Proteomics analysis of siRNA-mediated silencing of Wilms' tumor 1 in the MDA-MB-468 breast cancer cell line

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Abstract. The Wilms' tumor 1 (*WT1*) gene encodes a zinc finger which appears to be a transcriptional activator or repressor for many genes involved in cell differentiation, growth and apoptosis. In order to determine the relationship between WT1 and related proteins, WT1 was silenced with small interfering RNA (siRNA) and the protein expression pattern was analyzed by proteomics analysis including one-dimensional gel electrophoresis (1-DE) and LC-MS/MS mass spectrometry. The results revealed that 14 proteins were expressed in WT1-silenced cells ($siRNA_{WT1}$) and 12 proteins were expressed in the WT1-expressing cells ($siRNA_{neg}$), respectively. These proteins may be classified by their functions in apoptosis, cell signaling, protein folding, gene expression, redox-regulation, transport, structural and unknown functions. Mitogaligin, an apoptosis-related molecule, was identified when WT1 was silenced while the proteins related to the signaling pathway were detected in both $siRNA_{neg}$ and $siRNA_{WT1}$ but the type of proteins were different. For example, the IBtK protein and the SH2 domain-containing protein were present in $siRNA_{WT1}$ conditions, while the platelet-derived growth factor receptor α (PDGFRA) and Rho guanine nucleotide exchange factor 1 (Rho-GEF 1) were expressed in $siRNA_{neg}$. Of these, Rho-GEF was selected for validation by western blot analysis and demonstrated to be present only in the presence of WT1. In conclusion, WT1 is related to mitogaligin via EGFR and behaves as an anti-apoptotic molecule. Moreover, WT1 may be associated with PDGFRA and Rho-GEF 1 that activates proliferation in MDA-MB-468 cells.

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Introduction

Breast cancer is the most common cancer and the leading cause of mortality in women worldwide, accounting for 23% (1.38 million cases) of the total new cancer cases and 14% (458,400 cases) of all cancer deaths in 2008 (1). The development of breast cancer may result from interaction between the change in genetic elements, environmental factors and also the difference in ethnicity (2). There are several genes reported to be associated with breast cancer, such as *ERBB2*, *c-Myc*, *CCND1*, *TP53*, *PTEN* and *Wilms' tumor 1* (*WT1)* (3).

The human *WT1* gene is located at chromosome locus 11p13 comprising 10 exons. Alternative splicing occurs at exon 5 (plus or minus 17AA) and exon 9 (plus or minus KTS) in mRNA of WT1. These two alternative splicing sites yield four different isoforms: WT1+/+, WT1+/-, WT1-/+ and WT1-/- (4,5). Loeb *et al* demonstrated that WT1 mRNA and protein was detected in nearly 90% of breast cancers but not in most normal breast samples (6). Moreover, Navakanit *et al* reported that the siRNA against WT1 inhibited both WT1 protein expression level and growth of breast cancer cell line MCF-7, in a dose- and time-dependent manner. These results suggested that WT1 may act as an oncogene in the breast cancer cell line MCF-7. Furthermore, WT1 may play a role in the pathogenesis of breast cancer as an oncogene rather than a tumor suppressor gene as in leukemia (7). Additionally, the high level expression of WT1 mRNA detected by real-time RT-PCR can predict a poor prognosis in breast cancer patients (8) and the absence of mutations through the whole 10 exons of the *WT1* gene in the 36 cases of primary breast cancer (9). *WT1* encodes a zinc finger acting as a transcriptional activator or repressor for many genes involved in cell differentiation, growth and apoptosis. These functions depend on the type of cells, WT1 isoforms and the status of targeted molecules. There are several targeted molecules for WT1 including growth factor genes: IGF-II, PDGF-A, CSF-1 and TGF-β1, growth factor receptor genes: insulin receptor, IGF-1R and EGFR, and transcription factor and other genes, including: Egr1, PAX4, p53, c-myc, Bcl-2, cyclin E, Bak, Bax (10,11). However, the relationship between WT1 and the targeted molecules involved in breast cancer remains unclear

and the overview study of the relationship between WT1 and the related molecules has not been reported.

MDA-MB-468 breast cancer cells are estrogen receptor (ER), progesterone receptor (PR) and HER2-negative (12). The cells have a very high number of the epidermal growth factor receptors (EGFRs) which is growth inhibited by EGF and mediated apoptosis (13,14). Moreover, the cells have a p53 mutation, G -> A mutation in codon 273 of the p53 gene resulting in an Arg -> His substitution (15).

