the breast, and little is known about the molecular mechanisms in those tumor types. There are several types of benign lesions in the breast, which can be classified into three different subgroups as proposed by Dupont and Page (17): (i) non-proliferative lesions, (ii) proliferative lesions without atypia and (iii) atypical hyperplasia. Several studies have suggested that women with proliferative lesions without atypia and women with atypical hyperplasia are at greater risk of developing breast cancer compared with women with non-proliferative lesions in the breast (17–21). Fibroadenoma, in particular, has previously been shown to be a long-term risk factor for breast cancer, and the risk is increased in women with complex fibroadenomas, proliferative disease or family history of breast cancer (21–24). To study the role of miRNAs in benign and malignant human breast tissue, we first analyzed matched miRNA and mRNA expression profiles in breast fibroadenoma and fibroadenomatosis, malignant (infiltrating) breast tumors and normal breast tissue. Thereafter, *in vitro* miRNA functional studies were performed by transfecting the downregulated miRNAs into breast cancer cells in order to assess their effect on cell growth.

Materials and methods

Tissue specimens

Tumor tissue specimens, biopsies and associated clinical data were collected at Akershus University Hospital, Norway, between years 2003 and 2009. Normal tissue specimens were collected at the Colosseum Clinic, Oslo, Norway, in 2008, without any clinical data. All tumor cases have been histologically confirmed by a pathologist, and a written informed consent was obtained from each patient. The study was approved by the Regional Ethics committee.

In total, there were 29 normal breast tissue samples from women who had undergone reduction mammoplasty, 29 malignant breast tumor tissue samples from women with primary breast carcinomas and 21 core needle biopsies

Abbreviations: cPARP, cleaved PARP; DMEM, Dulbecco's modified Eagle's medium; FDR, false discovery rate; miRNA, microRNA; mRNA, messenger RNA; SAM, Significance Analysis of Microarrays.

[†]These authors contributed equally to this work.

from women with benign tumors, mainly fibroadenomas or fibroadenomatosis, with no previous history of breast cancer. Tissue samples and biopsies were stabilized in RNAlater (Sigma–Aldrich, St Louis, MO) and stored at −80°C. The clinical study cohort was selected randomly, and the clinicopathological data are summarized in Supplementary Table 1, available at *Carcinogenesis* Online.

RNA isolation

Total RNA was extracted from all specimens using TRIzol (Invitrogen, Carlsbad, CA) in combination with RNeasy Mini Kit (Qiagen, Valencia, CA). The method combines phenol/guanidine-based lysis and silica membrane column purification of total RNA (25). The quantity and purity of total RNA was assessed by using NanoDrop ND-1000® spectrophotometer (Thermo Scientific, Wilmington, DE), whereas the RNA quality was controlled by using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The integrity of total RNA was assessed using the RNA 6000 Nano Kit (Agilent), and the presence of miRNAs was confirmed with the Small RNA Assay (Agilent). Only extracts with RNA Integrity Number >6 were included in the analysis.

miRNA microarrays

miRNA expression profiling was performed on 79 breast tissue samples using Agilent Technologies 'Human miRNA Microarray Kit (V3)' (Agilent). For all samples, 100 ng of total RNA was used for the experiment. The microarray contained probes for 866 human and 89 human viral miRNAs from the Sanger database (26). Sample labeling, hybridization and scanning were performed according to the manufacturer's protocol (ver. 1.6). Features and local background were detected and analyzed with Agilent Feature Extraction 10.7.3.1., whereas raw data were analyzed using J-Express 2009 (27). The signal intensity of each probe was $\log_2$ transformed, and missing values were imputed by the program. Between-sample normalization was performed using the built-in method for scale normalization (28). In total, 328 miRNA probes were considered to be expressed in our dataset and used for further analysis. The miRNA microarray data are submitted to ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) with accession number E-MTAB-471.

mRNA microarrays

Gene expression analysis was performed using Agilent Technologies 'whole human genome 4 × 44K one-color oligo array' for 53 of the samples (see Supplementary Table 1, available at *Carcinogenesis* Online). For all samples, 250 ng of total RNA was used for the experiment. mRNA expression profiling was performed on 22 normal breast tissue samples, 21 breast cancer tissue samples and 10 biopsies of fibroadenomas and fibroadenomatosis. Sample labeling, hybridization and scanning were performed according to the manufacturer's protocol (ver. 5.7). Features and local background were detected and analyzed with Agilent Feature Extraction 10.7.3.1. For expression values, all data were $\log_2$ transformed and quantile normalized in J-Express 2009. Additionally, batch effects were removed using Partek Genomics Suite 6.6 (Partek, St Louis, MO). The mRNA microarray data are submitted to ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) with accession number E-MTAB-779.

Statistical analysis

For supervised analysis, Significance Analysis of Microarrays (SAM) (29), as implemented in J-Express 2009, was used to identify differentially expressed miRNAs. Only miRNAs with false discovery rate (FDR) <0.001 were chosen for further analysis, and for probes represented more than once on the array, the median value was used. FDR is computed as (median of the number of falsely called genes) divided by (the number of genes called significant) (29). Hierarchical clustering was performed using Euclidean correlation as a distance measure and complete linkage. Correspondence analysis was performed using J-Express 2009.

miRNA target enrichment

miRNA target prediction scores were obtained from Targetscan v.5.1 (30). We defined the targets of each miRNA to be the top 2000 scored genes based on the context score. Genes were ranked by differential expression *t*-test *P* value (e.g. normal versus cancer). miRNA target enrichment in the ranked list of genes was assessed by mHG statistics (31).

miRNA expression using reverse transcription–PCR

Quantification of 12 selected mature miRNAs was performed on 13 samples (four normal, five benign and four malignant samples) with TaqMan® MicroRNA Assays (Applied Biosystems, Foster City, CA). The miRNAs selected for this validation were let-7c, miR-21, miR-96, miR-126, miR-183, miR-193a-3p, miR-193b, miR-200b, miR-200c, miR-205, miR-224 and miR-551b. The endogenous control used for this experiment was RNU6B. Reverse transcription–PCR reactions were carried out using the manufacturer's recommendation. In brief, 10 ng of total RNA was reverse transcribed using the TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems) with miRNA specific RT primers (Applied Biosystems). Quantitative Real-Time PCR was performed following the manufacturer's recommendation in triplicates on a 7900 HT Fast Real-Time PCR System (Applied Biosystems) with a standard relative quantification thermal cycling program. Cycle threshold (*C*t) values were obtained using the SDS 2.3 software (Applied Biosystems). An average *C*t value was calculated for each sample on each miRNA, and the data were normalized by subtracting the average *C*t value of the endogenous control from the corresponding sample, thus obtaining the Δ*C*t value. Pearson correlation was used to investigate the correlation between Agilent array and TaqMan expression.

