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RETRACTED ARTICLE: Targeting of GIT1 by miR-149* in breast cancer suppresses cell proliferation and metastasis in vitro and tumor growth in vivo

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Abstract: Breast cancer remains a major cause of cancer-related deaths in women worldwide. Dysregulation of microRNAs (miRNAs) is involved in the initiation and progression of breast cancer. Moreover, it was found that GIT1 was widely involved in the development of many human cancers. Herein, we aimed to investigate the expression changes of miR-149* and GIT1 and the functional effects of miR-149*/GIT1 axis in breast cancer. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blot (WB) were used to examine the expression levels of miR-149* and GIT1. Dual luciferase reporter assay was utilized to confirm the target interaction between miR-149* and GIT1. The biological functions, including cell proliferation, invasion, and migration, of miR-149* and GIT1 were determined by MTT assay and Transwell assays, respectively. Eventually, the tumor xenograft model in nude mice injected with stable transfected MDA-MB-231 cells was established to verify the effects of miR-149* and GIT1 on tumor growth. Our results showed that miR-149* expression was decreased, whereas GIT1 expression was increased in clinical samples of breast cancer. Based on the inverse expression trend between miR-149* and GIT1, we further demonstrated that miR-149* indeed directly targets GIT1. Subsequently, it was observed that inhibition of miR-149* significantly promoted cell proliferation, invasion, and migration, but the ability of cell proliferation, invasion, and migration was obviously declined after silencing of GIT1 in MDA-MB-231 cells transfected with miR-149* mimic and/or si-GIT1. Finally, it was also found that elevated miR-149* decelerated the tumor growth, while restored GIT1 accelerated the tumor growth in nude mice after 35 days of tumor xenograft. Collectively, these findings concluded that miR-149* might exert a tumor suppressive role in breast cancer by targeting GIT1.

Keywords: microRNA 149*, miR-149*, G protein-coupled receptor kinase interacting protein 1, GIT1, tumor suppressive role, breast cancer

Introduction

Breast cancer is a major health problem and the most frequent malignancy that affects females around the world.[1] The disease shows increasing incidence rates over the past decades and accounts for more than 458,000 deaths annually worldwide, as it is the fifth most common cause of cancer-related deaths in the world.[2,3] Although the disease has been studied widely, local recurrence and distant metastasis remain main reasons for poor prognosis, thereby to improve clinical outcomes of breast cancer patients, determining the underlying molecular mechanisms of breast cancer progression, recurrence, and distant metastasis or identifying better therapeutic methods must be given priority.[4,5]

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microRNAs (miRNAs), a set of highly conserved, endogenous, small noncoding single-stranded RNA molecules consisting of ~22 nucleotides in length, negatively regulate the expression of target messenger RNAs (mRNAs) by binding to partially complementary sequences in their 3′-untranslated regions (3′-UTRs), ultimately leading to mRNA degradation and/or translation inhibition.[6] In recent years, a previous study has been reported that miRNAs may involve in the occurrence, development, and progression of various types of tumors by regulating the proliferation, differentiation, apoptosis, metastasis, angiogenesis, and chemoresistance of tumor cells.[7] Moreover, the significance of miRNAs in breast cancer has been documented by a study.[8] For example, miR-198 inhibited cell proliferation and migration and promoted cell adhesion in aggressive breast cancer by targeting CUB domain-containing protein 1 (CDCP1);[9] miR-340 suppressed the migration, invasion, and metastasis of breast cancer cells through modulating Wnt/β-catenin signaling pathway;[10] miR-148a accelerated cell apoptosis and restricted growth of breast cancer cells via regulating B-cell lymphoma 2 (Bcl-2).[11] Therefore, miRNAs are regarded as reliable prognostic markers, promising therapeutic targets, or new drugs, which would be essential in the prevention and treatment of breast cancer.[12]

Previous evidence showed that miR-149* was implicated in a variety of tumorigenesis processes.[13–16] For instance, miR-149* was found to be closely linked with clinical outcome in human neuroblastoma, meanwhile it could influence cancer cell proliferation through Ras-related protein 1 (Rap1) independent of v-myc-related oncogene (MYCN) amplification.[13] Furthermore, it discovered that miR-149* is a modulator of cancer chemoresistance by targeting GlcNAc *N*-deacetylase/*N*-sulfotransferase-1 (NDST1) in the treatment of human breast cancer.[17] However, the effects of miR-149* on breast cancer have not been completely understood. Additionally, GIT1 played a critical role in focal adhesion, cell invasion, cell migration, and cell mobility in many cancers. For example, GIT1 promotes lung cancer cell metastasis through modulating Rac1/Cdc42 activity and is associated with poor prognosis.[19] Furthermore, it presented a strong correlation with miR-149 to suppress integrin signaling in breast cancer metastasis.[20] Thus, in the present study, we attempted to further elucidate the involvement of the miR-149*/GIT1 interaction in breast cancer and their functional role, including cell proliferation, invasion, migration, and tumor growth, in the development of breast cancer, which might provide a novel useful strategy for effective clinical therapies in the future.

