Growth Inhibition of Breast Cancer Cell Line MCF-7 by siRNA Silencing of Wilm Tumor 1 Gene

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Background: *RNA interference (RNAi) is sequence-specific inhibition of gene expression induced by double-stranded RNA.*
Objective: *Define the role of Wilms tumor 1 gene (WT1) in breast cancer oncogenesis using RNAi.*
Material and Method: *MCF-7 breast cancer cells, which express a high level of WT1, were transfected with synthetic small interfering RNA (siRNA) targeting WT1 (siRNA$_{WT1}$) resulting in inhibition of WT1 expression, as well as growth, in a dose- and time-dependent manner.*
Result: *The minimum concentration of siRNA$_{WT1}$ for growth inhibition and WT1 silencing was 25 nM and 50 nM respectively. WT1 expression was completely abolished at 200 nM siRNA$_{WT1}$.*
Conclusion: *These data suggest that WT1 is indispensable for the survival of breast cancer MCF-7 cell line.*

Keywords: *RNA interference, WT1, MCF-7, Breast cancer*

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WT1 gene was originally identified as a tumor suppressor gene responsible for Wilms tumor, a kidney neoplasm of childhood[1,2]. *WT1* encodes a zinc finger protein that acts as a transcriptional regulator for many genes involved in cell differentiation, growth, and apoptosis. Its tumor-suppressive function is attributed to transcriptional repression of growth factors and growth factor receptors, IGF-II, IGF-IR, TGFβ, and EGFR[3-7].

The role of *WT1* in human cancer has been extensively examined in several types of human tumor. Recent studies have shown that *WT1* plays an important role in tumorigenesis and progression of leukemia[8-10]. Indeed, decreased expression of WT1 protein induced growth inhibition and apoptosis in leukemia cells both in vitro[11,12] and in vivo[13]. Therefore, *WT1* appears to act as a survival factor in leukemia. Inoue et al[8] reported that *WT1* mRNA expression is significantly up-regulated in leukemia cells compared with the normal hematopoietic cells. However, the role of *WT1* in breast cancer is largely unknown. Recently, Loeb et al[14] have demonstrated that *WT1* mRNA and protein was detected in nearly 90% of breast cancers but not in most normal breast samples. These results suggest that *WT1* plays a role in the pathogenesis of breast cancer as an oncogene rather than a tumor suppressor gene as in leukemia. However, Siberstein et al[15] studied WT1 expression by immunohistochemistry technique and demonstrated that WT1 was down-regulated in the majority of invasive cancer samples compared with benign counterparts. Interestingly, cytoplasmic localization and altered ratio of *WT1* mRNA spliced variants were also detected in some cancer samples. Intriguingly, WT1 protein expression was demonstrated to be more frequent in ER-negative cancers compared with ER-positive cancers[14]. The explanation for the discrepancies between these studies is not clear. However, differences in detection methods in these studies could be the possible attributable factor.

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Recently, a functional study showed that *WT1* silencing by antisence oligomer in breast cancer cells results in inhibition of growth stimulatory effect by estrogen. As the role of *WT1* in breast cancer is not established, specifically reducing its level by genetic means in established breast cancer cell lines is helpful for a better understanding of its role in the carcinogenesis of breast cancer. RNA interference (RNAi) has become an excellent approach for the specific silencing of gene expression in plants and invertebrates[16,17]. In this approach, nucleotide of 21-to 23-base pair in length, short interfering RNA (siRNA), complementary to the targeted gene are processed from a long dsRNA precursor by the Dicer enzyme and effectively inhibit target gene expression by binding to complementary mRNA and triggering mRNA degradation[17-21]. Elbashir et al[22] demonstrated that transfection of synthetic 21-nt siRNA duplexes into mammalian cells efficiently inhibited endogenous gene expression in a sequence specific manner.

Here, the authors show that siRNA directed against human *WT1* gene ($siRNA_{WT1}$) effectively inhibited WT1 expression and suppressed the growth of the breast cancer cells in both dose- and time-dependent manner. This finding suggests that *WT1* might serve as a molecular target for human breast cancer treatment and $siRNA_{WT1}$ could be used as therapeutic agent for a gene-targeted therapy approach for this cancer.

