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Expression of the *WT1* gene -KTS domain isoforms suppresses the invasive ability of human lung squamous cell carcinoma cells

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Abstract. Although the *WT1* gene was originally isolated as a tumor suppressor gene from Wilms' tumor, oncogenic roles for WT1 have been reported in several tumors. Here, we present new findings of high levels of *WT1* expression associated with the suppression of lymph node metastasis in patients with human lung squamous cell carcinoma (SCC). We investigated the effect of down-regulated *WT1* gene expression on the invasive phenotype of the SCC cell line RERF-LC-AI. Invasive ability was enhanced in *WT1*-specific siRNA-transfected cells, and a *WT1* target gene $p21^{Waf1/Cip1}$ was isolated by comprehensive gene expression analysis. As several isoforms are produced from the *WT1* gene, we isolated eight major WT1 isoforms from a cDNA library and cloned each variant into an expression vector. Luciferase reporter assays revealed that $p21^{Waf1/Cip1}$ expression was enhanced only by the *WT1* cDNA variants that included a three-amino acid deletion (-KTS). Our results suggested that the -KTS-containing variants of WT1 are directly involved in the regulation of $p21^{Waf1/Cip1}$ expression and the subsequent suppression of lymph node metastasis in human lung squamous cell carcinoma.

Introduction

The Wilms' tumor gene (*WT1*) was originally isolated as the gene responsible for the child neoplasm Wilms' tumor through its deletion in a subset of patients. The *WT1* gene maps to chromosomal band 11p13, spans approximately 50 kb, and is comprised of ten exons. The WT1 protein is a member of the zinc finger-type family of transcription factors and is responsible for the controlled expression of a variety of proliferation- and differentiation-associated target genes (1).

The role of *WT1* as a tumor suppressor gene has been established by studies that demonstrated inactivating point mutations and homozygous deletions in Wilms' tumor tissues (1). However, recent studies have shown that WT1 may also have oncogenic functions. In human myelocytic leukemia, acute lymphocytic leukemia, and chronic myelocytic leukemia, high levels of *WT1* expression have frequently been found and associated with the development of more primitive and refractory forms of disease (2-4). Furthermore, *WT1* over-expression has been reported in several human solid tumor specimens including ovarian cancer (5,6), breast cancer (7,8), lung cancer (9,10), esophageal cancer (11), colorectal adeno-carcinoma (12), head and neck squamous cell carcinoma (13), and osteogenic sarcoma (14).

Exons 5 and 9 of *WT1* are alternatively spliced, giving rise to four different splice isoforms. The inclusion of exon 5 inserts 17 amino acids, while the usage of an alternate splice donor site at the end of exon 9 results in the incorporation of three additional amino acids, lysine, threonine and serine (KTS). In addition to *WT1* isoforms translated from the authentic initiator AUG, smaller *WT1* isoforms have been described, through the usage of an internal translation initiation site at the in-frame AUG 127 codon downstream of the initiator AUG, generating truncated *WT1* isoforms (15). Furthermore, a short transcript that lacks 147 amino acids at the N-terminus has also been reported (16). Although all the isoforms are expressed in primary human solid tumors and leukemia, the functions of the individual WT1 isoforms in human cancer cells remain unclear.

Recently, we demonstrated that *WT1* expression correlated with lymph node metastasis in patients with lung adeno-carcinoma (AD), and that patients that expressed *WT1* showed significantly lower rates of disease-free survival compared with patients that did not express *WT1*. This tendency was reversed in patients with lung squamous cell carcinoma (SCC), whereby *WT1* non-expression was correlated with lymph node metastasis and recurrence. In an effort to explore this phenomenon in patients with lung SCC, we examined the effect of expression of each of the WT1 isoforms on metastasis development in human lung SCC cell lines.

Materials and methods

Cell line and cell culture. The human lung SCC cell line RERF-LC-AI was obtained from the Riken cell bank and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37˚C.

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siRNA transfection. Control siRNA (negative control siRNA), WT1 siRNA (Hs_WT1_1), and p21$^{Waf1/Cip1}$ siRNA (Hs_CDKN1A_7) were obtained from Qiagen (Hilden, Germany). Transfection into RERF-LC-AI cells was performed using a final concentration of 5 nM siRNA and HiPerFect transfection reagent (Qiagen).

RNA purification and preparation of cDNA. Total RNA was isolated from cells using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and 1 μg aliquots of total RNA were converted into cDNA using oligo dT primers (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed in 20 μl reaction mixtures containing 10 μl HotStarTaq master mix (Qiagen), 0.5 μM each forward and reverse primer, and 1 μl cDNA. Sequences of the primers used were as follows: WT1 forward primer, 5'-GATAACCA CACAACGCCCATC-3', and reverse primer, 5'-CACACGT CGCACATCCTGAAT-3'; p21$^{Waf1/Cip1}$ forward primer, 5'-TA TGGGGCTGGGAGTAGTTG-3', and reverse primer, 5'-AG CCGAGAGAAAACAGTCCA-3'; ß-actin forward primer, 5'-GACAGGATGCAGAAGGAGATTACT-3', and reverse primer, 5'-TGATCCACATCTGCTGGAAGGT-3'. RT-PCR was performed using a GeneAmp 9700 (Applied Biosystems, Foster, CA) with conditions of activation at 95˚C for 15 min, followed by 35 cycles of denaturation at 94˚C for 15 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec. For real-time RT-PCR analysis, the same primers and conditions were used except that the number of cycles was increased to 40. Standard curves for quantitation were constructed from the results of simultaneous amplification of serial dilutions of cDNA from non-treated RERF-LC-AI cells, from which the expression level was defined as 1.0.

