Abstract. We have previously shown that dysregulation of miR-21 functioned as an oncomiR in breast cancer. The aim of the present study was to elucidate the mechanisms by which miR-21 regulate breast tumor migration and invasion. We applied pathway analysis on genome microarray data and target-predicting algorithms for miR-21 target screening, and used luciferase reporting assay to confirm the direct target. Thereafter, we investigated the function of the target gene phosphoinositide-3-kinase, regulatory subunit 1 (α) (PIK3R1), and detected PIK3R1 coding protein (p85α) by immuno-histochemistry and miR-21 by RT-qPCR on 320 archival paraffin-embedded tissues of breast cancer to evaluate the correlation of their expression with prognosis. First, we found that PIK3R1 suppressed growth, invasiveness, and metastatic properties of breast cancer cells. Next, we identified the PIK3R1 as a direct target of miR-21 and showed that it was negatively regulated by miR-21. Furthermore, we demonstrated that p85α overexpression phenocopied the suppression effects of antimiR-21 on breast cancer cell growth, migration and invasion, indicating its tumor suppressor role in breast cancer. On the contrary, PIK3R1 knockdown abrogated antimiR-21-induced effect on breast cancer cells. Notably, antimiR-21 induction increased p85α, accompanied by decreased p-AKT level. Besides, antimiR-21/PIK3R1-induced suppression of invasiveness in breast cancer cells was mediated by reversing epithelial-mesenchymal transition (EMT). p85α downregulation was found in 25 (7.8%) of the 320 breast cancer patients, and was associated with inferior 5-year disease-free survival (DFS) and overall survival (OS). Taken together, we provide novel evidence that miR-21 knockdown suppresses cell growth, migration and invasion partly by inhibiting PI3K/AKT activation via direct targeting PIK3R1 and reversing EMT in breast cancer. p85α downregulation defined a specific subgroup of breast cancer with shorter 5-year DFS and OS, which may require more aggressive treatment.

Introduction

Breast cancer is a heterogeneous group of malignant tumors (1). Clinicopathological surrogate definitions of subtypes have been used for a long time. However, these subtypes even have subtypes considering their distinct responses to available therapy and clinical outcomes (1,2). Although accumulating evidence supports the use of multi-gene signatures to make distinctions among breast cancer patients, the cost of these assays remains prohibitive (3). The heterogeneity in tumor cell phenotypes make breast tumor categorization a challenging task (1).

The phosphoinositide 3-kinase (PI3K) pathway provides proliferative and migratory signals and is frequently activated in human breast cancer (4-7). The PI3K family of enzymes encompasses class I, II and III, with only class I being involved in human cancer (8-11). Class IA PI3K consists of a catalytic subunit (p110α as a key subunit) and a regulatory subunit (p85α as a key subunit decoded by PIK3R1) (11-13). When lacking upstream signals, p85 stabilizes p110 and suppress its catalytic activities (14). Uchino et al (7) reported that PIK3R1 was significantly downregulated in MDA-MB-231 cells and MCF-7 invasive clone compared with MCF-7 cells, thereby possibly contributing to metastasis development. Another study demonstrated that p85α downregulation was an inde-
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pendent prognostic marker in breast cancer (15). Although the importance of the PI3K/AKT pathway in breast cancer is well known, the function of p85α in breast cancer has not been widely studied.

miR-21-5p (previously named miR-21) is one of the most overexpressed miRNAs in numerous malignancies (16-19). miR-21 targets many important tumor suppressors to promote breast cancer growth, proliferation, migration and metastasis (20-22). We have previously shown that miR-21 was overexpressed in breast cancer and associated with inferior survival (23). We have reported on human genome microarray to screen potential targets of miR-21 (24).

In the present study, to elucidate the mechanisms by which miR-21 regulate breast tumor migration and invasion, we applied pathway enrichment analysis and target-predicting algorithms for the screening target of miR-21. PIK3R1 was predicted to be a functional target of miR-21. We further investigated the regulation of PIK3R1 coding protein p85α by miR-21, the impact of changes in antimiR-21 mediated p85α expression and the clinicopathological and prognostic significance of p85α in breast cancer patients.

Materials and methods

Cell lines. Human breast cancer cell lines (MCF-10A, MDA-MB-231 and BT-474) were purchased from the American Type Culture Collection and cultured according to specifications. Human breast cancer cell lines (MCF-7, BT-549, T47D and SK-BR-3) were purchased from the Cell Bank of Chinese Academy of Sciences. All cells were used within 2 months after resuscitation of frozen aliquots.

Quantification of miRNA and mRNA. Total RNA was isolated from cells and tissues using the Total RNA Purification kit (Norgen Biotek Corp., Thorold, ON, Canada). miR-21 expression was assessed by quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis using microRNA PCR system (Exiqon A/S) according to the manufacturer's instructions. RT-qPCR was utilized to analyze expression changes of potential miR-21 targets as previously described (23). Primers for PCR amplifications (Table I) were designed using Primer5.0 Input (version 0.4.0). Relative mRNA levels were calculated using the 2-ΔΔCT method (25).

