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Expression of the Wilms' Tumor Gene *WT1* in Solid Tumors and Its Involvement in Tumor Cell Growth

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To determine the role of the Wilms' tumor gene *WT1* in tumorigenesis of solid tumors, expression of the *WT1* gene was examined in 34 solid tumor cell lines (four gastric cancer cell lines, five colon cancer cell lines, 15 lung cancer cell lines, four breast cancer cell lines, one germ cell tumor cell line, two ovarian cancer cell lines, one uterine cancer cell line, one thyroid cancer cell line, and one hepatocellular carcinoma cell line) by means of quantitative reverse transcriptase-polymerase chain reaction. *WT1* gene expression was detected in three of the four gastric cancer cell lines, all of the five colon cancer cell lines, 12 of the 15 lung cancer cell lines, two of the four breast cancer cell lines, the germ cell tumor cell line, the two ovarian cancer cell lines, the uterine cancer cell line, the thyroid cancer cell line, and the hepatocellular carcinoma cell line. Therefore, of the 34 solid tumor cell lines examined, 28 (82%) expressed *WT1*. Three cell lines expressing *WT1* (gastric cancer cell line AZ-521, lung cancer cell line OS3, and ovarian cancer cell line TYK-nu) were further analyzed for mutations and/or deletions in the *WT1* gene by means of single-strand conformation polymorphism analysis. However, no mutations or deletions were detected in the region of the *WT1* gene ranging from the 3′ end of exon 1 to exon 10 (the *WT1* gene consists of 10 exons) in these three cell lines. Furthermore, when AZ-521, OS3, and TYK-nu cells were treated with WT1 antisense oligomers, the growth of these cells was significantly inhibited in association with a reduction in WT1 protein levels. Furthermore, constitutive expression of the transfected *WT1* gene in cancer cells inhibited the antisense effect of WT1 antisense oligomer on cell growth. These results indicated that the *WT1* gene plays an essential role in the growth of solid tumors and performs an oncogenic rather than a tumor-suppressor gene function.

Key words: Wilms' tumor gene — *WT1* — Tumor suppressor gene — Solid tumors

The Wilms' tumor gene *WT1* was isolated as a tumor-suppressor gene responsible for Wilms' tumor, a kidney neoplasm of childhood.[1, 2] The *WT1* gene encodes a zinc finger transcription factor that represses transcription of growth factor (PDGF-A chain, CSF-1, and IGF-II)[3–5] and growth factor receptor (*IGF-IR*)[6] genes and other genes (*RAR-α*, c-*myc*, and *bcl-2*).[7, 8]

We have previously reported high expression of wild-type *WT1* in fresh leukemia cells regardless of the disease type,[9] an inverse correlation between *WT1* expression levels and prognosis,[9] increased *WT1* expression at relapse in acute leukemia,[10] inhibition of leukemia cell growth by WT1 antisense oligomers,[11] and blocking of differentiation but induction of proliferation by constitutive expression of the *WT1* gene in 32D cl3 myeloid progenitor cells.[12] These results suggested that the *WT1* gene plays an essential role in leukemogenesis and performs an oncogenic rather than a tumor-suppressor gene function in hematopoietic progenitor cells.

Expression of the *WT1* gene in solid tumors was identified in 10 of 40 ovarian tumors,[13] in seven of 10 granulosa cell tumors[14] and in two of three Leydig cell tumors.[14] Furthermore, *WT1* expression was also found in 16 of 19 mesothelioma cell lines and in five of eight malignant mesothelioma tumors.[15, 16] Normal tissue expression of the *WT1* gene is restricted to gonads, uterus, kidney, and mesothelial structures.[15] Therefore, *WT1* expression in solid tumors which are derived from gonads or mesothelial structures is not surprising.

The present study was performed to test our hypothesis that the *WT1* gene performs an oncogenic function not only in leukemogenesis, but also in the tumorigenesis of solid tumors. *WT1* gene expression was examined in 34 solid tumor cell lines and detected in 28 (82%). Furthermore, suppression of *WT1* gene expression by WT1 antisense oligomers inhibited tumor cell growth, suggesting an oncogenic function of the *WT1* gene in solid tumors.

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MATERIALS AND METHODS

Cell lines Human gastric cancer cell lines (AZ-521, MKN1, and MKN28), human lung cancer cell lines (VMRC-LCP, LU99C, LU99B, RERF-LC-AI, CADO LC6, RERF-LC-MS, LU65B, LC-1F, LU65A, LC-2/ad, LC-1/sq, and PC-14), a human breast cancer cell line (YMB-1), a human germ cell tumor cell line (NEC8), human ovarian cancer cell lines (TYK-nu and TYK-nu.CP-r), a human uterine cancer cell line (HeLa AG), a human thyroid cancer cell line (8505C), and a human hepatocellular carcinoma cell line (HepG2) were kindly provided by Health Science Research Resources Bank (Tokyo). Human gastric cancer cell line GCIY was obtained from RIKEN Cell Bank (Tokyo). Human colon cancer cell lines, SW480, SW620, COLO320DM, LoVo, and HT29 were kindly given by Dr. M. Tsujie (Osaka University Medical School, Osaka). Human lung cancer cell lines, OS1, OS2R, and OS3 were kindly given by Dr. S. Hosoe (Osaka University Medical School). Human breast cancer cell lines, MDAMB231, T47D, and ZR75-1 were kindly provided by Dr. M. Koga (Osaka University Medical School). MKN1, MKN28, LU99C, LU99B, LU65B, LU65A, NEC8, SW480, SW620, COLO320DM, LoVo, and HT29 were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS). The remaining cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS.

RNA purification and reverse transcriptase-polymerase chain reaction (RT-PCR) Total RNA was isolated according to the acid-guanidine-phenol-chloroform method as described previously,[9] dissolved in diethylpyrocarbonate-treated water and quantitated spectrometrically based on the absorbance at 260 nm.