In vitro invasion assay. Following siRNA transfection, cells were incubated for 24 h, harvested, counted, and dispersed in FBS-free DMEM, and 5x10^4 cells/well were added to inserts (upper chamber). Culture medium containing 10% FBS as the chemotactic agent was added to the lower chamber. After 22 h incubation, non-invading cells were removed from upper filter surfaces and filters washed, fixed and stained using Diff-Quick kit (Sysmex, Kobe, Japan). Four randomly selected x200 fields were photographed and invading cells counted.

Cell growth assay. Cells were seeded into six-well plates at a density of 0.5x10^4 cells/well and counted after 27, 48, 72 and 94 h of incubation. Cell numbers were determined using a Coulter counter (Beckman Coulter, Fullerton, CA).

DNA microarray analysis. AceGene Human Oligo Chip 30K microarrays (DNA Chip Research Inc. & Hitachi Software Engineering, Yokohama, Japan) containing a total of 30,336 spots corresponding to 29,640 independent genes (gene list available at http://bio.hitachi-sk.co.jp/acegene/) were used for expression profiling. Total RNA (1 μg) was used for RNA amplification. Amino allyl-labeled antisense RNA (aRNA) was prepared using the Amino Allyl MessageAmp aRNA kit (Ambion, Austin, TX), and amino allyl-modified aRNA was then labeled with Cy5 or Cy3. Of labeled aRNA, 5 μg was used for each hybridization experiment. Probe purification, hybridization, and washing steps were performed according to the manufacturer's instructions. DNA microarray analysis was repeated using a dye-swap. Arrays were scanned using the Packard GSI Lumonics ScanArray 4000 (Perkin Elmer, Boston, MA), and data analyzed by DNASISarray software (Hitachi Software Engineering, Yokohama, Japan), which converted the signal intensity of each spot into a text format.

Statistical analyses of DNA microarray data. Mean and standard deviations (SD) of background levels were calculated, and genes with intensities less than the mean + 2SD of background level were excluded from further analysis. Cy3/Cy5 ratios for all spots on the microarray were normalized by global normalization. Mean and SD of log2-ratios were calculated, and genes with expression levels of log2-ratios that varied more or less than the mean ± 2 SD of all analyzed genes were subjected to further analysis.

WT1 cDNA gene cloning. *WT1* cDNA was generated by RT-PCR using primers that contained *Kpn*I and *Mlu*I sites (underlined) to facilitate cloning: short isoform forward primer, 5'-<u>GGTACC</u>ATGTTTCCTAACGCGCCCTACCTGCCCAG-3' and reverse primer, 5'-<u>ACGCGT</u>TTCCTTTTGAATAGAC TTTAATTGAGAGC-3', normal isoform forward primer, 5'-<u>GGTACC</u>CAGCAAATGGGCTCCGAC-3'. Products were subcloned into the pCMVFL3 expression vector (Toyobo, Osaka, Japan).

Western blot analysis. Protein was extracted in lysis buffer [50 mM Tris pH 8.0, 1% Triton-X, 450 mM NaCl, 100 μg/ml PMSF, 20 μl/ml Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)]. Whole cell lysates (10 μg) were separated by electrophoresis in 10% tris-glycine SDS polyacrylamide gels and then transferred onto nitrocellulose membranes. After blocking in TBST (50 mM Tris pH 7.6, 150 mM NaCl, and 0.1% Tween 20) plus 5% (wt/vol) nonfat milk, blots were incubated for 45 min at room temperature with primary antibodies, then incubated with HRP-conjugated secondary antibody, and finally visualized using ECL (GE Healthcare, Danbury, CT). Equivalent protein loading was evaluated using anti-WT1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) or anti ß-actin (1:1000; Sigma-Aldrich) antibodies.

Construction of the reporter plasmid harboring the 5' p21$^{Waf1/Cip1}$ promoter. The *p21$^{Waf1/Cip1}$* promoter (-2317 to +49 of the *p21$^{Waf1/Cip1}$* proximal promoter region) was generated by PCR with primers containing *Nhe*I and *Hind*III sites (underlined) to facilitate cloning: forward primer, 5'-<u>GCTA GC</u>CCCAGGAACATGCTTGGGCAGCAG-3' and reverse primer, 5'-<u>AAGCTT</u>AGCTCCGGCTCCACAAGGA-3'. The *p21$^{Waf1/Cip1}$* promoter fragment was subcloned into the luciferase reporter construct pGL3 (Promega, Madison, WI), and designated as pGL3-p21.

