miR-655 suppresses epithelial-to-mesenchymal transition by targeting Prrx1 in triple-negative breast cancer

Zhi-Dong Lv a, Bin Kong a, Xiang-Ping Liu b, Li-Ying Jia, Qian Dong d, Fu-Nian Lia, Hai-Bo Wang a, *

a Department of Breast Surgery, The Affiliated Hospital of Qingdao University, Qingdao, China
b Central Laboratory of Molecular Biology, The Affiliated Hospital of Qingdao University, Qingdao, China
c Cerebrovascular Disease Research Institute, The Affiliated Hospital of Qingdao University, Qingdao, China
d Department of Pediatric Surgery, The Affiliated Hospital of Qingdao University, Qingdao, China

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Abstract

Triple-negative breast cancer (TNBC) is a highly aggressive breast cancer subtype that lacks effective targeted therapies. The epithelial-to-mesenchymal transition (EMT) is a key contributor in the metastatic process. In this study, we found that miR-655 was down-regulated in TNBC, and its expression levels were associated with molecular-based classification and lymph node metastasis in breast cancer. These findings led us to hypothesize that miR-655 overexpression may inhibit EMT and its associated traits of TNBC. Ectopic expression of miR-655 not only induced the up-regulation of cytokeratin and decreased vimentin expression but also suppressed migration and invasion of mesenchymal-like cancer cells accompanied by a morphological shift towards the epithelial phenotype. In addition, we found that miR-655 was negatively correlated with Prrx1 in cell lines and clinical samples. Overexpression of miR-655 significantly suppressed Prrx1, as demonstrated by Prrx1 3’-untranslated region luciferase report assay. Our study demonstrated that miR-655 inhibits the acquisition of the EMT phenotype in TNBC by down-regulating Prrx1, thereby inhibiting cell migration and invasion during cancer progression.

Keywords: miR-655 ● triple-negative breast cancer ● Prrx1 ● epithelial-to-mesenchymal transition

Introduction

Triple-negative breast cancer (TNBC) is a highly aggressive tumour subtype associated with a poor prognosis, and most mortality and morbidity does not arise from the primary tumour, but from distant metastasis [1, 2]. To metastasize, a cancer cell must shed many of its epithelial characteristics, invade the surrounding tissue to enter the circulation, subsequently survive in the circulation, extravasate and proliferate in the metastatic niche [3, 4]. Invasion is therefore a key step in the metastatic cascade. Recent research has demonstrated that epithelial-to-mesenchymal transition (EMT) plays a key role in the early process of metastasis of cancer cells [5, 6]. During the acquisition of EMT characteristics, cancer cells lose the expression of genes that promote cell–cell contact, such as E-cadherin and the miR-200 family, and gain the expression of mesenchymal markers, such as vimentin, fibronectin and N-cadherin, leading to enhance cancer cell migration and invasion [7, 8] and to confer drug resistance [9]. Therefore, the development of EMT inhibitors may provide novel strategies for the prevention, diagnosis and treatment of cancers.

MicroRNAs (miRNAs) are a family of small, non-coding, endogenous RNAs (19–24 nucleotides in length), which inhibit gene expression by binding to the 3’-untranslated region (3’-UTR) of mRNA sequence, and leading to translational degradation or repression [10–12]. It has been demonstrated that miRNAs play crucial roles in cell biology such as proliferation, apoptosis, cell cycle, migration and invasion [13–15]. Increasing evidence shows the potential involvement of miRNAs in the development of various human cancers such as gastric cancer, bladder cancer, lung cancer, hepatocellular carcinoma, and breast cancer [16–19]. miRNAs can function as either tumour suppressors or oncogenes according to their target genes [20, 21]. A recent study had performed a miRNA chip-based expression analysis of tissues from primary oesophageal squamous cell carcinoma and found that the expression of miR-655 in tumour was lower than that in adjacent, and paired non-tumour tissues. Studies have also shown that miR-655 is an EMT-suppressive miRNA [22, 23]. However, the expression level of miR-655 and its role in breast cancer have not yet been determined.
Prrx1 is a newly identified EMT inducer conferring migratory and invasive properties [24]. In addition, high expression of Prrx1 was significantly correlated with metastasis and poor prognosis in colorectal cancer cases [25]. Recent studies have also shown that Prrx1 played a pivotal role in pancreatic regeneration and carcinogenesis [26]. In this study, we illustrated that miR-655 suppresses EMT by down-regulating Prrx1 in TNBC. These findings have been uncovered for the first time, thereby illustrating how miR-655 plays a key role in suppressing proliferation and metastasis in TNBC.

