**ORIGINAL ARTICLE**

**MiR-145-targeted HBXIP modulates human breast cancer cell proliferation**

Yang Jiang¹, Dan Wang², Hui Ren³, Ying Shi⁴ & Yufei Gao⁵

¹ Department of Gastrointestinal Surgery, China-Japan Union Hospital of Jilin University, Changchun, China
² Department of Breast Surgery, The Second Hospital of Jilin University, Changchun, China
³ Department of General Surgery, China-Japan Union Hospital of Jilin University, Changchun, China
⁴ Department of Breast-Thyroid Surgery, The Second Hospital of Jilin University, Changchun, China
⁵ Department of Neurosurgery, China-Japan Union Hospital of Jilin University, Changchun, China

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**Correspondence**
Yufei Gao, Department of Neurosurgery, China-Japan Union Hospital of Jilin University, 126 Xiantai Street, Changchun 130033, China.
Tel: +86 431 8464 1026
Fax: +86 431 8464 1026
Email: gaoyufei1975@126.com

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**Abstract**

**Background:** MiR-145 has been identified as a tumor suppressive microRNA in multiple cancers. In this current investigation, we searched for new direct targets of miR-145 and evaluated their effect on breast cancer development.

**Methods:** Targetscan was used to predict the target genes of miR-145. The targeting of miR-145 on oncogenic HBXIP was verified by luciferase reporter gene analysis. The effect of miR-145 on the level of messenger RNA and protein of HBXIP was evaluated by quantitative real-time PCR and immunoblotting. Correlations between miR-145 and HBXIP, as well as miR-145 expression, were analyzed in 30 paired breast cancer and noncancerous tissues by quantitative real-time PCR. Methyl thiazol tetrazolium and colony formation assays were applied to determine the cell proliferation ability.

**Results:** HBXIP was identified as a novel target gene of miR-145 in breast cancer. MiR-145 was found to dose-dependently decrease messenger RNA and protein expression of HBXIP in breast cancer MCF-7 cells. Notably, miR-145 expression was negatively related to HBXIP expression and was obviously reduced in breast cancer samples. Finally, miR-145 suppressed cell proliferation while its inhibitor, anti-miR-145, accelerated cell proliferation. Interestingly, silencing of HBXIP reversed the acceleration of cell proliferation induced by anti-miR-145 in breast cancer.

**Conclusion:** Oncogenic HBXIP is a new direct target of tumor suppressive miR-145. Our findings reveal that miR-145-targeting HBXIP could be a potential therapeutic target in breast cancer.

**Introduction**

Breast cancer is considered the most prevalent malignancy among women in the world. MicroRNAs (miRNAs), members of the non-coding RNA (ncRNA) family, play key roles in regulating gene expression by targeting and interacting with the 3’ untranslated regions (UTRs) of messenger RNAs (mRNAs) of target genes, in which the targeting usually directly induces mRNA degradation or translational repression.¹⁻³ Many investigations have revealed that miRNAs can affect multiple cellular processes, such as cell proliferation, differentiation, and apoptosis.⁴⁻⁶ MiRNAs can play oncogenic or tumor suppressive roles in various cancers.

As a tumor suppressor miRNA, miR-145 plays a significant role in several cancers. In non-small cell lung cancer (NSCLC) tumorigenesis, miR-145 could be sponged by a long ncRNA (lncRNA), LINC00339, in FOXM1 regulation.⁷ Both miR-145-5p and miR-145-3p modulate tumor-promoting genes in lung adenocarcinoma.⁸ During NSCLC development, miR-145, together with miR-497, directly target MTDH to restrain transforming growth factor-β-mediated epithelial-mesenchymal transition (EMT).⁹ In gastric cancer development, miR-145/SOX9 can be targeted by the lncRNA SNHG14.¹⁰ In addition, miR-145 can impact the anti-tumor effect of 5-aminolevulinic acid treatment for...
In osteosarcoma, micelleplexes containing miR-145 are used to suppress cell proliferation and migration. In ovarian cancer, the negative feedback loop of DNMT3A and miR-145 can affect the Warburg effect of cells. MI-145 has also been used to treat prostate cancer metastasis. During host immunity-associated tumor metastasis, miR-130a and miR-145 play crucial roles in the reprogramming of Gr-1+CD11b+ myeloid cells. During the progression of breast cancer, elevated miR-145 changes the transcriptome and plays a tumor suppressive role. In triple-negative breast cancer, miR-145 is involved in lincRNA-RoR/MUC1-induced cell invasion and metastasis. MiR-145 can also target transforming growth factor-β to modulate cell proliferation and migration in breast cancer.

