Overexpression of the Wilms' tumor gene *WT1* in primary thyroid cancer

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The expression levels of the Wilms' tumor gene *WT1* were examined in 34 primary thyroid cancers (24 papillary, 5 follicular, 1 anaplastic, and 4 medullary carcinomas), 17 thyroid follicular adenomas, and 6 normal-appearing thyroid tissues using quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). In 33 of 34 thyroid cancers, the *WT1* mRNA was expressed at levels ranging from 5.0×10^{-5} to 8.3×10^{-2} levels (*WT1* expression level in K562 leukemic cells was defined as 1.0). The *WT1* mRNA expression levels were significantly higher than those in either thyroid follicular adenomas ($P<0.001$) or normal-appearing thyroid tissues ($P<0.01$). Immunohistochemical analysis confirmed the expression of WT1 protein in 20 of 21 thyroid cancers with *WT1* mRNA expression. WT1 protein was also detected in 6 of 7 follicular adenomas with *WT1* mRNA expression. However, the intensity of staining of WT1 protein in adenoma cells was weaker than that in cancer cells and its expression was restricted to approximately 30–80% of adenoma cells in the tumors examined. The direct sequencing analysis of the *WT1* genomic DNA showed no mutations in any of the 10 exons of the *WT1* gene in all of the 9 different thyroid cancers. These findings indicate an important role of the wild-type *WT1* gene in the tumorigenesis of primary thyroid cancer. (Cancer Sci 2003; 94: 606–611)

The Wilms' tumor gene (*WT1*) was originally isolated as a tumor-suppressor gene that was inactivated in a subset of Wilms' tumors and mutated in the germline of children with genetic predisposition to this kidney neoplasm of childhood.[1–3] The *WT1* gene encodes a zinc finger transcription factor that represses transcription of growth factor (*PDGF-A chain*, *CSF-1*, and *IGF-II*)[4–6] and growth factor receptor (*IGF-IR*)[7] genes and other genes (*RAR-α*, *c-myc*, and *bcl-2*).[8, 9]

We and others have demonstrated that the wild-type *WT1* gene is expressed in cancer cells derived from various kinds of cancers[10–12] and overexpressed in primary leukemia,[13] breast cancer[14, 15] and lung cancer,[16] and bone and soft-tissue sarcoma.[17] Furthermore, growth of *WT1*-expressing cancer cells was inhibited by treatment with *WT1* antisense oligomers.[11, 18, 19] We have proposed that the wild-type *WT1* gene plays an oncogenic role rather than having tumor-suppressor properties in tumorigenesis of various types of cancers on the basis of these findings.[20]

In primary thyroid cancers, some of the genes, such as *PTC-ret, trk, met*, and *p53* have been reported to be involved in the pathogenesis of thyroid cancer.[21–25] However, the precise mechanism of tumorigenesis of thyroid cancer remains unclear.

In the present study, we analyzed the expression of the *WT1* gene in 34 primary thyroid cancers, 17 thyroid follicular adenomas, and 6 normal-appearing thyroid tissues to examine the involvement of the *WT1* gene in tumorigenesis of primary thyroid cancer, and found that the *WT1* gene was overexpressed at both mRNA and protein levels in the majority of thyroid cancers examined, and that the *WT1* gene overexpressed in thyroid cancer was the non-mutated, wild-type.

Materials and Methods

Tissue samples. Thyroid cancer tissues were obtained from 34 patients with thyroid cancer. They included 24 patients with papillary carcinoma, 5 with follicular carcinoma, 1 with anaplastic carcinoma, and 4 with medullary carcinoma. Paired thyroid adenoma tissues and normal-appearing tissues from the same patients were obtained from 17 patients with thyroid follicular adenoma. All samples were obtained with informed consent at Osaka University Hospital. Clinicopathological features of the patients are shown in Table 1.

