MiR-218-5p promotes breast cancer progression via LRIG1

Mingping Qian1,2, Hui Xu3, Hongming Song1, Hao Xi4, Lin Fang1*

1 Department of Thyroid and Breast Surgery, Shanghai Tenth People's Hospital, School of Medicine, Tongji University, Shanghai 200072, P.R. China
2 Department of General surgery, Yinshan Lake Hospital of Suzhou Wuzhong District, Jiangsu, 320506, China
3 Department of Thyroid and Breast Surgery, Affiliated hospital of Yangzhou University, Yangzhou, 225009, P.R. China
4 Department of Pathology, Shanghai Tenth People's Hospital, School of Medicine, Tongji University, Shanghai 200072, P.R. China

*To whom correspondence should be addressed. E-mail: fanglin2017@126.com
Abstract

Background: MiR-218-5p is a small non-coding RNA acting as either oncogenes or tumor suppressor genes in human cancer. The expression levels of some miRNAs in human breast cancer plays a potential role in disease pathogenesis.

Methods: Thirty pairs of invasive ductal carcinoma and adjacent specimens were included in the study. Breast tissues cell lines MCF-7 and MDA-MB-231 were identified as a breast cancer research cell line. MiR-218-5p mimics, miR-218-5p inhibitor, or negative controls were transfected. Specific antibodies were probed with LRIG1, ErbB2, and EGFR. Proliferation, migration, cell cycle and apoptosis, dual-luciferase reporter assay and immunohistochemistry were used to analyze miR-218-5p, LRIG1 and so on.

Results: It was shown that miR-218-5p expression was higher in 30 breast cancer specimens than adjacent normal breast tissues. In human breast cancer cells MCF-7 and MDA-MB-231, restoring miR-218-5p promoted cell proliferation and migration and inhibited cell apoptosis and cell cycle arrest in the G1 stage. Luciferase assays indicated miR-218-5p could bind with its putative target site in the 3'-untranslated region (3'-UTR) of LRIG1. RT-qPCR, western blot, and immunocytochemistry analyses all indicated miR-218-5p overexpression results in LRIG1 downregulation at the mRNA and protein levels. ErbB2 and EGFR were found to be downstream effectors of miR-218-5p.

Conclusion: MiR-218-5p promotes ErbB2 and EGFR expression by inhibiting LRIG1 in breast cancer cells, which suggests miR-218-5p and LRIG1 may act as an oncogene in breast cancer and it could be used as a therapeutic target for breast cancer treatments.

Keywords: Breast cancer; miR-218-5p; LRIG1; Oncogene
Introduction

MicroRNAs (MiRNAs) are a class of 19-24-nucleotide-long non-coding RNAs that repress gene expression in one of two manners: by inhibiting mRNA translation or promoting mRNA degradation. During the last few years, increasing evidence has indicated miRNAs are involved in a wide range of biological processes, including cell proliferation, apoptosis, and migration \[1-3\]. Regarding cancers, miRNAs have also been found to play important tumor suppressor or oncogene roles according to their expression levels and the involved downstream targets \[4-6\]. Recently, miR-218-5p was reported to act as a tumor suppressor in many human cancers, such as hepatocellular carcinoma, renal cell carcinoma, and gastric, oral, and bladder cancers \[7-11\]. However, the precise molecular mechanism through which miR-218-5p influences breast cancer progression remains largely unknown, indicating further investigation is required.

LRIG1, a member of the LRIG family of transmembrane leucine-rich repeat proteins, is a negative regulator of several oncogenic receptor tyrosine kinases, including all members of the ErbB family \[12-14\] as well as the Met \[15\] and Ret receptors \[16\]. LRIG1 is broadly expressed in healthy tissue \[17\], but its expression decreases in cancers such as renal cell carcinoma \[18\], cervical cancer \[19\], and breast cancer \[14\]. Relieving LRIG1-mediated negative regulation in LRIG1 knock-out mice results in ErbB \[20, 21\] and Met \[21\] receptor up-regulation in the intestinal epithelium, underscoring the physiological significance of the receptor negative regulation performed by LRIG1 \[21\].

This study first demonstrated that miR-218-5p expression was significantly elevated in breast cancer specimens relative to normal tissues. This overexpression promoted MCF-7 and MDA-MB-231 breast cancer cell proliferation and migration, as well as inhibiting cell apoptosis and disrupting the cell cycle by targeting LRIG1. These results indicated that miR-218-5p functions as a tumor promoter gene whose dysregulation may be involved in the development of human breast cancer.
**Materials and Methods**

**Human breast cancer specimens**

30 paired breast cancer specimens and adjacent normal breast tissues were collected from the Department of General Surgery of the Shanghai Tenth People's Hospital (Shanghai, China). One of these samples was immediately snap-frozen in liquid nitrogen. Other tissues were formalin-fixed and paraffin-embedded. All samples were confirmed as invasive ductal breast cancer by trained pathologists. No patients had received chemotherapy or radiotherapy prior to surgery. The investigation was approved by the ethics committee at Shanghai Tenth People's Hospital, and informed consent for the use of the postsurgery samples was obtained from the donors who were patients with breast invasive ductal carcinoma.