As a highly conserved protein across the mammalian species, HBXIP is widely expressed in many tissues. Overexpressed HBXIP has been found in breast cancer tissues, indicating that HBXIP is capable of serving as an oncoprotein to promote breast cancer progression, including growth and metastasis. As a regulatory member, HBXIP plays a pivotal role in the activation of mTORC1-mediated by amino acid. Some studies have revealed that HBXIP can function as an oncogenic co-activator of many transcription factors to play a key part in the transcription regulation of tumor-related genes in breast cancer. However, whether HBXIP is involved in the effect of miR-145 on breast cancer development remains unknown.

In this study, we investigate new target gene candidates of miR-145 and evaluate their effect on breast cancer development. We observe that miR-145 directly targets the 3'UTR of HBXIP mRNAs in breast cancer cells. We find that the miR-145/HBXIP axis affects breast cancer cell proliferation. Our findings raise the possibility of an miR-145/HBXIP axis for the treatment of breast cancer.

### Methods

#### Cell culture

Human breast cancer cell line MCF-7 was obtained from American Type Culture Collection (Rockville, MD, USA). RPMI-1640 (Invitrogen, Carlsbad, CA, USA) medium with 10% fetal bovine serum (FBS) was applied to cultivate the cells in a humidified incubator at 37°C with 5% CO₂.

#### Transfection of miRNA, miRNA inhibitor, and small interfering RNA

miR-145, its inhibitor (anti-miR-145), and small interfering HBXIP (si-HBXIP) were purchased from GenePharma (Shanghai, China). We used Lipofectamine 2000 (Invitrogen) as transfection reagent. The concentrations of miR-145, anti-miR-145, and the negative control were 50 nM or 100 nM.

#### Luciferase reporter gene analysis

The pGL3-control vector (Promega, Madison, WI, USA) containing the predicted miR-145 binding site of the 3'UTR of HBXIP mRNA and its mutant were transfected into the cells and seeded on 24-well plates. MiR-145, anti-miR-145, and the negative control were transfected into the cells with pGL3-HBXIP-wt or pGL3-HBXIP-mut reporter vectors. A Dual-Luciferase Reporter Assay System (Promega) was used to evaluate the luciferase activity using the cell extracts 48 hours post-transfection.

#### Quantitative real-time PCR

TRIzol reagent (Invitrogen) was used to extract total RNA from the cells. An MiRNeasy Minikit (Qiagen, USA) was used to prepare RNA from human clinical breast cancer tissues. Quantitative real-time PCR was performed using the QuantiNova SYBR Green PCR kit (Qiagen, Valencia, CA, USA). The HBXIP or miR-145 levels were normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or U6, respectively.

#### Immunoblotting

Breast cancer cells were lysed using a radiolabeled precipitation assay buffer. All protein samples were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, CA, USA). The primary antibodies used included anti-HBXIP (Abcam, Cambridge, UK) or anti-β-actin (Cell Signaling Technology, Danvers, MA, USA) antibodies. ImageQuant 5.2 software (GE Healthcare, Little Chalfont, UK) was used to quantify the density of the bands.

#### Patient samples

Thirty pairs of breast cancer and noncancerous tissues (Table S1) from the China-Japan Union Hospital of Jilin University were used. Patients approved tissue use and the Research Ethics Board at the China-Japan Union Hospital of Jilin University approved the investigation protocol.

#### Cell proliferation assay

Methyl thiazol tetrazolium (MTT) and colony formation assays were performed to show cell proliferation ability. For MTT assay, breast cancer MCF-7 cells treated with
miR-145, anti-miR-145, and/or si-HBXIP were cultured in 96-well plates. Twenty-four hours post-transfection, the cells were transferred onto six-well plates and maintained for approximately two weeks for the colony formation assay. The colonies were fixed with methyl alcohol, stained by crystal violet, and counted by at least three observers.