Luciferase reporter assay. The 3'-untranslated region (UTR) of PIK3R1 containing the putative miR-21 target sites was amplified by PCR from genome DNA derived from HEK293T cells. The synthetic mutant 3'-UTR of PIK3R1 was produced by PCR, and then the PCR products were cloned into pCI-neo vector. After digestion by XhoI and NolI, the fragment containing 3'-UTR of PIK3R1 was cloned into pCI-neo vector (Promega, Madison, WI, USA). All inserts were sequenced to verify polymerase fidelity. The PCR primers are listed in Table I. HEK293T cells were cultured in 24-well plates and cotransfected with 200 ng of pCI-neo vector containing 3'-UTR of PIK3R1 and 50 nM of miRNA mimic (Exiqon A/S) per well. Transfections were performed using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). The luciferase analysis was performed 48 h later using the Dual-luciferase reporter assay system (cat. no. E1910; Promega).

Table I. Sequences of RNA and DNA oligonucleotides.

| Name                  | Sense strand/sense primer (5'-3')   | Antisense strand/antisense primer (5'-3') |
|-----------------------|------------------------------------|-----------------------------------------|
| Primers for RT-PCR    | GGTTCGAGCTACATGCAGT               | GGCCGAACAATGACAGTCG                    |
| PIK3R1 18s rRNA      | CCCTGGCAGAGCCTAGA                 | GGCGGCGGCTGAGCCTAGA                    |
| PK3R1 primers        | TTTGCCGAGCCCTATAACT               | TGCATATACTGGGTAGGCTAGT                 |
| PK3R1 siRNA duplexes | CAAGG AGU UUC AGU UUC AGU ACT U   | CAAGG AGU UUC AGU UUC AGU ACT U        |
| Control siRNA        | UUCAU AAA UAC AUA UAA AAA UCA TT    | UUCAU AAA UAC AUA UAA AAA UCA TT        |
| RT, reverse transcription primer | F, forward primer; R, reverse primer | F, forward primer; R, reverse primer |
CO2 for 2 days post-transfection. After 2 weeks, plates were assayed. Briefly, cells were plated on 6-well plates at 100 and 200 cells/well in triplicate and incubated at 37˚C under 5% background absorbance (medium without cells) subtracted. For this assay cells were plated at 10,000 cells/well in triplicate for each transfection condition and time-point. Raw values were averaged, and background absorbance (medium without cells) subtracted. Absorbance was measured by MTS-formazan reduction using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) exhibiting no significant sequence similarity to human, mouse or rat gene sequence served as a negative control. Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For PIK3R1 overexpression, lentivirus was produced by transfecting HEK 293T packaging cells in DMEM (HyClone, Logan, UT, USA; cat. no. SH30022.01B) with a 3-plasmid system. DNA for transfection was prepared by mixing pHelper 1.0, pHelper 2.0 and pLVX-IRES-Neo-PIK3R1. The empty vector pLVX-IRES-Neo was purchased from Clontech Laboratories (Mountain View, CA, USA; cat. no. 632184), and the plasmid pLVX-IRES-Neo-PIK3R1 was generated by insertion of PIK3R1 sequence. MDA-MB-231 cells were transduced with lentivirus in the presence of 6 µg/ml polybrene (Sigma-Aldrich) for 24 h. Cells were then selected for 7 days in 2.5 mg/ml neomycin. Overexpression of PIK3R1 was confirmed by western blot analysis.

Cell viability and clonogenic assays. Cell growth and viability were measured by MTS-formazan reduction using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) at 24, 48, 72 and 96 h post-transfection with a vector (empty pcDNA3.1) or PIK3R1. Absorbance was measured at 490 nm using a Multiskan plate reader (Thermo Labsystems, Beverly, MA, USA). Raw values were averaged, and background absorbance (medium without cells) subtracted. For this assay cells were plated at 10,000 cells/well in triplicate for each transfection condition and time-point. Raw values were averaged, and background absorbance (medium without cells) subtracted. The cellular effects of these manipulations were further investigated in MDA-MB-231 and BT-474 cells using clonogenic assays. Briefly, cells were plated on 6-well plates at 100 and 200 cells/well in triplicate and incubated at 37˚C under 5% CO₂ for 2 days post-transfection. After 2 weeks, plates were washed, fixed in 50% methanol and stained with 0.1% crystal violet and then the number of colonies was counted.

In vivo tumorigenicity assays. Five-week-old female BALB/c nude mice, provided by Shanghai Laboratory Animal Center, Chinese Academy Sciences (Shanghai, China) were used. Equivalent amounts of MDA-MB-231 cells transected with PIK3R1 or vector were injected subcutaneously (10⁷ cells/tumor) into the left axilla of nude mice. Cells were weighed, and the longest and the shortest diameters of the tumor were measured every day. The tumor volume (V) was calculated according to the following equation: V = axb²/2, where a is the longest diameter and b is the shortest diameter of the tumor (26). Thirty-six days after the initial injection, the animals were sacrificed and tumors were extracted and weighed. The ethics guidelines for investigations in conscious animals were followed in all experiments.

Wound healing/migration assay. To assay the migratory response of breast cancer cells to miR-21 inhibitor or PIK3R1 expression, the cellular effects of these manipulations were further investigated using a wound healing assay as previously described (24). Cells were allowed to reach confluence before dragging a 1-ml sterile pipette tip (Axygen Scientific, Inc., Union City, CA, USA) through the monolayer. Cells were washed with PBS to remove cellular debris and allowed to migrate for 48 h. Images were acquired at 0, 6, 24 and 48 h post-wounding with a digital camera system (Leica DFC480; Leica Microsystems, Bannockburn, IL, USA). Cell-free areas were measured with ImageJ software (National Institutes of Health, Bethesda, MD, USA) and were expressed as the percentage of migration compared to control, arbitrarily set at 100% (27). All experiments were carried out in triplicate.