RNA was converted into cDNA as described previously[9] with a minor modification. In brief, 2 μg of total RNA in 12.5 μl of diethylpyrocarbonate-treated water was heated at 65°C for 5 min and then mixed with 17.5 μl of RT buffer (50 m*M* Tris-HCl [pH 8.3]; 70 m*M* KCl; 3 m*M* $MgCl_2$; 10 m*M* dithiothreitol) containing 600 U of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL, Gaithersburg, MD), 500 μ*M* of each deoxynucleotide triphosphate, 750 ng of oligo dT primers and 40 U of RNase inhibitor (Boehringer Mannheim, Mannheim, Germany). The reaction mixture was incubated at 37°C for 90 min, heated at 100°C for 5 min, and then stored at −20°C until use. PCR was performed for optimized cycles as described below with a DNA thermal cycler under the following conditions: denaturation at 94°C for 1 min, primer annealing at 64°C for 1 min, and then chain elongation at 72°C for 1.5 min. PCR products were separated in 1.3% agarose gels containing 0.05 μg/ml of ethidium bromide, and photographed with Polaroid 665 film. The negative film was developed at 25°C for 5 min, and band density (densitometric units) was measured by a densitometer (Image Quant, produced by Molecular Dynamics, Sunnyvale, CA). Optimal conditions for PCR to quantitate *WT1* expression levels were determined as follows. PCR was performed for various cycles using *WT1* primers (sense primer for exon 7, 5′-GGCATCTGAGACCAGTGAGAA-3′; antisense primer for exon 10, 5′-GAGAGTCAGACTTGAAAGCAGT-3′) with serial dilutions of the cDNA prepared from total RNA of K562 human leukemic cells which highly express *WT1*. PCR amplification for 35, 29, 25, and 22 cycles was exponential from 8×10^{-4} to 8×10^{-2} ng of RNA (equivalent to 10^{-5}–10^{-3} levels when *WT1* expression level of K562 leukemic cells was defined as 1.0), from 8×10^{-2} to 8×10^{-1} ng of RNA (10^{-3}–10^{-2} levels), from 8×10^{-1} to 8×10^{0} ng of RNA (10^{-2}–10^{-1} levels) and from 8×10^{0} to 8×10^{1} ng of RNA (10^{-1}–10^{0} levels), respectively. Therefore, PCR was performed for 35, 29, 25, or 22 cycles according to the *WT1* expression levels in the cell lines under exponential amplification conditions. Calibration curves to quantitate *WT1* expression levels are shown in Fig. 1, A–D. Similarly, to determine optimal conditions for PCR to quantitate *β-actin* expression levels, PCR was performed for various cycles using *β-actin* primers (sense primer, 5′-GTGGGGCGCCCCAGGCACCCA-3′; antisense primer, 5′-GTCCTTAATGTCACGCACGATTTC-3′) with serial dilutions of the cDNA prepared from total RNA of K562 leukemic cells. Expo-

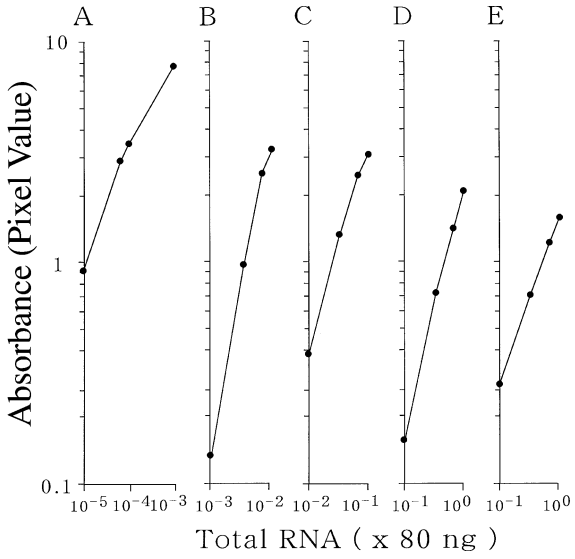

Fig. 1. Calibration curves for the quantitation of various levels of *WT1* expression (A–D) or for the quantitation of expression the *β-actin* gene (E). For calibration curves, serially diluted cDNA samples from K562 cells were amplified by PCR. Optimal calibration curves were used according to the *WT1* expression levels in cell lines. Pixel value was used as a densitometric unit.

nential amplification was observed for 16 cycles of PCR in the range from 8×10^0 to 8×10^1 ng of RNA, within which *β-actin* expression levels in all cell lines were included, as shown in Fig. 1E. To normalize the differences in RNA degradation for individual samples and in RNA loading for RT-PCR, the value of *WT1* gene expression divided by that of *β-actin* gene expression was defined as the *WT1* expression level in the samples. Calibration curves were obtained in every experiment and the *WT1* expression levels were quantitated according to the curves. The expression level of the *WT1* gene in K562 leukemic cells was defined as 1.0, and the values of *WT1* expression level in the samples are given relative to that in K562 cells.

Single-strand conformational polymorphism (SSCP) analysis of *WT1* cDNA PCR primers used for PCR-SSCP analysis are shown in Fig. 4. PCR was performed in the presence of [α-^{32}P]deoxycytidine triphosphate (Amersham, Buckinghamshire, UK). Thirty-five cycles of amplification were carried out, each cycle consisting of denaturation at 94°C for 1 min, primer annealing at optimized temperature for 1 min, and chain elongation at 72°C for 1.5 min. After PCR amplification, aliquots of 5 μl were mixed with 45 μl of loading buffer (95% formamide, 10 m*M* EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). The samples (1.5 μl) were denatured at 97°C for 5 min and rapidly cooled on ice. Samples were loaded onto a polyacrylamide gel (5% polyacrylamide, 5% glycerol, and 0.5× TBE [50 m*M* Tris, 49.5 m*M* boric acid, 1 m*M* EDTA]), and electrophoresis was carried out at 20°C for 150 min in 1× TBE buffer. The gel was dried and exposed to X-ray film (Fuji Corp., Tokyo) at −80°C for 6–18 h with an intensifying screen.

