Role of the Wilms' tumor 1 gene in the aberrant biological behavior of leukemic cells and the related mechanisms

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Received June 25, 2014; Accepted September 2, 2014

DOI: 10.3892/or.2014.3529

Abstract. The Wilms' tumor 1 (WT1) gene is one of the regulating factors in cell proliferation and development. It is a double-functional gene: an oncogene and a tumor suppressor. This gene was found to be highly expressed in many leukemic cell lines and in patients with acute myeloid leukemia. In the present study, we demonstrated that the WT1 gene was commonly expressed in leukemic cell lines apart from U937 cells. The K562 cell line which expresses WT1 at a high level (mRNA and protein) was used in the entire experiment. By MTT and colony formation assays, we found that curcumin, an inhibitor of the WT1 protein, inhibited cell proliferation and clonogenicity in a time- and dose-dependent manner. It also caused cell cycle arrest at the G_2/M phase. We then designed specific short hairpin RNAs (shRNAs) which could downregulate WT1 by 70-80% at the mRNA and protein levels. Reduction in the WT1 levels attenuated the proliferative ability and clonogenicity. Cell cycle progression analysis indicated that the proportion of cells in the G_0/G_1 phase increased while the proportion in the S phase decreased distinctively. ChIP-DNA selection and ligation (DSL) experiment identified a cohort of genes whose promoters are targeted by WT1. These genes were classified into different cellular signaling pathways using MAS software and included the Wnt/β-catenin pathway, MAPK signaling pathway, apoptosis pathway, and the cell cycle. We focused on the Wnt/β-catenin signaling pathway, and compared expression of several genes in the K562 cells transfected with the control shRNA and WT1-specific shRNA. β-catenin, an important gene in the Wnt canonical pathway, was downregulated after WT1 RNAi. Target genes of β-catenin which participate in cell proliferation and cell cycle regulation, such as CCND1 and MYC, were also significantly downregulated. Collectively, these data suggest that WT1 functions as an oncogene in leukemia cells, and one important mechanism is regulation of the Wnt/β-catenin pathway.

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Key words: leukemia, Wilms' tumor 1 gene, proliferation, cell cycle, RNAi technology

Introduction

The Wilms' tumor 1 (WT1) gene is one of the important regulators of cellular growth and development. It was originally identified as a gene that is deleted or rearranged in many cases of hereditary Wilms' tumor, a childhood kidney neoplasm. Located at 11p13, the wt1 gene encodes a zinc finger-containing nuclear protein with DNA- and RNA-binding activities (1,2). Its target genes are involved in cell growth, metabolism, differentiation and death, including CDKN1A, a negative regulating gene in cell cycle progression; MAPK signaling genes; and Wnt pathway genes (3-7). In many hematological malignancies, particularly in most acute leukemias, WT1 mRNA expression is high and is associated with poor prognosis (8-10).

In the present study, WT1 mRNA and protein expression levels were detected in several leukemic cell lines, particularly in K562 cells, a blast phase chronic myeloid leukemia (CML) cell line, where a higher expression was observed. Thus, we chose K562 cells as the main subject for further study. Curcumin is one of the WT1 protein inhibitors and was used to treat K562 cells (2). Correlation between expression of the WT1 gene and cell biological characteristics was analyzed. As curcumin is not a specific inhihitor of WT1 protein, we designed WT1-specific short hairpin RNA (shRNA) nucleotides. The WT1 gene in K562 cells was downregulated by WT1-specific shRNA nucleotides, and cell growth was inhibited, the numbers of colonies were obviously decreased and the cell cycle was arrested at the G_0/G_1 phase. These results indicate that the WT1 gene may play an important role in leukemia cell proliferation and cell cycle regulation. Yet, the mechanisms involved in the effects of WT1 on K562 cells remain unknown. Thus, CHIP-DSL was performed. Classes of the WT1 targets identified consisted of several vital signaling pathways, such as the Wnt pathway, the MAPK pathway, cell cycle, apoptosis, differentiation, cell adhesion and cell migration. The Wnt/β-catenin pathway plays an important role in tumorigenesis and progression of leukemia. Therefore, we detected the levels of major genes in the traditional canonical

and non-canonical pathways. We observed that when WT1 was downregulated, expression of several important genes such as β-catenin, SUZ12 and Wnt11 was decreased at the mRNA and protein levels, while expression of Wnt3a and Wnt5a was slightly increased. Meanwhile, the subcellular location of β-catenin was altered, and expression levels of the targets of β-catenin, CCND1 and MYC genes, were obviously decreased. Collectively, these data suggest that WT1 functions as an oncogene in leukemia cells, and one important mechanism is regulation of the Wnt/β-catenin pathway.