RNA purification and reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated from the frozen tissues using Trizol (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions, dissolved in diethylpyrocarbonate (DEPC)-treated water, and quantified by measuring the absorbance at 260 nm with a spectrophotometer. RNA was converted into cDNA as described previously.[16] In brief, 3 μg of total RNA in DEPC-treated water was incubated at 65°C for 5 min and then mixed with 25 μl of RT buffer (50 m*M* Tris-HCl, pH 8.3; 75 m*M* KCl; 3 m*M* $MgCl_2$; and 10 m*M* dithiothreitol) containing 600 U of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI), 500 μ*M* of each dNTP, 200 ng of oligo dT primers and 80 U of RNase inhibitor (Promega). The reaction mixture was then incubated at 37°C for 2 h, boiled for 5 min, and stored at −20°C until use.

To determine relative *WT1* expression levels, cDNA (3.0 μl for *WT1* and 2.0 μl for β-actin) was added to the PCR buffer (100 m*M* Tris-HCl, pH 8.3; 500 m*M* KCl; and 3 m*M* $MgCl_2$) containing 200 μ*M* of each dNTP, 1.25 U of Ampli*Taq* Gold (PE Applied Biosystems, Foster City, CA), 0.5 μ*M* forward and reverse primers, and 200 n*M* *Taq*Man probe in a total volume of 50 μl. The sequences of primers and probes used are as follows. *WT1*: forward primer (F1), 5′GATAACCACACAACGCCCATC3′; reverse primer (R1), 5′ CACACGTCGCACATCCTGAAT3′; probe, 5′FAM-ACACCGTGCGTGTGTATTCTGTATTGG-TAMRA3′. β-Actin: forward primer, 5′CCCAGCACAATGAAGATCAAGATCAT3′; reverse primer, 5′ATCTGCTGGAAGGTGGACAGCGA3′; probe, 5′FAM-TGAGCGCAAGTACTCCGTGTGGATCGGCG-TAMRA3′. After activation of Ampli*Taq* Gold polymerase at 95°C for 10 min, PCR was performed for 40 cycles (95°C for 30 s/63°C for 60 s). Sequences of *WT1* reverse and β-actin forward primers spanned two consecutive exons from exons 6 to 7 and from exons 4 to 5 of the respective gene in order to avoid amplification of the corresponding genome se-

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Table 1. Clinical features of patients with thyroid cancer or follicular adenoma

