RESEARCH ARTICLE

Tumour Suppressive Effects of WEE1 Gene Silencing in Breast Cancer Cells

Naghmeh Ghiasi[1,3], Mojtaba Habibagahi[2], Rozita Rosli[3,4], Abbas Ghaderi[1,2], Khatijah Yusoff[4,5], Ahmad Hosseini[1], Syahrilnizam Abdullah[3], Mansooreh Jaberipour[1]*

Abstract

Background: WEE1 is a G2/M checkpoint regulator protein. Various studies have indicated that WEE1 could be a good target for cancer therapy. The main aim of this study was to asssess the tumor suppressive potential of WEE1 silencing in two different breast cancer cell lines, MCF7 which carries the wild-type p53 and MDA-MB468 which contains a mutant type. Materials and Methods: After WEE1 knockdown with specific shRNAs downstream effects on cell viability and cell cycle progression were determined using MTT and flow cytometry analyses, respectively. Real-time PCR and Western blotting were conducted to assess the effect of WEE1 inhibition on the expression of apoptotic (p53) and anti-apoptotic (Bcl2) factors and also a growth marker (VEGF). Results: The results showed that WEE1 inhibition could cause a significant decrease in the viability of both MCF7 and MDA-MB-468 breast cancer cell lines by more than 50%. Interestingly, DNA content assays showed a significant increase in apoptotic cells following WEE1 silencing. WEE1 inhibition also induced up-regulation of the apoptotic marker, p53, in breast cancer cells. A significant decrease in the expression of VEGF and Bcl-2 was observed following WEE1 inhibition in both cell lines. Conclusions: In concordance with previous studies, our data showed that WEE1 inhibition could induce G2 arrest abrogation and consequent cell death in breast cancer cells. Moreover, in this study, the observed interactions between the pro- and anti-apoptotic proteins and decrease in the angiogenesis marker expression confirm the susceptibility to apoptosis and validate the tumor suppressive effect of WEE1 inhibition in breast cancer cells. Interestingly, the levels of the sensitivity to WEE1 silencing in breast cancer cells, MCF7 and MDA-MB468, seem to be in concordance with the level of p53 expression.

Keywords: WEE1 - Breast cancer - shRNA - viability inhibition - G2 arrest abrogation

Asian Pac J Cancer Prev, **14 (11)**, 6605-6611

Introduction

Breast cancer is the most common malignancy among women affecting more than a million women per year worldwide (Hortobagyi et al., 2005). Conventional treatments, including chemotherapy and radiotherapy, have been used as front line anti-cancer strategies in past decades. However, those therapies are involved with toxic side effects, as they do not discriminate effectively between normal and tumor cells. In contrast, biological therapies targeting tumor cells focus on molecular and cellular changes/pathways that are relatively specific to cancer cells. Such approaches may block molecules involved in signal transduction, or inhibit tumor cell invasion or angiogenesis. Some others may target specific cell cycle components to stop tumor cell proliferation or push them toward apoptosis (Schlotter et al., 2008). The development of an effective targeted therapy requires the identification of a good target, which plays a key role in cancer cell growth and survival but less affected in normal cells.

Regulation of the cell cycle is frequently distorted in human cancer cells which leading to uncontrolled proliferation. When normal cells are subject to DNA damage, the checkpoints of cell cycle control induce a series of DNA repair mechanisms. Several studies have shown defective G1 checkpoint in many cancer cells, including breast cancer cells. Such cells depend to a great extent on the G2 checkpoint for DNA repair mechanisms (Dixon and Norbury, 2002; Foulkes et al., 2003). That is why abrogation of the G2 checkpoint could reduce the time needed to repair sub-lethal DNA damages, motivated premature mitotic entry and consequent apoptosis. In this way, targeting G2 repair pathway can be exploited as a

*[1]Shiraz Institute for Cancer Research, [2] Department of Immunology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran, [3]Genetic Medicine Research Centre, Faculty of Medicine and Health Sciences, [4]UPM-MAKNA Cancer Research Laboratory, Institute of Bioscience, [5]Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Selangor, Malaysia *For correspondence: jaberim@sums.ac.ir*

potential anti-cancer strategy in such tumor cells (Bucher and Britten, 2008).

WEE1, a member of the tyrosine kinase family, is a G2/M checkpoint regulatory protein that plays a crucial role in controlling the timing of mitosis under normal circumstances. Activated WEE1 causes an inhibitory phosphorylation of CDC2/CDK1 on Tyr15, which prevents cells from entering mitosis in order to provide time for DNA repair (Igarashi et al., 1991; Wang et al., 2004). It has been shown that WEE1 gene silencing causes G2 arrest abrogation, premature termination DNA repair and cell death in Glioblastoma (Mir et al., 2010). It was also reported that inhibition of WEE1 could improve the effects of radiotherapy and chemotherapy to induce DNA damage in different cancer cells (Bridges et al., 2011; Carrassa et al., 2012). Suppression of WEE1 in leukemia cell lines or ex-vivo derived leukemia cells also made the cells more sensitive to conventional drugs (Tibes et al., 2012). Recently it was shown that pushing breast cancer cells through G2 arrest, which results from inhibition or loss of WEE1, may allow DNA damage to accumulate and induce programmed cell death in breast cancer cells (Murrow et al., 2010).