Oligodeoxynucleotides Unmodified oligodeoxynucleotides were synthesized by using an automated synthesizer (Applied Biosystems, Foster City, CA). The oligomers were purified by high-performance liquid chromatography (HPLC) and precipitated three times with ethanol, then resuspended in phosphate-buffered saline (PBS). The following sense (SE) and antisense (AS) *WT1* sequences were used[11]: SE1 (transcription cap 1 site), 5′-CCCACCGCATTCGACCCT-3′; AS1 (transcription cap 1 site), 5′-AGGGTCGAATGCGGTGGG-3′. Random sequences used as a control were 18-mer oligodeoxynucleotides, each deoxynucleotide of which was randomly synthesized.

Oligomer treatment of cells Oligomer treatment was performed as described previously[11] with a minor modification. Cells (5×10^3/well) were plated in a 96-well dish in Dulbecco's modified Eagle's medium without fetal bovine serum (FBS). The oligodeoxynucleotides were added to the culture medium in duplicate at the indicated concentration for each experiment, and 2 h later, FBS was added to the culture medium at a final concentration of 10%. The same oligodeoxynucleotides were added to each well to a final concentration equivalent to half of the initial concentration every 24 h for the days indicated for each experiment. The rate of cell growth was determined by counting the viable cells with the dye exclusion method. Each experiment was repeated more than three times.

Western blot analysis Western blot analysis was performed as described previously.[11] Cells were washed with PBS twice and lysed with Laemmli's sample buffer. The cell lysates were boiled for 5 min and applied to each lane in 7.5% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, the proteins were transferred to Immobilon polyvinylidene difluoride (Millipore Corp., Bedford, MA). The membrane was first probed with anti-*WT1* polyclonal antibody directed against a synthetic polypeptide (position 177 to 192: KHEDPMGQQGSLGEQQ) and then with horseradish peroxidase-linked anti-immunoglobulin antibodies (Amersham, Little Chalfont, UK). The filter was washed, immersed in detection reagents (Amersham, Little Chalfont, UK) for 1 min and autoradiographed for 1 to 5 min. The same filter was washed twice with TBST (Tris-buffered saline containing 0.05% Tween 20), then probed with anti-actin monoclonal antibody (Oncogene Science Inc., Cambridge, MA) and autoradiographed in the same manner as mentioned above. The density of the bands corresponding to the WT1 protein and actin was measured with a CS-9000 densitometer (Shimadzu, Kyoto).

Statistical analysis The percent growth inhibition was determined from the number of cells in oligomer-treated plates as a percentage of that in untreated controls. The statistical significance of differences between the arithmetical means of test groups was assessed by use of the unpaired *t* test.

RESULTS

***WT1* expression in a variety of solid tumor cell lines** *WT1* expression levels were examined in a variety of solid tumor cell lines by means of quantitative RT-PCR (Fig. 1) and shown in comparison with the *WT1* expression level (defined as 1.0) in human leukemia cell line K562, which expresses a high level of *WT1* ($>10^{-1}$ level) (Table I and Fig. 2). One (AZ-521) of four gastric cancer cell lines expressed a high level of *WT1* and two others (MKN1 and MKN28) expressed low levels (10^{-3} to 10^{-5} levels). In the remaining cell line (GCIY), *WT1* expression was undetectable ($<10^{-5}$). Out of five colon cancer cell lines, two (SW480 and SW620) expressed high levels of *WT1* and the other two (COLO320DM and LoVo), intermediate levels (10^{-1} to 10^{-3} levels). The remaining one (HT29) expressed a low level of *WT1*. Out of the 15 lung cancer cell lines, one (VMRC-LCP), six (LU99C, OS3, LU99B, OS1, OS2R, and RERF-LC-AI), and five (CADO LC6,

Table I. *WT1* Expression Levels in Solid Tumor Cell Lines

Origin	No.	Cell line	Histology[a)]	*WT1* expression level	Reference
Gastric cancer	1	AZ-521	ad	1.2×10^{0}	29
	2	MKN1	ad-sq	1.3×10^{-4}	30
	3	MKN28	ad	9.0×10^{-5}	31
	4	GCIY	p.d.ad	$<10^{-5}$	32
Colon cancer	5	SW480	p.d.ad	2.3×10^{-1}	33
	6	SW620	p.d.ad	1.0×10^{-1}	33
	7	COLO320DM	ad	7.2×10^{-3}	34
	8	LoVo	w.d.ad	1.1×10^{-3}	35
	9	HT29	w.d.ad	2.0×10^{-4}	36
Lung cancer	10	VMRC-LCP	sq	4.9×10^{-1}	37
	11	LU99C	gc	3.4×10^{-2}	unpublished
	12	OS3	sc	3.1×10^{-2}	38
	13	LU99B	gc	2.9×10^{-2}	unpublished
	14	OS1	sc	1.6×10^{-2}	38
	15	OS2R	sc	8.3×10^{-3}	38
	16	RERF-LC-AI	sq	1.2×10^{-3}	39
	17	CADO LC6	sc	7.8×10^{-4}	40
	18	RERF-LC-MS	ad	7.3×10^{-4}	41
	19	LU65B	sc	3.8×10^{-4}	42
	20	LC-1F	sq	1.3×10^{-4}	43
	21	LU65A	sc	9.1×10^{-5}	42
	22	LC-2/ad	ad	$<10^{-5}$	44
	23	LC-1/sq	sq	$<10^{-5}$	43
	24	PC-14	ad	$<10^{-5}$	45
Breast cancer	25	YMB-1	w.d.ad	5.2×10^{-2}	46
	26	MDAMB231	p.d.ad	3.3×10^{-3}	47
	27	T47D	p.d.ad	$<10^{-5}$	48
	28	ZR75-1	ad	$<10^{-5}$	49
Germ cell tumor	29	NEC8	tc	5.8×10^{-3}	50
Ovarian cancer	30	TYK-nu	undif	4.5×10^{-1}	51
	31	TYK-nu.CP-r	undif	2.5×10^{-1}	51
Uterine cancer	32	HeLa AG	ep	1.5×10^{-4}	52
Thyroid cancer	33	8505C	undif	8.9×10^{-4}	53
Hepatocellular carcinoma	34	HepG2		6.2×10^{-4}	54