Materials and methods

Ethics statement and clinical samples

Clinical specimens were collected from eight patients suffered from breast cancer, who underwent surgery at Jinshan Hospital, Fudan University, from January 2016 to December 2016. Adjacent nontumoral breast tissues (at least 5 cm away from the primary tumor) from these patients served as normal controls. None of the patients received radiation therapy and chemotherapy before surgery. Moreover, all patients have signed an written informed consent form ahead of surgery and been told that their tissues would be used in our research. After being taken out, tissue specimens were rinsed with cold phosphate-buffered saline (PBS) to reduce blood contamination and then immediately stored at −80°C for further miR-149* and GIT1 expressions' examinations by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blot (WB) assays. All protocols and procedures were approved by the Clinical Research Ethics Committee of Fudan University in this study.

Cell line culture and transfection

Human breast adenocarcinoma (MDA-MB-231) cell lines and 293T cells, obtained from American Type Culture Collection (ATCC; Manassas, VA, USA), were grown in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), as well as 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified chamber containing 5% CO_2. Cell medium was renewed every other day and cells were sub-cultured with 0.25% trypsin (Sigma-Aldrich Co., St Louis, MO, USA) upon reaching 80% confluence. Meanwhile, cells were regularly tested with MycoAlert (Lonza, Basel, Switzerland) to ensure the absence of mycoplasma contamination. MDA-MB-231 cells were used for whole experimental process, whereas 293T cells were only applied for dual luciferase reporter assay.

MDA-MB-231 cells were seeded in six-well plates at an intensity of 5×10^5 cells/well ~24 h before transfection at optimal confluence of ~70%. On the transfection day, the cells were randomly divided into six groups, namely, cell group, negative control (NC) group, miR-149* group, miR-149* inhibitor group, siGIT1 group, and miR-149* + siGIT1 group, which transfected with no treatment, NC plasmid, miR-149* mimic plasmid, miR-149* inhibitor plasmid,

small-interfering RNA (siRNA) plasmid of GIT1, and miR-149* mimic plasmid + GIT1-siRNA plasmid, respectively, using Lipofectamine 2000 (Promega Corporation, Fitchburg, WI, USA) according to the manufacturer's instructions. The above plasmids were synthesized from Sangon Biotech (Shanghai, China). Furthermore, the transfection efficiency of these plasmids was confirmed by qRT-PCR.

RNA extraction and qRT-PCR analysis

Total RNA was extracted from breast cancer tissue samples and adjacent nontumor tissue samples using TRIzol Reagent (Tiangen Biotech, Beijing, China) as recommended by the manufacturer, and the isolated RNA was cleared of contaminating genomic DNA by DNase treatment (Thermo Fisher Scientific). For miR-149* analysis, complementary DNA (cDNA) was first synthesized with a miRNA-specific stem-loop primer using an M-MLV reverse transcriptase kit (Promega Corporation) in accordance with the manufacturer's protocols. Then, the mature miR-149* expression level was quantified through PCRs with the corresponding miRNA primers on an ABI PRISM® 7500 Sequence Detection System (Thermo Fisher Scientific) using a SYBR Green qPCR SuperMix Kit (Thermo Fisher Scientific) following the manufacturer's guidelines. For GIT1 analysis, [illegible] transcription reaction was performed using an M-[illegible]LV reverse transcriptase kit, and subsequent amplified reac[illegible] was conducted with ABI PRISM 7500 S[illegible]quence [illegible]etecti[illegible] System using a SYBR Green qPCR S[illegible]erMix [illegible]ccord[illegible]ing to the manufacturer's recom[illegible]endati[illegible]. The relative expression levels of miR-149[illegible]nd GIT1 w[illegible] calculated by the $2^{-\Delta\Delta Ct}$ method and no[illegible]malize[illegible]to universal U6 small nuclear RNA (U6 snR[illegible]A) and 18S r[illegible]IA as endogenous controls for miRN[illegible]and mRNA, respectively. For each sample, miRNA a[illegible]mRN[illegible] expressions were measured in triplicates. The primers' sequences of miR-149* and GIT1 were designed and listed in Table 1.

WB detection

Clinical tissue samples were homogenized in 200 µL radioimmunoprecipitation assay (RIPA) lysis buffer (0.5 M Tris-HCl, pH 6.8, 2 mM EDTA, 10% glycerol, 2% sodium dodecyl sulfate [SDS], and 5% β-mercaptoethanol) for at least 20 min on ice. After samples centrifuged at 4°C for 15 min at 12,000 rpm, the supernatants of each sample were harvested. Subsequently, a BCA Protein Assay kit (Therm[illegible]isher Scientific) was utilized to determine the prot[illegible]n concen[illegible]tion following the manufacturer's instructio[illegible]. Equal amo[illegible]nts of protein extracts (30 µg total prot[illegible]n/lane) [illegible]re resol[illegible]d by 8%–10% SDS-polyacrylamide[illegible]gels for electro[illegible]esis (SDS-PAGE) for 60 min at 120 [illegible] and [illegible]ansferred onto polyvinylidene difluoride (PV[illegible]F) men[illegible]nes at a [illegible]nstant current of 200 mA for 60 mi[illegible]lowed by bl[illegible]ki[illegible]g with 5% nonfat dried milk in Tris-buffered [illegible]line with 0.1% Tween-20 (TBST) for 2 h at [illegible]emperature[illegible]. Afterward, the PVDF membranes were [illegible]ncubated with primary antibodies specific to GIT1 (1:1,500 [illegible]ilution; Ab[illegible]m, Cambridge, UK) and GAPDH (1:2,000 d[illegible]tion, a re[illegible]rence control; Abmart, Berkeley Heights, NJ, USA) [illegible]night at 4°C with gentle agitation. After extensive [illegible]hing with TBST three times, the membranes were probed with an antirabbit or antimouse IgG conjugated to horseradish peroxidase (HRP) (1:12,000 dilution; Boster, Shanghai, China) at 37°C for 1 h, and then washed thrice with TBST before protein visualization with enhanced chemiluminescence reagents (Beyotime, Jiangsu, China). Ultimately, the immune-reactive bands were scanned on a GelDocXR instrument (Bio-Rad Laboratories Inc., Hercules, CA, USA), and the relative intensities of the bands were analyzed by Quantitive One software (Bio-Rad Laboratories Inc).

