Pttg1 Promotes Growth of Breast Cancer through P27 Nuclear Exclusion

Yishan Xie\textsuperscript{a} Rui Wang\textsuperscript{b}

\textsuperscript{a}Department of Oncology, \textsuperscript{b}Department of Emergency, Remin Hospital of Wuhan University, Wuhan, China

Key Words
Pituitary Tumor Transforming Gene 1 (Pttg1) • p27 • Breast cancer (BC)

Abstract
Background/Aims: A role of Pituitary Tumor Transforming Gene 1 (Pttg1) in the carcinogenesis has been shown in some cancers, but not in BC (BC). Methods: We compared the levels of Pttg1 in the resected BC tissue with the adjacent normal breast tissue from the same patient. We modified Pttg1 levels in a BC cell line, MCF7, by either a Pttg1 transgene, or a Pttg1 shRNA. The cell growth was measured in an MTT assay. The cell apoptosis was measured by apoptosis assay. The nuclear protein of cell-cycle-related genes was examined in Pttg1-modified BC cells. Co-immunoprecipitation was performed to examine the association of Pttg1 and p27. Results: We detected significantly higher levels of Pttg1 in the resected BC tissue, compared to the adjacent normal breast tissue from the same patient. Overexpression or depletion of Pttg1 in MCF7 significantly increased or inhibited cell growth, respectively. Changes in Pttg1 levels, however, did not alter cell apoptosis, suggesting that Pttg1 increases cell growth through augmented cell proliferation, rather than decreased cell apoptosis. Among all examined cell-cycle-related proteins in Pttg1-modified BC cells, only nuclear p27 levels were significantly affected. Further, co-immunoprecipitation showed that Pttg1 directly associated with p27. Conclusion: Pttg1 may increase BC cell growth through nuclear exclusion of p27, which highlights a novel molecular regulatory machinery in tumorigenesis of BC.

Introduction
Breast cancer (BC) are fast growing, and frequently insensitive to chemotherapy and radiation therapy [1]. Thus, understanding of the mechanisms underlying the growth of BC is extremely important for its therapy [2-5].

Originally cloned from human fetal liver [6], the Pituitary Tumor Transforming Gene 1 (Pttg1, also known as securin) has been found to increase in an active cell cycle [7-10], suggesting a possible role of Pttg1 in the regulation of cell proliferation. The cell cycle
regulation relies on the interactions among a number of cell-cycle regulatory factors [7-10]. Pttg1 is known as a regulator of sister chromatid separation and transition from metaphase to anaphase [7-10]. However, increasing evidence suggests that the roles of Pttg1 in the cell-cycle regulation are much more profound [7-10], and the precise role of Pttg1 may be cell-type dependent. Pttg1 upregulation has been reported in a number of human tumors. However, the molecular mechanisms underlying Pttg1-induced cancer growth is not completely understood.

The cell cycle is regulated by a small number of protein kinases [11-14]. The regulatory subunits of these kinases are called Cyclins (e.g. CyclinB, CyclinD1, CyclinD2 and CyclinE), and the catalytic subunits are called Cyclin-dependent kinases (CDKs, e.g. CDK2, CDK4 and CDK6) [11-14]. Association of a CDK catalytic subunit with a specific Cyclin forms a CDK-Cyclin complex to phosphorylate certain proteins. Besides CDKs and Cyclins, there are also cell-cycle inhibitors, including the CDK interacting protein/kinase inhibitory protein family genes (p21, p27 and p57) and the Inhibitor of Kinase 4/alternative reading frame (p16INK4a) family genes [11-14]. These genes encode proteins that prevent the progression of the cell cycle. Cyclin-dependent kinase inhibitor 1B (p27) encodes protein that prevents the activation of Cyclin E-CDK2 or Cyclin D-CDK4 complexes after physical binding, which stops the cell cycle progression at G1 [11-14]. Transforming growth factor β (TGFβ) receptor signaling has been known to regulate p27 at different levels [15-22].