Luciferase reporter assays. Luciferase reporter assays were performed using the PicaGene Dual SeaPansy™ luminescence kit (Wako, Osaka, Japan). Of each of the pGL3-p21 reporter plasmid and the pCMVFL3 constructs expressing each WT1 isoform, 1 μg was co-transfected by Lipofectamine 2000

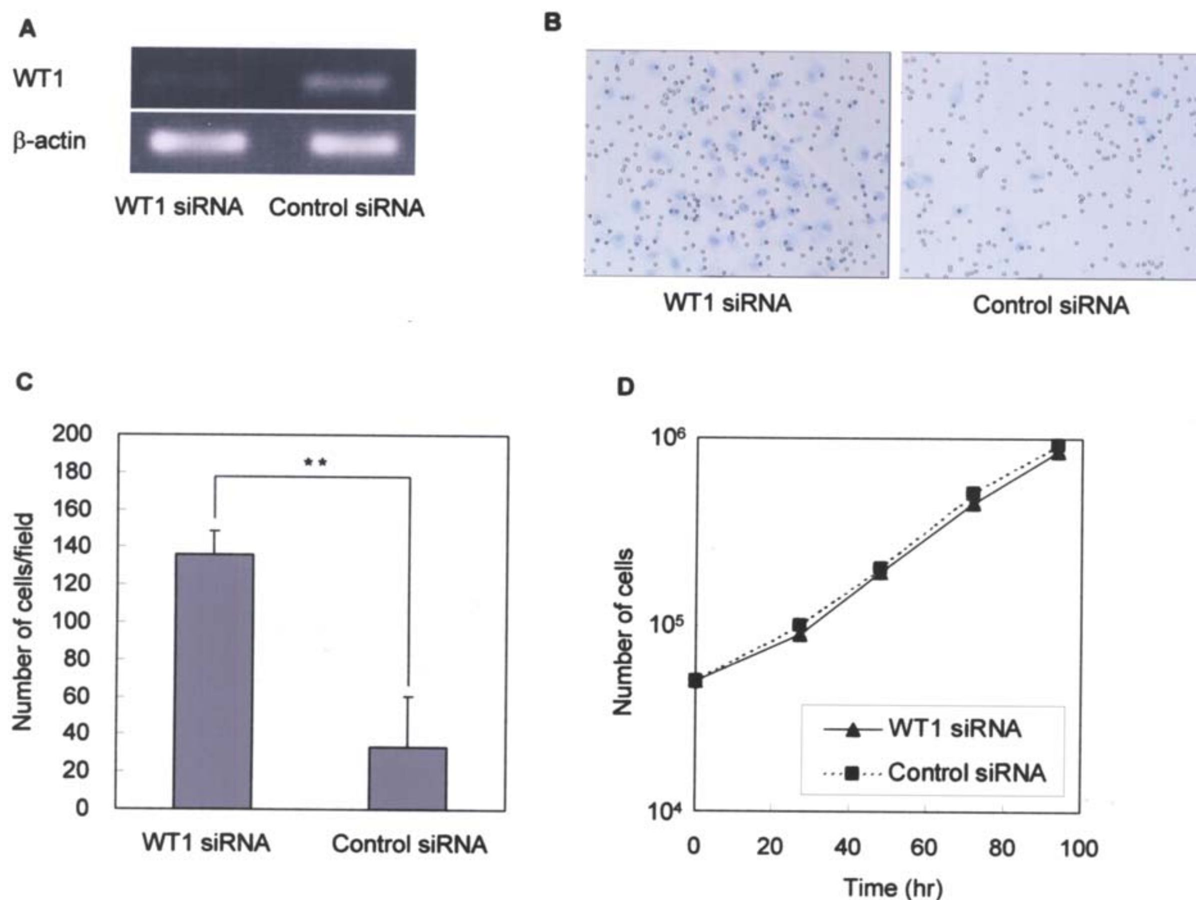

Figure 1. Effect of WT1-specific siRNA treatment on the invasiveness of RERF-LC-AI cells. (A) Down-regulation of *WT1*. Total RNA derived from RERF-LC-AI cells transfected with *WT1*-specific siRNA (WT1 siRNA) or negative control siRNA (control siRNA) were subjected to RT-PCR. (B) Invasion into Matrigel. Diff-Quick staining showed the invasion of cells transfected with WT1 siRNA or control siRNA in an invasion assay. Cells were collected 24 h after transfection, dispersed in FBS-free DMEM, and added to the insert. After 22 h, non-invading cells were removed from the upper filter surface and the filter washed, fixed and stained. (C) Quantification of invasiveness. WT1 siRNA-treatment of cells led to a significant increase in the number of invading cells compared with control siRNA-treated cells. **$P<0.01$. (D) Effect on RERF-LC-AI cell growth. Growth curves of cells transfected with WT1 siRNA or control siRNA showed no significant differences.

(Invitrogen). To monitor transfection efficiency, 10 ng pRL-CMV vector was co-transfected with each sample. After 48 h, luciferase reporter activity was measured according to the manufacturer's instructions.

Statistical analysis. Experiments were repeated at least three times. Statistical analysis was performed using Student's t-test. Differences were considered significant at $P<0.05$.

Results

Increased invasiveness of RERF-LC-AI cells following inhibition of WT1 expression. Effects of altered WT1 expression on invasion and cell growth were examined. Transfection of RERF-LC-AI lung SCC cells with *WT1*-specific siRNA (WT1 siRNA) resulted in decreased levels of *WT1* expression compared to cells transfected with negative control siRNA (control siRNA) (Fig. 1A). To assay for invasiveness, siRNA-treated cells were incubated in transwell plates coated with Matrigel for 22 h to allow cell invasion. WT1 siRNA-treated cells showed significant increases in invading cell numbers compared with control siRNA-treated cells ($P<0.01$) (Fig. 1B and C). WT1 siRNA treatment caused no significant changes in cell growth compared to the control siRNA treatment (Fig. 1D).