Table 1 [illegible]etails of [illegible]rimers' s[illegible]ences for miRNA and gene

miRNA [illegible]gene	Sequence
*miR-149**	RT primer: 5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGGCACAGCCC-3′ Forward primer: 5′-ACACTCCAGCTGGGAGGGAGGGACGGGGGC-3′ Reverse primer: 5′-CTCAACTGGTGTCGTGGA-3′
U6 snRNA	RT primer: 5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAAAAATATGG-3′ Forward primer: 5′-CTCGCTTCGGCAGCACA-3′ Reverse primer: 5′-AACGCTTCACGAATTTGCGT-3′
GIT1	Forward primer: 5′-ATGGTGCACACGCTTGCCAGC-3′ Reverse primer: 5′-TGCCTGTCCGCACGCTCGAGT-3′
18S rRNA	Forward primer: 5′-CCTGGATACCGCAGCTAGGA-3′ Reverse primer: 5′-GCGGCGCAATACGAATGCCCC-3′

Abbreviation: U6 snRNA, U6 small nuclear RNA.

Dual luciferase reporter assay

It was predicted that GIT1 had two miR-149*-binding sites at positions 206 and 970 bp, thereby the full-length sequence of GIT1 3′-UTR and two different mutant fragments of GIT1 3′-UTR were amplified and inserted into the psiCHECK-2 vector (Promega Corporation) downstream of the luciferase gene sequence to generate the recombinant vectors wild type (WT)-GIT1 and Mutant GIT1-1–2. Additionally, Mutant GIT1-3 was constructed with the miR-149*-binding sites at both positions. 293T cells were plated in 96-well plates (2×10^4 cells/well) 1 day before transfection. Then, cells were transiently cotransfected with WT-GIT1 or three Mutant-GIT1 plasmids, along with control psiCHECK-2 plasmid (blank group), miR-149* mimics, miR-149* inhibitor, NC plasmid, or NC inhibitor, using Lipofectamine 2000. Forty-eight hours later, cells were processed for the fluorescence values measurement with the Dual-Luciferase Reporter Assay System (Promega Corporation) in accordance with the manufacturer's protocols. For data analysis, firefly luciferase activity was normalized to the corresponding renilla luciferase activity and all the experiments were repeated three times.

Cell proliferation examination

A cell proliferation assay was carried out with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit (Promega Corporation) following manufacturer's recommendations. MDA-MB-231 cells were allowed to grow in a 96-well plate with 2×10^4 cells/well and then transfected with different plasmids for 24 h. Next, 20 μL MTT (5 mg/mL) was added and incubated for 4 h in a humidified atmosphere containing 5% CO_2 at 37°C. The reaction was then terminated by removal of the supernatant without disturbing the cells and 150 μL of dimethylsulfoxide (DMSO) solution was added to the well. After continuous shaking at room temperature for 15 min to dissolve the purple formazan crystals, the optical density (OD) at a wavelength of 490 nm of each well was examined with a microplate reader (Bio-Rad Laboratories Inc.).

Cell invasion and migration measurement

Cell invasion and migration were assessed using a transwell assay. For cell invasion experiment, 2×10^5 MDA-MB-231 cells transfected with different plasmids were trypsinized into a single-cell suspension and placed on top of 8 μm transwell inserts (BD Biosciences, San Jose, CA, USA) precoated with Matrigel basement membrane matrix (BD Biosciences). Additionally, the lower chamber was supplied with 600 μL of culture media containing 20% FBS as a chemoattractant, followed by an incubation at 37°C with 5% CO_2 for 24 h. Subsequently, the cells remaining on the apical side of each transwell membrane were carefully scraped off with a cotton swab, while cells that had invaded to the other side of the membrane were fixed with 4% paraformaldehyde at room temperature for 30 min, stained with 0.1% crystal violet for 10 min, washed with PBS three times, and dried at 80°C for 30 min. Finally, the number of invasive cells was counted from 4 to 5 randomly microscopic filed under an inverted microscope (×400 magnification; Nikon Corporation, Tokyo, Japan). However, for cell migration experiment, the concentration of MDA-MB-231 cells in each group was adjusted to 1×10^5 cells/well and similarly placed on top of 8 μm transwell inserts but without Matrigel basement membrane matrix and the other procedures were similar to invasion experiment.

Tumor xenograft model in nude mice

Stable transfected MDA-MB-231 cells (1.0×10^6 in 200 μL PBS) were subcutaneously injected into the right side of 3–4 week-aged male BALB/c nude mice, supplied by the Experimental Animal Center of Fudan University. All mice were randomly assigned to four groups (n=5 per group), including NC group without plasmid transfection, miR-149* mimic group, miR-149* inhibitor group, and GIT1 overexpression group. Thirty-five days after tumor cell implantation, all mice were sacrificed by CO_2 inhalation to ameliorate animal suffering and tumor xenografts were excised. Then, tumor sizes in each group were measured with a digital caliper.