In the present study, we used siRNA against WT1 mRNA to silence WT1 expression and the relationship between WT1 and related proteins in the breast cancer cell line MDA-MB-468 was investigated by proteomics analysis. The proteins were further identified by LC-MS/MS and database searching. These studies may provide further evidence to understand the relationship between WT1 and the related molecules in breast cancer.

Materials and methods

Cell culture. The human breast cancer cell line MDA-MB-468 was purchased from American Type Culture Collection. MDA-MB-468 was cultured in DMEM (Gibco-BRL) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin and 10% glutamine. Then, the cells were incubated in a 37˚C incubator with 5% CO_2 (7).

Small interfering RNA (siRNA) transfection. MDA-MB-468 at $1x10^5$ cells were seeded in each well of 24-well culture plates and incubated in a CO_2 incubator at 37˚C for 24 h. The cells were transfected using Lipofectamine® 2000 reagent (Invitrogen) in 24-well plates with 200 nM siRNA duplexes (optimal siRNA conditions performed in preliminary study and data not shown). The siRNA against WT1 ($siRNA_{WT1}$) (Invitrogen) consisted of a mixture of two 25-nt duplexes, i.e., $siRNA_{WT1}$R88 (5'-AAATATCTCTTATTGCAGCCT GGGT-3') and $siRNA_{WT1}$R90 (5'-TTTCACACCTGTATGTCT CCTTTGG-3'). To minimize the cytotoxicity of the reagent itself, the cells were washed once with PBS and the media was changed 6 h after transfection (7). After 72 h, the cells were harvested and the protein level was investigated by western blot analysis.

Western blot analysis. Cell pellets were harvested by trypsinization and extracted with radioimmunoprecipitation assay (RIPA) buffer (Pierce, USA). Then, the concentration of protein was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). The 50 μg of protein samples were loaded to 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked by blocking solution [5% low fat dry milk in 1X TTBS (0.1% Tween-20, 154 mM NaCl, 48 mM Tris-base)] for 1 h and washed 5 min for three times with washing solution (1% low fat dry milk in 1X TTBS buffer). After blocking, the blot was incubated with primary antibody anti-WT1 (1:200), anti-rho-GEF (1:1,000) and anti-actin (1:1,000) antibodies (diluted with 1% low fat dry milk in 1X TTBS) for 2 h and washed 5 min for three times with washing solution. The membrane was then incubated with secondary antibody polyclonal anti-IgG rabbit (1:10,000) antibody in 1% low fat dry milk in 1X TTBS for 1 h and washed three times (10 min/wash). The proteins were visualized using a chemiluminescent detection kit (Pierce) and exposed to X-ray film (7).

Proteomics analysis

One-dimensional electrophoresis (1-DE). After transfection, $siRNA_{neg}$- and $siRNA_{WT1}$-transfected cell pellets from MDA-MB-468 were extracted with 0.5% SDS. Protein quantification was calculated using the Lowry method. Total protein samples of 50 μg were loaded onto 12.5% SDS-polyacrylamide gel and a marker lane (low range marker; GE Healthcare) was added for calculation of the molecular weight of the protein bands. The gel was run at 20 mA/gel for 1.45 h. After electrophoresis, the gel was fixed in fixing solution (40% ethanol, 10% acetic acid) and stained with Colloidal Coomassie Blue G-250 (8% ammonium sulfate, 0.8% phosphoric acid, 0.08% Coomassie Brilliant Blue G-250 and 20% methanol). Then, the gel was scanned with EPS 601 scanner (Bio-Rad) and the gel bands were fractionated to 15 slices and excised from the bottom to the top of the gel lane and each slice was cut into 1 mm cubes. The gel pieces were transferred into a well of low binding 96-well plates.

In-gel digestion. The gel pieces were destained by washing twice with 25 mM ammonium bicarbonate in 50% methanol and further washed with 100% acetonitrile. Dried gel pieces were added with 20 μl of 10 mM DTT in 10 mM ammonium bicarbonate and incubated at 56˚C for 1 h, followed by the addition of 100 mM iodoacetamide in 10 mM ammonium bicarbonate. The gel pieces were then digested with 10 μl of 10 ng/μl sequencing grade modified porcine trypsin (Promega, USA) in 10 mM ammonium bicarbonate solution and incubated at 37˚C overnight. The peptides were extracted by addition of 30 μl of 50% acetonitrile in 0.1% formic acid and dried at 40˚C overnight.

