# Correlation of Wilms' Tumor 1 Isoforms with HER2 and ER-α and its Oncogenic Role in Breast Cancer

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**Abstract.** *Background: Wilms' tumor 1 (WT1) gene has different functional properties depending on the isoform type. This gene correlates with cell proliferation in various types of cancer. Here, we investigated the expression of WT1 isoforms in breast cancer tissues, and focused on the oncogenic role through estrogen receptor-alpha (ER-α) and human epidermal growth factor receptor 2 (HER2). Materials and Methods: Expression of WT1(17AA+) and (17AA−) was investigated in adjacent normal breast and breast cancer using Reverse transcription-polymerase chain reaction and western blotting. The correlation of WT1 isoforms with HER2 and ER-α was examined using MCF-7 cells stably-overexpressing WT1s and siRNA against WT1 gene. Results: The expression of WT(17AA−) was significantly found in adjacent normal breast tissues. A mixture of WT1(17AA+) and WT1(17AA−) were highly expressed in breast carcinoma tissues. MCF-7 cells overexpressing WT1+/+ and WT1+/− represented strong expression of ER-α and HER2. Moreover, the silencing of WT1+/+ and WT1+/− resulted in a decrease of both ER-α and HER2 and led to a decrease of cell numbers. Conclusion: Our results suggest that WT1(17AA+) was exhibited dominantly in breast carcinoma tissues. WT1+/+ and WT1+/− correlated with the high expression of ER-α and HER2, leading to cell proliferation and might be involved in cancer development and progression.*

The *WT1* gene was originally identified as a tumor suppressor gene in Wilms' tumor, a neoplasm that occurs primarily in childhood (1). Subsequent work has demonstrated that WT1 is overexpressed in various types of solid tumors, such as those of the lung (2) and breast (3-5), and other non-solid tumors such as leukemia (6-8). This has raised the possibility that WT1 could have tumorigenic activity rather than tumor-suppressor activity (9-13). Moreover, *WT1* mRNA and protein are expressed in nearly 90% of breast carcinoma tissues but with low detection in adjacent normal breast samples (3). High expression levels of *WT1* mRNA are related to poor prognosis of breast cancer (14) and leukemia (7). These phenomena could be due to a growth and survival effect from WT1. In addition, down-regulation of *WT1* inhibits breast cancer cell proliferation (11). WT1 is also expressed in malignant melanoma and directly involved in cell proliferation (15).

The *WT1* gene spans about 50 kb and is located at chromosome 11p13 (16). This gene encodes 10 exons and generates a 1.5 kb mRNA. *WT1* consists of two alternative splicing sites at exon 5 (17 amino acids, 17AA) and exon 9 [three amino acids: lysine (K), threonine (T) and serine (S); KTS] which generates two functional domains: a proline-rich domain at the *N*-terminus and a zinc finger domain at the C-terminus. Alternative splicing of these two sites gives rise to four main different protein isoforms designated as A, B, C and D, or *WT1*(17AA−/KTS−; *WT1*−/−), *WT1*(17AA+/KTS−; *WT1*+/−), *WT1*(17AA−/KTS+; WT1−/+) and *WT1*(17AA+/KTS+; *WT1*+/+), respectively (17). The presence or absence of 17AA or KTS results in different functions. For example, the absence of KTS, which is represented by *WT1*+/− and *WT1*−/− isoforms, up-regulates B-cell lymphoma-2 (BCL-2) expression (18) and the overexpression of *WT1*+/− decreases the expression of BCL-2 antagonist/killer (BAK) (19). In addition, expression of *WT1*(17AA+) is involved in cell proliferation, apoptosis and cancer development. *WT1*(17AA+)-specific small-interfering (siRNA) induces apoptosis through induction of caspase-3 and -9 in leukemia cell lines. On the contrary, *WT1*(17AA−)-specific siRNA does not induce apoptosis. Constitutive expression of *WT1*(17AA+) isoform protects cells against etoposide-induced apoptosis (19, 20). Our previous report showed that overexpression of *WT1*+/+ and *WT1*+/−

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isoforms in MCF-7 cells reduces pro-apoptotic BAK and caspase-7 proteins, and *p53* mRNA levels (21).

Estrogen, progesterone and HER receptors are prognostic and predictive for invasive breast carcinoma (22). Estrogen receptor (ER) functions as a ligand-dependent transcription factor and promotes expression of a variety of genes (23) which directly promotes breast cancer cell proliferation, survival and tumor progression (24). Overexpression of HER2 in cancer cells leads to increased cell proliferation and reduced cell death, as well as changes in cell motility (25). WT1 induces estrogen-independent growth and anti-estrogen insensitivity in ER-positive breast cancer MCF-7 cells through the mitogen-activated protein kinases (MAPK) pathway. Moreover, WT1 plays a role in the up-regulation of epidermal growth factor receptor (EGFR), ER-α and HER2 expression in breast cancer cells (8, 26). High expression levels of EGFR, HER2 and WT1 are found in tamoxifen-resistant MCF-7 cells (MCF-$7^{TAM}$). Furthermore, knock-down of *WT1* expression by short hairpin (shRNA) down-regulates expression of EGFR, ER-α and HER2 (27).

