Long non-coding RNA PVT1 regulates the proliferation and metastasis of human breast cancer via the Wnt/β-catenin signalling pathway

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Abstract

Introduction: The development of many human diseases has been implicated to be coupled by the dysregulation of long non-coding RNAs (lncRNAs). Considering this, the current study was aimed at identifying and then investigating the molecular role of a specific lncRNA from a set of such genetic elements in regulating the developmental aspects of human breast cancer.

Material and methods: The quantitative real-time polymerase chain reaction (qRT-PCR) method was used to deduce the gene expression levels. Proliferation of cancer cells was determined by the cell counting kit 8 (CCK8). The evaluation of apoptotic cell death in breast cancer cells was made through the acridine orange/ethidium bromide (AO/EB) and annexin V-FITC staining protocols. Transwell assays were used to monitor cell migration and invasion.

Results: Estimation of gene expression levels of a set of lncRNAs showed that lncRNA PVT1 is specifically overexpressed in the breast cancer tissues and cell lines. The downregulation of PVT1 in cancer cells negatively affected their proliferation rates, and cancer cells exhibited significantly lower viabilities due to induction of Bax/Bcl-2 signal arbitrated apoptotic cell death in the cancer cells. Moreover, the cancer cells showed significantly lower rates of migration and invasion when lncRNA PVT1 was repressed. The PVT1 repression-driven anti-cancer effects against the cancer cells were seen to be modulated through the Wnt/β-catenin signalling pathway.

Conclusions: The results of this work are indicative of the prognostic role of lncRNA PVT1 in breast cancer. Also, the molecular targeting of PVT1 might prove to be a vital step against the progression of human breast cancer.

Key words: long non-coding RNA, breast cancer, apoptosis, anti-cancer, migration, invasion.
of cancer-related deaths among women worldwide [4]. Data about cancer-related mortality suggest that in 2013 the deaths resulting from breast cancer accounted for about 15% of the total cancer-related deaths [5]. The most important hurdle in combating the disease of breast cancer is poor prognosis at the early stages of its incidence together with the lack of effective treatment measures [6, 7]. Research studies of recent times have laid profound stress on the vitality of the genetic factors regulating the progression of the development of many dominant human disorders, including different types of cancer. These genetic factors also include the category of long non-coding RNAs (lncRNAs). lncRNAs have been shown to have tremendous potential to act as molecular prognostic tools in the diagnosis of the human cancers, together with the possibility of the use of specific lncRNA molecule targeting as an efficient anticancer strategy against particular human cancers [8]. Among lncRNAs, lncRNA PVT1 has been shown to play a role in the development of different cancers. For instance, lncRNA PVT1 has been shown to promote the angiogenesis and invasion of gastric cancer [9, 10]. Similarly, it has been shown to regulate the development of triple-negative breast cancer through KLF5/β-catenin signalling [11]. In yet another study, lncRNA PVT1 has been shown to regulate the progression of human breast cancer [12]. Nonetheless, the information on the regulation of breast cancer growth and metastasis via modulation of the Wnt/β-catenin signalling pathway is still largely unknown. Considering this, the present study aimed to unravel the specific lncRNA molecule associated with the growth and progression of breast cancer together with its molecular characterization in regulating the proliferation and metastasis of breast cancer. The results showed that lncRNA PVT1 is specifically overexpressed in breast cancer tissues and cancer cell lines. Its downregulation led to a significant decline in the proliferation rate and viability of breast cancer cells. The reason behind this growth inhibitory effect was found to be the Bax/Bcl-2-driven apoptotic induction in the cancer cells. Moreover, it was shown that the migration and invasion of breast cancer cells declined severely under the transcriptional repression of lncRNA PVT1. The anti-cancer effects of downregulation of lncRNA PVT1 against breast cancer cells were found to be propagated through the inhibition of the Wnt/β-catenin signalling pathway in the cancer cells. Taken together, the results of this study indicate the molecular therapeutic potential of lncRNA PVT1 to aid in the prognosis of breast cancer. Furthermore, it is also evident that targeting of PVT1 might emerge as a vital step in the strategy against the proliferation and advancement of human breast cancer in the future.