Cell culturing

MCF-7 cells were obtained from the Interlab Cell Line Collection (ICLC, Genova, Italy) and cultured in Dulbecco's modified Eagle's medium (DMEM; 1 g/l glucose) (Sigma) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin/streptomycin. JIMT-1 cells were obtained from The German Collection of Microorganisms and Cell Cultures (DSMZ, Leibniz, Germany), and they were cultured in 1:1 Ham's F-12/DMEM (4.5 g/l glucose) supplemented with 10% fetal bovine serum, 10 µg/ml insulin, 2 mM L-glutamine and 1% penicillin/streptomycin. KPL-4 cells were a gift from Prof Junichi Kurebayashi (Kawasaki Medical School, Japan), and they were cultured in DMEM (4.5 g/l glucose) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin/streptomycin. Non-malignant cell lines, hTERT-HMEC and MCF-10A, were obtained from American Type Culture Collection (ATCC) and cultured according to ATCC's recommendations. The growth medium for HMEC cells was MEGM™ Mammary Epithelial Cell Growth Medium with BulletKit™ supplements (Lonza, Basel, Switzerland). For MCF-10A, DMEM/F12 supplemented with 5 ng/ml epidermal growth factor, 2% horse serum, 0.5 µg/ml hydrocortisone, 10 µg/ml insulin and 100 ng/ml cholera toxin was used as growth medium. Both ICLC and the DSMZ authenticate all human cell lines by DNA typing using short tandem repeats. The DSMZ uses additional cytogenetic and immunophenotypic tests, and ICLC uses isoenzyme analysis for determining the species of origin. ATCC performs authentication through short tandem repeat profiling, karyotyping and cytochrome C oxidase I testing. All cells were cultured for a maximum of 30 passages prior to use.

miRNA functional screening

For miRNA functional screening, cells were transfected with miRIDIAN microRNA mimics (library v. 10.1 with 810 miRNA mimics) (20 nM) (Dharmacon, Lafayette, CO) in 384-well plates using SilentFect (Bio-Rad Laboratories, Hercules, CA), as described previously (32). After 72 h incubation, cell viability was assayed by CellTiter-GLO cell viability assay (Promega Corp., Madison, WI). The results were $\log_2$ transformed and normalized using a Loess method (33). Alternatively, for lysate microarray analysis, cells were lysed and printed on nitrocellulose-coated microarray FAST™ slides (Whatman, Florham Park, NJ). Ki67 and cleaved PARP (cPARP) were detected by staining the slides with Ki67 antibody (#M7240; Dako, Glostrup, Denmark) and cPARP antibody (#ab32064; Abcam, Cambridge, UK), respectively, followed by exposure to Alexa Fluor 680-tagged secondary antibodies (Invitrogen). For total protein measurement, the arrays were stained with Sypro Ruby Blot solution (Invitrogen). The slides were scanned with Tecan LS400 (Tecan, Durham, NC) microarray scanner and Odyssey Licor IR-scanner (LI-COR Biosciences, Lincoln, NE) to detect the Sypro, Ki67 and cPARP signals. Array-Pro Analyzer microarray analysis software (Median Cybernetics, Bethesda, MD) was used for analyzing the data.

Growth curves

For assaying the cell growth, MCF-7 cells (8000 per well) were reverse transfected with 20 nM pre-miRNA constructs for miR-195, miR-497, negative control miRNA (Ambion, Austin, TX) or with siRNA for KIF11 (Qiagen) in 96-well plates using SiLentFect. The growth curves were generated during 156 h period of time by using the INCUCYTE™ Live-Cell Imaging System (Essen BioSciences, Ann Arbor, MI), which provides a time-lapse method for quantifying cell growth inside the cell culture incubator.

Cell cycle analysis

The cell cycle analyses were performed using the nuclei preparations of MCF-7 cells. Cells (30 000 per well on 24-well plates) were transfected with 20 nM pre-miR constructs and incubated for 48 h. Subsequently, the cells were trypsinized, processed for the cell cycle analysis with BD CycleTest Plus DNA reagent kit (BD Biosciences, San Jose, CA) and examined for the cell cycle distribution with FACSArray (BD Biosciences). The data were analyzed using Multicycle software (Phoenix FlowSystems, San Diego, CA).

Soft agar assay

MCF-7 cells were transfected with the pre-miR constructs. After 24 h of incubation, the cells were trypsinized and counted. Subsequently, 2500 cells were mixed 1:1 with 2× DMEM + 20% fetal calf serum + 0.7% agar so as

to result in a final agar concentration of 0.35%. Thereafter, 1 ml samples of this cell suspension were plated in six-well plates coated with 0.5% agar in 1× DMEM + 10% fetal calf serum (2 ml per well), allowed to solidify and then covered with the growth medium. The colony numbers were counted 2 weeks later.

Results

Sample classification based on miRNA expression profiles

miRNA expression profiles in benign and malignant breast tumors and in normal breast tissue were studied using microarrays. Unsupervised correspondence analysis of the expression of all miRNA probes showed a clear division of normal tissue from benign and malignant tumors (Figure 1A). The normal tissue samples clustered together, with the exception of a few samples that were assigned to the benign and malignant tumor group, which for the most clustered together. A total of 134 probes (58 miRNAs) were identified as significantly differentially expressed (FDR < 0.001) between the three histological tissue types (see Supplementary Table 2, available at *Carcinogenesis* Online). After supervised hierarchical clustering using only these 134 probes, the normal tissues clustered as in the unsupervised analysis, whereas the benign and malignant tumors remained unseparated but divided into several clusters (Figure 1B). The separation pattern obtained was independent of clustering method, gene filtering during supervised and unsupervised analysis or clinical factors such as age, receptor status (estrogen receptor, progesterone receptor and Her2), subtypes or pathological types. Additionally, there were no differences in miRNA expression obtained between fibroadenomas and fibroadenomatosis.

Deregulated miRNA expression in benign and malignant breast tumors

Comparing the miRNA expression profiles in benign and malignant tumors, we identified miRNAs that were similarly or differentially expressed in the different tissue types (Table I). A set of 33 miRNAs were identified as deregulated in a similar way in both benign and malignant tumors compared with those in normal tissue (Supplementary Table 3, available at *Carcinogenesis* Online). Of these, 19 miRNAs were downregulated, whereas 14 miRNAs were upregulated in both tumor types. The most strongly upregulated miRNAs were miR-21, miR-196a, miR-183, miR-96 and miR-200b/c, whereas the most strongly downregulated ones were miR-551b, miR-224, miR-193b, miR-139-5p, miR-365, miR-145 and miR-193a-3p. miRNA array expression validation was performed for 12 of the miRNAs that were significantly up/downregulated in benign and malignant tumors, compared with those in normal tissue, using reverse transcription–PCR (Supplementary Figure 1, available at *Carcinogenesis* Online). Good correlation (Pearson's $r > 0.65$) was observed between microarray and reverse transcription–PCR expression for most of the miRNAs. We also identified a set of 40 miRNAs that were significantly differentially expressed between benign and malignant tumors (FDR < 0.001) (Table I). Studying in detail the average miRNA expression levels of the three tissue types, the expression of miRNAs in benign tumors was generally intermediate compared with that of miRNAs in normal tissue and malignant tumors (Supplementary Figure 2A, available at *Carcinogenesis* Online). The same trend of up/downregulation and a gradient from normal/benign/malignant expression was confirmed for the reported miRNAs through miRNA expression profiling of non-malignant and malignant cell lines (Supplementary Figure 2B, available at *Carcinogenesis* Online). Therefore, certain miRNAs identified as differentially expressed between benign and malignant tumors were also identified as differentially expressed between tumors and normal tissue. Additionally, some of the miRNAs could separate between tissue types (see Table I), e.g. downregulation of miR-497 was only observed in malignant tumors, separating one tumor type from the other, and to the normal physiological state. On the other hand, miR-205 was downregulated in benign tumors and upregulated in malignant tumors compared with that in normal tissue.