Ethics permission

All the animal care and experimental procedures were approved by the Animal Care and Use Committee of Fudan University Committee Guidelines on the Use of Live Animals in Research, which is according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publications no 80-23), revised 2010.

Statistical analysis

Statistical testing was conducted with the assistance of Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA). All of the results are depicted as mean ± standard deviation (SD) from at least three biological repeated experiments. An unpaired, two-tailed Student's *t*-test was used to compare differences between two groups. A two-sided *P*-value of <0.05 was considered to indicate a statistically significant difference.

Results

miR-149* is downregulated, while GIT1 is upregulated, in human breast cancer tissues

To examine the expression signature of miR-149* in human breast cancer progression, we first performed miRNA-based qRT-PCR analysis in eight clinical tumor samples and matched adjacent tissues. Data showed that all breast cancer tissues presented lower miR-149* levels than the corresponding tumor-adjacent normal tissues controls (Figure 1A). Contrary to miR-149*, we observed that the mRNA and protein expressions of GIT1 in the breast cancer tissues, determined by qRT-PCR and WB, respectively, were higher than those in the corresponding tumor-adjacent normal tissues controls (Figure 1B and C). And the relative protein levels of GIT1 in carcinoma and para-carcinoma tissues of eight patients were calculated as shown in Figure 1D. Therefore, these data suggested that miR-149* and GIT1 might play an important role in breast cancer.

miR-149* directly targets the GIT1 3′-UTR

Based on the above result, we speculated that there was a target interaction between miR-149* and GIT1, thereby we predicted the binding sites between miR-149* and GIT1 by TargetScan online software (http://www.targetscan.org/vert_71/) and found that there were two putative binding sites, namely, positioned at 206–226 and 970–990 bp, for miR-149* in the 3′-UTR of GIT1 mRNA (Figure 2A). Results displayed that the luciferase renilla/firefly activity in mutant sites at positions 206–226 and 970–990 bp of GIT1 3′-UTR was not significantly changed. Furthermore, it was similarly not changed in mutant sites at both positions of GIT1 3′-UTR (Figure 2B), indicating that the GIT1 3′ UTR was the direct target of miR-149*.