Materials and methods

Cell culture. Human leukemia cell lines K562, HL60, NB4, U937 and Kasumi-1 were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum at 37˚C in a humidified environment of 5% CO_2. Proper cell densities were planted to ensure logarithmic growth. To assess the effect of colony formation under different conditions, aliquots containing 1,000 cells were plated into 48-well culture plates. At different time points, colonies containing approximately 50 cells were counted using an inverted phase contrast microscope.

Western blot assays. Cell pellets were lysed in lysis buffer [50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 25 mM Tris (pH 7.5), 1 μg/ml leupeptin, 10 μg/ml laprotinin, and 1 mM phenylmethylsulfonyl fluoride] on ice for 30 min and centrifuged for 5 min at 20,000 x g at 4˚C. The protein concentration of the supernatant was determined using BCA protein assay reagents (Pierce, USA). Equal amounts of protein (100 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted on nitrocellulose membranes, and immunostained with the primary antibodies followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody. Finally, protein densities were analyzed using the AlphaEaseFC software according to the manufacturer's instructions. Antibodies included rabbit polyclonal WT1 antibody, β-catenin monoclonal antibody, wnt3a, wnt5a, wnt11 (Abcam Bio Company) and SUZ12 (CST).

Lentivirus production and cell transduction. The WT1 shRNA sequence (obtained by the TRC consortium) was sense, 5'-CCGGGCAGCTAACAATGTCTGGTTACTCGAGTAACCAGACATTGTTAGCTGCTTTTTG-3' and antisense, 5'-AATTCAAAAAGCAGCTAACAATGTCTGGTTACTCGAGTAACCAGACATTGTTAGCTGC-3'; targeting sequence, GCAGCTAACAATGTCTGGTTA, and the control-shRNA sense, 5'-CCGGCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGTTTTT-3' and antisense, 5'-AATTAAAAACCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGGG-3'. By ligation, transformation, plasmid extraction and sequencing, plKO.1 vectors were obtained with the puromycin-selection marker. Then lentiviral vector production was performed by transient transfection of 293T cells using calcium phosphate. The viral supernatant was concentrated 200-fold by ultracentrifugation. K562 cells were plated at a density of 10^6/ml in serum-free medium, and then transduction was carried out for 12 h. Puromycin was then used to select the positive cells (the final concentration of puromycin was 2-5 μg/ml); using three selecting cycles, the positive cells were obtained.

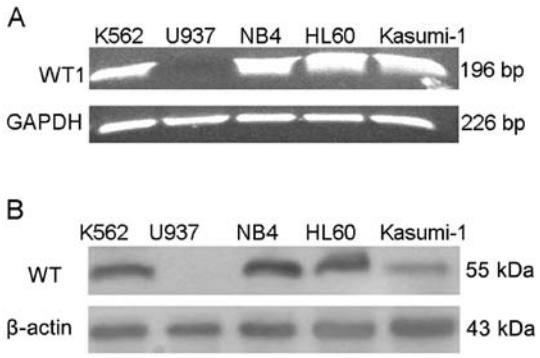

Figure 1. WT1 was highly expressed in most of the leukemia cell lines at the mRNA and protein levels. (A) Expression of the WT1 gene (RQ-PCR) was negative in the U937 cells, but positive in the other four cell lines. (B) WT1 protein was also expressed in the above four cell lines (except for U937 cells) by western blot assay, which was consistent with the mRNA levels. The Kasumi-1 cells expressed a lower level.