Pt. ID	Age	Sex	Disease	*WT1* levels in cancer/ adenoma tissues	WT1 protein
1	51	F	Papillary carcinoma	8.3×10^{-2}	n.d.
2	58	F	Papillary carcinoma	5.7×10^{-2}	positive
3	35	F	Papillary carcinoma	1.9×10^{-2}	positive
4	58	M	Papillary carcinoma	1.8×10^{-2}	positive
5	74	F	Papillary carcinoma	1.3×10^{-2}	n.d.
6	32	F	Papillary carcinoma	8.9×10^{-3}	n.d.
7	68	F	Papillary carcinoma	8.6×10^{-3}	n.d.
8	52	M	Papillary carcinoma	8.5×10^{-3}	positive
9	66	F	Papillary carcinoma	8.4×10^{-3}	n.d.
10	48	F	Papillary carcinoma	3.1×10^{-3}	positive
11	77	F	Papillary carcinoma	2.4×10^{-3}	n.d.
12	83	F	Papillary carcinoma	1.5×10^{-3}	positive
13	52	F	Papillary carcinoma	1.1×10^{-3}	positive
14	30	F	Papillary carcinoma	7.3×10^{-4}	n.d.
15	63	F	Papillary carcinoma	4.0×10^{-4}	positive
16	16	F	Papillary carcinoma	3.8×10^{-4}	n.d.
17	27	F	Papillary carcinoma	2.6×10^{-4}	positive
18	45	F	Papillary carcinoma	2.0×10^{-4}	positive
19	42	F	Papillary carcinoma	1.8×10^{-4}	positive
20	51	F	Papillary carcinoma	1.4×10^{-4}	positive
21	38	F	Papillary carcinoma	1.3×10^{-4}	positive
22	81	M	Papillary carcinoma	1.2×10^{-4}	positive
23	27	F	Papillary carcinoma	9.4×10^{-5}	positive
24	56	F	Papillary carcinoma	8.6×10^{-5}	positive
25	51	F	Follicular carcinoma	2.8×10^{-2}	n.d.
26	43	F	Follicular carcinoma	1.1×10^{-3}	positive
27	59	M	Follicular carcinoma	4.9×10^{-4}	positive
28	58	F	Follicular carcinoma	6.2×10^{-5}	n.d.
29	59	M	Follicular carcinoma	$<1.0\times10^{-5}$	negative
30	55	F	Anaplastic carcinoma	5.0×10^{-5}	negative
31	38	F	Medullary carcinoma	3.7×10^{-2}	positive
32	55	M	Medullary carcinoma	3.4×10^{-3}	n.d.
33	34	F	Medullary carcinoma	8.7×10^{-4}	negative
34	65	F	Medullary carcinoma	1.2×10^{-4}	n.d.
35	78	F	Follicular adenoma	8.9×10^{-4}	positive
36	49	F	Follicular adenoma	8.0×10^{-4}	positive
37	27	F	Follicular adenoma	7.5×10^{-4}	n.d.
38	55	F	Follicular adenoma	5.1×10^{-4}	n.d.
39	77	F	Follicular adenoma	4.3×10^{-4}	negative
40	25	F	Follicular adenoma	3.7×10^{-4}	n.d.
41	46	F	Follicular adenoma	3.1×10^{-4}	positive
42	49	F	Follicular adenoma	3.1×10^{-4}	n.d.
43	40	F	Follicular adenoma	2.9×10^{-4}	n.d.
44	36	F	Follicular adenoma	2.2×10^{-4}	n.d.
45	65	F	Follicular adenoma	7.2×10^{-5}	positive
46	52	F	Follicular adenoma	4.2×10^{-5}	positive
47	51	F	Follicular adenoma	3.6×10^{-5}	n.d.
48	49	F	Follicular adenoma	1.2×10^{-5}	positive
49	72	F	Follicular adenoma	$<1.0\times10^{-5}$	negative
50	77	F	Follicular adenoma	$<1.0\times10^{-5}$	n.d.
51	28	F	Follicular adenoma	$<1.0\times10^{-5}$	n.d.

WT1 mRNA expression levels were determined by real-time RT-PCR. *WT1* mRNA expression level in leukemic cell line K562 cells was defined as 1.0. WT1 protein expression was determined by immunohistochemistry.
n.d., not done.

quences. Standard curves for the quantitation of *WT1* and β-actin were constructed from the results of simultaneous amplification of serial dilutions of the cDNA from *WT1*-expressing K562 leukemic cells, whose *WT1* expression level was defined as 1.0 as described previously.[16] Real-time PCR and subsequent calculations were performed on an ABI Prism 7700 Sequence Detector System (PE Applied Biosystems). To normalize the difference in RNA degradation and in RNA loading for RT-PCR in individual samples, the values of levels of *WT1* gene expression divided by those of *β-actin* gene expression were defined as relative *WT1* expression levels in the samples. All experiments were performed in duplicate.

Sequencing analysis. Genomic DNA was isolated from the frozen thyroid cancer tissues with standard technique, dissolved in distilled water, and quantified in terms of the absorbance at 260 nm. For amplification of exons 2–10 of the *WT1* gene, 0.2 μg

