Analysis of MiR-195 and MiR-497 Expression, Regulation and Role in Breast Cancer

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Human Cancer Biology

Abstract

Purpose: To investigate expression, regulation, potential role and targets of miR-195 and miR-497 in breast cancer.

Experimental Design: The expression patterns of miR-195 and miR-497 were initially examined in breast cancer tissues and cell lines by Northern blotting and quantitative real-time PCR. Combined bisulfite restriction analysis and bisulfite sequencing were carried out to study the DNA methylation status of miR-195 and miR-497 genes. Breast cancer cells stably expressing miR-195 and miR-497 were established to study their role and targets. Finally, normal, fibroadenoma and breast cancer tissues were employed to analyze the correlation between miR-195/497 levels and malignant stages of breast tumor tissues.

Results: MiR-195 and miR-497 were significantly downregulated in breast cancer. The methylation state of CpG islands upstream of the miR-195/497 gene was found to be responsible for the downregulation of both miRNAs. Forced expression of miR-195 or miR-497 suppressed breast cancer cell proliferation and invasion. Raf-1 and Ccnd1 were identified as novel direct targets of miR-195 and miR-497. miR-195/497 expression levels in clinical specimens were found to be correlated inversely with malignancy of breast cancer.

Conclusions: Our data imply that both miR-195 and miR-497 play important inhibitory roles in breast cancer malignancy and may be the potential therapeutic and diagnostic targets. Clin Cancer Res; 17(7); 1722–30. ©2011 AACR.

Introduction

MicroRNAs (miRNA) are a class of highly conserved, small noncoding RNAs, which regulate gene expression in a variety of eukaryotic organisms and execute pivotal roles in physiological and pathological processes including development, differentiation, metabolism, immunity, cell proliferation and apoptosis (1–3). In general, these single-stranded miRNAs show a trend toward multiple targeting sites in the coding sequence (CDS) regions or the 3′ untranslated (3′UTR) regions of their target messenger RNAs (mRNAs) and cause translational repression or mRNA cleavage (4). There is growing evidence that differential expressions of miRNAs are associated with tumor types and cancer development (5).

Breast cancer is one of the leading causes of cancer death in women worldwide (6). miRNAs, such as miR-21, miR-27a, miR-17, miR-155, miR-10b, miR-125b, and miR-145, are found to be dysregulated and serve as oncogenic agent or tumor suppressor in breast cancer (6–11). It has been known that these small molecules are involved in tumor cell proliferation, migration, invasiveness and metastasis. Recently, blood-based miRNA profiling studies demonstrate that systemic miR-195 levels are increased before surgery and decreased after surgery in breast cancer patients (12). Interestingly, miR-497 has been found to be one of most prominently downregulated miRNAs in male breast cancer (13). As miR-195/497 is a highly conserved miRNA cluster located at Chromosome 17p13.1 (6), it would be interesting to simultaneously study the roles of miR-195 and miR-497 in breast cancer.
Human tissue samples

Human breast tumor samples and normal tissues were obtained from Peking Union Medical College Hospital and Shanghai Huashan hospital. All participants provided written informed consent following institutional review board approval at the participating hospitals. Tissue samples were collected and frozen in liquid nitrogen. These tissues were prepared in the hospital biorepository, and clinical annotation is available through a database. Cases were classified and selected based on diagnosis using the CoPath Anatomic Pathology system, and no information regulated by HIPPA was included in the study, which qualifies for the status of NIH Exemption #4.

Cell culture and RNA preparation

Human breast cancer cell lines MDA-MB-231, MDA-MB-435s, MDA-MB-453, ZR-75-30, SK-BR-3, T47D, and MCF7 were obtained from ATCC. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; MDA-MB-231, MDA-MB-435s, MDA-MB-453, and T47D) or RPMI 1640 medium (ZR-75-30, SK-BR-3) supplemented with 10% FBS. MCF7 cells were maintained in MEM supplemented with 10% FBS, and 0.01 mg/mL bovine insulin. Total RNAs were extracted from tissues and cells using Trizol reagent (Invitrogen).

Translational Relevance

In this study, we show that the promoter hypermethylation of miR-195/497 cluster causes their downregulation in breast cancer. The levels of miR-195/497 expression are inversely correlated with tumor malignancy, and Raf-1 is identified as a direct target of both miRNAs. Our data imply that both miR-195 and miR-497 may be of potential interest as therapeutic and diagnostic targets in breast cancer.