miRNAs are inversely correlated with their putative target mRNAs

To test whether miRNA differential expression is influencing gene expression programs, we examined the matched dataset of both miRNA and mRNA expression profiling. To estimate the putative regulatory activity of a particular miRNA, we tested whether upregulation of one miRNA is co-occurring with a significant downregulation of its predicted targets in all three tissue types. We identified 15 miRNAs as being upregulated in malignant tumors, compared with those in normal tissues, for which we were able to statistically validate a significant reduction in the expression levels of their predicted mRNA targets (Table II). For instance, targets of miR-96 were the most downregulated genes in malignant tumors (mHG $P <$ 2E-13; Figure 2), consistent with the significant upregulation of miR-96 in these tissues (SAM FDR < 0.001). miRNA target regulation activity was also observed for miR-21 that showed upregulation of the miRNA (SAM FDR < 0.001) and downregulation of its targets in malignant tumors (mHG $P <$ 2E-8; Figure 2). The same trend for downregulation of targets for these 15 miRNAs was also observed in benign tumors compared with the expression level in normal tissue samples. These results underlined the similarities between benign and malignant tumor samples with respect to miRNA and mRNA expression levels. For downregulated miRNAs, no such significant difference in miRNA target enrichment was observed between benign and malignant tumor samples (Table II).

miRNA gain-of-function assays identify candidate tumor suppressor miRNAs

To study the functional significance of the observed aberrant miRNAs, we performed miRNA gain-of-function studies with both non-malignant breast epithelial cells and breast cancer cell lines, focusing on the downregulated miRNAs in malignant versus normal tissue (Table I). miRNA mimics were transfected into three breast cancer cell lines and two non-malignant cell lines, and their effect on cell growth was measured by luminescent-based cell viability assay. Alternatively, the cells were lysed and printed as protein microarrays, which were stained with markers for proliferation (Ki67) or apoptosis (cPARP). Several of the downregulated miRNAs in breast cancer showed inhibitory effects on breast cancer cell growth when they were overexpressed, suggesting that they might have a tumor suppressive role in breast cancer (Figure 3A and B; Supplementary Table 4, available at *Carcinogenesis* Online). The most effective growth suppressors identified were miR-193b, miR-193a-3p, miR-126, miR-134, miR-132, miR-486-5p, miR-886-3p, miR-195 and miR-497 (Figure 3A and B). Of these, only miR-497 and miR-132 had a slightly significant effect on cell growth on one of the non-malignant cell lines (Figure 3C).

Identification of the tumor suppressive role of miR-195/497

miR-195 and miR-497 belong to the same miRNA cluster and are thought to act as tumor suppressors (34). Here, they were downregulated in malignant tumors compared with those in normal tissue, and efficiently inhibited breast cancer viability and proliferation when they were re-expressed in cancer cells (Figure 3A and B). To further study these putative tumor suppressor miRNAs, we transfected MCF-7 breast cancer cells with pre-miR-195, pre-miR-497 and pre-miR negative control and monitored cell growth over a 156 h period of time in INCUCYTE Live-Cell Imaging System. As shown in Figure 4A, miR-195 and miR-497 efficiently inhibited the growth of MCF-7 cells, similar to the positive control siRNA for KIF11. The predicted targets of miR-195/497 are enriched for cell cycle genes (data not shown), and accordingly, overexpression of miR-195 and miR-497 resulted in cell cycle arrest at the G_1 phase of the cell cycle (Figure 4B). Next, we studied the effect of miR-195 and miR-497 on the colony formation of MCF-7 cells. Anchorage-independent growth of cells in soft agar is one of the hallmark characteristics of cellular transformation and uncontrolled cell growth. Normal cells are typically not capable of growing in semisolid matrices. MCF-7 cells transfected with negative control miRNA formed colonies in soft agar, whereas miR-195 and miR-497 inhibited this when they were

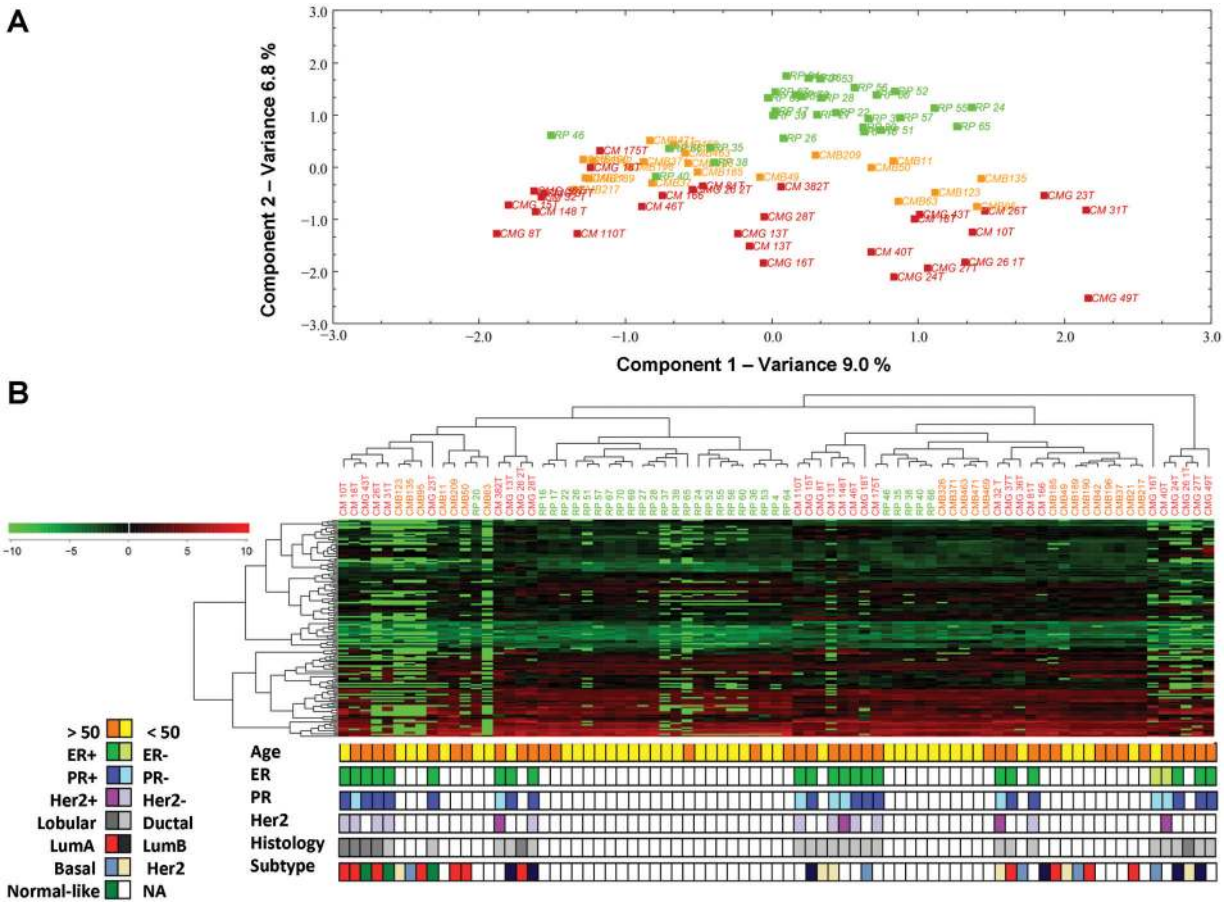

Fig. 1. miRNA expression profiles in normal, benign and malignant breast tissue. (**A**) Unsupervised correspondence analysis shows the distribution of samples according to their miRNA profiles in normal tissue (green), benign tumors (orange) and malignant tumors (red). In this plot, the normal tissue is distinct from benign and malignant tumors, but the benign and malignant tumors are not clearly defined in separate groups. (**B**) Supervised hierarchical clustering of the 58 most variable miRNAs (134 probes) across normal tissue (green), benign tumors (orange) and malignant tumors (red) at FDR <0.001 is shown. No clinical parameters or biological factor correlated with the observed clustering of samples. Several variables are categorized for illustration purposes. Age = age at time of inclusion.

transfected in the cells (Figure 4C). This provides further evidence for the tumor suppressive role of miR-195/497.

