Down-regulation of WT1/+17AA gene expression using RNAi and modulating leukemia cell chemotherapy resistance

We have shown that inhibition of WT1/+17AA protein expression following transfection with a vector-based small interfering RNA expression construct in K562 cell lines, leads to a decrease in MDR1 and P-glycoprotein levels, accumulation of Rh123, and enhancement of the doxorubicin cytotoxicity. Our findings suggest that WT1/+17AA exerts its oncogenic function by modulating multidrug resistance in leukemia cells.

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The Wilms' tumor gene (WT1) is now thought to be involved in the pathogenesis of leukemia as well as a wide variety of solid cancers. The WT1 gene is alternatively spliced at two main regions, exon 5 (17 amino acids: 17AA) and the last three codons of exon 9 (KTS), yielding four isoforms: (+17AA/+KTS), (+17AA/-KTS), (-17AA/+KTS), and (-17AA/-KTS).[1] All four WT1 isoforms are expressed in primary leukemia cells and each is considered to have a different function in interfering with signaling in cell differentiation, apoptotic and drug resistance pathways. However, the functions of each WT1 isoform in leukemia cells remains controversial. Recently, the co-expression of WT1 and the multidrug resistance related gene (MDR1) has been shown *in vitro* and *in vivo*.[2] The promoter region of MDR1 has been carefully mapped and an EGR1/SP1/WT1 site at positions -69 to -41 has been identified.[3] It is clear that the SP1/EGR1/WT1 site is a key regulatory region for MDR1 transcription, suggesting that WT1 is a potential modulator for leukemia cells' multidrug resistance.

In this study, a vector-based shRNA expression system was used (Figure 1A). We selected the target region in exon 5 of WT1/+17AA cDNA and designed WT1/+17AA ShRNA and nonspecific control ShRNA oligonucleotides according to Tushul's principles. Recombinant plasmids were transfected with the LIPOFECTAMINETM2000 transfection reagent into two paired erythroleukemia cell lines: K562 and K562/A02 (sensitive and resistant to doxorubicin, respectively). WT1/+17AA, WT1/-17AA, MDR1 and β2-MG genes were determined by quantitative real-time PCR using the SYBR green I dye. The primer pairs were designed using the Primer Premier software (version 5.0): WT1/+17AA forward primer 5'-AAAGGGAGTTGCTGCTGG-3', reverse primer 5'-TGCCGACCGTACAAGAGT-3'(202bp for the product) WT1/-17AA forward primer 5'-CACCTTAAAGGGCCACAG-3', reverse primer 5'-TGCCGACCGTACAAGAGT-3'(157 bp for the product) MDR1 forward primer5'-CCCATCATTGCAATAGCA-3', reverse primer 5'-GTTCAAACTTCTGCTCCTGA-3', (157 bp for the product) β2-MG forward primer 5'-CTCGCGCTACTCTCTCTTTC-3' reverse primer 5'-CATGTCTCGATCCCACTTAAC-3', (330 bp for product).

The products of WT1/+17AA, WT1/-17AA, MDR1 and β2-MG were ligated into pUCm-T vectors and serially 10-fold diluted in water with 1 to 10^8 copies to be a positive template. The normalized WT1 isoform expression levels (WT1/+17AAN and WT1/-17AAN) and the MDR1 expression level (MDR1N) were represented as a ratio of the WT1 isoform's copy number and MDR1 products to the control β2-MG product, determined by real-time PCR for each RNA sample. It has been suggested that the protein expression of WT1/+17AA and WT1/-17AA was measured using western blotting. The expression of P-gp was detected by flow cytometric analysis using fluorescein isothiocyanate-conjugated anti-P-gp.[4] P-gp function was examined for uptake, efflux and accumulation of Rh123. Doxorubicin cytotoxicity was determined by tetrazolium (MTT) assay. Normalized WT1 isoform WT1/+17AAN expression level in K562 and K562/A02 cells reduced rapidly from 182.45±42.32 to 92.77±27.38 (p<0.005, n=9) and from 243.77±56.93 to101.81±37.51 (p<0.001, n=9) respectively. The WT1/-17AAN expression remained relatively constant throughout (p>0.05, n=9). The ratios of