a) ad-sq, adenosquamous carcinoma; ad, adenocarcinoma; sq, squamous cell carcinoma; gc, giant cell carcinoma; sc, small cell carcinoma; p.d.ad, poorly differentiated adenocarcinoma; w.d.ad, well-differentiated adenocarcinoma; undif, undifferentiated carcinoma; tc, teratocarcinoma; ep, epitheloid carcinoma.

RERF-LC-MS, LU65B, LC-1F, and LU65A) expressed high, intermediate, and low levels of *WT1*, respectively. In the remaining three cell lines (LC-2/ad, LC-1/sq, and PC-14), *WT1* expression was undetectable. Of four breast cancer cell lines, two (YMB-1 and MDAMB231) expressed intermediate levels of *WT1*. In the remaining two cell lines (T47D and ZR75-1), *WT1* expression was undetectable. Both of the ovarian cancer cell lines (TYK-nu and TYK-nu.CP-r) expressed high levels of *WT1*, and one germ cell tumor cell line (NEC8) expressed an intermediate level. One uterine cancer cell line (HeLa AG), one thyroid cancer cell line (8505C), and one hepatocellular carcinoma cell line (HepG2) expressed low levels of *WT1*. Thus, of the 34 solid tumor cell lines examined, six cell lines (18%) expressed high, eleven (32%) intermediate, and eleven (32%) low levels of *WT1*. In the remaining six cell lines(18%), *WT1* expression was undetectable.

Relation between *WT1* expression level and histopathology of cancers Whether or not the *WT1* expression level in cancer cells correlated with the histopathology of the cancer was examined in the largest sample, i.e., lung cancer cell lines (Table I and Fig. 3). However, no clear

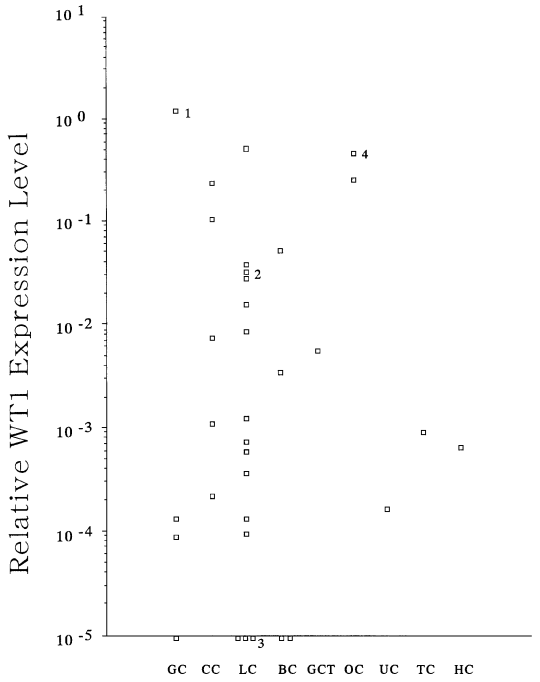

Fig. 2. Relative *WT1* expression levels in solid tumor cell lines. *WT1* expression levels are shown as relative values with the *WT1* expression level of human leukemic cells K562 defined as 1.0. GC, gastric cancer cell lines; CC, colon cancer cell lines; LC, lung cancer cell lines; BC, breast cancer cell lines; GCT, germ cell tumor cell lines; OC, ovarian cancer cell lines; UC, uterine cancer cell lines; TC, thyroid cancer cell lines; HC, hepatocellular carcinoma cell lines. 1, 2, 3, and 4, respectively, represent AZ-521, OS3, PC-14, and TYK-nu, which were selected for further studies.

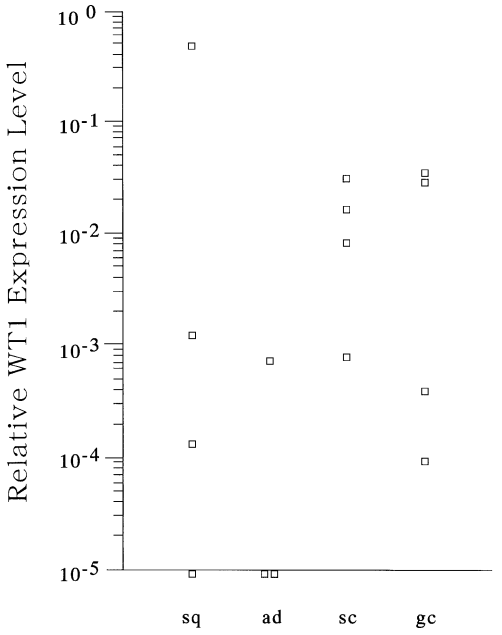

Fig. 3. No clear correlation between *WT1* expression level and the histopathology of lung cancers. sq, squamous cell carcinoma; ad, adenocarcinoma; sc, small cell carcinoma; gc, giant cell carcinoma.

correlation appeared to exist between *WT1* expression level and histopathology (squamous cell, giant cell, small cell, or adenocarcinoma). In colon cancers, however, *WT1* expression appeared to be higher in poorly differentiated than in well-differentiated adenocarcinoma, although this finding was not conclusive because of the small number of samples.

