Targeting of TLE3 by miR-3677 in human breast cancer promotes cell proliferation, migration and invasion

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Abstract. Numerous studies have indicated an important function of microRNAs (miRs) in breast cancer (BC) progression, oncogenesis and metastasis. However, the function of miR-3677, which has been revealed to be upregulated in BC [The Cancer Genome Atlas (TCGA) data], has not been investigated to date. In the present study, miR-3677 was revealed to be upregulated in BC as determined using TCGA. miR-3677 was significantly upregulated in BC tissues and cell lines compared with those noted in adjacent non-cancerous tissues and primary normal breast cells (P<0.05). The overexpression of miR-3677 promoted the cell proliferation, migration and invasion of BC cells. Using bioinformatics algorithms and luciferase assays, a novel target gene for miR-3677, namely transducin-like enhancer of Split3 (TLE3), was identified. Silencing of TLE3 in miR-3677-transfected BC cells suppressed their proliferation and migration. An inverse correlation was observed between miR-3677 and TLE3 expression levels in human BC tissues. In conclusion, the present study demonstrated that miR-3677 promoted BC cell proliferation, migration and invasion by inhibiting TLE3 expression, which provided a novel mechanism and a promising therapeutic target for patients with BC.

Introduction

Human breast cancer (BC) has been reported as one of the most common carcinomas in women (1,2). However, the causes of human BC development and progression are largely unknown. Therefore, understanding the molecular mechanisms of human BC is an active area of research, as they are important for developing better diagnostic strategies and novel approaches to molecular therapeutics for human BC.

MicroRNAs (miRNAs/miRs) are a class of small RNAs that serve essential functions in various physiological and pathological processes (3-5). A large body of evidence has demonstrated that the dysregulation of miRNA expression has been identified in a number of different types of cancer (6‑8). Compelling evidence has indicated that miRNAs are novel modulators of cancer progression and novel targets for cancer therapy, including BC treatment (9-11). miR-137 was reported to suppress the cell growth of BC by reducing the levels of epidermal growth factor receptor (12). miR-520c inhibits BC cell migration and invasion by suppressing the expression levels of transforming growth factor-β receptor II (13). Mecsi et al suggested that miR-330-3p promotes the metastasis of human BC by targeting collagen and calcium binding EGF domains 1 (14). Another study by Wang et al (15) indicated that miR-217 promotes the proliferation and invasion of BC by repressing tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein-γ. miR-3677 correlates significantly with the survival time of patients with hepatocellular carcinoma (16‑18). However, the biological function of miR-3677 in BC remains yet to be fully investigated.

The aim of the current study was to systematically explore the precise role of miR-3677 in BC and elucidate the underlying mechanism.

Materials and methods

The cancer genome atlas (TCGA) dataset analysis. For the TCGA dataset, the miRNA expression data were downloaded from TCGA (http://tcga-data.nci.nih.gov/tcga/) on May 2nd, 2018. The mRNA expression data included 1,041 BC tumor samples and 88 breast tissue samples.

Clinical specimens. A total of 10 paired human BC tissues (age, 45±5 years; Luminal A: 4 and Luminal B: 6) and their matched adjacent non-tumor tissues were obtained from patients with BC and confirmed by a pathologist. The patients who provided these specimens were recruited at...
the Guangzhou First People's Hospital (Guangzhou, China) between January 2017 and August 2017. The use of human breast tissues was ethically approved by the ethics committee of the Guangzhou First People's Hospital. Written informed consent was obtained from all patients prior to the study. The collection and use of tissues were conducted according to the ethical standards stated in the Declaration of Helsinki.

**Cell culture.** The human BC cell lines SKBR3, BT549, MDA-MB453, MCF-7, MDA-MB231, ZR-75-1 and T47D were purchased from the Type Culture Collection of the Chinese Academy of Sciences. The cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA), 100 U/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen; Thermo Fisher Scientific, Inc.). Primary normal breast cells (NBECs) from mammoplasty material of a 32-year-old woman collected with written informed consent at Guangzhou First People's Hospital were cultured in the keratinocyte serum-free medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with epithelial growth factor, bovine pituitary extract and antibiotics (120 µg/ml streptomycin and 120 µg/ml penicillin). All cells were cultured in an atmosphere of 5% CO2 and 95% air at 37°C.