Material and methods

Clinical specimens and maintenance of cell lines

The clinical specimens pertaining to breast cancer along with the surrounding normal tissues were obtained after obtaining informed consent and under standard ethical guidelines from breast cancer patients being treated at the First Affiliated Hospital of Shandong First Medical University, Shandong Provincial Qianfoshan Hospital, Jinan, Shandong, China. Immediately after their collection, the tissue specimens were frozen in liquid nitrogen and kept at −80°C for future experimentation. The normal human breast cell line (MB-157) and the cancer cell lines (MDA-MB-231, SK-BR-3, CAMA-1, and MDA-MB-436) were obtained from ATCC, USA. DMEM growth medium (Thermo Scientific) with 10% FBS was used for cell culturing, and a CO₂ incubator was used to maintain the cell lines in an environment of 5% CO₂ and 95% air at 37°C. The study was approved by the research ethics committee of The First Affiliated Hospital of Shandong First Medical University, Shandong Provincial Qianfoshan Hospital, Jinan, Shandong, China with approval number 6787FMU-2019.

Transfection

The transfection of cell lines was performed by using Lipofectamine 2000 reagent. The downregulation of the PVT1 gene was made by transfecting the cancer cells by its RNA interference construct (si-PVT1) using si-NC as the silencing control.

Gene expression analysis

A TRIzol reagent (Thermo Scientific)-based method was used for isolation of total RNA from the tissue specimens and the cell lines. DNase I treatment was employed for removal of DNA contamination, and the RNA was then used for cDNA synthesis with the help of a miScript First Strand cDNA synthesis kit (Qiagen). Using this cDNA as a template, SYBR Green mix was used to perform the quantitative real-time expression analysis, and the gene expression levels were ascertained by employing the 2^-ΔΔCt method. The human β-actin gene was used as an internal control in the gene expression studies. A heat map was generated from the expression values with the help of an online software tool (www.heatmapper.ca).

Assessment of cell proliferation

The SK-BR-3 breast cancer cells were transfected with si-NC or si-PVT1 for 24 h and then about 2 × 10⁴ cells/well were cultured in 96-well plates for 0, 12, 24, 48, or 96 h at 37°C. Each well of the
96-well plate was then supplied with 10 µl CCK8 reagent from the Cell counting kit 8 (CCK8, Thermo Scientific). The 37°C incubation was prolonged for a further 4 h. Following the kit protocol, each sample was then processed for estimation of cell proliferation rates by determining the absorbance at 570 nm wavelength.

**Colony-forming assay**

The viabilities of the SK-BR-3 cancer cells transfected with si-NC or si-PVT1 was inferred through their respective colony-forming potential. Briefly, a homogenized solution of about 200 cells was added to each well of the 6-well plate. The cells were allowed to grow for 4 days at 37°C, after which they were fixed with methanol, stained with crystal violet solution (0.1% w/v), and then observed for the colony formation under the light microscope.

**AO/EB and annexin V-FITC/PI staining assays**

To confirm if downregulation of IncRNA PVT1 inhibited the proliferation of the SK-BR-3 cancer cells, AO/EB and annexin V-FITC/PI staining assays were performed. Consequently, the cells were added to 12-well plates at densities of 10^6 cells/well. The cells were cultured at 37°C for 24 h. Centrifugation was used to collect the cells, which were fixed with 70% ethanol. The cells were then stained with acridine orange/ethidium bromide (AO/EB) or annexin V-FITC/PI solutions. The cancer cells stained with former staining mix were examined under the fluorescent microscope and those stained with the latter solution were assessed with the flow cytometric technique to determine the level of apoptotic cell death in breast cancer cells.

**Western blotting**

The SK-BR-3 cancer cells transfected with si-NC or si-PVT1 were lysed with the lysis buffer (20 mM HEPES, 350 mM NaCl, 20% glycerol, 1% Nonidet P 40, 1 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail, and phosphatase inhibitor cocktail) to extract the total cellular proteins, the concentrations of which were calculated by the Lowry method. From each protein extraction, about 40 µg proteins were separated on 10% SDS-PAGE. The gel contents were transferred by blotting method to PVDF membranes. Primary antibody exposure was given overnight at 4°C. Then, the membranes were treated with secondary antibodies. Finally, chemiluminescence reagent was used to visualize the protein bands for concentration estimations. The protein expressions were standardized using β-actin protein.

Estimation of cell migration and invasion

The transwell assay method was performed for the estimation of the migration and invasion of SK-BR-3 cancer cells transfected with si-NC or si-PVT1. For the estimation of the former parameter, the transwell lacked Matrigel but in the latter’s estimation, Matrigel-fitted transwell was used. The upper and lower chambers of the transwell plate were supplemented with the cancer cells and growth medium, respectively. Culturing of the cells was done for 24 h at 37°C, after which the filter of the transwell chamber was taken and cells sticking to its upper surface were cleared using cotton swabs. The cells from the lower surface were fixed with 70% methanol and stained with 0.1% crystal violet solution and then assessed under a light microscope at 100× magnification to assess the migration and invasion of the cancer cells.