**Cell lines and transfection**

Human breast cancer cell lines MDA-MB-231, MDA-MB-468, HCC1937 and MCF-7 were acquired from the American Type Culture Collection (ATCC), and MCF-10A cells were purchased from the Chinese Academy of Science at Shanghai (Shanghai, China). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Enpromise, China). Cells were incubated at 37 °C in a humidified chamber with 5% supplemental CO₂.

For transfections, 2×10⁵ cells were added to each well of a 6-well plate and cultured with DMEM medium without either serum or antibiotics. When MCF-7 and MDA-MB-231 breast cancer cells reached 30–40% density, miR-218-5p mimics/negative control (NC), miR-218-5p inhibitor/inhibitor negative control (GenePharma Co., Ltd., Shanghai, China), and Lipofectamine transfection reagent (Invitrogen, USA) were each diluted in 500 μl DMEM medium at a ratio of 1 μg : 3 μl and incubated for 5 min at room temperature (RT). The two mixtures were then gently combined and incubated for a further 20–30 min at RT. Subsequently, 1,000 μl of the complexes were added to each well. After 5–6 h of incubation, the DMEM medium was replaced by DMEM with 10% FBS. Cells were incubated at 37 °C in a CO₂ incubator for 48 h prior to further testing.

**RNA extraction and RT-qPCR assay**

Breast cancer specimens and adjacent normal breast tissues were prepared for miRNA
extraction using a miRNA rapid extraction kit (Tiangen, Beijing, China). Total RNA was isolated from harvested cells of the selected cell lines using Trizol reagent (Invitrogen, USA). Reverse transcription PCR and quantitative PCR (RT-qPCR) kits (TaKaRa, Japan) were used according to the manufacturer's instructions to detect the relative quantity of RNA. GAPDH was used as an endogenous control.

**Western-blot assay**

Whole cell proteins were extracted using a protein lysis buffer (Sigma-Aldrich, USA) and quantified via bicinchoninic acid assay (Pierce, USA). Protein samples were then electrophoresed in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (PVDF, EMD Millipore, MA, USA), which was probed with LRIG1-, ErbB2-, and EGFR-specific antibodies. Blots were subsequently detected and visualized using an enhanced chemiluminescence detection kit (Millipore, Billerica, MA, USA) according to protocols provided by the manufacturer. A Bio-RAD scanning system was used to detect immunoreactive protein bands.

**Proliferation assay**

Cell proliferation assays were monitored using cell-counting kit 8 (Invitrogen, Shanghai, China) according to the instructions provided by the manufacturer. Approximately 4–5 h after miR-218-5p mimics, miR-218-5p inhibitor, or negative controls were transfected, cells from each condition were plated (3,000/well) onto 96-well plates (BD Biosciences, USA) and incubated at 37 °C in a humidified chamber with 5% supplemental CO₂. Cell proliferation was assessed at 0, 24, 48, and 72 h. The optical density (OD) of each well was measured using a microplate spectrophotometer at 450 nm. All experiments were performed in biological triplicate.

**Migration assay**

Cell migration assays were conducted in transwell chambers with polycarbonate membrane inserts (Corning, NY, USA). Cells (6×10⁴ per well) were suspended in 180 μl of serum-free DMEM, and 600 μl of the same medium (containing 10% FBS) were placed in the lower chambers to stimulate cell migration. After 16 h incubation, cells adhering to the transwells were
fixed with 3% paraformaldehyde (30 min), then stained with 0.1% crystal violet (15 min). Cells in the upper chambers were wiped with a cotton ball, leaving migrating cells adhered to the bases of the chambers. Five random fields were picked at 200× by the camera of an inverted microscope (Thermo Fisher Scientific).

**Cell cycle and apoptosis assay**

Thirty-six hours after miR-218-5p mimic, miR-218-5p inhibitor, or negative control transfection, cells were trypsinized and centrifuged at 1,000 rpm for 5 min, then washed twice in cold PBS. Subsequently, 3 ml ice-cold ethanol was added in a dropwise fashion and cells were allowed to affix for ≥30 min. A total of 250 μl 0.05 g/l propidium iodide (PI) staining solution was added into each sample, after which samples were incubated for 30 min at RT. Cells were then analyzed using a flow cytometer (Beckman coulter, KBB, CA, USA).