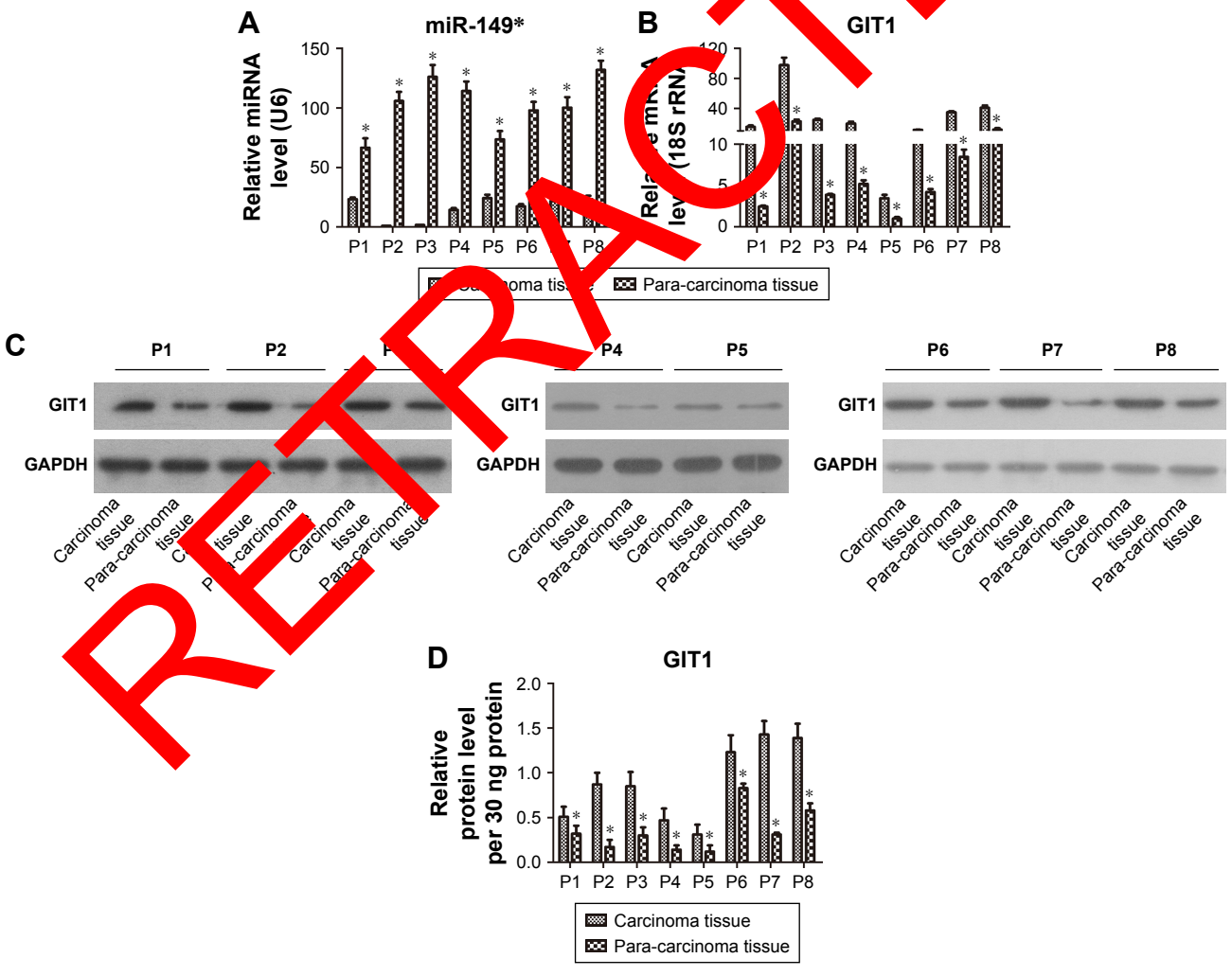

Figure 1 Comparisons of miR-149* and GIT1 expressions in breast tumor tissues and adjacent nontumor tissue.
Notes: (**A**) Relative quantification of miR-149* was determined by qRT-PCR. (**B**) Relative quantification of GIT1 was examined by qRT-PCR. (**C**) GIT1 protein expression was measured by WB. (**D**) The statistical histogram of GIT1 protein. *$P<0.05$. "P" denotes patients.
Abbreviations: qRT-PCR, quantitative reverse transcription-polymerase chain reaction; WB, Western blot.

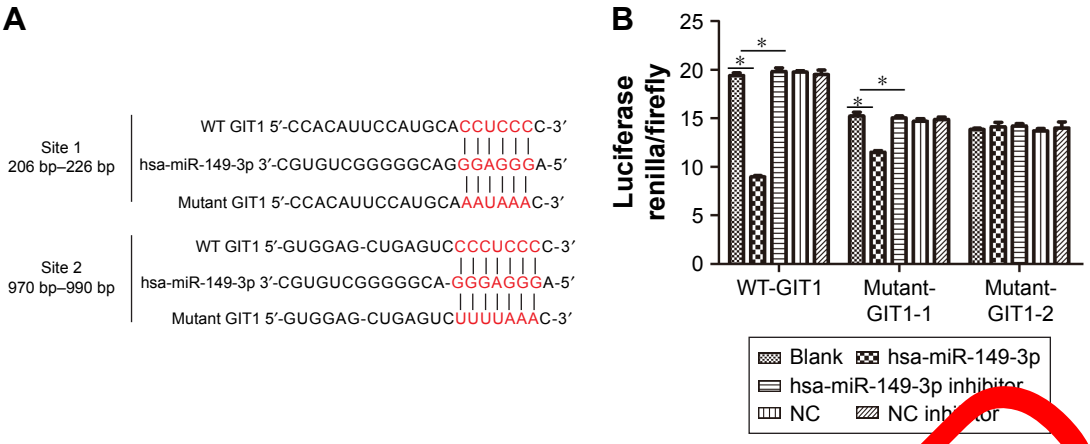

Figure 2 GIT1 is a direct target of miR-149*.
Notes: (**A**) Schematic representation of the GIT1 3′-UTRs with miR-149*-binding sites and sequence alignment of predicted miR-149*-binding sites on 3′-UTRs. (**B**) The target interaction between miR-149* and GIT1 was confirmed by dual luciferase report assay. *$P<0.05$.
Abbreviations: NC, negative control; 3′-UTRs, 3′-untranslated regions; WT, wild type.

Apparent transfection efficiency was tested by qRT-PCR

To confirm the transfection efficiency of NC, miR-149*, miR-149* inhibitor, si-GIT1, and miR-149* + siGIT1 plasmids, qRT-PCR was used to examine the expression levels of miR-149* and GIT1. The results showed that miR-149* mimic and miR-149* inhibitor significantly promoted and inhibited miR-149* expression in MDA-MB-231 cells, respectively (Figure 3A). In addition, siGIT1 transfection notably suppressed GIT1 expression (Figure 3A).

Increased miR-149* and decreased GIT1 levels repressed cell proliferation, invasion and migration

In order to further investigate the biological function of miR-149* and its target GIT1, we assessed the ability of cell proliferation, invasion, and migration in MDA-MB-231 cells treated with miR-149* mimic, miR-149* inhibitor, GIT1-siRNA, and miR-149* + GIT1-siRNA. The MTT assay revealed that downregulation of miR-149* remarkably enhanced the proliferation of MDA-MB-231 cells, whereas there were no significant differences in OD450 values among miR-149* mimic, GIT1-siRNA, and miR-149* + GIT1-siRNA groups (Figure 3B). Moreover, the ability of cell proliferation in downregulation of GIT1 was apparently lower than that in cell and NC groups. Hence, these results implied that miR-149* could promote MDA-MB-231 cell proliferation via regulating GIT1. Subsequently, transwell assay demonstrated that reduced miR-149* expression obviously increased the mean numbers of cells penetrating the membrane (Figure 3C and D). However, silencing of GIT1 in miR-149* mimic, GIT1-siRNA, and miR-149* + GIT1-siRNA group notably decreased the mean numbers of cells penetrating the membrane as compared to miR-149* inhibitor group, even cell and NC groups (Figure 3C and D). Thus, these findings concluded that miR-149* might restrict cell invasion and migration of MDA-MB-231 cell through modulating GIT1.