Statistical analysis

To minimize the experimental error, three replicates were used for every experimental set up. The average and standard deviation were taken from the obtained values and final representation was given as the average ± standard deviation (SD). Student’s t-test and ANNOVA were performed using GraphPad prism 7.0 software. The p-values ≤0.05 were taken as measure of a statistically significant difference between 2 experimental data points.

**Results**

PVT1 expression is downregulated in human breast cancer

Among the different long non-coding RNAs (lncRNAs) whose expression was analysed in different breast tissue specimens, only the lncRNA PVT1 expression was specifically seen to be over-expressed in the breast cancer tissues, whereas the expression of all other lncRNAs did not reveal any transcript level correlation (Figure 1 A). Moreover, the qRT-PR expression analysis of lncRNA PVT1 in normal breast cell line (MB-157) and the cancer cell lines (MDA-MB-231, SK-BR-3, CAMA-1, and MDA-MB-436) revealed that cancer cell lines exhibited significantly higher transcript levels of PVT1 (Figure 1 B). Amongst the cancer cell lines, the SK-BR-3 cells possessed the highest expression of PT1 and thus were used for further experimental characterization.

Inhibition of SK-BR-3 cell viability upon PVT1 repression

Utilizing the RNA interference approach, the IncRNA PVT1 was downregulated in the SK-BR-3 cancer cells, and the same was confirmed by the
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qRT-PCR method (Figure 1 C). The breast cancer cells transfected with si-NC or si-PVT1 constructs were processed for estimation of proliferation rates through a CCK8 kit-based method. The results showed that downregulation of lncRNA PVT1 in breast cancer cells significantly reduced the cell proliferation (Figure 1 D). Further, the downregulation of PVT1 also led to a significant decline in the colony-forming potential of the breast cancer cells, and si-PVT1-transfected cells exhibited only 47% of the number of colonies in comparison to the si-NC-transfected cancer cells (Figure 2). These effects are indicative of the therapeutic potential of lncRNA PVT1.

Suppression of IncRNA PVT1 triggers apoptosis in SK-BR-3 cells

The AO/EB and annexin V-FITC/PI staining assays revealed that the repression of PVT1 in SK-BR-3 cancer cells led to the induction of apoptosis of the cancer cells (Figures 3 A, B). The flow cytometry revealed that the percentage of apoptotic cells was 21.16% under PVT1 downregulation, while the control cells showed only 2.18% apoptosis. The western blotting of Bax and Bcl-2 proteins showed that downregulation of PVT1 in cancer cells led to an increase of the former protein and a decrease in the concentration of the latter (Figure 3 C). Thus, the results suggest that transcriptional repression of PVT1 in breast cancer cells induced Bax/Bcl-2-driven apoptotic cell death.

SK-BR-3 cell migration and invasion declines under PVT1 transcriptional repression

The migration and invasion of breast cancer cells transfected with si-NC or si-PVT1 was anal-
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**Figure 2.** Downregulation of IncRNA PVT1 decreases the viability of breast cancer cells. Analysis of the colony-forming ability of SK-BR-3 cancer cells transfected with si-NC or si-PVT1. Individual experiments were performed in triplicate, and the values are presented as mean ± SD (*p < 0.05).

**Figure 3.** Downregulation of IncRNA PVT1 induces apoptotic cell death in breast cancer cells. A – AO/EB staining procedure for the assessment of apoptosis of SK-BR-3 cancer cells transfected with si-NC or si-PVT1; B – Annexin V-FITC/PI staining procedure for the assessment of apoptosis of SK-BR-3 cancer cells transfected with si-NC or si-PVT1; C – Western blotting of Bax and Bcl-2 proteins from SK-BR-3 cancer cells transfected with si-NC or si-PVT1. Individual experiments were performed in triplicate.
ysed by the transwell assay. It was observed that repression of lncRNA PVT1 not only reduced the migration of cancer cells, but their invading potential was also negatively affected to a significant level (Figure 4). The percentage of cell migration and invasion declined to 32% and 25%, respectively, under PVT1 downregulation, which further highlights the anti-cancer potential of lncRNA PVT1 against breast cancer.

**LncRNA PVT1 exerts its effects via Wnt/β-catenin signalling pathway**

The western blotting study of SK-BR-3 breast cancer cells transfected with si-NC or si-PVT1 showed that repression of lncRNA PVT1 led to a decline in the concentrations of the vital signalling components of the Wnt/β-catenin signalling pathway, such as β-catenin, cyclin D1, and c-myc (Figure 5). Together, the results show that the anti-cancer effects of repression of lncRNA PVT1 against breast cancer are exerted through the blockage of the Wnt/β-catenin signalling pathway in cancer cells.