Materials and methods

Clinical samples

A total of 135 pairs of invasive breast carcinoma and adjacent non-cancerous tissue samples were obtained from patients who underwent modified radical mastectomy in the Affiliated Hospital of Qingdao University. The matched non-cancerous adjacent tissues were harvested at least 5 cm away from the tumour site. None of the patients underwent radiotherapy or chemotherapy prior to the surgery. Written informed consent was obtained from all participants, and research protocols for the use of human tissue were approved by and conducted in accordance with the policies of the Institutional Review Boards at Qingdao University. The histological subtype was determined according to the World Health Organization classification. The TNM stage was determined post-operatively according to the American Joint Committee on Cancer (7th edition), and the histological grade was determined according to the Scarff-Bloom-Richardson grading system.

Cell culture and transfections

Two breast cancer cell lines with a luminal transcriptional profile (MCF-7 and Bcap-37), three breast cancer cell lines with a basal-like transcriptional profile (MDA-MB-435, MDA-MB-231 and HCC-1937) and a normal mammary epithelial cell line (MCF-10A) were obtained from the Cancer Research Institute of Beijing, China. These cells were cultivated in T75 tissue culture flasks in DMEM supplemented with 10% foetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 20 mM hydroxyethyl piperazine ethanesulfonic acid, and incubated in humidified incubator containing 5% CO₂ at 37°C. The miR-655 mimics and a non-specific miR control were synthesized and purified by RiboBio (Guangzhou, China). miR-655 mimics were transfected at working concentrations using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The full-length Prrx1 cDNA was purchased from GeneCopeia™ (Genecopeia inc, Maryland, MD, USA), and was subcloned into the eukaryotic expression vector pcDNA3.1 (+). The vector pcDNA3.1 (+) was used as a negative control.

Western blot analysis

Tissues or cells were lysed in RIPA buffer supplemented with protease inhibitor mixture for 30 min. at 4°C. The cell lysates were then sonicated briefly and centrifuged (14,000 × g at 4°C) for 15 min. to remove insoluble materials. Equal amounts of protein were separated by SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked with 5% non-fat dry milk and then incubated with first antibody, followed by horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized by ECL chemiluminescence method.

Invasion assay

Invasion assays were performed with the Chemicon Cell Invasion Assay Kit (Millipore, Billerica, MA, USA) according to the manufacturer’s protocol. Briefly, cells (1 × 10⁵) were plated onto a Matrigel-coated transwell invasion chamber and incubated at 37°C for 24 hrs. Non-invading cells were removed by wiping the upper side of the transwell. Invading cells were fixed with methanol and stained with haematoxylin. Three independent invasion assays were performed in triplicate. Six random fields on average were counted using a light microscope.

MTT proliferation assay

The capability of cellular proliferation was measured by the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] MTT assay. Approximately, 5 × 10⁴ cells were seeded into 96-well culture plates, then cells were incubated with 20 μl MTT (10 mg/ml) for 4 hrs at 37°C and 200 μl Dimethyl sulfoxide (DMSO) was pipetted to solubilize the formazan product for 20 min. at room temperature. The optical density was determined using a spectrophotometer at a wavelength of 570 nm. The experiment was repeated three times in triplicate.

Luciferase assay

The full-length Prrx1 3’-UTR was amplified by PCR and cloned downstream of the firefly luciferase gene in the pGL3 vector (Promega, Madison, WI, USA). The vector was named wild-type (wt) 3’-UTR. The GeneTailor Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA, USA) was used to perform site-directed mutagenesis of the miR-655 binding site in Prrx1 3’-UTR: the resultant was named mutant (mt) 3’-UTR. These cells were transfected with reporter plasmids and placed in 96-well plates. After incubating for 48 hrs, the cells were harvested and assayed using the dual-luciferase reporter assay system (Promega, Beijing, China) according to the manufacturer’s instructions. Luciferase activities were normalized by β-galactosidase activity. Each experiment was performed in triplicate.