Although many scientists have reported the function of each *WT1* isoform, as far as we are aware, there are no reports on the expression of *WT1*(17AA+) and *WT1*(17AA–) in breast cancer tissue and the correlation between each *WT1* isoform with ER-α and HER2 remains unclear. Herein, we studied the expression of *WT1*(17AA+) and (17AA–) isoforms in mRNA and protein in both breast cancer and adjacent normal tissues breast from patients with breast cancer. We also focus on its potential oncogenic role through HER2 and ER-α using MCF-7 cell stably overexpressing *WT1* and using siRNA against *WT1* gene.

## Materials and Methods

*Patients and tissue specimens*. Tissue samples were obtained from 33 Thai patients who underwent surgery at Songklanagarind Hospital, Prince of Songkla University, Thailand. The collection and use of these samples were approved by the Medical Ethics Committee of Songklanagarind Hospital (approval number: EC 55-014-04-1-3 and EC 53-072-04-2-3). All samples were collected between 2010 and 2012. Tissues were examined by one pathologist. Grading was performed by an experienced gynecologic pathologist according to the Scarff-Bloom-Richardson system (28, 29). The adjacent normal breast tissues (30 samples from non-cancer and cancer patients) and breast cancer tissues (23 samples) were obtained at surgery. Small tissues were minced and washed twice with PBS and kept at −70˚C until use.

*Cell lines and culture conditions*. Four cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Two breast cancer cell lines, MDA-MB-231 and MDA-MB-468, were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen) and 2 mM L-glutamine (Invitrogen). The MCF-7 breast cancer cell line was maintained as previously described (20). The MCF-12A normal breast cell line was grown in a medium containing a 1:1 ratio mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (JR Scientific, CA, USA) supplemented with 20 ng/ml of human EGFR (Calbiochem, Darmstadt, Germany), 100 ng/ml of cholera toxin (Sigma-Aldrich, St. Louis, MO, USA), 0.01 mg/ml bovine insulin (Sigma-Aldrich), 500 ng/ml of hydrocortisone (Sigma-Aldrich) and 5% horse serum (Invitrogen). All cells were incubated at 37˚C in an incubator with a humidified atmosphere of 5% $CO_2$.

*Reverse transcription polymerase chain reaction (RT-PCR)*. Total RNA was isolated using Trizol reagent (Invitrogen) and the mRNA was determined using OneStep RT-PCR kit (Qiagen, Hilden, Germany) according to the instructions from the manufacturer. In brief, PCR was performed in a total volume of 25 μl with 500 ng of total RNA. The PCR conditions were started at one cycle at 50˚C for 50 min (cDNA synthesis), one cycle at 95˚C for 15 min (denaturation), followed by 35 cycles at 95˚C for 30 s (denaturation), 40˚C for 30 s (annealing), 72˚C for 45 s (extension) and one cycle at 72˚C for 10 min by using a thermal cycler. The sequence of primers was as follows: 17AA, forward: 5'-CCAGCTTGAATGCATGAC-3' and reverse: 5'-CACCGTGCGTG TGTATTC-3'; and GAPDH, forward: 5'-GAAGGTGAAGGTC GGAGT-3' and reverse: 5'-GAAGATGGTGATGGGATTTC-3'. PCR products were loaded onto a 2% agarose gel, separated by electrophoresis and visualized by ethidium bromide staining. The specific PCR bands were purified using the QIAquick® PCR purification kit (Qiagen) and sequenced using an ABI Prism 377 DNA sequencer.

*Establishment of cells stably overexpressing WT1 isoforms*. For generation of MCF-7 cells stably overexpressing WT1s, MCF-7 cells were transfected, using FuGENE6 reagent (Roche, IN, USA), with pcDNA6/V5, a mammalian expression vector containing a blasticidin selection marker (Invitrogen), containing the cDNA encoding *WT1*+/+ or *WT1*+/– or *WT1*–/+ or *WT1*–/–. These four isoform transcripts encode the exon1 to exon 10 but different in exon 5 (17AA + or –) and exon9 (KTS + or –). For a control, MCF-7 cells were transfected with empty pcDNA6/V5. Transfected cells were selected for at least one week by using 10 μg/ml blasticidin and characterized by Western blot analysis using antibody to WT1 and anti-antibody to glyceraldehydes 3-phosphate dehydrogenase (GAPDH). The resulting lines were named MCF-$7_{WT1+/+}$, MCF-$7_{WT1+/-}$, MCF-$7_{WT1-/+}$, MCF-$7_{WT1-/-}$ and MCF-$7_{Empty}$.

*siRNA transfection*. Transfection of siRNA was performed as previously described (21). siRNAs against *WT1* ($siRNA_{WT1}$) were designed and synthesized by Invitrogen. The targeted sequences were located in exon 7 and exon 8. siGENOME Non-Targeting siRNA#2 was used as a negative control (Dharmacon product;Thermo Fisher Scientific, Lafayette, CO, USA). All procedures were performed under an RNase-free environment. In brief, cells were grown in a 24-well plate to 30-40% confluence before transfection. Cells were transfected with 100 nM siRNA duplexes using Lipofectamine 2000 reagent (Invitrogen) at a final concentration of 0.2%. To minimize the cytotoxicity of the reagent itself, cells were washed once with PBS and the media were changed after transfection.