MTT assays. The MTT assay was performed as previously described (11). Briefly, $1x10^4$ cells were plated in each well of 96-well microtiter plates with 100 μl of fresh medium containing curcumin at various concentrations. After 24, 48 or 72 h of further incubation, 10 μl methylthiazol tetrazolium (MTT) solution (2.5 mg/ml) was added to each well, and the cells were incubated for another 4 h. The medium was then removed, and the formed formazan crystals were dissolved in 150 μl of dimethyl sulfoxide (Sigma, USA). The plates were placed on a plate shaker for 10 min, and the absorbance of the resulting solution was immediately measured at 546 nm using a microplate reader (SLT-Lab, Salzburg, Austria). The growth inhibition ratio was calculated using the formula: Growth inhibitory rate = (1 - T/C) x 100%, where T is the absorbance rate of the treatment group with curcumin and C is the absorbance rate of the control group. the IC_{50} value of curcumin was calculated using SPSS software (11). These experiments were repeated three times.

Cell cycle analysis. K562 cells treated with curcumin at different time points or cells which were silenced for WT1 were washed twice with cold PBS and then resuspended with ice-cold 70% ethanol overnight. After washing with cold PBS, the cells were treated with 10 mg/ml of RNase at 37˚C for 30 min and then stained using 40 mg/ml of propidium iodide (PI) for 30 min. DNA content was measured by flow cytometry (Beckman Coulter).

Real-time quantitative RT-PCR. Total RNA was extracted from $1x10^6$ cells using RNAsio (Takara, Japan) according to the protocol supplied with the reagent. Isolated RNA was resolved in 0.1% diethyl pyrocarbonate (DEPC)-treated water. Complementary DNA (cDNA) was synthesized from 2 μg RNA with N6 primers using murine myeloleukemia virus reverse transcriptase (M-MLV, Promega, USA), following the procedure provided by the manufacturer.

Real-time quantitative RT-PCR (RQ-PCR) was performed using SYBR-Premix Ex Taq (Takara, Japan) on a 7500 Thermo

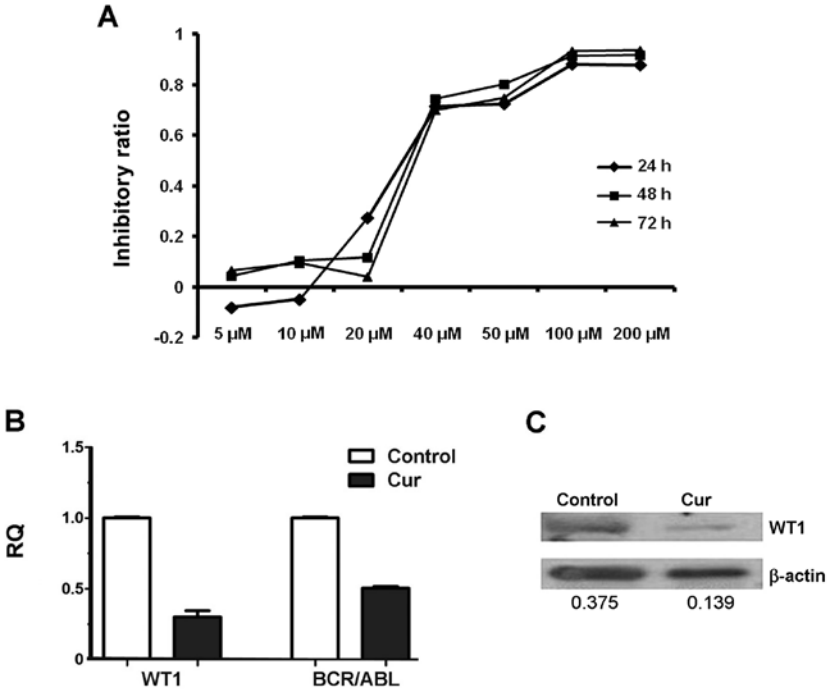

Figure 2. Curcumin inhibits cell proliferation and alters cell cycle progresssion. (A) MTT assay was used to assess cell proliferation. K562 cells were treated with curcumin at concentrations of 0, 5, 10, 20, 40, 50, 100 and 200 μM. At 24, 48 and 72 h time points, cell viability was assessed respectively, and the inhibition rate was calculated. The cell viability of the K562 cells treated with curcumin was decreased dose-dependently. (B) Relative WT1 mRNA and BCR/ABL mRNA levels in the K562 cells following curcumin treatment were estimated by real-time PCR normalized to endogeneous GAPDH. (C) WT1 protein in K562 cells treated with curcumin was markedly decreased when compared with the untreated cells as determined by western blot assay.