Wound healing assay

A wound healing assay was performed to examine cell migration. Briefly, after the cells grew to 90–95% confluence in six-well plates, a single scratch wound was generated with a 200 μl disposable pipette tip. The scratch wounds were photographed over a 24-hr period using a Nikon inverted microscope with an attached digital camera, and the widths of the wounds were quantified using Image software (Version 2.2.1, Nicon-BioImage Ltd, Japan).
Immunofluorescence microscopy

The cells were fixed with 4% paraformaldehyde, permeabilized in 0.5% Triton X-100 and then blocked with 10% goat serum. The cells were incubated for 1 hr with antibodies against vimentin and E-cadherin and then stained for 1 hr with a TRITC-conjugated secondary antibody (1:200). The cells were visualized by fluorescence microscopy.

Mouse xenograft model

The breast cancer model in nude mice was constructed as previously described [27]. A total of 2 × 10^6 MDA-MB-231 cells infected with miR-655 or scramble viruses were propagated and inoculated subcutaneously into the dorsal flanks of nude mice. The tumour size was measured every 4 days. After 28 days, the mice were killed, necropsies were performed, and the tumours were weighed. The tumour volumes were determined according to the following formula: \( V = \frac{1}{2} \times \text{length} \times \text{width}^2 \), where \( A \) is the largest diameter and \( B \) is the diameter perpendicular to \( A \). To assay the effect of miR-655 on tumour metastasis, 1 × 10^6 MDA-MB-231 cells infected with miR-655 or scramble viruses were injected into the tail vein of nude mice. After 60 days, necropsies were performed. The numbers of micrometastases in the lung per haematoxylin and eosin-stained section from individual mice were analysed by morphological observation. All of the animal procedures were performed in accordance with institutional guidelines.

Statistical analysis

SPSS V18.0 (SPSS inc, Chicago, IL, USA) was used for statistical analysis. Statistical analysis was carried out using the Student’s t-test for comparisons of two groups, and data with three groups were analysed using a one-way ANOVA. The F-test in one-way ANOVA is used to assess whether the expected values of a quantitative variable within several pre-defined groups differ from each other. Spearman’s correlation was used to identify the correlation between the expression of miR-655 and Prx1. All error bars represent the S.E. of three experiments. Differences were considered statistically significant when the \( P \)-value was <0.05.

Results

Down-regulation of miR-655 in TNBC and EMT model

We detected miR-655 in 63 pairs of TNBC tissues and their matched adjacent tissues and in 72 pairs of non-triple-negative breast cancer (NTNBC) tissues and their matched adjacent tissues. Among the 63 TNBC patients, approximately 87.3% (55/63) of the tumours revealed notable reductions in the miR-655 levels (\( P = 0.001 \)) (Fig. 1A). However, the miR-655 level was only slightly reduced in approximately 58.9% (43/73) of NTNBC tumours (Fig. 1B). In addition, the miR-655 level was notably reduced in TNBC compared with NTNBC (Fig. 1C). Clinicopathological analysis further revealed that the down-regulation of miR-655 was significantly associated with the molecular-based classification and lymph node metastasis (Table 1). We then detected miR-655 expression in different subtypes of mammary cell lines by qRT-PCR. We found that miR-655 is highly expressed in normal mammary cell lines and is expressed at low levels in some tumour cell lines, particularly those classified as basal-like (Fig. 1D). These results indicated that miR-655 had lower expression in basal-like cell lines compared to luminal cell lines. The data also indicated that human breast cancer cell lines, MDA-MB-231, of epithelial cell characteristics were induced to undergo EMT by transforming growth factor (TGF)-β1. Following TGF-β1 treatment, cells showed dramatic morphological changes assessed by phase contrast microscopy, accompanied by decreased epithelial marker and increased mesenchymal markers (Fig. 1E and F). Moreover, we found a correlation between loss of miR-655 expression and cellular differentiation in the EMT model. These findings led us to hypothesize that miR-655 overexpression may inhibit EMT and its associated traits (Fig. 1G).

