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WT1 silencing by RNAi synergizes with chemotherapeutic agents and induces chemosensitization to doxorubicin and cisplatin in B16F10 murine melanoma cells

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Abstract. The Wilm's tumor gene (WT1), encoding a transcription factor that modulates the expression of certain genes that are involved in proliferation and apoptosis, is overexpressed in numerous solid tumors. WT1 is important for cell proliferation and in the diagnosis of melanoma. The objectives of this study were to investigate whether WT1 silencing is capable of synergizing with chemotherapeutic agents and whether this silencing is capable of sensitizing cancer cells to doxorubicin and cisplatin in the B16F10 murine melanoma cell line. In the present study, B16F10 cells were simultaneously treated with median lethal doses (LD50s) of WT1-1 or WT1-2 small hairpin RNAs (shRNAs) and chemotherapeutic agents. A total of 24 h post-transfection, a [3-(4,5-dimethylthiazol-2yl)-2,5- diphenyl tetrazolium bromide assay] MTT assay was performed. To determine whether shRNA interference (shRNAi) is capable of sensitizing B16F10 cells to chemotherapeutic agents, cells were transfected with an LD50 of each of the recombinant plasmids, treated with varying concentrations of doxorubicin or cisplatin 24 h post-transfection, and analyzed 48 h later for inhibition of cell proliferation using the MTT assay. We observed that WT1-RNAi and the two chemotherapeutic agents acted synergistically to inhibit B16F10 cell proliferation. The greatest inhibition of cell proliferation was observed with the WT1-2/cisplatin (91%) and WT1-1/cisplatin combinations (85%). WT1 silencing using shRNAi induced the chemosensitization of cells to doxorubicin and cisplatin, with the greatest inhibition (85%) of cell proliferation being observed in the cells treated with the WT1-2/cisplatin 6 ng/μl combination. Our results provide direct evidence that WT1 gene silencing has a synergistic effect with chemotherapeutic drugs and sensitizes B16F10 melanoma cells to doxorubicin and cisplatin. This suggests that these combination strategies are potentially utilized in melanoma therapy.

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Introduction

The incidence of cutaneous melanoma is on the increase worldwide. Despite advances in diagnosis and conventional treatment, mortality remains high. Melanoma prognosis is poor once the tumor has metastasized, since no curative therapy exists (1,2). Numerous patients with melanoma are resistant to chemotherapy and do not respond to treatment. Therefore, new treatment alternatives are urgently required to reduce mortality in these patients (3). An alternative to conventional therapeutic strategies is the silencing of genes involved in the neoplastic processes, including genes involved in apoptosis, proliferation and angiogenesis. The silencing of genes has been used for therapeutic purposes as a single agent and to sensitize tumor cells to conventional chemotherapy (4). The RNA interference (RNAi) pathway is widely used for manipulating biological systems, and is a potent and specific pathway involved in post-transcriptional gene silencing. Thus, it is a promising pathway target for the development of tailored therapies (5,6).

The Wilm's tumor gene (WT1) modulates the expression of several genes involved in cell proliferation [e.g., cyclin D1 (7)] and in apoptosis [e.g., p21 (8) and Bcl-2 (9,10)]. In addition, it physically interacts with the p53 and Par-4, genes that are involved in apoptosis (11). These interactions make WT1 a good target for gene therapy and for the sensitization of tumor cells to chemotherapy (5).

WT1 encodes a transcription factor with zinc finger motifs, which is involved in gonadal development, sexual differentiation, cell proliferation and apoptosis (12,13). WT1

is considered an oncogene rather than a tumor suppressor gene (14) because its high expression levels are associated with the development and progression of a number of neoplasias, such as leukemia, as well as solid tumors, including mesothelioma (15), breast (16), colon (17), ovarian (18) and lung (19) cancer, and melanoma (20). High WT1 mRNA expression levels have been correlated with a biologically aggressive phenotype that has been associated with a poor prognosis for leukemia (21) and sarcoma (22). Leukemia patients who had high levels of WT1 mRNA had increased drug resistance and worse overall survival than patients who had low levels of WT1 mRNA (21,23).

Certain studies indicate that WT1 is a relatively reliable marker of malignancy in melanocytic lesions. Wilsher *et al* (20) demonstrated that WT1 expression is present in the majority of invasive primary cutaneous and metastatic melanomas. *In vitro*, WT1 wild-type transcripts have been detected in human melanoma cell lines but not in normal melanocyte lines. *In vivo*, WT1 expression clearly discriminates benign acquired nevi from malignant melanomas and appears to be correlated with melanocytic atypia and malignancy (24). Results of previous studies showed that WT1 expression is directly involved in melanoma cell proliferation (5,25).

