ARTICLE
Journal of Cellular Biochemistry 114:1457–1463 (2013)

Journal of Cellular Biochemistry

MiR-199b-5p Targets HER2 in Breast Cancer Cells

Chen Fang,[1] Yu Zhao,[2] and Baoyu Guo[1*]

[1] *Department of Biochemical Pharmacy, School of Pharmacy, Second Military Medical University, Shanghai 200433, China*

[2] *Shanghai Key Laboratory of Gastric Neoplasms, Department of Surgery, Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China*

ABSTRACT

HER2 (ErbB2) has been reported to be overexpressed in 20–30% of breast cancer and confers poor survival because of high proliferation and metastasis rates. MicroRNAs are small noncoding RNAs that are responsible for the post-transcriptional regulation of target genes. We found miR-199b-5p inhibited HER2 expression by direct targeting its 3′-untranslated region (3′UTR) in breast cancer cells. In addition, miR-199b-5p inhibited HER2 downstream signaling by ERK1/2 and AKT pathways in breast cancer cells. Besides, transwell migration, wound healing, and clonogenicity were obviously inhibited by overexpression of miR-199b-5p in HER2-positive breast cancer cells. We also found that miR-199b-5p could enhance the suppression of trastuzumab on cell migration and clonogenicity. These results suggest that miR-199b-5p may have the potential to be a novel important alternative therapeutic target for HER2-positive breast cancer. J. Cell. Biochem. 114: 1457–1463, 2013.

KEY WORDS: HER2; microRNA; miR-199b-5p; BREAST CANCER

HER2 (ErbB2) protooncogene is a member of the epidermal growth factor receptor (EGFR) family of transmembrane tyrosine kinase type receptors, and is reported to be overexpressed in 20–30% of breast cancer [Slamon et al., 1987]. HER2 confers a more aggressive tumor phenotype and associates with a poor prognosis in patients with breast cancer [Slamon et al., 1987; Kallioniemi et al., 1991]. Therefore, agents targeting HER2 have been clinically used for breast cancer treatment. There are two therapeutic agents approved by US Food and Drug Administration (FDA), the monoclonal antibody trastuzumab and the tyrosine kinase inhibitor lapatinib [Ryan et al., 2008]. However, most patients with HER2-positive breast cancer treated with these agents eventually relapse or develop resistance later in the therapy [Smith et al., 2007; Eichhorn et al., 2008; Martin et al., 2008; Trowe et al., 2008; Liu et al., 2009; Xia et al., 2010]. Mechanisms of resistance to anti-HER2 therapies studied over the last decade include: impaired access of trastuzumab to HER2 by overexpression of MUC4 or p95 HER2 (an extracellular domain-truncated form of HER2) [Molina et al., 2002; Scaltriti et al., 2007]; aberrant activation of signaling from insulin-like growth factor-1 receptor (IGF-1R), other EGFR family members, or MET; continued activation of downstream signaling caused by phosphatase and tensin homologs (PTEN) mutation, PIK3CA mutation, or downregulation of p27; FCGR3A polymorphisms [Mukohara, 2011]. Therefore, the need for developing added therapies against HER2-positive breast cancer is vital.

MicroRNAs (miRNAs) are a class of small (18–25 nucleotides), endogenous, noncoding, single-stranded RNAs which bind partially to complementary sequences in the 3′-untranslated regions (3′UTRs) of target genes, causing either target mRNA degradation or reduced protein translation [Ambros, 2001; Bartel, 2004]. Growing evidence has proved that miRNAs regulate major cellular processes involved in tumor biology, including cell proliferation, differentiation, apoptosis, and metastasis [Croce, 2009; Lynam-Lennon et al., 2009]. Further, it has been reported that some miRNAs can serve as either oncogenes or oncosuppressors by targeting oncogenes or oncosuppressor genes [Esquela-Kerscher and Slack, 2006; Stahlhut

Conflict of interest: nothing to declare.

Chen Fang and Yu Zhao contributed equally to this work.

Additional supporting information may be found in the online version of this article.

Grant sponsor: National Natural Science Foundation of China; Grant number: 30572200.

*Correspondence to: Baoyu Guo, Department of Biochemical Pharmacy, School of Pharmacy, Second Military Medical University, Shanghai 200433, China. E-mail: byguo1632000@yahoo.com.cn

Manuscript Received: 7 November 2012; Manuscript Accepted: 18 December 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 7 January 2013

DOI 10.1002/jcb.24487 • © 2013 Wiley Periodicals, Inc.

Espinosa and Slack, 2006]. For example, miR-17-92, miR-21, and miRNA-372/373, which are usually upregulated in tumors, have been shown to inhibit tumor suppressor genes such as PTEN, Bim, and LATS2 [Lewis et al., 2003; Zhu et al., 2008]. In contrast, tumor suppressor miRNAs such as let-7, miR-15, and miR-16, which are frequently downregulated in tumors, can inhibit oncogenes such as RAS, MYC, and BCL2, respectively [Cimmino et al., 2005; Johnson et al., 2005]. Overexpression of miR-125a or miR-125b in SK-BR-3 cells, produced suppression of cell anchorage-dependent growth and even greater inhibition of cell motility and invasive abilities by direct targeting HER2 or HER3 [Scott et al., 2007].