Expression analysis by microarray. As significant changes in invasiveness were observed in WT1 siRNA-treated cells (Fig. 2A), the effects of altered WT1 levels on patterns of gene expression were examined. Total RNA extracted from cells 24 h after WT1 siRNA treatment or control siRNA treatment was subjected to DNA microarray analysis, which was repeated using a dye-swap. Changes in gene expression were considered meaningful if expression levels varied by more or less than the mean ± 2SD over all analyzed genes for both dye configurations. By these criteria, 11 genes were up-regulated over the mean + 2SD (Table I), while 23 genes were down-regulated under the mean - 2SD (Table II). Cyclin-dependent kinase inhibitor 1A ($p21^{Waf1/Cip1}$) was selected for further analysis, and down-regulation of $p21^{Waf1/Cip1}$ expression in WT1 siRNA-treated cells was confirmed by real-time RT-PCR (Fig. 2B).

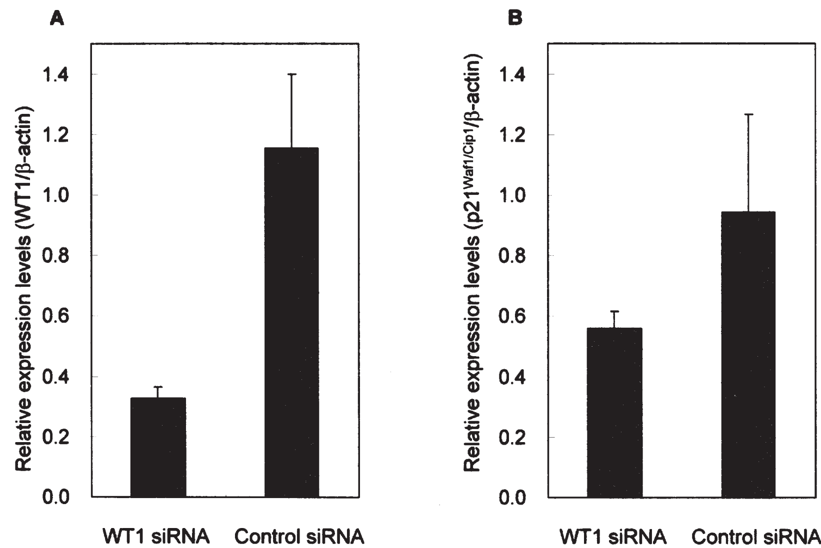

Figure 2. *WT1* and $p21^{Waf1/Cip1}$ expression in *WT1*-specific siRNA-transfected RERF-LC-AI cells. (A) *WT1* expression was quantified by real-time RT-PCR in RERF-LC-AI cells transfected with WT1 siRNA or control siRNA as in Fig. 1. (B) $p21^{Waf1/Cip1}$ expression. Down-regulation of $p21^{Waf1/Cip1}$ in WT1 siRNA-transfected RERF-LC-AI cells was confirmed.

Table I. Genes up-regulated above the mean + 2SD in *WT1* down-regulated cells.

Gene name	Symbol	Accession no.	Gene ID	Location	Median	Function
secretogranin III	SCG3	NM_013243	29106	15q21	2.625	Other
hypothetical protein xp_051447; loc95256	-	-	-	-	2.133	Unknown
h4 histone family, member c	HIST1H4F	NM_003540	8361	6p21.3	1.912	Other
aspartate ß-hydroxylase	ASPH	NM_004318	444	8q12.1	1.909	Signal transduction
tropomyosin 4	TPM4	NM_003290	7171	19p13.1	1.860	Cytoskeleton/cell membrane-linked
acetyl-Coenzyme A carboxylase α	ACACA	NM_198834	31	17q21	1.776	Metabolism
hypothetical protein xp_030958; loc90333	-	-	-	-	1.759	Unknown
hypothetical protein mgc5384; mgc5384	-	-	-	-	1.749	Unknown
chorionic somatomammotropin hormone 1 (placental lactogen)	CSH1	NM_001317	1442	17q24.2	1.722	Signal transduction
chromosome 11 open reading frame 16	C11orf16	NM_020643	56673	11p15.3	1.637	Unknown
WD repeat domain 5B	WDR5B	NM_019069	54554	3q21.1	1.596	Signal transduction

Increased invasiveness via inhibition of $p21^{Waf1/Cip1}$ expression. To confirm the involvement of $p21^{Waf1/Cip1}$ in invasion, $p21^{Waf1/Cip1}$-specific siRNA ($p21^{Waf1/Cip1}$ siRNA)-transfected RERF-LC-AI cells were compared to control siRNA-treated cells in the invasion assay. Transfection with $p21^{Waf1/Cip1}$ siRNA led to decreased $p21^{Waf1/Cip1}$ expression levels (Fig. 3A), and significantly increased invading cell numbers compared with control siRNA-treated cells ($P<0.01$) (Fig. 3B and C). No significant differences in cell growth were observed between the $p21^{Waf1/Cip1}$ siRNA-treated and control siRNA-treated RERF-LC-AI cells (Fig. 3D).

