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Proteomics evaluation of MDA-MB-231 breast cancer cells in response to RNAi-induced silencing of hPTTG

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ABSTRACT

Aims: Breast cancer is the most common cancer in women worldwide. Several genes are up-regulated in breast cancer such as human pituitary tumor transforming gene (hPTTG). This study aims to evaluate cell proliferation and the downstream expression pattern of hPTTG1 gene at the mRNA and protein levels after specific down-regulation of hPTTG1 by siRNA.

Main methods: The human breast cancer MDA-MB-231 cell line was transfected with siRNA against hPTTG1. The mRNA and protein expression levels were examined by Real-time PCR and Western blot, respectively. The cell proliferation was assayed by MTS. To investigate the pattern of protein expression, total cellular protein was analyzed by 2D gel electrophoresis and mass spectroscopy. Subsequently, the possible biological consequences were determined by the bioinformatics databases.

Key findings: Subsequent of hPTTG1 silencing in the MDA_MB-231 cells, the proliferation of cells decreased obviously. In response to hPTTG1 silencing, the levels mRNA and protein were effectively down-regulated 80% and 50%, respectively, at 48 h post-transfection. The proteomics evidenced that PTTG1 increased the expression of 5 proteins. The reduced expression of PTTG1 was functionally involved in hypoxia (NPM1, ENO1), cell proliferation and apoptosis (ENO1, NPM1, NME1, STMN1), and metastasis (NPM1, NME1).

Significance: We identified the hPTTG1-regulated proteins and its molecular mechanism in pathogenesis of breast cancer. Further study emphasis is to understand the association of hPTTG1 with other genes in cancer progression. This novel modality might also consider for identification of targeted drugs, prognosis and follow up in breast cancer gene therapy.

1. Introduction

Breast cancer is the leading cause of cancer death in women aged from 20 to 59 years [1]. More than 20 million people are living with cancer all over the world and its number is expected to increase to > 30 million by 2020 CE. According to the National Cancer Institute, every 2 min one women is diagnosed with breast cancer and every 13 min a woman dies of breast cancer in US [2].

Breast cancer reflects the molecular dysregulation of cell cycle as its fundamental characteristic influences the cell growth and mitotic cell division [3]. Human pituitary tumor-transforming gene 1 (hPTTG1; NM_004219.4) as an oncogene, is located on chromosome 5q33.3 and encodes Securin protein with 202 amino acids (O95997). Securin inhibits premature sister chromatid separation during cell cycle [4]. PTTG1 is involved in several pathways including cell proliferation, DNA repair, angiogenesis and apoptosis [5–7].

Several studies have shown high PTTG expression in breast cancer tumors and this overexpression accelerates invasion and metastasis. Furthermore, up-regulation of this gene in different cancers was associated with metastasis and tumor recurrence [8,9].

Abundantly expression of PTTG1 occurs in wide variety of tumors and regulates tumor cell proliferation. For instance, it induces the expression of EMMPRIN that promotes invasion in MCF-7 breast cancer cell line through Akt/mTOR signaling pathway [10]. Also, it functions as one of the STAT3 target genes in colorectal cancer, suggesting the requirement of PTTG1 for STAT3-promoted metastasis [11]. The

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PTTG1 interacting partner, PBF, influences the expression of p53 and its related apoptosis activity in head and neck [12] and colorectal [13] cancers.

Moreover, the high expression of PTTG1 was identified in breast carcinoma especially in high tumor grade and negative estrogen receptor tissues by several bioinformatics tools such as Gene Expression Profiling Interactive Analysis (GEPIA) [14], CANCERTOOL, and ARCHS4 resources. Therefore, MDA-MB-231 breast cancer cell line was selected. To explore the role of PTTG1 in breast tumor growth, we utilized small interfering RNA to avoid non-specific gene silencing induced by longer double-stranded RNA. Finally, downstream mechanism of PTTG1 silencing was identified by screening of proteome pattern in MDA-MB-231 breast cancer cells.

2. Materials and methods

2.1. Cell culture and transfection

The human MDA-MB-231 breast cancer cell line was obtained from Pasteur Institute (Tehran, Iran). The cells were cultured at 37 °C in 5% CO_2 in RPMI-1640 medium (Invitrogen; Carlsbad, CA) containing 10% FBS (Gibco) and 1% penicillin/streptomycin antibiotic solution (Sigma, St. Louis, Mo). The cells were seeded in 6-well plates (2×10^5 cells/well) and incubated for 18 h. They were then transfected with 20 pmols siRNA against PTTG1 (Santa Cruz Biotechnology, Inc.) by lipofectamin 2000 according to the instruction. Then cells were incubated for 24, 48, and 72 h. Similarly, control cells were transfected with scrambled siRNA as a control (si-NEG) (Santa Cruz Biotechnology, Inc.).