RNAi technology has been used as a strategy in a number of studies in melanoma cell lines to silence genes involved in tumor progression and metastasis, including C-MYC (26) and WT1 (27). WT1 silencing significantly reduced the expression of Nestin and Zyxin genes, inhibited cell proliferation (24) and induced apoptosis by caspase-3 and poly-ADP-ribose polymerase activation (5).

In this investigation, a B16F10 murine melanoma cell line was used since this cell line is considered to be a good model for melanoma lung metastasis. Our proposal is based on the results obtained from this investigation using an animal model of melanoma and the relevant therapeutic strategy (28-30).

The purpose of this study was to investigate whether WT1 silencing by RNAi exhibited a synergistic effect with chemotherapeutic drugs, such as cisplatin and doxorubicin, and whether WT1 silencing is capable of sensitizing cancer cells to these agents in the B16F10 murine melanoma cell line.

Materials and methods

Cell line and cell culture. The B16F10 murine melanoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured and maintained in Dulbecco's modified Eagle's medium (DMEMF-12) (Life Technologies, Invitrogen, Burlington, Ontario, Canada), supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 g/ml of streptomycin (Gibco, Grand Island, NY, USA). Cells were incubated in a humidified chamber at 37˚C in a 95% O_2 and 5% CO_2 atmosphere.

WT1-RNAi expression constructs. The WT1-1 and WT1-2 DNA vectors used to produce RNAi by expressing small hairpin RNA (shRNA) have been previously described by Zamora-Avila *et al* (5). The constructs were tested and produced on a large scale and purified with the Endo free plasmid Giga kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

RNAi and drugs screening to median lethal dose (LD50)%. The [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide assay] MTT assay was performed to determine the LD50s of the recombinant plasmids and chemotherapeutic agents, cisplatin and doxorubicin. Cell survival was determined using the MTT cell proliferation assay (Sigma, St. Louis, MO, USA). Briefly, 24 h prior to transfection, the B16F10 cells were seeded at $3x10^3$ cells/well on a 96-well plate. To determine LD50s, cells were transfected with different concentrations of WT1-1 or WT1-2 recombinant plasmids or doses of drugs. Cells were transfected with 0.05 μg of pEGFP-N2 plasmid as a negative control (Clontech, Palo Alto, CA, USA). B16F10 cells were transfected with WT1-1, WT1-2 and pEGFP-N2 plasmids (Clontech) using 25 kDa of cationic polymer branched polyethylenimine (PEI). The complex PEI: DNA was performed as described by Zamora-Avila *et al* (27). Following 72 h, a MTT assay was performed. The MTT solution was created by mixing 0.025 g of MTT (Sigma) with 5 ml of phosphate-buffered saline (PBS) for a final MTT concentration of 5 mg/ml, and 200 μl of this solution was added to each well. The cells were then incubated with the MTT solution at 37˚C for 1 h, followed by removal of the medium, addition of 100 ml dimethylsulfoxide to each well, and rocking of the samples for 10 min. The optical density (OD) was determined at 570 nm using a microplate reader (Microplate Autoreader EL311, BioTek Instruments Inc., Winooski, VA, USA). Data are presented as the percentage viability ± the standard error.

Synergistic effect and sensitization to drugs. To determine whether the recombinant plasmids (WT1-1 and WT1-2) synergize with chemotherapeutic drugs to inhibit cell proliferation, the B16F10 cells were seeded as described above, transfected with the LD50 of the recombinant plasmids and simultaneously treated with the LD50 of the chemotherapeutic drugs. As a control, B16F10 cells, which received chemotherapy alone also received the empty vector. Twenty-four hours following treatment, the MTT assay was performed to determine cell viability as previously mentioned.

To determine whether the recombinant plasmids (WT1-1 and WT1-2) are capable of sensitizing the B16F10 cancer cells to chemotherapeutic drugs, B16F10 cells were seeded as described above, followed by transfection 24 h later with the LD50 of the recombinant plasmids, and treated with various doses of the drugs 24 h post-transfection. Cell viability was then determined 24 h later using the MTT assay.

WT1 protein expression. To analyze WT1 protein expression, B16F10 cells were plated at $1x10^6$ cells/25 cm^2 flask in 5 ml DMEMF-12 supplemented with 10% FBS, and incubated overnight. The cells were harvested, lysed in 100 μl of lysis buffer (1% Triton, 150 mmol/l NaCl, 25 mmol/l Tris pH 7.6), and protein concentrations were measured using the DC Protein Assay kit (Bio-Rad, Hercules, CA, USA). Approximately 50 μg of protein from each sample was separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by western blotting with WT1-C19 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Samples were normalized using anti-β-actin antibody (Sigma). Proteins were visualized using the Lumi-Light western blotting system (Roche, Pleasanton, CA, USA).