Discussion

miRNAs are important regulators of the cell, controlling gene expression in a subtle, yet not fully understood way. Their deregulation has been identified in many cancer types with high clinical relevance (12). In this study, our objective was to investigate the molecular profiles in benign breast tumors in comparison with those in malignant tumors and normal breast tissue.

Firstly, we wanted to determine whether benign tumors differ in miRNA profile from normal tissue on one hand and malignant tumors on the other. For many of the miRNAs, we observed a gradient from normal/benign/malignant expression. This may either suggest a role for those miRNAs in proliferation, but not malignancy, or offer us a more subtle view of miRNA driven regulation where quantity (expression levels) rather than quality (which miRNAs) may determine the phenotype (malignant or benign).

According to our results, neither supervised nor unsupervised analysis with miRNA expression profiles showed any clear separation of benign and malignant tumor samples in correlation to different biological or clinical parameters, indicating that benign and malignant tumors share some similarities in miRNA expression profiles. In this study, many cancer-related miRNAs were not only identified in malignant tumors but also in two types of benign breast tumors: fibroadenomas and fibroadenomatosis. A set of 33 miRNAs were similarly deregulated in benign and malignant tumors, and amongst them were miR-21, miR-145, miR-155 and members of the let-7 family, all previously found to be deregulated in breast cancer (10). In addition, the expression pattern of the studied cancer-related miRNAs in non-malignant breast epithelial cell lines and cancer cell lines is comparable with that observed in patient material.

miR-21 acts as an oncogene and is highly upregulated in malignant tumors, including preinvasive tumors (10,35,36). Its overexpression has previously been associated with several clinicopathological features (37,38) and targets several tumor suppressor genes important for several cellular processes (39). In this study, miR-21 was significantly upregulated in both tumor tissues, with its gene targets being significantly downregulated in both benign and malignant tumors, confirming the close relationship between the two pathological states. Previous studies have suggested miR-21 as a good marker of malignancy as it is upregulated in both tissue and serum of breast cancer patients compared with the expression level in healthy individuals (40). However, the upregulation of miR-21 in benign tumors renders a further consideration of its usefulness as a marker of progression of malignancy.

Another miRNA of interests that was similarly deregulated in benign and malignant tumors is miR-96. Previously, miR-96 has been identified as being overexpressed in eight different cancer types, including breast cancer (11). Upregulation of miR-96 in breast cancer is associated with enhanced proliferation and tumorigenicity through downregulation of *FOXO3a* (41). miRNA/mRNA correlation studies identified miR-96 gene targets as the most statistically significantly enriched miRNA target set in the downregulated genes in both malignant and benign tumors. Downregulation of *FOXO3a* by miR-96 was

Table I. Differentially expressed miRNAs at FDR <0.001

Benign versus normal		Malignant versus normal		Benign versus malignant	
miRNA	LFC (relative to benign expression)	miRNA	LFC (relative to malignant expression)	miRNA	LFC (relative to benign expression)
hsa-let-7a[a,b]	1.56	hsa-let-7a[a]	1.84	hsa-let-7c[a]	1.97
hsa-let-7b[b]	1.7	hsa-let-7c[a]	2.81	hsa-miR-100[b]	3.67
hsa-let-7c[a,b]	1.67	hsa-let-7d[b]	1.94	hsa-miR-10a[b]	−2.24
hsa-miR-10a[b]	1.76	hsa-let-7g[b]	1.72	hsa-miR-1202[b]	−2.73
hsa-miR-1202[b]	2.7	hsa-miR-100[b]	2.54	hsa-miR-1225-5p[b]	−2.73
hsa-miR-1207-5p[b]	1.98	hsa-miR-106b[c]	−2.07	hsa-miR-1246[b]	−2.66
hsa-miR-1225-5p[b]	2.67	hsa-miR-10b[b]	2.57	hsa-miR-125b[b]	3.15
hsa-miR-1234[a]	−1.73	hsa-miR-1228	−2.49	hsa-miR-1260[b]	2.5
hsa-miR-1246[b]	2.47	hsa-miR-1234[a]	−1.69	hsa-miR-127-3p[b]	2.63
hsa-miR-126[a,b]	2.37	hsa-miR-125b[b]	3.42	hsa-miR-1274b[b]	2.97
hsa-miR-1260[b]	−2.13	hsa-miR-126[a,b]	2.6	hsa-miR-1308[b]	−2.58
hsa-miR-1274b[b]	−4.96	hsa-miR-1280[a]	−1.74	hsa-miR-130a[b]	2.7
hsa-miR-1280[a]	−1.9	hsa-miR-130a[b]	2.01	hsa-miR-130b[a,c]	−1.84
hsa-miR-1308[b]	2.58	hsa-miR-130b[a,c]	−2.69	hsa-miR-132[b]	2.36
hsa-miR-130b[a,c]	−1.69	hsa-miR-139-5p[a,b]	3.91	hsa-miR-135a[d]	−2.32
hsa-miR-134[b]	1.7	hsa-miR-140-3p[a,b]	3.15	hsa-miR-139-5p[a,b]	1.84
hsa-miR-135a[d]	1.91	hsa-miR-141[a,c]	−3.04	hsa-miR-145[a,b]	2.58
hsa-miR-139-5p[a,b]	2.41	hsa-miR-143[b]	1.85	hsa-miR-150[d]	−1.99
hsa-miR-140-3p[a,b]	1.94	hsa-miR-145[a,b]	4.24	hsa-miR-155[a,c]	−2.36
hsa-miR-141[a,c]	−2.63	hsa-miR-146a[c]	−2.04	hsa-miR-15a	−1.78
hsa-miR-145[a,b]	1.71	hsa-miR-146b-5p[a,c]	−2.18	hsa-miR-15b	−1.87
hsa-miR-146b-5p[a,c]	−2.36	hsa-miR-149[a,c]	−2.8	hsa-miR-183[a]	−2.88
hsa-miR-149[a,c]	−2.12	hsa-miR-150[d]	−1.94	hsa-miR-188-5p	−2.21
hsa-miR-150	1.77	hsa-miR-151-3p	−1.69	hsa-miR-199b-5p	1.95
hsa-miR-151-5p	1.36	hsa-miR-155[a,c]	−3.09	hsa-miR-205[b]	5.9
hsa-miR-155[a,c]	−1.49	hsa-miR-181b	−1.43	hsa-miR-21[a,c]	−5.72
hsa-miR-183[a,c]	−2.74	hsa-miR-183[a,c]	−6.5	hsa-miR-210[c]	−2.66
hsa-miR-193a-3p[a,b]	3.06	hsa-miR-193a-3p[a,b]	2.04	hsa-miR-22[b]	−1.99
hsa-miR-193a-5p[a,b]	2.6	hsa-miR-193a-5p[a,b]	3.02	hsa-miR-328[b]	1.93
hsa-miR-193b[a,c]	4.18	hsa-miR-193b[a,b]	2.83	hsa-miR-331-3p	−1.59
hsa-miR-196a[a]	−3.55	hsa-miR-195[b]	2.37	hsa-miR-342-3p	−2.28
hsa-miR-19b	−2.83	hsa-miR-196a[a]	−5.92	hsa-miR-425	−2.04
hsa-miR-200b[a,c]	−2.58	hsa-miR-199a-5p[b]	1.75	hsa-miR-486-5p[b]	3.31
hsa-miR-200c[a,c]	−2.69	hsa-miR-200b[a,c]	−4.18	hsa-miR-497[b]	2.37
hsa-miR-205[b]	−2.38	hsa-miR-200c[a,c]	−3.65	hsa-miR-572[b]	2.29
hsa-miR-21[a,c]	−2.53	hsa-miR-205[b]	2.48	hsa-miR-663[b]	−2.99
hsa-miR-22[b]	1.85	hsa-miR-20a[c]	−1.68	hsa-miR-886-3p[b]	1.8
hsa-miR-224[a,b]	4.11	hsa-miR-21[a,c]	−14.69	hsa-miR-96[a,c]	−2
hsa-miR-23a[b]	1.64	hsa-miR-210[c]	−3.03	hsa-miR-99a[a,b]	2.4
hsa-miR-27a[b]	2.02	hsa-miR-224[a,b]	2.96		
hsa-miR-29a[a,b]	1.86	hsa-miR-28-5p	−1.62		
hsa-miR-30b	−2.1	hsa-miR-29a[a,b]	2.1		
hsa-miR-320c[a,b]	2.09	hsa-miR-30d	−1.53		
hsa-miR-342-5p	−1.68	hsa-miR-320c[a,b]	1.93		
hsa-miR-34a[a,c]	−1.62	hsa-miR-331-3p	−1.66		
hsa-miR-365[a,b]	3.1	hsa-miR-342-3p	−2.84		
hsa-miR-551b[a,b]	5.13	hsa-miR-342-5p	−1.85		
hsa-miR-574-3p	−1.63	hsa-miR-34a[a,c]	−1.89		
hsa-miR-575[a,b]	2.59	hsa-miR-365[a,b]	3		
hsa-miR-630[a,b]	1.81	hsa-miR-425	−2.31		
hsa-miR-652[a,b]	2.5	hsa-miR-451[b]	3.54		
hsa-miR-663[b]	1.89	hsa-miR-486-5p[b]	4.86		
hsa-miR-671-5p[b]	1.58	hsa-miR-497[b]	3.79		
hsa-miR-720	−2.29	hsa-miR-551b[a,b]	5.58		
hsa-miR-766[a]	−1.76	hsa-miR-575[a,b]	2.26		
hsa-miR-886-3p[b]	−1.82	hsa-miR-630[a,b]	2.02		
hsa-miR-96[a,c]	−2.55	hsa-miR-652[a,b]	1.98		
hsa-miR-99a[a,b]	1.48	hsa-miR-766[a]	−2.08		
kshv-miR-K12-3[b]	1.66	hsa-miR-92a[b]	1.75		
		hsa-miR-940	−2.08		
		hsa-miR-96[a,c]	−5.11		
		hsa-miR-98	−1.88		
		hsa-miR-99a[a,b]	3.57		