Therefore, WEE1 could be a potential molecular target for breast cancer treatment. But the basis for this toxicity in different cell lines needs to be more clarified. Here, to do more investigation, we modeled to knockdown WEE1 with specific shRNAs and assessed the downstream effects in the two different breast cancer cells, MCF7, which carries the wild-type p53 and MDA-MB-468 which contains a mutant type.

Materials and Methods

Cell lines and Cell culture

MCF7 and MDA-MB-468 cell lines were purchased from the National Cell Bank of the Institute Pasteur of Iran. The cell lines were cultured in RPMI-1640 (Sigma-Aldrich, Honkong, China) medium supplemented with 10% fetal bovine serum and contained 100 unit/ml penicillin and 100 μg/ml streptomycin under standard conditions (37°C incubator with 95% humidified air and 5% CO_2).

shRNA plasmids and transfection

Plasmids encoding specific shRNA against WEE1 were purchased from Origene Company (Beijing, China). The sequence of each 29mer shRNA constructs and the target locations are listed in Table 1. In order to investigate the effect of WEE1 inhibition on breast cancer cells, MCF7 and MDA MB-468 cells were transfected with these plasmids separately and in combination (pooled). Briefly, 5×10^5 cells were transfected with 4μg of each WEE1 specific shRNA construct separately with 8μL of Lipofectamine 2000 (Invitrogen, Grand Island, New York, USA) in a 6 well plate according to the manufacturer's protocol. For transfection with pooled shRNAs, 1μg of each construct was used in combination.

Quantitative real-time PCR

Quantification of gene expression at the mRNA level was performed using quantitative real-time PCR (quantitative RT-PCR). Total RNA was extracted from transfected cells using TRIzol reagent at different time points (24, 48, and 72h) following transfection. Reverse transcription to cDNA was performed using Revert AidTM HMinus First Strand cDNA Synthesis Kit (Fermentas, Helsinki, Finland). Real-Time PCR was carried out to determine the expression levels of target genes using specifically designed primers and probes (Table 2). Each sample was normalized based on β-actin expression.

Table 1. The Sequence of Each 29mer shRNA Construct and Its Target Location

ShRNAs	29mer shRNA sequence	Target location
ShRNA1	GCTGGCGAACAAATGTAAACACGGAGATC	5'UTR
ShRNA 2	GCCAGTGTCCAGCCTAAGCACATCGGCTC	5'UTR
ShRNA 3	TGATGTGCGACAGACTCCTCAAGTGAATA	CDS
ShRNA 4	TGTTGCTTCATTCCTCAGGACAGTGTCGT	CDS

Table 2. Sequences of Primers and Probes Used

Gene		Sequence
β-actin	Forward	GGCGGCACCACCATGTACCC
	Reverse	GGAGGGGCCGGACTCGTCAT
	Probe	CGGCGGCTCCATCCTGGCCTCGC
WEE1	Forward	GGCTCTGTTGATGAGCAGAACGCTT
	Reverse	CTCAAGCCTCGGCGGCCAACTTGC
	Probe	TGCTCATGCAGTGCTTGGACAGCATTCTCATGT
VEGF	Forward	CACAGCCCGAGCCGGAGAG
	Reverse	CAGCCTGGGACCACTTGGCA
	Probe	GGCCCCGGTCGGGCCTCCGAAACCATG
Bcl-2	Forward	ACGGAGGCTGGGATGCCTTT
	Reverse	CAAGCTCCCACCAGGGCCAA
	Probe	TGTACGGCCCCAGCATGCGGCCTCTGT
MDM2	Forward	TCCTTTGATGAAAGCCTGGCTCTGT
	Reverse	TCACCTGAATGTTCACTTACACCAGCA
p53	Forward	GGCCCACTTCACCGTACTAA
	Reverse	GTGGTTTCAAGGCCAGATGT

Western blotting

Protein lysates from transfected (24, 48, 72 hours post-transfection) and untreated cells were prepared using RIPA lysis buffer (150 mMNaCl, 0.1% SDS, 50 nMTris pH 8.0, 1% TritonX-100, 0.1% DOC). Total cell lysate from each condition (40μg) was loaded onto 6-12% polyacrylamide SDS gels and subjected to electrophoresis. Molecular weight markers (Fermentas, Helsinki, Finland) were run on each gel for a size reference. Proteins were transferred onto PVDF membranes (BioRad, Hercules, California, USA), blocked and probed with primary antibody. After overnight incubation in 4ºC, the membrane was washed 3 times with wash buffer (PBS containing 0.05% Tween 20) and then probed with secondary antibody for 2 h in 4ºC. All primary and secondary antibodies were diluted in blocking buffer (1X PBS with 5 % skim milk) to the suggested concentrations in their datasheets. Protein bands were visualized using ECL (Thermo SIENTIFIC, Waltham, Massachusetts, USA) and XAR film (Kodak, Rochester, New York, USA). The following antibodies were used: β-actin (ab20272), WEE1 (ab37597), CDC2 (phospho Y15) (ab47594), VEGF (ab46154), p53 (ab1101), Bcl-2 (ab692), goat polyclonal secondary antibody to rabbit IgG-H&L (HRP-conjugated) (ab6721) and rabbit polyclonal secondary antibody to mouse IgG-H&L (HRP-conjugated) (ab6728).