miR-655 inhibited the EMT process of TNBC

To explore the relationship between miR-655 and the biological behaviour of breast cancer cells, we up-regulated miR-655 by using gene transfection technology in the MDA-MB-231 cell line which has low endogenous miR-655 expression (Fig. 2A). As shown in Figure 2B, cells overexpressed miR-655 exhibited epithelial morphology. To further investigate the effects of miR-655 up-regulation on the EMT phenotype in breast cancer cells, we measured the relative protein expression of EMT markers by Western blot analysis. Up-regulation of miR-655 expression in MDA-MB-231 cells led to a significant increase in cytokeratin expression and decreased expression of α-SMA and vimentin in the cells (Fig. 2D). Moreover, we used immunofluorescence microscopy to confirm those protein data (Fig. 2C). These findings strongly indicate that the up-regulation of miR-655 inhibits the EMT phenotype in TNBC.

Ectopic expression of miR-655 suppressed proliferation, migration and invasion ability of MDA-MB-231 in vitro

To determine whether miR-655 functionally behaved as a tumour suppressor, we next examined whether the up-regulation of miR-655 could inhibit cell migration using an in vitro transwell chamber assays. We demonstrated that the up-regulation of miR-655 expression inhibited the invasiveness of breast cancer cells compared with control cells, as indicated by a marked decrease in the number of cells that invaded the bottom well (Fig. 3A). To further determine whether miR-655 affected the invasive ability of breast cancer cells, we performed cell invasion assays using a wound healing assay. One day after the breast cancer cells transfected with the negative controls or miR-655 mimics, a single scratch wound was created in the well, and the wound closure was monitored over time (Fig. 3C). We also found that overexpression of miR-655 significantly suppressed the proliferation of breast cancer cells (Fig. 3B). These results suggest
that the up-regulation of miR-655 expression decreased proliferation, migration and invasion ability of MDA-MB-231.

**miR-655 suppressed tumourigenesis and metastasis in a xenograft model**

To directly evaluate the role of miR-655 in tumour formation and growth in vivo, the xenograft model of human TNBC cells in nude mice was adopted. Briefly, MDA-MB-231 cells infected with miR-655 or scramble lentivirus were injected subcutaneously into each flank of nude mice. After the cells injected, the tumour volume was monitored every 4 days, and the growth curves of the tumours were plotted accordingly. Finally, all of the mice were killed to harvest the xenograft. It is obvious that the mean volume and weight of the tumours generated from the miR-655 overexpression group was significantly lower compared with the control group (Fig. 4A-C). We then studied the effect of miR-655 on tumour metastasis in vivo. MDA-MB-231 cells infected with miR-655 or scramble lentivirus were transplanted into the nude mice via tail vein injection. After 60 days, the mice were anaesthetized, and their lungs were dissected. As shown in Figure 4E, a significantly lower number of macroscopic lung metastases could be observed in cells infected with miR-655 lentivirus. These results indicate that miR-655 may repress TNBC proliferation and metastasis in vivo.

**miR-655 was negatively correlated with Prx1 in cell lines and clinical samples and can function as a tumour suppressor**

To further validate that miR-655 suppressed invasion and metastasis by targeting Prx1, the expression levels of miR-655 and Prx1 were detected in a variety of breast cancer cell lines and clinical samples. As shown in Figure 5A, higher Prx1 expression was
detected in the breast cancer tissues, whereas lower Prrx1 expressions was detected in the adjacent normal tissues. We also found that the relative expression level of Prrx1 was plotted against that of miR-655 in each sample. Lower miR-655 expression was observed in TNBC tissues with higher immunostaining of Prrx1 (Fig. 5C), and a moderate negative correlation of Prrx1 with miR-655 was observed (Fig. 5D and E).