Forced expression of miR-149* prohibited tumor growth in vivo but elevated expression of GIT1 facilitated tumor growth in vivo

To validate our findings biologically, we administered subcutaneous injections of MDA-MB-231 cells stably transfected with miR-149* mimic, miR-149* inhibitor, and GIT1 overexpression plasmids. As illustrated in Figure 4A and B, the sizes of tumor xenograft in miR-149* group were dramatically diminished when compared to NC group. On the contrary, forced expression of GIT1 in miR-149* inhibitor and GIT1 overexpression groups had an enhanced effect on tumor growth. Taken together, these data hinted that miR-149* might delay tumor growth by targeting GIT1.

Discussion

Epidemiological statistical data revealed that breast cancer remained the leading cause of cancer deaths in women all over the world.[3] Growing evidence has demonstrated that miRNAs are key players in the initiation of oncogenesis, as well as the progression, invasion, and metastasis of breast cancer, and thus, understanding their mechanisms is of utmost importance to reduce the mortality of breast cancer

patients.[21] In the current study, we found that miR-149* expression was decreased, while GIT1 expressions in mRNA and protein levels were increased in tumor tissues of breast cancer patients. Previous analysis illustrated that miR-149 was significantly downregulated in various primary human cancers, such as lung cancer,[22] glioblastoma,[23] and neck and head cancer,[24] which was consistent with our result. Moreover, it was reported that GIT1 was upregulated in oral,[25] liver, and colon cancer,[26] which was also in agreement with our result. Therefore, these results suggested that the alterations of miR-149* and GIT1 might play a vital role in the development and progression of breast cancer. Subsequently, based on the opposite expressions of miR-149* and GIT1, we performed a bioinformatics analysis about the relationship between miR-149* and GIT1. Surprisingly, there might be a target interaction between miR-149* and GIT1 due to two different binding sites of miR-149* on the GIT1 3′-UTR. Furthermore, dual luciferase reporter assay revealed that miR-149* could directly target GIT1 3′-UTR.

In order to further explore the functional mechanisms of miR-149* and GIT1 in breast cancer, we carried out the examinations of cell proliferation, invasion, and migration in MDA-MB-231 cells. Cell proliferation, a complicated process, which is always regulated by a series of genes, is one of the most important malignant characteristics in cancer.[27] Moreover, enhanced cell survival signals usually represent a poor prognosis in cancer. Our results showed that inhibition of miR-149* promoted cell proliferation in MDA-MB-231 cells, but silencing of GIT1 by transfecting with miR-149* mimic or si-GIT1 dramatically suppressed cell proliferation in MDA-MB-231 cells, indicating that miR-149* might be a tumor suppressor to hinder cell proliferation by regulating GIT1. Additionally, miR-149* has been validated to exert a tumor suppressive role in gastric cancer and pancreatic cancer through inhibiting Wnt1 signaling pathway[28] and Akt1 signaling pathway,[29] respectively. Metastasis is the most troublesome complication that is the major clinical obstacle to the successful treatment of cancer, accounting for >90% of cancer patients' death resulting from breast cancer.[30,31] Furthermore, invasion and migration are two metastatic traits of cancer cells.[32] Our data displayed that downregulation of miR-149* obviously increased the number of invasive and

