

Function of miR-152 as a Tumor Suppressor in Human Breast Cancer by Targeting PIK3CA

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miR-152, as a tumor suppressor, has been reported to be downregulated in a number of cancer cell lines and tumor tissues, including breast cancer. This study aimed to investigate the role of miR-152 in human breast cancer and its underlying mechanisms. Human breast cancer cell line HCC1806 was transfected with hsa-miR-152-3p mimic, inhibitor, or scrambled negative controls. The efficiency of miR-152-3p transfection was evaluated by quantitative real-time PCR, and the effects on cell viability and apoptosis as well as on the PI3K/AKT signaling pathway were investigated by MTT assay, flow cytometry, and Western blot analysis, respectively. The binding effect of miR-152-3p on PIK3CA 3'-UTR was also investigated. The results suggested that miR-152-3p mimic transfection inhibited cell viability while inducing apoptosis of HCC1806 cells. Furthermore, miR-152-3p negatively regulated PIK3CA expression via binding to the 3'-UTR of PIK3CA and decreased the phosphorylation levels of AKT (Ser473) and RPS6 (Ser235/236) in HCC1806 cells. miR-152-3p inhibitor transfection showed the opposite effects. In conclusion, miR-152-3p might serve as a tumor suppressor in human breast cancer cells via negatively regulating PIK3CA expression to inhibit the activation of AKT and RPS6, leading to suppression of HCC1806 cell proliferation.

Key words: miR-152; Tumor suppressor; Breast cancer; PIK3CA; Proliferation; Apoptosis

INTRODUCTION

According to recently reported statistics, breast cancer has become the second most common cancer worldwide, accounting for 11.9% of all cancers, excluding nonmelanoma skin cancers. The incidence of breast cancer is increasing in many regions, as well as in China, and it has been reported to exceed 200,000 by 2015. It was also predicted to reach 230,000 by 2025¹. Despite advances in the prevention and detection of and adjuvant therapy for breast cancer, a substantial number of patients are diagnosed with metastatic breast cancer, with 5% at initial presentation and 30% during the course of treatment². Therefore, a comprehensive understanding of the mechanisms of pathogenesis and metastasis in breast cancer is required to develop more effective treatments.

MicroRNAs (miRNAs) are short (19–25 nt), endogenous, single-stranded, and noncoding RNAs that function as posttranscriptional regulators via binding to the 3'-untranslated regions (3'-UTRs) of classic protein-coding genes. These interactions result in the inhibition

of target gene expression by degrading the mRNA or inhibiting the translation. miRNAs are involved in the regulation of a wide variety of biological processes such as differentiation, proliferation, and apoptosis³. miRNAs have also been shown to have an important role in tumorigenesis by acting as oncogenes (oncomiRNAs) or tumor suppressors^{4,5}. Recent reports have suggested that miR-152 acts as a tumor suppressor. Downregulation of miR-152 has been reported in a number of cancer cell lines and tumor tissues, including gastrointestinal cancer⁶, endometrial cancer⁷, ovarian cancer⁸, hepatocellular carcinoma⁹, colorectal cancer¹⁰, and breast cancer¹¹.

The phosphatidylinositol 3-kinases (PI3K)/AKT signaling pathway is tightly regulated to control the balance between cell proliferation and apoptosis. A number of miRNAs have been investigated to regulate the target genes that were involved in these processes^{12,13}. PI3K is a lipid kinase family that is involved in many cellular processes, such as cell growth, proliferation, differentiation, motility, and survival. PI3K is a heterodimer composed

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of an 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit, which is encoded by the phosphatidylinositol-4,5-bisphosphate 3-kinase-catalytic subunit α (PIK3CA) gene. Recently, gain-of-function mutations of PIK3CA, which resulted in the elevated activity of lipid kinase, have been found in several cancers, including breast cancer, indicating that (PIK3CA) acted as an oncogene. AKT and RPS6 are two well-known downstream targets of PIK3CA that control the proliferation of human cancer cells.

In this study, we aimed to investigate the molecular mechanisms of miR-152 as a tumor suppressor to affect human breast cancer via transfecting the human breast cancer cell line HCC1806 with miR-152 mimics or inhibitors, as well as to evaluate the effects of miR-152 on cell viability, apoptosis, and the PI3K/AKT signaling pathway.

MATERIALS AND METHODS

Cell Culture

The human breast cancer cell line HCC1806 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C.

miRNA Precursors

The human miR-152 gene was first transcribed in the nucleus as pre-miR-152 and transported into the cytoplasm following cleavage by the ribonuclease III enzyme Droscha. In the cytoplasm, pre-miR-152 is processed by Dicer to form an miR-152 duplex. Two mature forms of miR-152 are excised from this duplex: miR-152-5p and miR-152-3p. The latter form appears to be more commonly found in a number of species. Therefore, this form was investigated in the present study. The hsa-miR-152-3p mimic, inhibitor, or scrambled negative control was purchased from GenePharma (Shanghai, P.R. China). The sequence of the hsa-miR-152-3p miRNA is UCAGUGCAUGACAG AACUUGG (No. MIMAT0000438, miRBase, NCBI).