Cycler (Applied Biosystems, USA). Each sample was run in triplicate. Relative quantification of WT1, BCR/ABL, wnt3a, wnt5a, wnt11, SUZ12, β-catenin, CCND1 and MYC mRNA expression was conducted by the comparative Ct method established by Livak and Schmittgen (12). SDS 2.1 software (Applied Biosystems, USA) was used. The output relative expression value was normalized to the endogenous reference GAPDH. The primer sequences used are as follows: WT1 forward, 5'-CACGAGGAGCAGTGCCTGAG-3' and reverse, 5'-AACCCTGATTGCGAATAGCG-3'; GAPDH forward, 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse, 5'-GAA GATGGTGATGGGATTTC-3'; BCR/ABL forward, 5'-gggctc tatgggtttctgaatg-3' and reverse, 5'-cgctgaagggcttttgaact-3'; wnt3a forward, 5'-TGGTGTCTCGGGAGTTCGC-3' and reverse, 5'-CCGTGGCACTTGCACTTGA-3'; wnt5a forward, 5'-CATACCTTGAGCACGAC-3' and reverse, 5'-TCTTAA CGTCCATGTCTAT-3'; wnt11 forward, 5'-GACCTCAAG ACCCGATACCT-3' and reverse, 5'-GGAGCCCACCTT CTCATTC-3'; SUZ12 forward, 5'-CCTATTGCCAAAC CTC-3' and reverse, 5'-TTGCCTTGTATTGTTGT-3'; β-catenin forward, 5'-GGCAGCAACAGTCTTA-3' and reverse, 5'-GTCTCAGGGAACATAGC-3'.

Immunofluorescence assay. Cells were washed 3 times with PBS buffer and then fixed with 4% paraformaldehyde solution at RT for 10 min. The cells were washed 3 times with PBS buffer, and permeabilized for 10 min at RT with 0.1% Triton and 0.2% BSA-PBS. Cell were then washed 3 times with PBS buffer, and non-specific protein binding was blocked with 10% goat serum-PBS or with 2% BSA-PBS for 30 min. Goat serum or BSA was removed and without washing a coverslip was added. The primary antibody was diluted in 2% goat serum-PBS or 1% BSA-PBS for 45 min at RT. Washing was carried out 5 times with PBS, added to coverlip. The secondary antibody was diluted in 2% goat serum-PBS or 1% BSA-PBS for 30 min at RT. Washing was carried out 5 times with PBS. The coverslip was placed on the slide very carefully and observed under a confocal laser scanning scope immediately.

Statistical analysis. Three repetitions were performed for each experiment. The significance of the differences in the relative mRNA expression levels and the percentages of cell growth inhibition between two groups was determined using the paired t-test. All analyses were carried out using the SPSS software package. $P<0.05$ was deemed to indicate a statistically significant difference.

Results

The WT1 gene is highly expressed in most leukemic cell lines. In order to determine the expression of the WT1 gene in the AML cell lines, we designed primers of the WT1 gene which could cover the four main spliceosomes. As shown in Fig. 1, WT1 mRNA was expressed highly in the K562, HL60, NB4 and Kasumi-1 cells, but was negative in the U937 cells. Approximately 100 μg of the protein extract was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Fig. 1, apart from U937, WT1 was highly expressed in the other four cell lines.