Accumulating evidence also demonstrates that genetic and epigenetic alterations cause the dysregulation of subsets of miRNAs (14, 15). Among them, promoter hypermethylation is known for silencing the specific miRNA expression to be involved in tumorigenesis (15). Mammalian DNA is predominantly methylated at the C-5-position of complimentary CpG bp by DNA methyltransferases (16). The aberrations of this epigenetic modification may lead to various diseases including cancer (15, 17). For miRNA expression, DNA methylation of CpG islands was found to be present in the promoter region of miRNAs with tumor suppressor features in human cancer such as miR-127, miR-124a, miR-1, as well as miR-148a and miR-34b (18–21). Thus, such DNA methylation might also play a role in tumorigenesis. The objective of this study was to reveal the roles of miR-195 and miR-497 in breast cancer.

Materials and Methods

Northern blot analysis

Total RNAs (25 μg each) were electrophoresed on 15% acrylamide and 8 M urea denature gels, and transferred onto Hybond N+ membrane (Amersham Biosciences). The membranes were washed at 42°C for 2 hours before hybridization, then hybridized with oligo-nucleotide probes corresponding to the complementary sequences of the following mature miRNAs: miR-195, 5’-GCCCAG-GATTTCTGCTGCTGCA-3’ and miR-497, 5’-ACAACCCACTGTGTGTGCTGCTG-3’. Probes were 5-end labeled using the polynucleotide kinase in the presence of [γ-32P] ATP. Hybridization was performed at 39°C in ULTRAhyb™ Oligo Hybridization Buffer (Ambion) for 16 hours. Membranes were washed at 42°C, 3 times with 2× SSC with 0.1% SDS. The membranes were rehybridized after stripping the oligo nucleotides used as probes in 1% SDS for 30 minutes at 65°C. The U6 RNA (5’-GCTATCCTTCCGATATTT-3’) was used as an internal control.

Real-time polymerase chain reaction assays for mature miRNAs

Total RNAs were prepared from tissue samples and cell lines for the analysis. The expression levels of miR-195 and miR-497 were assayed using the primers designed by Biomics Company. Reverse transcription reaction was

Combined bisulfite restriction analysis and bisulfite sequencing

The University of California Santa Cruz (UCSC) database was used to identify CpG islands (CGI) spanning miR-195 and miR-497 genes. Genomic DNA was isolated using Universal Genomic DNA Extraction Kit Ver.3.0 (Takara), and bisulfite conversion was performed using EZ DNA Methylation-Gold Kit™ (Zymo Research). Bisulfite-converted genomic DNA that converts only unmethylated cytosines to uracils, was amplified with specific primers. The purified PCR fragments were cloned into a pMD19-T vector (Takara Code: D102A), and individual clones were sequenced. For combined bisulfite restriction analysis (COBRA), PCR fragments were digested with Taq I (New England Biolabs Inc.) for 3 hours at 37°C. The restriction products were electrophoresed on a 3% agarose gel, and visualized by staining with Gold View (SBS Genetech). The primers used for amplification of CGI on miR-195 and miR-497 genes are: miR-195 and miR-497-BSF1: GTGTATTTTGTAGTAGATT; miR-195 and miR-497-BSF2: TAACTCCCTCAATCTTATCTCCTATTCTT.

5-Aza-dC treatment and precursor transfection

ZR-75-30 cells were seeded 24 hours before treatment with 5 μM 5-aza-2'-deoxycytidine (5-Aza-dC; Sigma-Aldrich). After 5 days of treatment, the cells were harvested and total RNAs were prepared and used for quantitative real-time PCR analysis.