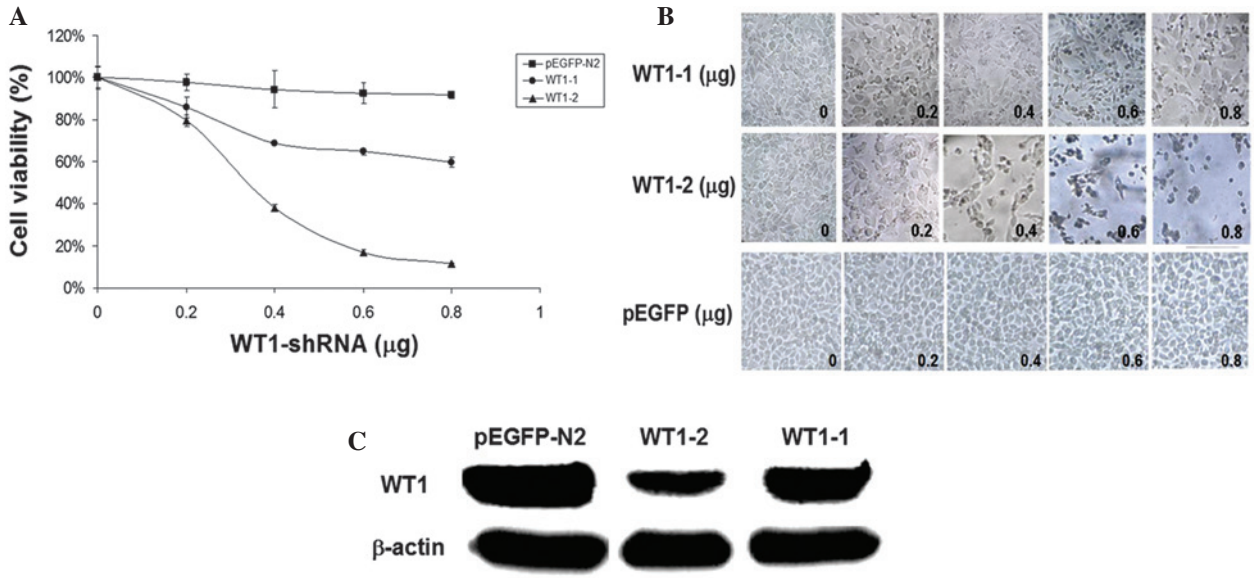

Figure 1. Silencing of WT1 by RNAi results in inhibition of cell proliferation in the B16F10 cancer cell line. (A) MTT cell growth assay. Transfection with WT1-1 and WT1-2 recombinant plasmids results in an inhibition of cell proliferation in a dose-dependent manner. Values are the means of the average cell viability for three independent experiments ± standard deviation (SD) (*P<0.01). (B) Reduction in the number of B16F10 cells treated with WT1-1 and WT1-2 for 3 days as observed by light microscopy. (C) Downregulation of WT1 protein analyzed by western blotting using the WT1-1 and WT1-2 RNAi construct, compared with the pEGFP-N2 control plasmid. MTT, 3-(4, 5-dimethylthiazol-2yl)-2,5- diphenyl tetrazolium bromide assay; RNAi, RNA interference; shRNA, small hairpin RNA; WT1, Wilm's tumor gene.

Statistical analysis. Experiments were performed in triplicate and statistical analysis was performed using one-way analysis of variance. Differences were considered significant at P<0.01.

Results

Inhibition of WT1 with RNAi. It was previously reported that the B16F10 melanoma cell line has high WT1 expression and that downregulation of this protein by RNAi is capable of inhibiting its growth [Zamora-Avila *et al* (27)]. To determine the LD50s to WT1-1 and WT1-2, we used a MTT assay and western blot analysis to characterize the effect of the downregulation of WT1 expression in the B16F10 cell line. The inhibition of cell proliferation was dose-dependent in B16F10 cells treated with the two RNAi expression plasmids (Fig. 1A); however, the two constructs had different LD50s when analyzed by MTT assay and light microscopy (Fig. 1A and B). The LD50% for WT1-1 was 0.8 μg, while the LD50% for WT1-2 was 0.35 μg. Down-modulation of the WT1 protein was observed in the WT1-2 RNAi transfected cells but not in those transfected with the control plasmid (Fig. 1C).