Cell Transfection

HCC1806 cells were seeded into six-well plates (5 \times 10⁴ cells/well) and cultured overnight to approximately 70% confluence in a humidified atmosphere containing 5% CO₂ at 37°C. The cells were then transfected with 100 pmol of miR-152-3p mimic, inhibitor, or scrambled negative control and/or siRNA for PIK3CA (si-PIK3CA) purchased from GenePharma by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the

manufacturer's instructions. At 48 h after transfection, the transfected cells were collected to analyze the efficiency of transfection by quantitative real-time polymerase chain reaction (qPCR).

RNA Extraction and qPCR

Total RNA was extracted by TRIzol reagent (Invitrogen, Grand Island, NY, USA), and the quality of RNA was evaluated by the standard method¹⁴. A sample of RNA (500 ng) was reverse transcribed to cDNA using the NCode miRNA First-Strand cDNA Synthesis Kit (Invitrogen). qPCR was performed using the FastStart Universal SYBR Green Master (Roche, Mannheim, Germany) with the universal reverse primer provided in the kit and the prepared cDNA as the template. qPCR was performed on the Applied Biosystems real-time detection system (Applied Biosystems, Foster City, CA, USA) using the following thermocycling conditions: 95°C for 3 min, 40 cycles of 95°C for 15 s followed by 60°C for 30 s. Each sample was run in triplicate, and expression levels were normalized against U6 snRNA levels [U6: 5'-CTTCGGCAGCACATATACT-3' (forward) and 5'-AAAATATGGAACGCTTC ACG-3' (reverse)]. Melting curve analysis was performed to confirm the specificity of the PCR products. The replicates were then averaged, and fold induction was analyzed according to the $\Delta\Delta$ CT method.

Cell Viability Assay

After transfection with miR-152-3p mimic, inhibitor, or scrambled negative control and/or si-PIK3CA, cell viability was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays¹⁵. Suspended cells (100 μ l) were plated in triplicate into a 96-well plate at a density of 2 \times 10³ cells/well, transfected, and then incubated. After transfection, MTT (50 μ g; Sigma-Aldrich, St. Louis, MO, USA) was added to each well on days 1–5, respectively. Cells were incubated for another 4 h at 37°C. Subsequently, the reaction was terminated by the addition of 100 μ l of 10% sodium dodecyl sulfate (SDS)/12 nM HCl/5% isopropanol. Plates were read on a Molecular Devices microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 570 nm, and background reading of 650 nm was subtracted.

Apoptosis Analysis by Annexin V/FITC/PI Staining

The transfected cells (at a density of 3 \times 10⁶) were collected at 48 h posttransfection and stained using FITC Annexin-V/Dead Cell Apoptosis Kit (V13242; Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. Stained cells were suspended in annexin V-binding buffer (Invitrogen) for flow cytometric analysis using a BD FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany). In total,

1×10^4 cells were analyzed per measurement, and data were analyzed using FlowJo 10.0.7 software (Treestar, Ashland, OR, USA).

Dual-Luciferase Activity Assay

The PIK3CA 3'-UTR, which contains the putative binding site of miR-152-3p, was cloned into the pGL2-basic vector (NEB) as wild type (WT) or mutant type. One hundred nanograms of each recombinant construct was then cotransfected with 50 nM miR-152-3p mimic, inhibitor, or a negative control into cells in a 24-well plate using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The cell extracts were then screened and harvested, and the firefly and *Renilla* luciferase activities were measured after 24 h using the dual-luciferase reporter system (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Western Blot Analysis

Total proteins were isolated from transfected cells and washed twice with PBS. The protein concentration was determined using a bicinchoninic acid (BCA) kit (Solarbio, Beijing, P.R. China). The samples were then prepared lysed with $1 \times$ SDS loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, and 0.1% bromophenol blue). All protein samples were resolved by 10–12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in 5% nonfat milk for 2 h at room temperature and then incubated with the following corresponding primary antibodies overnight at 4°C: PIK3CA (sc8010), AKT (sc5298), phosphorylated (p)-AKT (sc135651), RPS6 (sc248489), p-RPS6 (sc293144), and tubulin (sc53646) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were then washed and incubated with the horseradish peroxidase-conjugated secondary antibodies (1:5,000; Santa Cruz Biotechnology) for 1 h at room temperature. The immunoblotting of bands was enhanced by chemiluminescence (ECL Plus; Amersham Pharmacia Biotech, Piscataway, NJ, USA) and quantified based on density measurements using ImageJ software (NIH, Bethesda, MD, USA). The tested protein expression was determined as a ratio relative to the expression of internal reference.