Pre-miR-195 and pre-miR-497 and pre-miR™ miRNA precursors were purchased from Ambion. Cells were transfected with the precursors at 100 nM using LipofectAMINE 2000 reagent (Invitrogen).
performed using 30 ng of total RNAs and 1 μM looped primers. Real-time PCR was performed using the standard SYBR Green Assay protocol by the MX3000p Real-Time PCR Detection System (Stratagene). The 25 μL PCR reaction included 2 μL reverse transcription product, 1× PCR Master Mix (TaKaRa), 1.5 μmol/L forward primer, and 0.7 μmol/L reverse primer. The reactions were incubated in a 96-well plate at 95°C for 10 seconds, followed by 40 cycles of 95°C for 5 seconds, and 60°C for 30 seconds. The 2^−ΔΔCt method for relative quantification of gene expression was used to determine miRNA expression levels. Each sample was analyzed in triplicate, and U6 RNA was used to normalize the miRNA levels.

Western blot analysis
For each sample, 25 μg of total protein extracts were separated on SDS-PAGE gels, and transferred to PVDF membrane. All the primary antibodies were incubated overnight at 4°C, followed by the incubation with AP-conjugated goat anti-rabbit secondary antibody and detection with BCIP/NBT (Amresco). Bands were quantified with Labworks Instrument software (UVP LLC).

Soft agar colony-formation assays
MCF7 and ZR-75-30 cells were transfected with miR-195, miR-497, or negative control precursor miRNAs. After 24 hours of transfection, cells were suspended in complete medium containing 0.35% agar, and overlaid on 0.6% agar for 2 days. The plates were maintained for up to 2 weeks. The number of colonies (>50 μm in diameter) was counted under a microscopic field at 10× magnification.

Fluorescence-activated cell sorting (FACS) cell cycle analysis
MCF7 and ZR-75-30 cells were seeded at 30% confluence in 12-well plate 24 hours before transfection. Cells were treated with nocodazole for 16 to 20 hours after the transfection of miR-195, miR-497, or negative control precursor at 100 nM; and fixed in 70% ethanol at 4°C for 2 hours. Fixed cells were then washed once with PBS, resuspended in 500 μL PBS containing 10 mg/mL propidium iodide and 50 mg/mL RNase, and incubated for 30 minutes at room temperature. The cells were then analyzed with Fluorescent-Activated Cell Sorter (BD FACSaria cell sorter, BD Biosciences).

Lentivirus preparation, titration and infection
HEK293T cells were seeded in 10 cm plate at 6 × 10^6 cells and transfected with 9 μg of pLemir-195 or pLemir-497 plasmid and 26 μL of the packaging mix stock using Arrest-In transfection reagent (Openbiosystems). The culture supernatants were collected 48 and 72 hours after transfection, filtered through 0.45-μm filters, and stored as virus stocks. For virus titration, TLA-HEK293T cells were seeded at 5 × 10^4 cells per well with 24-well tissue culture plate in DMEM. The diluted virus were added to each well, and incubated at 37°C for 4 hours. Then, the transduction mix was removed from cultures, cells were cultured for 48 hours for the virus titration. For virus infection, cells were incubated at 37°C with virus and polybrene (8 μg/mL) for 6 hours, then for 2 days to select puromycin-resistant cells.

MiRNA target prediction and luciferase activity assay
MiRNA target prediction was performed by in-lab developed algorithm KeyTar. KeyTar miRNA target prediction is based on miRNA: target sequence, structure, and function (unpublished). The human 3′ UTR region of Ccn1 gene was amplified by PCR using the following primers: Ccn1-3UTR-F: 5′-GGTACGGCCAAAGGGATAGAAA-3′, and Ccn1-3UTR-R: 5′-CGTCTAGATGGCTAAGTGAAGCAT-GAGG-3′, and cloned into the KpnI and XbaI sites of the pGL3-control vector (Promega). The human 3′ UTR of Raf-1 gene was amplified by PCR using the following primers: Raf-1-3UTR-F: 5′-GAATTCGCAATGAAGGCTGGTA-3′, and Raf-1-3UTR-R: 5′-CTCGAGGAGCGCAACGATAGGAAGTG-3′, and cloned into the EcoRI and XhoI sites of the pGL3-control vector. Nucleotide-substitution mutations were carried out using PCR-based methods at the 3′ UTR regions of Ccn1 and Raf-1. Primers were as below: Mut 3′UTR of Ccn1: 5′-CGACGAGCAATGGTGACCTCGAGCT-3′ and 5′-CGAACATTGGCAGCTCGGCGC-3′, and Mut 3′UTR of Raf-1: 5′-CGACGAGCAATGGTGACCTCGAGCT-3′ and 5′-TCTCTAGGAAAATGTGTTTTCGTC-3′. All constructs were verified by sequencing. The underlined nucleotides indicate the bases where mutations were made. For luciferase assay, the 293A cells were cultured in 24-well plates, and transfected with 100 ng luciferase reporter plasmid, 5 ng pRL-TK vector expressing the Renilla luciferase (Promega), and 50 pmoles of miR-195, miR-497, or miRNA negative control precursor. Transfection was performed using Lipofectamine 2000 (Invitrogen). After transfection for 36 hours, firefly and renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay (Promega). Each transfection was repeated twice in triplicate.

