Original Article
Long noncoding RNA PDIA3P promotes breast cancer development by regulating miR-183/ITGB1/FAK/PI3K/AKT/β-catenin signals

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Abstract: Aberrantly expressed long noncoding RNAs (lncRNAs) play crucial roles in the process of breast cancer (BC). This research aims to dig the possible roles and regulatory mechanism of lncRNA protein disulfide isomerase family A member 3 pseudogene 1 (PDIA3P1) in BC. The mRNA level of PDIA3P both in BC tissues and in cells were determined, followed by the investigation of the effects of PDIA3P suppression on cell biological processes including the viability, apoptosis, migration and invasion. Furthermore, whether PDIA3P modulated the expression of integrin β1 (ITGB1) expression by competitively sponging miR-183 and then regulated the activation of FAK/PI3K/AKT/β-catenin pathway was explored. PDIA3P was discovered as up-regulated in BC tissues and cells. PDIA3P suppression markedly decreased cell viability, promoted apoptosis, and inhibited migration and invasion in MCF-7 cells. In addition, PDIA3P was found to be negatively interacting with miR-183, and PDIA3P regulated tumor growth and metastasis through negatively regulating miR-183. Moreover, ITGB1 was targeted by miR-183 and involved in tumor growth and metastasis. Lastly, PDIA3P suppression markedly inhibited the activation of FAK/PI3K/AKT/β-catenin pathway, which was significantly reversed after simultaneous inhibition of miR-183. Our results reveal that PDIA3P may forpromote BC development by sponging miR-183 to regulate ITGB1, thus inducing the activation of FAK/PI3K/AKT/β-catenin signals. PDIA3P may serve as a promising biomarker or target for the detection or treatment of BC.

Keywords: Breast cancer, long noncoding RNA, protein disulfide isomerase family A member 3 pseudogene 1, miR-183, integrin β1

Introduction
Breast cancer (BC) remains one of the most frequently diagnosed cancer in women worldwide, with an estimated 232670 newly diagnosed cases and 40000 deaths in the United States [1]. Most patients with BC are diagnosed at an advanced stage due to difficulties in early detection, greatly reducing the quality of the patients’ lives [2]. The five-year survival rate is less than 10% because of the predilection for bone metastasis [3]. For improving the clinical outcome, it is still imperative to deepen understanding of the pathogenesis of BC.

Increasing evidence proves the significant roles of long non-coding RNAs (lncRNAs), which are more than 200 nucleotides in length, are widely involved in diverse cellular processes [4, 5]. Aberrantly expressed lncRNAs are frequently observed in various tumors and may be used for cancer diagnosis and prognosis due to their diverse functions in mediating tumor initiation and metastasis [6-8]. IncRNA DSCAM-AS1 plays a crucial role in the process of disease [9]. Other lncRNAs, such as LncRNA Inhibiting Metastasis (LIMT) [10], Growth arrest-specific 5 (GAS5) [11], and Esrp2-antisense [12] have been implicated in progression of BC.

Protein disulfide isomerase family A member 3 pseudogene 1 (PDIA3P1) is a 2099-bp IncRNA that located on chromosome 1q21.1. Recently, PDIA3P up-regulation was found contributing to the development of oral squamous cell carcinoma [13]. Yang demonstrated that PDIA3P could regulate cell proliferation in multiple myeloma through inducting pentose phosphate pathway [14]. Despite the above studies, the status of PDIA3P in BC still remains largely unknown.
In the current study, we detected the mRNA level of PDIA3P in cancer tissues and cells and also investigated the effects of PDIA3P suppression on tumor cell biological processes. Furthermore, IncRNAs are emerging as important regulators of gene expression during tumorigenesis through serving as competing endogenous RNAs (ceRNAs). We explored whether PDIA3P modulated the expression of integrin β1 (ITGB1) by competitively sponging miR-183 and then regulated the activation of FAK/PI3K/AKT/β-catenin signals. All of these findings were to disclose the detailed role and molecular mechanisms of PDIA3P in BC development.