Statistical Analysis

Data were presented as mean \pm standard deviation (SD), which were representative at least in triplicate. The two-tailed Student's *t*-test was used to evaluate the significance of differences between two groups; one-way analysis of variance (ANOVA) was used to evaluate the significance of differences in mean values within and between multiple groups. Statistical analysis was performed using GraphPad Prism 6.01 (GraphPad Software

Inc., San Diego, CA, USA). A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

miR-152-3p Overexpression Inhibited Cell Viability While Inducing Apoptosis of HCC1806 Cells

HCC1806 cells were transfected with miR-152-3p mimics, inhibitor, or scrambled control. qPCR was performed to determine transfection efficiency (Fig. 1A). The upregulated expression of miR-152-3p was detected after transfection with the miR-152-3p mimic, while it was downregulated after transfection with the miR-152-3p inhibitor ($p < 0.01$).

The effects of miR-152-3p on HCC1806 cell viability were determined over a period of 5 days posttransfection by MTT assays (Fig. 1B). Transfection with the miR-152-3p mimic resulted in a clear reduction in cell viability of HCC1806 cells compared with the control groups from day 3 to day 5 after transfection. In contrast, transfection with the miR-152-3p inhibitor showed the opposite effect, which increased cell viability compared with the control. The mechanism by which miR-152-3p inhibited HCC1806 cell apoptosis was investigated by flow cytometry assay after transfection (Fig. 1C and D). Annexin V/FITC/PI staining of apoptotic cells showed a significantly higher percentage of miR-152-3p mimic-transfected cells compared with the control ($p < 0.001$). In contrast, transfection with the miR-152-3p inhibitor showed no significant effect on the percentage of apoptotic cells compared with the control (ns).

miR-152-3p Negatively Regulated PIK3CA Expression via Binding Effect on the 3'-UTR of PIK3CA in HCC1806 Cells

The mechanism by which miR-152-3p mimic transfection inhibited the cell viability of HCC1806 cells was further investigated by Western blot (Fig. 2A–C). The PIK3CA expression in HCC1806 cells after miR-152-3p transfection is shown in Figure 2A and B. Quantitative analysis results show that PIK3CA expression was significantly reduced after transfection with the miR-152-3p mimic compared with the control ($p < 0.001$) (Fig. 2C). In contrast, PIK3CA expression was significantly increased after transfection with the miR-152-3p inhibitor ($p < 0.001$) (Fig. 2C).

As miR-152-3p was negatively related to the expression of PIK3CA in miR-transfected HCC1806 cells, we assessed the relationship between miR-152-3p and the PIK3CA promoter. The putative binding site of miR-152-3p in PIK3CA 3'-UTR was obtained via TargetScan (Fig. 2D). The luciferase reporter assay results suggest that in the miR-152-3p mimic-administrated groups, the fluorescence signal intensity of PIK3CA (WT) was