LC-MS/MS and protein identification. The dried extracted peptides were resuspended with 12 μl of 0.1% formic acid and transferred to low binding microcentrifuge tube. Solution was centrifuged at 10,000 rpm for 10 min and transferred to vial tube. The resuspended peptide was injected to LC-MS/MS (ESI-QUAD-TOF mass spectrometry). The peptide sequences from LC-MS/MS were analyzed by Mascot Search and identified by NCBInr database.

Results and Discussion

siRNA against WT1 transfection in MDA-MB-468 cell line. The MDA-MB-468 breast cancer cell line was transfected with 200 nM of siRNA against WT1 ($siRNA_{WT1}$) compared to control ($siRNA_{neg}$) for 72 h. After transfection of the cells we detected WT1 level by western blot analysis. The results showed that knockdown of WT1 led to decrease in WT1 protein expression in MDA-MB-468 (Fig. 1A).

1-DE. The quantitative proteomic, one-dimensional gel electrophoresis (1D-PAGE) was carried out to determine the protein expression patterns between $siRNA_{neg}$ compared to $siRNA_{WT1}$ in MDA-MB-468. Fig. 1B1 represents the protein patterns obtained from 1D-PAGE. Lane 1 and 2 show the

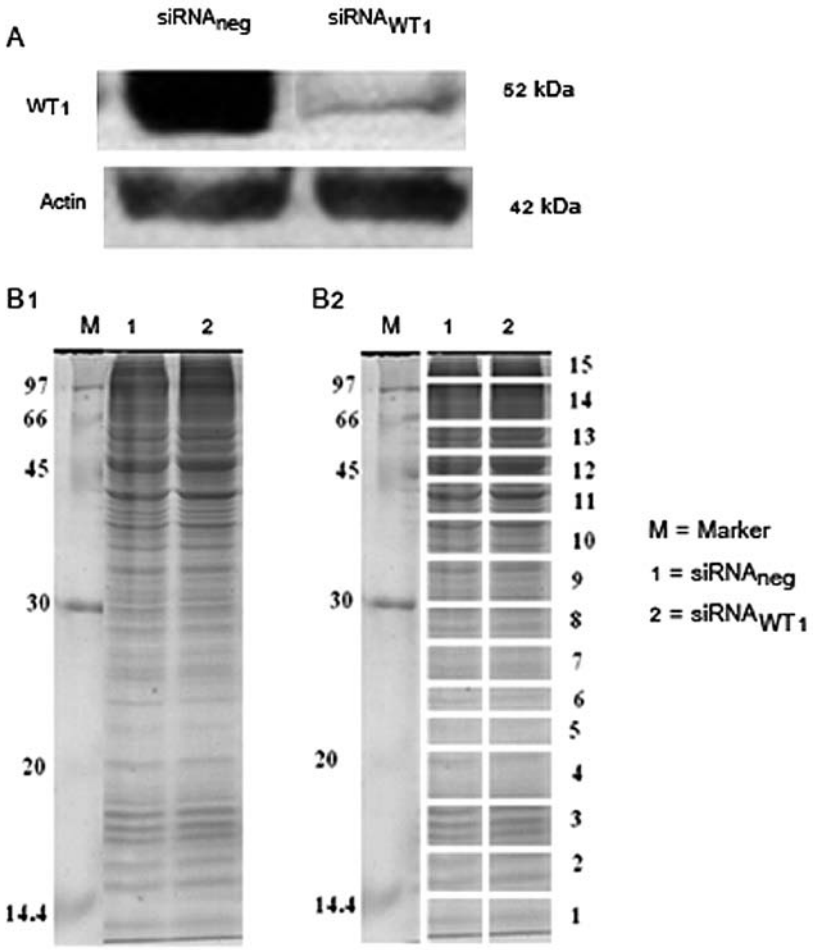

Figure 1. (A) siRNA$_{WT1}$ transfection in the MDA-MB-468 cell line. MDA-MB-468 was transfected with 200 nM of siRNA$_{WT1}$ for 72 h. Total protein (50 μg) was detected by western blot analysis; (B) SDS-gel image (12.5%) of protein pattern between siRNA$_{neg}$ compared to siRNA$_{WT1}$ in MCF-7 and MDA-MB-468 (B_1) and gel after fractionation into 15 slices (B_2). siRNA, small interfering RNA; WT1, Wilms' tumor 1.

protein bands of siRNA$_{neg}$ and siRNA$_{WT1}$, respectively. After 1D-PAGE, the gels were cut into 15 slices as shown in Fig. 1B2.