*Trypan blue exclusion assay*. Trypan blue exclusion assay was used for estimating the number of viable cells present in the population

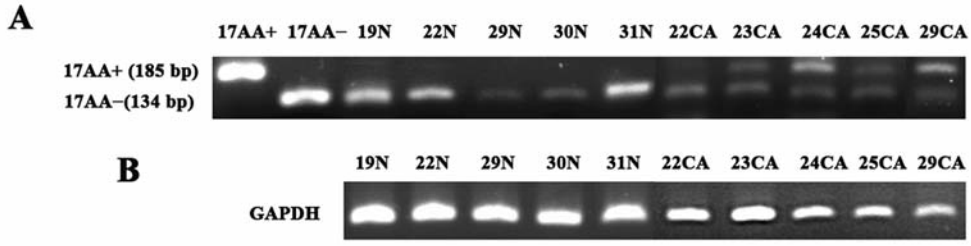


Figure 1. *Expression of Wilms' tumor 1, WT1(17AA+) and (17AA−). A: Reverse transcription polymerase chain reaction (RT-PCR) was performed on total RNA from breast tissues using primer that amplified regions surrounding exon 5. B: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal controls. PCR products were run on 2% agarose gel electrophoresis. 17AA+ and 17AA− at lane 1 and lane 2 served as a positive control. The gel image presented is representative of PCR product from 53 samples including adjacent normal breast and breast cancer tissues. N, Adjacent normal breast tissues; CA, breast cancer tissues.*

and was performed as previously described (21). The stably WT1-overexpressing cells were transfected with 100 nM $siRNA_{WT1}$. The cells were then harvested and transferred into a 1.5-ml microcentrifuge followed by centrifugation at 3,000× *g* for 7 min. The floating and attached cells were counted by a hemocytometer after adding trypan blue stain solution. The average cell count of four fields represented the number of cells per ml of cell solution and was used to determine the total number of cells from each well.

*Protein extraction*. The cells were harvested and lysed in RIPA lysis buffer [150 mM NaCl, 50 mM Tris, pH 7.4, 1% (v/v) NP-40, 0.25% (w/v) sodium deoxycholate and 1 mM EDTA] containing a cocktail of protease inhibitors (Sigma-Aldrich). The cells were then vigorously vortexed and subsequently incubated on ice for 10 min followed by centrifugation at 14,000 ×*g* for 10 min at 4˚C to collect the protein lysate. The tissue specimens were ground in a liquid nitrogen-cooled mortar. The tissue powder was suspended in RIPA buffer and incubated on ice for 45 min. Then the mixture was incubated in liquid nitrogen for 10 min followed by thawing in a water bath at 42˚C. This step was repeated two to three times. The solution was cleared by centrifugation at 14,000 ×*g* for 30 min at 4˚C. The protein concentration in the supernatant from the cells and tissues was determined by Bradford's method (Bio-Rad, Hercules, CA, USA).

*Western blotting*. Equal amounts of protein lysates were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Bio-Rad) which were then blocked with 5% non-fat milk in TBST (0.5% Tween 20, 154 mM NaCl, 40 mM Tris-HCl, 48 mM Tris-base) for one hour. Membranes were incubated with primary antibodies against WT1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), ER-α (1:500, Santa Cruz Biotechnology), HER2 (1:1,000; Cell Signaling Technology, La Jolla, CA, USA) and GAPDH (1:400; Calbiochem). Bound antibodies were detected by donkey anti-rabbit or sheep anti-mouse conjugated antibodies and visualized with chemiluminescence detection using the SuperSignal West Dura substrate (Pierce, Rockford, IL, USA). The intensity of the band was analyzed by Scion image software.

*Statistical analysis*. Student's *t*-test was used to analyze inter-group differences. Experiments were repeated at least three times and data are represented as mean±SD. A *p*-value of less than 0.05 was considered statistically significant.

## Results

*Expression of WT1(17AA+) and WT1(17AA−) mRNA in breast cancer.* To evaluate the expression of WT1 at splicing variant 17AA at exon 5 in breast cancer, we determined mRNA expression in breast cancer tissues in comparison to adjacent normal tissue and using RT-PCR technique. PCR primers were designed to span the exon 5 and cover 17AA. PCR product sizes were 185 bp for WT1(17AA+) and 134 bp for WT1(17AA−) (Figure 1). A summary of 17AA mRNA expression is shown in Table I. The *WT1* mRNA either 17AA+ or 17AA− was found in both adjacent normal breast tissue (83%; 25 out of 30 samples) and breast cancer tissue (100% of 23 samples). However, adjacent normal breast tissue highly expressed WT1(17AA−) mRNA at 53.33% (16 out of 30 samples), whereas breast carcinoma grade 1 and 2, and 3 represented 13.33% (2 out of 15 samples) and 12.5% (1 out of 8 samples), respectively. Mixed WT1(17AA+) and WT1(17AA−) mRNA was highly detectable in breast carcinoma grade 1 and 2 at 73.33% (7 out of 8 samples) and breast carcinoma grade 3 at 87.5% (11 out of 15 samples) but rarely detectable in adjacent normal breast tissue (13.33%; 4 out of 30 samples). PCR products were sampled to confirm the sequence.