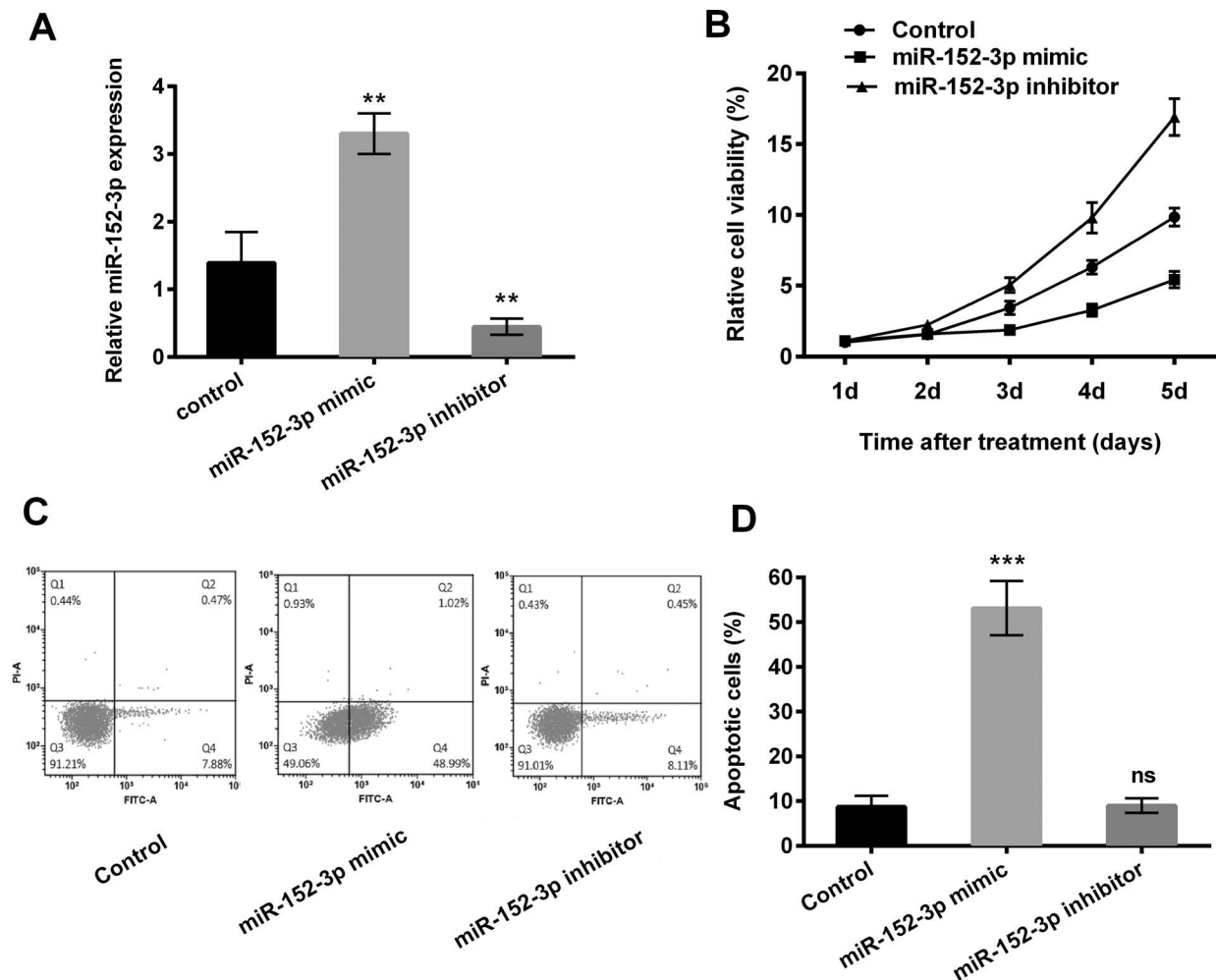


Figure 1. miR-152-3p overexpression inhibited cell viability while inducing apoptosis of human breast cancer cells HCC1806. HCC1806 cells were transfected with miR-152-3p mimic, inhibitor, or scrambled control. (A) The expression levels of miR-152-3p were measured by quantitative real-time polymerase chain reaction (PCR). (B) Cell viability was determined using MTT assays. (C, D) Related apoptotic cells were measured by flow cytometry. Data represent the mean \pm standard deviation (SD) of triplicate readings. ns, nonsignificant; ** $p < 0.01$; *** $p < 0.001$.

significantly decreased, while showing crosscurrent in the miR-152-3p inhibitor-administered groups ($p < 0.05$) (Fig. 2E and F). There were no significant effects (ns) of miR-152-3p mimic cotransfection on the PIK3CA (Mut) group. These results suggest that miR-152-3p could efficiently bind with FIK3CA 3'-UTR and thus regulate the expression of PIK3CA in HCC1806 cells.

Knockdown of PIK3CA Inhibited Cell Viability While Inducing Apoptosis of HCC1806 Cells

The above results suggest that PIK3CA expression is regulated by miR-152-3p, so the effect of PIK3CA knockdown on HCC1806 cells was also investigated. The transfection efficiency of PIK3CA siRNA (si-PIK3CA) in HCC1806 cells was assessed and suggests that mRNA

and protein expression levels of PIK3CA were both decreased after siRNA transfection ($p < 0.01$) (Fig. 3A).

Cells were then transfected with the miR-152-3p inhibitor and/or si-PIK3CA. Cell viability assay results suggest that si-PIK3CA transfection decreased cell viability and reversed the promoting effect of miR-152-3p inhibitor on HCC1806 cell viability ($p < 0.05$ or $p < 0.01$) (Fig. 3B). The results of apoptotic cells were increased after si-PIK3CA transfection alone compared with the control ($p < 0.001$). Even the miR-152-3p inhibitor decreased cell apoptosis compared with the control ($p < 0.01$). While transfection with both the miR-152-3p inhibitor and si-PIK3CA offsets these effects, no significant (ns) effect was shown when compared to the control group, suggesting that knockdown of PIK3CA might act as a miR-152-3p mimic