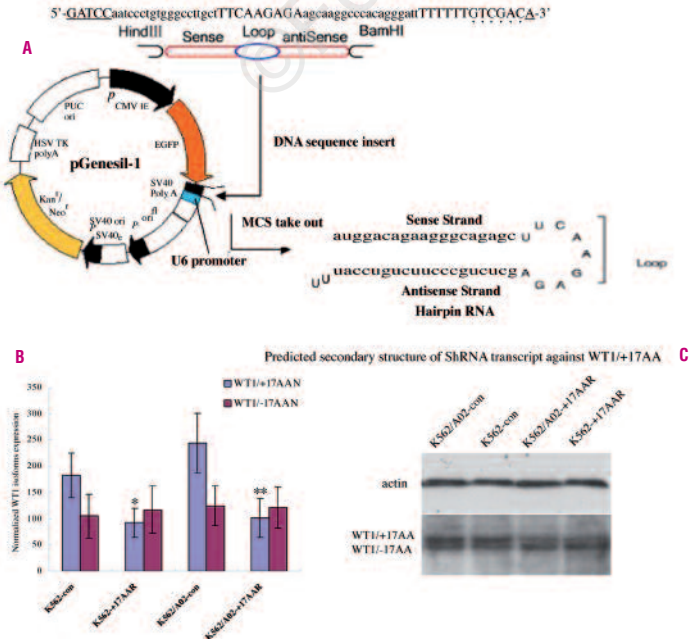

Figure 1. Down-regulation of WT1 exon 5 transcript expression level following treatment with WT1/+17AA ShRNA. **A.** Schematic presentation of U6 promoter-based ShRNA expression vector. Sequences encoding ShRNA with 19-nt of homology to the target gene are synthesized as 65 bp double-stranded DNA oligonucleotides and inserted downstream of U6 promoter. **B.** WT1 isoforms mRNA expression was determined by real-time RT-PCR using SYBR green I dye. It was showed that the expression levels of WT1/+17AAN in K562-+17AAR and K562/A02-+17AAR were statistically lower than that in K562-con (*p<0.005) and K562/A02-con (**p<0.001) controls, respectively. **C.** WT1 isoform protein expressions were detected by Western blotting using monoclonal anti-WT1 antibody and horseradish peroxidase-conjugated goat anti-mouse secondary antibody. When compared with K562-con and K562/A02-con, there was significant reduction of WT1/+17AA isoform production in both K562-+17AAR (p<0.005) and K562/A02-+17AAR (p<0.001), while there was no significant change in WT1/-17AA expression (p>0.05). There was no difference in actin production among the these groups (p=0.453).

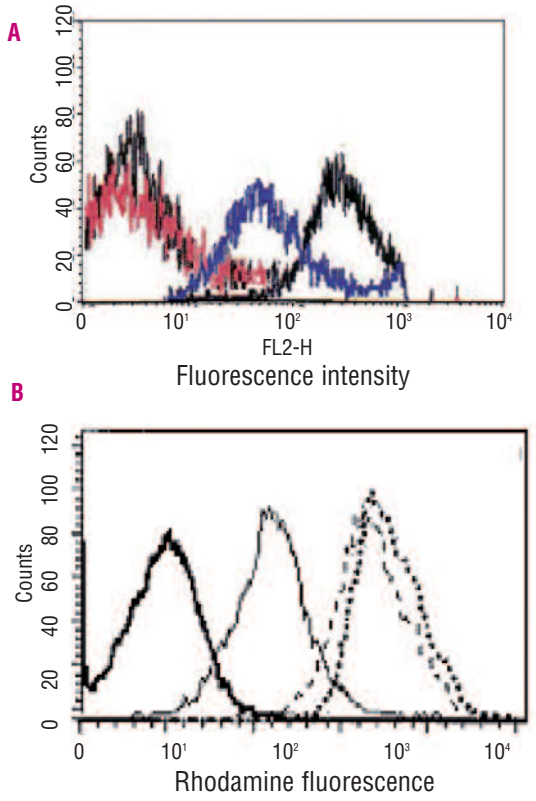

Figure 2. Representative flow cytometric histograms show the effect of WT1/+17AA ShRNA on the levels of cell surface P-glycoprotein expression and P-glycoprotein mediated rhodamine 123 efflux in both K562 and K562/A02 cells. A. P-glycoprotein is present at very high levels in drug-resistance K562/A02-con cells (53.92) when compared with parental drug-sensitive K562-con cells (4.78) (dark lines). The expression rates of P-gp in K562/A02-+17AAR cells (21.47, blue line) decreased obviously compared to K562/A02-con cells, whereas K562-+17AAR cells (4.54, red line) showed similar results with the K562-con cells. B. The uptake processes in K562 cells were unaffected by WT1/+17AA ShRNA transfection, the K562/+17AAR cells (dashed line) displayed uptake kinetics similar to that of the parental K562-con cells (dotted line), whereas Rh123 uptake was dramatically altered upon transfection with WT1/+17AA ShRNA in K562/A02 cells, the K562/A02-+17AAR cells (solid line) displayed rates of Rh123 accumulation between those of K562-con and K562/A02-con (heavy solid line).