miRNAs are sorted in alphabetical and numerical order. LFC, log fold change.
[a]miRNAs similarly deregulated in both benign and malignant tumors.
[b]Functionally validated miRNAs.
[c]miRNA hubs with multiple targets identified through miRNA/mRNA analysis.
[d]Antisense miRNA.

Table II. miRNA target enrichment *P* values in downregulated mRNAs

miRNA	Normal versus cancer	Normal versus benign	Benign versus cancer
hsa-miR-96	1.54198E-13	4.22337E-12	0.220095012
hsa-miR-34a	1.72717E-08	6.20594E-06	0.296446004
hsa-miR-130b	1.56126E-07	3.12501E-05	0.481349583
hsa-miR-21	1.74673E-08	0.013739705	0.029005476
hsa-miR-106b	2.15914E-09	2.71309E-06	1
hsa-miR-20a	2.50501E-08	4.18106E-05	1
hsa-miR-200b	7.16424E-08	0.00076323	1
hsa-miR-200c	1.05827E-07	0.00076323	1
hsa-miR-149	1.72906E-05	0.008602671	1
hsa-miR-141	0.000184525	2.88998E-05	1
hsa-miR-183	0.000300593	0.057011491	1
hsa-miR-146a	6.55016E-05	0.979109429	1
hsa-miR-146b-5p	0.000114438	0.979109429	1
hsa-miR-155	7.64221E-06	0.003484386	1
hsa-miR-210	4.55662E-05	0.001083796	0.885474951