Curcumin, an inhibitor of the WT1 gene, inhibits cell growth by decreasing WT1 expression. As shown in Fig. 2A, the cells

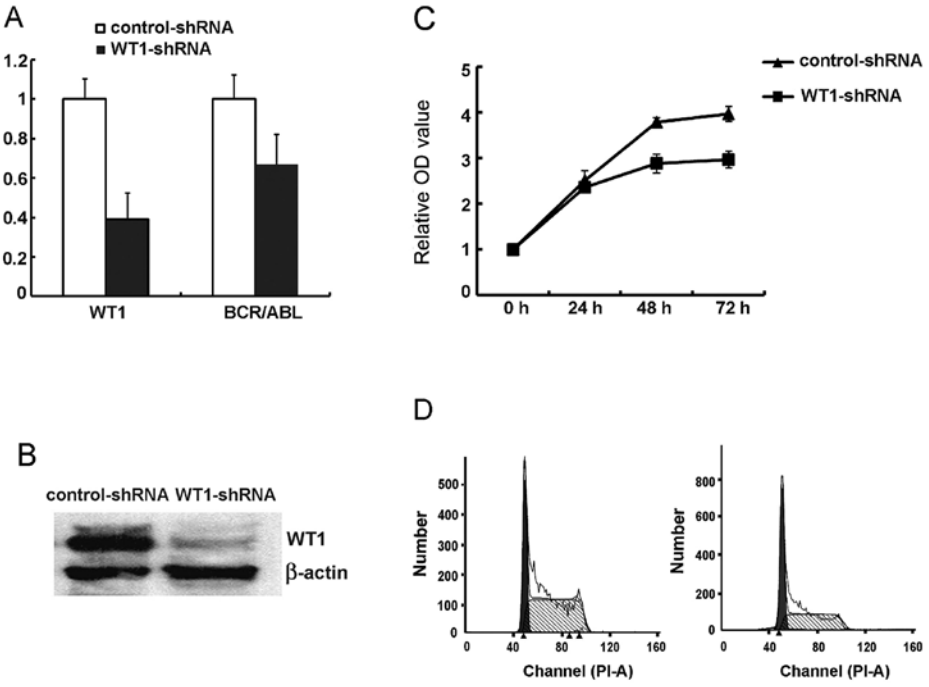

Figure 3. WT1 silencing in K562 cells. Cells transduced with WT1-shRNA exhibited reduced *in vitro* cell proliferation and cell cycle progression. (A) Real-time PCR assay of WT1 and BCR/ABL mRNA levels normalized to endogeneous GAPDH expression. (B) WT1 protein levels in the K562 cells transduced with WT1-shRNA were markedly decreased as determined by western blot analysis. (C) K562 cells with WT1 silencing showed slower proliferation than the control group as determined by MTT assay. (D) FACS assay by PI staining showed a significant increase in the proportion of cells in the G_0/G_1 phase and a decrease in the proportion of cells in the S phase in the K562 cells transduced with WT1-shRNA, when compared to the control-shRNA group.

treated with curcumin showed a dose-dependent decrease in cell viability. The IC_{50} values of curcumin in the K562 cells were 28.15, 33.79 and 38.12 μM at time points 24, 48 and 72 h, respectively. For the subsequent studies, we used curcumin at a concentration of 20 μM.

As shown in Fig. 2B, WT1 mRNA was decreased by approximately 80% and simultaneously WT1 protein (Fig. 2C) was obviously reduced after curcumin treatment for 24 h. We also detected the expression of BCR/ABL and found that BCR/ABL copies were markedly decreased, which indicated that curcumin also affected the BCR/ABL pathways in addition to downregulating the WT1 gene.

In addition, the colony formation assays showed a marked reduction in colony formation (458±50.4 vs. 260±18.7, P<0.01) 4 days after treatment with 20 μM curcumin, when compared to the cells treated with DMSO alone. Cell cycle analysis showed that curcumin also induced cell cycle arrest in the G_2/M phase (data not shown).