In vitro invasion assay. Invasion of cells in vitro was assayed using the BD BioCoat Matrigel Invasion Chambers and Control Inserts (BD Biosciences, Bedford, MA, USA) respectively. Each well of a 24-well plate contained an insert with an 8-µm pore size polyethylene terephthalate membrane. Cells (1x10⁵ per Transwell) were suspended in serum-free DMEM and seeded into the upper chamber. DMEM containing 2% fetal bovine serum was then added to the bottom chamber of 24-well plates to serve as a chemoattractant. After 48 h of incubation, cells on the upper surface of the filter were removed, and cells that migrated to the lower surface were fixed and stained with 1% toluidine blue. For quantification of cell invasion, 10 fields per experimental condition were randomly selected as previously described (28) and micrographed with IX71 microscope (Olympus, Tokyo, Japan). Images are representative of at least three independent experiments.

Western blots. Cells were harvested and lysed in radioimmunoprecipitation buffer (Upstate Biotechnology, Inc., Lake Placid, NY, USA). Antibodies used for immunoblot analysis were p85α 1:1,000 (Cell Signaling Technology, 13666), p110α 1:1,000 (Cell Signaling Technology, 42336), p-AKT (Ser473) 1:2,000 (Cell Signaling Technology, 4060), AKT 1:1,000 (Cell Signaling Technology, 9272), E-cadherin 1:1,000 (Cell Signaling Technology, 3193), N-cadherin 1:1,000 (Cell Signaling Technology, 1316), vimentin 1:1,000 (Abcam, 92547), FSP1 1:1,000 (Cell Signaling Technology, 8457) were used as loading controls. All bands were detected using a SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

Cell transfection and transduction. For transient miR-21 knockdown, the LNA-anti-miR-21 or LNA-control (Exiqon A/S, Vedbaek, Denmark) were delivered at a final concentration of 50 nM using Lipofectamine 2000 (Invitrogen). For PIK3R1 knockdown, three siRNAs (Sigma-Aldrich, St. Louis, MO, USA) designed against PIK3R1 (GenBank accession no. NM_181523) were included (Table I). One control siRNA (Sigma-Aldrich) exhibiting no significant sequence similarity to human, mouse or rat gene sequence served as a negative control. Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

In vitro invasion assay. Invasion of cells in vitro was assayed using the BD BioCoat Matrigel Invasion Chambers and Control Inserts (BD Biosciences, Bedford, MA, USA) respectively. Each well of a 24-well plate contained an insert with an 8-µm pore size polyethylene terephthalate membrane. Cells (1x10⁵ per Transwell) were suspended in serum-free DMEM and seeded into the upper chamber. DMEM containing 2% fetal bovine serum was then added to the bottom chamber of 24-well plates to serve as a chemoattractant. After 48 h of incubation, cells on the upper surface of the filter were removed, and cells that migrated to the lower surface were fixed and stained with 1% toluidine blue. For quantification of cell invasion, 10 fields per experimental condition were randomly selected as previously described (28) and micrographed with IX71 microscope (Olympus, Tokyo, Japan). Images are representative of at least three independent experiments.

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Tissue specimens. Eligible patients were women with invasive breast cancer, no special type; operable; no previous chemotherapy; adequate formalin-fixed paraffin-embedded (FFPE) tumor specimens from the pre-treatment biopsy or surgery sample for representation in tissue microarrays (TMAs); outcome data available. Patients with distant metastases or a
history of a previous or concomitant malignancy were excluded. The archived FFPE tissues were obtained from the Department of Pathology, Guangdong General Hospital between 2009 and 2012. A consensus diagnosis of invasive breast cancer was confirmed by two expert pathologists according to the fourth edition of the World Health Organization (WHO) classification of tumors of the breast, published in 2012 (29). The surrogate definition of intrinsic subtypes of breast cancer was according to the St Gallen International Expert Consensus 2013 (3). The clinicopathological characteristics of the patients are summarized in Table II. Median follow-up time was 36 months (range, 5-68 months). The Research Ethics Committee of Guangdong General Hospital and Guangdong Academy of Medical Science reviewed and approved the study (no. GDREC2012022H) according to the principles expressed in the Declaration of Helsinki. The Research Ethics Committee specifically waived the need for informed consent for this retrospective study.

TMA construction and immunohistochemistry (IHC). TMA slides containing three representative 2.0-mm cores from each tumor of the cases were prepared with a tissue microarrayer (Beecher Instruments, Silver Spring, MD, USA). Immunohistochemical staining was performed using Real EnVision kit (K5007; Dako, Carpinteria, CA, USA) on an automated immunostaining instrument (Leica Bond-Max; Leica Microsystems GmbH, Wetzlar, Germany) according to the manufacturer’s instructions. Internal control cores were present in each TMA. Sections were subjected to staining protocols with the anti-PI3 kinase p85α antibody (EP380Y) (Abcam; cat. no. ab40755). A negative control was performed in all cases by omitting the primary antibody, which in all instances resulted in negative immunoreactivity. Positive immunohistochemical staining was defined as a brown cytoplasmic staining for p85α. A semi-quantitative intensity scale ranging from 0 (no staining) to 3+ (the most intense staining) was used by comparing neoplastic cells to adjacent breast cells belonging to normal terminal duct lobular units as previously described (15). p85α downregulation was defined by an IHC score 0, and p85α overexpression by an IHC score 1+ to 3+ (15). The localization and intensity of staining were assessed by two independent pathologists. Hormonal receptors were evaluated with the 1D5 antibody for the estrogen receptor (ER; Dako) and antibody PGR-1A6 for the progesterone receptor (PR; Dako). The human epidermal growth factor receptor 2 (HER2/neu) was detected with CB11 (Dako). Hormonal receptors and gene copy number of HER2 were assessed by IHC staining on 4-μm thick tumor sections from FFPE blocks.