No detection of mutations or deletions in *WT1* transcripts To determine whether *WT1* transcripts expressed in cancer cells have deletions and/or mutations, SSCP analysis was performed for sequences from the 3′ end of exon 1 through to exon 10 in *WT1* transcripts from gastric cancer cell line AZ-521, lung cancer cell line OS3, or ovarian cancer cell line TYK-nu (Figs. 4 and 5). Sequences (the majority of exon 1) ranging from bp 381 to 780 could not be subjected to SSCP analysis because of their high GC content. When SSCP analysis was performed using six different primer pairs, which covered sequences from the 3′ end of exon 1 to the whole of exon 10, the SSCP patterns of *WT1* transcripts from these three cell lines were the same as those of wild-type *WT1* transcripts with all six primer pairs. Representative patterns of SSCP analysis are shown in Fig. 5. It should be noted that in SSCP analysis using primer pair E, SSCP patterns were formed by both KTS+ and KTS− *WT1* transcripts (Fig. 5B). These results thus showed that in these cell lines no mutations and/or deletions were detected from the 3′ end of exon 1 through to exon 10.

Growth inhibition of solid tumor cells by WT1 antisense oligonucleotides To determine whether *WT1* exerts an essential role in tumor cell growth, three cell lines (gastric cancer cell line AZ-521, lung cancer cell line OS3, and ovarian cancer cell line TYK-nu) expressing high or intermediate levels of *WT1* and one lung cancer cell line, PC14, which did not express detectable *WT1*, were treated with WT1 antisense oligomers (AS1) at a concentration of 400 μg/ml. As shown in Fig. 6, in the three cell lines expressing *WT1*, cell growth was significantly inhibited by WT1 antisense oligomers, but not by random or WT1 sense oligomer, whereas the growth of cells not expressing a detectable level of *WT1* was not inhibited by treatment with WT1 antisense oligomers. When WT1 antisense oligomers were added to the culture medium of AZ-521 cells at varying concentrations, they inhibited cell growth dependently on the concentration, whereas random or WT1 sense oligomer did not significantly inhibit the cell growth even at a high concentration

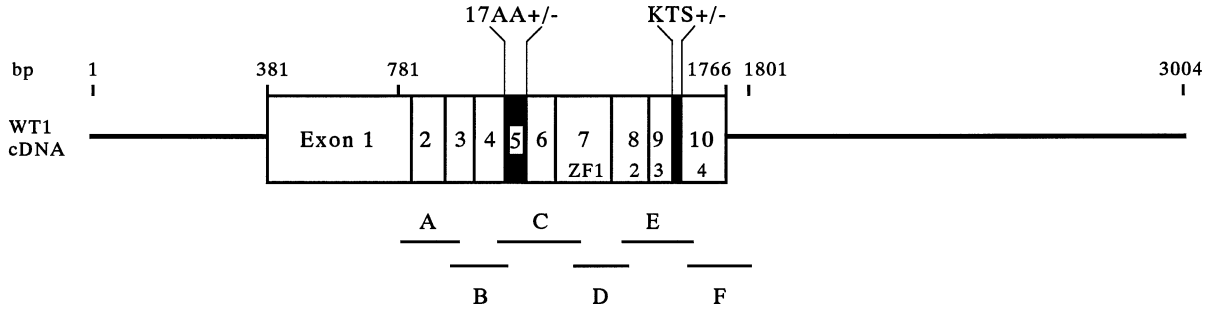

Fig. 4. Structure of *WT1* cDNA and regions of PCR-SSCP analysis. Exon 5, which encodes 17 amino acids (17AA), and the 3′ end of exon 9, which encodes three amino acids (KTS), are alternatively spliced, resulting in production of at least four different *WT1* transcripts (17AA+/KTS+, 17AA+/KTS–, 17AA–/KTS+ and 17AA–/KTS–). ZF1–4 represent four regions that encode four zinc finger domains. Primers for PCR-SSCP analysis of A–F regions. A, sense primer: 5′-TGCCCAGCTGCCTCGAGA-3′, antisense primer: 5′-ACCGAGTACTGCTGCTCACC-3′ (position 781–964 bp); B, sense primer: 5′-GGTGAGCAGCAGTACTCGGT-3′, antisense primer: 5′-GGCTCCTAAGTTCATCTGATT-3′ (position 945–1115 bp); C, sense primer: 5′-CAGCTTGAATGCATGACCTG-3′, antisense primer: 5′-TTCTCACTGGTCTCAGATGCC-3′ (position 1074–1339 bp); D, sense primer: 5′-GGCATCTGAGACCAGTGAGAA-3′, antisense primer: 5′-GAAGTCACACTGGTATGGTTTC-3′ (position 1319–1451 bp); E, sense primer: 5′-GAAACCATACCAGTGTGACTTC-3′, antisense primer: 5′-GACAGCTGAAGGGCTTTTCAC-3′ (position 1430–1629 bp); F, sense primer: 5′-GTGAAAAGCCCTTCAGCTGTC-3′, antisense primer: 5′-GACAGCTGAAGGGCTTTTCAC-3′ (position 1609–1801 bp).