Statistical analysis
The results are expressed as mean ± SD. Data comparisons were performed using analysis of variance followed by Dunnett’s method using Microsoft Excel. The Mann–Whitney test was used to compare depot-specific differences between cancer tissues and normal controls. The results were considered statistically significant at P < 0.05.

Results
MiR-195 and miR-497 are downregulated in human breast cancer tissues and cell lines
Initially, we examined the expression levels of miR-195 and miR-497 in breast cancer tissues. The expression levels of both miR-195 and miR-497 in breast cancer tissues were greatly decreased when compared with those in the normal adjacent tissue (Fig. 1A and B). Similarly the levels of miR-195 and miR-497 were lower in different breast cancer cell lines including MCF7, ZR-75-30, MDA-453, MDA-435, and MDA-231 (Fig. 1B and C), indicating that miR-195 and
miR-497 are decreased in both human breast cancer tissues and cell lines.

DNA methylation is responsible for miR-195 and miR-497 downregulation

We sequentially examined the methylation status of CpG islands in human breast cancer tissues and matching normal tissue by COBRA. Digestion of the amplicon by methylation-sensitive enzyme Taq I indicates that genomic DNA contains methylation. Analysis of 5 pairs of human breast cancer tissues showed significant methylation at Taq I sites in 4 of 5 tumors, T1–T4 (Fig. 2A). The same methylation site was observed in MCF7 and ZR7530 cells. In contrast, there is no obvious CpG methylation in the normal tissue, suggesting that CpG islands are specifically methylated in human breast cancer. To determine the methylation status of CpG islands, we performed bisulfite sequencing analysis. The results showed that CpG islands were methylated in MCF7 and ZR7530 cells but not in normal tissue.

Figure 1. miR-195 and miR-497 are underexpressed in human breast cancer tissues and cell lines. A, Pathological examination of normal breast tissues and tumor tissues using H&E staining. B, Northern blot analysis of miR-195/497. miR-195/497 were highly expressed in normal breast tissue, but less expressed in breast cancer tissues (T1 to T7). C, Detection of mature miR-195, miR-497 and miR-21 by real-time PCR. U6 RNA was used for normalization control. Each data point was obtained in triplicate. Values (mean ± SD) are shown. *, P < 0.01 (t-test).

Figure 2. DNA Methylation analysis of miR-195 and miR-497 in human breast cancer tissues and cell lines. A, Combined bisulfite restriction analysis of human breast cancer and normal tissue and breast cancer cell lines. Genomic DNA was treated with bisulfite, PCR-amplified, and digested with TaqI enzyme. B, Bisulfite sequencing analysis of normal and tumor tissue and cell lines to determine the methylation state of CpG nucleotides upstream of the miR-195/497 gene on Chromosome 17. Filled circle, methylated CpG; open circle, unmethylated CpG. C, RT-PCR analysis of miR-195/497 in 2 breast cancer cell lines MCF7 and ZR7530 untreated or treated with DNA demethylating agent 5-aza-2-deoxycytidine (AZA).
islands in the nucleotide sequence upstream of miR-195 and miR-497 promoters on Chromosome 17, we carried out bisulfite sequencing analysis of genomic DNA isolated from normal and tumor tissues, and breast cancer cell lines. The results showed dense methylation of certain CpGs located in the region upstream of miR-195 and miR-497 promoters in the tumor tissues and cancer cells MCF7 and ZR7530, whereas only a few scattered CpGs were methylated in the region upstream of miR-195 and miR-497 promoters from normal breast tissue (Fig. 2B). To study whether CpG methylation affects miR-195 and miR-497 expression, the cancer cells were treated with DNA demethylating agent 5-aza-2-deoxycytidine (5-Aza-dC). The cells treated with 5-Aza-dC increased the expression of both miR-195 and miR-497 (Fig. 2C). Thus, DNA methylation is responsible for the low expression of miR-195 and miR-497 in breast cancer cells.