2.2. Real-time PCR

Total RNA was extracted by TRIZOL reagent (Invitrogen, Italy) according to the manufacturer's instructions. To eliminate genomic DNA contamination, extracted RNA was treated with RQ1 RNase-Free DNAse (Promega Corporation, USA). Then, cDNA was synthesized with 1 μg DNase-treated RNA using the Transcriptor First Strand cDNA Synthesis Kit according to the manufacturer's instructions (Roche Applied Science). To explore the expression of PTTG1 and GAPDH genes, Real-time PCR assay was performed using ABI 7300 real-time PCR systems (Applied Biosystems, Foster City, CA). The Real-Time PCR products were amplified by SYBR®Green PCR Master Mix (Applied Biosystems, Foster City, CA) using the following parameters: 50 °C for 2 min 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min.

The oligonucleotide primer sequences were designed by the AlleleID and Oligo 7 Primer Analysis software at 3′-UTR region of each gene and presented in Table 1. All SNPs for each 3′-UTR were identified at *Homo sapiens* - Ensembl genome browser (https://asia.ensembl.org/Homo_sapiens/Info/Index) and these regions avoided to enter the primer (Supplementary data). Finally, all data were analyzed by the REST (Relative Expression Software Tool) software. GAPDH gene was applied as a housekeeping gene in this assay.

Table 1
The applied primer oligonucleotide in the quantitative real-time PCR.

Gene name	Sequence	Amplicon size (bp)
hPTTG1	F: 5′-TGATCCTTGACGAGGAGAGAG-3′ R: 5′-GGTGGCAATTCAACATCCAGG-3′	144
NPM1	F: 5′-TAGTAGCGGTGGTCAGA-3′ R: 5′-GGGCAGGGACATTCT-3′	190
ENO1	F: 5′-TGTCAGCTCAGGCAG-3′ R: 5′-TGCCAACAGGGCTC-3′	135
NME1	F: 5′-TACCTTCATTGCGATCAAACCA-3′ R: 5′-AAAACGCTTGATAATCTCTCCC-3′	67
NR3C1	F: 5′-TGGGGACAAATCTATATTATACT-3′ R: 5′-GGTTTTACAGTCTGACATTTC-3′	212
STMN1	F: 5′-ATCCAAAGACCCTGCTGA-3′ R: 5′-CTGGATCTACCTATACAGTCCTA-3′	170
GAPDH	F: 5′-TCGTGGAAGGACTCATGACCACA-3′ R: 5′-AGGCAGGGATGATGTTCTGGAGAG-3′	120

Data were reported as “fold change” compared to the expression level of GAPDH mRNA. The mean Ct value for all experimental groups were analyzed according to the formula: The relative quantities (Q) = $2^{-\Delta\Delta Ct}$ [15]. Whereas $\Delta Ct_{t\ \mathrm{PTTG1}} = Ct_{control\ (without\ treatment)} - Ct_{treatment\ \mathrm{(with\ PTTG1\ siRNA)}}$ and $\Delta Ct_{si\text{-}NEG} = Ct_{control\ (without\ treatment)} - Ct_{treatment\ \mathrm{(with\ si\text{-}NEG)}}$. Subsequently, the $\Delta\Delta Ct$ is calculated by $\Delta Ct_{si\text{-}NEG} - \Delta Ct_{t\ \mathrm{PTTG1}}$.

2.3. Western blotting

Total protein was extracted by chilled lysis buffer (contain 5% SDS, Triton X100, 100 mM Tris-HCl (pH 4.7), sodium deoxycholate 150 mM NaCl, 5 mM EDTA, 10% glycerol) supplemented with 1% protease inhibitor Cocktail (Sigma-Aldrich). The equal amounts of extracted protein supernatant (100 μg) were resolved on SDS-PAGE electrophoresis gel (12%) and transferred to a nitrocellulose membrane. The membranes were incubated with anti-PTTG1 antibody (sc-22772, Santa Cruz Biotechnology, USA) at a dilution of 1:800 and subsequently re-probed with 1:500 β-actin (A5441, Sigma-Aldrich, USA) at 4 °C overnight. Then, blots were incubated with goat anti-mouse IgG horseradish peroxidase (sc-2005, Santa Cruz Biotechnology, USA) for 2 h. The proteins were visualized by the Enhanced Chemiluminescent Detection kit (ECL, ab65623, Abcam) according to the instructions provided. Band densities were determined using NIH ImageJ software (US National Institutes of Health, Bethesda, MA, USA).

2.4. 2D electrophoresis and image analysis

Total proteins were extracted as earlier explained and quantified by the 2D Quant Kit (Amersham Biosciences) using BSA as standard. The entire 2D protocol was performed as previously described [16]. Briefly, IPG strips (18 cm 3–10 NL, Immobiline Dry Strip; Bio-Rad) were transferred onto vertical 12.5% SDS-PAGE gels and second dimension was performed at a constant current of 7.5 mA per gel for 30 min and 20 mA per gel for 5 h at 20 °C. Following gel fixation by methanol and acetic acid solution for 1 h, the gels were stained with colloidal Coomassie Brilliant Blue G-250 [17]. Image analysis, spot detection, and pairing were carried out using Image Master 2D Platinum 6.0 (Amersham Biosciences). The spot intensity of three different experiments was measured and subsequently, the induction ratios were determined by comparing gel intensities between inoculated gels and control.

