Original article

Anti-apoptotic function of T-KTS+, T-KTS-, WT1+/+ and WT1+/- isoforms in breast cancer

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Background: WT1 was originally identified in Wilms tumor, a childhood kidney cancer. This gene was expressed in wide variety of solid cancers. Alternative splicing of WT1 transcript generates four major protein isoforms and thirty-six minor protein isoforms, each having different functional properties. WT1 gene has been considered as a tumor suppressor gene and anti-apoptotic protein. However, the mechanism of WT1 in breast cancer remains unclear.

Objective: Evaluate the role of truncated WT1 isoforms (T-KTS+ and T-KTS-) and two major WT1 isoforms (+/+ and +/-) in apoptosis in breast cancer cell line, MCF-7.

Materials and methods: RNA interference (RNAi) was employed in an attempt to define the role of WT1 in a breast cancer cell line (MCF-7). Furthermore, MCF-7 over-expressing cells that stably expressed two truncated WT1 isoforms (T-KTS+ and T-KTS-) or two major WT1 isoforms (+/+ and +/-) were generated and exposed to Doxorubicin. The mortality of cells was determined as a percentage of trypan blue-stained cells in total cells. The apoptotic molecules in apoptosis pathway were detected using RT-PCR, caspase-7 activity assay and Western blot analysis techniques.

Results: Transfection of siRNA_{WT1} into MCF-7 cells resulted in decreasing of WT1 protein and related to the increasing in number of cell death and caspase-7 activity. Over-expression of T-KTS+, T-KTS-, WT1+/+ and WT1+/- isoforms protected cells from cell death induced by apoptosis-inducing agent, doxorubicin. Moreover, the expression of apoptotic p53, Bak and caspase-7 were decreased by the expression of all four WT1 isoforms, especially T-KTS- and T-KTS+ isoforms.

Conclusion: T-KTS+ and T-KTS- isoforms as well as WT1+/+ and WT1+/- isoforms could function as an anti-apoptotic protein in breast cancer cell line, MCF-7.

Keywords: Anti-apoptosis, MCF-7, truncated WT1, WT1

The WT1 gene was originally identified as a tumor suppressor gene responsible for Wilms' tumor, a renal neoplasm of childhood [1]. The WT1 protein can act as a transcriptional activator or repressor for many genes involved in cell differentiation, growth and apoptosis. WT1 plays an important role in the pathogenesis and progression of leukemia [2-4].

Moreover, the decreased expression of WT1 protein levels led to growth inhibition and apoptosis in leukemia cells in vitro [5, 6] and in vivo [7]. Inoue et al. [2] reported that the WT1 mRNA expression was significantly up-regulated in leukemia as compared with the normal hematopoietic cells. Moreover, Loeb et al. [8] have demonstrated that WT1 mRNA and WT1 protein was expressed in nearly 90% of breast cancers but not in most normal breast tissue. These results suggest that WT1 plays a role in the pathogenesis of breast cancer as an oncogene rather than a tumor suppressor gene. Recently, we showed

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that down regulation of WT1 by RNAi led to the growth arrest of breast cancer cell line (MCF-7) [9] and enhanced doxorubicin-induced cytotoxicity.