*Expression of WT1(17AA+) and WT1(17AA−) protein in breast cancer.* To confirm the results of RT-PCR experiments, Western blot analysis was performed using selected tissue specimens from RT-PCR experiments. Upper and lower bands represented (17AA+) and (17AA−), respectively (Figure 2). Similar to the results obtained by RT-PCR, *WT1*(17AA−) isoform alone dominantly expressed in adjacent normal breast tissue in 15 out of 17 samples but not detected in breast cancer grade 1, 2 and 3. However, both present (+) and absent (–) 17 amino acid significantly expressed in breast carcinoma grade 1, 2 and 3 (100% of 17 samples) but low levels in adjacent normal breast tissue (11.76%, 2 out of 17 samples); the variation from sample to sample may reflect the varying

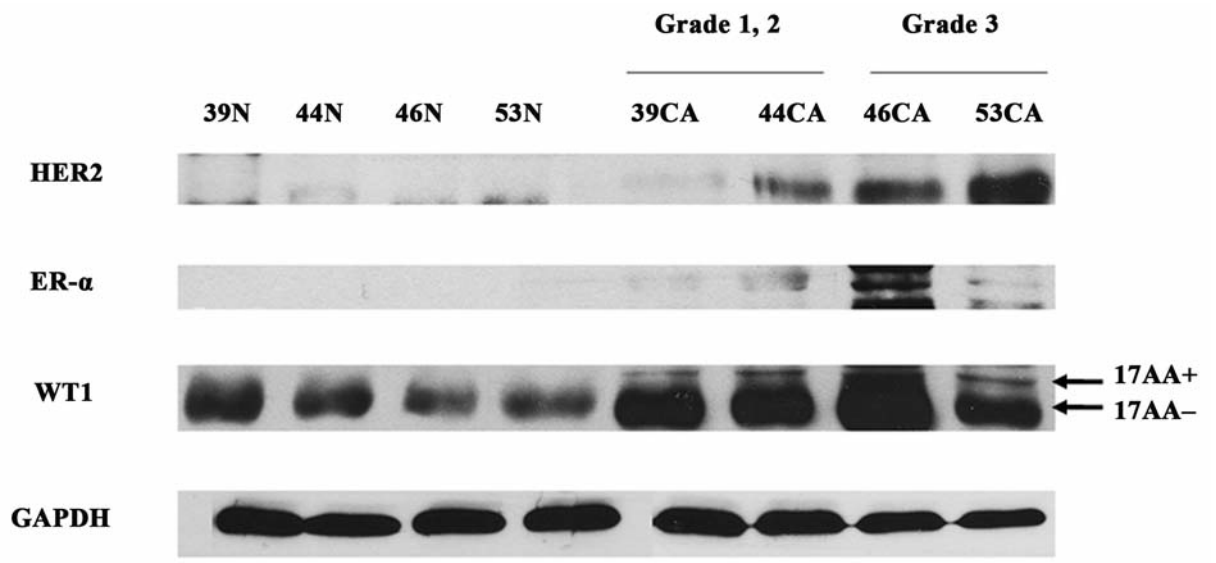


Figure 2. *Expression of Wilms' tumor 1 (WT1), estrogen receptor-alpha (ER-α) and human epidermal growth factor receptor-2 (HER2) using western blot analysis. Proteins were extracted from adjacent normal breast and breast cancer tissues. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. N, Adjacent normal breast tissues; CA, breast cancer tissues.*

Table I. *Breast cancer patients' clinical and histopathological characteristics.*

| Parameter | Number of patients (%) |
|---|---|
| Age at diagnosis (years) | |
| ≤40 | 3 (13) |
| 41-50 | 7 (31) |
| 51-60 | 9 (39) |
| 61-70 | 4 (17) |
| Tumor size (cm) | |
| ≤2 | 14 (61) |
| >2 | 9 (39) |
| Histological type | |
| Invasive ductal carcinoma | 23 (100) |
| Tumor grade | |
| 1 | 4 (17) |
| 2 | 11 (48) |
| 3 | 8 (35) |
| Lymph node metastasis | |
| Positive | 11 (48) |
| Negative | 12 (52) |

No patients were treated with chemotherapeutic drugs.

amounts of cancer *versus* the adjacent normal cells in each sample, or differences in the biological behavior of these cancer cells (Table II). The results from mRNA and protein demonstrate that *WT1*(17AA+) and *WT1*(17AA–) play an oncogenic role in breast cancer.