2.5. Protein identification by LC-ESI-MS/MS

To identify the differentially expressed proteins, protein spots were manually excised from the gels (1 mm^3) and destained with a solution of acetonitrile (1:1 v/v) and ammonium bicarbonate (25 mM). Then, the destained pieces were equilibrated in 5 mM ammonium bicarbonate, and digested in ammonium bicarbonate (5 mM) containing 10 ng/μl trypsin at 37 °C for 16 h. Following digestion, trifluoroacetic acid (10%) was added and supernatant was collected. Finally, the samples were dissolved in 2% acetonitrile, 0.1% TFA. Protein sequence analysis was performed by Q-TRAP (LC packings and Applied Biosystems/MDS Sciex) nLC-MS/MS system. The criteria for searching including up to two missed cleavages and 0.5 Mass accuracy, allowed for parent and the fragment ions, and variable modifications set to deamidated arginine or glutamine and oxidation of methionine were considered. To validate the MASCOT scores for protein identification, the MFPaQ program version 4 was used.

2.6. Cell proliferation assay

To determine the effect of PTTG down-regulation on cell viability, MTS assay was performed according to the manufacturer's instructions (Promega, CellTiter 96 Aqueous, USA). Cells were seeded at a density of 2×10^3 cells/well in 96-well plates and transfected with siRNA when the cells were nearly 70% confluent as described above. Subsequently, the MTS viability assay performed at 0, 24 and 48 h after transfection (Each test was repeated 4 times). The absorbance was determined by the BioTek plate reader at 490 nm.

2.7. System biology tools

The possible biological consequences were determined following PTTG1 silencing in MDA-MB-231 cells, protein classification analysis was performed using the bioinformatics databases. PANTHER (v 14.0), KEGG, and STRING (v 11.0) resources were employed to identify the functional annotation, significant pathways, and protein interactions as a signature to predict the clinical outcomes. The partner of NME1, STMN1, ENO1, NR3C1, and NPM1 was retrieved with at least medium confidence (score 0.4) and Kmeans clustering based exclusively on experimental, co-expression, text mining, and database knowledge.

2.8. Statistical analysis

The statistical analysis was carried out using the SPSS software, version 18.0 (SPSS Inc., Chicago, IL). The results were presented as mean value ± SD. The $p \leq 0.05$ was considered as statistically significant in all tests.

3. Results

3.1. PTTG siRNA decreases PTTG mRNA and Securin expression

The expressions of PTTG1 at mRNA and protein levels were measured after PTTG siRNA transfection in MDA-MB-231 breast cancer cell line. As shown in Fig. 1A, a relative expression that examined by REST software indicated that the PTTG gene was successfully down-regulated at 24, 48 and 72 h post-transfection at the mRNA level.

To evaluate the effect of PTTG siRNA on the expression of Securin protein, Western blot assay was performed (Fig. 1B). Subsequently, the quantification of protein band density in radiography films was carried out by the imageJ software (Fig. 1C). Our results displayed reduction in Securin at all times of 24, 48 and 72 h. The main reduction was indicated at 48 h after PTTG silencing.

3.2. Cell proliferation reduction following PTTG siRNA transfection

We recorded decreased cell proliferation after application of PTTG siRNA in MDA-MB-231 cell line compared to the si-scramble treated cells (Fig. 2). Two-way ANOVA analysis revealed that proliferation of cells decreased during 48 h post-siRNA transfection in PTTG siRNA-treated cells than treated cells with the si-scramble and blank ($p < 0.05$). The significant difference was not observed between the untreated cells and si-scramble treated cells.

3.3. Identification of PTTG silencing-regulated proteins

Since the lowest amount of Securin protein was identified at 48 h post-transfection by Western blotting, we analyzed the expression pattern of Securin downstream proteins at this time. In total, 7 proteins consistently appeared to be differentially expressed between silenced and siNEG treated cells, of which 6 up-regulated and 1 down-regulated (Table 2). The nature of differentially expressed proteins was identified by mass spectrometry. The theoretical masses and the isoelectric points of these proteins were almost the same, suggesting that they could be closely located on 2D-PAGE. The downstream up-regulated proteins by PTTG1 silencing are NPM1 (nucleophosmin, +15.7 fold), NME1 (nucleoside diphosphate kinase, +9.48 fold), STMN1 (stathmin, +5.36 fold), ENO1 (Alpha enolase, +5.65 fold), and NR3C1 (+3.51 fold) (Table 2). NPM1 and NME1 showed higher fold change expression among these proteins.