Materials and methods

Human tissue samples

In total, 50 patients with BC from December 2012 to January 2018 were enrolled in this study. Tumor tissues and the adjacent non-tumor tissues were collected by surgical resection, and then were quickly preserved in liquid nitrogen following resection and stored at -80°C. This study was proved by the ethics committee of our hospital and informed consents for research were obtained from all patients.

Cell lines and cell transfection

Human BC cell lines MCF-7 and MDA-MB-231 as well as normal human mammary epithelial cell line MCF10A, obtained from American Type Culture Collection (ATCC), were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco) in a 37°C humidified incubator with 5% CO₂. Cells were passaged every 2-3 days for maintaining exponential growth. For cell transfection, sh-PDIA3P, sh-NC, miR-183 mimic, mimic NC, miR-183 inhibitor, si-NC and/or si-ITGB1 were transfected into MCF-7 cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions.

Quantitative real-time reverse transcription PCR (qRT-PCR)

Total RNA from tissues/cells was isolated using Trizol (Invitrogen), and reverse transcription to cDNA was subsequently conducted using the Omniscript RT Kit (Qiagen). The PCR reaction mixture was then prepared, including 0.3 mM of each primer, 1 Fast SYBR® Green Master Mix (Applied Biosystems), and 50 ng of cDNA. qRT-PCR analyses were performed on a StepOnePlus™ (Applied Biosystems). For relative quantification, the threshold cycle values were normalized to GAPDH, and the relative gene expression was quantitated by 2-ΔΔCt method.

Cell viability assay

Cell viability was assessed using MTT assay (Roche Applied Science, Penzberg, Germany). Cells with different transfections were grown in 96-well plates. After 24 h of transfection, cell viability in each group was assessed by measuring the absorbance (470 nm) with a MRX II absorbance reader (DYNEX Technologies, Chantilly, Virginia, USA).

Cell apoptosis

Cells were harvested after 48 h of different transfections. The apoptotic cells were analyzed using the FITC-Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer’s recommendations, cells were double stained with FITC-Annexin V and propidium iodide (PI), and then analyzed with a flow cytometry (FACScan; BD Biosciences) equipped with a CellQuest software (BD Biosciences). Viable cells, early/late apoptotic cells, and dead cells were distinguished, and the percentage of apoptotic cells was calculated.

Cell migration and invasion assays

Cell migration and invasion assay was performed using Transwell assay. For the migration assay, 5 × 10⁴ cells were harvested after 48 h of transfection, then were suspended in serum-free medium, and placed into the upper chamber of an insert (8-mm pore size; Millipore, Billerica, MA, USA). For cell invasion assay, cells were treated the same with that treated of migration, but the upper chamber of an insert coated with Matrigel. Medium containing 10% FBS was added to the lower chamber. After 24 h of incubation, cells that had migrated or invaded through the membrane were fixed with methanol, stained 0.1% crystal violet, and counted using an IX71 inverted microscope (Olympus, Tokyo, Japan).
Western blot

The total protein from MCF-7 cells with different transfections was extracted by lysing with cell lysis buffer (Beyotime, Haimen, China). After being quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA), the proteins (30 μg per lane) were separated on 12% SDS-PAGE gels and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were then incubated with the primary antibodies to pro-caspase-3, cleaved-caspase-3, Bax, Bcl-2, pro-caspase-9, cleaved-caspase-9, ITGB1, FAK, p-FAK, PI3K, AKT, p-AKT, β-catenin, and β-actin (1:1000 dilution; Abcam, Cambridge, UK) overnight at 4°C and recommended secondary antibodies (1:5000 dilution; Abcam) for 2 h. β-actin was used as the control. The protein signals were revealed using ECL detection kit (Pierce, Rockford, IL, USA) and analyzed using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA).

Luciferase reporter assay

The pMIR-REPORT-ITGB1-WT/MUT (Obio, Shanghai, China) were constructed and then co-transfected into MCF-7 cells with miR-183 mimic or mimic control. After 48 h of transfection, luciferase activity of each group was detected using the Dual-Luciferase Reporter Assay System (Promega).

Statistical analysis

All experiments were independently repeated three times. Statistical analyses were performed with SPSS version 18.0 (SPSS, Chicago, IL). Statistical differences between groups were compared by Student’s t test or one-way ANOVA, and P < 0.05 was considered as statistically significant.