WT1 gene contains 10 exons that are alternatively spliced at two sites (plus or minus 17AA in exon 5 and plus or minus KTS in exon 9), and yield four isoforms; +/+, +/-, -/+ and -/- [10]. The carboxyl terminus contains four zinc finger domains that mediate DNA binding, RNA recognition and nuclear localization signal [10, 11]. The amino terminus comprises of a transcriptional repression domain, RNA recognition, self-association, and repression domain and activation domain [11, 12]. In addition, a novel truncated WT1 was found in breast cancer cell line (MCF-7), leukemia cell line (K562), and blood samples from patients with acute leukemia but was not found in normal blood samples. This 2.1 kb transcript consisted of the coding region (exon 6-10) of the zinc finger domain of WT1, together with a portion of intron 5 at the 5' end of the transcript and unknown peptide (approximately 38 amino acids) [13]. However, the function of this truncated WT1 remains unknown. As WT1 has four major isoforms and thirty-six minor isoforms and each form considers a different function and protein partner, it difficult to clarify the role of each WT1 isoform. However, all of the four major WT1 isoforms have an apoptotic function such as WT1+/+, WT1+/- and WT1-/- isoforms act as anti-apoptotic molecule through the suppressing the expression of caspase-3, caspase-9 and Bax [14]. Moreover, WT1+/+ and WT1+/- isoforms significantly inhibited etoposide and doxorubicin induced apoptosis by blocking cytochrome *c* release in K562 cells [15]. WT1+/+ isoform has characteristics as an oncogene through the up-regulation of RPS6K, accounting for the lack of cell cycle arrest [16]. WT1+/- isoform down-regulated Bak (proapoptotic Bcl-2 family member) and up-regulated Bcl-2 [14, 15]. WT1-/- isoform induced Bcl-2 and Bfl-1/A1 promoter [14, 17].

Since truncated WT1 isoforms (T-KTS+ and T-KTS-) expressed in MCF-7 and the function of these isoforms are not established yet [13], we generated MCF-7 over-expressing cells that stably expressed T-KTS+ and T-KTS-. We also generated MCF-7 overexpressing WT1+/+ and WT1+/- to test the hypothesis that WT1+/+ and WT1+/- isoforms function as an anti-apoptotic protein not only in leukemia cell but also in breast cancer cell. Another hypothesis of this work is to study if the truncated WT1 isoforms function as an anti-apoptotic protein.

As described above, WT1+/+ and WT1+/- isoforms contain the same amino acid in N-terminal and different amino acid in C-terminal (KTS+ and KTS-), and act as an anti-apoptotic molecule in K562. Therefore, these two isoforms were used as a positive control to evaluate the anti-apoptotic function of truncated WT1 isoforms. In this study, we report that two truncated WT1 isoforms (T-KTS+ and T-KTS-) and two major WT1 isoforms (+/+ and +/-) played an anti-apoptotic role in the breast cancer cell line, MCF-7. In addition, T-KTS- isoform was the powerful molecule to protect cell against doxorubicin-induced apoptosis.

Materials and methods

Cell line and culture condition

A breast cancer cell line (MCF-7) was purchased from the American Type Culture Collection (Manassas, USA). The cells were grown in RPMI 1640 (Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (Invitrogen), 50 units/ml of penicillin, and 50 µg/ml of streptomycin (Invitrogen) and maintained in a humidified 37°C incubator with 5% CO₂.

Small interference RNA (siRNA) transfection

siRNA against WT1 (siRNA_{WT1}) (Invitrogen) consist of a mixture of three 25-nt siRNA duplexes targeting different non-overlapping regions of WT1 mRNA, namely, siRNA_{WT1}R88 (5'-AAATATCTCTT ATTGCAGCCTGGGT-3'), siRNA_{WT1}R89 (5'-TTCACAGTCCTTGAAGTCACACTGG-3') and siRNA_{WT1}R90 (5'-TTTCACACCTGTATGTCTCC TTTGG-3'). The first sequence is located in exon 7 and the last two sequences are located in exon 8. The non-specific sequence (siRNA_{neg}) was used to evaluate the potential presence of the non-specific effects of irreverent siRNA. All procedures were performed under an RNase-free environment. In brief, cells were transfected with 100 nM siRNA duplexes using Lipofectamine 2000 reagent (Invitrogen) at a final concentration of 0.2%, according to the manufacturer's instructions. To minimize the cytotoxicity of the reagent itself, cells were washed once with PBS and the media was changed five hours after transfection.