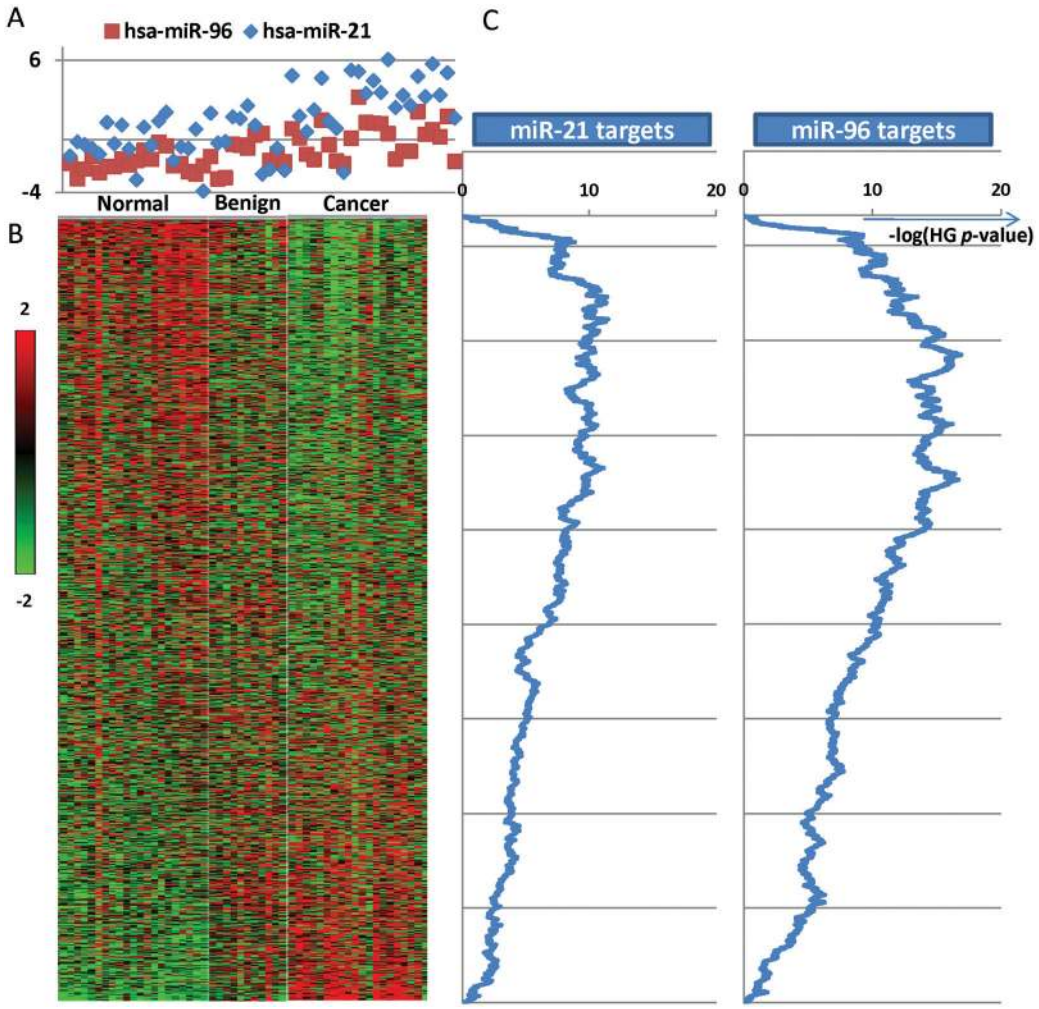

Fig. 2. miRNA regulation. mRNAs were tested for reciprocal expression levels to their regulating miRNA. (**A**) Expression levels of miR-96 and miR-21 across the cohort of normal, benign and malignant tumors samples. (**B**) Heatmap of mRNA expression levels ranked according to their level of downregulation in the tumors compared with the expression levels in normal tissue. (**C**) Enrichment levels of miR-96 and miR-21 targets plotted in –log10 (hypergeometric *P* value). In both cases, we see a significant enrichment of the targets in the top of the ranked list of genes. The figure was generated with the help of VistaClara (50).

identified as a downregulated target in our data set, supporting previous findings, that miR-96 plays an important role in the growth of tumors, possibly also during the benign stage.

A candidate for a true tumor suppressor oncomir is miR-205, which was significantly downregulated in malignant breast tumors and significantly upregulated in benign tumors. miR-205 has been found to be involved in epithelial to mesenchymal transition (37). Studies have shown that expression of miR-200 family members and miR-205 are lost in invasive breast cancer cell lines with mesenchymal phenotype, suggesting that downregulation of these miRNAs may be

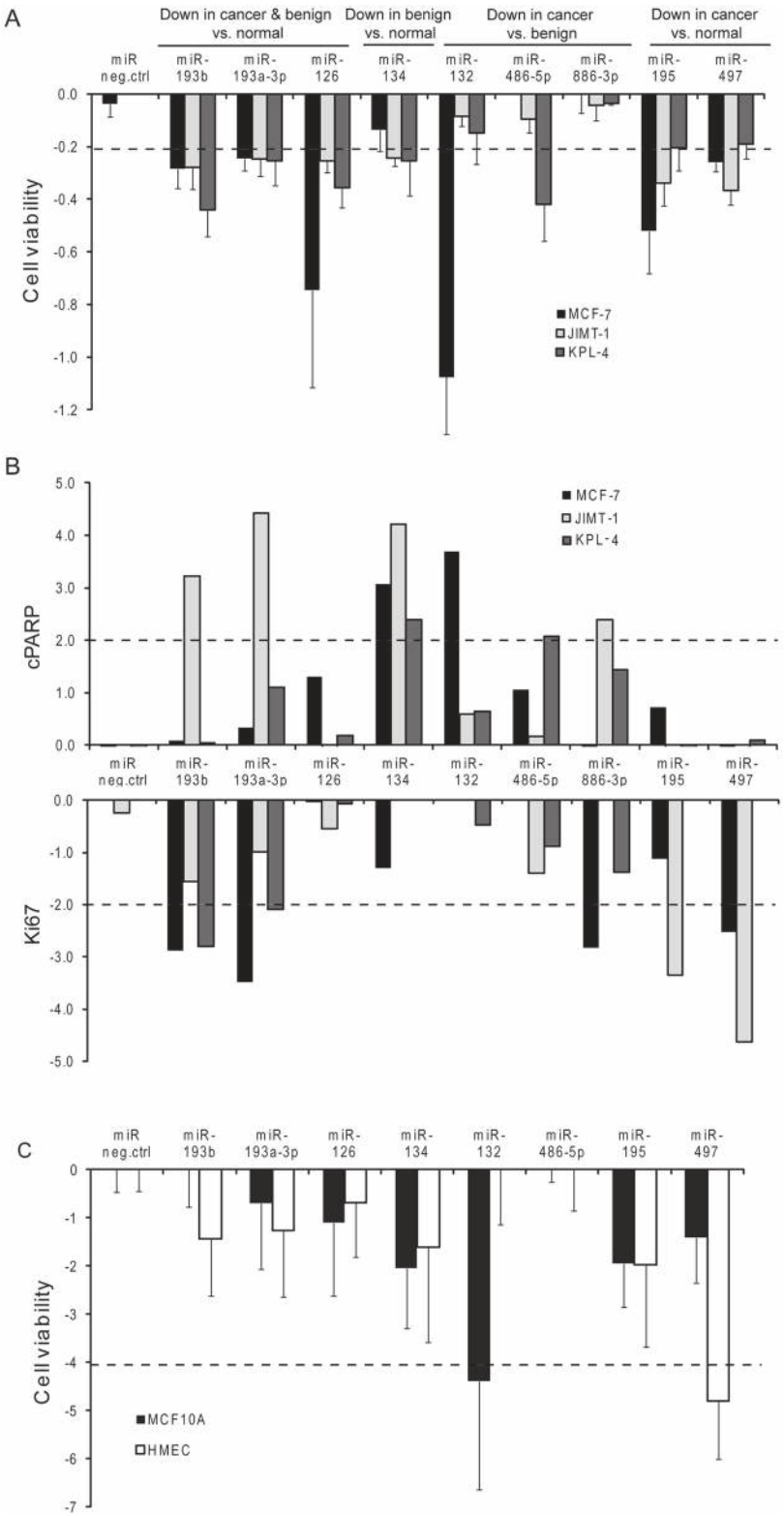

Fig. 3. miRNAs downregulated in benign or cancer tissue inhibit breast cancer cell growth. (**A**) MCF-7, JIMT-1 and KPL-4 cells were transfected with Dharmacon miRNA mimics (20 nM). After 72 h incubation, cell viability was measured with CellTiter-GLO assay (mean of two biological replicate experiments). (**B**) The cells were lysed 72 h after miRNA transfection and printed on nitrocellulose-coated microarray slides as protein arrays, which were stained with cPARP or Ki67 antibodies to measure apoptosis or proliferation, respectively. (**C**) MCF-10A and HMEC non-malignant control cells were transfected with miRNA constructs and the cell viability was assayed after 72 h incubation (n = 4–6). The dashed lines indicate cutoff values, which were considered as significant (±2 SD).