Material and Method

Cell culture

MCF-7 breast cancer cell line was obtained from Assoc. Prof. Dr. Arunporn Itharat, Faculty of Pharmacology, Prince of Songkla University, Thailand[23]. Cells were grown in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Gibco BRL), 50 units/ml of penicillin, and 50 µg/mL of streptomycin in a humidified 37ºC incubator with 5% CO_2.

siRNA transfection of breast cancer cells

A total of 5 x 10^4 cells were seeded into each well of a 24-well tissue plate. The next day when cells were 40-50% confluent, cells was transfected with siRNA. siRNA against WT1 ($siRNA_{WT1}$) (Invitrogen) consisted of a mixture of three 25-nt siRNA duplexes targeting different non-overlapping regions of WT1 mRNA, labeled as $siRNA_{WT1}$HSS111388 ($siRNA_{WT1}$R88), $siRNA_{WT1}$HSS111389 ($siRNA_{WT1}$R89) and $siRNA_{WT1}$HSS111390 ($siRNA_{WT1}$R90). The first region is located in exon 7 and the last two regions are located in exon 8. Transfection agent plus nonspecific sequence (Invitrogen) was used as a negative control (siRNAneg) (Cat. No.12935-200). All procedures were performed in an RNase-free environment. In brief, transfection of cells with siRNA duplexes was performed using transfection reagent, LipofectAMINE 2000, at a final concentration of 0.2%. To minimize the cytotoxicity of the reagent itself, cells were washed once with PBS and media were changed 5 h after transfection. Cells were harvested at different time points after trasfection (12, 24, 48, 72, 96, and 120 h) with different concentrations of siRNA (0, 25, 50, 100, 200, 400, and 800 nM).

Western blot analysis

Cells were lysed in mammalian cell lysis buffer (10% glycerol, 2% SDS and 10 µg/mL of proteinase inhibitor) and heated for 5 min at 100ºC. Protein concentration was determined by Lowry method (ABC Kit, BioRAD). Protein samples were then subjected to 12% SDS-PAGE, followed by electrophoretic transfer of

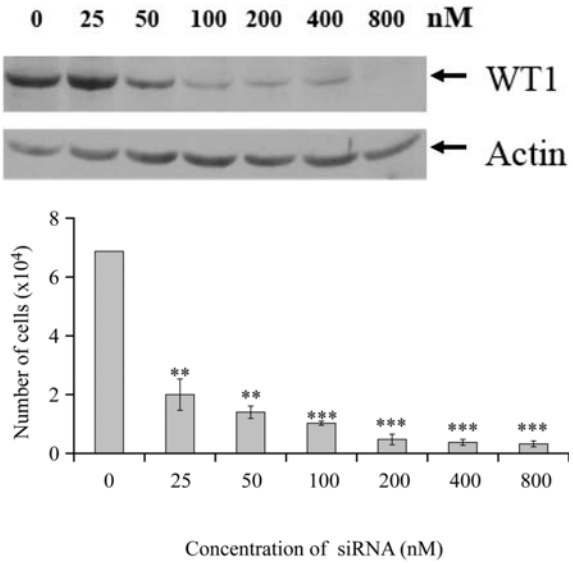

Fig. 1 Dose-dependent $siRNA_{WT1}$ inhibition of MCF-7 breast cancer cell proliferation and WT1 level MCF-7 cells were transfected with 0, 25, 50, 100, 200, 400, and 800 nM $siRNA_{WT1}$ for 72 h. The data represent the average value for three independent transfection experiments, each performed in triplicate, 50 µg of total protein extracted from the cells were subjected to Western immunoblotting for WT1 protein detection, ** $p < 0.01$ and *** $p < 0.005$ by two-sample *t* test, comparing with control

the protein to nitrocellulose membrane. Membrane was incubated with in 5% skim milk in TBS-T (0.1% Tween 20, 154 mM NaCl, 48 mM Tris-base, pH 6.8) and then treated with a polyclonal anti-WT1 antibody (C19) or a polyclonal anti-actin antibody (Santa Cruz Biotechnology, Inc) at 1:200 and 1:5000 dilution respectively in TBS-T buffer for 1 h. Membrane was then washed with TBS-T and incubated with a horseradish peroxidase-conjugated polyclonal IgG-anti-rabbit antibody (GE bioscience). Bands corresponding to WT1 and actin protein were detected using Super-Signal West Pico chemiluminescence substrate (Pierce).