**Figure 1** MiR-145 directly targets the 3' untranslated region (UTR) of HBXIP messenger RNAs (mRNAs). (a) Targetscan was used to predict the target genes of miR-145. (b) Wild-type (wt) and mutant-type (mut) vectors containing 3' UTR of HBXIP mRNA with miR-145 were constructed. (c,d) Luciferase reporter gene assay was used to evaluate the effect of miR-145 (or anti-miR-145) on the luciferase activities of the 3' UTR of HBXIP mRNA in MCF-7 cell lines. **P < 0.01; ns, not significant.

**Figure 2** MiR-145 reduced the level of HBXIP in breast cancer cells. (a,b) MiR-145 and HBXIP levels were determined by quantitative real-time PCR assay or immunoblotting after miR-145 or anti-miR-145 was introduced into breast cancer MCF-7 cells. **P < 0.01. a: [miR-145], [HBXIP]. b: [anti-miR-145], [HBXIP].
Statistics

Data are shown as mean values with standard error of the mean (SEM). *P* < 0.05 was considered statistically significant. The Student’s *t*-test was used to analyze statistical significance in the two different groups. Pearson’s correlation coefficient was applied to evaluate the correlation between miR-145 and HBXIP levels in clinical breast cancer samples.

Results

**MiR-145 directly targets the 3’ untranslated region of HBXIP messenger RNAs**

As a tumor suppressor, miR-145 is implicated in the development of various types of cancers. In this study, we were interested in screening the novel candidate target miR-145 in breast cancer. The online database TargetScan (http://www.targetscan.org/) was used to predict the potential target genes of miR-145. HBXIP attracted our attention because of its pivotal role in the promotion of breast cancer progression. We observed that the 3’UTR of HBXIP mRNAs own a putative site for binding to the seed region of miR-145 (Fig 1a). To investigate whether HBXIP is a new direct target of tumor suppressive miR-145, we constructed luciferase reporter vectors in which wide-type (wt) and mutant (mut) binding sites of HBXIP 3’UTR with miR-145 in HBXIP mRNA were cloned into the pGL3-Control vector (Fig 1b). Luciferase reporter gene analysis revealed that miR-145 markedly decreased luciferase activity in the wild-type reporter of HBXIP, while the suppressive effect on the mutant reporter was lost (Fig 1c). Furthermore, anti-miR-145 decreased the level of endogenous miR-145 to upregulate the luciferase activities of the wild-type reporter of HBXIP, but it had no effect on the luciferase activities of the mutant reporter of HBXIP (Fig 1d). Our findings showed that oncogenic HBXIP could serve as a novel target gene of tumor suppressive miR-145 in breast cancer.

**MiR-145 can reduce the HBXIP level in breast cancer cells**

We then attempted to more clearly show miR-145 modulation of HBXIP in the cells. We used immunoblotting to analyze HBXIP alteration in breast cancer MCF-7 cells after miR-145 was induced or reduced by the transfection of miR-145 or its inhibitor (anti-miR-145). We observed that miR-145 transfection increased the miR-145 level 4–8-fold in the cells. At the same time, the HBXIP protein level and mRNA was decreased in a dose-dependent manner (Fig 2a). To decrease the endogenous levels of miR-145, we transfected the inhibitor, anti-miR-145, into breast cancer cells. Interestingly, miR-145 inhibition mediated by anti-miR-145 efficiently elevated the HBXIP level. We observed that anti-miR-145 increased the HBXIP level at least 2–6-fold in the MCF-7 cell line (Fig 2b). Thus, we hypothesize that miR-145 may play a pivotal role in breast cancer progression.

**Figure 3** Reduced MiR-145 is related to overexpressed HBXIP in clinical breast cancer patients. (a) Pearson *r* analysis revealed a correlation between miR-145 and HBXIP at the RNA level. (b,c) The Wilcoxon signed rank test was used to analyze miR-145 and HBXIP levels in clinical breast cancer and paired noncancerous tissues. **P** < 0.01; ***P*** < 0.001; mRNA, messenger RNA.
conclude that the protein and mRNA levels of HBXIP can be reduced by miR-145 in breast cancer cells.