### Table 1 Characteristic and miR-655 expression in total breast cancer

| Clinicopathological variables | Cases | miR-655 expression level | P-value |
|------------------------------|-------|--------------------------|---------|
|                              |       | No. of low expression    | No. of high expression |
| Age (years)                  |       |                          |                     |
| ≤45                          | 65    | 47                       | 18                  | 0.943   |
| >45                          | 70    | 51                       | 19                  |
| Tumour size (cm)             |       |                          |                     |
| ≤2                           | 40    | 26                       | 14                  | 0.072   |
| 2–5                          | 63    | 45                       | 18                  |
| >5                           | 32    | 27                       | 5                   |
| Histological grade           |       |                          |                     |
| I                            | 39    | 29                       | 10                  | 0.997   |
| II                           | 46    | 32                       | 14                  |
| III                          | 50    | 37                       | 13                  |
| Lymph node status            |       |                          |                     |
| 0                            | 68    | 44                       | 24                  | 0.021*  |
| 1–3                          | 42    | 32                       | 10                  |
| >3                           | 25    | 22                       | 3                   |
| TNM stage                    |       |                          |                     |
| I                            | 45    | 32                       | 13                  | 0.583   |
| II                           | 67    | 48                       | 19                  |
| III                          | 23    | 18                       | 5                   |
| Molecular-based classification|       |                          |                     |
| TNBC                         | 63    | 55                       | 8                   | 0.000*  |
| Non-TNBC                     | 72    | 43                       | 29                  |

*P < 0.05.

Indeed, there was perfect base pairing between the seed sequence of mature miR-655 and the 3’-UTR of Prrx1 mRNA, and these seed sequences were conserved across species (Fig. 6A). To determine whether the 3’-UTR of Prrx1 mRNA is a functional target of miR-655 in breast cancer cells, the target sequence of Prrx1 3’-UTR (wt 3’-UTR) or the mutant sequence (mt 3’-UTR) were cloned into a luciferase reporter vector (Fig. 6B). Thereafter, HEK293 cells were transfected with wt or mt 3’-UTR vector and miR-655 mimics. The results showed a significant decrease in luciferase activity compared with control. As shown in Figure 5C and E, the ectopic overexpression of miR-655 led to the inhibition of Prrx1 expression. By contrast, miR-655 inhibitor dose-dependently restored Prrx1 expression (Fig. 6D).

**miR-655 directly targeted the Prrx1 3’-UTR**

We performed a bioinformatics analysis using TargetScan and PicTar and predicted that miR-655 may target Prrx1 3’-UTR region.
Exogenous Prrx1 overturned the inhibitory effects of miR-655 on breast cancer cells in vitro and in vivo

We wondered whether miR-655 brought about this effect by down-regulating Prrx1. Thereafter, we transfected pcDNA3.1-Prrx1 into miR-655-overexpressed breast cancer cell lines MDA-MB-231. Western blot analysis showed that Prrx1 could reverse the expression of EMT-related genes caused by overexpression of miR-655 (Fig. 7A). We then attempted to test whether restoration of Prrx1 could reverse the miR-655-mediated inhibition of invasion ability of breast cancer cells. As shown in Figure 7B, overexpression of Prrx1 with a cDNA without 3'UTR could partially abrogate the miR-655-mediated suppression of the migration and invasion of breast cancer cells. Furthermore, miR-655-Prrx1 cotransfected MDA-MB-231 cells were injected subcutaneously into nude mice. We found that the re-expression of Prrx1 rescued the growth defects of miR-655 (Fig. 7C-E). We then studied the effect of Prrx1 on tumour metastasis in vivo. miR-655-Prrx1 cotransfected MDA-MB-231 cells were transplanted into the nude mice via tail vein injection. After 60 days, the mice were anaesthetised, and their lungs were dissected. As shown in Figure 7F, a significantly higher number of macroscopic lung metastases could be observed in cells re-expression of Prrx1.

Discussion

Epithelial-to-mesenchymal transition plays a crucial role in many stages not only in embryonic development but also in cancer progression [28]. Cancer cells undergoing EMT are endowed with more aggressive phenotypes, such as mesenchymal and stem cell-like features, resulting in the acquisition of malignant properties, such as invasion, metastasis, recurrence and drug resistance [9, 29]. The evidence for EMT, including our own [6], led us to consider that the development of EMT inhibitors might provide opportunities for both prevention and treatment of cancer. It has known that miR-655 identified as a novel EMT-suppressive miRNA [22]. In addition, miR-655 is markedly down-regulated in oesophageal squamous cell carcinoma and tissues and that it inhibited the proliferation and invasion of oesophageal squamous cells [23]. In the current study, we showed that the miR-655 levels were down-regulated in breast cancer cells, particularly in TNBC cells, and the miR-655 levels in TNBC tissues were significantly lower than those in NNBC as determined by qRT-PCR. Moreover, the miR-655 levels were associated with lymph node metastasis and molecular-based classification. Furthermore, we found a correlation between loss of miR-655 expression and cellular differentiation in the EMT model. These findings led us to hypothesize that miR-655 overexpression may inhibit EMT and its associated traits of TNBC.