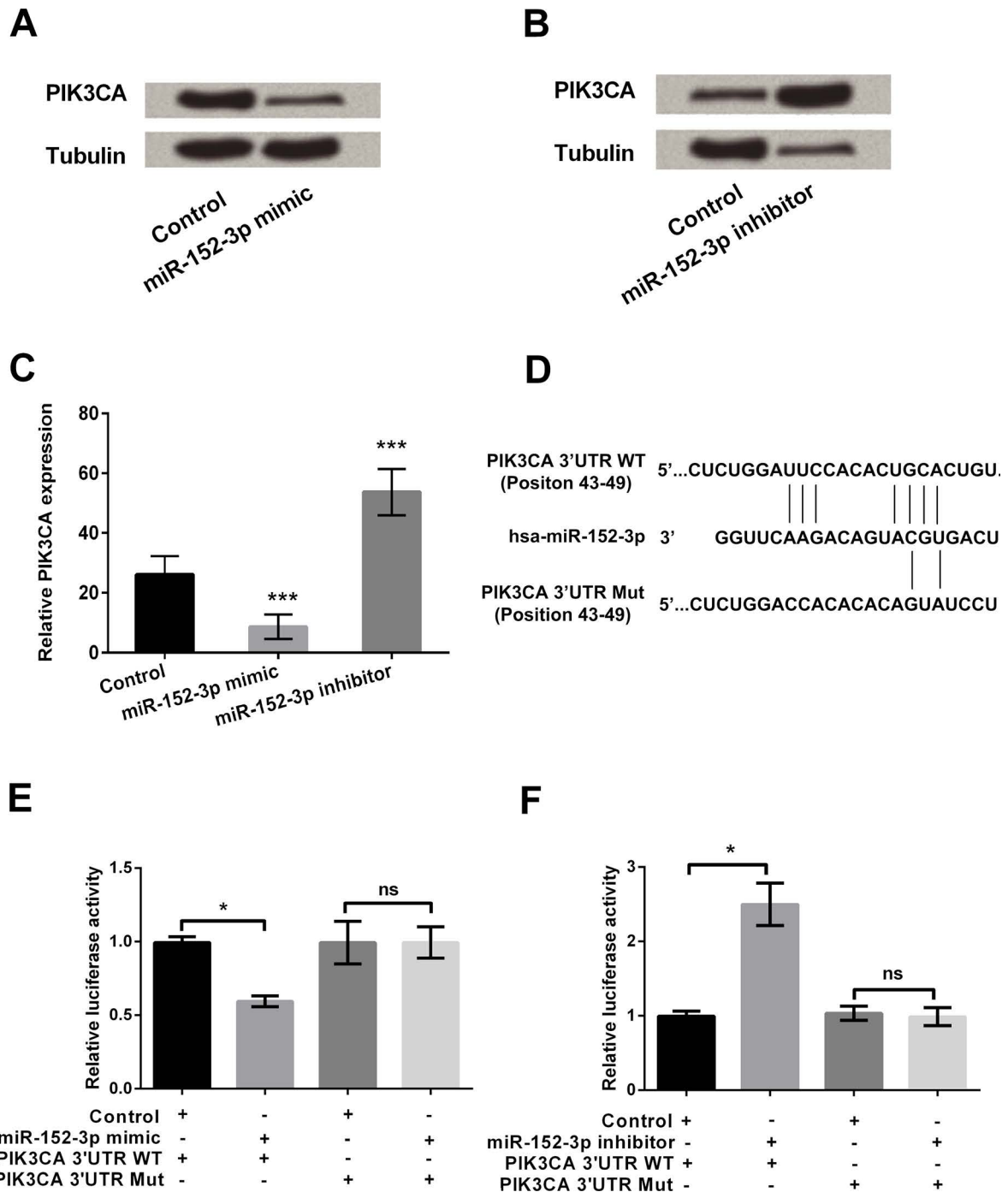


Figure 2. miR-152-3p negatively regulated the expression of phosphatidylinositol-4,5-bisphosphate 3-kinase-catalytic subunit α (PIK3CA) in HCC1806 cells. (A, B) Expressions of PIK3CA in HCC1806 cells transfected by miR-152-3p mimic, inhibitor, or scrambled control were determined by Western blot analysis. (C) Quantitative analysis of the immunoreactive bands was performed by densitometry. The protein tubulin acted as an internal reference. (D) The putative binding site for the miR-152-3p in PIK3CA 3'-UTR wild type (WT) and mutant (Mut) was predicted by TargetScan. (E, F) The targeting effect of miR-152-3p on PIK3CA was detected by dual-luciferase activity assay. Data represent the mean \pm SD of triplicate samples. * p < 0.05; *** p < 0.001.