*Correlation of WT1 isoforms with ER-α and HER2 in breast cancer.* To investigate the role of *WT1* in breast cancer progression, we performed a Western blot analysis using ER-α and HER2 antibodies (Figure 2). ER-α protein was detected in breast carcinoma grade 1 and 2, and 3 at 60% (6 out of 10 samples) and 100% (7 out of 7 samples), respectively (Table III). It was expressed at low levels in adjacent normal breast tissues (23.5%; 4 out of 17 samples). HER2 protein was detected in breast carcinoma grade 1 and 2, and 3 at 80% (8 out of 10 samples) and 71.4% (5 out of 7 samples) but was expressed at low levels in adjacent normal breast tissues (11.8%; 2 out of 17 samples). In addition, mixture of *WT1*(17AA+) and *WT1*(17AA–) expression was found in all breast cancer tissues (Table II). These results support our hypothesis that a mixture of *WT1*(17AA+) and *WT1*(17AA–) expression is involved in breast cancer development and up-regulation of ER-α and HER2 expression.

*Knock-down of WT1 down-regulates the expression of ER-α and HER2 in MCF-7 cells.* To explore the relation between WT1, ER-α and HER2 in breast cancer, we used breast cancer cell lines as a model. We first detected these three proteins in three breast cancer cell lines (MCF-7, MDA-MB-468 and MDA-MB-231) and one normal breast cell line (MCF-12A). We found that the WT1 level in MDA-MB-468 cells was significantly higher than in MCF-7 and MDA-MB-231 cells, but WT1 was not expressed in MCF-12A cells. Moreover, ER-α and HER2 proteins were detected in MCF-

Table II. *Expression of Wilms' tumor 1, WT1(17AA+) and WT1(17AA−), isoforms in adjacent normal breast and breast cancer tissues.*

| *WT1* | Adjacent normal breast | Invasive carcinoma | |
|---|---|---|---|
| | | Grade 1 and 2 | Grade 3 |
| No band | 5 | 0 | 0 |
| 17AA− | 16 | 2 | 1 |
| 17AA+ | 5 | 2 | 0 |
| 17AA− and 17AA+ | 4 | 11 | 7 |
| Total cases | 30 | 15 | 8 |

*Table III. The relation between Wilms' tumor 1 (WT1(17AA+) and WT1(17AA−)), estrogen receptor-alpha (ER-α) and human epidermal growth factor receptor-2 (HER2) in adjacent normal breast and breast cancer tissues.*

| *WT1* | Adjacent normal breast | Invasive carcinoma | |
|---|---|---|---|
| | | Grade 1 and 2 | Grade 3 |
| 17AA− | 15 | 0 | 0 |
| 17AA+ | 0 | 0 | 0 |
| 17AA+ and 17AA− | 2 | 10 | 7 |
| ER-α | 4 | 6 | 7 |
| HER2 | 2 | 8 | 5 |
| Total cases | 17 | 10 | 7 |

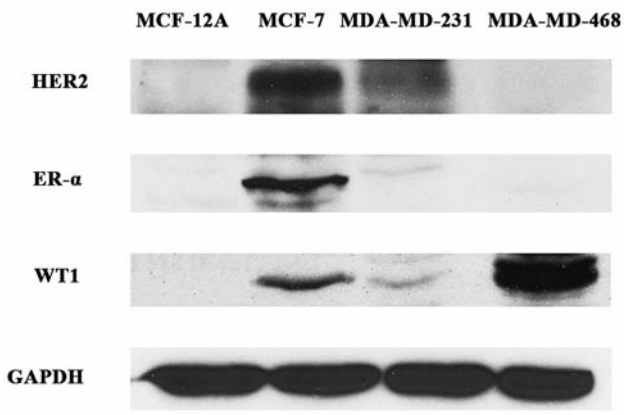


Figure 3. *Expression of Wilms' tumor 1 (WT1), estrogen receptor-alpha (ER-α) and human epidermal growth factor receptor-2 (HER2) using western blot analysis. One hundred micrograms of proteins from three breast cancer cell lines (MCF-7, MDA-MB-231 and MDA-MB-468) and one normal breast cell line (MCF-12A) were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis.*

7 cells but were not found in MDA-MB-468 and MCF-12A cells. HER2 and ER-α were slightly expressed in MDA-MB-231 cells (Figure 3). We then chose MCF-7 cells to produce stable cell lines expressing *WT1* isoforms because these cells expressed both ER-α and HER2. MCF-7 also is the cell line that has been studied for the growth and proliferation *via* HER2 and ER (26, 27).

In our next study, we determined the WT1 function using $siRNA_{WT1}$ in MCF-7 cells that stably expressed each of four WT1 isoforms: *WT1*+/+, *WT1*+/−, *WT1*−/+ and *WT1*−/− isoforms (Figure 4A). The effect on cell proliferation of MCF-7 cells overexpressing *WT1* isoforms was investigated. Cells grown in a 24-well plate were treated with 100 nM of $siRNA_{WT1}$ for four days. The number of cells was assessed using trypan blue exclusion assay. Colonies of cells that were stably transfected with *WT1*+/+ and *WT1*+/− exhibited a higher number of cells than those transfected with *WT1*−/+, *WT1*−/− and empty vector. Overexpression of *WT1*−/− led to slow growth. Moreover, the knocking down of *WT1* in MCF-$7_{+/+}$, MCF-$7_{+/-}$, MCF-$7_{-/+}$ and MCF-$7_{-/-}$ cells led to the same results, *i.e.* reduced the number of cells. These observations provide evidence that *WT1*+/+ and *WT1*+/− isoforms may contribute to cell proliferation and growth. The results are shown in Figure 4B.