Emerging evidence suggests that the EMT play important roles in tumour metastasis and the recurrence. Understanding molecular mechanisms that regulate EMT process is crucial for improving treatment of breast carcinoma. This study showed that the ectopic expression of miR-655 in TNBC cells impaired invasion, proliferation and growth. Meanwhile, high expression of miR-655 resulted in increased cytokeratin expression and decreased vimentin expression. Moreover, in vivo data demonstrated that miR-655 inhibited tumour growth and metastasis. These in vitro and in vivo data further indicated that miR-655 inhibited cell migration, invasion and the EMT phenotype in TNBC. miR-655 play important roles in TNBC; however, the mechanisms of miR-655 on the EMT and their therapeutic potential remains largely unknown. We performed a bioinformatics analysis using
TargetScan and Pictar and predicted that miR-655 may target Prrx1 3'-UTR region. Prrx1 functions as a transcription coactivator, enhancing the DNA-binding activity of serum response factor [30]. Prrx1 regulates the differentiation of mesenchymal precursors and plays a central role in pancreatic regeneration and carcinogenesis [31]. More importantly, Prrx1 promotes metastasis through the
Fig. 5 miR-655 was negatively correlated with Prrx1 in cell lines and TNBC samples. (A) Immunohistochemistry results of Prrx1 expression in paired breast cancer tissue sample (40×). (B) RT-PCR analysis demonstrated the Prrx1 expression in TNBC tissues and matched distal normal tissues. (C) miR-655 was down-regulated in breast cancer tissues. (D) Diagram represents the relative miR-655 and Prrx1 mRNA expression levels in breast cancer cells. (E) In TNBC tissues, lower miR-655 expression was accompanied by higher immunostaining of Prrx1.

Fig. 6 Oncogene Prrx1 was specifically targeted by miR-655. (A) The predicted binding sequences for miR-655 within the human Prrx1 3′-UTR. Seed sequences were highlighted and underlined. (B) Luciferase activity assays using a luciferase reporter with wild-type or mutant human Prrx1 3′-UTR were performed after co-transfection of miR-655 mimics or control into HEK293 cells. And mt 3′-UTR had a significantly increase compared with wt 3′-UTR. (C and E) Prrx1 expression was determined in breast cancer cells stably overexpressed miR-655 and cells transfected with miR-655 inhibitors, or anti-miR-control Western blot analysis. (D) Different concentrations of miR-655 inhibitor transfection gradually increased Prrx1 expression in MDA-MB-231 cells.
induction of the EMT in hepatocellular carcinoma and breast cancer [32]. Here, Prrx1 was identified as an important downstream target of miR-655. miR-655 directly bound to the 3'-UTR of Prrx1, which contained a miR-655-binding site using a dual-luciferase reporter assay. Up-regulation of miR-655 significantly reduced the Prrx1 protein level in MDA-MB-231, and the inhibitory effects of miR-655 overexpression on breast cancer cell proliferation and invasion were reversed by up-regulation of Prrx1. Together, these data suggest that miR-655 might inhibit MDA-MB-231 proliferation and metastasis through regulating Prrx1.

In conclusion, the present study demonstrated that miR-655 levels were down-regulated in TNBC tissues and cell lines. Overexpression of miR-655 suppressed the cell proliferation, invasion and EMT of MDA-MB-231 through targeting Prrx1. To the best of our knowledge, this is the first study to demonstrate that the miR-655/Prrx1 axis regulates the proliferation, invasion and EMT of breast cancer cells. Our results suggest the potential of the EMT-suppressor miR-655 targeting Prrx1 as a prognostic marker and therapeutic agent for breast cancer.

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Conflicts of interest

The authors declare that they have no competing interests exist.

Author contribution

ZDL, BK and XPL participated in and performed the in vivo assays. ZDL, LYJ and QD performed the western blot assays and qRT-PCR assays. LYJ and FNL performed the pathological assessment of tumours. ZDL and HBW conceived the study and participated in its design and co-ordination, ZDL, FNL and HBW drafted the manuscript. All of the authors read and approved the final manuscript.
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