The quantification of protein from 1D-PAGE was analyzed by the DeCyder™ MS 2.0 Differential Analysis Software (GE Healthcare). The protein expressions of siRNA$_{WT1}$ and siRNA$_{neg}$ were compared. The different intensity of protein expression in both conditions is shown in Venn's diagram (Fig. 2A). These demonstrated all possible relations of protein expressions in two conditions. The protein names and their biological functions of expressed proteins found only in siRNA$_{WT1}$ are listed in Table I and the expressed proteins found only in siRNA$_{neg}$ are listed in Table II. Table III shows the protein names and the biological functions of expressed proteins found in siRNA$_{WT1}$ and siRNA$_{neg}$. Rho guanine nucleotide exchange factor 1 (Rho-GEF) was selected to validate by western blot analysis. The result showed the presence of Rho-GEF only in WT1 presence in the cell (Fig. 2B).

Due to p53 mutation in MDA-MB-468 (15), the apoptosis pathway may occur via p53 independently. Notably, a novel target protein of WT1, mitogaligin, was found when WT1 was silenced. Mitogaligin is a 96 amino acid protein highly cationic and rich in tryptophan (16). This protein contains two localization signals, mitochondria and nucleus. Mitogaligin is mainly localized in mitochondria and promotes the release

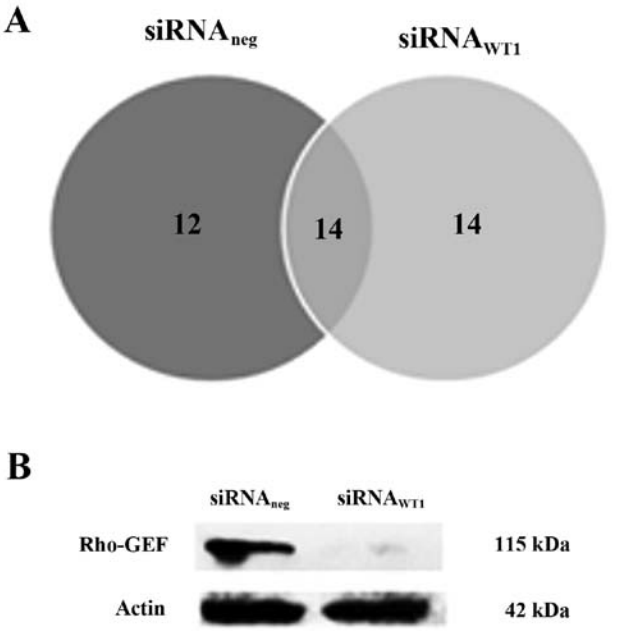

Figure 2. (A) Venn's diagram of protein expression with different intensity between siRNA$_{WT1}$ transfection and siRNA$_{neg}$ control of MDA-MB-468; (B) Rho-GEF was validated by western blot analysis. siRNA, small interfering RNA; WT1, Wilms' tumor 1; Rho-GEF, Rho guanine nucleotide exchange factor 1.

Table I. Identification of expressed proteins found only in MDA-MB-468 $siRNA_{WT1}$ using DeCyder™ MS 2.0 Differential Analysis Software.

Protein name	Accession no.	Peptide	Mowse score
Apoptosis			
Mitogaligin	gil12005991	AWRMGEPACWGR	9.50
Cell signaling			
IBTK protein, partial	gil34192875	SLDVLSDGVLK	27.56
SH2 domain-containing protein 3C isoform a	gil41281821	RSSASISR	11.47
Structural protein			
Cytokeratin 9	gil435476	GGSGGSYGGGGSGGGYGGGSGSR	91.06
Keratin 10	gil21961605	SQYEQLAEQNRK	50.17
Keratin, type II cytoskeletal 1	gil119395750	SLNNQFASFIDK	98.71
Type I keratin 16	gil1195531	APSTYGGGLSVSSR	30.64
Protein folding			
Ankyrin repeat domain-containing protein 62	gil302393830	LNDLNDRDK	13.03
Gene regulation			
SON DNA binding protein isoform E	gil17046381	NRDKGEKEK	10.73
Redox-regulation			
Selenoprotein I	gil119621096	KMAASTRVEASR	5.30
Transport			
Synaptosomal-associated protein 23 isoform SNAP23A	gil18765729	KLIDS	4.17
Unknown			
hCG2042301	gil119611404	TGGDRTKAQRHEIISLS	11.14
Unknown protein IT12	gil2792366	SGARAMAKAKK	7.15
Unnamed protein product	gil21757251	LINDSTNK	19.40

siRNA, small interfering RNA; WT1, Wilms' tumor 1.