To further characterize WT1, ER-α and HER2 protein expression levels in the MCF-7 cells overexpressing *WT1*s, Western blot analysis was performed. All the four WT1 isoforms expressed in MCF-7 cells showed the same result of down-regulation of ER-α and HER2 only when WT1 was down-regulated using $siRNA_{WT1}$. Interestingly, overexpression of *WT1*+/+, *WT1*+/−, *WT1*−/+ and *WT1*−/− significantly induced the expression of ER-α at 0.53-, 0.57-, 0.44- and 0.29-fold, respectively. In addition, overexpression of *WT1*+/+ and *WT1*+/− significantly induced the expression of HER2 at 0.81- and 0.83-fold, respectively. These results indicate that the increase of *WT1* isoforms resulted in a significant increase of ER-α and HER2 protein levels, especially in MCF-7 cells overexpressing *WT1*+/+ and *WT1*+/− isoforms (Figure 4C and D).

## Discussion

Our first finding is the study of growth inhibition of MCF-7 cells by silencing of the *WT1* gene by siRNA. The results show the effect of siRNA against the *WT1* gene ($siRNA_{WT1}$) on cell proliferation. The level of WT1 protein in MCF-7 cells transfected with $siRNA_{WT1}$ decreased in a dose-dependent manner and is related to the decrease in the number of MCF-7 cells. Moreover, the number of cells also decreased in a time-dependent manner. These findings

suggest that *WT1* has a key role in mediating proliferation and WT1 might serve as a molecular target for human breast cancer treatment (30). Furthermore, we investigate the anti-apoptotic function of *WT1*+/+ and *WT1*+/− isoforms in MCF-7 cells. The results showed that *WT1*+/+ and *WT1*+/− isoforms prevent MCF-7 cells from doxorubicin-induced cell death (21). It also has been reported that WT1 are expressed at high levels in almost all types of solid tumors (31). Loeb *et al*. reported that *WT1* mRNA and protein is expressed in nearly 90% of breast cancer cases but are detected at low levels in adjacent normal breast samples (3). On the other hand, our present study showed that *WT1* mRNA expressed in adjacent normal breast and breast cancer tissues. However, expression of *WT1*(17AA−) mRNA and protein dominantly present in adjacent normal breast, but a mixture of *WT1*(17AA+) and *WT1*(17AA−) mRNA and protein is highly expressed in breast cancer. Similarly, Oji *et al*. found that the expression of *WT1*+/+ isoform rescued the growth-inhibitory effect of WT1 antisense oligomers on cancer cells (8). In addition, Ito *et al*. demonstrated the anti-apoptotic function of *WT1*(17AA+) isoforms (*WT1*+/+ and *WT1*+/−) using shRNA targeting exon 5 (19). It showed that both *WT1*+/+ and *WT1*+/− inhibited apoptosis though the intrinsic pathway (19, 20).

Cancer grading is classified from cytological images based on tubule formation of the tumor tissues, nuclear grade evaluation of the size and shape of the nucleus in the tumor cells and mitotic rates. There are three grades of cancer: low grade (grade 1), moderate or intermediate grade (grade 2) and high grade (grade 3). Grade 1 is the initiation stage of cancer. Cells in this grade consist of well-differentiated cells which look similar to normal cells and grow very slowly. Grade 2 has moderately differentiated cells and these cells grow slightly faster. Cancer cells in both grade 1 and 2 do not spread to the lymph nodes or surrounding breast tissue and distance organs. However, grade 3 is an aggressive and advanced stage. This grade can spread into the surrounding tissue, lymph nodes and other organs of the body either through the blood stream or the lymphatic system (32, 33). Here, we found that mixed *WT1*(17AA+) and (17AA−) mRNA was significantly expressed in normal tissue and breast carcinoma grades 1, 2 and 3 at 11.76, 73 and 87%, respectively. This observation indicated that the mixed *WT1*(17AA+) and (17AA−) mRNA level of normal breast tissues was lower than in the initiation and advanced stages of cancer. The protein level of WT1 was confirmed using Western blotting. Consistent with mRNA experiments, the expression of mixed *WT1*(17AA+) and (17AA−) protein was dominantly expressed in all grades of cancer but with low expression in adjacent normal breast tissues. Taken together, our finding offers evidence that the high expression of mixed *WT1* isoforms might indicate an increase of aggression in breast cancer rather than normal tissue. Interestingly, we found *WT1*(17AA−) mRNA and protein highly expressed in adjacent normal tissue. These results suggest that *WT1*(17AA+) might play as a crucial isoform in cancer progression and development, and might work together with *WT1*(17AA−) as a protein partner.