Cell viability assay

The effect of WEE1 silencing on the viability of the breast cancer cell lines was determined by performing MTT assays. Cells (15×10^3) were seeded in 96 well plates overnight, transfected with 200ng of each shWEE1 plasmid using 0.4μl Lipofectamine™ 2000 per well according to the manufacturer's protocol. Media was replenished with 150μl of tetrazolium bromide solution (5 mg/ml in complete media) at 24, 48, 60 and 96h following transfection. Plates were incubated in the dark at room temperature for 4h and subsequently, the supernatant was removed and the crystal products were dissolved in 150μl DMSO (Merck, Darmstadt, Germany). After an overnight incubation in the dark at room temperature, colorimetric evaluation was performed using a spectrophotometer at 490 nm. All MTT measurements were done in replicates of 4 wells and each experiment was carried out in triplicate. The absorbance at each condition was compared with the control and the percentage of viable cells was calculated based on the following formula: ***% cell growth=(OD490 transfected/OD490 untreated)×100***

DNA content assay

Cells (5×10^5) were plated in 6 well plates overnight and then transfected with combination of 4 specific shRNA plasmids against WEE1 (pooled) and the control plasmid separately using 8μl Lipofectamine™ 2000 for each well. They were trypsinized and washed with cold PBS after 24, 48 and 60 h post-transfection. Then, 1 ml cold ethanol 75% was added to the cell plate drop by drop to fix them. The cells were incubated for10 min at -20ºC and centrifuged at 1000 rpm for 5 min, after which, the supernatant was aspirated. The cells were re-suspended in 500 μl cold PBS and 5 μl of RNaseA (Fermentas, Helsinki, Finland) was added to each tube then staining was performed with 5 μg/ml propidium iodide (PI) (P4170-100MG SIGMA). Analysis was done on the Becton Dickinson FACS Calibur using FLOWJO software.

Statistical analysis

The expression levels of target genes were determined from the ΔCt and $2^{-\Delta Ct}$ formulae. Data were analyzed with the Mann-Whitney U test to compare the means of cell viability percentages and also gene expression levels between transfected and untreated cells. All data are presented as mean±standard error. A p value of <0.05 was considered statistically significant.

Results

WEE1 gene silencing

The evaluated transfection efficiency using flow cytometry analysis was about 60% in MCF7 and MDA-MB-468. Quantitative RT-PCR and western blot analysis showed a reduction in WEE1 expression at both mRNA and protein levels, 24h post-transfection by WEE1 specific shRNAs (Figure 1). WEE1 gene expression showed 16%, 63%, 78%, 83% and 84% down regulation after shRNA1, 2, 3, 4 and pooled shRNAs transfection, respectively, in MCF7 cells compared with control-shRNA (Figure 1A). In addition, in MDA-MB-468 cells WEE1 transcript levels were reduced to 12%, 43%, 66%, 79% and 81% in the conditions, respectively, compared to the control (Figure 1B).

The expression level of phosphorylated CDC2 (p-CDC2) was considered to evaluate the efficiency of WEE1 silencing, since p-CDC2 is a major determinant of the DNA damage induced G2 arrest. In both breast cancer cells, WEE1 inhibition led to decreased level of p-CDC2 (Figure 5). This data suggests that both MCF7 and MDA-MB-468 cell lines could be sensitive to the downstream effects of WEE1 inhibition.

WEE1 inhibition decreased the viability of breast cancer cell lines

To investigate the effect of WEE1 silencing on the viability of breast cancer cells, MTT assay was performed. The shRNA 2, 3, 4 indicated significant inhibitory effects on cell viability in comparison with the control shRNA, 60 and 90 h post-transfection, in MCF7 (p≤0.02) and

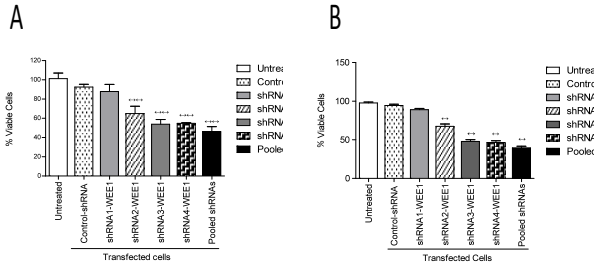

Figure 2. MTT Assay Analysis Following WEE1 Silencing in Breast Cancer Cells. A) Transfected MCF7 cells with shRNA 2, 3, 4 and pooled shRNAs show significant decrease (p≤0.02) in cell viability compared with control shRNA 60 h post-transfection; **B)** Transfected MDA-MB-468 cells with shRNA 2, 3, 4 and pooled shRNAs show significant decrease (p≤0.05) in cell viability compared with control shRNA, 90 h post-transfection. The data is presented as mean±SEM *p<0.05, **p<0.02