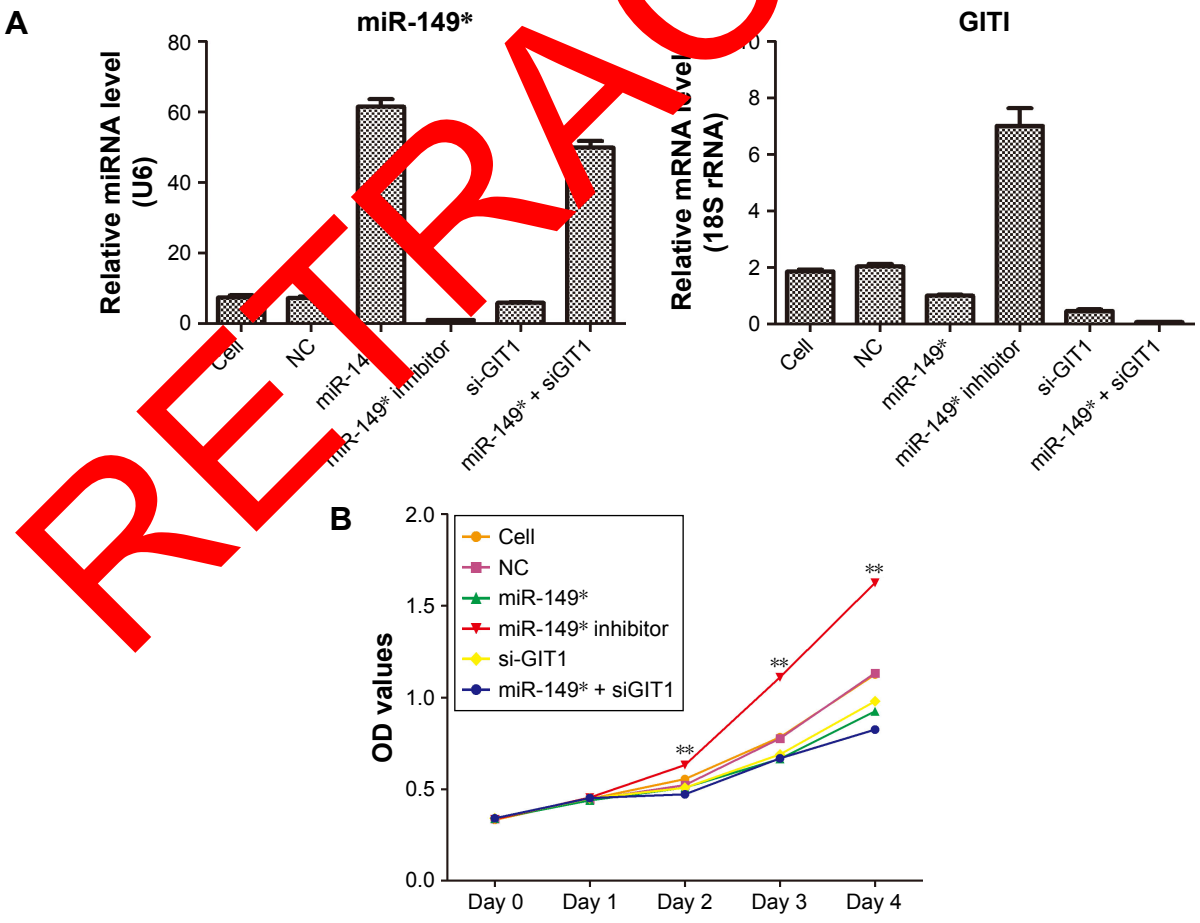

Figure 3 (*Continued*)

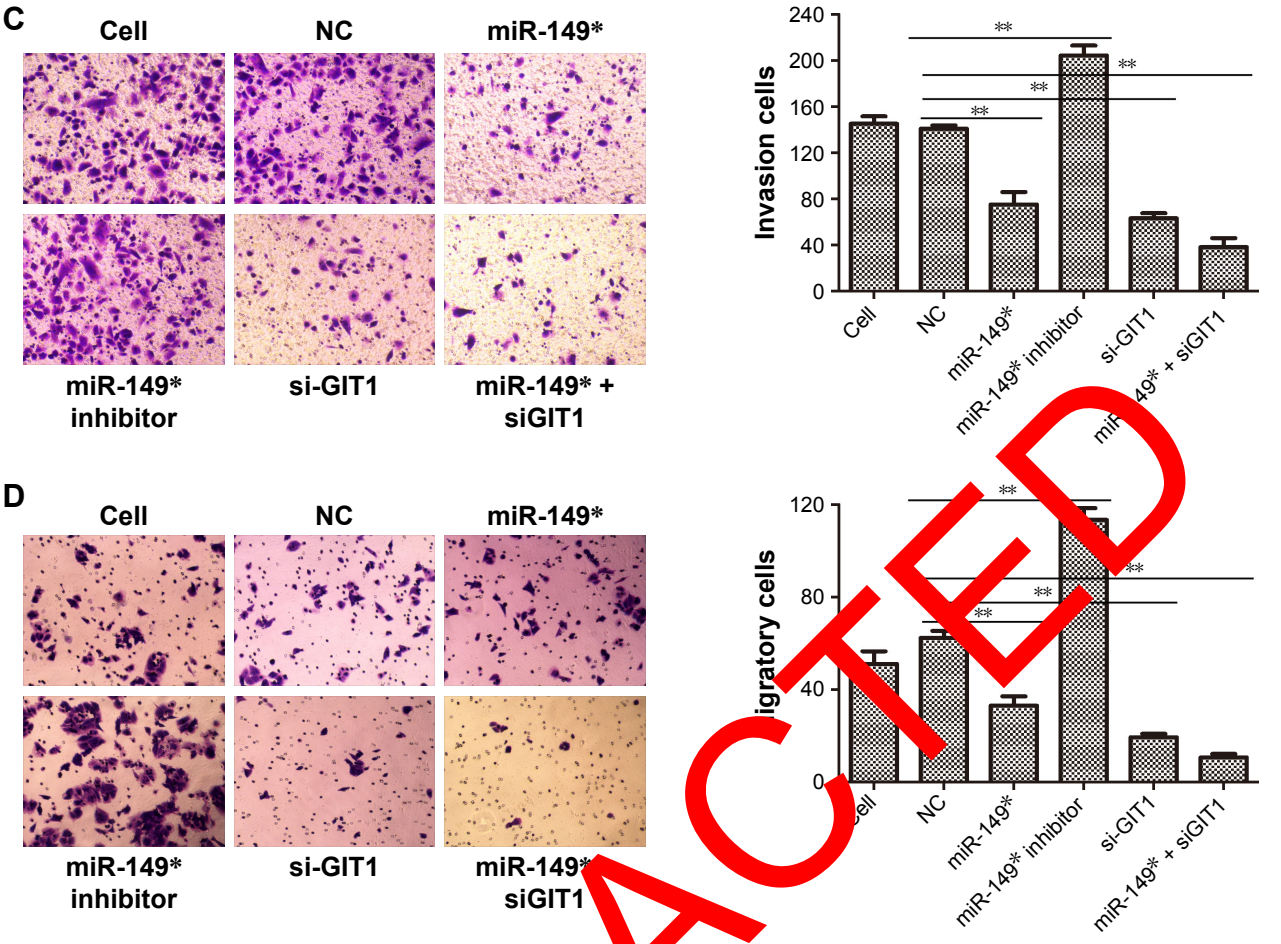

Figure 3 The effects of miR-149* and GIT1 on cell proliferation, i[illegible]d migrat[illegible].