Cell proliferation assay

After transfection, siRNA-transfected cells were harvested at specific time points. The total viable cell number was assessed by trypan blue exclusion assay and counted by a hemacytometer under an inverted microscope (Olympus).

Results

To determine the effects of $siRNA_{WT1}$ on cells proliferation, MCF-7 cells were transfected with 0, 25, 50, 100, 200, 400 and 800 nM for 72 h. The authors found that 25 nM $siRNA_{WT1}$ were sufficient to inhibit the proliferation of MCF-7 cells (71% inhibition). In addition, the numbers of cells also decreased in a dose-dependent manner reaching a steady stage at 200 nM (Fig. 1). Furthermore, the levels of WT1 protein in $siRNA_{WT1}$-transfected MCF-7 cells decreased in dose-dependent manner (Fig. 1). The inhibitory effect of $siRNA_{WT1}$ on WT1 protein expression was clearly observed at 50 nM $siRNA_{WT1}$ and WT1 protein was completely abolished at the 800 nM $siRNA_{WT1}$.

To investigate the effects of $siRNA_{WT1}$ on the proliferation of MCF-7 cells with time, cells were transfected with 100 nM $siRNA_{WT1}$ or siRNAneg and the cells were harvested at various time points (12, 24, 48, 72, 96 and 120 h) for cell proliferation and gene