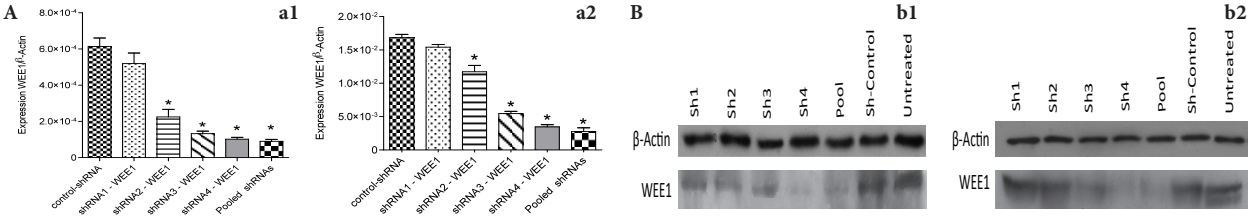

Figure 1. WEE1 Expression in Transfected Cells with Specific WEE1 shRNAs. A) Real time PCR analysis shows WEE1 expression decreased 24 h post-transfection in MCF7 (a1) and MDA-MB-468 (a2) cells; **B)** Western blot analysis also shows WEE1 efficiently silenced in MCF7 (b1) and MDA-MB-468 (b2) cell lines after transfection by different WEE1 shRNA constructs in comparison with control shRNA. The data is presented as mean±SEM *p<0.05

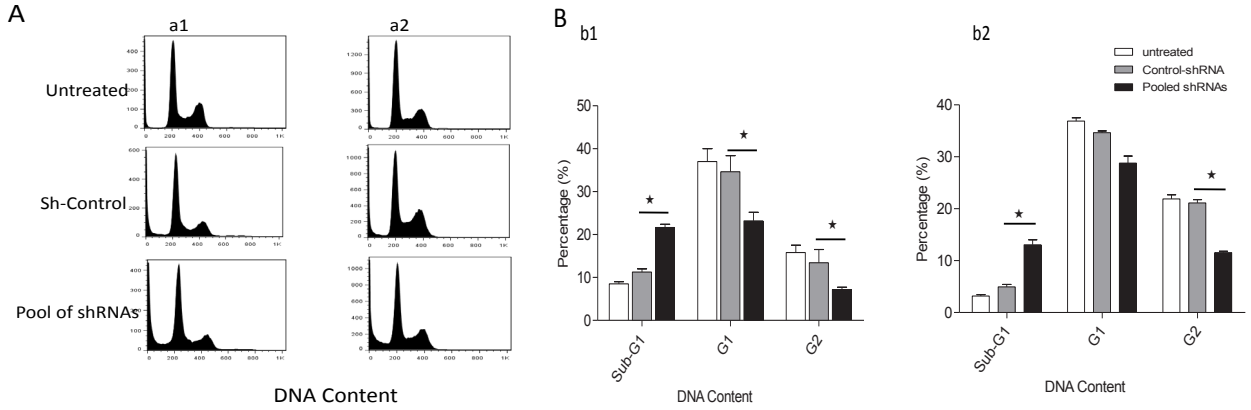

Figure 3. Cell cycle analysis after WEE1 silencing (A) DNA content histogram of MCF7 (a1) and MDA-MB-468 (a2) cells, 48 h and 60 h post-transfection, respectively. (B) The percentage of cells in sub-G1-, G1- and G2-phase of cell cycle in MCF7 (b1) and MDA-MB-468 cells (b2). DNA content assay shows WEE1 silencing caused a significant increase in the fraction of cells with sub-G1 DNA content (which could be the indicative of apoptosis) and abrogation of G2 arrest in both cell lines. The graph represents the mean values ± SEM of 3 independent experiments *p< 0.05

MDA-MB-468 cells (p≤0.05), respectively. The highest percentage of viability reduction was 50% upon WEE1 silencing using pooled shRNAs (Figure 2). WEE1 shRNA1 had no significant effect on viability of both breast cancer cell lines which probably was not functional.

WEE1 gene silencing induced cell-cycle arrest abrogation and cell death in breast cancer cells

As WEE1 is a gatekeeper of the DNA damage-induced G2 arrest which controls mitotic entry of the cells, we investigated the effect of WEE1 inhibition on the cell cycle profile of breast cancer cell lines, MCF7 and MDA-MB-468, using propidium iodide (PI) staining by FACS analysis (Figure 3A). Results summarized in Figure 3B show that the population of cells with sub-G1 DNA content (which is indicative of apoptosis) increased up to 2 fold 48 h and 60 h following WEE1 silencing in MCF7 and MDA-MB-468 cells, respectively. The 50% reduction in the fraction of cells in G2 phase, indicate that WEE1 silencing could significantly abrogate G2 arrest in both cell lines and push them toward premature mitosis.