Fluorescein in situ hybridization (FISH). HER2 amplification status was detected by PathVysion kit (Abbott) according to the manufacturer’s instructions. HER2 was defined as amplified when the FISH ratio was 2 or greater.

Statistical analysis. Statistical analysis was prepared using the Statistical Package of MedCalc statistical software (version 12.7.4; MedCalc Software, Mariakerke, Belgium) and Social Sciences (version 20.0; SPSS, Inc., Chicago, IL, USA). The receiver operating characteristic curves were constructed to estimate the optimal cut-off points for of p85α protein and miR-21 as the predictors for disease-free survival (DFS) and

| Characteristics | Patients (N=320) |
|-----------------|-----------------|
| Median age (range), years | 50 (25-91) |
| Clinical stage at diagnosis | |
| I | 109 | 34.1 |
| II | 144 | 45.0 |
| III | 67 | 20.9 |
| Tumor stage (size cm) | |
| T1 (≤2.0) | 157 | 49.1 |
| T2 (>2.0 to ≤5.0) | 131 | 40.9 |
| T3 (>5.0) | 26 | 8.1 |
| T4a | 6 | 1.9 |
| Nodal stage | |
| N0 (node negative) | 178 | 55.6 |
| N1 (1-3 positive nodes) | 83 | 25.9 |
| N2 (4-9 positive nodes) | 36 | 11.3 |
| N3 (≥10 positive nodes) | 23 | 7.2 |
| Histological grade | |
| Grade 1 | 16 | 5.0 |
| Grade 2 | 176 | 55.0 |
| Grade 3 | 128 | 40.0 |
| Subtypes of breast cancer | |
| Luminal A-like | 71 | 22.2 |
| Luminal B-like | 186 | 58.1 |
| HER2 positive (non-luminal) | 27 | 8.4 |
| Triple negative (ductal) | 31 | 9.7 |
| Not known | 5 | 1.6 |
| ER status | |
| Negative | 66 | 20.6 |
| Positive | 254 | 79.4 |
| PR status | |
| Negative | 78 | 24.4 |
| Positive | 242 | 75.6 |
| HER2 status | |
| Negative | 238 | 74.4 |
| Positive | 69 | 21.6 |
| Not known | 13 | 4.1 |
| Surgery | |
| Mastectomy | 281 | 87.8 |
| Breast conservation | 39 | 12.2 |
| Chemotherapy | |
| Neoadjuvant chemotherapy | 70 | 21.9 |
| Adjuvant chemotherapy | 165 | 51.6 |
| Not given | 85 | 26.6 |
| Targeted therapy | |
| Herceptin | 12 | 3.8 |
| No herceptin | 308 | 96.3 |

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2. "T4, tumor of any size with direct extension to the chest wall and/or to the skin."
Overall survival (OS). Pearson's Chi-square test and Spearman rank correlation analysis were used to determine association and correlation between variables. Survival analyses were plotted using Kaplan-Meier curves and compared using the log-rank test. Univariate and multivariate survival analyses were analyzed by Cox proportional hazards regression models. The results were considered statistically significant when two-sided \( P < 0.05 \).

**Results**

PIK3R1 suppresses growth, invasiveness and metastatic properties of breast cancer cells. PIK3R1 overexpression significantly reduced proliferation and colony formation capabilities in MDA-MB-231 and BT-474 cell lines as compared to control cells (Fig. 1A-C). In vivo study showed that at the 36th day, the average tumor volume in the PIK3R1 group...
PIK3R1 is a direct target of miR-21. We have previously identified miR-21 as an oncomiR in breast cancer and have used human genome microarrays to identify potential targets of miR-21 (24). In the present study, to biologically and metabolically interpret the array data, we applied pathway enrichment analysis with KEGG (http://www.genome.jp/kegg/), GenMAPP (http://www.genmapp.org/), and BioCarta (http://www.biocarta.com/), and identified a set of interesting gene sets including PIK3R1, NFKB2, STAT3, AK3, and AK4 (Table III).

To narrow down candidate target genes, we applied mRNA target-predicting algorithms (TargetScan, picTar, miRDB, PITA, and microRNA.org) based on the presence of binding sites in the 3'-UTR. All the five algorithms identified PIK3R1 as the potential target of miR-21.