Cloning of WT1 isoforms. To examine the effect on endogenous $p21^{Waf1/Cip1}$ expression in RERF-LC-AI cells after exposure to different WT1 isoforms, 8 *WT1* isoform cDNA sequences (Fig. 4A) were cloned and sequence-confirmed. Each isoform was named according to a scheme whereby WT1 $17AA^+/KTS^+$ or WT1s $17AA^-/KTS^-$ referred to 'WT1' as the translational start from the authentic initiator AUG, while 'WT1s' referred to translation from the downstream in-frame AUG 127 codon; '$17AA^+$' indicated insertion of exon 5; '$17AA^-$', deletion of exon 5; 'KTS^+', insertion of KTS at the end of exon 9, and 'KTS^-', deletion of KTS. Each cDNA was inserted into the

Table II. Genes down-regulated below the mean - 2SD in *WT1* down-regulated cells.

Gene name	Symbol	Accession no.	Gene ID	Location	Median	Function
hairy and enhancer of split 1, (Drosophila)	HES1	NM_005524	3280	3q28-q29	0.370	Transcription and processing
dj345p10.3 (3' end of a novel gene)	-	-	-	-	0.437	Unknown
v-abl Abelson murine leukemia viral oncogene homolog 2	ABL2	NM_005158	27	1q24-q25	0.440	Signal transduction
growth arrest and DNA-damage-inducible, α	GADD45A	NM_001924	1647	1p31.2-p31.1	0.469	Cell-cycle regulation
UDP-N-acetyl-α-D-galactosamine:polypeptide-N acetylgalactosaminyl-transferase 14 (GalNAc-T14)	GALNT14	NM_024572	79623	2p23.1	0.494	Other
chemokine (C-X-C motif) ligand 9	CXCL9	NM_002416	4283	4q21	0.499	Signal transduction
MCF.2 cell line derived transforming sequence	MCF2	NM_005369	4168	Xq27	0.521	Signal transduction
neuronal PAS domain protein 3	NPAS3	NM_022123	64067	14q12-q13	0.541	Transcription and processing
polycystic kidney disease 2-like 2	PKD2L2	NM_014386	27039	5q31	0.542	Cytoskeleton/cell membrane-linked
fibroblast growth factor receptor substrate 2	FRS2	NM_001042555	10818	12q15	0.548	Cell differentiation
spermidine/spermine N1-acetyltransferase	SAT	NM_002970	6303	Xp22.1	0.560	Metabolism
tetraspanin 16	TSPAN16	NM_012466	26526	19p13.2	0.561	Cytoskeleton/cell membrane-linked
cyclin-dependent kinase inhibitor 1A ($p21^{Waf1/Cip1}$)	CDKN1A	NM_000389	1026	6p21.2	0.565	Cell-cycle regulation
hypothetical protein pro1097; pro1097	-	-	-	-	0.572	Unknown
α-1,3-d-mannoside ß-1,4-n-acetylglucosaminyltransferase iv-homologue	-	-	-	-	0.576	Unknown
sodium channel, voltage-gated, type X, α	SCN10A	NM_006514	6336	3p22-p21	0.582	Cytoskeleton/cell membrane-linked
protein kinase (cAMP-dependent, catalytic) inhibitor α	PKIA	NM_006823	5569	8q21.11	0.583	Signal transduction
hypothetical protein xp_043007; loc92132	-	-	-	-	0.584	Unknown
connective tissue growth factor	CTGF	NM_001901	1490	6q23.1	0.585	Cell adhesion/ surface-linked
retinoic acid receptor responder (tazarotene induced) 1	RARRES1	NM_002888	5918	3q25.32	0.605	Cell proliferation
armadillo repeat containing 4	ARMC4	NM_018076	55130	10p12.1-p11.23	0.607	Cytoskeleton/cell membrane-linked
G protein-coupled receptor 45	GPR45	NM_007227	11250	2q11.1-q12	0.613	Signal transduction
putative GR6 protein	C3orf27	NM_007354	23434	3q21	0.636	Unknown

pCMVFL3 expression vector, and resultant constructs transfected into RERF-LC-AI cells transiently using Lipofectamine 2000 (Invitrogen). WT1 expression was confirmed by Western blotting (Fig. 4B).

Up-regulation of $p21^{Waf1/Cip1}$ expression by WT1 isoforms. Changes in endogenous *$p21^{Waf1/Cip1}$* expression were examined by real time RT-PCR (Fig. 5). *$p21^{Waf1/Cip1}$* mRNA levels were significantly up-regulated in four transfectants: WT1s $17AA^{+}$/