**MiR-195 and miR-497 inhibit breast cancer cell colony formation and invasion in vitro**

The breast cancer cells were transiently transfected with miR-195, miR-497 or negative control precursors to study their effects in colony formation. Overexpression of miR-195 and miR-497 in both ZR-75-30 and MCF7 cells exhibited a significant reduction of colony number when compared with the cells transfected with the negative control (Fig. 3A). To analyze the role of miR-195 in cell invasion, ZR-75-30 and MCF7 cells were transfected with miR-195 or miR-497, and analyzed by Transwell assay. MiR-195 or miR-497 transfection significantly inhibited cell invasion when compared with the control (Fig. 3B). To understand the potential mechanism of miR-195, miR-497 in inhibiting these biological effects, we found that miR-195 and miR-497 overexpression induced cell cycle G1 arrest 48 hours after transfection (Fig. 3C), and 24 and 36 hours after transfection (Supplementary Fig. S1).

**MiR-195 and miR-497 directly target Raf-1**

To further understand molecular mechanism of miR-195 and miR-497 in inhibiting breast cancer cells, we searched for potential targets of miR-195 and miR-497 by the KeyTar miRNA target prediction algorithm that is based on sequences and real physiological situation. This algorithm

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**Figure 3.** miR-195/497 inhibit breast cancer cell growth and invasion in vitro. A, Colony formation in soft agar of MCF7 and ZR-75-30 cells transfected with miR-195, miR-497 or the negative control oligo (a mismatched miRNA oligo, mir-mm). Colonies >50 μm in diameter were counted under a microscopic field at 10x magnifications. Values (mean ± SD, n = 3) are shown. *, P < 0.05; **, P < 0.01 (t-test). B, Matrigel invasion assay of MCF7 and ZR-75-30 cells that were transfected with control (upper) or miR-195/497 oligo (lower; >200). A significant decrease was observed in miR-195- or miR-497-transfected breast cells. *, P < 0.05; C, Cell cycle analysis by FACS in MCF7 and ZR-75-30 cells transfected with miR-195 or miR-497. Mean ± SD were shown. *, P < 0.01 (t-test).
suggested that Raf-1 is a novel direct target of both miR195 and miR-497 (Fig. 4B). The potential sites of miR195 and miR-497 in Raf-1 3' UTR were even more conserved than those in Ccnd1 3' UTR, a known target of miR-195 and miR-497. Luciferase assays showed that miR-195 and miR-497 significantly reduced the activity of the Raf-1 gene reporter, but not of the mutated gene reporter (Fig. 4C). Western blot analysis of Raf-1 and Ccnd1 in ZR-75-30 and MCF7 cell lines showed that miR-195 or miR-497 transfection inhibited Raf-1 and Ccnd1 expression and ERK1/2 phosphorylation (Fig. 4D and E). To confirm the data further, we subsequently established stable-expression of miR-195 and miR-497 breast cancer cells by lentivirus transfection (Fig. 4F). Similarly, we found that miR-195 and miR-497 inhibited cell growth and survival (data not shown), and led to significant downregulation of both Raf-1 and Ccnd1 in vitro (Fig. 4G). The results further demonstrate that Raf-1 is a novel target of miR-195 and miR-497 in different experimental systems.