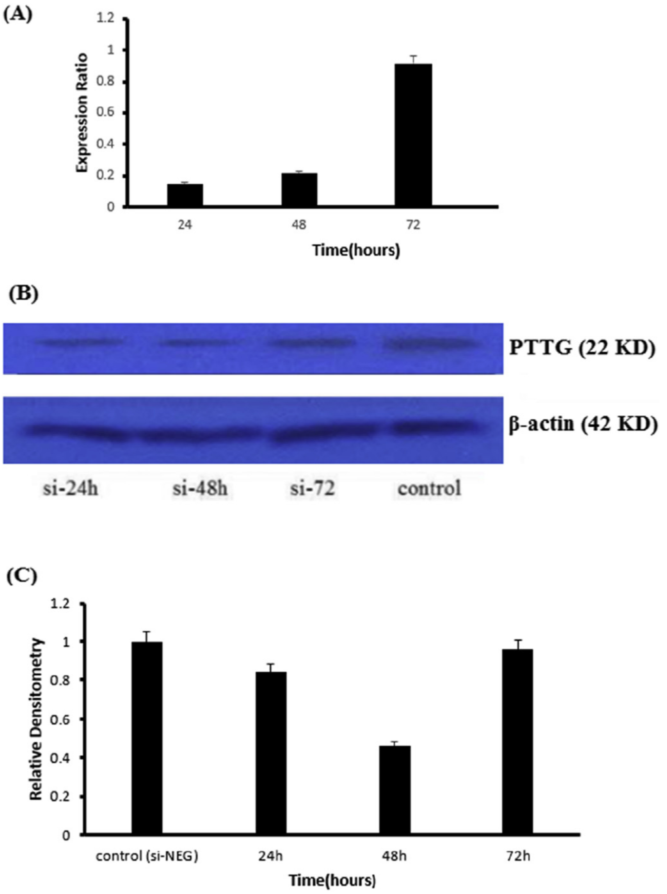


Fig. 1. siRNA against PTTG1 reduces its mRNA and protein expression. After transfection of MDA-MB-231 cells with PTTG1 siRNA, its expression in mRNA and protein levels were assessed by real-time PCR and western blot, respectively. (A) Significant down-regulation of PTTG1 was observed at 24 and 48 h post transfection. The lowest expression was seen after 24 h PTTG1 siRNA induction. (B) Radiography bands of Securin (22 kDa) shows PTTG1 siRNA reduced the expression of PTTG1 protein after 24 and 48 h post-transfection. (C) The western blot bands were quantified by ImagJ software. The results showed the highest down-regulation of PTTG1 at 48 h after cell transfection.

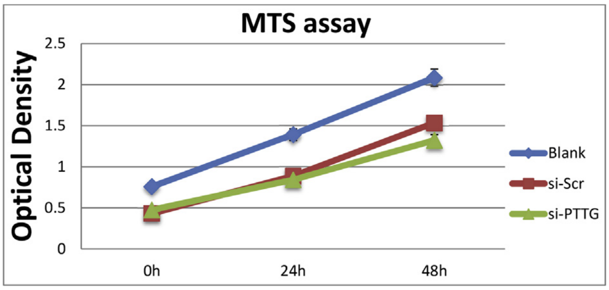


Fig. 2. PTTG1 gene suppression inhibits MDA-MB-231 cell line proliferation. Si-PTTG1: siRNA against PTTG1; si-Scr: Random siRNA as a control; Blank: non-siRNA treated cells.

In order to verify the proteomics results, further, the transcription level of these proteins was assessed by the real-time PCR. The ratio of RNA expression level at the PTTG1 siRNA treated line in compared to the control line revealed that NPM1 (+8.61 fold), NME1 (+1.72 fold), STMN1 (+3.10 fold), ENO1 (+7.83 fold), and NR3C1 (+7.21 fold) up-regulated within 48 h post-transfection (Table 2).

Table 2
Differentially expressed proteins following PTTG1 siRNA post-transfection in MDA-MB-231 breast cancer cells.

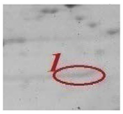
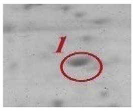
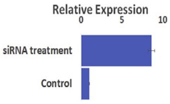
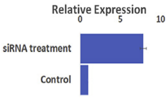
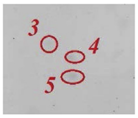
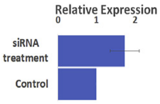
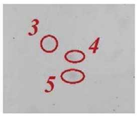
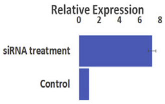
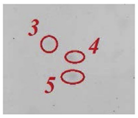
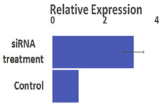
Spot ID	Gene name	Accession number	Chromosome	Theoretical MW (kDa)/pI	On gel MW (kDa)/ pI	Ratio for protein spot (treatment/ control)	Status	Control	Treatment	Ratio for RNA	Histogram of relative gene expression (RNA)
1	NPM1	4869	5	32,726/4.64	43.87/ 4.8	15.77	Up			8.61	
2	ENO1	2023	1	47,481/7.01	48/6.05	5.65	Up			7.83	
3	NME1	4830	17	17,309/5.83	22/5.46	9.48	Up			1.72	
4	NR3C1	2908	5	86,745/6.00	21/5.4	3.51	Up			7.21	
5	STMN1	3925	1	17,292/5.76	19/5.43	5.36	Up			3.10	
6	–	–	–	–	50/5.78	3.51	Up				
7	–	–	–	–	49/5.6	0.37	Down				