Here, we reported significantly higher levels of Pttg1 in the resected BC tissue, compared to the adjacent normal breast tissue from the same patient. The Pttg1 levels in a BC cell line, MCF7, were modified by either overexpression of Pttg1 transgene, or Pttg1 shRNA, which significantly increased or inhibited cell growth, respectively. Changes in Pttg1 levels, however, did not alter cell apoptosis, suggesting that Pttg1 increases cell growth through augmented cell proliferation, rather than decreased cell apoptosis. We then examined nuclear protein of cell-cycle-related genes in Pttg1-modified BC cells, and found that only nuclear p27 levels were significantly affected, which seemed to result from its nuclear exclusion induced by Pttg1. Further, co-immunoprecipitation showed that Pttg1 directly associated with p27.

Materials and Methods

Specimens from patients

A total of 27 BC (PC) patients were included in the study. The resected specimens (paired BC tissue and the adjacent normal breast tissue (NBT)) from the patients were used for analyzing Pttg1 protein. All specimens had been histologically and clinically diagnosed at Remin Hospital of Wuhan University from 2008 to 2013. For the use of these clinical materials for research purposes, prior patient’s consents and approval from the Institutional Research Ethics Committee were obtained.

Cell Line culture and transfection

Human BC cell line MCF7 was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO, USA) in a humidified chamber with 5% CO₂ at 37 °C. The overexpressing or shRNA plasmids (2µg) were kindly provided by Dr. Shengquan Huang (Xinqiao Hospital, Third Military Medical University, China), as has been described before [23]. Transfection was performed using the Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). The plasmid construct also contained a GFP reporter. Thus, the transfection efficiency could be evaluated by GFP expression.

Cell growth assay

For assay of cell growth, cells were seeded into 24 well-plate at 10⁴ cells per well and subjected to a Cell Proliferation Kit (MTT, Roche, Indianapolis, IN, USA), according to the instruction of the manufacturer. The MTT assay is a colorimetric assay for assessing viable cell number, taking advantage that NADPH-dependent
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Cellular oxidoreductase enzymes in viable cells reduce the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to its insoluble formazan in purple readily being quantified by absorbance value (OD) at 570 nm. Experiments were performed three times.

Cell apoptosis assay
Two days after transfection, apoptosis of transfected MCF7 cells was measured using the Annexin V Apoptosis Kit following the manufacturer's instructions (Becton Dickinson Biosciences, San Diego, CA, USA). The cells were stained with fluorescein isothiocyanate-conjugated Annexin V and PI solution (20μg/mL) for 15 min in the dark. Apoptotic cells (annexin V-positive) were analyzed by flow cytometry.

RNA extraction, reverse transcription and quantitative RT-PCR
Total RNA was extracted from the cultured cells using RNaseasy kit (Invitrogen), according to the manufacturer's instruction. For mRNA analysis, complementary DNA (cDNA) was randomly primed from 2μg of total RNA using the Omniscript reverse transcription kit (Qiagen, Hilden, Germany). Real-time PCR was subsequently performed in triplicate with a 1:4 dilution of cDNA using the Quantitect SyBrgreen PCR system (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed with the Rotorgene software accompanying the PCR machine, using 2-△△Ct method for quantification of the relative mRNA expression levels. Values of genes were first normalized against α-tubulin, and then compared to controls.

Western blot
Total Protein was extracted from the cultured cells by RIPA buffer (Sigma-Aldrich), and nuclear protein was isolated with Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, St Jose, CA, USA). Equal amount of proteins was loaded in the gel. Primary antibodies for Western Blot are rabbit Pttg1, CDK2, CDK4, CDK6, CyclinB1, CyclinD1, CyclinD2, CyclinE, p21, p27, LaminB1 (nuclear protein loading control) and α-tubulin (total protein loading control) (all purchased from Cell Signaling, St Jose, LA, USA). Secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). Images shown in the figure were representative from 5 repeats. Densitometry of Western blots was quantified with NIH ImageJ software.

Immunocytochemistry
Primary antibody is rabbit anti-p27 (Cell Signaling). Indirect fluorescent staining was performed with Cy3-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Labs).