Results

PDIA3P is upregulated in BC tissues and cells

PDIA3P was up-regulated in BC tissues in relation to that in adjacent normal tissues (P < 0.01, Figure 1A), indicating a possible oncogenic role of PDIA3P in BC. Moreover, PDIA3P was also highly expressed in both MCF-7 and MDA-MB-231 cells in comparison with that in normal mammary MCF10A cells (P < 0.05, Figure 1B). We found that PDIA3P expression in MCF-7 cells was higher than MDA-MB-231 cells, so MCF-7 cells were selected for following experiments.

Suppression of PDIA3P decreases cell viability, promotes apoptosis, and inhibits migration and invasion

PDIA3P was suppressed in MCF-7 cells, followed by detection of the effects of PDIA3P suppression on cell viability, apoptosis, migration and invasion. In comparison with sh-NC transfection, transfection with sh-PDIA3P#1 or sh-PDIA3P#2 resulted in the significantly decreased expression of PDIA3P in MCF-7 cells (P < 0.05, Figure 1C), and sh-PDIA3P#2 was selected for subsequent experiments due to the stronger transfection efficiency. Subsequently, we found that the cell viability of sh-PDIA3P#2-transfected groups was significantly reduced compared with sh-NC transfected or control cells (P < 0.05, Figure 1D). Next, flow cytometry showed that the percentage of apoptotic cells was clearly increased after transfection with sh-PDIA3P#2 relative to sh-NC (P < 0.01, Figure 1E). Moreover, the expression of apoptosis-related proteins also exhibited consistent changes after different transfection. Lastly, the transwell assay was performed to study the effects of PDIA3P suppression on cell migration and invasion. It was revealed that the number of migrated or invaded cells was distinctly decreased following PDIA3P suppression (P < 0.05, Figure 1F, 1G). Taken together, these results suggested that suppression of PDIA3P decreased cell viability, promoted apoptosis, and inhibited migration and invasion.

PDIA3P regulates tumor growth and metastasis through negatively regulating miR-183

LncRNAs are shown to function as a ceRNA to influence the expression of miRNA. Thereby, we explored the regulatory relationship between PDIA3P and miR-183. miR-183 expression in sh-PDIA3P#2-transfected cells was observably increased in relation to that in sh-NC-transfected cells (P < 0.01, Figure 2A), indicating the negative regulatory relationship between PDIA3P and miR-183. Moreover, we found that miR-183 expression was markedly downregulated in BC tissues compared to that in adjacent normal tissues (P < 0.01, Figure 2B), as well as in BC cells (MCF-7 and MDA-MB-231 cells) relative to that in MCF10A cells (P < 0.01, Figure 2C), indicating that miR-183 was also involved in the process of BC. To further reveal the role of PDIA3P in BC was medi-
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Figure 1. LncRNA PDIA3P expression is upregulated in breast cancer (BC) tissues and cells. A: qRT-PCR showed the PDIA3P expression in BC tissues and adjacent non-tumor tissues. B: qRT-PCR showed the PDIA3P expression in BC cells (MCF-7 and MDA-MB-231 cells) and normal mammary MCF10A cells. *P < 0.05 and **P < 0.01 compared to corresponding control. C-G. Suppression of PDIA3P in MCF-7 cells decreased cell viability, promoted apoptosis, and inhibited migration and invasion. C: qRT-PCR showed the PDIA3P expression in MCF-7 cells after transfection with sh-NC, sh-PDIA3P#1 or sh-PDIA3P#2. D: MTT assay showed the MCF-7 cell viability after different transfections. E: Flow cytometry showed that the percentage of apoptotic MCF-7 cells after different transfections and western blot revealed the expression of apoptosis-related proteins after different transfections. F, G: Transwell assays showed the migration and invasion of MCF-7 cells after different transfections. *P < 0.05 and **P < 0.01 compared to corresponding control.
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Figure 2. PDIA3P regulated tumor growth and metastasis through negative regulating miR-183. A: qRT-PCR showed the miR-183 expression in MCF-7 cells after transfection with sh-NC, or sh-PDIA3P#2. B: qRT-PCR showed the miR-183 expression in BC tissues and adjacent non-tumor tissues. C: qRT-PCR showed the PDIA3P expression in BC cells (MCF-7 and MDA-MB-231 cells) and normal mammary MCF10A cells. D: qRT-PCR showed the miR-183 expression in MCF-7 cells after transfection with miR-183 mimic, mimic control, miR-183 inhibitor, and inhibitor control. E: MTT assay showed the MCF-7 cell viability after different transfections. F: Flow cytometry showed that the percentage of apoptotic MCF-7 cells after different transfections and western blot revealed the expression of apoptosis-related proteins after different transfections. G, H: Transwell assays showed the migration and invasion of MCF-7 cells after different transfections. *P < 0.05, **P < 0.01, and ***P < 0.001 compared to corresponding control.