of cytochrome *c* resulting in the induction of cell death (17). Moreover, it can also be directed to the nucleus and can play a role in apoptotic properties leading to cell death (18). The STRING shows the correlation between WT1 and mitogaligin via EGFR (Fig. 3A). WT1 may act as negative regulator of mitogaligin through the EGFR leading cell death.

WT1 interacts with many genes involved in the cell signaling pathway. In the present study, the proteins involved in the cell signaling pathway, platelet-derived growth factor receptor α (PDGFRA) and Rho-GEF were found when WT1 was present in MDA-MB-468, while SH2 domain-containing protein and IBtK were found when the cell was without WT1. However, the STRING 9.05 showed that these molecules were not associated with WT1 (Fig. 3B).

The signal transduction pathway in the MDA-MB-468 breast cancer cell line was related to the mTOR signaling pathway that regulates cell growth, proliferation, differentiation and survival (19). The mTOR protein exists in two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 contains the protein *raptor* while mTORC2 contains the protein *rictor*. In the presence of growth factors, activated Akt phosphorylates and inhibits tuberous sclerosis protein 2 (Tsc2), thereby promoting the activation of Rheb. Activated Rheb (Rheb-GTP) helps activate mTORC1, which in turn stimulates cell growth. Furthermore, mTORC2 phosphorylates Akt at Ser473 and regulates the actin cytoskeleton and cell motility (20). Recently, Razmara *et al* demonstrated that PDGFRs are essential for multiple growth factor signaling pathways that lead to PI3K/Akt activation. The pathway from PDGFR leads to phosphorylation of Akt which is involved in both the mTORC2 and PLCγ/PKC pathways (21).

The WT1 protein has two nuclear localization domains: within zinc fingers I and within zinc fingers II and III. It is responsible for transcription and RNA processing (22). However, WT1 can be detected in the cytoplasm of various cell lines including breast cancer and shuttles the nucleus and the cytoplasm (23,24). PDGFRA is a tyrosine-protein kinase that acts as a cell surface receptor for PDGFA and plays a role in the regulation of cell proliferation and survival (25). Rho-GEF is an intracellular signaling molecule that regulates cytoskeleton organization, gene expression, cell cycle progression, cell motility and other cellular processes. It represents the activating enzymes of Rho GTPases by serving to relay a variety of signals to catalyze GDP/GTP exchange of specific Rho GTPases (26).

Table II. Identification of expressed proteins found only in MDA-MB-468 siRNA$_{neg}$ using DeCyder™ MS 2.0 Differential Analysis Software.

Protein name	Accession no.	Peptide	Mowse score
Cell adhesion			
Vang-like protein 1 isoform 1	gil20373171	HMAGLK	12.95
Cell differentiation			
METRNL protein, partial	gil30047763	VFEPVPEGDGHWQGR	10.04
Cell signaling			
PDGFRA protein	gil39645305	VPSIKLVYTLTVPEATVK	11.73
Rho guanine nucleotide exchange factor 11 isoform 1	gil7662086	SSNSK	6.04
Structural			
Keratin 5	gil18999435	LAELEEALQK	23.61
Peroxisome assembly protein 26 isoform a	gil8923625	KSDSSTSAAPLR	6.59
hHa7 protein	gil50949256	NTLNGHEK	12.35
Transport			
Na+/K+-ATPase α 3 subunit variant	gil62898870	LNIPVSQVNPR	14.46
Unknown function			
Unnamed protein product	gil194390014	MFHLAAFKLK	22.44
hCG2042050	gil119579649	ASTVPDLK	7.42
Chromosome 9 open reading frame 39	gil119579068	LLEGQSLALSPR	11.96
Hypothetical protein LOC286076	gil119602615	DVGDALPR	29.47

siRNA, small interfering RNA; WT1, Wilms' tumor 1. PDGFRA, platelet-derived growth factor receptor α.