of genomic DNA was added to the PCR buffer (100 m*M* Tris-HCl, pH 8.3; 500 m*M* KCl; and 3 m*M* $MgCl_2$) containing 250 μM of each dNTP, 1.25 U of Ex*Taq* polymerase (TaKaRa, Shiga), 0.5 μM forward and reverse primers[16, 26] (Table 2) in a total volume of 50 μl. For amplification of exon 1 of the *WT1* gene, which has a high GC content, 0.2 μg of genomic DNA was added to the PCR buffer (1× Pfx Amplification buffer with 2.5 m*M* $MgCl_2$ and 1× PCRx Enhancer solution) containing 250 μM of each dNTP, 1.25 U of PLATINUM Pfx DNA polymerase (Invitrogen Corp.), 0.5 μM forward (A-1) and reverse (AA-2) primers (Table 2) in a total volume of 50 μl. PCR amplification was carried out using thermal cycler TP-3000 (TaKaRa) for 35 cycles; each cycle consisting of 60 s at 94°C, 60 s at 53°C for exon 1 or 55°C for exons 2–10, and 90 s at 72°C. PCR products were separated on 2% agarose gel, cut from the gel, and purified using Qiaquick gel extraction kit (QIAGEN, Valencia, CA). After ethanol precipitation, the PCR-amplified DNA fragments were directly sequenced in both directions by an ABI Prism 377 sequencer (Perkin Elmer Life Science, Boston, MA) using appropriate primers (Table 2) and a Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer Life Science).

Immunohistochemistry. Formalin-fixed tissue sections of 4-μm thickness were cut from each paraffin block. After dewaxing with xylene and rehydration through a graded series of ethanol, the sections were microwaved for 15 min in 10 m*M* citrate buffer (pH 6.0) for antigen retrieval, incubated in phosphate-buffered saline containing goat serum albumin, reacted with anti-WT1 rabbit polyclonal antibody C-19 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100 at 4°C overnight and then reacted with biotinylated goat anti-rabbit IgG antibody (Vector Labs., Burlingame, CA) diluted 1:100 at 37°C for 30 min. After treatment with 3 % H_2O_2 solution to reduce endogenous peroxidase activity, immunoreactive WT1 protein was visualized using a Vectastain ABC kit (Vector Labs.) according to the manufacturer's instructions. The sections were then counterstained with methyl green.

Western blot analysis. Frozen tissues were hammered into small pieces and lysed with SDS sample buffer (0.125 *M* Tris-HCl, pH 6.8; 100 m*M* dithiothreitol; 4% SDS; 10% sucrose; and 0.004% bromophenol blue) on ice. After separation of proteins by SDS-PAGE followed by electrophoretic transfer to Immobilon polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA), nonspecific binding of antibody to the proteins was blocked with Tris-buffered saline containing 1% gelatin and 1% Tween 20, incubated with an anti-WT1 antibody C-19 diluted 1:100 or anti-glyceraldehyde phosphate dehydrogenase (GAPDH) antibody (Chemicon, Temecula, CA) diluted 1:500 in the blocking solution, and then with alkaline phosphatase-conjugated antibody against rabbit or mouse IgG (Santa Cruz Biotechnology) diluted 1:1000, respectively. WT1 or GAPDH protein was visualized using a BCIP-NBT solution kit (Nacalai Tesque, Kyoto).

Statistical analysis. The statistical significance of differences in *WT1* mRNA expression levels between thyroid cancer, thyroid follicular adenoma, and normal-appearing thyroid tissues was assessed by use of one-way factorial analysis of variance (ANOVA). Fisher's protected least significant difference (PLSD) was used as a post hoc test.