WEE1 inhibition induced up-regulation of p53 (apoptotic marker) and down-regulation of Bcl-2 (anti-apoptotic marker)

p53 is a nuclear transcription factor which trans-activates many target genes involved in the cell cycle arrest induction or apoptosis (Chen et al., 1990). We investigated the effect of WEE1 inhibition on the expression of p53 at the both mRNA (Figure 4) and protein (Figure 5) levels in transfected MCF7 and MDA-MB-468 cells. The results showed that the expression level of p53 significantly increased in transfected cells after 48 h (p≤0.05). In transfected MCF7 cells, p53 mRNA level increased up to 23%, 38%, 61%, and 70% in shRNA 2, 3, 4 and pooled conditions, respectively, compared to the control (Figure 4, a1). The MDA-MB-468 cells showed 30%, 38%, and 41% increase in p53 mRNA level after shRNA 3, 4, and pooled transfection conditions, respectively, in comparison to control [Figure 4 (b1)]. ShRNA1 in both cell lines and shRNA 2 in MDA-MB-468 cells showed no significant effect on p53 transcripts level. These results indicate that

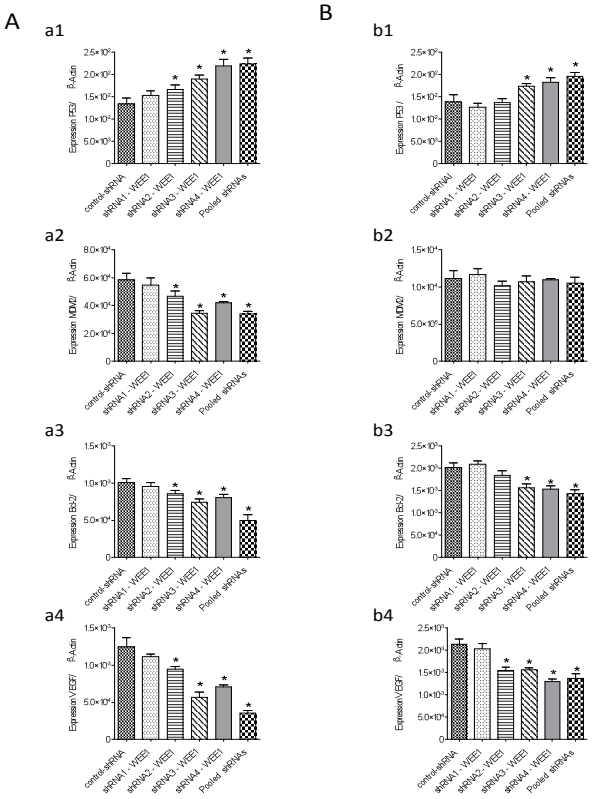

Figure 4. Real time PCR analysis 48 h post-transfection in MCF7 (A) and MDA-MB-468 (B) cell lines. The data is presented as mean±SEM *p< 0.05

WEE1 silencing could induce p53 accumulation, which could exert the pro-death function and eliminate cancer cells with serious DNA damage.

MDM2 mRNA level (the major cellular antagonist of p53) was determined using real time PCR in both cell lines 48 hours post-transfection. Transfected MCF7 cells with shRNA 2, 3, 4 and pooled of shRNAs showed 10%, 40%, 22% and 42% down regulation in MDM2 gene expression level, respectively, in comparison to the control [Figure 4 (a2)]. On the other hand, MDA-MB-468 cells did not show any significant change in MDM2 mRNA level post-transfection (Figure 4, b2).

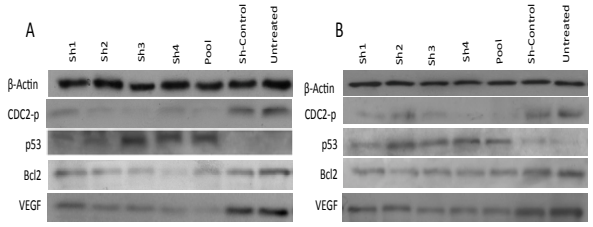

Figure 5. Western blot analysis 48 h post-transfection in MCF7 (A) and MDA-MB-468 (B) cell lines.

Bcl-2 is unique amongst pro-oncogenes. Increased expression of Bcl-2 as an anti-apoptotic protein has been reported in many tumors (Kelly and Strasser,2011). In the current study, Bcl-2 decreased at both mRNA (Figure4) and protein (Figure 5) levels in breast cancer cell lines 48 h following transfection with specific WEE1 shRNAs ($p \leq 0.05$). The gene expression level of Bcl-2 was down-regulated 18%, 30%, 22%, and 60% in transfected MCF7 cells using shRNA 2, 3, 4, and pooled, respectively, in comparison with control shRNA (Figure 4, a3). MDA-MB-468 cells showed 25%, 26%, and 32% reduction in Bcl-2 mRNA level after 48 h transfection with shRNA 3, 4, and pooled, respectively compared to control (Figure 4, b3). shRNA 2 in MDA-MB-468 cells and shRNA1 in the both cell lines, caused no significant change in the Bcl-2 transcript level.