Cell apoptosis was performed using an Annexin V-FITC apoptosis detection kit (BestBio, Shanghai, China) according to protocols provided by the manufacturer: cells were seeded onto 6-well plates at a 1.5×10^5 cells-per-well density, then, 48 h after transfection, cells were digested by 0.08% EDTA-free trypsin and washed twice with ice-cold PBS. Propidium iodide (PI) and Annexin V-fluoresceinisothiocyanate (FITC) stainings were applied to determined the percentage of cells undergoing apoptosis or necrocytosis. Cell apoptosis was measured via flow cytometry (Beckman coulter, KBB, CA, USA), and data were analyzed using FlowJo software.

**Dual-luciferase reporter assay**

LRIG1 3’-UTR fragments containing predicting miR-218-5p binding sites were amplified via PCR using PrimerSTAR Max DNA polymerase (Takara, Japan) and the following primers: 5’-GCGGAGCTCAACCAGAAGGCCAAGTC-3’ (FORWARD), 5’-GCGTCTAGAAAATGGACAAAGTGGGTGG-3’ (REVERSE), both designed and synthesized by Biosune (Jinan, China). The LRIG1 3’-UTR were then inserted at the XbaI and SacI sites of the pmirGLO target expression vector (Promega, San Luis Obispo, CA, USA.). Breast cancer cell MCF-7 were seeded on 24-well plates at a density of 5×10^4 per well 12 h before transfection. The cells were then transfected with 1 μg LRIG1 3’UTR fragment reporter vector containing the predicted miR-218-5p binging sites, which was co-transfected with 30nM miR-218-5p mimics or negative controls (NC)
using the Tuberfect transfection reagent (Roche, USA). After 48 h incubation, cells were lysed by PLB and placed on an orbital shaker (QILINBEIER, Jiangsu, China) at 100 rpm for 30 min before being placed in a -80 °C refrigerator overnight. Renilla luciferase and firefly luciferase signals were measured using a dual-luciferase reporter assay kit (Promega, Madison, WI, USA) according to instructions provided by the manufacturer.

**Immunohistochemistry**

Paraffin-embedded blocks were cut into 4 μm thick sections and subjected to deparaffinized re-hydration, then immersed in twice-distilled water with hydrogen peroxidise (3%) to reduce endogenous oxidise activity. Tissue immunohistochemistry samples were then incubated with primary antibodies at 4 °C for 1 h, and, subsequently, a secondary antibody was applied to the cells at RT for 30 min. The degree of staining was developed by diaminobenzidine (DAB) chromogen (Dako, Inc, Carpinteria, CA, US). Subsequently, the tissues were dehydrated and sealed with gum. According to the modified guideline recommendations for LRIG1 Testing in breast cancer, immunoreactivity was graded by scoring the percentage of positive membrane staining: negative (0, 1+), equivocal (2+), and positive (3+), as previously reported.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). Comparisons between two groups were subject to t-testing, whereas those between multiple groups underwent an analysis of variance (ANOVA). All experiments were performed in triple replicate, and the data below are presented in the mean±standard deviation (S.D) format. A p-value < 0.05 was considered statistically significant. According to the modified guideline recommendations for LRIG1 testing in breast cancer, immunoreactivity was graded by scoring the percentage of positive membrane staining:
Results

miR-218-5p expression increased in human breast cancer tissues and cell lines

To investigate the expression level of miR-218-5p in breast cancer, miR-218-5p expression was investigated in 30 pairs of breast cancer and normal adjacent tissues using RT-qPCR. As depicted in Fig. 1A, the $2^{-\Delta\Delta C_t}$ value of miR-218-5p was significantly increased in breast cancer tissues relative to that of normal adjacent tissues ($p<0.05$). This finding was consistent with an in vitro expression analysis comparing miR-218-5p in MDA-MB-231, MDA-MB-468, HCC1937, and MCF-7 cells to MCF-10A, an immortal mammary epithelial cell line. It was found that the miR-218-5p expression was higher in MCF-7 and MDA-MB-231 cells, above that of MDA-MB-468 and MCF-10A cells (Figure 1B). Therefore, MCF-7 and MDA-MB-231 breast cancer cells were selected as the focus of this study.