Generation of cells stably over-expressing WT1 isoforms

For the generated MCF-7 cells stably over-

expressing WT1s, MCF-7 cells were transfected, using FuGENE6 reagent (Roche, Indianapolis, USA), with pcDNA6/V5, a mammalian expression vector containing a blasticidin selection marker (Invitrogen), containing the cDNA encoding T-KTS+ or T-KTS- or WT1+/+ or WT1+/- . This truncated WT1 transcript encodes a protein that retains an intact DNA-binding zinc finger domain but lacks the exon1 to exon5 for transcription repression [13], whereas WT1+/+ and WT1+/- transcript encode the whole WT1 protein (exon1 to exon10). For a control, MCF-7 cells were transfected with empty pcDNA6/V5. Transfected cells were selected for at least one week by using 5 µg/mL blasticidin and characterized by Western blot analysis using anti-WT1 antibody (Santa Cruz Biotechnology, Santa Cruz, USA) and anti-actin antibody (Sigma-Aldrich, St.-Louis, USA). The resulting lines were named MCF-7_{T-KTS+}, MCF-7_{T-KTS-}, MCF-7_{WT1+/+}, MCF-7_{WT1+/-} and MCF-7_{Empty}, respectively.

Western blot analysis

Cells were harvested by trypsinization and lysed in radio immunoprecipitation assay (RIPA) buffer (Pierce, Rockford, USA) followed by the determination of the protein concentration using the Bradford method (Bio-Rad, Hercules, USA). The samples were then subjected to 12% SDS-polyacrylamide gel electrophoresis and the proteins were transferred to a nitocellulose membrane (Bio-Rad). After blocking, the immunoblot was incubated with anti-WT1 (1:200) (Santa Cruz Biotechnology), anti-caspase-7 (1:1,000) (Cell Signaling, Boston, USA) anti-Bak (1:20), anti-Bax (1:500) (Calbiochem, La Jolla, USA) and anti-actin (1:1,000) antibodies (Sigma), as described previously [9].

Trypan blue exclusion assay

Trypan blue assay was performed as previously described [18]. Cells were harvested after the addition of doxorubicin (Sigma), by a brief trypsinization. Cells were transferred into 1.5 mL microcentrifuge tube and centrifuged at 1,500 xg for seven minutes. Both floating and attached cells were subjected to the assay. After centrifugation, the pellet was re-suspended with 100 µL of new medium and 100 µL of 0.4% of trypan blue stained solution (Invitrogen). At least 150 cells were counted per treatment after being stained by trypan blue at the final concentration of 0.2%. The assays were performed in triplicate.

Caspase-7 activity assay

The Caspase-Glo 3/7 assay (Promega, Madison, USA) was used to measure caspase-7 activity. MCF-7 cell clones that stably expressed each WT1 isoform were cultured in 96-well plates. Cells were challenged with 1 µM doxorubicin for 24 hours. After treatment, caspase-Glo 3/7 reagent was added to each well according to the manufacturer's instructions (Promega). The plate was mixed on a plate shaker for 30 seconds and incubated at room temperature for one hour. Luminescence was measured using the luminometer. The assay was performed in triplicate.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen) and the mRNA level was determined using OneStep RT-PCR kit (Qiagen, Valencia, USA) according to the instructions from the manufacturer. In brief, the PCR was performed in a total volume of 25 µl with 0.5 µg of total RNA. The PCR condition was started at 50°C for 30 minutes followed by an initial PCR activation step at 95°C for 15 minutes followed by 35 cycles of 95°C denaturation for one minute, 58°C annealing for one minute and 72°C extension for one minute. PCR primer was as follows, p53: forward primer, 5'-GCTCTGTTTACTGTACCA CCATCC-3', reverse primer, 5'-CTCTCGGAACA TCTCGAAGCG-3'; GAPDH: forward primer, 5'-GAAGGTGAAGGTCGGAGT-3', reverse primer, 5'-GAAGATGGTGGTGGGATTTC-3'. The PCR products were loaded on a 1.8% agarose gel, stained with ethidium bromide, and photographed with a digital camera under UV illumination.