Down-regulation of the angiogenesis marker, VEGF, upon WEE1 silencing

VEGF is the most important angiogenesis marker and known to be a powerful growth factor playing a central role in promoting tumor angiogenesis via activation of VEGFR (Hirakawa et al., 2005). When examined whether WEE1 inhibition could affect VEGF expression, a significant decrease in the expression of VEGF at both mRNA (Figure 4) and protein (Figure 5) levels was observed after 48 h transfection with specific shRNAs ($p \leq 0.05$). The VEGF transcript levels were reduced 10%, 50%, 30%, and 70% in shRNA 2, 3, 4, and pooled conditions, respectively, in transfected MCF7 cell line (Figure 4, a4). It was down-regulated by 26%, 25%, 40%, and 38%, respectively, in transfected MDA-MB-468 cells compared to the control (Figure 4, b4). shRNA1 in both cell lines, caused no significant change in the VEGF transcript level.

Discussion

In the present study; we investigated the effects of WEE1 silencing on breast cancer cell lines. In this regard, its effect on breast cancer cell's viability was first determined by MTT assay. The results showed WEE1 inhibition could significantly decrease the viability of the breast cancer cells. The observed viability reduction was more than 50 % upon WEE1 silencing using pooled shRNAs, 60 and 90 h post-transfection in MCF7 and MDA-MB-468, respectively, compared to the control shRNA (Figure 2). To investigate functional impact of WEE1 silencing on the cell cycle, we performed cell cycle DNA analysis following WEE1 silencing. As shown in Figure 3, WEE1 inhibition led to the abrogation of G2 arrest, and significant increase in cells with sub-G1 DNA content (which could be the indicative of apoptosis). Our results are consistent with previous studies which reported the sensitivity to WEE1 inhibition in different breast cancer cell lines (Iorns et al., 2009; Murrow, Garimella et al., 2010). Although they have shown pushing breast cancer cells through G2 arrest, which results from inhibition or loss of WEE1, may allow DNA damage to accumulate and induce programmed cell death in breast cancer cells, but they have not clarified the basis for this toxicity in different cell lines. To do more investigation, we also examined the effect of WEE1 inhibition on the expression of apoptotic (p53) and anti-apoptotic (Bcl-2) and also growth marker (VEGF), in the two different breast cancer cells, MCF7, which carries the wild-type p53 and MDA-MB-468 which contains a mutant type. The results of real-time PCR and western blotting analysis showed that the expression of p53 significantly increased at both mRNA (Figure 4a1, b1) and protein (Figure 5) levels. In a recent study done by Mir et al (2010), WEE1 inhibition in irradiated glioblastoma cells could abrogate G2-arrest and induce mitotic catastrophe and cell death. Mitotic catastrophe constitutes a special case of apoptosis and occurs in a p53-independent manner (Castedo et al., 2004). In our study, WEE1 inhibition induced G2 arrest abrogation and caused a significant increase in the fraction of sub-G1 cells (Figure 3, A and B). However, based on these results, the induced cell death in the breast cancer cells might be as a result of pushing breast cancer cells toward premature mitosis and induction of mitotic catastrophe, but the observed significant increase in p53 level following WEE1 inhibition in breast cancer cells should be also noted. The maximum level of this increase was 70% and 41% in MCF7 and MDA-MB-468, respectively. p53 has a crucial role in the maintenance of genomic integrity and in the response to cellular DNA damage accumulation, can stimulate apoptosis (Amundson et al., 1998). MDA-MB-468 cells, which carry a mutant type of p53, can undergo a change to the pseudo-normal wild-type species under certain circumstances (Prasad and Church,1997; Mandal et al., 2007). Therefore, MDA-MB-468 cell line was used to study the status of p53 following WEE1 gene silencing. In normal conditions, p53 is expressed at an extremely low level (Vousden and Lu,2002). Accumulation of DNA damage is a p53-activating signal which leads to a marked increase of active p53 protein to exert its pro-death function. This activation occurs mainly through post-translational mechanisms that cause an increase in the protein half-life of p53 (Lacroix et al., 2006). Time course experiments which done by Murrow et al (2010) suggested that WEE1 inhibition causes DNA damage followed by caspase activation. The observed increase in the amount of p53 in this study following WEE1 silencing could be explained through this effect of WEE1 inhibition on DNA damage induction. On the other hand, pro-death activity of p53 could be inhibited by increase transcription from MDM2 promoter; MDM2 protein can bind to p53 and inactivates it through enhancing the association of p53 with the proteasome (Lai et al., 2001). In the current study,

as shown in Figure 4 (a2 and b2), MDM2 mRNA levels decreased up to 40% in transfected MCF7 cells while no significant changes were observed in transfected MDA-MB-468 cells in comparison with the control.

Interactions between pro- and anti-apoptotic proteins are critical for susceptibility to apoptosis. The anti-apoptotic protein, Bcl-2, has been shown to effectively confer resistance to programmed cell death. High expression of Bcl-2 prevents cells from apoptosis caused by cellular stress and contributed to the DNA damage accumulation (Jiang and Milner, 2003). There are established evidences that activated p53 function has the capacity to repress the expression ratio of Bcl-2 (anti-apoptotic protein) and also to up-regulate the pro-apoptotic marker, Bax, in favor of apoptosis (Porebska et al., 2006). Our results indicated that Bcl-2 is down-regulated up to 60% and 32% in MCF7 and MDA-MB-468 cells, respectively, upon WEE1 silencing [Figure 4 (a3 and b3)]. These outcomes also can suggest that loss of WEE1 function might stimulate the pro-death activity of p53.