miR-218-5p promotes breast cancer cell proliferation and migration

To further explore the possibility miR-218-5p affected breast cancer cell tumorigenesis, cell proliferation (Cell Counting Kit 8, CCK8) and migration (transwell) assays were performed on MCF-7 and MDA-MB-231 cells. The CCK8 assays revealed that cell proliferation abilities were significantly inhibited following an overexpression of miR-218-5p in the MCF-7 and MDA-MB-231 cells (Figure 2A), whereas the knockdown of miR-218-5p in MCF-7 and MDA-MB-231 cells promoted cell growth rates (Figure 2B). Furthermore, a transwell assay revealed the migratory capabilities of MCF-7 and MDA-MB-231 cells were greatly decreased when they transfected with the miR-218-5p mimic and increased when transfected with the antisense miR-218-5p (Figures 2C–2F). Overall, these results suggest miR-218-5p exerts proliferative and migratory effects on breast cancer cells and therefore may act as a tumor promoter in breast cancer.

miR-218-5p disrupts the cell-cycle progression of breast cancer cells in different phases

As cell growth inhibition can result from cell cycle arrest, the possibility that miR-218-5p affects cell-cycle progression was examined. First, miR-218-5p mimics and inhibitor were transfected into MCF-7 and MDA-MB-231 cell respectively. These cells were then analyzed via flow cytometry. Results showed that miR-218-5p mimic transfection arrested significantly more
MCF-7 and MDA-MB-231 cells in the G2/M-phase than transfection with NC did (Figures 3A to 3D). Furthermore, transfecting MCF-7 cells with miR-218-5p mimics resulted in a significant increase in the percentage of S-phase cells relative to NC. However, miR-218-5p inhibitor transfection arrested less MCF-7 and MDA-MB-231 cells at G2/M-phase than NC transfection (Figures 3A to 3D). These findings suggest miR-218-5p can initiate S phase arrest, and upregulating miR-218-5p expression could lead to an increase in S- and G2/M-phase cells.

miR-218-5p inhibits apoptosis in breast cancer cells

A flow cytometry assay was performed to further detect the effect of miR-218-5p on breast cancer cell apoptosis. Results demonstrated that the group transfected with miR-218-5p mimics had a significantly lower proportion of late apoptotic cells than the NC group in both MCF-7 and MDA-MB-231 cells (Figures 4A to 4D), whereas miR-218-5p inhibitor increased both of early and late apoptosis rates in transfected cells (Figures 4A to 4D). Taken together, these data indicate that miR-218-5p could inhibit breast cancer cell apoptosis.

miR-218-5p directly targeted LRIG1 to inhibit its expression

To further explore the role of LRIG1 in miR-218-5p-induced cellular apoptosis sensitization, the effects of miR-218-5p on LRIG1 expression were examined. Two putative miR-218-5p target sites were identified in LRIG1 3'-UTR using TargetScan Release 6.2 software (Figure 5A), indicating LRIG1 was a potential miR-218-5p target. The target sites in the Homo sapiens LRIG1 3'-UTR are shown in Figure 5B. To determine whether LRIG1 was a direct target of miR-218-5p, reporter vectors containing either the wild-type full-length 3'-UTR (WT-UTR) or mutant miR-218-5p binding sites were constructed (Figure 5C). miR-218-5p reduced LRIG1 WT-UTR luciferase plasmid activity by up to 60%, but had no effect on LRIG1 mut-UTR luciferase plasmid activity (Figure 5D). Combined, these results indicate miR-218-5p inhibited LRIG1 expression by directly targeting LRIG1.

To confirm miR-218-5p directly targets LRIG1, miR-218-5p mimics, miR-218-5p inhibitor, and the corresponding NC were transfected into MCF-7 cells. miR-218-5p transfection decreased LRIG1 mRNA levels, whereas miR-218-5p inhibitor reversed this inhibition effect (Figures 5E and 5F). These results confirmed that miR-218-5p inhibited LRIG1 expression.
ERBB2, EGFR in LRIG1-mediated signaling pathway were downstream effectors of miR-218-5p

ERBB2 and EGFR have been reported as primary downstream messengers in LRIG1 signaling. To elucidate whether miR-218-5p could regulate ERBB2 and EGFR via LRIG1 inhibition, the protein levels of LRIG1, ERBB2, and EGFR in MCF-7 cells (transfected with quantified miR-218-5p mimics or miR-218-5p inhibitor and the corresponding NC) via western blot. Results demonstrated that miR-218-5p overexpression could suppress the mRNA expression of LRIG1 (Figures 6A and 6B). Furthermore, miR-218-5p was proven to increase the protein expression of ERBB2 and EGFR (Figures 6A, 6C and 6D). Western blot and immunocytochemistry assay results showed that miR-218-5p significantly reduced the protein expression of LRIG1 (Figure 6E and 6F). As LRIG1 is a direct target gene of miR-218-5p, all data demonstrated that ERBB2 and EGFR were downstream effectors of miR-218-5p, at least in part induced by LRIG1 targeting (Figure 6G).
Discussion