Synergistic effect of the combination of WT1 RNAi/doxorubicin and cisplatin. WT1 has been associated with malignancy and melanoma proliferation and modulates the expression of several genes that are involved in apoptosis. For this reason, we investigated the feasibility of a combined treatment of B16F10 melanoma cells with WT1 shRNA and doxorubicin or cisplatin. The B16F10 cells were simultaneously transfected with the LD50 of WT1-1 or WT1-2 and treated with the LD50 of doxorubicin (100 pg/μl) or cisplatin (12 ng/μl). Forty-eight hours later, we determined the inhibition of cell proliferation. WT1-2/cisplatin and WT1-2/doxorubicin combinations had the greatest effect with 91 and 82% inhibition of cell proliferation, respectively, whereas theWT1-1/cisplatin and WT1-1/doxorubicin combinations yielded 85 and 69% inhibition of cell proliferation, respectively (Fig. 2). However, contrary to expectations, the combination of shRNA WT1-1/WT1-2 increased cell proliferation. These results demonstrate a synergistic effect on the inhibition of cell proliferation when cells are transfected with WT1-RNAi and the chemotherapeutic agents, doxorubicin or cisplatin.

WT1 silencing sensitizes B16F10 cells to doxorubicin and cisplatin. To determine whether WT1 RNAi sensitizes the melanoma cell line to doxorubicin and cisplatin, we treated B16F10 cells with the LD50s of WT1-1 and WT1-2 and added various concentrations of doxorubicin or cisplatin 24 h following transfection, followed by determination of the inhibition of cell proliferation 48 h later. The greatest chemosensitization was observed in the cells treated with the WT1-2/cisplatin combination. Treatment with the WT1-2/cisplatin combination increased the cell death percentage from 20% in cells treated with cisplatin alone to 80% using a WT1-2/0.6 ng/μl cisplatin dose. Cells treated with the WT1-2/doxorubicin combination at a concentration of 100 pg/μl resulted in 60% inhibition compared to 38% inhibition in cells treated with doxorubicin alone (Fig. 3). These results indicate that WT1 gene silencing by RNAi sensitizes cancer cells to chemotherapeutic agents, such as doxorubicin and cisplatin, increasing the cytotoxicity rates beyond those obtained with the drugs alone.

Discussion

The balance between life and death is important in tissue homeostasis. The majority of the mechanisms that regulate cell proliferation and homeostasis are linked to apoptosis. Tumor cells often express a variety of genes that lead to uncontrolled

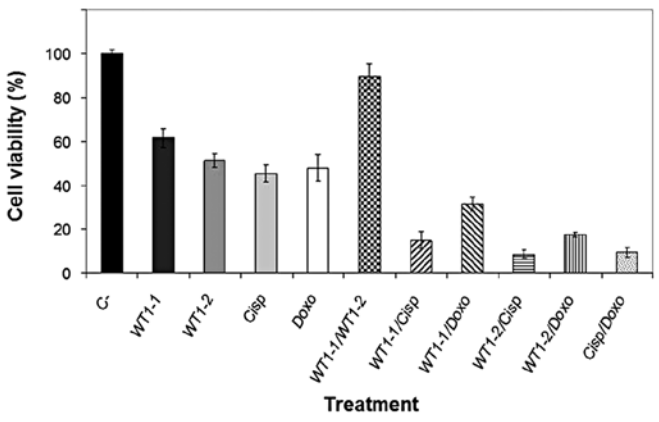

Figure 2. Synergistic effect between WT1 gene silencing and chemotherapeutic agents. Cells were simultaneously treated with a LD50 of recombinant plasmids (WT1-1, 0.8 μg and for WT1-2, 0.35 μg) and a LD50 of the drugs (doxorubicin, 100 pg/μl and cisplatin, 12 ng/μl). Cell proliferation was determined 48 h following treatment by a MTT assay. The graph shows the cell viability percentages for the various combinations tested. Values are presented as the means of the average cell viability for three independent experiments ± standard deviation (SD) (*P<0.01). WT1, Wilm's tumor gene; LD50, median lethal dose; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5- diphenyl tetrazolium bromide assay.