In the present study, we also assessed the effect of WEE1 inhibition on VEGF expression. Interestingly, real-time PCR (Figure 4) and western blotting analysis (Figure 5) showed a significant decrease in VEGF expression in both breast censer cells. The maximum level of this reduction was 70% and 38% in MCF7 and MDA-MB-468 cells, respectively [Figure 4 (a4 and b4)]. VEGF is a survival factor for tumor cells and plays a major role in the progression of different cancers, including breast cancer, by modulating tumor proliferation through its promotion of tumor angiogenesis. Different studies suggested a significant correlation between VEGF and p53 expression, they described a regulatory role for the tumor suppressor gene p53 in angiogenesis by down-regulating VEGF mRNA level, as well as VEGF promoter activity (Mukhopadhyay et al., 1995; Fujisawa et al., 2003). It has been reported that p53 has a central inhibitory role on the transcriptional regulation of VEGF. They suggested that p53 makes a complex with the transcription factor sp1 and thereby inhibits the transcriptional activation of VEGF promoter in mammary carcinoma (Pal et al., 2001). Based on these available data, the observed decrease in the VEGF transcript level may be as a result of the increased p53 protein expression following WEE1 inhibition. Notably, it has been reported that the absence of growth factors could be a major signal to trigger p53-dependent apoptosis following p53 activation and accumulation (Canman et al., 1995; Gottlieb and Oren,1998). However, additional studies will be necessary to clarify the exact signaling pathway.

Although in this study, the tumor suppressive effects of WEE1 inhibition was observed in both breast cancer cell line, but MDA-MB-468 cells showed a considerable delay in cell viability reduction after WEE1 inhibition and the levels of observed decrease in the Bcl-2 and VEGF expression after WEE1 inhibition was also lower in compare to MCF7.

It is important to note that p53 is haploinsufficient for tumor suppression. It is suggested that mere reduction in p53 dosage is sufficient to promote cancer formation (Gottlieb et al., 1997). In an interesting research, Vankatachalam et al. (2001) studied the p53 function for various parameter of growth control and stress response such as induction of G1 arrest or apoptosis, in p53+/+, p53+/-, p53-/- cells. They showed that although in p53-/- cells, growth control and stress response was completely eliminated, p53+/- cells showed the reduced parameters compared to their p53+/+ counterparts. They hypothesized that the reduced p53 dosage in p53+/- cells, provided a reduction in their ability to response to DNA damage (Venkatachalam et al., 2001). It could be suggested that, the lower p53 dosage might be expected to result in less efficient biological effects in MDA-MB-468 following WEE1 inhibition.

In the present study, we documented the inhibitory effects of WEE1 gene knockdown onto breast cancer cell lines using shRNAs against WEE1. Our results are in concordance with the previous studies which showed WEE1 inhibition could decrease breast cancer cell's viability; induce G2 arrest abrogation and an increase in apoptotic cells (sub-G1) following WEE1 silencing. Moreover, in this study, the observed interactions between the pro- and anti-apoptotic proteins and decrease in the angiogenesis marker expression confirm the susceptibility to cell death and validate the tumor suppressive effect of WEE1 inhibition in breast cancer cells. Although, the levels of the sensitivity to WEE1 silencing in breast cancer cells, MCF7 and MDA-MB-468, seem to be in concordance with the level of p53 expression.

Acknowledgements

This study was financially supported by the Novin Committee of Shiraz University of Medical Sciences, the Institute for Cancer Research (Grant No.ICR-100-505). The study is part of a PhD thesis project (N. Ghiasi) submitted to the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. N.Ghiasi, acknowledges the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia for support.

References

Amundson SA, Myers TG, Fornace AJ (1998). Roles for p53 in growth arrest and apoptosis: putting on the brakes after genotoxic stress. *Oncogene,* **17**, 3287-99.

Bridges KA, Hirai H, Buser CA, et al (2011). MK-1775, a novel Wee1 kinase inhibitor, radiosensitizes p53-defective human tumor cells. *Clin Cancer Res*, **17**, 5638-48.

Bucher N, Britten CD (2008). G2 checkpoint abrogation and checkpoint kinase-1 targeting in the treatment of cancer. *Br J Cancer*, **98**, 523-8.

Canman CE, Gilmer TM, Coutts SB, et al (1995). Growth factor modulation of p53-mediated growth arrest versus apoptosis. *Genes Dev,* **9**, 600-11.

Carrassa L, Chila R, Lupi M, et al (2012). Combined inhibition of Chk1 and Wee1: *in vitro* synergistic effect translates to tumor growth inhibition *in vivo*. *Cell Cycle,* **11**, 2507-17.