Co-immunoprecipitation
The co-immunoprecipitation (IP) procedure was described previously [24]. Briefly, cells were lysed in 5ml of lysis buffer (50mmol/l Tris–HCl, pH 7.5, 200mmol/l NaCl, 0.5% Nonidet P40, protease inhibitor cocktail) for 30 min at 4 °C. Lysates were cleared using centrifugation at 15,000 rpm for 10 min; the supernatant was then subjected to IP with anti-FLAG M2 affinity resin (Sigma-Aldrich) overnight. Resin containing immune complexes were washed with ice cold lysis buffer five times. Proteins were eluted with Flag-peptide (Sigma-Aldrich) in TBS for 30 min. Proteins were precipitated with cold acetone, and the resulting pellet washed two times with cold acetone. IP samples were analyzed by immunoblot (IB).

Statistical analysis
All statistical analyses were carried out using the SPSS 18.0 statistical software package. All values are depicted as mean ± standard deviation and are considered significant if p < 0.05. All data were statistically analyzed using one-way ANOVA with a Bonferroni correction.

Results
Overexpression of Pttg1 was detected in BC specimen
The resected specimens (paired BC tissue and the adjacent normal breast tissue (NBT)) from the 27 BC patients were analyzed for Pttg1 levels by Western blot. We found that
compared to NBT, BC tissue had a significantly higher Pttg1 levels (Fig. 1, p<0.05, more than 7-fold increase). These data suggest a role of Pttg1 in the pathogenesis of BC.

**Pttg1 increased BC cell proliferation**

Then, we aimed to examine whether modification of Pttg1 levels in BC cells may alter cell growth. We used either a Pttg1-overexpressing plasmid, or a plasmid carrying short hairpin small interfering RNA (shRNA) for Pttg1 (shPttg1), to transfect a human BC cell line, MCF7, to increase or decrease Pttg1 levels respectively. MCF7 cells were also transfected with a plasmid carrying a scrambled sequence (scr) as controls. The modifications of Pttg1 levels in MCF7 cells were confirmed by RT-qPCR (Fig. 2A), and by Western blot (Fig. 2B). We found that overexpression of Pttg1 in MCF7 cells (MCF7-Pttg1) significantly increased cell growth in an MTT assay (Fig. 2C), while depletion of Pttg1 in MCF7 cells (MCF7-shPttg1) significantly decreased cell growth (Fig. 2C). To figure out whether the effects of Pttg1 on cell growth are contributable to alterations in cell proliferation or cell apoptosis, we examined cell apoptosis in these Pttg1-modified MCF7 cells. We found that the changes in Pttg1 levels in MCF7 cells did not significantly alter the apoptosis of the cells (Fig. 2D), suggesting that the effects of Pttg1 on cell growth are contributable to alterations in cell proliferation, rather than the alterations in cell apoptosis. In another word, Pttg1 increased BC cell proliferation.

**Pttg1 decreased nuclear p27 levels to increase BC cell proliferation**

We next aimed to find out the molecular mechanisms underlying the Pttg1-induced BC cell growth. We examined the levels of all CDKs, Cyclins and cell-cycle inhibitors that had been shown to be involved in the pathogenesis of BC in these Pttg1-modified MCF7 cells by Western blot. Since only nuclear forms of these proteins are critical for the regulation of cell-cycle-associated genes, while cytosol forms are not, we isolated nuclear proteins to analyze. We did not find changes in CDKs, Cyclins and p21 in these Pttg1-modified BC cells, but detected a significant increase in p27 in Pttg1-deprived cells and a significant decrease in p27 in Pttg1-overexpressing cells (Fig. 3A). Immunocytochemistry confirmed this finding, suggesting that the nuclear exclusion of p27 may be induced by Pttg1 (Fig. 3B). Together, these data suggest that Pttg1 may prevent nuclear retention of p27 and induce its nuclear exclusion to allow cells to proliferate.

**Pttg1 directly binds to p27**

To find out whether the effects of Pttg1 on p27 are direct or indirect, we performed co-immunoprecipitation (IP) to detect the physical interaction between Pttg1 and p27. Our results showed that endogenous Pttg1 co-precipitated with p27 and vice versa in MCF7 cells.