Interestingly, p85α has previously been shown to exert tumor suppressor properties through negative regulation of growth factor signaling (30). PIK3R1 expression was significantly decreased by 18% in breast cancer tissues (31) and cell lines (7), and was associated with decreased survival in breast cancer patients (15). Therefore, we conducted analyses to determine whether miR-21 might target PIK3R1. First, we examined miR-21 and PIK3R1 mRNA in a range of metastatic (BT-474, MDA-MB-231 and BT-549), and non-metastatic (MCF-7, SK-BR-3 and T-47D) human breast cancer cell lines and breast epithelial cell line MCF-10A. All breast cancer lines tested, except SK-BR-3 and T-47D, exhibited elevated levels of miR-21 compared to MCF-10A cells, with the lowest levels in breast cancer cell lines and breast epithelial cell line MCF-10A. The number of invaded cells vs. controls, with the lowest levels of miR-21 (Fig. 1D; 3.46±1.43 g, P=0.046). Moreover, the average tumor weight in the mice expressing the invasive capabilities of MDA-MB-231 and BT-474 cell lines was significantly smaller than in the control group (Fig. 1D; 3.46±1.43 g, P=0.008). Moreover, the average tumor weight in the PIK3R1 group (1.78±0.105 g) was lower than that in the control group (1.82±0.102 g, P=0.046). PIK3R1 overexpression reduced the average percentage of wound healed in both MDA-MB-231 and BT-474 cell lines as measured at 48 h (Fig. 1F and G; P<0.001 for both lines as compared to control lines). We used the BD Biocoat Matrigel Invasion Assay to test the invasive capabilities of MDA-MB-231 and BT-474 cells expressing PIK3R1. For the two lines, PIK3R1 strongly reduced the number of invaded cells vs. controls, with the lowest percent invasion in PIK3R1-BT-474 lines (Fig. 1H and I; 4.4%). These data suggest that PIK3R1 plays an important role in the suppression of cell proliferation, migration and invasion of breast cancer cells.

Table III. Top three signaling pathways for PIK3R1 in breast cancer cells.

| Pathway analysis | Pathway name | Total | P-value | Q-value | Gene |
|------------------|--------------|-------|---------|---------|------|
| KEGG             | Regulation of actin cytoskeleton | 7     | 0.012   | 0.002   | LIMK1, SLC9A1, GNG12, WASF2, PIK3R1, ARPC4, ACTN4 |
|                  | Insulin signaling pathway | 5     | 0.024   | 0.003   | FLT1, PRKCI, PPP1R3C, PIK3R1, PHKA2 |
|                  | Apoptosis | 4     | 0.025   | 0.003   | APAF1, TRAF2, NFkB2, PIK3R1 |
| GenMAPP          | Lipid binding | 9     | 0.001   | <0.001  | PRKCI, ANXA6, SCP2, STARD3, WDFY1, ANXA2, PXIN, PIK3R1, BPI |
|                  | Kinase activity | 5     | 0.044   | 0.005   | ADCK4, GALK1, AK3, CARK1, PIK3R1, APAF1, IRF1, TRAF2, PIK3R1 |
|                  | Apoptosis | 4     | 0.023   | 0.003   | APAF1, IRF1, TRAF2, PIK3R1 |
| BioCarta         | Role of PI3K subunit p85 in regulation of actin organization and cell migration | 3     | 0.001   | <0.001  | ACTR2, PIK3R1, ARPC4 |
|                  | EGF signaling pathway | 3     | 0.004   | 0.001   | STAT3, PIK3R1, MEF2D |
|                  | PDGF signaling pathway | 3     | 0.004   | 0.001   | STAT3, PIK3R1, MEF2D |
|                  | Signaling of hepatocyte growth factor receptor | 3     | 0.009   | 0.002   | STAT3, PIK3R1, MEF2D |
|                  | Mechanism of gene regulation by peroxisome proliferators via PPARα | 3     | 0.020   | 0.003   | PPARBP, EHHADH, PIK3R1 |
transfected them with LNA-antimiR-21. Indeed, inhibition of miR-21 in breast cancer cells resulted in a 7- to 9-fold increase in \( \text{PIK3R1} \) mRNA levels (Fig. 3A) and an approximate 3-fold increase in protein (p85\( \alpha \)) levels (Fig. 3B). Furthermore, overexpression of miR-21 resulted in a 30-50% reduction in \( \text{PIK3R1} \) mRNA levels (Fig. 3A) and an approximate 30% reduction in protein levels (Fig. 3B) in both MDA-MB-231 and BT-474 cells. Concomitant with the increase in p85\( \alpha \), a decrease in
Figure 4. AntimiR-21-induced suppression of proliferation, clonogenicity, invasiveness, and metastatic properties of breast cancer cells is mediated by direct repression of PIK3R1. (A) MTS assays were conducted on breast cancer cells after transfection with antimiR-21 (50 nmol/l), antimiR-21 + PIK3R1-shRNA or control. At 48, 72 and 96 h, the antimiR-21 lines showed significantly reduced levels of proliferation as compared to control lines. PIK3R1-shRNA reversed the effect of antimiR-21 on cells. (B) Representative images depicting clonogenic assays performed with cells plated at 200 cells/well. (C) In MDA-MB-231 and BT-474 lines, antimiR-21 resulted in a decrease in colony number as compared to control lines. PIK3R1-shRNA reversed the effect of antimiR-21 on cells. (D) Representative images depicting cell migration assays. (E) Cell migration was quantitated as percentage of wound-healed area from corresponding control and transfected cells. (F) Invasion assays in these control and transfected cells. (G) For each cell line, antimiR-21 resulted in reduced invasion as compared to controls. PIK3R1 knockdown reversed the effect of antimiR-21 on cell migration in both cell lines. (H) MDA-MB-231 and BT-474 lines were transfected with PIK3R1, antimiR-21, antimiR-21 + PIK3R1-shRNA or control, followed by western blot analysis of the indicated EMT-related proteins. Relative E-cadherin, N-cadherin, vimentin, FSP1, snail and slug levels were normalized to the β-actin level. (I) Breast cancer lines were transfected with PIK3R1, antimiR-21, antimiR-21 + PIK3R1-shRNA or control, followed by RT-qPCR analysis of the indicated EMT-related mRNAs. Data represent mean ± SD. *P<0.05; **P<0.01.
PI3K pathway activation was observed, as evidenced by decreased p-AKT expression (Fig. 3C). These results suggest that miR-21-dependent proto-oncogene PI3K/AKT pathway is active in breast cancer cell lines.