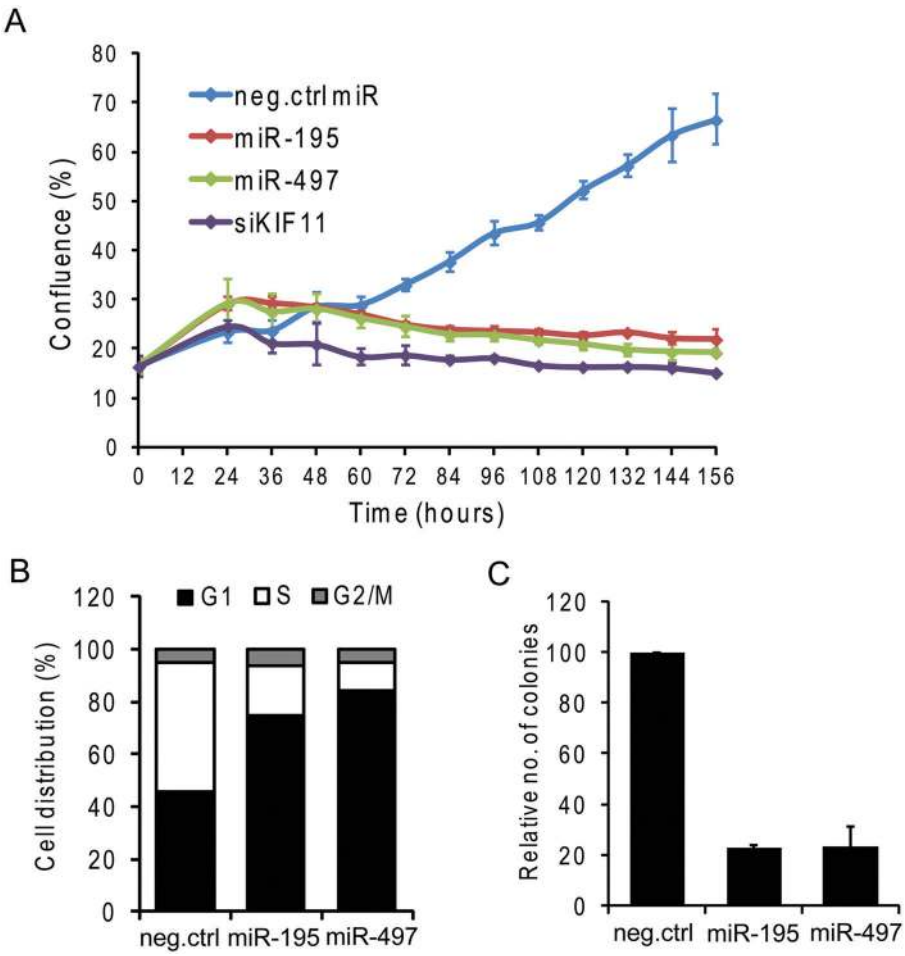

Fig. 4. miR-195 and miR-497 inhibit breast cancer cell growth and colony formation. (**A**) MCF-7 cells were transfected with pre-miR-195, pre-miR-497 and pre-miR negative control. Cell growth was monitored over 156 h period of time in INCUCYTE Live-Cell Imaging System. As a positive control, siRNA for KIF11 was used. (**B**) MCF-7 cells were transfected with the pre-miR constructs and incubated for 48 h. Thereafter, the cell cycle distribution was analyzed from the nuclei preparations with fluorescence-activated cell sorting. The distribution of cells in each phase of the cell cycle is shown. (**C**) MCF-7 cells were transfected with the pre-miR constructs. After 24 h incubation, the cells were seeded in 0.35% agarose containing growth medium. The colony numbers were counted 2 weeks later. The data represent mean of two biological replicates.

an important step in tumor progression (42). Since miR-205 is downregulated in malignant tumors but upregulated in benign tumors and in normal tissue, one might postulate that miR-205 serves a protective role in the cells, and downregulation of its expression may be specific to malignancy.

Several of the miRNAs described here as being downregulated in both benign and malignant tumor tissues, compared with those in normal tissue, had antiproliferative or proapoptotic effects when they were overexpressed in breast cancer cells. Specifically, miR-193b, miR-193a-3p and miR-126, similarly downregulated in both benign and malignant tumors, were the most efficient growth inhibitors. This is in concordance with several previous studies suggesting tumor suppressive role for these miRNAs. miR-193b is downregulated in breast cancer and this results in upregulation of its target genes, such as urokinase-type plasminogen activator, which is associated with increased invasive, migratory and proliferative capacity of breast cancer cells (43). Urokinase-type plasminogen activator is involved in extracellular matrix degradation, and high levels may, therefore, enhance the migratory capacity of breast cancer cells. miR-193b directly targets the estrogen receptor-alpha, and is thereby able to inhibit estrogen-induced growth of breast cancer cells (32). It also targets several other breast cancer-associated genes as has been described previously (44).

miR-126 expression is lost in the majority of primary breast tumors from patients who relapse, and its loss of expression is associated with poor distal metastasis-free survival (45). miR-126 is also frequently lost in colon cancer tissue and it is thought to suppress growth by targeting phosphatidylinositol 3-kinase signaling. Loss of this miRNA has, therefore, been thought to promote growth during carcinogenesis (46). miR-193a-3p has recently been shown to co-target epidermal growth factor-driven cell cycle network proteins and inhibit cell cycle progression and proliferation in breast cancer (47). All these findings confirm the tumor suppressive role played by these miRNAs in cancerous tissue, and possibly also in the benign tissue.

miR-195/497 were downregulated in malignant tumors, and when they were re-expressed in breast cancer cells, cell growth and colony formation was inhibited very efficiently. In non-malignant cells (HMEC and MCF-10A), miR-195 had no significant effect on the growth, whereas miR-497 showed some degree of inhibition of HMEC cell growth, but not in MCF-10A. This suggests that at least for miR-195, its growth inhibition effects were cancer specific. miR-195/497 also inhibited cell cycle in cancer cells, which is in accordance with previous studies reporting that they target several cell cycle genes (48,49). Li *et al*. showed that the promoter of the miR-195/497 cluster is hypermethylated and this leads to their downregulation in breast cancer. Interestingly, these miRNAs have been shown to be repressed by Myc (34), which is constitutively active in many cancers and promotes tumorigenesis. Chang *et al*. showed that re-expression of Myc-repressed miRNAs, among them miR-195/497, inhibited lymphomagenesis (34). This suggests that miR-195 and miR-497 may be potential therapeutic and diagnostic

targets in breast cancer. Furthermore, we identified many miRNAs with deregulated expression being the hallmark of either benign or malignant tumors. It remains to clarify whether these miRNAs can be used in the clinic to differ between two disease states or to diagnose patients with either benign or malignant tumors simply by miRNA expression levels.

In conclusion, the results described in this study show that malignant primary breast tumors and two types of benign tumors are more similar to each other than to normal tissue, with respect to the expression of certain miRNAs. Our findings suggest that several miRNAs, previously considered to be 'cancer-related' miRNAs, may in fact be markers of proliferation and growth rather than markers of malignancy, as these miRNAs are deregulated in benign tumors in the same way as in breast carcinomas. Alternatively, this may suggest that certain early events of malignancy-related deregulation of miRNA expression can also be seen in benign tumors. All in all, the present study contributes to the general understanding of miRNAs in breast cancer. Our data strongly suggests that miRNAs may be used as biological markers in all stages of the disease.

Supplementary material

Supplementary Tables 1–4 and Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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References