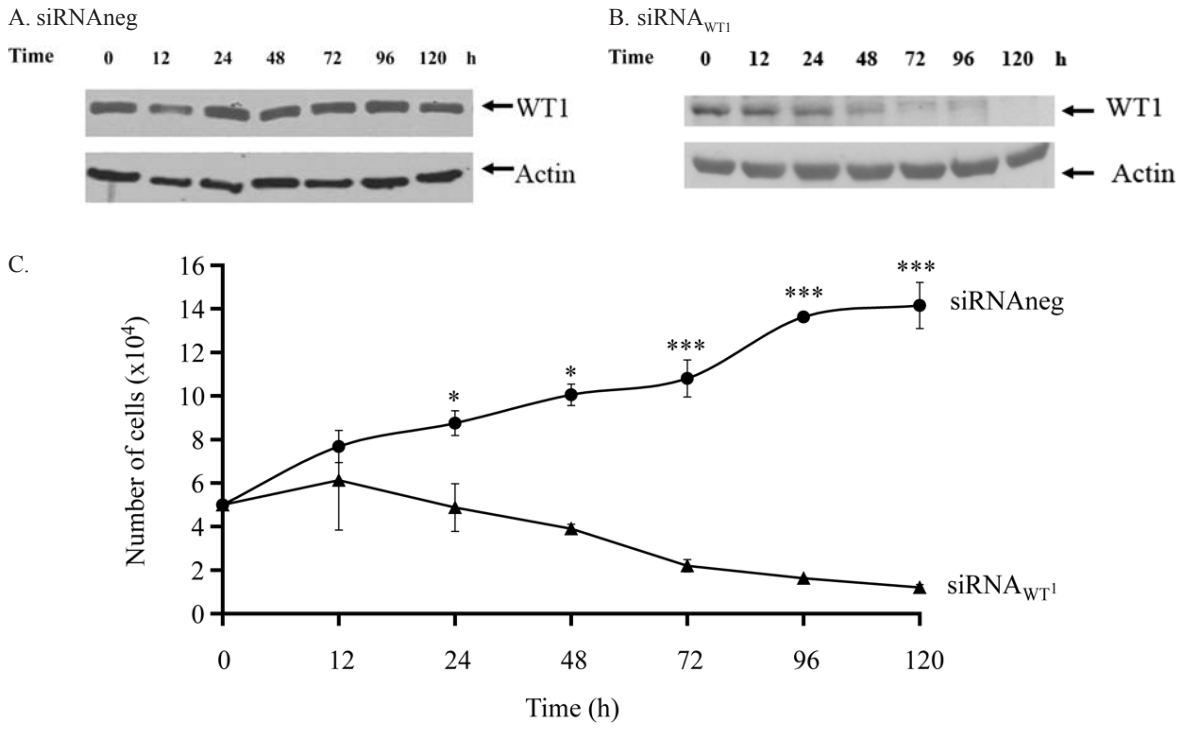

Fig. 2 Time-dependent $siRNA_{WT1}$ inhibition of MCF-7 breastcancer cell proliferation and WT1 level MCF-7 cells were transfected with 100 nM of $siRNA_{WT1}$ and cells were harvested at 12, 24, 48, 72, 96, and 120 h after transfection for cell proliferation assay and Western blotting, the data represented the average value of three independent transfection experiments, each performed in triplicate, 50 μg of total protein extracted from the cells were subjected to Western immunoblotting for WT1 protein detection, *** $p < 0.005$; * $p < 0.05$ by two-sample *t* test, comparing with control, (panel A = siRNAneg-transfected cell, panel B = $siRNA_{WT1}$-transfected cell)

expression detection assay. The present data showed that $siRNA_{WT1}$ inhibited the growth of MCF-7 cells in time-dependent manner (Fig. 2). Significant growth inhibitory effect of $siRNA_{WT1}$ compared with control cells was observed at 24 h after transfection (44% inhibition) and reached the maximum effect (92% growth inhibition) at 120 h after transfection. To support that this effect was attributable to WT1 down-regulation, the levels of WT1 protein were monitored by Western immunoblotting along the experimental time course (Fig. 2). 100 nM $siRNA_{WT1}$ the inhibitory effect on WT1 protein expression was observed 24 h after transfection and increased with experimental time course reaching a maximum effect at 120 h after transfection, when no WT1 protein was detected.

Discussion

The *WT1* gene was originally isolated as a tumor-suppressor gene responsible for Wilms tumor, a kidney neoplasm of childhood[1,2]. Subsequently, a growing body of evidences suggests that WT1 can act as a survival factor in various types of human cancer. In breast cancer, Silberstein et al[15] reported that WT1 protein is decreased during malignant transformation. However, other studies analyzing human breast cancer samples for WT1 expression by RT-PCR demonstrated that *WT1* is over expressed in the majority of cancer samples compared with benign counterparts[14]. Moreover, the prognostic value of *WT1* gene expression was also demonstrated in the present study, in which high levels of *WT1* transcript as detected by real time RT-PCR were found to be associated with adverse clinical outcome of breast cancer patients[24].

To address this controversial issue, the authors employed RNAi to specifically inhibit *WT1* gene expression in a breast cancer cell line (MCF-7) to determine this effect on cell growth and WT1 protein level. The authors selected siRNA for this purpose as the amount can be accurately determined allowing a dose titration study. The present study used a mixed population of $siRNA_{WT1}$ consisting of three siRNAs targeting three different regions of *WT1* transcript, as this strategy is believed to minimize the non-specific cytotoxic effect of siRNA. The present study showed that the $siRNA_{WT1}$ inhibited both WT1 protein expression level and growth of breast cancer cells in dose - and time-dependent manner. This growth inhibitory effect was likely to be due to WT1 protein down-regulation as no effect was observed in control cells in which no change in WT1 protein level was detected. The presented data are in line with earlier studies of Zapata-Benacides et al[25] who showed that WT1 protein expression levels increase when proliferation of breast cancer cells are stimulated by 17β-estradiol, but decrease when inhibited by tamoxifen or all-trans-retinoic acid and that growth of breast cancer cells is inhibited by *WT1* antisense oligomer treatment which induces WT1 protein downregulation.