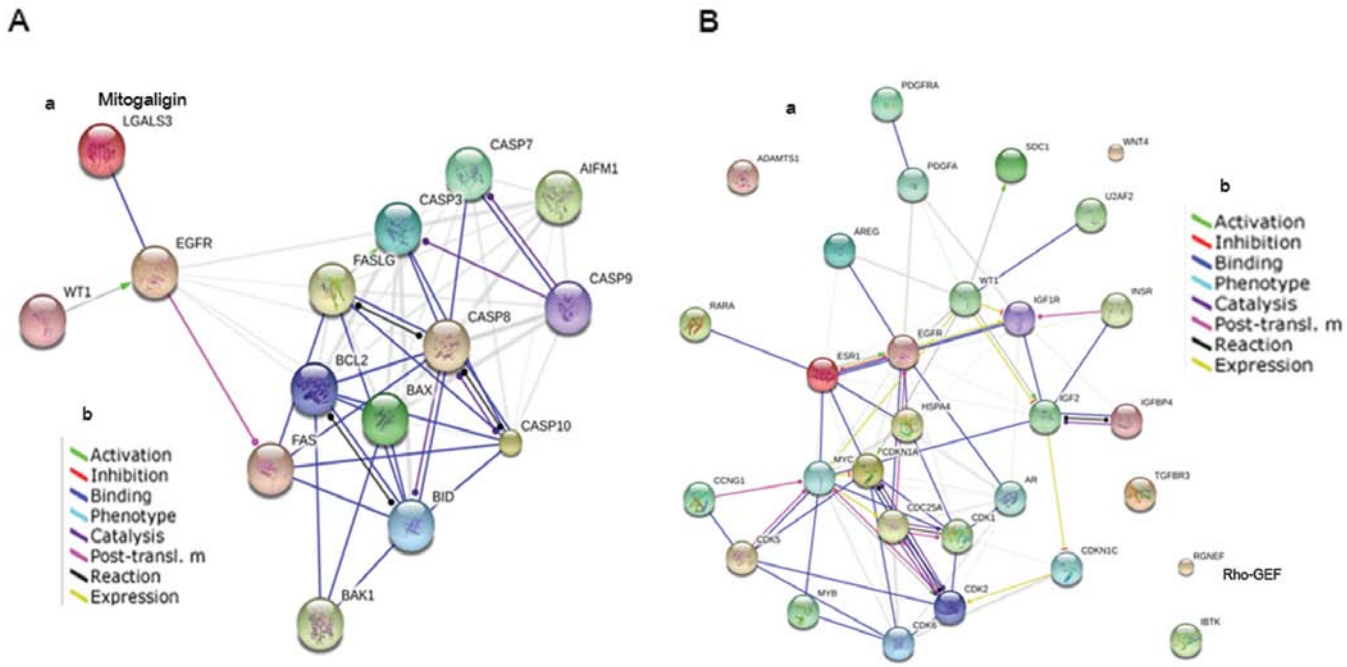

Figure 3. (A-a) The involvement of WT1 and p53-independent apoptosis pathway in MDA-MB-468 (STRING 9.05). (b) Modes of action are shown in different colors. The red circle shows the proteins found in this study. (B-a) The involvement of WT1 and proteins in signal transduction pathway in MDA-MB-468 (STRING 9.05). (b) Modes of action are shown in different colors. The red circle shows the proteins found in this study. WT1, Wilms' tumor 1; BAK1, Bcl-2 homologous antagonist killer; BID, BH3 interacting domain death agonist; FASLG, Fas ligand; BCL-2, B-cell lymphoma-2; BAX, Bcl-2-associated X protein; LGALS3, mitogaligin; EGFR, epidermal growth factor receptor; PDGFRA, platelet-derived growth factor receptor α; RARA, retinoic acid receptor α; IBtK, inhibitor of Bruton's tyrosine kinase.

Table III. Identification of expressed proteins found in MDA-MB-468 $siRNA_{WT1}$ and MDA-MB-468 $siRNA_{neg}$ using DeCyder™ MS 2.0 Differential Analysis Software.