Among an expanding list of cancer related miRNAs, miR-199b-5p has been documented involved in various types of cancers including medulloblastoma, choriocarcinoma, chronic myeloid leukemia, colorectal cancers, and renal cell carcinoma [Garzia et al., 2009; Chao et al., 2010; Flamant et al., 2010]. Based on microarray analysis of breast cancer tissues, miR-199b-5p was down-expressed in the more aggressive, grade 3, HER2-positive, ER-negative samples [Blenkiron et al., 2007]. A recent report also showed that miR-199b-5p was down-expressed in the tumor tissue compared with tumor-adjacent samples in breast cancer patients [Persson et al., 2011]. Therefore, further analysis for the significance of miR-199b-5p misregulation in breast cancer remains to be done.

In this report, we discovered, for the first time, that miR-199b-5p inhibited HER2 gene expression by direct targeting its 3′UTR and inhibited the activation of the HER2 downstream signal pathway mediators ERK1/2 and AKT. We also found overexpression of miR-199b-5p not only inhibited migration and clonogenicity of BT-474 and SK-BR-3 cells, but also enhanced the suppression of trastuzumab on cell migration and clonogenicity. These results strongly suggest that overexpression of miR-199b-5p in HER2-positive breast cancer cells can interfere with the aggressive phenotype by direct suppressing HER2 expression.

MATERIALS AND METHODS

CELL LINES AND CELL CULTURE

Human breast cancer cell lines SK-BR-3 and BT-474 were originally purchased from the American Type Culture Collection (Manassas, VA) and stored, recovered, and used at early passage from cryopreservation in liquid nitrogen. Cells were maintained in RPMI-1640 medium plus 10% fetal bovine serum (FBS) and cultured in 5% CO_2 humidified atmosphere.

CELLULAR TREATMENT AGENTS

Trastuzumab (Herceptin; Roche Pharmaceutical) was dissolved in PBS. Human immunoglobin G (hIgG, reagent grade; Sigma–Aldrich), which served as control for trastuzumab, was dissolved in PBS. Trastuzumab or hIgG was added to medium, respectively, at the final concentration 20 μg/ml.

RNA ISOLATION AND qRT-PCR

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. Concentrations and purity of the RNA samples were measured by electrophoresis and spectrophotometric methods. For quantitation of mRNA, 2 μg of total RNA were reverse transcribed with random primers following the manufacturer's instruction (MBI Ferments, Vilnius, Lithuania). Subsequently, the PCR amplifications were performed in triplicate using the SYBR® Premix ExTaq™ (Takara) following the manufacturer's instruction. The thermal cycling conditions were, an initial a hot start step at 95°C for 10 min, followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. Dissociation curve analysis was also performed for all the samples following the completion of amplification to rule out the presence of nonspecific amplifications. Specificity of PCR-products was also checked on agarose gel. Quantification was done using the DDCt relative quantification method with Human β-actin as an internal control. The following primers were used: HER-2 (sense: 5′-CCATCTGCACCATTGATGTC-3′; antisense: 5′-ATGCGGGAGAATTCAGACAC-3′) and β-actin (sense: 5′-ATTGCCGACAGGATGCAGA-3′); antisense: 5′-GAG-TACTTGCGCTCAGGAGGA-3′) [Adachi et al., 2011].

The microRNA was extracted from cells using a mirVana™ MiRNA Isolation Kit (Applied Biosystems, Foster City, CA) following the manufacturer's instruction. For quantitation of miRNA, 10 ng of the microRNA were reverse transcribed using Taq-Man MicroRNA Reverse Transcription Kit (Applied Biosystems) with specific primers for miR-199b-5p and U6 small nuclear RNA (RNU6B; Applied Biosystems). Subsequently, the PCR amplifications were performed in triplicate according to Taqman MicroRNA Assays protocol (Applied Biosystems). The thermal cycling conditions were, a hot start step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Relative miRNA expression of miR-199b-5p was normalized against the endogenous control, U6, using the DDCt method.

TRANSIENT TRANSFECTION OF miRNA MIMICS

MiR-199b-5p mimics (dsRNA oligonucleotides), negative control mimics (NC) (sense: 5′-UUC UCC GAA CGU GUC ACG UTT-3′, antisense: 5′-ACG UGA CAC GUU CGG AGA ATT-3′), miR-199b-5p inhibitors (anti-miR-199b-5p), inhibitor negative control (anti-NC, CAG UAC UUU UGU GUA GUA CAA) were purchased from GenePharma (Shanghai, China). Cells were seeded into 6- or 24-well plates the day before transfection to ensure 50% cell confluence at the moment of transfection. Transfection was carried out using Lipofectamine 2000 (Invitrogen) follow the manufacturer's procedure. The oligonucleotides were used at a final concentration of 100 nM. Western blot and qRT-PCR were performed at 48-h post-transfection.

VECTOR CONSTRUCTION AND LUCIFERASE REPORTER ASSAY

A 618-bp full-length of the wild-type (WT) HER2-3′UTR or mutant HER2-3′UTR (mut) containing the putative miR-199b-5p binding site were synthesized (Sangon, Shanghai, China). After digestion by *Spe*I and *Hin*dIII, the full-lengths of wild-type and mutant HER2-3′UTR were cloned into the *Spe*I and *Hin*dIII sites of pMIR-Report Luciferase vector (Applied Biosystems) and named pMIR/HER2 and pMIR/HER2/mut, respectively. Sequencing was used to corroborate the constructs (Supplementary Figs. S1 and S2).

For the relative luciferase reporter assay, cells were seeded in a 24-well plate 24 h before. In each well, 100 ng pMIR/HER2 or pMIR/HER2/mut and 100 nM microRNA mimics were cotransfected using

Lipofectamine™ 2000 reagent. The Renilla expression vector pRL-TK (Promega, Madison, WI) was cotransfected in each experiment to normalizing transfection efficiency. Firefly and Renilla luciferase activities were quantitated 48 h after cotransfection using Dual-Glo Luciferase assay. Results were calculated by relative luciferase activity of firefly to Renilla.