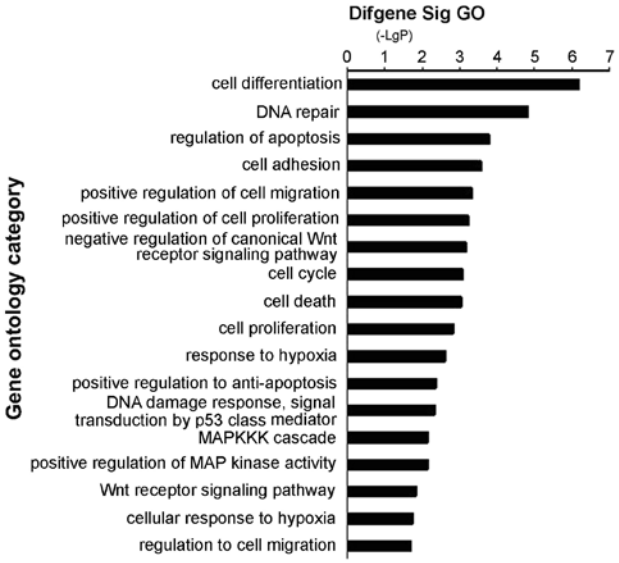

Figure 4. As determine by CHIP-DSL assay, many vital signaling pathway genes were found to be regulated directly by WT1 at the transcription level.

WT1 expression is related to cell proliferation and cell cycle progression in the AML cell lines. To further analyze the exact role of the WT1 gene in leukemia cell biology, we downregulated WT1 expression specifically using RNA interference technique. pLKO.1 lentiviral shRNA constructs generated by the RNAi consortium with the fragments expressing puromycin were used to identify and sort the transduced cells. The efficiency of WT1 downregulation by the different shRNA vectors was investigated by RNA and protein expression of WT1 in the leukemic cells. TRC0063 shRNA was confirmed to be the most efficient oligonucleotide; it downregulated WT1 mRNA and protein by over 70 and 90% respectively (Fig. 3A and B) and was selected for further studies (referred to here as WT1-shRNA).

Cell proliferation, colony formation and cell cycle following WT1 downregulation were examined. We observed that WT1-shRNA-transduced K562 cells showed decelerated cell proliferation *in vitro* particularly at the 48- and 72-h time points when compared with cells transduced with the non-specific sequence (Fig. 3C). To investigate whether the reduced