The evolution and progression of breast cancer are governed by complex interactions between steroid receptors (*i.e*. ER and progesterone receptor) and growth factor receptor signaling (24). ER-α plays a crucial role in breast cancer malignancies (34, 35). ER is known for its nuclear-initiated steroid signaling (36) and functions as a ligand-dependent transcription factor and promotes expression of insulin-like growth factor receptor (IGFR), the cell cycle regulator cyclin D1, the antiapoptotic factor BCL-2 (37-39), and proangiogenic vascular endothelial growth factor (40, 41). In addition, nuclear ER also induces the expression of transforming growth factor alpha (TGFα) and amphiregulin (42). A recent study of endogenous membrane ER in breast cancer culture models illustrates directly or indirectly the activation of EGFR, HER2, and IGFR1 (43). This process involves the activation of the cellular tyrosine kinase SRC (44), matrix metalloproteinases (MMPs) 2 and 9, and the release of EGFR ligand hairpin binding epidermal growth factor-like growth factor (HB-EGF), which in turn activates the EGFR downstream kinase cascades (*i.e*. rat sarcoma viral oncogene homolog (small GTP binding protein, RAS)/mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK)/MAPK and phosphatidylinositide-3 kinase (PI3K)/serine-theronine protein kinase (AKT) (45-46). The relationship of ER with the EGFR/HER2 pathway, and presumably with additional growth factor receptor pathways, leads to promotion of breast cancer cell proliferation and survival, and tumor progression. Furthermore, the expression levels of HER2 and EGFR increase in high-passage MCF7 cells ($MCF^H$, >75 passages) compared to low-passage MCF7 cells ($MCF7^L$, <35 passages), indicating high passage MCF7H cells gain expression of two members of the EGFR family, EGFR and HER2. In $MCF7^H$ cells, the level of ER-α phosphorylation at Ser118 is also increased, while the expression level of ER-α does not significantly change compared to $MCF7^L$ cells. The expression level of WT1 greatly increases in $MCF7^H$ cells compared to MCF7L cells (27). These findings support our hypothesis that *WT1* plays an oncogenic role in ERα and HER2 protein regulation. In this present study, we observed that ER-α and HER2 proteins were highly expressed in breast cancer but expressed at low levels in adjacent normal breast tissues, while *WT1*(17AA+) was strongly expensed in breast cancer and slightly in adjacent normal breast tissues.