Notes: (**A**) The miR-149* and GIT1 expressions were measur[illegible]by qRT-P[illegible] to demo[illegible]trate the transfection efficiency of NC, miR-149*, miR-149* inhibitor, si-GIT1, and miR-149* + siGIT1 plasmids. (**B**) Cell proliferation was perf[illegible]ed by MT[illegible]ssay. $^{**}P<0$[illegible]. (**C**) A Matrigel transwell invasion assay was used to evaluate the invasive ability of MDA-MB-231 cells subjected to different plasmids' transfe[illegible]n. Re[illegible]s of the invasion assays for MDA-MB-231 cells are displayed in the left panel, while statistical histogram is exhibited in the right panel. $^{**}P$[illegible].01. (**D**) [illegible]answell migration assay was applied to evaluate the migrative ability of MDA-MB-231 cells subjected to different plasmids' transfection. Representative i[illegible]ges of the inva[illegible]n assays for MDA-MB-231 cells are displayed in the left panel, while statistical histogram is exhibited in the right panel. $^{**}P<0.01$.

Abbreviations: MTT, 3-(4,5-dimethylthiazol[illegible]yl)-2,5-[illegible]enyltetrazolium bromide; NC, negative control; OD, optical density; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

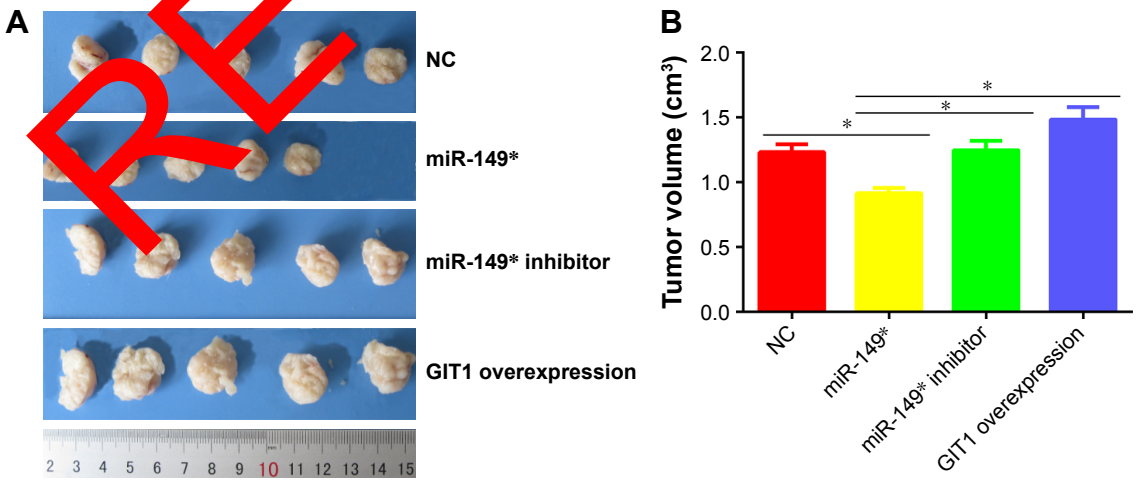

Figure 4 The growth of tumors in different groups.

Notes: (**A**) The tumor sizes were measured at the 35th day after being inoculated with stable transfected MDA-MB-231 cells. (**B**) The statistical histogram of tumor volume. $^{*}P<0.05$.

Abbreviation: NC, negative control.

migratory cells, whereas the number of invasive and migratory cells after miR-149* induction or GIT1 knockout was apparently decreased. Currently, accumulating researchers uncovered that abnormal expression of miRNAs contributed to tumor metastasis, which further resulted in the deterioration of breast cancer.[33,34] For example, miR-340 impeded cell invasion and migration by targeting Rho-associated protein kinase 1 (ROCK1), catenin beta 1 (CTNNB1), and c-MYC in breast cancer, which implied the possible inhibitory effect of miR-340 on breast cancer metastasis.[10] In addition, GIT1 has been known to have a positive correlation with tumor metastasis characterized by cell migration, invasion, focal adhesion, and angiogenesis mainly due to its structural properties, which contained coiled-coil, Spa homology, focal adhesion targeting (FAT) domain of focal adhesion kinase (FAK), FAT homology (FAH), paxillin, and Hic-5 LD3-binding domains that control the rearrangement of the actin cytoskeleton and eventually mediate cell invasion, migration, and adhesion.[35,36] For instance, miR-34c downregulation in breast cancer cells resulted in the upregulation of GIT1, which in turn enhanced the migration and invasion of breast cancer and ultimately caused poor survival rates of breast cancer patients.[37] Thus, these findings concluded that restored miR-149* might conspicuously ameliorate the [illegible] metastasis via the modulation of GIT1. Finally, to further confirm the influences of miR-149*/GIT1 link in breast cancer growth, tumor xenograft model in nude mice was established by injecting stable transfected MDA-[illegible]B-231 cells. It was observed that augmented miR-149* remarkably inhibited tumor growth, while elevated GIT1 with miR-149* mimic and GIT1 overexpression treatment markedly improved tumor growth. Hence, our data indicated that miR-149* may be participated in breast tumorigenesis via regulating GIT1.

Conclusion

We have established a new miR-149*/GIT1 link in breast cancer, which demonstrated that the loss of miR-149* directly caused the high expression level of GIT1, which in turn endowed breast cancer cells with rapidly improved cell proliferation, cell invasion, and migration capacity in vivo, as well as tumor growth in vitro. Taken together, the restoration of miR-149* and/or inhibition of GIT1 expression had the potential to serve as a novel therapeutic target in breast cancer.

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Disclosure

The authors report no conflicts of interest in this work.

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