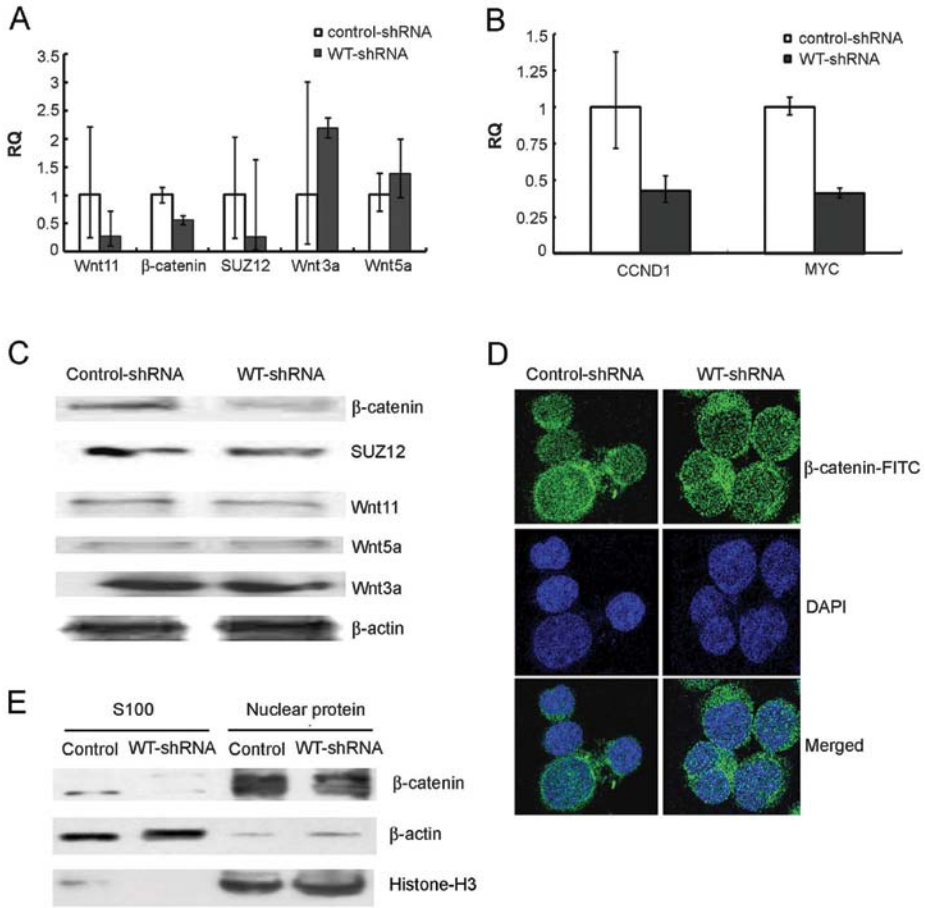

Figure 5. WT1 silencing directly affects the wnt/β-catenin signaling pathway. (A) Reduced relative wnt11, SUZ12, and β-catenin mRNA levels and increased levels of wnt3a, wnt5a were noted following WT1 downregulation in K562 cells as estimated by real-time PCR. (B) mRNA levels of the functional downstream genes CCND1 and MYC were reduced following WT1 downregulation. (C) Western blot assay showed that wnt11 and β-catenin protein levels were reduced obviously, while other proteins were changed slightly following WT1-shRNA transduction in K562 cells. (D) In K562 cells transduced with WT1-shRNA, β-catenin protein translocated from the nucleus to the cytoplasm when compared to the control group, as observed under confocal laser scanning microscope. (E) As determined by western blot assay, following WT1 silencing, β-catenin was increased in the cytoplasmic extracts (S100) and was decreased in nuclear extracts normalized to β-actin and histone-H3, separately.

cell proliferation was due to cell death, the levels of apoptosis were measured in cells with reduced WT1 levels at various time points after transduction by flow cytometry. The percentage of $PI^-/Annexin\ V^+$ cells was not different when compared to this percentage in the control cells. We subsequently analyzed the cell cycle distribution of WT1-shRNA- and control-shRNA-transduced K562 cells and observed an increase in the proportion of cells in the G_0/G_1 phase (from 27.7 to 44.7%) while the proportion of S phase cells was decreased (71.9 vs. 55.3% in control cells; Fig. 3D). These data suggest that WT1 is implicated in the cell cycle progression, proliferation and self-renewal of AML cells.

Exploration of the mechanism and function of WT1 in leukemia. We analyzed the genome-wide transcriptional targets of the WT1 gene using the chromatin immunoprecipitation-DNA selection and ligation (ChIP-DSL) approach. DNA from K562 cells was amplified and marked, and then the data were analyzed using MAS software (http://bioinfo.capitalbio.com/mas) with a P-value cutoff of 10^{-3} (13). CHIP-CHIP analysis identified 2034 promoters by single array and 207 promoters by 2/2 arrays. As shown in Fig. 4, WT1-bound targets were enriched in cell apoptosis, Wnt signaling pathway, MAPK signaling, cell migration, cell proliferation and cell adhesion. The key genes of the different pathways are shown in Table I.

Table I. Gene ontology of the genes bound by WT1.

GO names	Genes
Wnt signaling pathway	AES, TGFB111, SFRP4, GSK3A, LRRFIP2, WNT2B, WNT11, GRK5, NKX2-5, CBY1, AXIN1
MAPK signaling pathway	PDGFB, PDCD10, VEGFA, EDN1, MAP3K5, SMAD1, CAMKK2, DUSP4, MAPKAPK2, SHC1
Cell migration	CCL26, PIK3R1, VEGFA, SPAG9, PDPN, WNT11, PLD1, EDN1, FER, FGF1, RRAS2, PDGFB

As WT1 and the Wnt pathway are important factors in the progression of CML (14-16), we speculated that WT1 participates in the pathogenesis of leukemia mainly by directly regulating genes of the wnt pathway. First, we found that β-catenin, an essential gene of the wnt pathway, was noticeably decreased at the RNA and protein levels in the K562 cells after WT1 was silenced (Fig. 5A and C). Confocal immunofluorescence and western blot analyses demonstrated that β-catenin translocated from the nucleus to the cytoplasm following WT1 downregulation (Fig. 5D and E).