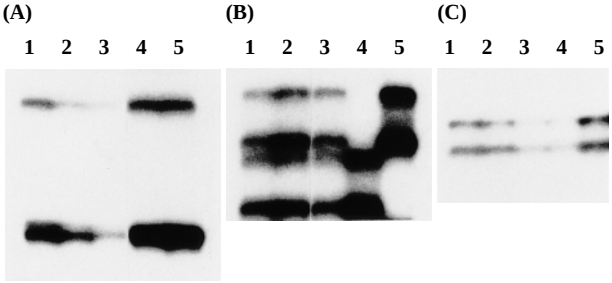

Fig. 5. PCR-SSCP analysis of *WT1* transcripts from solid tumor cells. PCR-SSCP analysis was performed as described in "Materials and Methods." Representative SSCP patterns are shown. (A) bp 945–1115 (region B in Fig. 4), (B) bp 1430–1629 (region E in Fig. 4), (C) bp 1609–1801 (region F in Fig. 4). 1, AZ-521; 2, TYK-nu; 3, OS3; 4, 17AA+/KTS+ nonspliced, wild-type *WT1* cDNA; 5, 17AA+/KTS– spliced, wild-type *WT1* cDNA.

(Fig. 7). Next, gastric cancer cell line AZ-521 expressing a high level of *WT1* was treated with WT1 antisense oligomer (AS1) at a concentration of 400 μg/ml and the number of viable cells was counted every 24 h (Fig. 8). Antisense effects were observed from day 3 of treatment and continued thereafter. These results showed that *WT1* plays an important role in tumor cell growth.

Reduction in WT1 protein levels by treatment with WT1 antisense oligomer To confirm that growth inhibition of cancer cells is due to the specific inhibitory effect of WT1 antisense oligomers, WT1 protein levels were analyzed after treatment with WT1 antisense oligomer

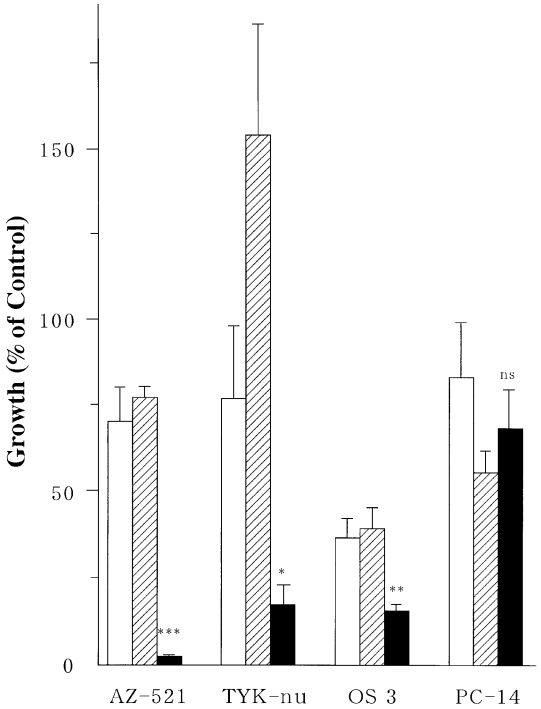

Fig. 6. Growth inhibition of solid tumor cells by WT1 antisense oligomers. AZ-521 gastric cancer-derived cells (*WT1* highly expressed), OS3 lung cancer-derived cells (*WT1* intermediately expressed), TYK-nu ovary cancer-derived cells (*WT1* highly expressed), and PC-14 lung cancer-derived cells (*WT1* undetectable) were treated with oligomers, and the number of viable cells was counted after 6 days. * $P<0.05$, ** $P<0.01$, *** $P<0.0001$. □ random oligomer, ▨ SE1 oligomer, ■ AS1 oligomer.

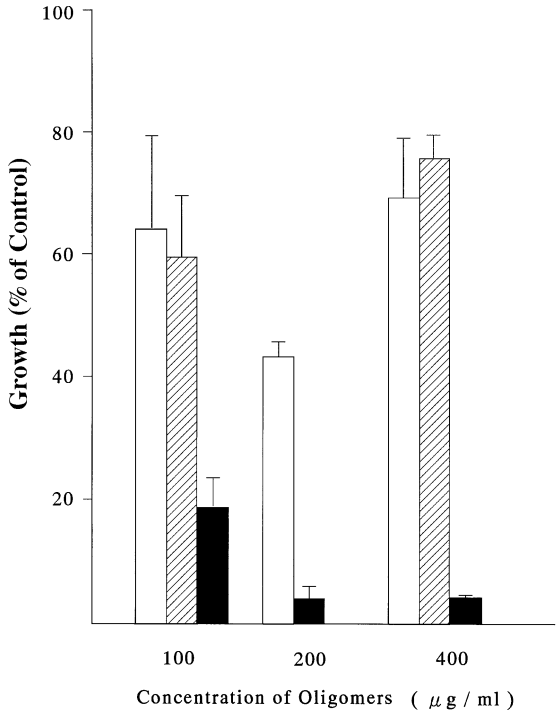

Fig. 7. Concentration-dependent growth inhibition of AZ-521 gastric cancer cells by WT1 antisense oligomers. Cells were treated with oligomers, and the number of viable cells was counted after 6 days. □ random oligomer, ▨ SE1 oligomer, ■ AS1 oligomer.

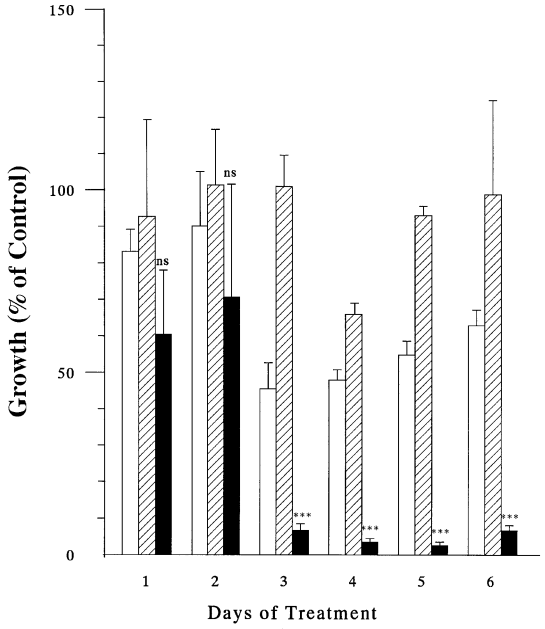

Fig. 8. Time course of growth inhibition of AZ-521 gastric cancer-derived cells by WT1 antisense oligomer. AZ-521 cells were treated with oligomers at the concentration of 400 μg/ml, and half of the initial dose of the oligomers was added every 24 h. The number of viable cells was counted every 24 h and the growth-inhibitory effect of WT1 antisense oligomer was evaluated compared with that of random oligomer. *** $P<0.001$. □ random oligomer, ▨ SE1 oligomer, ■ AS1 oligomer.