Table 3
Biological and cancer associated function of differentially expressed proteins in response to PTTG1 silencing.

Gene name	Protein name/ID	Molecular & cellular function	Cancer-associated function
ENO1	Alpha-enolase (P06748)	It is a multifunctional enzyme implicated in growth control, hypoxia tolerance and allergic responses. Myc promoter-binding protein-1 and ENO1 originate from a single gene by the alternative use of translational starting sites. It may be a tumor suppressor through the binding to the MYC promoter [36].	Lack of ENO1 promotes oxidative stress in various cancer cells. It also induces autophagy and fatty acid oxidation. ENO1 silencing reduced cancer cell growth and survival *in vitro* [37].
NPM1	B23 nucleophosmin (P06748)	Maintenance of genomic stability and the regulation DNA transcription	In cancer, it regulates the activity of tumor suppressors such as p53 and ARF. Its oncogenic function is attributed to the cytoplasmic localization. The enhanced level of NPM reduced breast tumor cell growth, metastasis, and invasion *in vivo* by γ-catenin [38].
NME1	Nucleoside diphosphate kinase A (P15531)	It has nucleoside-diphosphate kinase and serine/threonine-specific protein kinase that implicated in cell proliferation, differentiation and development and gene expression [24].	High expression of NME1 induces apoptosis and its deletion suppresses apoptosis by increasing of Bcl2L10 in cervical and ovarian carcinoma [24]. The regulated genes by NME1 are involved in metastasis and known as prognostic value for the outcome of survival in breast cancer and melanoma patients [39].
STMN1	Stathmin 1 (P06748)	STMN1 is a phosphoprotein that known as a destabilizer of microtubule. It possesses a critical role in cell cycle, chromosome segregation, cell motility and survival. This protein is contributed to the MAPK, PI3K, CDKs, and AURKB pathways [40].	Reduction in STMN1 expression occurs through the activation of p21 and EGR-1 by p53 induction. Both STMN1 over- and down-regulation cause abnormalities in progression of M phase. Silencing of STMN1 results in cell cycle arrest and induction of apoptosis in breast and lung cancers [29,41]. The binding of STMN1 to p27Kip1 inhibits cell migration associated with extracellular matrix.
NR3C1	Glucocorticoid receptor (P04150)	It is a transcription factor that binds to glucocorticoid response elements of nuclear and mitochondrial DNA. It involves in glucocorticoid-induced apoptosis and accurate chromosome segregation during M phase. It may play as a tumor suppressor [30,42].	This protein with dual activity involved in mRNA degradation by interaction of PNRC2. The reduced level of glucocorticoid receptor in various human cancers suggesting its tumor suppressor function [30].