WESTERN BLOT ANALYSIS

Cells in culture were lysed using M-PER reagents and Halt Protease Inhibitor Cocktail kits (Pierce, Appleton, WI). The protein concentrations of the cells lysates were quantified using a BCA Protein Assay Kit (Pierce). Aliquots containing 60 μg of protein were prepared for loading with Laemmli sample buffer and run on SDS–PAGE gels. Separated proteins were then transferred to nitrocellulose membrane (Amersham, Buckinghamshire, United Kingdom) in transfer buffer [25 mmol/L Tris, 192 mmol/L glycine, 20% (v/v) methanol (pH 8.3)] at 300 mA at 4°C for 1 hour and 20 min. Membranes were blocked with 5% milk in TBS containing 0.5% Tween 20 (TBS-T) for 60 min at room temperature and incubated with following specific antibodies: HER2 (1:1,000, cs-2242, Cell Signaling Technology), phospho-AKT (Ser 473, 1:1,000, cs-4058, Cell Signaling Technology), total AKT (1:1,000, cs-4685, Cell Signaling), phospho-ERK1/2 (Thr202/Tyr204, 1:1,000, cs-4377, Cell Signaling Technology), ERK1/2 (1:1,000, cs-4695; Cell Signaling) in 5% bovine serum albumin (BSA) or β-actin (1:5,000; AC-74; Sigma) in 5% milk in TBS-T at 4°C overnight. Then the membranes were washed three times with TBS-T and incubated with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody at room temperature for 60 min. Finally, the membranes were washed three times with TBS-T buffer, and signals were detected using the ECL chemiluminescent kit (Pierce).

CELL MIGRATION ASSAY

At 16-h post-transfection with miRNA mimics, 2×10^5 of BT-474 cells or 5×10^4 SK-BR-3 cells in 0.5 ml of serum-free medium with or without trastuzumab (Tras) or control hIgG (20 mg/ml) were introduced into the upper compartment of the BD BioCoat control inserts (Cat. # 354578; BD Discovery Labware) fitted with membranes of 8 micron porosity separating the upper and lower compartments. The lower compartment was filled with normal culture medium supplemented with 10% FBS. After 24 h of incubation, cells were wiped off from the upper surface of each insert. The cells on the lower surface, which represented the cells that migrated through insert membrane, were fixed and stained with hematoxylin–eosin and counted by microscopic examination in 10 representative fields. Cell migration was expressed as relative to the migration of each control group. Each condition was tested in triplicate and each experiment was repeated at least three times.

WOUND HEALING ASSAY

At 16-h post-transfection with miRNA mimics, BT-474 (5×10^6 cells/well) or SK-BR-3 (2×10^6 cells/well) cells were seeded to 90% confluence in a six-well plate for overnight culture. The following day a scratch was made through the center of each well using a pipette tip, creating an open "scratch" or "wound" that was clear of cells. The dislodged cells were removed by three washes with complete culture media, and cells were incubated under standard conditions with or without trastuzumab (Tras) or control hIgG (20 mg/ml). Migration into the open area was documented at different time points post-scratching. Each condition was tested in triplicate and each experiment was repeated at least three times.

CELL COLONY-FORMING ASSAY

At 16-h post-transfection with miRNA mimics, BT-474 (5×10^3 cells/well) or SK-BR-3 (2×10^3 cells/well) cells were seeded in six-well plate in triplicate. Cells were incubated with normal culture medium supplemented with 10% FBS with or without trastuzumab (Tras) or control hIgG (20 mg/ml) and continuously treated during the experiment. Media were changed every 3 days. Fourteen days later, the cells were fixed and stained in crystal violet. Colonies containing at least 50 cells were counted. Each condition was tested in triplicate and each experiment was repeated at least three times.

STATISTICAL ANALYSIS

All experiments were repeated at least three times on different occasions. The results are presented as the mean ± SD for all values. The differences between two groups were analyzed using Student's *t*-test. All statistical analyzes were performed using the SPSS 15.0 software. $P < 0.05$ was considered statistically significant. All statistical tests and corresponding P values were two-sided.

RESULTS

MiR-199b-5p DIRECT TARGETS HER2 IN BREAST CANCER CELL

According to recent studies of breast cancer miR signature, we selected miR-199b-5p, by means of bioinformatics analysis of those miRNAs which may target the 3′UTR sequences of HER2, using algorithms by miRanda and RNAhybrid simultaneously (Supplementary Fig. S3). To validate this hypothesis experimentally, we first set up a luciferase reporter assay. The wild 3′UTR of HER2, including miR-199b-5p target sites, was cloned into the downstream of the luciferase open reading frame (Fig. 1A) of pMir-Reporter vector, along with corresponding point mutations within the miR-199b-5p seed predicted site. The reporter vector and pRL-TK were cotransfected in BT-474 cells with miR-199b-5p or negative control mimics (verified by real-time PCR in Fig. 1B), and the luciferase activity was markedly decreased (50% reduction) after miR-199b-5p overexpression (Fig. 1C). Similar results were observed in SK-BR-3. Moreover, the mutation of the miR-199b-5p binding site in HER2-3′UTR prevented the down-regulation of luciferase expression, supporting the evidence that miR-199b-5p can direct bind 3′UTR of HER2 in breast cancer cells, and that the "seed site" was necessary for the binding.