Table 2. Primers for amplification and sequencing of exons of *WT1* genomic DNA

Exons	Primers	
	Names	Sequences
1	A-1	5′-GGAATTCAGCAAATGGGCTCCGACGTG-3′
	A-2	5′-GTAAGCCGAAGCGCCCG-3′
	AA-1	5′-CCGGTGCTGGACTTTGCG-3′
	AA-2	5′-CCTGAATTCCCGGCCTACTTACCC-3′
2	B-1	5′-CCCAAGCTTCCGTCTTGCGAGAGCACC-3′
	B-2	5′-CCCCGAATTCAATTTGCTGTGGGTTAGG-3′
3	C-1	5′-CCCCAAGCTTCTCGTGTCTCCCCCAAC-3′
	C-2	5′-CGAATTCAGCCTCCAAGACCCAGCATGC-3′
4	DD-1	5′-GTGTATAACTGTGCAGAGATCAGTGG-3′
	DD-2	5′-GTCACAGAGAGCTTTGCCCTTTCTTC-3′
5	E-1	5′-CCTGAATTCCACTCCCCACCTCTTC-3′
	E-2	5′-CCTGAATTCGCCATTTGCTTTGCC-3′
6	F-1	5′-CCTGAATTCCTTTTTCCCTTCTTTG-3′
	F-2	5′-CCTGAATTCCTTCCGCTGGGGCC-3′
7	G-1	5′-CCTGAATTCGCTTAAAGCCTCCCTTC-3′
	G-2	5′-CCTGAATTCTTGAACCATGTTTGCCC-3′
8	H-1	5′-CCTGAATTCGAGATCCCCTTTTCCAGT-3′
	H-2	5′-CCTGAATTCACAGCTGCCAGCAATG-3′
9	I-1	5′-CCTGAATTCTCACTGTGCCCACATTG-3′
	I-2	5′-CCTGAATTCAATTTCATTCCACAATAG-3′
10	J-1	5′-CCTGAATTCCTGTCTCTTTGTTGC-3′
	J-2	5′-GTCCCCGAGGGAGACCCC-3′

DD-1 and DD-2 primers were newly designed. A-1 primer was described previously.[16] All primers but A-1, DD-1 and DD-2 were reported by others.[26]

Results

***WT1* gene overexpression in primary thyroid cancer.** The *WT1* mRNA expression levels in primary thyroid cancers, follicular adenomas, and normal-appearing tissues were examined by means of quantitative real-time RT-PCR. As shown in Table 1 and Fig. 1, all of the 24 papillary carcinomas expressed the

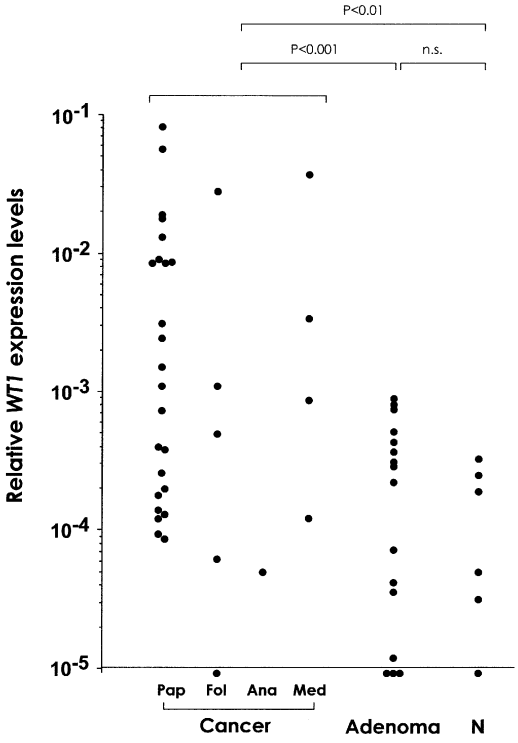

Fig. 1. Overexpression of the *WT1* mRNA in primary thyroid cancer. Relative *WT1* mRNA expression levels in primary thyroid cancer, thyroid follicular adenoma and normal-appearing thyroid tissues were examined by quantitative real-time RT-PCR. *WT1* mRNA expression level in leukemia cell line K562 was defined as 1.0. Pap, papillary carcinoma; Fol, follicular carcinoma; Ana, anaplastic carcinoma; Med, medullary carcinoma; Adenoma, follicular adenoma; and N, normal-appearing thyroid tissues from patients with thyroid follicular adenoma.