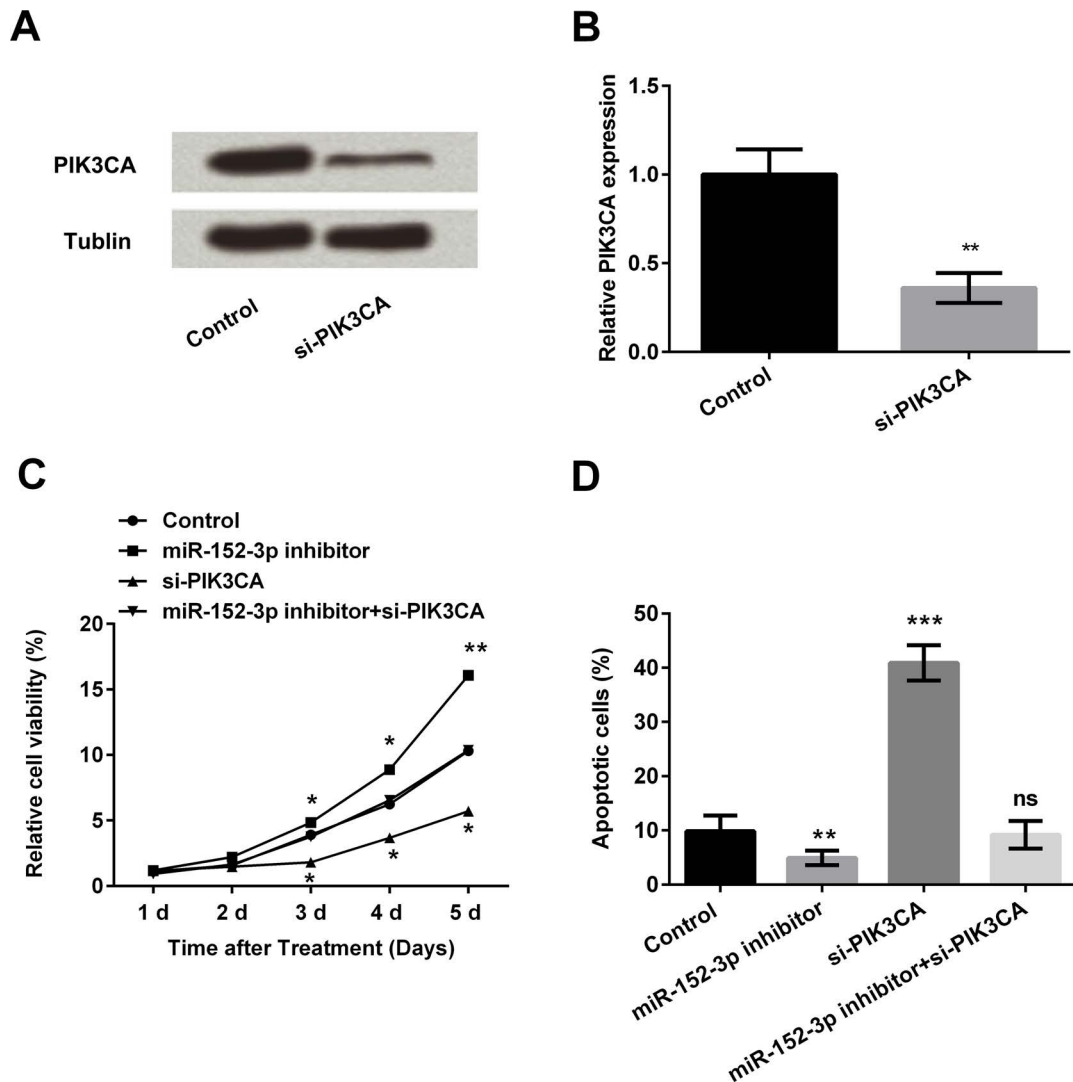


Figure 3. Knockdown of PIK3CA reversed the effect of the miR-152-3p inhibitor on cell viability and apoptosis of HCC1806 cells. (A, B) Expressions of PIK3CA in HCC1806 cells transfected by siRNA and negative control were determined by Western blot analysis. HCC1806 cells were transfected with the miR-152-3p inhibitor and/or PIK3CA siRNA (si-PIK3CA) as well as the corresponding control. (C) Cell viability was determined using MTT assays. (D) Related apoptotic cells were measured by flow cytometry. Data represent the mean \pm SD of triplicate samples. * p <0.05; ** p <0.01; *** p <0.001.

transfection and reverse the effect of miR-152-3p inhibitor transfection on HCC1806 cell viability and apoptosis.

miR-152-3p Overexpression Decreased the Expression Levels of Phosphorylated AKT (Ser473) and RPS6 (Ser235/236) in HCC1806 Cells

AKT (Ser473) and RPS6 (Ser235/236) are two well-known downstream targets of PIK3CA. Therefore, to further investigate the signaling pathways involved in the mechanism of miR-152-3p inhibiting the proliferation of HCC1806 cells, we analyzed the effects of miR-152-39 transfection on the expression (total and phosphorylated forms) of these molecules by Western blotting (Fig. 4A).

Quantitative analysis of the immunoreactive bands by densitometry (Fig. 4B) showed that the p-AKT/AKT ratio was significantly increased in miR-152-3p inhibitor-transfected cells compared with the control (p <0.01), whereas the ratio was significantly decreased in miR-152-3p mimic-transfected cells (p <0.001). The p-RPS6/RPS6 ratio showed a similar trend after miR-152-39 transfection (p <0.01 or p <0.001).