Furthermore, to determine at mRNA level or protein level miR-199b-5p down-regulated HER2, we detected HER2 expression by qRT-PCR and Western blot assays. Overexpression of miR-199b-5p (verified by real-time PCR in Fig. 1B) decreased HER2 protein expression in SK-BR-3 and BT-474, respectively (Fig. 1D). Nevertheless, qRT-PCR analysis of HER2 showed that miR-199b-5p had no effect on the HER2 mRNA level (Supplementary Fig. S4a,b). These results strongly suggest that miR-199b-5p negatively regulates HER2 expression at the translational level.

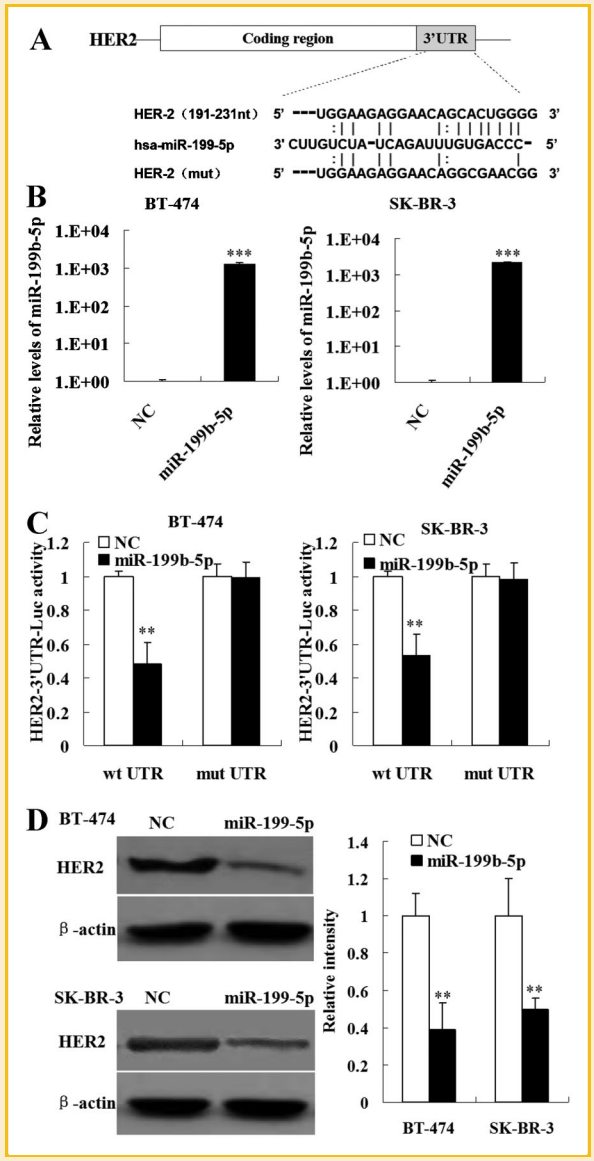

Fig. 1. MiR-199b-5p direct targets HER2 in breast cancer cells. A: Sequence of the HER2 3′UTR showing the miR-199b-5p binding seed region and mutation of the HER2 3′UTR seed region to create HER2-mut. B: qRT-PCR analysis of miR-199b-5p expression in indicated cells. C: miR-199b-5p mimics down-regulated luciferase activities controlled by wild-type HER2 3′UTR, but did not affect luciferase activity controlled by mutant HER2 3′UTR. D: Representative Western blotting of HER2 protein in indicated cells 48 h after miR-199b-5p mimics and negative control mimics (NC) transfection, β-actin expression is shown for loading normalization (left panels), with relevant quantification (right panel). The results are means of three independent experiments ± SD. Columns, mean; bars, SD. $^{**}P < 0.01$; $^{***}P < 0.001$.

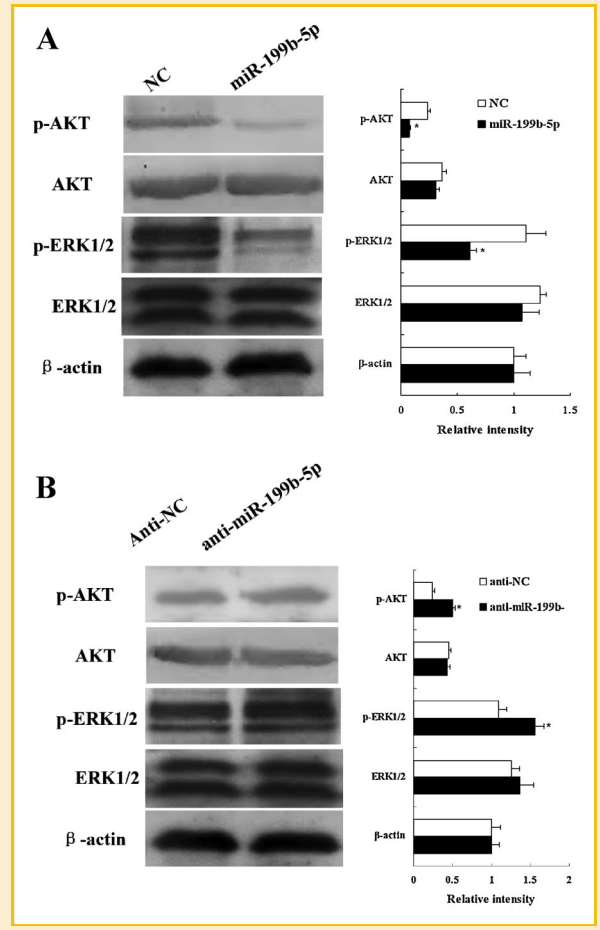

Fig. 2. MiR-199b-5p inhibits signaling pathways by ERK1/2 and AKT in breast cancer cells. A: Representative Western blotting of proteins (as indicated) in SK-BR-3 cells transfected with miR-199b-5p mimics and negative control mimics (NC), β-actin expression is shown for loading normalization (left panels), with relevant quantification (right panel). B: Representative Western blotting of proteins (as indicated) in SK-BR-3 cells transfected with miR-199b-5p inhibitors (anti-miR-199b-5p), inhibitor negative control (anti-NC), β-actin expression is shown for loading normalization (left panels), with relevant quantification (right panel). The results are means of three independent experiments ± SD. Columns, mean; bars, SD. $^{**}P < 0.01$.