Statistic analysis

Two-sample *t*-test was used to evaluate the difference in apoptotic and survival indices among the over-expressing cell (MCF-7_{WT1}) and the control cell (MCF-7_{Empty}). A *p*-value of less than 0.05 was considered to be statistically significant.

Results

Knock down of WT1 induces apoptosis in MCF-7

To examine the roles of WT1 in breast cancer, 100 nM siRNA_{WT1} was transfected into MCF-7. After 48 hours of transfection, cells were treated with 0, 1, 10, and 20 µM doxorubicin and incubated for another 24 hours. Trypan blue assay showed that suppression of WT1 resulted in an increasing number of cell deaths

related to the decrease of WT1 protein, as shown in **Fig. 1(A)**. The treatment of cells with siRNA_{neg} did not affect the WT1 protein level and the number of cell deaths was significantly less than siRNA_{WT1} treated cells ($p < 0.01$ by two-sample t -test). Furthermore, we performed caspase-7 assay to

evaluate the anti-apoptotic function of WT1. As shown in **Fig. 1(B)**, caspase-7 was increased in siRNA_{WT1} treated cells in a dose dependent manner, but not in the cells that were treated with siRNA_{neg}. These results indicate that WT1 prevents breast cancer cells from undergoing apoptosis.

Fig. 1 WT1 prevent doxorubicin-induced apoptosis, as shown by siRNA gene knockdown. (A) The reduction of WT1 protein level in cells treated with 100 nM siRNA_{WT1}. (B) After 24 hours of siRNA transfection, MCF-7 cells were challenged with 0, 1, 10, 20 μM doxorubicin and trypan blue assay was performed to determine death cell. (C) The depletion of WT1 in cells treated with siRNA_{WT1} increased caspase-7 activity upon doxorubicin stimulation. These results suggest that WT1 protect cells against doxorubicin-induce apoptosis. Assay was performed in triplicate. (***) $p < 0.005$.

Over-expression of T-KTS+, T-KTS-, WT1+/+ and WT1+/- isoforms prevent cell from doxorubicin induced cell death

To confirm that WT1 plays an important role in apoptosis in breast cancer, we considered four WT1 isoforms that are characterized as anti-poptotic molecule. MCF-7 over-expressing one each of four WT1 isoforms: T-KTS+, T-KTS-, WT1+/+ and WT1+/- were generated and subjected into a cell death assay. Ten polyclonal populations that harbored each WT1 fragment were used to determine the protein expression level. The protein expression level was characterized by Western blot analysis using anti-WT1 and anti-actin antibodies. The highest expression clones were selected as shown in **Fig. 2(A)**. MCF-7

cell that stably expressed one each of four WT1 isoforms at high levels were exposed to apoptosis-inducing agents, doxorubicin. The mortality of cells was determined as the percentage of trypan blue-stained cells in the total number of cells. The results of the three independent experiments are shown in **Fig. 2(B)**. Consistent with the results of the siRNA experiments described above, all four isoforms (T-KTS+, T-KTS-, WT1+/+ and WT1+/-) made the cells more resistant to doxorubicin treatment than the control (MCF-7_{Empty}) ($p < 0.05$ by two-sample *t*-test). These results showed that two truncated WT1 isoforms and two major WT1 isoforms prevent cells from doxorubicin induced cell death.

Fig. 2 Protection from doxorubicin induced cytotoxicity in MCF-7 cells overexpressing truncated WT1s, WT1+/+ and WT1+/- isoforms. (A) MCF-7 cells overexpressing four WT1 isoforms were evaluated by Western blot analysis. (B) MCF-7 cell that stably expressed WT1s were challenged with 1 μ M doxorubicin for 24 hours and subjected to trypan blue assay. Overexpression of truncated WT1 isoforms (MCF-7_{T-KTS+} and MCF-7_{T-KTS-}) and WT1 isoforms (MCF-7_{WT1+/+} and MCF-7_{WT1+/-}) were associated with significantly less doxorubicin-induced cell death. Assay was performed in triplicate. (*, $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ by two-sample *t* test, comparing MCF-7_{Empty} and MCF-7 cells overexpressing truncated WT1s, WT1+/+ and WT1+/- isoforms.) Empty, pcDNA6 empty vector; T-KTS+, truncated WT1(KTS+) isoform; T-KTS-, truncated WT1(KTS-) isoform.