Recently, miRNAs have been revealed to play an important role in the genesis and progression of human cancers has emerged. Many researchers have reported the extensive alteration of miRNA expression in the initial and developmental stages of human cancers, as well as the effects of miRNAs in tumor suppression and promotion \[22-24\]. The importance of miRNA function and dysfunction in various human cancers suggests that modulating miRNA expression may serve as a novel therapeutic modality for such diseases. To date, three main approaches have been used in potential miRNA-targeting therapy: expression vectors (miRNA sponges), small-molecule inhibitors, and antisense oligonucleotides. Chemically synthesized miRNAs and oligonucleotides that target miRNAs have already been proven to efficiently inhibit cancer development \[25, 26\]. At this time, several preclinical and clinical miRNA-targeting therapy trials which may pave the way for cancer therapy are in-progress \[27, 28\].

Several miRNAs are essential for tumor development in breast cancer, including miR-9, let-7, and miR-193a-3p \[29-31\]. This study examined the expression levels of miR-218-5p in human breast cancer and its potential role in disease pathogenesis. First, miR-218-5p expression levels in human breast cancer specimens were detected via RT-qPCR. These results showed miR-218-5p was significantly downregulated in breast cancer tissues relative to normal breast tissues. Similar findings have been reported in other cancer types \[32,33\], indicating decreases in miR-218-5p expression are common in human cancer specimens and cell lines. Next, miR-218-5p mimics were transfected into MCF-7 cells, simulating overexpression. This led to a significant inhibition of cellular proliferation, measured by CCK8, as well as a reduction in the colony number, determined by clone formation assay. Both experiments indicated miR-218-5p repressed the growth of breast cancer cells. Using a transwell migration assay, it was discovered that overexpressing miR-218-5p in breast cancer cells could suppress their migratory ability. miR-218-5p was definitively found to arrests cancer cells in the G1 phase relative to the cell cycle of NC groups. However, no significant differences in apoptosis were found between the miR-218-5p and NC groups in this study. The strong vitality of cancer cells was speculated to be one possible reason why miR-218-5p could not promote apoptosis.

To investigate the downstream targets of miR-218-5p that may play a role in mediating its cell function, putative targets were searched for in the miRanda, targetscan, and miRBase databases.
Through luciferase assays, LRIG1 was predicated as a direct target of miR-218-5p in MDA-MB-231 cells.

Additionally, both the mRNA and protein levels of LRIG1 were found to be significantly lower in miR-218-5p than in the NC groups. These findings support the assumption that LRIG1 is a downstream target of miR-218-5p.

According to reports, LRIG1 plays an important role in regulating cell surface levels of ErbB family RTKs \(^{[34]}\). In tamoxifen-treated luminal breast cancers, up-regulation of LRIG1 suppresses ErbB RTK family expression and signaling in luminal breast cancers, including EGFR, ErbB2, ErbB3 and ErbB4 \(^{[35]}\). Our study found that miR-218-5p could up-regulate the protein expression of ErbB2 and EGFR by targeting LRIG1, suggesting that miR-218-5p may promotes the biological function of breast cancer through LRIG1-mediated signaling pathway.

**Conclusions**

Collectively, these findings suggest that miR-218-5p can disrupt the cell cycle by targeting LRIG1 in MDA-MB-231 and MCF-7 cells. Its overexpression was shown to stimulate cell proliferation and migratory ability of cancer cells. As such, it may be concluded that miR-218-5p acts as a tumor promoter gene in breast cancer. Furthermore, luciferase, RT-qPCR, and western blot assays identified LRIG1 as a downstream target of miR-218-5p. Artificially decreasing miR-218-5p or upregulating LRIG1 as new therapeutic agents could thus offer a promising new direction in future breast cancer treatment.

**Abbreviations**

miRNA: micro ribonucleic acid; LRIG1: leucine-rich repeats and immunoglobulin-like domains1; RT: room temperature; CCK8: Cell Counting Kit 8; RT-qPCR: quantitative reverse transcription polymerase chain reaction; EGFR: epidermal growth factor receptor.
Ethical Approval and Consent to participate

The investigation was approved by the ethics committee at Shanghai Tenth People’s Hospital, School of Medicine, Tongji University, and informed consent for the use of the postsurgery samples obtained from the donors who were breast cancer patients.

Consent for publication

All patients have been informed that the individual data for publication will be freely available on the internet and may be seen by the general public. All patients consent the material to publish.

Availability of supporting data

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

The study has been supported by the National Natural Science Foundation of China (No. 82073204).