**Discussion**

The human genome is very complex, and more than 90% of human DNA does not code for proteins [13]. This major portion of human genome was previously referred as “junk DNA” with apparently no known function [14]. However, the

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**Figure 4.** Downregulation of lncRNA PVT1 restricts the metastasis of breast cancer cells. Transwell assay for analysis of migration and invasion of SK-BR-3 cancer cells transfected with si-NC or si-PVT1. Individual experiments were performed in triplicate, and the values are presented as mean ± SD (*p* < 0.05)

**Figure 5.** Downregulation of lncRNA PVT1 exerts anti-cancer effects through inhibition of Wnt/β-catenin signalling pathway. Western blotting of β-catenin, cyclin D1, and c-myc proteins from SK-BR-3 cancer cells transfected with si-NC or si-PVT1. Individual experiments were performed in triplicate.
advancement in the genomic studies has led to the understanding that the non-protein coding DNA is actively transcribed into a huge number of genetically crucial RNA elements [15]. The long non-coding RNAs (lncRNAs) also fall into the same category of RNA entities, which, although not translated, serve important functions in the cellular development and play a role in cellular differentiation [16]. It has been noted that lncRNAs regulate the development of many human diseases and are critical to the advancement of human cancer [17]. The dysregulation of lncRNAs has been implicated to govern the advancement of a number of human cancers including breast cancer [18, 19]. Thus, lncRNAs have been valued to serve as prognostic biomarkers against human cancers [8]. In the present research work, the role of lncRNA plasmacytoma variant translocation 1 (PVT1) was ascertained in regulating the growth and progression of human breast cancer. The PVT1 gene has been reported to play a role in physiological as well as pathological processes [20]. The cancer-regulatory role of PVT1 is also well established [21]. However, the regulatory effects of PVT1 have not been elucidated in human breast cancer. Human breast cancer is the most dominant female cancerous malignancy in terms of both its incidence as well as the level of mortality associated with this disorder [1, 4]. The results of the present study showed that PVT1 is specifically upregulated in breast cancer, which suggests its possible influence on the onset and proliferation of this malignancy because it has been shown to be upregulated in other cancer types in previous studies [22]. Surprisingly, the silencing of PVT1 at the transcriptional level caused a sufficient decline in the proliferation and viability of breast cancer cells. Whether or not this growth inhibition resulted from the induction of cell apoptosis, the assessment of nuclear viability of cancer cells was made, and it was observed that downregulation of PVT1 activates the Bax/ Bcl2 apoptotic cell signal to induce apoptosis in breast cancer cells. A similar mechanism of decline in cancer cell proliferation has been reported in previous investigations of PVT1 [23]. PVT1 transcriptional repression not only reduced the proliferation of breast cancer cells, but the results indicated that it also negatively affected the cancer cell migration and invasion rates. Taken together, it provides insight into the regulatory control of lncRNA PVT1 in breast cancer metastasis and hence indicates the molecular anti-cancer therapeutic potential of PVT1 against human breast cancer, which further strengthens such previous statements about PVT1 [24]. The Wnt/b-catenin signalling is one of the best-characterised cancer drivers. It has been reported to promote cancer progression by controlling the tumour-immune cycle in most of the nodes, including dendritic cells, T cells, and tumour cells [25]. Specifically, abnormal Wnt/b-catenin signalling directly alters the number of regulators critical for the antitumor activities of T cells, especially effector T cells, T helper cells, and regulatory T cells [26]. At the molecular level, the lncRNA PVT1 was shown to target one of the critical signalling pathways, the Wnt/b-catenin signalling pathway, to exert its regulatory role in breast cancer. The Wnt/b-catenin signalling pathway is highly overexpressed in cancer cells and enables them to proliferate at greatly elevated levels through over-expression of crucial regulators like cyclin D1 and c-myc [27]. The direct activation of c-myc by PVT1 has already been confirmed in previous reports [28]. Summing up, the results of this study indicate that the lncRNA PVT1 regulates the growth, proliferation, and metastasis of breast cancer in humans through the Wnt/b-catenin signalling pathway and thus lightens the possibility of molecular targeting of PVT1 to more effectively restrict the advancement of human breast cancer in the future.

In conclusion, the present study reveals the molecular therapeutic anticancer potential of lncRNA PVT1 against human breast cancer. The intracellular targeting of PVT1 might prove effective in better restricting the advancement of the breast cancer, which will invoke many future research studies. Future studies should be directed to investigate the prognostic value of lncRNA PVT1 and its effects on stemness markers. Additionally, in vivo studies need to be initiated to establish PVT as a therapeutic target for the treatment of breast cancer.

Conflict of interest

Authors declare that there are no conflicts of interest.

References


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