**Plasmids, small interfering RNA (siRNA) and transfection.** The miR-3677 mimic (HmiR0994-MR04), miR-3677 inhibitor (HmiR-AN1958-AM02) and their corresponding controls were purchased from GeneCopoeia, Inc. For the ectopic expression of transducin-like enhancer of Split3 (TLE3), TLE3-siRNAs (TLE3 siRNA#1: 5'-CCACACGTTCGACCCAA-3'; TLE3 siRNA#2: 5'-CCTCTGGATTCTGAACCA-3') and their negative controls (NC) were purchased from Guangzhou RiboBio Co., Ltd. MCF-7 and ZR-75-1 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences. The PCR primers were synthesized by GeneCopoeia, Inc. and the sequences used were as follows: Cyclin D1 forward, 5'-TCTCTTCCA AAAATGCCAGAG-3' and reverse, 5'-GGCGGATTGGAATGAACTT-3'; c-myc forward, 5'-TCAAGAGGCGCAACAC-3' and reverse, 5'-GGCCTTTTCTATTTGTTCCA-3'; and GAPDH forward, 5'-GACGGGCCGATCTTCTTGT-3' and reverse, 3'-CACACCCACCTACATT-5'. GAPDH was used as the reference gene used for normalization purposes. The relative expression levels were calculated using the 2^{-ΔΔCt} method. All experiments were performed in triplicate.

**Colony formation assay.** The transfected cells (5x10^5) were seeded on the top of an agar layer consisting of 0.5 ml agarose and cultured in RPMI-1640 supplemented with 10% FBS in the presence of 5% CO2 at 37°C. Following two weeks of incubation, the cell colonies were fixed in 4% paraformaldehyde at room temperature for 1 h and stained with 1% crystal violet (Sigma-Aldrich; Merck KGaA). Colony formation efficiency was examined by counting the number of colonies at 4°C overnight with BrdU antibodies (1:500; cat no. 61273; Upstate Biotechnology, Inc.) according to the manufacturer's protocol. After incubation for 1 h at 37°C with horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat. no. ab150077; Abcam), image gray images were acquired using a laser scanning microscope (Axioskop 2 plus; Carl Zeiss AG).

**RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from samples and cells using the TRIzol® kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was synthesized using the miScript II RT kit (Qiagen, Inc.) according to the manufacturer's instructions. The mRNA expression was quantified with the miRNA-specific TaqMan miRNA assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The relative miR-3677 expression levels following normalization to U6 small nuclear RNA were calculated using the following formula: 2^{-ΔΔCt} = 2^{-[(Ct_{target} - Ct_{U6})_treatment] - (Ct_{target} - Ct_{U6})_control].}

To examine the mRNA expression levels, total RNA was isolated from fresh tissues and cells using the TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was synthesized from the extracted RNA using Promega M-MLV cDNA synthesis kit (Promega Corporation) according to the manufacturer's instructions. The gene expression levels were examined using SYBR kit (Qiagen, Inc.) using a Light Cycler system (Roche Diagnostics). The thermocycling conditions were as follows: 95°C for 30 sec, followed by 40 cycles of amplification at 95°C for 5 sec, 59°C for 30 sec and 72°C for 30 sec. The PCR primers were synthesized by GeneCopoeia, Inc. and the sequences used were as follows: Cyclin D1 forward, 5'-TCTCTTCCA AAAATGCCAGAG-3' and reverse, 5'-GGCGGATTGGAATGAACTT-3'; c-myc forward, 5'-TCAAGAGGCGCAACAC-3' and reverse, 5'-GGCCTTTTCTATTTGTTCCA-3'; and GAPDH forward, 5'-GACGGGCCGATCTTCTTGT-3' and reverse, 3'-CACACCCACCTACATT-5'. GAPDH was used as the reference gene used for normalization purposes. The relative expression levels were calculated using the 2^{-ΔΔCt} method. All experiments were performed in triplicate.