WT1 gene at levels ranging from 8.6×10^{-5} to 8.3×10^{-2}. Four of 5 follicular carcinomas expressed the *WT1* gene at levels ranging from 6.2×10^{-5} to 2.8×10^{-2}. In the remaining one follicular carcinoma, the *WT1* gene was undetectable. One anaplastic carcinoma expressed the *WT1* gene at the level of 5.0×10^{-5}. All of the 4 medullary carcinomas expressed the *WT1* gene at levels ranging from 1.2×10^{-4} to 3.7×10^{-2}. Thus, the *WT1* gene was expressed in 33 (97%) of 34 thyroid cancers at levels ranging from 5.0×10^{-5} to 8.3×10^{-2}. In thyroid follicular adenomas, the *WT1* gene was detected in 14 (82%) of 17 cases at the levels ranging from 1.2×10^{-5} to 8.9×10^{-4}. In normal-appearing thyroid tissues, the *WT1* gene was detected in 5 (83%) of 6 cases at levels ranging from 3.2×10^{-5} to 3.3×10^{-4}. The *WT1* mRNA expression levels in thyroid cancer were significantly higher than those in either follicular adenoma ($P<0.001$) or normal-appearing thyroid tissues ($P<0.01$).

Expression of WT1 protein in primary thyroid cancers. Expression of WT1 protein was examined by immunohistochemistry in 22 thyroid cancers and 8 follicular adenomas. In all sections, normal thyroid cells could be seen adjacent to carcinoma or adenoma cells. In 20 of 21 thyroid cancer tissues with *WT1* mRNA expression, WT1 protein was detected in almost all of the carcinoma cells observed in the specimens. In 6 of 7 thyroid follicular adenomas with *WT1* mRNA expression, WT1 protein was detected in approximately 30–80% of adenoma cells in the tumors. However, the intensity of staining in adenoma cells was weaker than that in carcinoma cells. In normal thyroid cells, WT1 protein was undetectable or only faintly stained. In one each of thyroid cancer and follicular adenoma with negative *WT1* mRNA expression, WT1 protein was also undetectable. These results are summarized in Table 1 and representative results are shown in Fig. 2. Furthermore, expression of WT1 protein in paired thyroid cancer and normal-appearing thyroid tissues were examined by western blot analysis in 2 cases of papillary carcinomas (patients 4 and 20). The expression of WT1 protein was increased compared to that in normal-appearing thyroid tissue (Fig. 3). These results indicated overexpression of the *WT1* gene in thyroid cancer cells at the protein level.

Absence of mutation in the *WT1* gene in primary thyroid cancer. To determine whether or not the *WT1* gene overexpressed in these thyroid cancers had mutations, the *WT1* genomic DNAs from 9

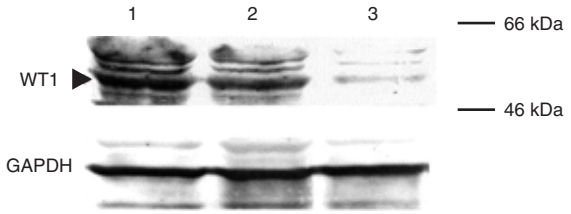

Fig. 3. Western blot analysis of WT1 protein expression in thyroid papillary cancer. Expression of WT1 protein in thyroid cancer and normal-appearing thyroid tissue was examined by western blot analysis using anti-WT1 antibody. 1 and 2, thyroid papillary carcinoma (patients 4 and 20, respectively); 3, normal-appearing thyroid tissue (patient 20).