Authors' contributions

MQ designed and carried out the experiments, analyzed the data, and wrote the manuscript; HS performed cell culture, transfection, migration assays, flow cytometry, real-time PCR, Western blotting assays, and dual-luciferase reporter assays. HX collected the clinical samples and performed immunohistochemistry. LF provided overall supervision. The authors read and approved the final manuscript.

Acknowledgements

We thank all patients who contributed to this study. We thank all of the medical doctors and nurses from Department of thyroid and breast surgery of Shanghai Tenth People's Hospital in China for their contributions.

Author details

1 Department of Thyroid and Breast Surgery, Shanghai Tenth People's Hospital, School of Medicine, Tongji University, Shanghai 200072, P.R. China

2 Department of General surgery, Yinshan Lake Hospital of Suzhou Wuzhong District, Jiangsu ,320506, China
References

1. Mohr AM, Mott JL. Overview of microRNA biology. Semin Liver Dis. 2015;35(1):3-11.
2. Lu TX, Rothenberg ME. MicroRNA. J Allergy Clin Immunol. 2018;141(4):1202-7.
3. Hayes CN, Chayama K. MicroRNAs as Biomarkers for Liver Disease and Hepatocellular Carcinoma. Int J Mol Sci. 2016;17(3):280.
4. Detassis S, Grasso M, Del Vescovo V, Denti MA. microRNAs Make the Call in Cancer Personalized Medicine. Front Cell Dev Biol. 2017;5:86.
5. Kittelmann S, McGregor AP. Modulation and Evolution of Animal Development through microRNA Regulation of Gene Expression. Genes (Basel). 2019;10(4):321.
6. Chen S, Xue Y, Wu X, Le C, Bhutkar A, Bell EL, Zhang F, Langer R, Sharp PA. Global microRNA depletion suppresses tumor angiogenesis. Genes Dev. 2014;28(10):1054-67.
7. Li L, Yu H, Ren Q. MiR-218-5p Suppresses the Progression of Retinoblastoma Through Targeting NACC1 and Inhibiting the AKT/mTOR Signaling Pathway. Cancer Manag Res. 2020;12:6959-67.
8. Zhu H, Wang X, Chen S. Downregulation of MiR-218-5p Protects Against Oxygen-Glucose Deprivation/Reperfusion-Induced Injuries of PC12 Cells via Upregulating N-myc Downstream Regulated Gene 4 (NDRG4). Med Sci Monit. 2020;26:e920101.
9. Tang S, Wang D, Zhang Q, Li L. miR-218 suppresses gastric cancer cell proliferation and invasion via regulation of angiopoietin-2. Exp Ther Med. 2016;12(6):3837-42.
10. Wang Y, Jiang Y, Chen L. Role of miR-218-GREM1 axis in epithelial-mesenchymal transition of oral squamous cell carcinoma: An in vivo and vitro study based on microarray data. J Cell Mol Med. 2020 Oct 27;24(23):13824–36.
11. Marrone AK, Ho J. MicroRNAs: potential regulators of renal development genes that contribute to CAKUT. Pediatr Nephrol. 2014;29(4):565-74.
12. Rafidi H, Mercado F 3rd, Astudillo M, Fry WH, Saldana M, Carraway KL 3rd, Sweeney C. Leucine-rich repeat and immunoglobulin domain-containing protein-1 (Lrig1) negative regulatory action toward ErbB receptor tyrosine kinases is opposed by leucine-rich repeat and immunoglobulin domain-containing protein 3 (Lrig3). J Biol Chem. 2013;288(30):21593-605.

13. Oh YM, Lee SB, Choi J, Suh HY, Shim S, Song YJ, Kim B, Lee JM, Oh SJ, Jeong Y, Cheong KH, Song PH, Kim KA. USP8 modulates ubiquitination of LRIG1 for Met degradation. Sci Rep. 2014;4:4980.

14. Yu S, Yang M, Lim KM, Cho Y, Kim H, Lee K, Jeong SH, Coffey RJ, Goldenring JR, Nam KT. Expression of LRIG1, a Negative Regulator of EGFR, Is Dynamically Altered during Different Stages of Gastric Carcinogenesis. Am J Pathol. 2018;188(12):2912-23.

15. Neirinckx V, Hau AC, Schuster A, Fritah S, Tiemann K, Klein E, Nazarov PV, Matagne A, Szpakowska M, Meyrath M, Chevigné A, Schmidt MHH, Niclou SP. The soluble form of pan-RTK inhibitor and tumor suppressor LRIG1 mediates downregulation of AXL through direct protein-protein interaction in glioblastoma. Neurooncol Adv. 2019;1(1):vdz024.

16. Faraz M, Herdenberg C, Holmlund C, Henriksson R, Hedman H. A protein interaction network centered on leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1) regulates growth factor receptors. J Biol Chem. 2018;293(9):3421-35.