To the best of the authors' knowledge, the present study is the first demonstration of the effectiveness of siRNA for *WT1* silencing as well as its potent cytotoxic effect on breast cancer cells. This finding suggests that *WT1* is indispensable for the survival of breast cancer cells and siRNA can be used to effectively inhibit WT1 gene expression. For a mechanistic point of view, this approach would be the powerful tool to dissect the molecular pathway involved in *WT1*-mediated carcinogenesis. As *WT1* is also found to be over expressed in several types of human cancer cells but not in most of the benign cells, anti-WT1 approach by siRNA may be one of the molecular-based treatments for human cancers. Further studies in animal models would be required to evaluate the effectiveness of this measure.

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References

1. Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, et al. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell 1990; 60: 509-20.
2. Gessler M, Poustka A, Cavenee W, Neve RL, Orkin SH, Bruns GA. Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. Nature 1990; 343: 774-8.
3. Drummond IA, Madden SL, Rohwer-Nutter P, Bell GI, Sukhatme VP, Rauscher FJ III. Repression of the insulin-like growth factor II gene by the Wilms tumor suppressor WT1. Science 1992; 257: 674-8.
4. Werner H, Re GG, Drummond IA, Sukhatme VP, Rauscher FJ III, Sens DA, et al. Increased expression of the insulin-like growth factor I receptor gene, IGF1R, in Wilms tumor is correlated with modulation of IGF1R promoter activity by the WT1 Wilms tumor gene product. Proc Natl Acad Sci U S A 1993; 90: 5828-32.

5. Gashler AL, Bonthron DT, Madden SL, Rauscher FJ III, Collins T, Sukhatme VP. Human platelet-derived growth factor A chain is transcriptionally repressed by the Wilms tumor suppressor WT1. Proc Natl Acad Sci U S A 1992; 89: 10984-8.
6. Dey BR, Sukhatme VP, Roberts AB, Sporn MB, Rauscher FJ III, Kim SJ. Repression of the transforming growth factor-beta 1 gene by the Wilms' tumor suppressor WT1 gene product. Mol Endocrinol 1994; 8: 595-602.
7. Hewitt SM, Hamada S, McDonnell TJ, Rauscher FJ III, Saunders GF. Regulation of the proto-oncogenes bcl-2 and c-myc by the Wilms' tumor suppressor gene WT1. Cancer Res 1995; 55: 5386-9.
8. Inoue K, Sugiyama H, Ogawa H, Nakagawa M, Yamagami T, Miwa H, et al. WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. Blood 1994; 84: 3071-9.
9. Inoue K, Tamaki H, Ogawa H, Oka Y, Soma T, Tatekawa T, et al. Wilms' tumor gene (WT1) competes with differentiation-inducing signal in hematopoietic progenitor cells. Blood 1998; 91: 2969-76.
10. Chen Z. The possible role and application of WT1 in human leukemia. Int J Hematol 2001; 73: 39-46.
11. Algar EM, Khromykh T, Smith SI, Blackburn DM, Bryson GJ, Smith PJ. A WT1 antisense oligonucleotide inhibits proliferation and induces apoptosis in myeloid leukaemia cell lines. Oncogene 1996; 12: 1005-14.
12. Yamagami T, Sugiyama H, Inoue K, Ogawa H, Tatekawa T, Hirata M, et al. Growth inhibition of human leukemic cells by WT1 (Wilms tumor gene) antisense oligodeoxynucleotides: implications for the involvement of WT1 in leukemogenesis. Blood 1996; 87: 2878-84.
13. Smith SI, Down M, Boyd AW, Li CL. Expression of the Wilms' tumor suppressor gene, WT1, reduces the tumorigenicity of the leukemic cell line M1 in C.B-17 scid/scid mice. Cancer Res 2000; 60: 808-14.
14. Loeb DM, Evron E, Patel CB, Sharma PM, Niranjan B, Buluwela L, et al. Wilms' tumor suppressor gene (WT1) is expressed in primary breast tumors despite tumor-specific promoter methylation. Cancer Res 2001; 61: 921-5.
15. Silberstein GB, Van Horn K, Strickland P, Roberts CT Jr, Daniel CW. Altered expression of the WT1 wilms tumor suppressor gene in human breast cancer. Proc Natl Acad Sci U S A 1997; 94: 8132-7.
16. Hannon GJ. RNA interference. Nature 2002; 418: 244-51.
17. McManus MT, Sharp PA. Gene silencing in mammals by small interfering RNAs. Nat Rev Genet 2002; 3: 737-47.
18. Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature 2000; 404: 293-6.
19. Nykanen A, Haley B, Zamore PD. ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell 2001; 107: 309-21.
20. Parrish S, Fleenor J, Xu S, Mello C, Fire A. Functional anatomy of a dsRNA trigger: differential requirement for the two trigger strands in RNA interference. Mol Cell 2000; 6: 1077-87.
21. Zamore PD, Tuschl T, Sharp PA, Bartel DP. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 2000; 101: 25-33.
22. Elbashir SM, Harborth J, Weber K, Tuschl T. Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods 2002; 26: 199-213.
23. Itharat A. ATCC; MCF-7 breast cancer cell line. Faculty of Pharmacology, Prince of Songkla University, Thailand
24. Miyoshi Y, Ando A, Egawa C, Taguchi T, Tamaki Y, Tamaki H, et al. High expression of Wilms' tumor suppressor gene predicts poor prognosis in breast cancer patients. Clin Cancer Res 2002; 8: 1167-71.
25. Zapata-Benavides P, Tuna M, Lopez-Berestein G, Tari AM. Downregulation of Wilms' tumor 1 protein inhibits breast cancer proliferation. Biochem Biophys Res Commun 2002; 295: 784-90.
26. Reizner N, Maor S, Sarfstein R, Abramovitch S, Welshons WV, Curran EM, et al. The WT1 Wilms' tumor suppressor gene product interacts with estrogen receptor-alpha and regulates IGF-I receptor gene transcription in breast cancer cells. J Mol Endocrinol 2005; 35: 135-44.
27. Burwell EA, McCarty GP, Simpson LA, Thompson KA, Loeb DM. Isoforms of Wilms' tumor suppressor gene (WT1) have distinct effects on mammary epithelial cells. Oncogene 2007; 26: 3423-30.