**Bromodeoxyuridine (BrdU) labeling and immunofluorescence.** Transfected cells were grown on cover slips (Thermo Fisher Scientific, Inc.), fixed with 4% paraformaldehyde at room temperature for 1 h and incubated with 10 µM BrdU for 1 h Subsequently, the cells were stained at 4°C overnight with BrdU antibodies (1:500; cat no. 61273; Upstate Biotechnology, Inc.) according to the manufacturer's protocol. After incubation for 1 h at 37°C with horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat. no. ab150077; Abcam), gray images were acquired using a laser scanning microscope (Axioskop 2 plus; Carl Zeiss AG).

**Transwell assay.** At 48 h following transfection, 1x10^5 transfected BC cells were seeded in 8-µm pore size Transwell chambers (Corning, Inc.) for the Transwell assay. The cells were cultured in RPMI-1640 medium supplemented with 2% FBS, and 600 µl RPMI-1640 medium supplemented with 10% FBS was added to the lower chamber. The chambers were incubated at 37°C for 24 h. The cells on the lower surface of the inserts were fixed with methanol for 15 min at room temperature and subsequently stained with 1% crystal violet for 15 min at room temperature. The number of cells were counted using a light microscope (Olympus Corporation) at x100 magnification A total of 5 fields from each well were randomly selected for quantification.

**Wound healing assay.** Transfected MCF-7 and ZR-75-1 cells (5x10^5) were seeded into six-well plates, and grown to 100% confluence. The confluent monolayer of the cells was scratched with a 200 µl tip. Subsequently, the cells were incubated with serum-free medium for 24 h after being gently washed with PBS at 37°C. Wound closure was measured at
different intervals using Image Pro-Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

**Western blotting.** Equal quantities of protein were extracted from MCF-7 and ZR-75-1 cells using RIPA buffer (Beyotime Institute of Biotechnology). The protein concentration was measured by bicinchoninic acid assay and equal amounts of protein (50 μg) were separated using 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked in TBS containing 0.5% Tween-20 with 5% milk for 2 h, and subsequently incubated overnight at 4°C using anti-TLE3 (cat. no. ab94972; 1:1,000; Abcam, Cambridge, UK), anti-cyclin D1 (cat. no. 2978; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-α-tubulin antibodies (cat. no. T6199; 1:5,000; Sigma-Aldrich; Merck KGaA). Following washing with Tris-buffered saline with 0.5% Tween-20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. sc-51948; 1:5,000; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. The membranes were visualized using an enhanced chemiluminescence detection reagent kit and analyzed with ImageJ 1.8.0 (National Institutes of Health) according to the manufacturer's protocol.

**Luciferase assays.** TargetScan 6.2 (http://www.targetscan.org/vert_61/) was used to identify the prospective targets of mir-3677. TLE3 was selected as a potential target of mir-3677. The wild-type 3′-untranslated region (UTR) of TLE3 mRNA was subcloned into the pGL3 vector (Promega Corporation, Madison, WI, USA). The cells were co-transfected with 80 nmol/l mir-3677 or 80 nmol/l mir-3677-mut and 200 ng wild-type vectors in the presence of 1 ng pRL-TK Renilla plasmid. The transfections were performed using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Following 48 h of incubation at 37°C, the activities of Renilla and Firefly luciferase in the cell lysates were measured using the Dual Luciferase Reporter Assay System (Promega Corporation).

**Statistical analysis.** Data are presented as the mean ± SD. All statistical analyses were performed using the SPSS 17.0 (SPSS, Inc.) or GraphPad Prism software (version 6.0; GraphPad Software, Inc.). A two-tailed paired Student's t-test were used to evaluate the differences between two groups of data. One-way analysis of variance followed by Tukey's test for multiple comparisons. Spearman's correlation was used to analyze the relationship between miR-3677 and TLE3 expression. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Upregulation of miR-3677 expression in BC tissues and BC cell lines.** In order to investigate the miR-3677 expression in BC, data from TGCA database were obtained for BC (n=1,041) and normal (n=88) samples. Following data analysis, miR-3677 was revealed to be significantly upregulated in BC samples compared with the corresponding expression in normal tissues (P<0.001; Fig. 1A). The data from the RT-qPCR assays demonstrated that the mRNA expression levels of miR-3677 in BC tissues were considerably higher compared with those noted in the corresponding non-tumor tissues (Fig. 1B). Subsequently, the miR-3677 expression was investigated in BC cells (SKBR3, BT549, MDA-MB453, MCF-7, MDA-MB231, ZR-75-1 and T47D). The results indicated that the miR-3677 expression levels in all eight tested BC cell lines were significantly higher compared with those noted in NBECs (P<0.05; Fig. 1C). These results suggested that miR-3677 was upregulated in BC, which may be associated with BC development.