17. Simion C, Cedano-Prieto ME, Sweeney C. The LRIG family: enigmatic regulators of growth factor receptor signaling. Endocr Relat Cancer. 2014;21(6):R431-43.

18. Thomasson M, Hedman H, Ljungberg B, Henriksson R. Gene expression pattern of the epidermal growth factor receptor family and LRIG1 in renal cell carcinoma. BMC Res Notes. 2012;5:216.

19. Hyunji K, Sungsook Y, Yejin C, et al. Sa1613-Expression of Lrig1, a Negative Regulator of Egfr, is Dynamically Altered in Different Stages of Gastric Carcinogenesis. Gastroenterology. 2018;154(6):S-330-.

20. Wong VW, Stange DE, Page ME, Buczacki S, Wabik A, Itami S, van de Wetering M, Poulsom R, Wright NA, Trotter MW, Watt FM, Winton DJ, Clevers H, Jensen KB. Lrig1 controls intestinal stem-cell homeostasis by negative regulation of ErbB signalling. Nat Cell Biol. 2012;14(4):401-8.
21. Powell AE, Wang Y, Li Y, Poulin EJ, Means AL, Washington MK, Higginbotham JN, Juchheim A, Prasad N, Levy SE, Guo Y, Shyr Y, Aronow BJ, Haigis KM, Franklin JL, Coffey RJ. The pan-ErbB negative regulator Lrig1 is an intestinal stem cell marker that functions as a tumor suppressor. Cell. 2012;149(1):146-58.

22. Witwer KW, Halushka MK. Toward the promise of microRNAs - Enhancing reproducibility and rigor in microRNA research. RNA Biol. 2016 Nov;13(11):1103-16.

23. Oom AL, Humphries BA, Yang C. MicroRNAs: novel players in cancer diagnosis and therapies. Biomed Res Int. 2014;2014:959461.

24. Chen PS, Su JL, Hung MC. Dysregulation of microRNAs in cancer. J Biomed Sci. 2012;19(1):90.

25. Iorio MV, Croce CM. MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. EMBO Mol Med. 2012 Mar;4(3):143-59.

26. Kumar MS, Erkeland SJ, Pester RE, Chen CY, Ebert MS, Sharp PA, Jacks T. Suppression of non-small cell lung tumor development by the let-7 microRNA family. Proc Natl Acad Sci U S A. 2008;105(10):3903-8.

27. Gambari R, Brognara E, Spandidos DA, Fabbri E. Targeting oncomiRNAs and mimicking tumor suppressor miRNAs: New trends in the development of miRNA therapeutic strategies in oncology (Review). Int J Oncol. 2016;49(1):5-32.

28. Lindow M, Kauppinen S. Discovering the first microRNA-targeted drug. J Cell Biol. 2012;199(3):407-12.

29. Chen X, Zhu LJ, Ma Z, Sun G, Luo X, Li M, Zhai SL, Li P, Wang XR. Oncogenic miR-9 is a target of erlotinib in NSCLCs. Sci Rep. 2015; 5:17031.

30. Stahlhut C, Slack FJ. Combinatorial Action of MicroRNAs let-7 and miR-34 Effectively Synergizes with Erlotinib to Suppress Non-small Cell Lung Cancer Cell Proliferation. Cell Cycle. 2015;14(13):2171-80.

31. Xie ZC, Tang RX, Gao X, Xie QN, Lin JY, Chen G, Li ZY. A meta-analysis and bioinformatics exploration of the diagnostic value and molecular mechanism of miR-193a-5p in lung cancer. Oncol Lett. 2018;16(4):4114-28.

32. Sampath D, Liu C, Vasan K, Sulda M, Puduvalli VK, Wierda WG, Keating MJ. Histone deacetylases mediate the silencing of miR-15a, miR-16, and miR-29b in chronic lymphocytic
leukemia. Blood. 2012;119(5):1162-72.

33. Luu HN, Lin HY, Sørensen KD, Ogunwobi OO, Kumar N, Chornokur G, Phelan C, Jones D, Kidd L, Batra J, Yamoah K, Berglund A, Rounbehler RJ, Yang M, Lee SH, Kang N, Kim SJ, Park JY, Di Pietro G. miRNAs associated with prostate cancer risk and progression. BMC Urol. 2017;17(1):18.

34. Morrison MM, Williams MM, Vaught DB, Hicks D, Lim J, McKernan C, Aurisicchio L, Ciliberto G, Simion C, Sweeney C, Cook RS. Decreased LRIG1 in fulvestrant-treated luminal breast cancer cells permits ErbB3 upregulation and increased growth. Oncogene. 2016;35(9):1206.