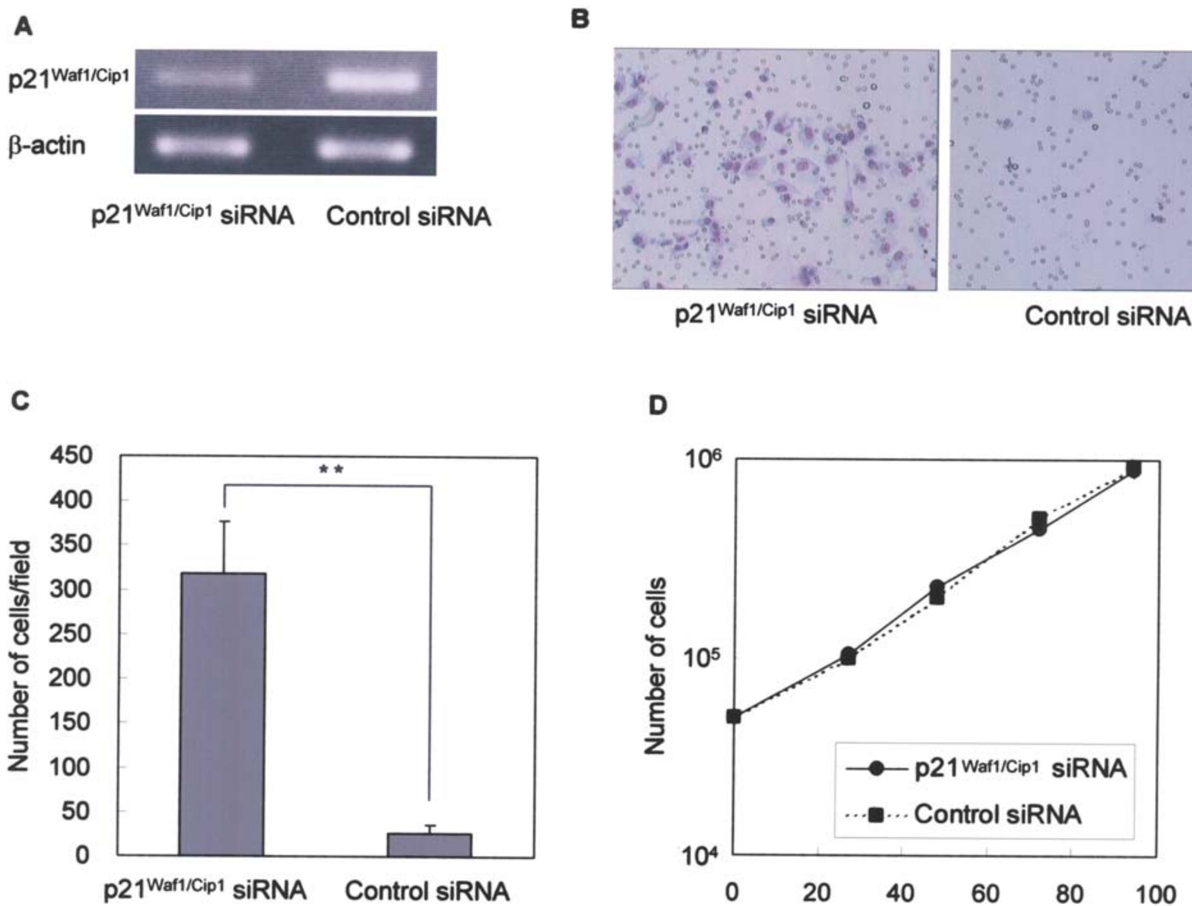

Figure 3. Effect of $p21^{Waf1/Cip1}$-specific siRNA treatment on the invasiveness of RERF-LC-AI cells. (A) Down-regulation of $p21^{Waf1/Cip1}$. Total RNA derived from RERF-LC-AI cells transfected with $p21^{Waf1/Cip1}$-specific siRNA ($p21^{Waf1/Cip1}$ siRNA) or negative control siRNA (control siRNA) were subjected to RT-PCR. (B) Invasion into Matrigel. Diff-Quick staining showed the invasiveness of cells transfected with $p21^{Waf1/Cip1}$ siRNA or control siRNA in an invasion assay. Cells were collected 24 h after transfection, dispersed in FBS-free DMEM, and added to the insert. After 22 h, non-invading cells were removed from the upper filter surface and the filter was washed, fixed and stained. (C) Quantification of invasiveness. Compared with control siRNA-treated cells, $p21^{Waf1/Cip1}$ siRNA-treated cells showed significantly increased numbers of invading cells. $^{**}P<0.01$. (D) Effect on RERF-LC-AI cell growth. Growth curves of cells transfected with $p21^{Waf1/Cip1}$ siRNA or control siRNA showed no significant differences.

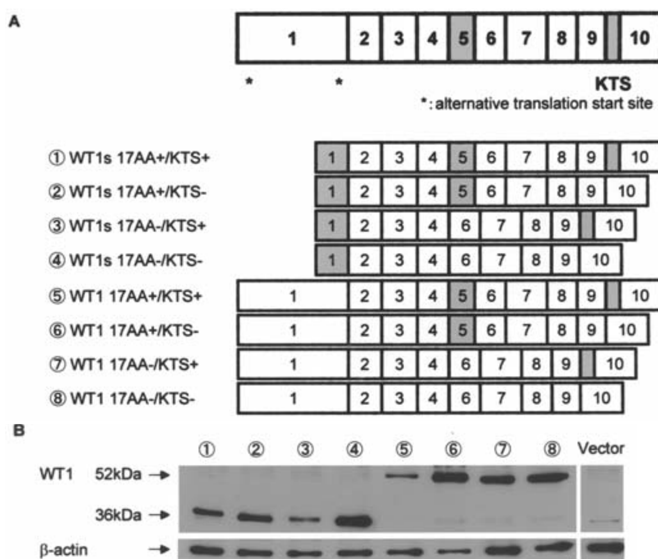

Figure 4. cDNA cloning of WT1 isoforms and protein expression. (A) Scheme used for the cloning of the WT1 isoforms. The eight WT1 isoforms were cloned into the pCMVFL3 expression vector. (B) WT1 isoform expression. RERF-LC-AI cells transiently transfected with each isoform WT1 expression vector or the empty expression vector pCMVFL3 (vector) were subjected to Western blot analysis.