**miR-3677 promotes BC cell proliferation and cell migration.** To further investigate the biological function of miR-3677 in BC, MCF-7 and ZR-75-1 cells were used to construct miR-3677-overexpressing and knockdown cell lines. These tools were used for further phenotypic and functional studies. Following incubation of the cells for 48 h post-transfection, the transfection efficiency was confirmed by RT-qPCR (Figs. 2A and 3A). The colony formation assay indicated that overexpression of miR-3677 significantly promoted the cell colony formation activity of BC compared with the vector, while miR-3677 inhibition exhibited the opposite results (P<0.05; Figs. 2B and 3B). The BrdU proliferation assay indicated that miR-3677 overexpressing cells resulted in a significantly increased number of BrdU-positive BC cells compared with that of the miR-control group, which was consistent with the colony formation results (P<0.05). In contrast to miR-3677 overexpression, miR-3677 inhibition exerted the opposite effects in BC cells (P<0.05; Figs. 2C and 3C). Furthermore, miR-3677 overexpression in MCF-7 and ZR-75-1 BC cells significantly facilitated cell migration (P<0.05; Fig. 2D and E), whereas miR-3677 inhibition in those cells resulted in a significantly decreased migratory activity compared with that of the control cells (P<0.05; Fig. 3D and E). Taken collectively, these data indicated that miR-3677 exhibited a tumor promoting role in BC.

**miR-3677 directly targets TLE3 by binding to its 3′-UTR.** To investigate the mechanism underlying the effects of miR-3677 on cell proliferation and cell metastasis, TargetScan 6.2 was used to identify the prospective targets of miR-3677. TLE3 was selected as a potential target of miR-3677 (19,20) (Fig. 4A). RT-qPCR and western blotting indicated that TLE3 expression levels were significantly downregulated in the miR-3677-overexpressing MCF-7 and ZR-75-1 cells compared with the vector cells (P<0.05), whereas they were significantly upregulated in the cells transfected with the miR-3677 inhibitor compared with the NC cells (P<0.05; Fig. 4B and C). To further confirm the regulation of TLE3 by miR-3677, luciferase reporter assays were used to examine whether miR-3677 directly binds to the TLE3 3′UTR sequence. Co-transfection of miR-3677 with the pGL3-TLE3-3′UTR luciferase reporter plasmid caused a significant decrease in luciferase activity compared with the vector cells, whereas miR-3677 inhibition resulted in significantly increased luciferase activity in MCF-7 and ZR-75-1 cells compared with the NC cells (P<0.05; Fig. 4D). These results suggested that miR-3677 directly targeted TLE3 in BC cells.

The effects of miR-3677 on the expression levels of genes that regulate cell proliferation and migration, including cyclin D1 and c-myc, were examined. RT-qPCR and western
blotting revealed that the mRNA and protein expression levels of cyclin D1 and c-myc were significantly upregulated in miR-3677-transfected cells, whereas they were downregulated in the cells transfected with the miR-3677 inhibitor compared with those in NC-transfected cells (P<0.05; Fig. 4E and F).
Silencing of TLE3 reverses the suppression of cell proliferation and cell migration in BC cells with the miR-3677 inhibitor.

To confirm the contribution of miR-3677 to suppressing TLE3 levels in BC, loss-of-function studies were performed by transfecting siRNA-TLE3 into miR-3677 inhibitor-transfected MCF-7 and ZR-75-1 cells. Western blotting indicated that the knockdown of TLE3 suppressed miR-3677 inhibitor-induced TLE3 expression (Fig. 5A). A colony formation assay illustrated that miR-3677 inhibitor-transfected MCF-7 and ZR-75-1 cells that were also transfected with TLE3 siRNAs formed a significantly greater number of colonies compared with those transfected with NC (P<0.05; Fig. 5B). Furthermore, the BrdU assays indicated a significant increase in the positive MCF-7 and ZR-75-1 cells that were transfected with the miR-3677 inhibitor following additional transfection with TLE3 siRNAs compared with those transfected with NC (P<0.05; Fig. 5C). The result of the migration assays indicated significantly increased migratory activity in miR-3677 inhibitor-transfected MCF-7 and ZR-75-1 cells that were also transfected with TLE3 siRNAs compared with those transfected with NC (P<0.05; Fig. 5D). The data confirmed that miR-3677 promoted BC cell proliferation and cell migration by repressing TLE3 expression.