Next, we detected expression of other genes of the canonical and noncanonical wnt pathway in the WT1-shRNA- and control-shRNA-transduced K562 cells. As shown in Fig. 5A and B, the typical canonical genes, such as wnt3a remained unchanged after WT1 interference. However, expression levels of the noncanonical genes, wnt11 and SUZ12, were significantly decreased at the RNA and protein levels.

As wnt11 is possibly a transcriptional target of WT1, we speculated that expression of wnt11 would be directly affected by the downregulation of WT1, and subsequently the expression and sublocation of β-catenin changed. In addition, the expression of β-catenin transcriptional targets, MYC and CCND1, was also obviously reduced, in the cells transduced with WT1-shRNA compared to the control cells (Fig. 5B).

Discussion

The WT1 gene is one of the important regulators of cell growth and development. WT1 mRNA has two important splicing sites, the fifth exon (which comprises 17 amino acids) and the ninth exon (which encodes three amino acids, lysine tyrosine and tyrosine, also called KTS); it can encode four major protein isomers. The COOH-terminal of WT1 protein contains a Zinc finger domain and plays an important role in transcription (1-3,5).

The WT1 gene is expressed at a low level in $CD34^+$ cells of normal bone marrow (9,10). An animal experiment demonstrated that WT1 was necessary for cell self-repopulation. Dysregulation of WT1 was found to promote stem cell proliferation and obstruction of differentration, consequently increasing the risk of leukemogenesis. As reported, WT1 was highly expressed in AML patients, and levels of WT1 in chronic myeloid leukemia patients are related to disease progression (14-17). In our study, we found that WT1 was highly expressed in different types of leukemia cell lines. Using the CML blastic cell line K562, we illustrated that treatment with an inhibitor of WT1, curcumin, a chemical extracted from plants could decrease the cell growth and colony formation and arrest the cell cycle at the G_2/M phase. This indicates that curcumin has antitumor activity partially by inhibiting the WT1 gene and causing cell cycle arrest. Furthermore, we also noted that curcumin interferes with the expression of BCR/ABL fusion genes. This phenomenon is extremely important in the treatment of CML.

To identify the transcriptional targets of the WT1 gene, we performed CHIP-DSL analysis and found that the target genes included wnt/β-catenin signaling pathway genes, MAPK pathway genes, cell cycle regulation, cell adhesion and cell apoptosis genes. These results are consistent with those reported by Kim *et al* in Wilms' tumor cell lines (18). Among these pathways, the wnt pathway may be a major target of WT1.

shRNAs can specifically and stably downregulate gene expression. We downregulated the WT1 gene in K562 cells using lentiviral-mediated expression of shRNAs. Reduction of WT1 levels in K562 cells decelerated their *in vitro* proliferative ability by cell cycle arrest, without affecting cell viability. Reduction in the levels of WT1 caused an increased proportion of cells in the G_0/G_1 phase while a decreased ratio of S phase cells. To elucidate the mechanisms of WT1 alteration involved in the biology of leukemia cells, we focused on the wnt/β-catenin pathway. We found that WT1 regulated the wnt pathway mainly by the wnt11-related non-canonical pathway genes but not the wnt-related canonical pathway. We demonstrated that downregulation of WT1 caused wnt11 to decrease at the mRNA and protein levels, and consequently the changes in wnt11 affected the expression of protein β-catenin causing it to translocate from the nucleus to the cytoplasm. Meanwhile, the target genes of β-catenin, MYC and CCND1, also decreased. As MYC and CCND1 are important genes in cell growth and cell cycle progession, downregulation of MYC and CCND1 caused the alteration of the cell cycle and cell growth (19,20). Finally, we can deduce that by targeting wnt11, WT1 participates in regulating the wnt/β-catenin signaling pathway, leading to uncontrolled cell cycle progression, cell growth and self-repopulation, resulting in leukemogenesis. Moreover, CHIP-DSL results demonstrated that WT1 may also regulate cell adhesion and migration, affect the bone marrow microenvironment and chemosensitivity. All these may contribute to leukemogenesis.

In conclusion, the WT1 gene is an oncogene and plays an important role in leukemogenesis. Therapy targeting the WT1 gene will be a significant strategy for leukemia.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (30870913) and Tianjin Major Science and Technology Project (12ZCDZSY17500).

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