KTS^- ($P<0.05$), WT1s $17AA^-/KTS^-$ ($P<0.05$), WT1 $17AA^+/KTS^-$ ($P<0.05$) and WT1s $17AA^-/KTS^-$ ($P<0.05$), compared with vector only-transfected cells, with no significant change in expression observed for the WT1s $17AA^+/KTS^+$, WT1s $17AA^-/KTS^+$, WT1 $17AA^+/KTS^+$, or WT1s $17AA^-/KTS^+$ isoforms. The four isoforms that led to $p21^{Waf1/Cip1}$ up-regulation commonly lacked the KTS domain situated at the end of exon 9, such that the alternative translation initiation sites and inclusion or exclusion of exon 5 did not appear to result in significant differences in expression.

Transactivation of the $p21^{Waf1/Cip1}$ promoter by WT1 isoforms. The transcriptional activity of the WT1 isoforms was compared according to the ability to transactivate a reporter gene in RERF-LC-AI cells that had been transiently transfected with the WT1 isoforms (Fig. 6). The WT1s $17AA^+/KTS^-$ ($P<0.01$), WT1s $17AA^-/KTS^-$ ($P<0.05$), WT1 $17AA^+/KTS^-$ ($P<0.01$) and WT1 $17AA^-/KTS^-$ ($P<0.05$) isoforms were significantly more effective than the empty vector in transactivation of the $p21^{Waf1/Cip1}$ promoter-driven reporter gene. In contrast, WT1s $17AA^+/ KTS^+$, WT1s $17AA^-/KTS^+$, WT1 $17AA^+/KTS^+$ and WT1 $17AA^-/KTS^+$ isoforms exhibited no significant differences compared to the control. Therefore, only the -KTS

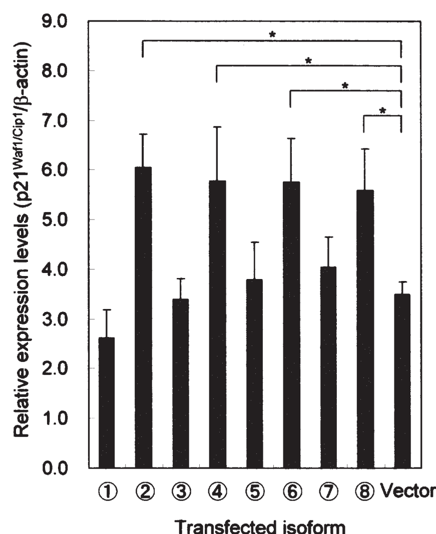

Figure 5. Effect of WT1 isoforms on endogenous *p21*$^{Waf1/Cip1}$ expression. *p21*$^{Waf1/Cip1}$ expression was quantified by real-time RT-PCR. *p21*$^{Waf1/Cip1}$ mRNA level in non-treated RERF-LC-AI cells was defined as 1.0. *p21*$^{Waf1/Cip1}$ mRNA levels were significantly up-regulated by -KTS isoforms: (2) WT1s 17AA$^+$/KTS$^-$, (4) WT1s 17AA$^-$/KTS$^-$, (6) WT1 17AA$^+$/KTS$^-$ and (8) WT1s 17AA$^-$/KTS$^-$. *P<0.05.

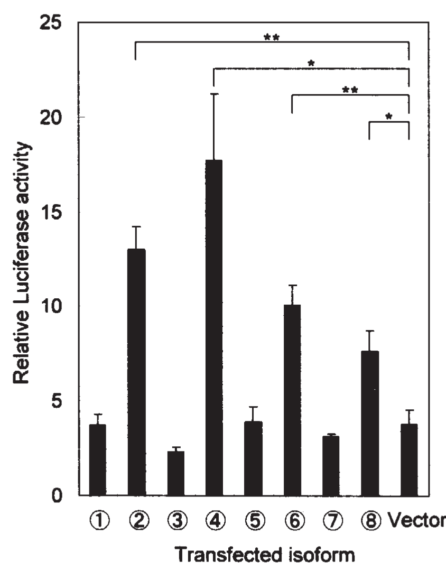

Figure 6. Transactivation of *p21*$^{Waf1/Cip1}$ promoter by WT1 isofoms. RERF-LC-AI cells were transfected with luciferase reporter constructs driven by the *p21*$^{Waf1/Cip1}$ promoter and WT1 isoform expression constructs or the empty expression vector pCMVFL3 (vector). WT1 -KTS isoforms [(2) WT1s 17AA$^+$/KTS$^-$, (4) WT1s 17AA$^-$/KTS$^-$, (6) WT1 17AA$^+$/KTS$^-$ and (8) WT1 17AA$^-$/KTS$^-$] significantly transactivated reporter gene expression. *P<0.05, **P<0.01.

isoforms significantly induced transactivation of the *p21*$^{Waf1/Cip1}$ promoter, while translation initiation site and presence or absence of exon 5 had no effect on transactivating activity.

Discussion

The *WT1* gene was first isolated as a tumor suppressor gene responsible for Wilms' tumor (1). However, recent studies have shown that the wild-type *WT1* gene is overexpressed in several types of leukemia (2-4) and solid tumors (5-14), and so may also have some oncogenic functions. The WT1 protein contains four zinc-finger domains and is involved in the transcriptional regulation of various genes (1). The presence of several WT1 isoforms has been reported and complicates explanations for its possible functions (1,15).