WT1/+17AA and WT1/-17AA in paired cell lines were rapidly reduced, dropping from 1.71±0.37 and 1.90±0.41 to 0.77±0.21 and 0.82±0.30 (Figure 1B). Compared with control cells, the WT1/+17AA isoform protein expression significantly decreased in WT1/+17AA ShRNA transfected K562-+17AAR (p<0.005, n=6) and K562/A02-+17AAR (p<0.001, n=6). There was no change in the WT1/-17AA expression in these two cell lines (p>0.05) (Figure 1C). The MDR1 gene expressions were quantified at 8.72±2.93 and 272.54±42.73 in the control ShRNA transfected K562-con and K562/A02-con cells by real-time RT-PCR. In K562-+17AAR and K562/A02-+17AAR cell lines, MDR1 gene expressions decreased to 7.43±2.23 and 105.73±27.73 respectively. The MDR1 transcript expression was markedly down-regulated in the multidrug resistant K562/A02 cell line (p=0.017) following WT1/+17AA ShRNA transfection. The fluorescent intensity of P-gp was 53.13±7.56 in K562/A02-con cells and 4.39±2.32 in K562-con cells (p<0.001). The P-gp expression in K562/A02 cells clearly decreased, obviously, falling to 19.12±4.21, after transfection with WT1/+17AA ShRNA (p<0.005) (Figure 2A). However, the MDR1 gene and P-gp expression levels in K562-+17AAR cells showed qualitatively similar results with those in the K562-con cells (p>0.05). Similarly, Rh123 uptake was dramatically altered after transfection with WT1/+17AA ShRNA in K562/A02 cells. The K562/A02-+17AAR cells displayed rates of Rh123 accumulation in between those of K562-con and K562/A02-con, while the K562/+17AAR cells displayed uptake kinetics similar to that of the parental K562-con cells (Figure 2B). The K562-+17AAR cells also displayed doxorubicin dose-response similar to K562-con parental cells, with an IC_{50} values of 0.79 and 0.94 μM. After transfection of WT1/+17AA ShRNA, the dose response curve of the K562/A02+17AA cells shifted significantly to the left, reducing the IC_{50} value from 78.67μM to 22.59 μM. Thus, transfection of WT1/+17AA ShRNA led to a substantial reversal of doxorubicin resistance in K562/A02 cells but had no effect on the doxorubicin dose response profile of the parental K562 cells.

It has been suggested that the presence of the exon 5 insert and the maintenance of the correct balance between WT1/+17AA and WT1/-17AA isoforms are essential for the regulation of critical cellular functions including proliferation, differentiation, and resistance to chemotherapeutic drugs.[5-7] The changes in exon 5 splicing are also believed to affect the regulation of downstream gene expression profile in the WT1 pathway.[8] The MDR1 gene transcription is regulated by the combined actions of several transcription factors that bind to its promoter region.[9,10] We suggest that ShRNA, when directed to WT1 exon 5, might inhibit WT1/+17AA isoform expression and strongly influence expression and function of the MDR1 gene in drug-resistant leukemia cells. To our knowledge, this is the first report of the relationship between the WT1/+17AA isoform and multidrug resistance in leukemia cells. These results provide novel insights into the role of WT1 exon 5 splicing transcript in the regulation of leukemia cells' survival signaling pathways. This suggests that a designed RNAi targeted WT1 isoform could be a very effective strategy for MDR1 regulation.

Huiling Shen, Wenlin Xu,* Zhaoyang Wu,° Huarong Tang,° Yanyan Xie,* Ximing Zhong**

**Department of Oncology, the Affiliated of People's Hospital, Jiangsu University, Zhenjiang, Jiangsu, China; °Central Laboratory of Experimental Hematology, the Affiliated of People's Hospital of Jiangsu University, Zhenjing, Jiangsu, China*

Huiling Shen and Wenlin Xu contributed equally to this study and should be considered as co-first authors.

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Correspondence: Professor Wenlin Xu, the Affiliated of People's Hospital of Jiangsu University, 8 Dianli Road, Zhenjiang, Jiangsu 212002, P.R.China. Phone: international +86.511.5246930. Fax: international +86.511.5234387. E-mail: XWL0806@163.com

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