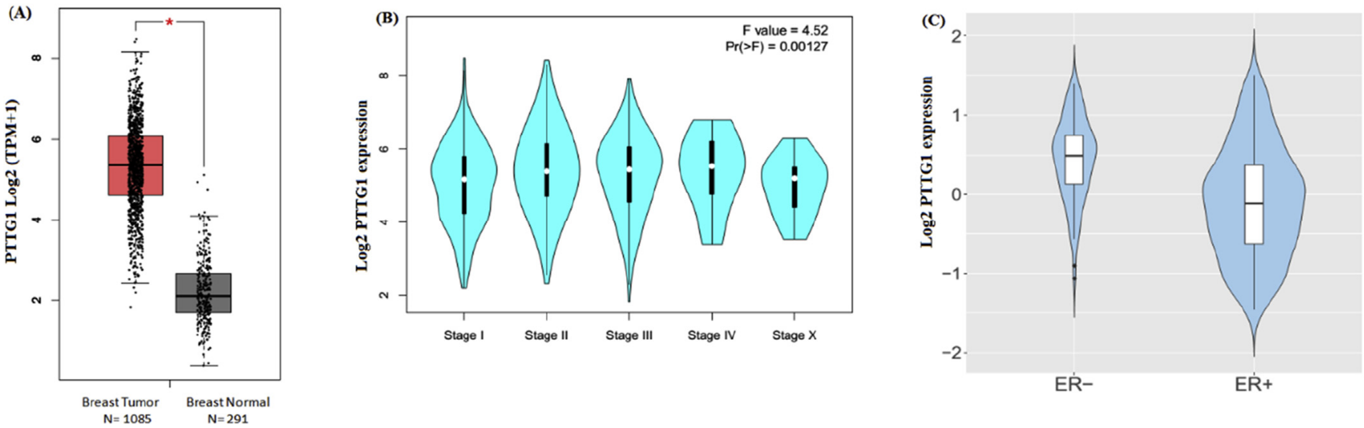


Fig. 3. Box, stage and surface receptor plots analysis of PTTG1 expression in breast cancer. The expression of PTTG1 is significantly increased in breast cancer than normal tissues. The increased expression level of PTTG1 represents in advanced stages of breast cancer. Moreover, the high expression of this gene is not related to the presence of estrogen receptor. Data retrieved from GEPIA and CANCERTOOL resources.

The biological and cancer associated function of differentially expressed proteins in response to PTTG1 silencing are listed in Table 3. They are mostly involved in the cancer related pathways.

3.4. *In silico expression profile of PTTG1*

The expression pattern of PTTG1 gene across breast carcinoma samples and paired normal tissues was investigated by the GEPIA database. PTTG1 gene expression is significantly increased in breast tumor tissues than normal tissues (Fig. 3A). Further comparison using violin plot depicts the expression of PTTG1 among different stages of breast cancer specimens (Fig. 3B). The median expression of PTTG1 was higher in stages II, III, and IV than stages I and X.

Moreover, data analysis of The Cancer Genome Atlas database (TCGA) demonstrated that PTTG1 was highly expressed in negative estrogen receptor breast cancer tissues compared to the positive estrogen receptor tissues ($p = 2.617\mathrm{e}-15$; Fig. 1C). Also, ARCHS4 resource indicated that the expression of this gene in different MDA-MB cell lines was higher than MCF-7 cell line.

3.5. *Gene ontology and protein interactions analysis*

Using PANTHER, we found that proteins regulated by PTTG1 were mostly involved in biological regulation (GO: 0065007; 27.3%), metabolic process (GO: 0008152; 27.3%), and cellular component organization or biogenesis (GO: 0071840; 18.2%). The GO enrichment analysis suggested three major molecular function categories: (1) binding (GO: 0005488; 50%), (2) catalytic activity (GO: 0003824; 33.3%), and (3) transcription regulator activity (GO: 0140110; 16.7%) (Fig. 4A).

Generally, cancer cells are identified by imbalance of cell proliferation and cell death (apoptosis), increasing angiogenesis, activation of oncogenes and presence of hypoxia. For further identification of functionally linked proteins, the proteins associated with cell proliferation (TP53, MAPK1, BCl2), angiogenesis (VEGF) and hypoxia (HIF1A) were added to the STRING network analysis. Fig. 4B illustrates a core network of PTTG-regulated proteins. According to the STRING analysis, these proteins were mostly implicated in metabolic process (NME1, NR3C1), cell migration (ENO1, NPM1), and apoptosis (NME1, ENO1) suggesting a remarkable task of PTTG1 in these processes.

4. Discussion

Breast cancer is still a growing health concern in female worldwide [18]. Several genetic changes occur in multiple steps of breast cancer initiation, progression and metastasis. Frequent up-regulation of PTTG have been presented in different cancer types [4,19]. Also, its expression has been shown to strongly promote the proliferation of several breast cancer cell lines such as MCF-7, MCF-10, MDA-MB-231, and SKBR3 [20]. However, the molecular mechanisms elucidating the PTTG1 action in breast cancer are mostly unknown. This study, therefore, was designed to determine the molecular targets and/or proteins regulated by PTTG in the progression of MDA-MB-231 breast cancer cells.

According to the GEPIA resource, PTTG is up-regulated in most of the cancers except leukemia and testicular cancer cells [14]. We showed that specific down-regulation of PTTG by siRNA inhibits the proliferation of MDA-MB-231 breast cancer cell line, supporting the efficient delivery of this siRNA into the cells. Also, this siRNA targeting PTTG remarkably reduces the expression of PTTG in both mRNA (~80%) and protein (~50%) levels.

Based on our investigation, down-regulation of PTTG decreased cell proliferation and changed the expression profile of downstream proteins. Proteomics in accompany with genomics provides a complement network of molecular pathways underlying the cellular response exposed to PTTG siRNA. The proteomics results showed 7 differentially expressed proteins within 48 h of breast cancer cells transfection by siRNA against PTTG1.

By silencing of PTTG1, NPM1 was the first highly expressed protein. However, up-regulation of NPM1 has been reported in various cancers. The previous research reported its positive correlation with migration of colon [21] and melanoma [22] cancer cells. This protein is implicated in chromosome maintenance, DNA repair (GO: 0006281), response to DNA damage by TP53 (GO: 0006977), nucleocytoplasmic transport (GO: 0006913), response to stress (GO: 0006950), and HIF1 pathway. It has been demonstrated that its expression is regulated by ARNT as a transcription factor for HIF1. Furthermore, the role of NPM1 in the metastasis was demonstrated by its interaction with CDK2 and RB1 that associated with MTOR and AKT1 [22].