The effect of PIK3CA knockdown on the expression levels of phosphorylated AKT (Ser473) and RPS6 (Ser235/236) in HCC1806 cells was also assessed. si-PIK3CA transfection increased the phosphorylation levels of AKT and RPS6 and reversed the inhibitory effect of

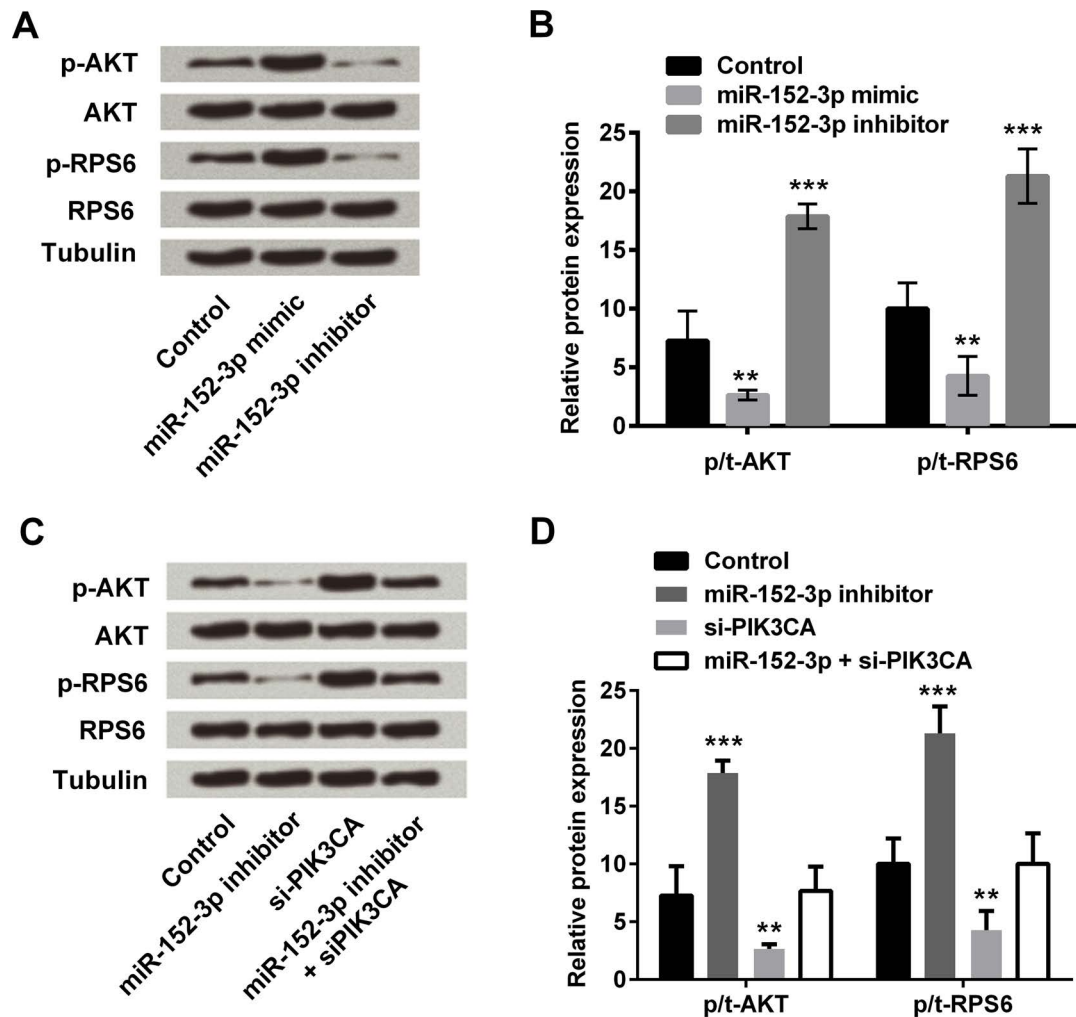


Figure 4. miR-152-3p overexpression decreases the levels of phosphorylated AKT (Ser473) and RPS6 (Ser235/236) in HCC1806 cells. (A) Expressions of the p/t-AKT (Ser473) and RPS6 (Ser235/236) (two well-known downstream targets of PIK3CA) in HCC1806 cells transfected with miR-152-3p mimic, inhibitor, or scrambled control (miR-NC) were determined by Western blot analysis. Tubulin served as an internal control. (B) Quantitative analysis of the immunoreactive bands was performed by densitometry. (C) Expressions of the p/t-AKT (Ser473) and RPS6 (Ser235/236) (two well-known downstream targets of PIK3CA) in HCC1806 cells transfected with miR-152-3p inhibitor and/or PIK3CA siRNA (si-PIK3CA), as well as the corresponding negative controls (Control) were determined by Western blot. Tubulin served as an internal control. (D) Quantitative analysis of the immunoreactive bands was performed by densitometry. Expressions of the phosphorylated forms [AKT (Ser473) and RPS6 (Ser235/236)] were determined as a ratio of the total expression. Data represent the mean \pm standard error (SE) of triplicate samples. ** $p < 0.01$; *** $p < 0.001$.

miR-152-3p inhibitor transfection on these two factor expressions ($p < 0.01$ or $p < 0.001$) (Fig. 4C and D).