Figure 3. ITGB1 was a target of miR-183. A: The complementary pairing relationship between miR-183 and ITGB1 according to the information of TargetScan software. B: Luciferase activity assay confirmed that miR-83 mimic inhibited the luciferase activity of ITGB1-WT. C, D: qRT-PCR and western blot showed the ITGB1 expression in MCF-7 cells after transfection with miR-183 mimic, mimic control, miR-183 inhibitor, and inhibitor control. *P < 0.05 and **P < 0.01 compared to corresponding control.

ated by miR-183, the miR-183 was dysregulated in MCF-7, followed by detection of the effects of co-transfection of sh-PDIA3P#2 and miR-183 inhibitor in MCF-7 cells. As shown in Figure 2D, miR-183 expression was prominently overexpressed and suppressed in MCF-7 cells by transfection of miR-183 mimic and miR-183 inhibitor (P < 0.001), respectively. Subsequently, we found that cotransfection of sh-PDIA3P#2 and miR-183 inhibitor significantly reversed the effects of sh-PDIA3P#2 alone by enhancing cell viability (P < 0.05, Figure 2E), decreasing apoptosis (P < 0.05, Figure 2F), and prompting migration and invasion (P < 0.05, Figure 2G, 2H).

ITGB1 is a target of miR-183

ITGB1 is one member of the integrins that are crucial in cancers [15], we predicted the interaction between miR-183 and ITGB1 using TargetScan software (version 7.1). There was a complementary pairing relationship between miR-183 and ITGB1 (Figure 3A). Moreover, luciferase activity assay further confirmed that
miR-83 mimic significantly inhibited the luciferase activity of ITGB1-WT (P < 0.05), but showed no effects on ITGB1-MUT (Figure 3B). These data indicated that ITGB1 was a target of miR-183. Furthermore, the expression of ITGB1 at both mRNA and protein levels was markedly downregulated in miR-183 mimic group and dramatically upregulated in miR-183 inhibitor group (P < 0.05, Figure 3C and 3D), confirming that ITGB1 expression was negatively regulated by miR-183.

miR-183 regulates tumor growth and metastasis through targeting ITGB1

To verify the regulatory interaction between miR-183 and ITGB1 in BC cells, ITGB1 was suppressed in MCF-7 cells by transfection with si-ITGB1. The level of ITGB1 was significantly decreased in si-ITGB1-transfected cells in comparison with that in si-NC-transfected cells (P < 0.01, Figure 4A). Moreover, in relation to transfection with miR-183 inhibitor alone, co-transfection with miR-183 inhibitor and si-ITGB1 in MCF-7 cells significantly decreased cell viability (P < 0.05, Figure 4B), promoted apoptosis (P < 0.001, Figure 4C) and suppressed migration (P < 0.0A, Figure 4D) and invasion (P < 0.01, Figure 4E). These data indicated that miR-183 regulates tumor growth and metastasis through targeting ITGB1.

PDIA3P mediates the process of BC cells through FAK/PI3K/AKT/β-catenin pathway

Previous evidence reported that ITGB1 is involved in the development of tumors including BC by FAK/PI3K/AKT/β-catenin pathway [16, 17], we investigated whether FAK/PI3K/AKT/β-catenin signal was involved in the process of PDIA3P-mediated BCPDIA3P suppression distinctly decreased the expression levels of p-FAK, p-PI3K, p-AKT and β-catenin, which was significantly reversed after simultaneous inhibition of miR-183 (Figure 4F). Moreover, the combined effects of simultaneous suppression of PDIA3P and inhibition of miR-183 on the expression of key FAK/PI3K/AKT/β-catenin-related proteins were reversed again after further suppression of ITGB1 at the same time (Figure 4F).