The second up-regulated protein, NME1, is a metastasis suppressor that is involved in metabolic process (GO: 0008152) with catalytic activity (GO: 0003824). It is introduced as a granzyme A that induces caspase-independent cell death pathway [23]. Also, this protein acts as a negative regulator in the regulation of Diva-mediated cell death activity as a negative regulator in SKOV3 and Hella cells. The over-expression of NME1 in these cell lines induced apoptosis [24].

We found that ENO1 production increased by PTTG1 siRNA treatment. ENO1 is involved in cell-cell adhesion (GO: 0098609). Moreover, it plays as a transcription repressor for MYC promoter [25] which is a regulator of cell progression and apoptosis. It participates in the PI3K/AKT pathway that correlates with tumor cell invasion [26,27]. This protein also participates in HIF1 signaling pathway to promote anaerobic metabolism (hsa04066).

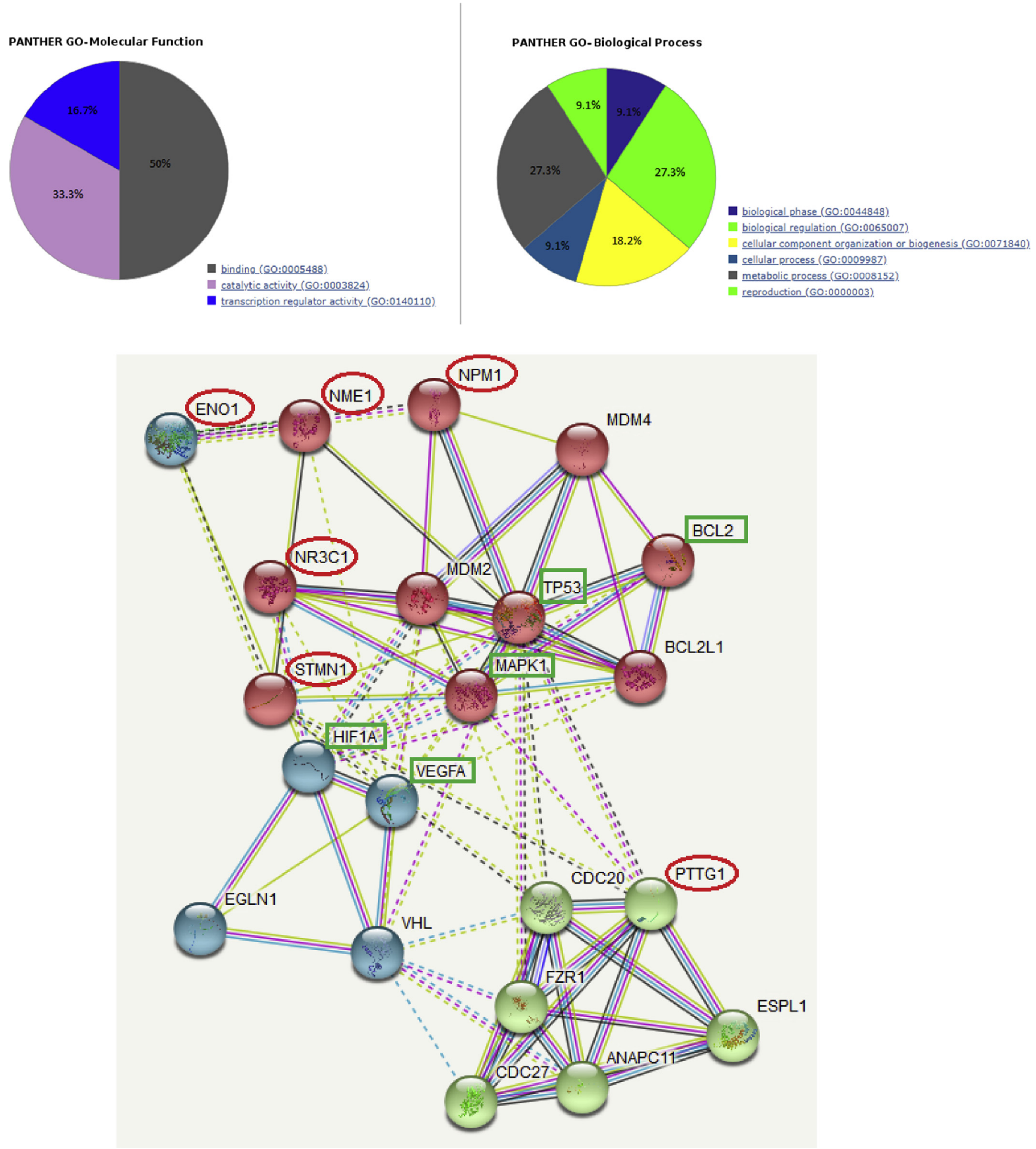


Fig. 4. Functional classification of differentially expressed proteins in MDA-MB-231 cells exposed to RNAi-induced silencing of hPTTG1. (A) Pie charts showing classification according to molecular function and biological process. Data retrieved from PANTHER classification system v14.0. (B) The physical and functional interactions of identified proteins with medium score (0.400) are selected based on the STRING database v 11.0. The lines indicate experimental (pink), co-expression (grey), textmining (green), and database (cyan) evidences. The identified and added proteins are highlighted in red and green, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Another PTTG1-regulated protein was STMN1 which acts in the microtubule organization and cytoskeletal signaling pathway. This protein is engaged in MAPK pathway (GO: 0000165) and protein phosphorylation (GO: 0006468). The over-expression of STMN1 has been reported in melanoma that contributes to the reduced level of miR-193b. Also, P21 and p27 are introduced as downstream effectors of STMN1 signaling [28]. Moreover, repression of STMN1 expression by siRNA decreased cell growth and induced tumor-suppressor activity in mutant p53 breast cancer cells [29].

The last regulated protein by PTTG1 was NR3C1. Transcription regulator activity (GO: 0140110) and binding (GO: 0005488) are molecular functions of this protein. It involves in biological regulation (GO: 0065007) and metabolic process (GO: 0008152). This bi-functional glucocorticoid receptor protein has a role in mRNA decay by PNRC2 interaction and the 5′-UTR binding. To date, the reduced expression of glucocorticoid receptor was reported in various human cancers including lung, liver, colon and breast. It may act as tumor suppressor as suggested by its reduced expression level [30].

Proteomic results of the current study, however, showed little overlap with previous research in lung cancer. They demonstrated that PTTG1 reduction was accompanied with suppression of Ki67, bFGF and CD34 in H1299 tumor cells that are associated with tumor progression and angiogenesis [31]. Irrespective of cell type difference, this disagreement may depend on the limited capacity of 2D gel for detection of proteins with specific molecular weight. Although, proteomics could expand our knowledge by figuring out unexplored molecular mechanisms [32]. The identified PTTG1-regulated proteins in breast and lung cancers are associated with cell growth and blood vessel development.