MiR-199b-5p INHIBITS ERK1/2 AND AKT SIGNALING PATHWAYS IN BREAST CANCER CELLS

The downstream signaling induced by HER2 includes serine phosphorylation of ERK1/2 and AKT pathway. Because HER2 was direct targeted by miR-199b-5p, we investigated whether miR-199b-5p was able to interfere with these pathways using Western blotting. As shown in Figure 2A, phospho-ERK1/2 (p-ERK1/2) and phospho-AKT (p-AKT) levels relative to total ERK1/2 and total AKT levels were significantly reduced in the SK-BR-3 cells transfected with miR-199b-5p mimics. A reverse result was observed when miR-199b-5p was knocked down in SK-BR-3 cells transfected with miR-199b-5p inhibitors (anti-miR-199b-5p) compared to inhibitor negative control (anti-NC, Fig. 2B).

OVEREXPRESSION OF miR-199b-5p SIGNIFICANTLY INHIBITS MIGRATION AND CLONOGENICITY OF BREAST CANCER CELLS

To study the role of miR-199b-5p in the migration of breast cancer cells, we transfected the miR-199b-5p mimic into SK-BR-3 and BT-474 cells. As shown in Figure 3A, overexpression of miR-199b-5p significantly inhibited the ability of SK-BR-3 and BT-474 cells to migrate through insert membrane. Furthermore, cell migration measured using the wound healing assay was significantly inhibited

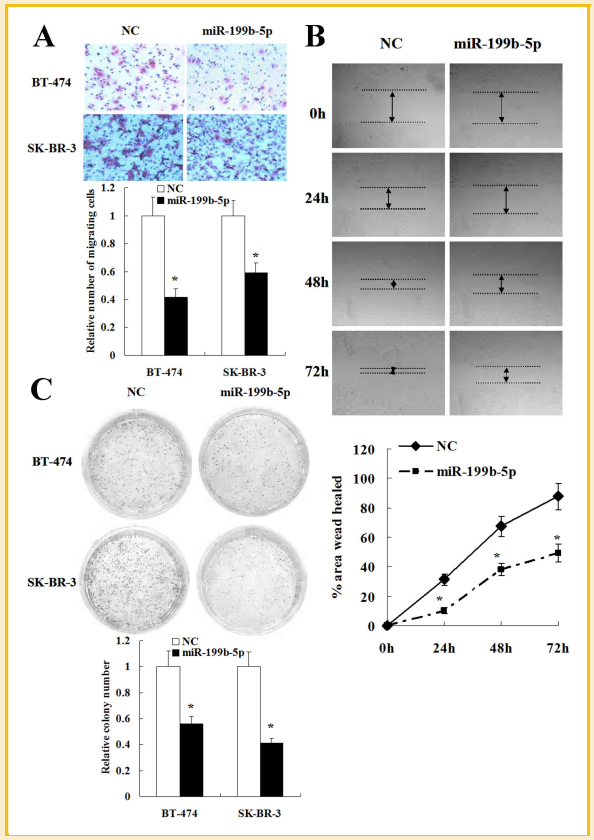

Fig. 3. MiR-199b-5p inhibits migration and clonogenicity of breast cancer cells. A: Representative images of transwell assays of membrane penetration in SK-BR-3 and BT-474 cells transfected with the miR-199b-5p mimics and negative control mimics (NC) (upper panels), with relevant quantification (lower panel). B: Representative images of wound healing assays of SK-BR-3 cells transfected with the miR-199b-5p mimics and negative control mimics (NC) (upper panels), with relevant quantification (lower panel). C: Representative images of cell colony-forming assays in SK-BR-3 and BT-474 cells transfected with miR-199b-5p mimics and negative control mimics (NC) (upper panels), with relevant quantification (lower panel). The results are means of three independent experiments ± SD. Columns, mean; bars, SD. $^{**}P < 0.01$.

in SK-BR-3 cells transfected with miR-199b-5p mimics compared with negative controls (Fig. 3B). These results demonstrate that miR-199b-5p inhibits migration of breast cancer cells.

Then we investigated the possibility that overexpression of miR-199b-5p might inhibit the survival ability of breast cells. Cell colony-forming assays clearly showed that SK-BR-3 cells transfected with miR-199b-5p mimics yielded decreased colony formation compared with negative controls (Fig. 3C). Similar results were observed in BT-474 cells (Fig. 3C).

MiR-199b-5p ENHANCES THE SUPPRESSION OF TRASTUZUMAB ON CELL MIGRATION AND CLONOGENICITY

The inhibition of miR-199b-5p on HER2-mediated aggressive phenotype also raised the intriguing possibility of a role of this microRNA in enhancing the antitumor efficacy of trastuzumab therapies in breast cancer. We investigated the effect of miR-199b-5p on trastuzumab-inhibited cell migration and clonogenicity. As shown in Figure 4A, cell migration of BT-474 measured using transwell was potently suppressed by trastuzumab, and was suppressed more potently when combined with miR-199b-5p. Similar results were observed in SK-BR-3 (Fig. 4A). Consistent with the results from transwell, cell migration measured using the wound healing assay also showed the enhanced effect of miR-199b-5p on trastuzumab-inhibited cell migration (Fig. 4B). Furthermore, cell colony-forming assay showed the enhanced effect of miR-199b-5p on trastuzumab-inhibited cell clonogenicity (Fig. 4C).