Over-expression of T-KTS+, T-KTS-, WT1+/+ and WT1+/- isoforms prevent cell from doxorubicin undergoing apoptosis

To examine the anti-apoptotic function of T-KTS+, T-KTS-, WT1+/+ and WT1+/- isoforms, we determined caspase-7 activity by Western blot analysis using anti-caspase-7 antibody and caspase-Glo 3/7 assay kit. MCF-7 cell that stably expressed one each of the four WT1 isoforms at high levels were exposed to 1 μ M doxorubicin, for 24 hours and analyzed for

caspase-7. As shown in **Fig. 3**, results from caspase-7 activity that were measured by caspase-7/Glo assay correlated with results from Western blot analysis. WT1 isoforms inhibited the cleavage of procaspase-7. Moreover, most of MCF-cells over-expressing WT1 isoforms significantly exhibited caspase-7 lower than the control (MCF-7_{Empty}). Surprisingly, T-KTS-isoform dramatically prevented cells from undergoing apoptosis.

Fig. 3 Expression of truncated WT1s, WT1+/+ and WT1+/- isoforms reduced caspase-7 activity. **(A)** Overexpressing cells that stably WT1 isoforms were challenged with 1 μ M doxorubicin for three hours and incubated with caspase-7 substrate for one hour. **(B)** Cell clones were exposed to 1 μ M doxorubicin for 24 hours. After harvesting, lysates were then separated by 12% SDS-PAGE and caspase-7 was characterized by Western blotting. MCF-7_{Empty} cells significant exhibited caspase-7 higher than MCF-7 cells overexpressing truncated WT1 isoforms (MCF-7_{T-KTS+} and MCF-7_{T-KTS-}) or WT1 isoforms (MCF-7_{WT1+/+} and MCF-7_{WT1+/-}) upon doxorubicin stimulation. These results suggest that truncated WT1s, WT1+/+ and WT1+/- isoforms protects MCF-7 against doxorubicin-induced apoptosis. Assay was performed in triplicate. (*p < 0.05, ***p < 0.005 by two-sample t test).

Inhibition of apoptosis molecule by WT1 isoforms

Since WT1 isoforms (T-KTS+, T-KTS-, WT1+/+ and WT1+/-) showed to play anti-apoptotic roles in apoptosis pathway, the effect of the suppression of these four isoforms on p53, which is considered to act as a key apoptotic signaling molecule, was analyzed. After six hours of challenging with 1 μ M doxorubicin, MCF-7 cells over-expressing WT1 isoforms were harvested, and the total RNA was extracted. RT-PCR was performed to determined *p53* mRNA level (**Fig. 4A**). Semi-quantitative analysis was used to evaluate the expression of *p53* (**Fig. 4(B)**). We found that the *p53* expression

decreased by the stable expression of WT1 isoforms. T-KTS- and T-KTS+ isoforms showed the highest potential in p53-down-regulation.

To understand the mechanism of WT1 isoforms, we further studied gene expression (Bak and Bax) which related to the intrinsic pathway and regulated by p53 by Western blot analysis. The expression of proapoptotic Bak was significantly decreased by the expression of T-KTS+, T-KTS-, WT1+/+ and WT1+/- isoforms. In addition, the expression level of Bax was significantly decreased by the expression of both truncated WT1 isoforms, T-KTS+ and T-KTS- (**Fig. 5**).

Fig. 4 Expression of p53 (Apoptotic protein) after challenging with 1 μ M doxorubicin for six hours. (**A**) RT-PCR product. The gel image presented is representative of three independent experiments. (**B**) Semi-quantitative analysis of agarose gel densitometry data. Each PCR band on photographs of the three agarose gels was quantitated using densitometry and mean $\pm$ SD. of these values were calculated. WT1 isoforms overexpression significantly reduced p53 (* p < 0.05, *** p < 0.005 by two-sample t test).