Moreover, PIK3R1 overexpression phenocopied the suppression effects of LNA-antimiR-21 on cell proliferation and colony formation capabilities. Notably, PIK3R1 knockdown abrogated LNA-antimiR-21-induced suppression of cell proliferation and colony formation capabilities (Fig. 4A-C). LNA-antimiR-21 reduced the average percentage of wound healed in both cell lines as measured at 48 h (P<0.001). In BT-474 cells, PIK3R1 knockdown significantly abrogated LNA-antimiR-21-mediated cell migration (P=0.007). Although not in a statistically significant manner, PIK3R1 knockdown also abrogated LNA-antimiR-21-mediated cell migration in MDA-MB-231 cells (Fig. 4D and E). We used the BD Biocoat Matrigel Invasion Assay to test the invasive capability of MDA-MB-231 and BT-474 cells lacking miR-21. For these lines, LNA-antimiR-21 strongly reduced the number of invaded cells vs. controls, with the lowest percent invasion in the PIK3R1-BT-474 line (Fig. 4F and G; 4.4%). Furthermore, PIK3R1 knockdown significantly abrogated LNA-antimiR-21-mediated cell invasion in MDA-MB-231 (P=0.004) and BT-474 lines (P<0.001). Together, these data support the hypothesis that miR-21 by targeting PIK3R1 promotes breast cancer cell growth, invasion and migration.

Figure 5. Tissue microarray based immunohistochemical analysis of p85α expression in breast cancer tissues. (A) Representative sections for staining intensity -, +, ++ and +++ of p85α protein are shown. Images were taken at x40 and x200 magnification. (B) Breast cancer cells exhibited a weaker p85α expression (staining intensity ++) than surrounding residual normal duct lobular units (staining intensity +++). (C) miR-21 expression in p85α overexpression (p85α+) and p85α downregulation (p85α-) breast cancers was analyzed by RT-qPCR.

AntimiR-21 reverses the epithelial-mesenchymal transition (EMT) target PIK3R1 suppression of invasiveness in breast cancer. To determine whether antimiR-21/PIK3R1-induced suppression of invasiveness in breast cancer cells is mediated by reversing EMT, we transfected the MDA-MB-231 and BT-474 cell lines, which exhibit a mesenchymal phenotype, with antimiR-21 or PIK3R1. Transfection of breast cancer cells with antimiR-21 or PIK3R1 resulted in reversal of EMT, as evidenced by repression of the mesenchymal markers N-cadherin, vimentin, FSP1, snail and slug and induction of the epithelial marker E-cadherin. Furthermore, PIK3R1 shRNA reversed the effect of antimiR-21 or PIK3R1 on EMT (Fig. 4H and I).

downregulation in patient tumor specimens. To establish the relevance of our findings in the patient tumors, we analyzed the expression of miR-21 by RT-qPCR and p85α by IHC in 320 primary human invasive breast cancers, and the adjacent non-tumor-affected epidermis. Alteration of p85α was also
verified at the protein level by IHC staining on TMAs. Positive staining of p85α was found in the cytoplasm (Fig. 5A). Tumor cells showed p85α moderate expression, while residual normal mammary epithelial cells presented strong IHC staining intensity (Fig. 5B).

Staining scores and log2 of CT values were analyzed using MedCalc statistical software to determine the optimal survival cut-off points for dichotomizing expression of p85α protein and miR-21. The cut points correspond to the maximum Chi-square value of the Kaplan-Meier test for OS between groups above and below the cut-point threshold. p85α downregulation was found in 25 (7.8%) of the 320 breast cancer patients. miR-21 high expression was found in 119 (37.2%) of 320 patients. Next, we investigated the negative regulation of endogenous p85α protein by endogenous miR-21. Correlation analysis demonstrated that endogenous p85α protein levels were not statistically correlated with miR-21 in the patient tumor specimens (Fig. 5C; rs=-0.109, P=0.052, Spearman's correlation analysis).

**Correlation of p85α expression with breast cancer clinicopathological characteristics and prognosis.** p85α downregulation was associated with PR positive status (Table IV; P=0.047). No significant correlation was observed between p85α and