Castedo M, Perfettini JL, Roumier T, et al (2004). Mitotic catastrophe constitutes a special case of apoptosis whose suppression entails aneuploidy. *Oncogene*, **23**, 4362-70.

Chen PL, Chen YM, Bookstein R, et al (1990). Genetic mechanisms of tumor suppression by the human p53 gene. *Science*, **250**, 1576-80.

Dixon H, Norbury CJ (2002). Therapeutic exploitation of checkpoint defects in cancer cells lacking p53 function. *Cell Cycle*, **1**, 362-8.

Foulkes WD, Stefansson IM, Chappuis PO, et al (2003). Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. *J Natl Cancer Inst,* **95**, 1482-5.

Fujisawa T, Watanabe J, Kamata Y, et al (2003). VEGF expression and its reguration by p53 gene transfection in endometrial carcinoma cells. *Hum Cell*, **16**, 47-54.

Gottlieb E, Haffner R, King A, et al (1997). Transgenic mouse model for studying the transcriptional activity of the p53 protein: age- and tissue-dependent changes in radiation-induced activation during embryogenesis. *EMBO J*, **16**, 1381-90.

Gottlieb E, Oren M (1998). p53 facilitates pRb cleavage in IL-3-deprived cells: novel pro-apoptotic activity of p53. *EMBO J*, **17**, 3587-96.

Hirakawa S, Kodama S, Kunstfeld R, et al (2005). VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. *J Exp Med*, **201**, 1089-99.

Hortobagyi GN, de la Garza Salazar J, Pritchard K, et al (2005). The global breast cancer burden: variations in epidemiology and survival. *Clin Breast Cancer,* **6**, 391-401.

Igarashi M, Nagata A, Jinno S, et al (1991). Wee1(+)-like gene in human cells. *Nature*, **353**, 80-3.

Iorns E, Lord CJ, Grigoriadis A, et al (2009). Integrated functional, gene expression and genomic analysis for the identification of cancer targets. *PLoS One*, **4**, 5120.

Jiang M, Milner J (2003). Bcl-2 constitutively suppresses p53-dependent apoptosis in colorectal cancer cells. *Genes Dev,* **17**, 832-7.

Kelly PN, Strasser A (2011). The role of Bcl-2 and its pro-survival relatives in tumourigenesis and cancer therapy. *Cell Death Differ*, **18**, 1414-24.

Lacroix M, Toillon RA, Leclercq G (2006). p53 and breast cancer, an update. *Endocr Relat Cancer*, **13**, 293-325.

Lai Z, Ferry KV, Diamond MA, et al (2001). Human mdm2 mediates multiple mono-ubiquitination of p53 by a mechanism requiring enzyme isomerization. *J Biol Chem*, **276**, 31357-67.

Mandal S, Berube G, Asselin E, et al (2007). A new platinum complex of triazine demonstrates G1 arrest with novel biological profile in human breast cancer cell line, MDA-MB-468. *Bioorg Med Chem Lett*, **17**, 2139-45.

Mir SE, De Witt Hamer PC, Krawczyk PM, et al (2010). In silico analysis of kinase expression identifies WEE1 as a gatekeeper against mitotic catastrophe in glioblastoma. *Cancer Cell*, **18**, 244-57.

Mukhopadhyay D, Tsiokas L, Sukhatme VP (1995). Wild-type p53 and v-Src exert opposing influences on human vascular endothelial growth factor gene expression. *Cancer Res*, **55**, 6161-5.

Murrow LM, Garimella SV, Jones TL, et al (2010). Identification of WEE1 as a potential molecular target in cancer cells by RNAi screening of the human tyrosine kinome. *Breast Cancer Res Treat* **122**, 347-57.

Pal S, Datta K, Mukhopadhyay D (2001). Central role of p53 on regulation of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) expression in mammary carcinoma. *Cancer Res*, **61**, 6952-7.

Porebska I, Wyrodek E, Kosacka M, et al (2006). Apoptotic markers p53, Bcl-2 and Bax in primary lung cancer. *In Vivo*, **20**, 599-604.

Prasad KA, Church JG (1997). EGF effects on p53 in MDA-468 human breast cancer cells: implications for G1 arrest. *Cell Prolif,* **30**, 81-94.

Schlotter CM, Vogt U, Allgayer H, et al (2008). Molecular targeted therapies for breast cancer treatment. *Breast Cancer Res*, **10**, 211.

Tibes R, Bogenberger JM, Chaudhuri L, et al (2012). RNAi screening of the kinome with cytarabine in leukemias. *Blood*, **119**, 2863-72.

Venkatachalam S, Tyner SD, Pickering CR, et al (2001). Is p53 haploinsufficient for tumor suppression? Implications for the p53+/- mouse model in carcinogenicity testing. *Toxicol Pathol*, **29**, 147-54.

Vousden KH, Lu X (2002). Live or let die: the cell's response to p53. *Nat Rev Cancer,* **2**, 594-604.

Wang Y, Decker SJ, Sebolt-Leopold J (2004). Knockdown of Chk1, Wee1 and Myt1 by RNA interference abrogates G2 checkpoint and induces apoptosis. *Cancer Biol Ther,* **3**, 305-13.