In summary, in this work, we describe miR-199b-5p as a new oncosuppressor gene in breast cancer, able to interfere with the aggressive phenotype mediated by the HER2. Our study also provides experimental evidence that miR-199b-5p can enhance the effect of trastuzumab treatment, raising the intriguing possibility of using this microRNA as a tool of an innovative and promising therapy.

DISCUSSION

In this report, we found that miR-199b-5p targeted HER2 in breast cancer cells. We discovered, for the first time, that miR-199b-5p inhibited HER2 gene expression by direct targeting its 3′UTR. In addition, miR-199b-5p inhibited HER2 downstream signaling pathways by ERK1/2 and AKT in breast cancer cells. Moreover, we also found overexpression of miR-199b-5p not only inhibited migration and clonogenicity of BT-474 and SK-BR-3 cells, but also enhanced the suppression of trastuzumab on cell migration and clonogenicity. These results strongly suggest that overexpression of miR-199b-5p in HER2-positive breast cancer cells can interfere with the aggressive phenotype by direct suppressing HER2 expression.

The HER2 oncogene encodes a 185 kDa transmembrane tyrosine kinase receptor and has been associated with aggressive phenotype of breast cancer [Slamon et al., 1987; Suda et al., 1990; Kallioniemi et al., 1991]. Targeted cancer therapies require development of approaches to inhibiting expression of tumor protein. MicroRNAs have been found altered in human cancer, which interfere with endogenous gene expression [Ambros, 2001]. It was recently reported microRNAs play essential role in Her2-induced breast cancer. MiR-205 [Adachi et al., 2011], miR-194 [Le et al., 2012], miR-26a, and miR-30b [Ichikawa et al., 2012] are downstream of Her2 in breast cancer. Although, HER2 has been reported to been direct targeted by upstream miR-125a or miR-125b in SK-BR-3 cells [Scott et al., 2007]. One gene can be targeted by hundreds of microRNAs. So we try to find another microRNA that can inhibit the expression of HER2. According to resent studies of breast cancer miR signature [Blenkiron et al., 2007; Persson et al., 2011] and bioinformatics analysis, we selected miR-199b-5p. In the Her2 3′UTR, the binding site position of miR-125a (17-44nt) is near to miR-125b (19-44nt), but all are far away from miR-199b-5p (191-231nt). It suggests that miR-135a/b and miR-199b-5p are able to bind 3′UTR of Her2 mRNA at the same time.

We confirmed our hypothesis that miR-199b-5p can direct suppress expression of HER2 in BT-474 and SK-BR-3 cells, which happened at the translational level. We also found that miR-199b-5p inhibited HER2 downstream signaling pathways by ERK1/2 and AKT in breast cancer cells.