การยับยั้งการแสดงออกของจีน WT1 โดยการใช้ siRNA ส่งผลต่อการยับยั้งการเจริญของเซลล์มะเร็งเต้านม

รักษพรรณ นวคณิต, พจนพร ไกรดิษฐ์, วิไลรัตน์ ลี้อนันต์ศักดิ์ศิริ, ชวบูลย์ เดชสุขุม

RNA interference (RNAi) คือวิธีการยับยั้งการแสดงออกของจีน โดยใช้ RNA สายคู่ที่จำเพาะต่อลำดับเบสของจีนเป้าหมาย ในการศึกษานี้จึงได้นำวิธีการดังกล่าวเพื่อศึกษาบทบาทของจีน WT1 ต่อกระบวนการเป็นมะเร็งเต้านม โดยใช้ small interfering RNA (siRNA) ในการยับยั้งการแสดงออกของจีนเป้าหมาย WT1 ($siRNA_{WT1}$) กับเซลล์เพาะเลี้ยงมะเร็งเต้านมชนิด MCF-7 ซึ่งมีการแสดงออกของจีน WT1 สูง พบว่า $siRNA_{WT1}$ สามารถยับยั้งการแสดงออกของจีน WT1 และส่งผลยับยั้งการเจริญของเซลล์มะเร็งเต้านม โดยผลการยับยั้งดังกล่าวจะขึ้นกับความเข้มข้นและเวลาที่ใช้ในการนำ $siRNA_{WT1}$ เข้าสู่เซลล์ ซึ่งความเข้มข้นน้อยที่สุดของ $siRNA_{WT1}$ ที่สามารถยับยั้งการเจริญของเซลล์และยับยั้งการแสดงออกของจีน WT1 คือ 25 nM และ 50 nM ตามลำดับ นอกจากนี้พบว่าที่ความเข้มข้น 200 nM สามารถยับยั้งการแสดงออกของจีน WT1 ได้อย่างสมบูรณ์ จากข้อมูลดังกล่าวนี้ แสดงให้เห็นว่า WT1 น่าจะเป็นจีนที่มีความสำคัญต่อการเจริญเติบโตของ เซลล์มะเร็งเต้านม MCF-7