Fig. 5 Expression of apoptotic-related proteins in MCF-7 cell clones that stably expressed WT1 isoforms. Cell clones were treated with 1 μ M doxorubicin for 24 hours. Then, apoptotic proteins were examined by Western blot analysis with anti-Bak, Bax and actin antibodies.

Discussion

As the mechanism of WT1 in breast cancer remains unclear, two possible functions, tumor suppressor and oncogene, are raised. In a previous study, we reported that the transfection of siRNA_{WT1} into MCF-7 cells resulted in the silencing of WT1 expression as well as growth inhibition in both dose- and time-dependent manners [9]. This present study showed that decreasing the WT1 protein resulted in an increasing number of cell deaths and increased caspase-7 activity. These results suggest that WT1 could function as an anti-apoptotic protein. As the WT1 gene encodes a transcription factor of the zinc-finger family and interacts with growth factors, growth receptors, transcription factors and apoptotic protein either by activation or suppression, we generated MCF-7 over-expressing WT1 isoforms (T-KTS+, T-KTS-, WT1+/+ and WT1+/-), to test the hypothesis of the WT1 role in the anti-apoptosis function. It is known that the KTS+ and KTS- can bind overlapping DNA sequences in the promoter of growth factors including the insulin-like growth factor II (IGF-II) [19-20] and platelet-derived growth factor A (PDGF-A) [12]. Similarly, Caricasole et al. [21] reported that both WT1(KTS+) and WT1(KTS-) isoforms can bind to the murine IGF-II transcript in exon2. Moreover, WT1(KTS+) isoform represses transcription from the IGF-II P3 promoter and had a greater affinity for IGF-II exon 2 RNA than WT1(-KTS) isoform [19].

Alternative splicing of WT1 transcript generates four major protein isoforms: +/+, +/-, -/+ and -/-, and thirty-six minor protein isoforms, each having different functional properties [22]. The expression and role of WT1 also varies by cell type and interacting proteins. Among the four WT1 isoforms, the WT1+/+ isoform was expressed dominantly in all of the cancers

examined [23]. Although, the WT1 gene has been considered as a tumor suppressor gene, wild type WT1 gene is expressed in several cancers including leukemia, breast and colon cancer. In this present study, we determined the anti-apoptosis function of two major WT1 isoforms: +/+ and +/- and two truncated WT1 isoforms: T-KTS+ and T-KTS-. Our data indicate that WT1 exerts an anti-apoptosis function in the breast cancer cell line (MCF-7), especially WT1+/- and T-KTS- isoforms. Similarly, Oji et al. [24] showed that the constitutive expression of the WT1+/+ isoform rescued the growth inhibitory effect of WT1 antisense oligomers on cancer cells. Furthermore, short hairpin RNAs targeting WT1 exon 5 (shWTE5) induced apoptosis through the suppression of the WT1 isoform with exon 5 resulting in an increasing expression of proapoptotic Bak and Bax proteins and decreasing anti-apoptotic Bcl-xL and Bcl-2 proteins in HT-1080 cells [25]. In addition, Ito et al. [15] reported that WT1+/+ and WT1+/- isoforms inhibited apoptosis induced by apoptosis-inducing agents, etoposide and doxorubicin. The expression of proapoptotic Bak was decreased by the expression of WT1+/- isoform.

Conclusion

T-KTS+ and T-KTS- isoforms function in an anti-apoptotic role as well as WT1+/+ and WT1+/- isoforms in the breast cancer cell line (MCF-7). The present results will be the foundation for the development of new molecular-based diagnostic and therapeutic approaches in the management of breast cancer as well. However, further studies to address more deeply in the apoptosis pathway should be both important.

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The authors have no conflict of interest to report.

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