### Table IV. Correlation between p85α protein expression and clinicopathological parameters of breast cancer patients.

| Characteristics | p85α | miR-21 |
|-----------------|------|--------|
|                 | Overexpression (n=295) | Downregulation (n=25) | Low (n=201) | High (n=119) | P-value |
| **Clinical stage** | N (%) | N (%) | N (%) | N (%) | P-value |
| I               | 100 (34) | 9 (36) | 73 (36) | 36 (30) | 0.016 |
| II              | 134 (45) | 10 (40) | 96 (48) | 48 (40) | 0.213 |
| III             | 61 (21) | 6 (24) | 32 (16) | 35 (29) | 0.213 |
| **Tumor size (cm)** | | | | | |
| ≤2              | 144 (49) | 13 (52) | 97 (48) | 66 (55) | 0.213 |
| >2              | 151 (51) | 12 (48) | 104 (52) | 53 (45) | 0.213 |
| **Node** | | | | | |
| Negative | 164 (56) | 14 (56) | 118 (59) | 60 (50) | 0.149 |
| Positive | 131 (44) | 11 (44) | 83 (41) | 59 (50) | 0.149 |
| **Histological grade** | | | | | |
| 1               | 13 (4) | 3 (12) | 13 (6) | 3 (3) | 0.110 |
| 2               | 163 (55) | 13 (52) | 103 (51) | 73 (61) | 0.110 |
| 3               | 119 (40) | 9 (36) | 85 (42) | 43 (36) | 0.110 |
| **Subtypes of breast cancer** | | | | | |
| Luminal A-like | 63 (21) | 8 (32) | 43 (21) | 28 (23) | 0.095^a |
| Luminal B-like | 170 (58) | 16 (64) | 113 (56) | 73 (61) | 0.095^a |
| HER2 positive | 27 (9) | 0 (0) | 18 (9) | 9 (8) | 0.110 |
| Triple negative | 30 (10) | 1 (4) | 24 (12) | 7 (6) | 0.110 |
| Not known | 5 (2) | 0 (0) | 3 (2) | 2 (2) | 0.110 |
| **ER** | | | | | |
| Negative | 64 (22) | 2 (8) | 47 (23) | 19 (16) | 0.113 |
| Positive | 231 (78) | 23 (92) | 154 (77) | 100 (84) | 0.113 |
| **PR** | | | | | |
| Negative | 76 (26) | 2 (8) | 55 (27) | 23 (19) | 0.106 |
| Positive | 219 (74) | 23 (92) | 146 (73) | 96 (81) | 0.106 |
| **HER2** | | | | | |
| Negative | 218 (74) | 20 (80) | 154 (77) | 84 (71) | 0.591^b |
| Positive | 65 (22) | 4 (16) | 42 (21) | 27 (23) | 0.591^b |
| Not known | 12 (4) | 1 (4) | 5 (2) | 8 (7) | 0.591^b |

P-values were derived from Pearson's Chi-square test. Italics indicate significance. ^P-value Luminal A and B vs. ^P-value HER2-negative vs. HER2-positive and triple negative.
clinical stage, tumor size, node status, histological grade, ER or HER2 status. miR-21 overexpression was associated with high clinical stage (Table IV; P=0.016). No correlation was observed between miR-21 and other characteristic.

Next, we investigated the prognostic impact of p85α and miR-21 expression on breast cancer patients. The survival curves showed that p85α downregulation was significantly associated with inferior 5-year DFS and OS of breast cancer patients (Fig. 6A and B; DFS: P=0.005, OS: P=0.021; log-rank tests). Within early stage stratum, patients with p85α downregulation had inferior 5-year DFS and OS compared to those with p85α overexpression (Fig. 6C and D; P<0.001 for DFS, P=0.004 for OS, log-rank test). However, within the late stage stratum, p85α expression was not related with the patient survival (Fig. 6E and F). Consistent with our previous study in another cohort, high miR-21 expression was significantly associated with inferior 5-year DFS and 5-year OS in this cohort (DFS: P=0.035; OS, P=0.028).

In univariate analysis, p85α downregulation, high miR-21, high clinical stage, tumor size >2 cm, node positive, high histological grade and breast conservation were associated with inferior 5-year DFS and 5-year OS of the breast cancer patients (Table V). While, subtypes of breast cancer, hormone receptor status, HER2 status, and chemotherapy were not associated with inferior 5-year DFS or 5-year OS. Multivariate Cox regression model that incorporated significant factors in the univariate analyses showed that only p85α downregulation and high clinical stage maintained independent prognostic factors for both inferior 5-year OS and DFS (Table VI).

**Discussion**

In the present study, we present evidence that PIK3R1 is a direct miR-21 target. PIK3R1 phenocopies the effect of miR-21 knockdown. Furthermore, we expanded our previous findings that miR-21 knockdown suppresses cell growth, migration and invasion by inhibiting PI3K/AKT activation via targeting PIK3R1. AntimiR-21/PIK3R1-induced suppression of invasiveness in breast cancer cells is mediated by reversing
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Table V. Univariate Cox models for patients with invasive breast cancer.

| Characteristics                                      | OS                  | DFS                 |
|------------------------------------------------------|---------------------|---------------------|
|                                                      | HR  | 95% CI   | P    | HR  | 95% CI   | P-value |
| p85α overexpression vs. downregulation                | 3.06 | 1.13-8.31 | 0.028 | 2.68 | 1.30-5.54 | 0.008   |
| miR-21 low vs. high                                   | 2.47 | 1.08-5.65 | 0.033 | 1.80 | 1.03-3.12 | 0.038   |
| Clinical stage I vs. II vs. III                       | 4.20 | 2.15-8.20 | <0.001 | 3.59 | 2.35-5.50 | <0.001   |
| Tumor size (cm) ≤2 vs. >2                            | 3.44 | 1.28-9.27 | 0.015 | 3.82 | 1.95-7.46 | <0.001   |
| Node negative vs. positive                           | 5.38 | 2.00-14.45 | 0.001 | 3.76 | 2.02-6.98 | <0.001   |
| Histological grade 1 vs. 2 vs. 3                      | 2.57 | 1.19-5.55 | 0.017 | 2.25 | 1.47-3.45 | <0.001   |
| Subtypes of breast cancer                             | 1.25 | 0.77-2.04 | 0.371 | 1.01 | 0.72-1.43 | 0.944   |
| ER negative vs. positive                              | 0.63 | 0.25-1.59 | 0.327 | 0.95 | 0.46-1.97 | 0.891   |
| PR negative vs. positive                              | 0.96 | 0.36-2.58 | 0.933 | 1.19 | 0.57-2.46 | 0.646   |
| HER2 negative vs. positive                            | 0.70 | 0.20-2.39 | 0.577 | 0.99 | 0.48-2.07 | 0.991   |
| Mastectomy vs. breast conservation                    | 0.50 | 0.26-0.95 | 0.033 | 0.63 | 0.42-0.95 | 0.026   |
| Neoadjuvant chemotherapy vs. adjuvant chemotherapy vs. not given | 0.33 | 0.05-2.48 | 0.282 | 0.55 | 0.20-1.52 | 0.246   |

Italics indicate significance. *Sample sizes differ due to complete data set per Cox model.