Protein name	Accession no.	Peptide	Mowse score
Structural protein			
LMNA protein	gil21619981	SGAQASSTPLSPTR	44.82
Cell differentiation and survival			
Nance-Horan syndrome protein isoform 2	gil42384238	KTISGIPR	26.98
Sestrin-2	gil13899299	KLSEINK	21.68
Cell signaling			
S100 calcium binding protein A10 [Annexin II ligand, calpactin I, light polypeptide (p11)], isoform CRA_b	gil119573783	NALSGAGEASAR	11.49
Chain A, catalytic domain of human phosphodiesterase 4b in complex with piclamilast	gil58177395	GMEISPMXDK	8.66
Protein S100-A6	gil7657532	LQDAEIAR	43.91
Hormone			
C-type natriuretic peptide precursor	gil13249346	YKGANKKGLSK	10.08
Protein folding			
Heat shock protein	gil4204880	IINEPTAAAIAYGLDKK	27.1
Transport			
Ras association domain-containing protein 9	gil114155158	ADAFLPVPLWR	6.35
Gene regulation			
TTLL5 protein	gil33877151	MGNTMDKR	10.31
39S ribosomal protein L15, mitochondrial	gil7661806	CGRGHK	16.37
Unknown function			
hCG16415, isoform CRA_f	gil119611935	GAECCPGGPVK	10.83
FLJ00258 protein	gil18676718	GSMSR	8.83
Pyruvate dehydrogenase E1 α subunit	gil861534	EEIPPHSYR	6.28

siRNA, small interfering RNA; WT1, Wilms' tumor 1.

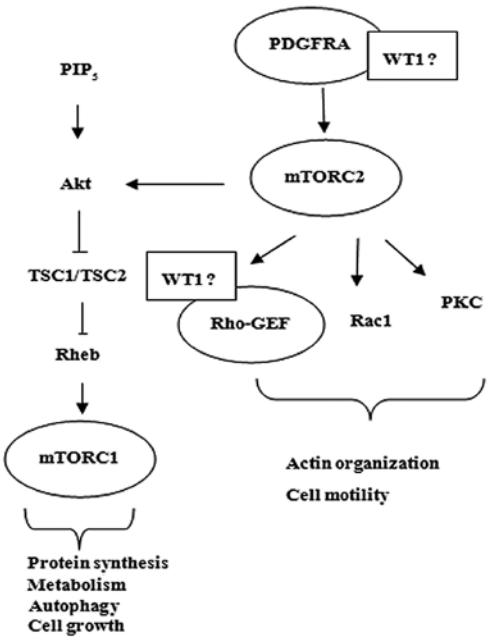

Figure 4. The possible relationship between WT1 and PDGFRA, Rho-GEF in signal transduction pathway in MDA-MB-468 [adapted from Zhou and Huang (20)]. WT1, Wilms' tumor 1; PDGFRA, platelet-derived growth factor receptor α; Rho-GEF, Rho guanine nucleotide exchange factor 1.

WT1 may be related to PDGFRA leading to activation of Akt/TSC1, TSC2/mTOR2 pathway resulting in cell growth. Moreover, WT1 may also be associated with mTOR2/Rho-GEF resulting in cell motility (Fig. 4).

Thus, WT1 plays an oncogenic role in MDA-MB-468. Moreover, when WT1 was silenced with $siRNA_{WT1}$, IBtK, SH2 domain-containing protein were upregulated. The relationship between WT1 and these proteins in the signaling pathway in MDA-MB-468 has not previously been elucidated. WT1 may behave as a negative-regulator of IBtK that binds to SH2 domain of BtK tyrosine kinase receptor resulting in IBtK inactivate leading to B-cell differentiation (27).

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
2. Adami HO, Signorello LB and Trichopoulos D: Towards an understanding of breast cancer etiology. Semin Cancer Biol 8: 255-262, 1998.
3. Dumitrescu R and Cotarla I: Understanding breast cancer risk - where do we stand in 2005? J Cell Mol Med 9: 208-221, 2005.
4. Gessler M, Poustka A, Cavenee W, Neve RL, Orkin SH and Bruns GA: Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. Nature 343: 774-778, 1990.