1. Sotiriou,C. *et al.* (2009) Gene-expression signatures in breast cancer. *N. Engl. J. Med.*, **360**, 790–800.
2. Parker,J.S. *et al.* (2009) Supervised risk predictor of breast cancer based on intrinsic subtypes. *J. Clin. Oncol.*, **27**, 1160–1167.
3. Pedraza,V. *et al.* (2010) Gene expression signatures in breast cancer distinguish phenotype characteristics, histologic subtypes, and tumor invasiveness. *Cancer*, **116**, 486–496.
4. Perou,C.M. *et al.* (2000) Molecular portraits of human breast tumours. *Nature*, **406**, 747–752.
5. van 't Veer,L.J. *et al.* (2002) Gene expression profiling predicts clinical outcome of breast cancer. *Nature*, **415**, 530–536.
6. Enerly,E. *et al.* (2011) miRNA-mRNA integrated analysis reveals roles for miRNAs in primary breast tumors. *PLoS One*, **6**, e16915.
7. Verghese,E.T. *et al.* (2008) Small is beautiful: microRNAs and breast cancer-where are we now? *J. Pathol.*, **215**, 214–221.
8. Lu,J. *et al.* (2005) MicroRNA expression profiles classify human cancers. *Nature*, **435**, 834–838.
9. Zhang,B. *et al.* (2007) microRNAs as oncogenes and tumor suppressors. *Dev. Biol.*, **302**, 1–12.
10. Iorio,M.V. *et al.* (2005) MicroRNA gene expression deregulation in human breast cancer. *Cancer Res.*, **65**, 7065–7070.
11. Navon,R. *et al.* (2009) Novel rank-based statistical methods reveal microRNAs with differential expression in multiple cancer types. *PLoS One*, **4**, e8003.
12. Ferracin,M. *et al.* (2011) MicroRNAs: toward the clinic for breast cancer patients. *Semin. Oncol.*, **38**, 764–775.
13. Sharma,S.V. *et al.* (2007) Oncogene addiction: setting the stage for molecularly targeted cancer therapy. *Genes Dev.*, **21**, 3214–3231.
14. Harbst,K. *et al.* (2012) Molecular profiling reveals low- and high-grade forms of primary melanoma. *Clin. Cancer Res.*, **18**, 4026–4036.
15. Romero-Cordoba,S. *et al.* (2012) Identification and pathway analysis of microRNAs with no previous involvement in breast cancer. *PLoS One*, **7**, e31904.
16. Farazi,T.A. *et al.* (2011) MicroRNA sequence and expression analysis in breast tumors by deep sequencing. *Cancer Res.*, **71**, 4443–4453.
17. Dupont,W.D. *et al.* (1985) Risk factors for breast cancer in women with proliferative breast disease. *N. Engl. J. Med.*, **312**, 146–151.
18. McDivitt,R.W. *et al.* (1992) Histologic types of benign breast disease and the risk for breast cancer. The Cancer and Steroid Hormone Study Group. *Cancer*, **69**, 1408–1414.
19. Fitzgibbons,P.L. *et al.* (1998) Benign breast changes and the risk for subsequent breast cancer: an update of the 1985 consensus statement. Cancer Committee of the College of American Pathologists. *Arch. Pathol. Lab. Med.*, **122**, 1053–1055.
20. Cole,P. *et al.* (1978) Incidence rates and risk factors of benign breast neoplasms. *Am. J. Epidemiol.*, **108**, 112–120.
21. Dupont,W.D. *et al.* (1994) Long-term risk of breast cancer in women with fibroadenoma. *N. Engl. J. Med.*, **331**, 10–15.
22. El-Wakeel,H. *et al.* (2003) Systematic review of fibroadenoma as a risk factor for breast cancer. *Breast*, **12**, 302–307.
23. Ashbeck,E.L. *et al.* (2007) Benign breast biopsy diagnosis and subsequent risk of breast cancer. *Cancer Epidemiol. Biomarkers Prev.*, **16**, 467–472.
24. Worsham,M.J. *et al.* (2009) Risk factors for breast cancer from benign breast disease in a diverse population. *Breast Cancer Res. Treat.*, **118**, 1–7.
25. Wei,J.S. *et al.* (2002) Purification of total RNA from mammalian cells and tissues. In Bowtell,D. and Sambrook,J. (eds) *DNA Microarrays: A Molecular Cloning Manual*. Cold Spring Harbor Laboratory Press, New York, NY, pp. 110–119.
26. Griffiths-Jones,S. (2006) miRBase: the microRNA sequence database. *Methods Mol. Biol.*, **342**, 129–138.
27. Dysvik,B. *et al.* (2001) J-Express: exploring gene expression data using Java. *Bioinformatics*, **17**, 369–370.
28. Yang,Y.H. *et al.* (2002) Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Res.*, **30**, e15.
29. Tusher,V.G. *et al.* (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl Acad. Sci. USA*, **98**, 5116–5121.
30. Friedman,R.C. *et al.* (2009) Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.*, **19**, 92–105.
31. Eden,E. *et al.* (2007) Discovering motifs in ranked lists of DNA sequences. *PLoS Comput. Biol.*, **3**, e39.
32. Leivonen,S.K. *et al.* (2009) Protein lysate microarray analysis to identify microRNAs regulating estrogen receptor signaling in breast cancer cell lines. *Oncogene*, **28**, 3926–3936.
33. Boutros,M. *et al.* (2006) Analysis of cell-based RNAi screens. *Genome Biol.*, **7**, R66.
34. Chang,T.C. *et al.* (2008) Widespread microRNA repression by Myc contributes to tumorigenesis. *Nat. Genet.*, **40**, 43–50.
35. Lee,J.A. *et al.* (2011) Prognostic implications of MicroRNA-21 overexpression in invasive ductal carcinomas of the breast. *J. Breast Cancer*, **14**, 269–275.
36. Hannafon,B.N. *et al.* (2011) Expression of microRNA and their gene targets are dysregulated in preinvasive breast cancer. *Breast Cancer Res.*, **13**, R24.
37. Sempere,L.F. *et al.* (2007) Altered MicroRNA expression confined to specific epithelial cell subpopulations in breast cancer. *Cancer Res.*, **67**, 11612–11620.
38. Yan,L.X. *et al.* (2008) MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. *RNA*, **14**, 2348–2360.
39. Baffa,R. *et al.* (2009) MicroRNA expression profiling of human metastatic cancers identifies cancer gene targets. *J. Pathol.*, **219**, 214–221.
40. Wang,F. *et al.* (2010) Correlation and quantitation of microRNA aberrant expression in tissues and sera from patients with breast tumor. *Gynecol. Oncol.*, **119**, 586–593.
41. Lin,H. *et al.* (2010) Unregulated miR-96 induces cell proliferation in human breast cancer by downregulating transcriptional factor FOXO3a. *PLoS One*, **5**, e15797.
42. Gregory,P.A. *et al.* (2008) The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat. Cell Biol.*, **10**, 593–601.
43. Li,X.F. *et al.* (2009) Downregulation of miR-193b contributes to enhance urokinase-type plasminogen activator (uPA) expression and tumor progression and invasion in human breast cancer. *Oncogene*, **28**, 3937–3948.

44. Leivonen,S.K. *et al.* (2011) Identification of miR-193b targets in breast cancer cells and systems biological analysis of their functional impact. *Mol. Cell. Proteomics*, **10**, M110.005322.
45. Tavazoie,S.F. *et al.* (2008) Endogenous human microRNAs that suppress breast cancer metastasis. *Nature*, **451**, 147–152.
46. Guo,C. *et al.* (2008) The noncoding RNA, miR-126, suppresses the growth of neoplastic cells by targeting phosphatidylinositol 3-kinase signaling and is frequently lost in colon cancers. *Genes. Chromosomes Cancer*, **47**, 939–946.
47. Uhlmann,S. *et al.* (2012) Global microRNA level regulation of EGFR-driven cell-cycle protein network in breast cancer. *Mol. Syst. Biol*., **8**, 570.
48. Li,D. *et al.* (2011) Analysis of MiR-195 and MiR-497 expression, regulation and role in breast cancer. *Clin. Cancer Res*., **17**, 1722–1730.
49. Xu,T. *et al.* (2009) MicroRNA-195 suppresses tumorigenicity and regulates G1/S transition of human hepatocellular carcinoma cells. *Hepatology*, **50**, 113–121.
50. Kincaid,R. *et al.* (2008) VistaClara: an expression browser plug-in for Cytoscape. *Bioinformatics*, **24**, 2112–2114.

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