Table VI. Multivariate Cox model for patients with invasive breast cancer.

| Characteristics                                      | 5-year OS            | 5-year DFS           |
|------------------------------------------------------|----------------------|----------------------|
|                                                      | HR  | 95% CI   | P-valuea | HR  | 95% CI   | P-valuea |
| p85α overexpression vs. downregulation                | 3.42 | 1.24-9.41 | 0.017 | 2.90 | 1.39-6.04 | 0.004   |
| Clinical stage I vs. II vs. III                       | 4.59 | 2.27-9.31 | <0.001 | 3.34 | 2.19-5.10 | <0.001   |

Italics indicate significance. *Cox regression forward LR method.

EMT. Additionally, we show an inverse correlation between p85α expression levels and PR expression in patient tumors. Finally, we demonstrate that p85α is downregulated in patients with invasive breast cancer, indicating an inferior prognosis. Taken together, our data provide novel insight into the regulation of p85α expression in breast cancer and its potential role on prognosis predication.

miR-21 is an oncomiR in breast cancer and targets several tumor suppressor genes important for various cellular processes (22). Here, we show that p85α is downregulated in 7.8% of breast cancer tumors, and is a direct target of miR-21. This finding is consistent with a recent study by Toste et al (32). They demonstrated a direct regulation of p85α by miR-21 and an inverse correlation between miR-21 and p85α expression levels in human pancreatic tumors. However, we did not find a statistically significant correlation between miR-21 and p85α expression levels in patient tumors (P=0.052). We speculate that patient tumor sections for quantitative detection of miR-21, which inevitably contain both normal and malignant cells, are the most possible reason for this inconsistent result.

The protein p85α is necessary for stabilization and membrane recruitment of the p110α subunit of PI3K (6). Loss of the p85α protein leads to downstream PI3K pathway activation (30,32-35). Therefore, the impact of p85α down-regulation on pathway signaling could be caused by the loss of the inhibitory effect of p85α on p110α and PI3K pathway activity (33,36). p85α protein has also been reported to be a positive regulator of PTEN via stabilization of this protein (37,38). Besides, several studies evidenced that PTEN is one of miR-21 targets (21,38,39). These studies support the notion that miR-21 actives PI3K pathway via multiple targets. Our finding that p-AKT levels are decreased after p85α overexpression in breast cancer cells is consistent with these previous observations. In addition, PIK3R1 overexpression phenocopies the effect of miR-21 knockdown on breast cancer cells and PIK3R1 knockdown inversely abrogates LNA-antimiR-21-mediated cell growth and invasion suppression. These findings suggest that PIK3R1 exerts tumor suppressor properties in breast cancer. Furthermore, the concept that p85α downregulation can be protumorigenic (30) is supported by our finding that p85α downregulation is seen in breast cancer tissues when compared with normal tissues. In the present study, this newly identified p85α downregulation by miR-21 has significant importance for interpretation of miR-21 promoting breast cancer cells growth, migration and invasion through the PI3K/AKT pathway.

Prognosis of invasive breast cancer, no special type, is influenced by the classical variables of histological grade,
tumor size, lymph node status and clinical stage (14,29,40,41). However, heterogeneity in tumor cell phenotypes make breast tumor categorization a challenging task, especially as it is relevant to therapeutic responses and patient prognosis (1). Our previous study and other research demonstrated that elevated miR-21 could predict unfavorable prognosis in breast cancer patients (23,42-44). In this study, we performed an evaluation of the prognostic significance of p85α, as well as miR-21, in a 320 patient cohort, and confirmed that miR-21 was a prognostic marker for inferior 5-year DFS and 5-year OS in breast cancer patients. Noticeably, p85α downregulation was a prognostic marker for inferior clinical stage. This finding is consistent with the association between p85α downregulation and an inferior prognosis not only in breast cancer (15) but also pancreatic cancer (32,45), hepatocellular cancers (30), neuroblastoma (46) and lung cancers (47). All these results support the notion that p85α plays as a tumor suppressor gene in invasive breast cancer tumors. Additional in vivo studies will be necessary to confirm the relationship between miR-21 and p85α, and the role of p85α in breast cancer.

In conclusion, we provided evidence that PIK3R1 is a direct target of miR-21. miR-21 knockdown induced increased p85α level, accompanied by decreased p-AKT level. miR-21 may play a role in breast cancer development by promoting breast cancer cell growth, migration and invasion partly by inhibiting PI3K/AKT activation via targeting PIK3R1 and reversing EMT. Furthermore, alterations in miR-21 and p85α had a complementary impact on breast cancer patient survival. Finally, p85α downregulation defined a specific subgroup of breast cancer with shorter 5-year DFS and OS, which may require more aggressive treatment.

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