5. Haber DA, Park S, Maheswaran S, *et al*: WT1-mediated growth suppression of Wilms tumor cells expressing a WT1 splicing variant. Science 262: 2057-2059, 1993.
6. Loeb DM, Evron E, Patel CB, *et al*: Wilms' tumor suppressor gene (*WT1*) is expressed in primary breast tumor despite tumor-specific promoter methylation. Cancer Res 61: 921-925, 2001.
7. Navakanit R, Graidist P, Leeanansaksiri W and Dechsukum C: Growth inhibition of breast cancer cell line MCF-7 by siRNA silencing of Wilm tumor 1 gene. J Med Assoc Thai 90: 2416-2421, 2007.
8. Miyoshi Y, Ando A, Egawa C, *et al*: High expression of Wilms' tumor suppressor gene predicts poor prognosis in breast cancer patients. Clin Cancer Res 8: 1167-1171, 2002.
9. Oji Y, Miyoshi Y, Kiyotoh E, *et al*: Absence of mutations in the Wilms' tumor gene *WT1* in primary breast cancer. Jpn J Clin Oncol 34: 74-77, 2004.
10. Yang L, Han Y, Saurez Saiz F and Minden M: A tumor suppressor and oncogene: the WT1 story. Leukemia 21: 868-876, 2007.
11. Graidist P: The role of WT1 in breast and other cancers: oncogene or tumor suppressor gene? Songkla Med J 27: 435-449, 2009.
12. Subik K, Lee JF, Baxter L, *et al*: The expression patterns of ER, PR, HER2, CK5/6, EGFR, Ki-67 and AR by immunohistochemical analysis in breast cancer cell lines. Breast Cancer 4: 35-41, 2010.
13. Filmus J, Pollak MN, Cailleau R and Buick RN: MDA-468, a human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified EGF receptor gene and is growth inhibited by EGF. Biochem Biophys Res Commun 128: 898-905, 1985.
14. Armstrong DK, Kaufmann SH, Ottaviano YL, Furuya Y, Buckley JA, Isaacs JT and Davidson NE: Epidermal growth factor-mediated apoptosis of MDA-MB-468 human breast cancer cells. Cancer Res 54: 5280-5283, 1994.
15. Holiday LD and Speirs V: Choosing the right cell line for breast cancer research. Breast Cancer Res 13: 215, 2011.
16. Guittaut M, Charpentier S, Normand M, Dubois J, Raimond J and Legrand A: Identification of an internal gene to the human Galectin-3 gene with two different overlapping reading frames that do not encode Galectin-3. J Biol Chem 276: 2652-2657, 2001.
17. Duneau M, Boyer-Guittaut P, Gonzalez P, *et al*: *Galig*, a novel cell death gene that encodes a mitochondrial protein promoting cytochrome *c* release. Exp Cell Res 302: 194-205, 2005.
18. Robinet O, Mollet L, Gonzalez P, Normand T, Charpentier S, Brulé F, *et al*: The mitogaligin protein is addressed to the nucleus via a non-classical localization signal. Biochem Biophys Res Commun 392: 53-57, 2010.
19. Yu K, Toral-Barza L, Discafani C, Zhang WG, Skotnicki J, Frost P and Gibbons JJ: mTOR, a novel target in breast cancer: the effect of CCI-779, an mTOR inhibitor, in preclinical models of breast cancer. Endocr Relat Cancer 8: 249-258, 2001.
20. Zhou H and Huang S: mTOR signaling in cancer cell motility and tumor metastasis. Crit Rev Eukaryot Gene Expr 20: 1-16, 2010.
21. Razmara M, Heldin CH and Lennartsson J: Platelet-derived growth factor-induced Akt phosphorylation requires mTOR/Rictor and phospholipase C-γ1, whereas S6 phosphorylation depends on mTOR/Raptor and phospholipase D. Cell Commun Signal 11: 3, 2013.
22. Bruening W, Moffett P, Chia S, Heinrich G and Pelletier J: Identification of nuclear localization signals within the zinc fingers of the WT1 tumor suppressor gene product. FEBS Lett 393: 41-47, 1996.
23. Silberstein GB, Van Horn K, Strickland P, Roberts CT Jr and Daniel CW: Altered expression of WT1 Wilms tumor suppressor gene in human breast cancer. Proc Natl Acad Sci USA 94: 8132-8137, 1997.
24. Niksic M, Slight J, Sanford JR, Caceres FJ and Hastie ND: The Wilms' tumor protein (WT1) shuttles between nucleus and cytoplasm and is present in functional polysomes. Hum Mol Genet 13: 464-471, 2004.
25. Alberts B, Johnson A, Lewis J, Raff M, Roberts K and Walter P: Molecular Biology of the Cell. 5th edition. Garland Science, New York, 2008.
26. Shang X, Marchioni F, Evelyn CR, *et al*: Small-molecule inhibitors targeting G-protein-coupled Rho guanine nucleotide exchange factors. Proc Natl Acad Sci USA 110: 3155-3160, 2013.
27. Janda E, Palmieri C, Pisano A, Pontoriero M, Laccino E, Falcone C, *et al*: Btk regulation in human and mouse B cells via protein kinase C phosphorylation of IBtkγ. Blood 117: 6520-6531, 2011.