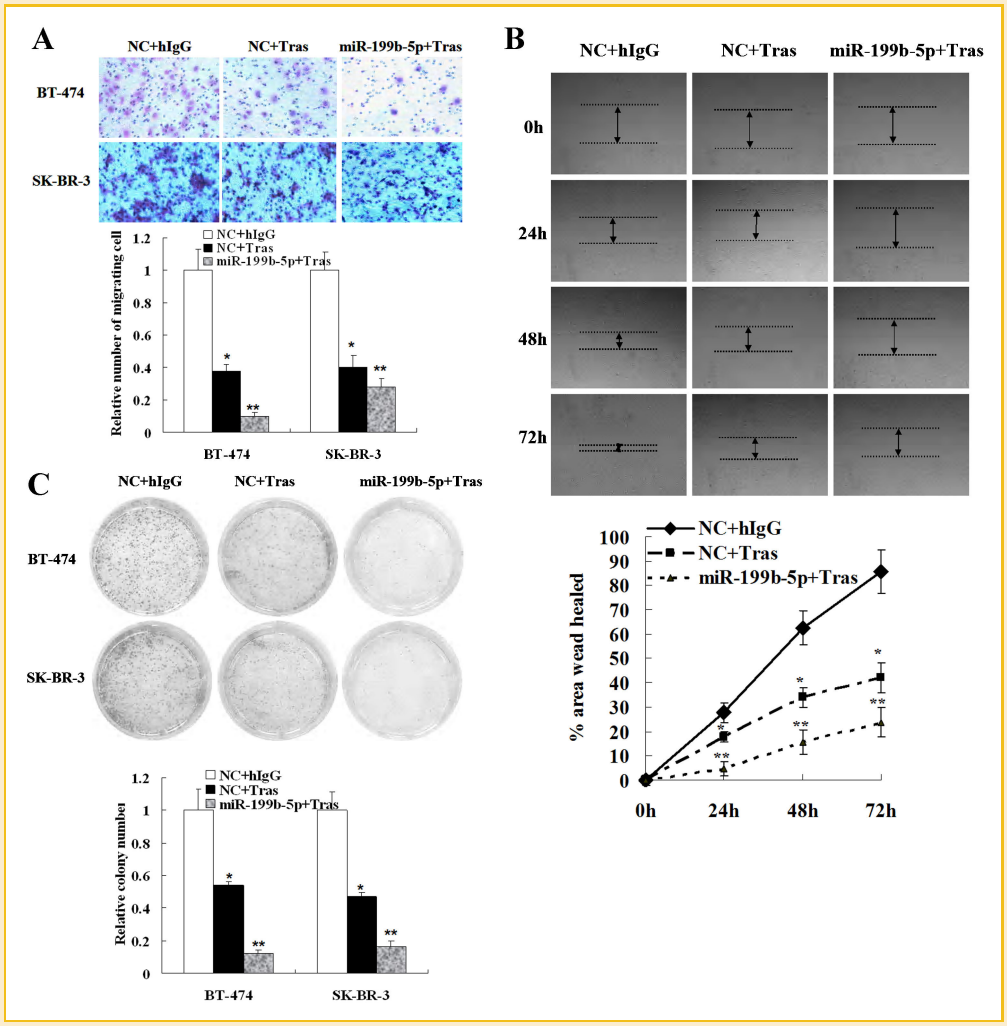

Fig. 4. MiR-199b-5p enhanced the suppression of trastuzumab on cell migration and clonogenicity. A: Representative images of transwell assays of membrane penetration (upper panels), with relevant quantification (lower panel). SK-BR-3 and BT-474 cells were transfected with microRNA mimics for 16 h, then seeded and treated with trastuzumab (Tras) or control hIgG (20 mg/ml) for 24 h. B: Representative images of wound healing assays (upper panels), with relevant quantification (lower panel). SK-BR-3 cells were transfected with the microRNA mimics for 16 h, then seeded and treated with trastuzumab (Tras) or control hIgG (20 mg/ml) for 72 h. C: Representative images of cell colony-forming assays (upper panels), with relevant quantification (lower panel). Cells were transfected with microRNA mimics for 16 h, then seeded and treated with trastuzumab (Tras) or control hIgG (20 mg/ml) for 14 days. The results are means of three independent experiments ± SD. Columns, mean; bars, SD. $^{**}P < 0.01$.

Although miR-199b-5p has been documented involved in various types of cancers [Garzia et al., 2009; Chao et al., 2010; Flamant et al., 2010], its biological function in breast cancer has not been reported. SK-BR-3 and BT-474 cells are classical models in the research of HER2-positive breast cancer. Data in these two cell lines show that overexpression of miR-199b-5p could significantly inhibit migration and clonogenicity of breast cancer cells. These results suggest that miR-199b-5p may have the potential to be a novel alternative therapeutic target for HER2-positive breast cancer.

Since trastuzumab is the antibody agent approved targeting direct against HER2 and its effect is limited to the time it remains present about the tumor cells at a therapeutic level, we next to demonstrate whether miR-199b-5p affect the antitumor efficacy of trastuzumab therapies. Results show the enhanced effect of miR-199b-5p on trastuzumab-inhibited cell migration and clonogenicity. It suggests miR-199b-5p as a novel therapeutic target combined with trastuzumab therapy for HER2-positive breast cancer.

In conclusion, our results strongly suggest that miR-199b-5p may as a new oncosuppressor gene in breast cancer, able to interfere with the aggressive phenotype mediated by HER2. Our study also provides experimental evidence that miR-199b-5p can improve the effect of trastuzumab treatment in breast cancer cells, raising the intriguing possibility of using this microRNA as a tool of an innovative and promising therapy.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (No: 30572200). The authors thank Dr. Zhao Zhisong for his help in revision of the manuscript.

REFERENCES

Adachi R, Horiuchi S, Sakurazawa Y, Hasegawa T, Sato K, Sakamaki T. 2011. ErbB2 down-regulates microRNA-205 in breast cancer. Biochem Biophys Res Commun 411:804–808.

Ambros V. 2001. microRNAs: Tiny regulators with great potential. Cell 107:823–826.

Bartel DP. 2004. MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116:281–297.

Blenkiron C, Goldstein LD, Thorne NP, Spiteri I, Chin SF, Dunning MJ, Barbosa-Morais NL, Teschendorff AE, Green AR, Ellis IO, Tavare S, Caldas C, Miska EA. 2007. MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. Genome Biol 8:R214.

Chao A, Tsai CL, Wei PC, Hsueh S, Chao AS, Wang CJ, Tsai CN, Lee YS, Wang TH, Lai CH. 2010. Decreased expression of microRNA-199b increases protein levels of SET (protein phosphatase 2A inhibitor) in human choriocarcinoma. Cancer Lett 291:99–107.

Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, Wojcik SE, Aqeilan RI, Zupo S, Dono M, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM. 2005. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci USA 102:13944–13949.

Croce CM. 2009. Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet 10:704–714.

Eichhorn PJ, Gili M, Scaltriti M, Serra V, Guzman M, Nijkamp W, Beijersbergen RL, Valero V, Seoane J, Bernards R, Baselga J. 2008. Phosphatidylinositol 3-kinase hyperactivation results in lapatinib resistance that is reversed by the mTOR/phosphatidylinositol 3-kinase inhibitor NVP-BEZ235. Cancer Res 68:9221–9230.

Esquela-Kerscher A, Slack FJ. 2006. Oncomirs—microRNAs with a role in cancer. Nat Rev Cancer 6:259–269.

Flamant S, Ritchie W, Guilhot J, Holst J, Bonnet ML, Chomel JC, Guilhot F, Turhan AG, Rasko JE. 2010. Micro-RNA response to imatinib mesylate in patients with chronic myeloid leukemia. Haematologica 95:1325–1333.

Garzia L, Andolfo I, Cusanelli E, Marino N, Petrosino G, De Martino D, Esposito V, Galeone A, Navas L, Esposito S, Gargiulo S, Fattet S, Donofrio V, Cinalli G, Brunetti A, Vecchio LD, Northcott PA, Delattre O, Taylor MD, Iolascon A, Zollo M. 2009. MicroRNA-199b-5p impairs cancer stem cells through negative regulation of HES1 in medulloblastoma. PLoS ONE 4:e4998.