The *WT1* transcript is spliced alternatively at two sites: the 17AA site, which consists of exon 5, and the KTS site, located between zinc fingers 3 and 4. Alternative splicing at these sites yields four isoforms (17AA$^+$/KTS$^+$, 17AA$^+$/KTS$^-$, 17AA$^-$/KTS$^+$ and 17AA$^-$/KTS$^-$). In addition to these WT1 isoforms, smaller WT1 isoforms have been described that are derived from internal translation initiation at the in-frame AUG 127 codon downstream of the initiator AUG. This generates a smaller isoform for each of the four isoforms described above (15). Lastly, a short transcript that lacks the 147 N-terminal amino acids has also been reported (16). While these isoforms are expressed in various tumors, the function of each isoform in cancer cells remains unclear.

The *WT1* gene is reported to be involved in the suppression of cancer cells. Transfection of four wild-type *WT1* isoforms (17AA$^+$/KTS$^+$, 17AA$^+$/KTS$^-$, 17AA$^-$/KTS$^+$, 17AA$^-$/KTS$^-$) suppressed the growth of RM1 cells derived from Wilms' tumor (17), while the 17AA$^-$/KTS$^-$ isoform suppressed the growth of ras-transformed NIH3T3 cells *in vitro* and *in vivo* (18). However, there are several reports that *WT1* can also function as an oncogene. For example, the growth of *WT1*-expressing leukemia and solid cancer cells can be inhibited by treatment with WT1 antisense oligomers. Ambiguity in the function of *WT1* is due at least in part to the lack of knowledge around its target genes as a regulator of transcription. Recently, Jomgeow *et al* demonstrated that the WT1 17AA$^-$/KTS$^-$ isoform induced morphological changes, allowing cancer cells to acquire a more invasive phenotype *in vitro*, and that these phenotypic changes were induced through the altered expression of cytoskeletal regulatory proteins such as a-actinin 1, cofilin and gelsolin (19).

Examining the effect of constitutive expression of each of the WT1 isoforms in various cancer cell types can be used to find the target genes of WT1. In the present study, we carried out microarray analysis on human lung SCC cells treated with specific siRNA for WT1, and found that *WT1* gene down-regulation caused decreased expression of 23 genes, including the connective tissue growth factor gene (CTGF) (20) and the p53-regulated growth arrest and DNA damage-inducible gene (GADD45A) (21), which have been reported as metastasis-suppressor genes. These results suggested that invasiveness in lung squamous cell carcinoma is controlled by WT1 through the regulation of several genes.

We focused on the *p21*$^{Waf1/Cip1}$ gene for further analysis, as *p21*$^{Waf1/Cip1}$ was found to be transactivated by the WT1 17AA$^+$/KTS$^-$ isoform (16) and thought to be involved in the metastasis of colorectal carcinomas (22). p21$^{Waf1/Cip1}$ siRNA treatment caused no apparent change in cell growth, although p21$^{Waf1/Cip1}$ is known as a cell cycle inhibitor. Sequence analysis revealed no mutations in any of the *p21*$^{Waf1/Cip1}$ exons in the RERF-LC-AI cell line (data not shown). Weiss and Randour (23) showed no significant growth inhibition by treatment of p53-inactivated human SCC A431 cells with antisense p21$^{Waf1/Cip1}$ oligodeoxynucleotides. RERF-LC-AI cells are also p53-inactivated,

as direct sequencing of p53 exon 4 indicated a C/T mutation at codon 104 (data not shown) that results in the change of a glutamine to a stop codon. Thus, low-level $p21^{Waf1/Cip1}$ may play a function in addition to the control of cell growth.

In this study, $p21^{Waf1/Cip1}$ siRNA treatment enhanced invasion *in vitro*, consistent with the notion that $p21^{Waf1/Cip1}$ inhibits invasion. Interaction between $p21^{Waf1/Cip1}$ and Rho kinase (ROCK) is of interest as there is increasing evidence that Rho proteins are overexpressed in tumors (24-26), and overexpression of RhoC causes experimental metastasis in A375 melanoma cells (27). Rho signaling triggered by ROCK promotes cell motility through reorganization of actin filaments (28), and ROCK is thought to play an essential role in tumor cell invasion, since a specific ROCK inhibitor blocks invasive activity (29). However, cytoplasmic $p21^{Waf1/Cip1}$ forms a complex with ROCK and inhibits its activity (30,31), while Coqueret (32) suggested that cytoplasmic $p21^{Waf1/Cip1}$ inhibits cell migration. Therefore, it is possible that RERF-LC-AI cell invasiveness is also inhibited by $p21^{Waf1/Cip1}$-ROCK complexes.

In summary, down-regulated *WT1* expression enhanced the invasiveness of lung SCC RERF-LC-AI cells. Down-regulation of $p21^{Waf1/Cip1}$, selected as a target gene of WT1 by DNA microarray analysis, also enhanced RERF-LC-AI cell invasiveness. Endogenous $p21^{Waf1/Cip1}$ expression was up-regulated in cells transfected with either of the four WT1 -KTS isoforms. These four -KTS isoforms transactivated the $p21^{Waf1/Cip1}$ promoter-driven reporter gene expression. Thus, it appears that the WT1 -KTS isoforms suppress the invasiveness of lung SCC through the transactivation of $p21^{Waf1/Cip1}$.

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