DISCUSSION

There is accumulating evidence suggesting that miR-152 acts as a tumor suppressor via regulating the expression of target genes involved in cell proliferation and apoptosis as well as migration and invasion. In this study, we demonstrated that miR-152-3p might serve as a tumor suppressor in human breast cancer via negative regulation of PIK3CA expression and inhibiting the activation of

downstream targets, AKT and RPS6, leading to suppression of human breast cancer cell proliferation.

miR-152 has been reported to be downregulated in breast tumor tissue compared with adjacent nontumor tissue as well as in a number of breast cancer cell lines¹¹. Furthermore, Xu¹¹ showed that stable abnormal overexpression of miR-152 in a breast cancer cell line attenuated the capacity for proliferation, colony formation, and angiogenesis by targeting insulin-like growth factor receptor 1 (IGF-1R) and insulin receptor substrate 1 (IRS1) and suppressing the downstream AKT and MAPK/ERK signaling pathways. Thus, these findings suggest

that miR-152 acted as a tumor suppressor in breast cancer; however, the signaling pathways involved in the underlying mechanism still need to be fully elucidated.

In this study, we showed that miR-152-3p mimic transfection inhibited cell viability and induced apoptosis of the human breast cancer cell line HCC1806. In contrast, transfection with the miR-152-3p inhibitor resulted in increased proliferation, but with no significant effect on the percentage of apoptotic cells. These results were also consistent with a study by Zhou et al., which showed that proliferation of the ovarian cancer cell line was significantly inhibited after transfection with the miR-152 mimic⁸. Furthermore, Li et al. reported that restoring the expression of miR-152 in colorectal cancer cells reduced cell proliferation and migration, while apoptosis and caspase 3 activity were promoted¹⁰. However, previous studies have shown that antisense oligonucleotide-mediated inhibition of miR-152 resulted in decreased proliferation in HeLa cells¹⁶. miR-152 was found to be upregulated in neuroblastoma cells and resulted in mediating negative control of apoptosis via downregulating the expression of proapoptotic genes¹⁷. Thus, the role of miR-152 as a tumor suppressor remains controversial and may be cell type dependent.

The PI3K/AKT signaling pathway is critical in the control of cell proliferation, survival, and metabolism, and it is dysregulated in the development of human cancers¹⁸. Oncogenic mutations in the PI3K pathway result in hyperactivation, which in turn leads to increased proliferation, decreased apoptosis, and tumor formation¹⁹. Mutations in the PIK3CA gene occur in human cancers with a high frequency²⁰. PIK3CA is mutated in approximately 30% of all human breast cancers, with approximately 15% of human cancers expressing a mutation in the p110 α catalytic domain²¹. These mutations result in the expression of a constitutively active form of the enzyme, which has been shown to transform cells in vitro and increase tumorigenicity in xenograft models in vivo^{22–24}.

In this study, we demonstrated that transfection of the miR-152-3p mimic in HCC1806 cells resulted in a significant reduction in PIK3CA expression, while transfection with the corresponding inhibitor had the opposite effect. The binding effect of miR-152-3p on PIK3CA 3'-UTR in HCC1806 cells was also investigated. Thus, we demonstrated that miR-152-3p negatively regulates the expression of PIK3CA via targeting the 3'-UTR of PIK3CA in HCC1806 cells. Similarly, miR-422a has been shown to act as a tumor suppressor in glioblastoma by targeting PIK3CA²⁵. Our study suggests that miR-152 might directly mediate the expression of PIK3CA. Whether this effect is related to the upstream components of the PI3K/AKT signaling pathway still needs exploration.

Although numerous genes have been predicted to be targets of miR-152, only DNA methyltransferase I (DNMT1), IGF-1R, and IRS1 have been experimentally confirmed to be targets of miR-152 in breast cancer¹¹. DNMT1 catalyzes DNA methylation, which is important in the epigenetic control of many cellular functions. Aberrant hypermethylation of miR-152 has been proven to be correlated with poor clinical outcome in mixed-lineage leukemia-rearranged infant acute lymphoblastic leukemia²⁶. The epigenetic regulation of miR-152/DNMT1 might be involved in tumorigenesis¹¹. Furthermore, miR-152 has been suggested to inhibit the AKT and ERK signaling pathways via targeting IGF-1R and IRS1, which were commonly upregulated in breast cancer. Thus, it could be hypothesized that miR-152 might act as a tumor suppressor by targeting PIK3CA via its interaction with IGF-1R and IRS1, which were upstream components of the PI3K signaling pathway.

Some limitations of this study should be noted. First, this study was conducted in vitro using the breast cancer cell line HCC1805, and the results remain to be confirmed using in vivo models and human breast cancer tissues. Furthermore, the possible influence of transfected miR-152 on other miRNA species should be investigated to clarify the specificity of our results.

In conclusion, the results of this study suggested that miR-152-3p might act as a tumor suppressor in HCC1806 breast cancer cells via targeting PIK3CA. These findings might provide a further understanding about the mechanism of underlying tumorigenesis of breast cancer and the development of new therapeutic strategies.

ACKNOWLEDGMENT: The authors declare no conflicts of interest.

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