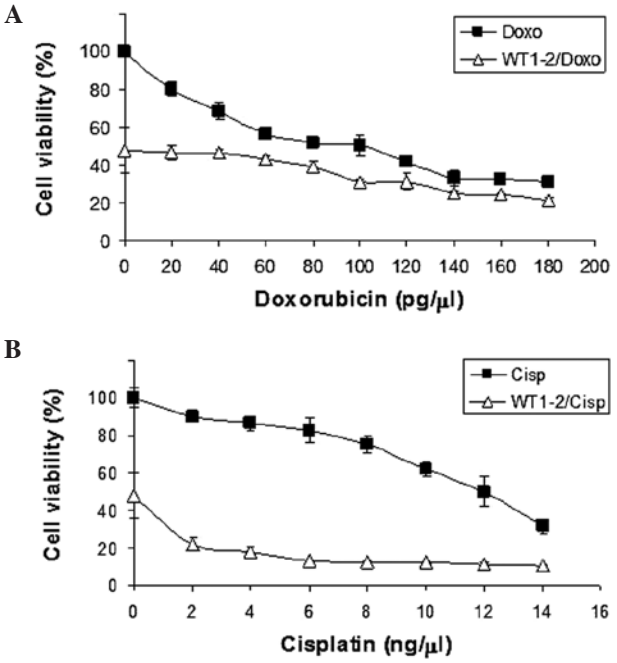

Figure 3. WT1 gene silencing sensitizes B16F10 cells to doxorubicin and cisplatin. The B16F10 cell line was transfected with the recombinant plasmids. A total of 24 h following transfection, various concentrations of doxorubicin or cisplatin were added, followed by determination of the cell viability by the MTT assay 48 h later. (A) Cells treated with WT1-2/doxorubicin induced greater cell inhibition (60%) compared to doxorubicin alone (38%). (B) Cells treated with WT1-2/cisplatin vs. cisplatin alone resulted in an increase in the mortality percentage from 20 to 80%. Values are presented as the means of the average cell viability for three independent experiments ± standard deviation (SD) (*P<0.01). WT1, Wilm's tumor gene.

cell growth or block the apoptotic process. Cytotoxic therapy is used to induce apoptosis in the treatment of a large majority of tumors. One anticancer strategy is to block the expression of genes involved in cell proliferation. The antisense oligonucleotides or RNAi are promising tools for use in cancer therapy. The antisense elements have been used with excellent results to silence various genes involved in cell proliferation, including PCDGF (31) and IGFR-1 (32), as well as genes involved in apoptosis, including Bcl-2 (33). RNAi is a powerful tool in gene therapy technology that has been widely used in recent years (34,35). In this study, we used RNAi technology to silence WT1 gene expression and inhibit cell proliferation to investigate whether its silencing synergizes with chemotherapeutic drugs in the induction of cell death, and also whether its silencing sensitizes cells to chemotherapy.

Mayo *et al* reported that WT1 induced apoptosis resistance, which may be a result of increases in Bcl-2 and the ability of WT1 to inhibit p53 tumor suppressor function (10). WT1 suppresses apoptosis in response to vincristine or doxorubicin *in vitro* by transcriptionally upregulating the Bcl-2 proto-oncogene. Bcl-2 overexpression reduces basic apoptosis and sensitivity of melanoma cells to proapoptotic stimuli. Proapoptotic Bcl-2 proteins may also be upregulated in the course of chemotherapy (36).

Our results, as well as those of other authors (15-20), indicate that WT1 is important in cell proliferation in a large number of tumors. In addition, WT1 downregulation results in the inhibition of cell proliferation and apoptosis induction by caspase 3 expression and PARP cleavage, suggesting that WY1 plays a distinct role in B16F10 melanoma growth (5,37).

In certain studies, antisense oligonucleotides and RNAi have been used in combination with conventional chemotherapy to determine whether they synergize or sensitize cells to chemotherapeutic agents (38-40). In the present study, the use of WT1-RNAi, combined with doxorubicin and cisplatin, induced a synergistic effect on the inhibition of cell proliferation; similar results have been observed *in vitro* by other investigators with different antisense oligonucleotides and siRNA in different cell lines (31,38,39). Moulder *et al* reported that the combination of a Bcl-2-specific antisense oligodeoxyribonucleotide (G3139) with doxorubicin and docetaxel is feasible but has limited efficacy in patients with LABC, most likely due to the inability of G3139 to induce a biologically meaningful downregulation of Bcl-2 levels in primary breast tumors in a Phase I/II study (33). The combination therapy of asODNs and cytotoxic drugs has several potential advantages, including the ability to use lower doses of chemotherapeutic drugs, fewer side effects on normal cells and loss of chemoresistance.

In conclusion, the results of this study provide direct evidence that WT1 gene silencing through RNAi, in combination with cisplatin and doxorubicin, has a synergistic effect and sensitizes cells to chemotherapy. WT1 is potentially a powerful therapeutic target for the gene therapy of melanoma with the use of effective drug delivery systems. Moreover, WT1 gene silencing by RNAi may be of significant therapeutic value in combination with existing cytotoxic agents.

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