**Reduced MiR-145 is related to overexpressed HBXIP in breast cancer**

Taken a step further, we explored the clinical link between miR-145 and HBXIP in 30 pairs of clinical breast cancer and noncancerous samples by quantitative real-time PCR. We found a negative correlation between miR-145 and HBXIP in breast cancer tissues ($R^2 = 0.598, P < 0.01$) (Fig 3a). Evidence suggests that miR-145 is decreased in cancer and plays a significant role by suppressing the development of various types of cancers. We further confirmed low miR-145 expression and high HBXIP expression in 30 breast cancer tissues compared to paired noncancerous tissues (Fig 3b,c). Our findings indicate a negative relationship between miR-145 and HBXIP in breast cancer progression.

**MiR-145-targeted HBXIP modulates breast cancer cell growth**

To elucidate the role of miR-145-regulated HBXIP in breast cancer growth, we introduced miR-145, anti-miR-145, and/or si-HBXIP into breast cancer MCF-7 cells. To avoid off-target RNA interference of HBXIP, we designed two different siRNAs targeting HBXIP mRNA (si-HBXIP-1 and si-HBXIP-2) and tested their efficiency by immunoblotting assay. Si-HBXIP-2 showed better effects of...

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**Figure 4** MiR-145-targeted HBXIP modulates breast cancer cell growth. (a) Immunoblotting assay was used to test the RNA interference efficiency of two different small interfering RNAs (siRNAs) targeting HBXIP messenger RNA (si-HBXIP-1 and si-HBXIP-2). (b,c) Methyl thiazol tetrazolium (MTT) or colony formation assays were used to analyze breast cancer cell proliferation after miR-145, anti-miR-145, or anti-miR-145/si-HBXIP was transiently transfected into breast cancer MCF-7 cells (Mock, miR-145, anti-miR-145, anti-miR-145+si-HBXIP-2). The colony formation data from three independent experiments was quantified. *$P < 0.05$; **$P < 0.01$. OD, optical density.
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plays a tumor suppressive role. In this study, we investigated elevated miR-145 changes the transcriptome and deregulation of miRNAs can inhibit or promote the initiation or progression of numerous cancers. As a tumor suppressor miRNA, miR-145 plays a significant role in several cancers, including lung, gastric, oral, ovarian, and prostate cancers, and osteosarcoma. During breast cancer progression, elevated miR-145 changes the transcriptome and plays a tumor suppressive role. In this study, we investigated new target gene candidates of miR-145 and evaluated their effect on breast cancer development.

Oncogenic HBXIP serves as a regulator, leading to amino acid-induced activation of mTORC1. HBXIP is frequently overexpressed in breast cancer and can participate in cell proliferation, invasion, and metabolism reprogramming. HBXIP can also bind to the anti-apoptosis factor, survivin, to play a part in the regulation of cell division and apoptosis. However, post-transcription regulation of HBXIP still needs to be further explored.

Using the Targetscan database, we revealed that HBXIP could be a candidate target of miR-145. We then confirmed that miR-145 could directly bind to the 3'UTR of HBXIP mRNA, resulting in the inhibition of mRNA and protein levels of HBXIP in breast cancer cells. We used clinical breast cancer tissues to determine whether there was a pathological link between miR-145 and HBXIP and found a negative relationship. To determine the function of the miR-145/HBXIP axis, we introduced miR-145, anti-miR-145, and/or HBXIP siRNA into breast cancer cells and used MTT and colony formation assays to test cell proliferation. Our data was consistent with previous reports, indicating that miR-145 plays a suppressive role in tumor development. Importantly, when endogenous miR-145 is reduced, cell proliferation is accelerated. Furthermore, anti-miR-145-induced cell proliferation was reversed by HBXIP knockdown.

In conclusion, miR-145 can directly bind to and target the 3'UTR of HBXIP mRNA to regulate HBXIP expression at the post-transcription level in breast cancer cells. The miR-145/HBXIP axis plays a significant role in breast cancer cell proliferation. Therapeutically, the miR-145/HBXIP axis could serve as a potential target for breast cancer.

Discussion

Dysregulation of miRNAs can inhibit or promote the initiation or progression of numerous cancers. As a tumor suppressor miRNA, miR-145 plays a significant role in several cancers, including lung, gastric, oral, ovarian, and prostate cancers, and osteosarcoma. During breast cancer progression, elevated miR-145 changes the transcriptome and plays a tumor suppressive role. In this study, we investigated new target gene candidates of miR-145 and evaluated their effect on breast cancer development.

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Disclosure

No authors report any conflict of interest.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher’s website.

**Table S1.** Clinical characteristics of 30 breast cancer tissue samples.