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**Fig. 1.** Overexpression of Pttg1 is detected in BC specimen. The resected specimens (paired BC tissue and the adjacent normal breast tissue (NBT)) from the 27 BC patients were analyzed for Pttg1 levels by Western blot. We found that compared to NBT, BC tissue had a significantly higher Pttg1 levels (Fig. 1, p<0.05, more than 7-fold increase). *p<0.05. n=27. Statistics: one-way ANOVA with a Bonferroni correction.
Fig. 2. Pttg1 increases BC cell proliferation. We used either a Pttg1-overexpressing plasmid, or a plasmid carrying short hairpin small interfering RNA (shRNA) for Pttg1 (shPttg1), to transf ect a human BC cell line, MCF7, to increase or decrease Pttg1 levels respectively. MCF7 cells were also transfected with a plasmid carrying a scrambled sequence (scr) as controls. (A-B) The modifications of Pttg1 levels in MCF7 cells were confirmed by RT-qPCR (A), and by Western blot (B). (C) We found that overexpression of Pttg1 in MCF7 cells (MCF7-Pttg1) significantly increased cell growth in an MTT assay, while depletion of Pttg1 in MCF7 cells (MCF7-shPttg1) significantly decreased cell growth. (D) We examined cell apoptosis in these Pttg1-modified MCF7 cells and found that the changes in Pttg1 levels in MCF7 cells did not significantly alter the apoptosis of the cells. *p<0.05. NS: non-significant. n=5. Statistics: one-way ANOVA with a Bonferoni correction.

Fig. 3. Pttg1 decreases nuclear p27 levels to increase BC cell proliferation. (A) We examined the nuclear protein levels of all CDKs, Cyclins and cell-cycle inhibitors that had been shown to be involved in the pathogenesis of BC in these Pttg1-modified MCF7 cells by Western blot. We did not find changes in any CDKs, Cyclins and p21 in these Pttg1-modified PC cells, but detected a significant increase in p27 in Pttg1-deprived cells and a significant decrease in p27 in Pttg1-overexpressing cells. (B) Immunocytochemistry for p27 in Pttg1-modified MCF7 cells.
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Fig. 4. Pttg1 directly binds to p27. Co-immunoprecipitation (IP) was performed, showing direct association between Pttg1 and p27. IB: immunoblot.

Fig. 5. Schematic of the model. Pttg1 may directly bind to p27, which induces nuclear exclusion of p27 to allow the BC cells to grow.

(Fig. 4). These data suggest that Pttg1 and p27 physically associate with each other in BC cells. Thus, Pttg1 may directly bind to p27, which induces nuclear exclusion of p27 to allow the cells to growth (Fig. 5).

Discussion

The molecular mechanisms that underlie the regulation of cancer growth by Pttg1 in BC are far from clarified. A recent study has shown that the key downstream component of TGFβ receptor signaling pathway SMAD3 is regulated by Pttg1 in a prostate cell line [23]. Activated by TGFβ receptor signaling pathway, SMAD3 is phosphorylated and then forms heteromeric complexes with SMAD4 to translocate to the nucleus to inhibit cell proliferation [23]. Together with our findings here, it appeared that the roles of Pttg1 on cell growth of cancer cells are cancer-cell-type independent.

In the present study, we used BC cell line MCF7, and reproduced the effects of Pttg1 on cell proliferation. Moreover, we found out that p27, a cell-cycle inhibitor, was directly associated with Pttg1, which affected the nuclear localization of p27. Of note, p27 is also directly regulated by TGFβ receptor signaling pathway [15-22]. Therefore, our study suggests that the findings in the previous study of Pttg1/SMAD3 on cell proliferation may be transduced through their downstream target p27, although this cascade has not been examined in the previous study [23]. Moreover, since we detected a direct association of p27 with Pttg1, the effects of Pttg1 on cell proliferation may transduce indirectly via SMAD3/p27 signaling, and directly via p27. We have checked several other lines and got essentially same results.
Our study should well associate the previous findings of Pttg1/SMAD3 signaling [23] and SMAD3/p27 signaling with each other [15-22]. Although our study highly suggests a molecular regulatory model illustrated in Fig. 5, this model needs further confirmation and extensive clarification. Additional gain-of-function and loss-of-function experiments should be performed in future to confirm the detail of this regulatory machinery. To summarize, our study sheds light on targeting interaction of Pttg1 and p27 as a therapeutic target for BC therapy.

Disclosure Statement

The authors have declared that no competing interests exist.

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