Discussion

The current research explored the roles of PDIA3P in the process of BC. We discovered that PDIA3P was up-regulated in BC tissues and cells. Suppression of PDIA3P markedly decreased the cell viability, promoted apoptosis, and inhibited migration and invasion. In addition, PDIA3P was negatively interacted with miR-183 in regulating tumor growth and metastasis. Moreover, miR-183 regulated tumor growth and metastasis through targeting ITGB1. Lastly, PDIA3P distinctly inhibited the activation of FAK/PI3K/AKT/β-catenin pathway, which was significantly reversed after simultaneous inhibition of miR-183 and further reversed after suppression of ITGB1 at the same time.

Increasing evidences have confirmed that IncRNAs can function as ceRNAs to modulate gene expression through sponging miRNAs, thus contributing to the development of many diseases [18, 19]. Our findings showed that PDIA3P modulated the expression of ITGB1 by competitively sponging miR-183. Besides, miR-183 is located on chromosome 7q32 and is dysregulated in numerous cancers [20, 21]. Lowery demonstrated that miR-183 overexpression could suppress the cell migration [22], which was consistent with our findings. miR-183/-96/-182 cluster is crucial to the development of breast tumor [23]. Furthermore, ITGB1 was targeted by miR-183, and aberrant expression of ITGB1 was also revealed in a variety of malignancies, such as prostate cancer [24], oral squamous cell carcinoma [25], and gastric cancer [26]. Moreover, a gene expression profiling combined with functional analysis has revealed that ITGB1 function as a promising prognostic biomarker for triple negative breast cancer [27]. The kinase LMTK3 promoted the invasion of BC cells by GRB2-mediated induction of ITGB1 [28]. Given the key role of ITGB1 in BC, we speculated that PDIA3P may contribute to BC development by modulating the expression of ITGB1 by competitively sponging miR-183.

Furthermore, ITGB1 is a key activator for FAK signaling through phosphorylating FAK [29]. Twist is shown to induce epithelial-mesenchymal transition and regulate the motility of BC cells through ITGB1-FAK/ILK signaling axis [30]. A diterpenoid compound, excisin A, repressed the invasive behavior of BC cells by regulating the ITGB1/FAK/PI3K/AKT/β-catenin signaling [17]. In this study, of PDIA3P suppression distinctly decreased the protein levels of p-FAK, p-PI3K, p-AKT and β-catenin by miR-183. Also,
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Figure 4. miR-183 regulated tumor growth and metastasis through targeting ITGB1. A: qRT-PCR and western blot showed the ITGB1 expression in MCF-7 cells after transfection with si-NC or si-ITGB1. B: MTT assay showed the MCF-7 cell viability after different transfections. C: Flow cytometry showed that the percentage of apoptotic MCF-7 cells after different transfections and western blot revealed the expression of apoptosis-related proteins after different transfections. D, E: Transwell assays showed the migration and invasion of MCF-7 cells after different transfections. F: The expression of key FAK/PI3K/AKT/β-catenin pathway-related proteins after dysregulation of PDIA3P, miR-183, and/or ITGB1 in MCF-7 cells. *P < 0.05, **P < 0.01, and ***P < 0.001 compared to corresponding control.
the combined effects of simultaneous suppression of PDIA3P and inhibition of miR-183 on the expression of key FAK/PI3K/AKT/β-catenin-related proteins were reversed again after further suppression of ITGB1 at the same time. Taken together, our results reflected that PDIA3P may promote BC development by regulating the activation of ITGB1/FAK/PI3K/AKT/β-catenin signals.

To sum up, our findings reflected that PDIA3P may contribute to the process of BC by sponging miR-183/ITGB1 through the activation of FAK/PI3K/AKT/β-catenin signals. PDIA3P may serve as a promising biomarker or targets for the detection or treatment of BC.

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Disclosure of conflict of interest

None.

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