35. Namjoshi P, Showalter L, Czerniecki BJ, Koski GK. T-helper 1-type cytokines induce apoptosis and loss of HER-family oncodriver expression in murine and human breast cancer cells. Oncotarget. 2019;10(57):6006-20.
Figure 1. Expression of miR-218-5p is upregulated in breast cancer tissues and cell lines. (A) miR-218-5p expression analyzed in breast cancer tissues and adjacent normal breast tissue via qRT-PCR. (B) miR-218-5p expression analyzed in non-malignant epithelial MCF-10A cells and four breast cancer cell lines, MCF-7, MDA-MB-231, MDA-MB-468, and HCC1937, by qRT-PCR. The average miR-218-5p expression was normalized using GAPDH expression. *P<0.05, **P<0.01.
Figure 2. miR-218-5p promotes breast cancer proliferation and migration in cell lines. The CCK8 assay was performed to monitor the proliferation level of MCF-7 (A) and MDA-MB-231 (B) breast cancer cells after transfection with blank, control (NC), miR-218-5p mimics or miR-218-5p inhibitor at the indicated concentrations. The optical density of each well was measured at the indicated time-points at 450 nm with a microplate spectrophotometer. Cell migration ability was analyzed via Transwell chamber assay 24 h after NC, miR-218-5p mimics or miR-218-5p inhibitor transfection. Representative images of crystal violet stained MCF-7 (C) and MDA-MB-231 (D) breast cancer cells. Quantification of MCF-7 (E) and MDA-MB-231 (F) breast cancer cells by solubilization of crystal violet. *P<0.05, **P<0.01. Bars represent means±SEM.
Figure 3. miR-218-5p promotes breast cancer cell cycle at S- and G2-stage. Cell cycle distribution was analyzed by flow cytometry 36 h after transfection with MCF-7 (A) and MDA-MB-231 (B) breast cancer cells with NC, miR-218-5p mimics, or miR-218-5p inhibitor. The respective proportion of the G1-, S-, and G2-phases of blank, NC, miR-218-5p mimics, or miR-218-5p inhibitor groups in MCF-7 (C) and MDA-MB-231 (D) breast cancer cells were then analyzed.

*P<0.05, **P<0.01.
Figure 4. miR-218-5p inhibits breast cancer cell apoptosis at both early and late stages. Apoptotic analyses of MCF-7 (A) and MDA-MB-231 (B) breast cancer cells transfected with NC, miR-218-5p mimics, or miR-218-5p inhibitor were performed. These cells were stained with PI and analyzed by flow cytometry. The respective proportion of early and later stage of blank, NC, miR-218-5p mimics, or miR-218-5p inhibitor groups in MCF-7 (C) and MDA-MB-231 (D) breast cancer cells were analyzed. Values are represented as mean±SEM (N=6). *P<0.05, **P<0.01.
Figure 5. miR-218-5p directly binds to LRIG1, inhibiting its expression. (A) and (B) putative miR-218-5p target sites were identified in the 3′-UTR of LRIG1 using TargetScan Release 6.2. A bioinformatics analysis revealed two miR-218-5p binding sites on LRIG1 3′-UTR. (C) luciferase reporter construct containing both LRIG1 3′-UTR and miR-218-5p binding sites on LRIG1 3′-UTR. (D) MCF-7 cells were transfected with LRIG1 WT-UTR (LRIG1 3′-UTR promoter) or LRIG1 mut-UTR (LRIG1 3′-UTR promoter with mutated miR-218-5p binding sites) together with increasing amounts of miR-218-5p mimics or NC. Luciferase activities were analyzed. Forty-eight hours after MCF-7 (E) and MDA-MB-231 (F) breast cancer cells were transfected with NC, miR-218-5p mimics, or miR-218-5p inhibitor. The expression of miR-218-5p and LRIG1 were then analyzed via qRT-PCR. GAPDH was used as a loading control. *P<0.05, **P<0.01.
Figure 6. miR-218-5p promotes ErbB2 and EGFR expression by inhibiting LRIG1 in breast cancer cells. Western blotting analyses were performed to evaluate the expression of the LRIG1, ErbB2, and EGFR protein in MCF-7 cells transfected with NC, miR-218-5p mimics, or miR-218-5p inhibitor. (A): representative images; (B)-(D): quantitative analysis. (E) Immunohistochemical analysis of LRIG1 expression in breast cancer patients. (F) LRIG1 expression was analyzed in breast cancer tissues and adjacent normal breast tissue via qRT-PCR. (G) Model of miR-218-5p function in breast cancer cells. *P<0.05, **P<0.01.