**MiR-195 and miR-497 levels correlate inversely with malignancy of human breast tumors**

We also investigated the relationship between the expression levels of miR-195 and miR-497 and the malignant stages of human breast tumors. As shown in Figure 5A, miR-195 and miR-497 levels were significantly downregulated in malignant tumors (n = 71, Stage I-III) when compared with those in the normal tissues (P < 0.0001), and to those in the benign tumors, known as fibroadenoma (P < 0.05). No significant difference existed between normal and benign tumor. Such data suggested that levels of miR-195 and miR-497 are inversely associated with...
malignancy of human breast cancer. But there were no significant differences between each stage of breast cancer (P > 0.05), indicating that miR-195 and miR-497 could not be used in staging of malignant breast tumors. In addition, malignant breast tumors showed much higher expression levels of Raf-1 and Erk1/2 when compared with the normal tissues (Fig. 5B). Thus, the expression levels of Raf-1 are inversely correlated with malignancy in human breast tumors. Such expression pattern could be used in staging of malignant breast tumors. In addition, forced expression of miR-195 and miR-497 had similar effect to suppress breast cancer cell proliferation, blocked cell cycle G1 progression, and induced apoptosis. Such results suggest that miR-195 and miR-497 have similar effects to play a tumor-suppressor role in breast cancer by the same cluster of gene regulation. Furthermore, we predicted and confirmed that Raf-1 is a novel target of both miR-195 and miR-497. Raf-1 has been found to be overexpressed or overactivated in a variety of cancers, including renal cell carcinoma, hepatocellular carcinoma, non–small cell lung cancer, melanoma, and papillary thyroid carcinoma (32). Recently, Raf-1 was found to be a target of miR-7 and miR-125b (33, 34). In breast cancer, miR-125b could target the same region of c-Raf-1 (34). In addition, Raf-1 was identified as a direct target of miR-7 in several cancer cell lines, including breast cancer (33). Here, we present the first report that Raf-1 is a direct target of both miR-195 and miR-497 in breast cancer, extending the potential target network of miRNAs. It would be interesting to test whether this novel regulation mechanism of miR-195 and miR-497 has a functional role to decrease their expression. Other than DNA methylation, p53 inactivation or genetic deletion could lead to miRNA deregulation (28, 29). In chronic lymphocytic leukemia patients, chromosome 17p deletion may influence a set of miRNAs, such as miR-21 and miR-34a (30, 31).

In addition, our data showed that expression levels of miR-195 and miR-497 correlated inversely with malignancy of human breast tumors. Such expression pattern could potentially identify malignant tumors from normal or benign tumors. Recent finding in miRNA study suggests some miRNAs to be used for diagnosis or prognosis of cardiac hypertrophy, and its overexpression led to pathological heart growth and heart failure in transgenic mice (22). Recently, miR-195 was found to be downregulated in a variety of cancers, including gastric cancer, liver cancer, bladder cancer, and adrenocortical cancer (23–26). Introduction of miR-195 markedly suppressed colony formation in vitro and tumor development in nude mice (24). MiR-497 expression was found to be downregulated in different cancers, such as primary peritoneal carcinoma, gastric cancer and breast cancer (23, 27). However, the role of miR-497 in human cancer is not clear yet. Our data suggested that both miR-195 and miR-497 were significantly downregulated in breast cancer, and that they were regulated by a common mechanism of CpG methylation upstream of miR-195 and miR-497 promoters. Recent studies showed that certain miRNA genes such as miR-1, miR-127, miR-148a, and miR-34b/c contain CpGs that are susceptible to DNA methylation for decreasing their expression (18, 20, 21). To understand whether CpG methylation was responsible for downregulation of miR-195 and miR-497, we found that DNA demethylating agent treatment restored the expression of miR-195 and miR-497 in breast cancer cells, suggesting that DNA methylation in CpG nucleotides upstream of miR-195 and miR-497 promoters has a functional role to decrease their expression. Other than DNA methylation, p53 inactivation or genetic deletion could lead to miRNA deregulation (28, 29). In chronic lymphocytic leukemia patients, chromosome 17p deletion may influence a set of miRNAs, such as miR-21 and miR-34a (30, 31).

Finally, our data showed that expression levels of miR-195 and miR-497 correlated inversely with malignancy of human breast tumors. Such expression pattern could potentially identify malignant tumors from normal or benign tumors. Recent finding in miRNA study suggests some miRNAs to be used for diagnosis or prognosis of
breast cancer in the future. MiR-21 was significantly correlated with advanced clinical stage, lymph node metastasis, and poor survival of the patients (35). As miRNAs have unique expression profile in cancer tissues, and their expression is more stable than mRNA, miRNAs are promising for application as biomarkers. Future work should focus on the evaluation of the diagnostic or prognostic value of miR-195/497. In summary, the results suggest that both miR-195 and miR-497 are downregulated in breast cancer, and their silence is caused by DNA methylation. Our data imply that miR-195/497 may be potential therapeutic targets in breast cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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