Ichikawa T, Sato F, Terasawa K, Tsuchiya S, Toi M, Tsujimoto G, Shimizu K. 2012. Trastuzumab produces therapeutic actions by upregulating miR-26a and miR-30b in breast cancer cells. PLoS ONE 7:e31422.

Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D, Slack FJ. 2005. RAS is regulated by the let-7 microRNA family. Cell 120:635–647.

Kallioniemi OP, Holli K, Visakorpi T, Koivula T, Helin HH, Isola JJ. 1991. Association of c-erbB-2 protein over-expression with high rate of cell proliferation, increased risk of visceral metastasis and poor long-term survival in breast cancer. Int J Cancer 49:650–655.

Le XF, Almeida MI, Mao W, Spizzo R, Rossi S, Nicoloso MS, Zhang S, Wu Y, Calin GA, Bast RC, Jr. 2012. Modulation of MicroRNA-194 and cell migration by HER2-targeting trastuzumab in breast cancer. PLoS ONE 7:e41170.

Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. 2003. Prediction of mammalian microRNA targets. Cell 115:787–798.

Liu L, Greger J, Shi H, Liu Y, Greshock J, Annan R, Halsey W, Sathe GM, Martin AM, Gilmer TM. 2009. Novel mechanism of lapatinib resistance in HER2-positive breast tumor cells: Activation of AXL. Cancer Res 69:6871–6878.

Lynam-Lennon N, Maher SG, Reynolds JV. 2009. The roles of microRNA in cancer and apoptosis. Biol Rev 84:55–71.

Martin AP, Miller A, Emad L, Rahmani M, Walker T, Mitchell C, Hagan MP, Park MA, Yacoub A, Fisher PB, Grant S, Dent P. 2008. Lapatinib resistance in HCT116 cells is mediated by elevated MCL-1 expression and decreased BAK activation and not by ERBB receptor kinase mutation. Mol Pharmacol 74:807–822.

Molina MA, Sáez R, Ramsey EE, Garcia-Barchino MJ, Rojo F, Evans AJ, Albanell J, Keenan EJ, Lluch A, García-Conde J. 2002. NH2-terminal truncated HER-2 protein but not full-length receptor is associated with nodal metastasis in human breast cancer. Clin Cancer Res 8:347–353.

Mukohara T. 2011. Mechanisms of resistance to anti-human epidermal growth factor receptor 2 agents in breast cancer. Cancer Sci 102:1–8.

Persson H, Kvist A, Rego N, Staaf J, Vallon-Christersson J, Luts L, Loman N, Jonsson G, Naya H, Hoglund M, Borg A, Rovira C. 2011. Identification of new microRNAs in paired normal and tumor breast tissue suggests a dual role for the ERBB2/Her2 gene. Cancer Res 71:78–86.

Ryan Q, Ibrahim A, Cohen MH, Johnson J, Ko CW, Sridhara R, Justice R, Pazdur R. 2008. FDA drug approval summary: Lapatinib in combination with capecitabine for previously treated metastatic breast cancer that overexpresses HER-2. Oncologist 13:1114–1119.

Scaltriti M, Rojo F, Ocaña A, Anido J, Guzman M, Cortes J, Di Cosimo S, Matias-Guiu X, y Cajal SR, Arribas J. 2007. Expression of p95HER2, a truncated form of the HER2 receptor, and response to anti-HER2 therapies in breast cancer. J Natl Cancer Inst 99:628–638.

Scott GK, Goga A, Bhaumik D, Berger CE, Sullivan CS, Benz CC. 2007. Coordinate suppression of ERBB2 and ERBB3 by enforced expression of micro-RNA miR-125a or miR-125b. J Biol Chem 282:1479–1486.

Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. 1987. Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177–182.

Smith I, Procter M, Gelber RD, Guillaume S, Feyereislova A, Dowsett M, Goldhirsch A, Untch M, Mariani G, Baselga J, Kaufmann M, Cameron D, Bell R, Bergh J, Coleman R, Wardley A, Harbeck N, Lopez RI, Mallmann P, Gelmon K, Wilcken N, Wist E, Sanchez Rovira P, Piccart-Gebhart MJ. 2007. 2-Year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: A randomised controlled trial. Lancet 369:29–36.

Stahlhut Espinosa CE, Slack FJ. 2006. The role of microRNAs in cancer. Yale J Biol Med 79:131–140.

Suda Y, Aizawa S, Furuta Y, Yagi T, Ikawa Y, Saitoh K, Yamada Y, Toyoshima K, Yamamoto T. 1990. Induction of a variety of tumors by c-erbB2 and clonal nature of lymphomas even with the mutated gene (Val659–Glu659). EMBO J 9:181–190.

Trowe T, Boukouvala S, Calkins K, Cutler RE, Jr., Fong R, Funke R, Gendreau SB, Kim YD, Miller N, Woolfrey JR, Vysotskaia V, Yang JP, Gerritsen ME, Matthews DJ, Lamb P, Heuer TS. 2008. EXEL-7647 inhibits mutant forms of ErbB2 associated with lapatinib resistance and neoplastic transformation. Clin Cancer Res 14:2465–2475.

Xia W, Bacus S, Husain I, Liu L, Zhao S, Liu Z, Moseley MA III, Thompson JW, Chen FL, Koch KM, Spector NL. 2010. Resistance to ErbB2 tyrosine kinase inhibitors in breast cancer is mediated by calcium-dependent activation of RelA. Mol Cancer Ther 9:292–299.

Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY. 2008. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. Cell Res 18:350–359.