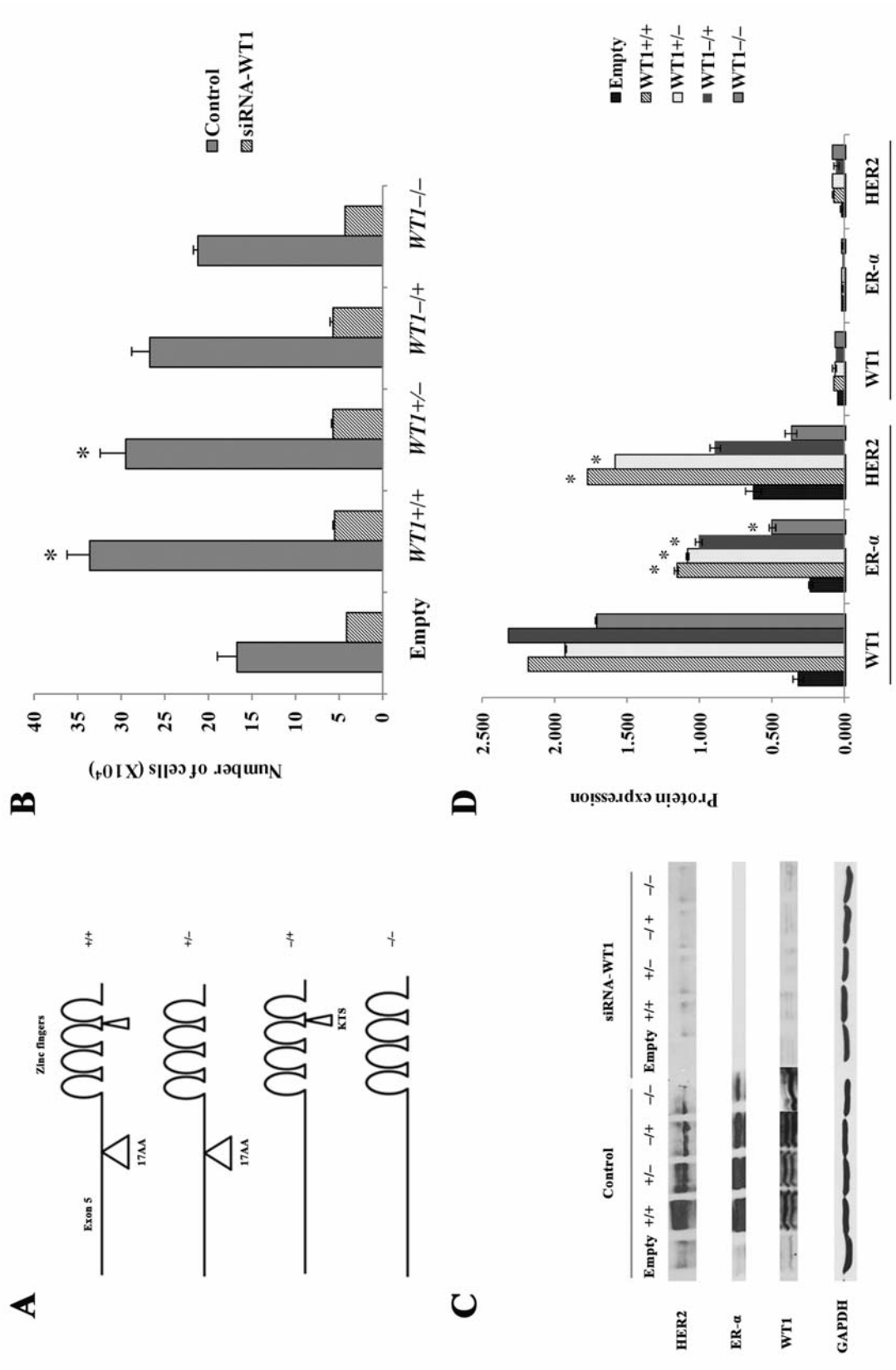


Figure 4. *MCF-7 cells overexpressing Wilms' tumor 1 (WT1). A: WT1 gene contains 10 exons that are alternatively spliced at two sites (plus or minus 17AA in exon5 and plus or minus KTS in exon 9), yield four isoforms: +/+, +/–, –/+ and –/–. These four different isoforms were constructed into pcDNA6/V5 vector. The recombinant plasmids were transfected into MCF-7 cells to generate cells overexpressing WT1s. B: The stably-overexpressing cells were transfected with 100 nM siRNA$_{WT1}$ and siRNA$_{neg}$ (control). After 96 h of treatment, living cells were counted using the trypan blue exclusion assay. The number of cells in the average value of transfection experiments are represented as mean±S.D. (*p<0.05) and each was performed in triplicate. C: Control and siRNA$_{WT1}$-transfected cells were harvested and lysed in RIPA buffer. The protein solution was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The level of Wilms' tumor 1 (WT1), estrogen receptor-alpha (ER-α) and human epidermal growth factor receptor-2 (HER2) protein expression was assessed by western blot analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. D: Each protein band was quantified using Scion Image software. Results were normalized with the intensity of GAPDH expression. Assays were performed in three independent experiments. Results (protein expression level) represent the average of three independent experiments and are shown as mean±S.D. (*p<0.05).*

The result of *WT1*(17AA+) mRNA expression was confirmed at the protein level. We also demonstrated the relationship of each *WT1* isoform with ER-α and HER2 in MCF-7 cells overexpressing WT1s using siRNA. The results indicate that *WT1*+/+ and *WT1*+/− are associated with expression of ER-α and HER2. Our results provide strong evidence to confirm the oncogenic roles of *WT1*(17AA+) in regulating ER-α and HER2 expression and the progression in breast cancer.

Herein, we observed the effect of each WT1isoform on cell proliferation using trypan blue assay. MCF-$7_{WT1+/+}$ and MCF-$7_{WT1+/-}$ exhibited faster cell growth than MCF-$7_{Empty}$. In addition, all overexpressing and control cells treated with $siRNA_{WT1}$ demonstrated an equal number of cells. This proliferation effect of *WT1*(17AA+) and growth-inhibitory effect of $siRNA_{WT1}$ supported our hypothesis that *WT1*(17AA+) may contribute to the cell growth and plays oncogenic functions by up-regulation of ER-α and HER2. Our results also supported the previous evidence that the *WT1*+/+ isoform promotes a transformed phenotype in mammary epithelial cells. High levels of *WT1*(KTS+) isoform contribute to the pro-proliferative effects in breast cancer cells, and consequently to a role as potential breast oncogenes (47, 48). In addition, MCF-7 cells overexpressing WT1−/− showed a slight increase in the number of cells. Besides, overexpression of *WT1*−/− illustrated lower expressions of ER-α and HER2 than *WT1*+/+ and *WT1*+/−. These results can be explained by previous findings concerning overexpression of the *WT1*−/− isoform that show tumor-suppressor effects in mammary epithelial cells (47-49). The *WT1*−/− isoform also induces cytoskeletal changes in gastric cancer, esophageal cancer, breast cancer and fibrosarcoma cell lines, and promote ovarian cancer cells (50). Taken together, *WT1*+/+ and *WT1*+/− isoforms might be key molecules in cancer progression rather than *WT1*−/−.

## Conclusion

In this study, *WT1*(17AA+) seemed to be involved in the expression and regulation of ER-α and HER2 in breast cancer. *WT1*(17AA+) mRNA and protein levels were significantly high in breast carcinoma tissues. In addition, overexpression of *WT1*+/+ and *WT1*+/− related to high expression of ER-α and HER2 and cell proliferation. Since *WT1* plays a role in up-regulation of EGFR, ER-α and HER2 expression, we can conclude that *WT1*(17AA+) may be an important molecule which plays an oncogenic role in regulating ER-α and HER2 expression and cancer development in breast cancer cells. Further dissection of the relationship of *WT1*+/+ and *WT1*+/− with ER-α, EGFR and HER2 will be necessary and useful for diagnostic and treatment in patients with breast cancer.

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