As shown in Table 3, these proteins are participated in cancer associated cellular processes including cell proliferation and apoptosis. For instance, NME1 and NR3C1 play as an inducer of apoptosis and tumor suppressor, respectively. While, STMN1 promotes cell growth by inhibiting the function of nuclear p27, which could support and explained previous findings that revealed the role of PTTG1 in the reduction of p27 in MCF-7 cells [33].

To investigate the involvement of differentially expressed proteins in breast cancer, a preliminary analysis was performed by the open-resource STRING tool. This analysis provided that up-regulated genes by PTTG1 repression take part in cell growth, hypoxia, and angiogenesis conditions. For instance, NR3C1, NPM1, and PTTG1 were directly interacted with TP53 [12,34], while ENO1 indirectly interacted with TP53 through NPM1. Previous research in melanoma have demonstrated a functional interaction of STMN1 and NPM1 with TP53 as well as NPM1 and ENO1 with MYC protein that are associated with apoptosis pathway. NPM1 with CASP6 and STMN1 with MAPK1 were also involved in apoptosis [22].

Functional interactions network of these proteins by KEGG analysis suggested that these proteins are involved in gluconeogenesis, RNA degradation, biosynthesis of amino acids, HIF1 signaling pathway, metabolic and cell cycle pathways [35].

5. Conclusion

The purpose of this study was to determine the molecular targets of PTTG1 in breast cancer by integrating proteomics and bioinformatics approaches. Only gene expression analysis could not explore and define the molecular changes and signaling pathways under the control of a target gene. This research, therefore, extended our knowledge of molecular mechanisms involved in the pathogenesis of breast cancer by PTTG1 influencing. Herein, we demonstrated that suppression of breast cancer cell viability using PTTG1 siRNA is associated with NPM1, NME1, ENO1, STMN1 and NR3C1 genes. The products of these genes are involved in cell proliferation, apoptosis, and angiogenesis, which are related to the cancer progression. The identification of PTTG-regulated proteins could be considered for the effectively targeted drug design, prognosis and follow up in breast cancer therapy.

Contributors

Majid Shahbazi initiated the research program and supervised the project. Ghasem khazaei established and carried out lab works. Masoud Golalipour involved in cell culture establishment method. Ahad Yamchi involved in 2D electrophoresis establishment. Fatemeh T. Shamsabadi analyzed the proteomics and bioinformatics data as well as wrote the paper. Gagan Deep Jhingan involved in Mass Spectrometry establishment. Majid Shahbazi revised the paper.

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116873.

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