Expression levels of miR-3677 and TLE3 exhibit an inverse correlation in human BC tissues. A total of 20 clinical human BC tissues and 20 adjacent samples were used in order to assess the expression levels of TLE3. The mean expression levels of TLE3 were significantly downregulated in human BC tissues compared with those noted in non-cancerous biopsy samples (P<0.001; Fig. 6A). A correlation analysis was performed between TLE3 and miR-3677 expression levels in the BC tissues. The results revealed a significant inverse correlation between TLE3 and miR-3677 expression levels (2-tailed Spearman's correlation, r=-0.705; P<0.001; Fig. 6B).

Discussion

miRNAs bind to the 3'-UTR of their target mRNAs and subsequently repress their expression. Accumulating evidence suggests that they may serve an essential function in the cellular processes associated with tumor progression,
including cell proliferation, migration, invasion and apoptosis (21-24).

In the present study, the function of miR-3677 in BC cell proliferation and migration was investigated, and the potential underlying molecular mechanism was identified. One previous study has reported that miR‑128 regulates glucose metabolism and cell proliferation in triple-negative BC (25). Lu et al (26) indicated that miR-140-5p was significantly downregulated in BC, and that it may suppress invasion and angiogenesis by targeting vascular endothelial growth factor A. miR-190 has been demonstrated to inhibit BC metastasis by regulating transforming growth factor-β-induced epithelial-mesenchymal transition (27). However, the underlying mechanism by which miR-3677 modulates BC carcinogenesis remains obscure. The
The present study indicated that the expression levels of miR-3677 were increased significantly in BC tissues and cells compared with those noted in adjacent non-cancerous tissues and NBECs, suggesting that miR-3677 may function as a potential oncogene in this type of cancer. Furthermore, the overexpression of miR-3677 promoted the cell proliferation and colony formation activity of BC, suggesting that miR-3677 promoted BC cell migration and metastasis.

To add insight into the molecular mechanisms of miR-3677, bioinformatics analysis was performed and TLE3 was identified as a potential target of miR-3677. TLE3 is a full-length member of the human TLE family and functions as a transcriptional co-repressor during cell differentiation, cell metabolism and tumorigenesis (28-30). In colorectal cancer, TLE3 is able to suppress colorectal cancer proliferation, partly via inhibition of the mitogen-activated protein kinase (MAPK) pathway.
kinase and protein kinase B signaling pathways (31). The ubiquitination and degradation of TLE3 by ring finger protein 6 results in the activation of the Wnt/β-catenin pathway in colorectal carcinogenesis (32). In addition, patients with ovarian carcinoma with a high TLE3 expression indicated a favorable response to taxane-containing chemotherapy regimens (33). In the present study, TLE3 was identified as the target of miR-3677 by luciferase reporter assays. Furthermore, a negative correlation between miR-3677 and TLE3 expression levels was identified in human BC tissues, indicating that miR-3677 may suppress the cell proliferation and metastasis of BC, at least in part, by downregulating the levels of TLE3.

In the present study, evidence was revealed that indicated miR-3677 may be involved in BC cell proliferation and metastasis by targeting TLE3. Therefore, these results provide novel insight into the function of miR-3677 in BC and imply that miR-3677 may be a potential therapeutic agent for BC treatment.

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Availability of data and materials
All data generated and analyzed during this study are included in this published article.

Authors' contributions
LP and BX conceived the study. LP wrote the manuscript. LP and BX designed and revised the manuscript. XD, XG, JZ, GR and FS analyzed and interpreted the data. FS, JF and WC assisted in data analysis. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate
The present study was approved by the Ethics Committee of the Guangzhou First People's Hospital (Guangzhou, China). Written informed consent was obtained from all patients prior to the study.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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