(AS1). When added to the culture medium of AZ-521 cells, WT1 antisense oligomer produced a significant reduction of 65% in WT1 protein level (Fig. 9). These results showed that the WT1 antisense oligomer specifically inhibits cell growth through a decrease of the WT1 protein level.

Constitutive expression of the *WT1* gene inhibits antisense effect of WT1 antisense oligomer on growth of cancer cells To confirm further that the inhibition of cell growth by WT1 antisense oligomers occurred as a result of specific suppression of *WT1* gene expression, gastric cancer cell line AZ-521 was transfected with the *WT1* gene (full-sized *WT1* cDNA) driven by long terminal repeat (LTR) of murine leukemia virus, and the antisense effect of WT1 antisense oligomer was examined. Percent growth with respect to the random oligomer-treated control (which represents the nonspecific effect of oligomer) is shown in Fig. 10. These results showed that constitutive expression of the *WT1* gene inhibited the antisense effect, although not completely, confirming that inhibition of cell growth by WT1-antisense oligomers resulted from specific suppression of *WT1* gene expression. One explanation for the incomplete restoration might be that since

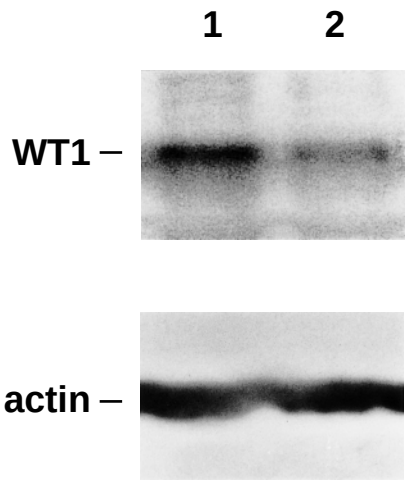

Fig. 9. Reduction in WT1 protein levels by WT1 antisense oligomers. AZ-521 gastric cancer-derived cells were treated with WT1 antisense oligomer (AS1) at the concentration of 400 μg/ml, and half of the initial dose of the oligomer was added to the culture every 24 h. The cells were harvested on day 7, lysed, and assayed for WT1 protein levels by western blot analysis. 1, random oligomer; 2, WT1 AS1 oligomer.

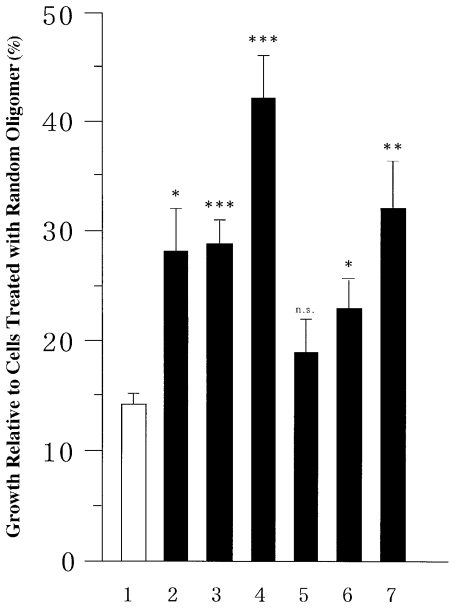

Fig. 10. Inhibition of antisense effect by constitutive expression of the *WT1* gene. AZ-521 gastric cancer cells were transfected with an LTR-driven *WT1* construct containing full-sized (nonspliced) *WT1* cDNA (closed columns) or backbone vector alone (open columns). Transfectants were then selected on the basis of G-418 resistance. Independent transfectants were treated with random oligomer or with the WT1 AS1 oligomer at the concentration of 50 μg/ml for 6 days, after which the number of viable cells was counted. The percentage growth of WT1 antisense oligomer-treated cells compared to that of random oligomer-treated cells is shown. 1, backbone vector-transfected AZ-521; 2–7, independent AZ-521 cell clones transfected with an LTR-driven *WT1* construct containing full-sized *WT1* cDNA. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ (backbone vector-transfected cells vs. LTR-driven *WT1* cDNA-transfected cells).

WT1 antisense oligomers could have an antisense effect on at least four different *WT1* transcripts (one nonspliced and three spliced), constitutive expression of a full-sized (nonspliced) *WT1* cDNA alone could not completely inhibit the antisense effect.

DISCUSSION

In the present study, we clearly demonstrated that the *WT1* gene is expressed at a variety of levels in various kinds of cancer cell lines derived from solid tumors in stomach, colon, lung, breast, testis, ovary, uterus, thyroid, or liver. *WT1* expression in solid tumors derived from stomach, colon, lung, thyroid, and liver is reported here for the first time. Furthermore, in three cell lines examined (gastric cancer cell line AZ-521, ovarian cancer cell line TYK-nu, and lung cancer cell line OS3) no mutations or deletions were detected in the *WT1* gene from the 3′ end of exon 1 through to exon 10, and suppression of *WT1* gene expression by WT1 antisense oligomers inhibited growth of these cancer cells.

Furthermore, we examined the expression levels of the *WT1* gene in fresh lung cancer tissues (data not shown). Tissue masses resected from lung cancer patients were separated into two parts: normal-appearing tissues and cancer cell-rich tissues. *WT1* expression levels in three paired normal-appearing and cancer cell-rich tissues obtained from three patients were $<10^{-5}$ and 3.9×10^{-3}, 4.1×10^{-4} and 3.6×10^{-2}, and 1.4×10^{-4} and 1.2×10^{-3}, respectively. The *WT1* expression level of cancer cell-rich tissues from another lung cancer patient was 1.4×10^{-3}. These results demonstrated that *WT1* expression is significantly higher in cancer cell-rich tissues than in normal-appearing tissues, suggesting abnormal expression of the *WT1* gene not only in cultured cells, but also in fresh lung cancer cells.

Silberstein *et al*. have reported that *WT1* immunostaining revealed little or no *WT1* expression in a high percentage of breast tumor cells.[17] Thus, it might appear that there is a discrepancy between our and their results. However, whether our results are compatible or imcompatible with their results can not be determined at the present time, because we can not estimate the difference in detection sensitivity for *WT1* between our quantitative RT-PCR and their immunostaining, and because *WT1* expression levels undetectable by immunostaining may be detectable by our RT-PCR method.

Our present results indicate that expression of the wild-type *WT1* gene plays an important role in tumorigenesis in solid tumors expressing *WT1* and that the *WT1* gene performs an oncogenic rather than a tumor-suppressor function in these tumors. We have recently proposed that the *WT1* gene has basically two functional aspects, namely that of a tumor-suppressor gene and that of an oncogene, but that in leukemic cells it performs an oncogenic rather than a tumor-suppressor gene function[12] on the basis of the following data: high levels of expression of wild-type WT1 in leukemic blast cells,[9] a clear inverse correlation between *WT1* expression level and prognosis,[9] an increase in *WT1* expression level at relapse,[10] inhibition of leukemic cell growth by WT1 antisense oligomers,[11] and blocking of differentiation but induction of proliferation in response to granulocyte-colony stimulating factor (G-CSF) in 32D cl3 myeloid progenitor cells, which constitutively express *WT1*, by transfection with the *WT1* gene.[12] Therefore we suggest that the wild-type *WT1* gene exerts an oncogenic function not only in leukemogenesis, but also in tumorigenesis.

WT1 expression levels in various cancer cell lines were widely distributed over a 5-log range. The biological significance of this wide distribution range remains undetermined. However, the growth of cancer cells expressing

high (AZ-521 and TYK-nu) or intermediate (OS3) levels of *WT1* was inhibited by WT1 antisense oligomers, whereas the growth of cancer cells (PC-14) not expressing *WT1* was not. This suggested an essential role for *WT1* in the proliferation of cancer cells expressing *WT1*. Therefore, at least two mechanisms of tumorigenesis may operate, i.e., one involving *WT1* and one not. The *WT1* gene was originally isolated from Wilms' tumor as a tumor-suppressor gene. However, in the former category of tumorigenesis the *WT1* gene appears to perform an oncogenic function.

Transfection of each of four wild-type *WT1* isoforms (17AA+/KTS+, –/–, +/–, –/+) suppressed the growth of RM1 cells, which were derived from human Wilms' tumor.[18] Furthermore, it has recently been reported that transfection of a *WT1* isoform (17AA–/KTS–) suppressed *in vitro* cell growth and *in vivo* tumor growth of *ras*-transformed NIH3T3 cells.[19] On the other hand, Menke *et al.* demonstrated that the *WT1* isoform (17AA–/KTS–) promoted *in vivo* tumor growth of adenovirus-transformed baby rat kidney cells.[20] These results and our present data indicate that the *WT1* gene has basically two functional aspects, i.e., tumor suppressor gene and oncogenic functions. Which function is exerted would depend on the cell type. In hematopoietic progenitor cells and cells from which the *WT1*-expressing solid tumors described here were derived, the *WT1* gene could exert its oncogenic function. Differences in the interactions of the WT1 protein with other regulatory proteins might determine whether the *WT1* gene acts as a tumor-suppressor gene or performs an oncogenic function, because the WT1 protein does interact with regulatory proteins such as P53[21] and par-4.[22]

As for the relation between *WT1* expression level and histopathology, no clear correlation was found between *WT1* expression levels and histology in the 15 lung cancer cell lines. In the breast cancer cell lines, MDAMB231 cells (expressing an intermediate level of *WT1*) did not have estrogen receptor, whereas YMB-1 (expressing an intermediate level of *WT1*) and two cancer cell lines, T47D and ZR75-1, which did not express detectable *WT1*, carried the estrogen receptor. Since it is well known that estrogen receptor appears with the differentiation of mammary glands, YMB-1, T47D, and ZR75-1 appear to be more differentiated than MDAMB231. This finding may suggest that there is no correlation between *WT1* expression level and differentiation stage of breast cancer cells. In colon cancers, however, *WT1* expression levels appeared to be higher in poorly differentiated than in well-differentiated adenocarcinoma, although this finding is not conclusive because of the small number of samples. In this context, *WT1* gene expression is downregulated along with differentiation of hematopoietic progenitor cells.[9, 23–28] As $CD34^+$ hematopoietic progenitor cells differentiate into more mature cells, the *WT1* expression is downregulated, implying that *WT1* may play an important role in the regulation of self-renewal and differentiation of multipotent hematopoietic stem cells.[21–24] Similarly, the *WT1* expression was downregulated during differentiation of HL60 myeloid progenitor cells by dimethyl sulfoxide or retinoic acid [23] and during induction of erythroid or megakaryocytic differentiation of K562 human leukemic cells.[24] At present, it is difficult to reach a conclusion as to the relation between *WT1* expression level and histopathology or differentiation stage of cancer cells. Further studies to address these issues should be both important and interesting.

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