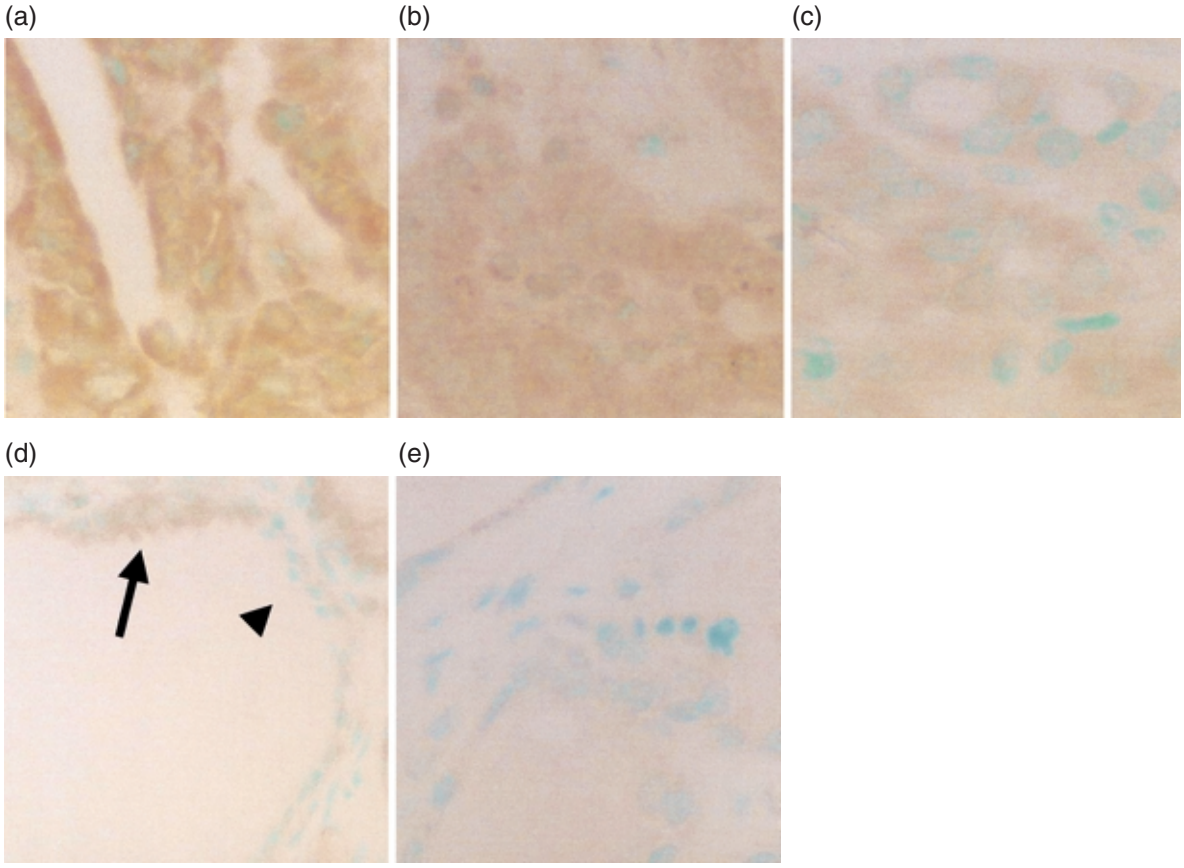

Fig. 2. Expression of WT1 protein in thyroid cancer and follicular adenoma cells. Section from thyroid papillary carcinoma (patients 8 and 10), thyroid follicular adenoma (patients 36 and 41), and normal thyroid tissue (patient 41) were stained with an anti-WT1 C-19 antibody. WT1 protein appears as brown in thyroid cancer cells (a and b). WT1 protein was also detected in thyroid follicular adenoma cells (c and d). However, the intensity of staining of WT1 protein was weaker than that in cancer cells and its expression was restricted to a part of adenoma cells in tumors (arrow and arrowhead indicate stained and non-stained adenoma cells, respectively) (d). In normal thyroid cells, WT1 protein was undetectable (e).

thyroid cancers with overexpression of *WT1* mRNA (patients 1, 2, 3, 4, 6, 7, 8, 9 and10) were PCR-amplified and examined for mutations by direct sequencing. The sequencing analysis showed the absence of mutations in all 10 exons of the *WT1* gene in all of these 9 different cases of primary thyroid cancers (data not shown). Two different single nucleotide polymorphisms (SNP) without an amino acid change were detected in the *WT1* gene. Pro42, C→T in exon 1 and Arg300, A→G in exon 7 were detected in 5 (patients 3, 4, 7, 9 and 10) of the 9 cases of primary thyroid cancer. In another case (patient 8), a polymorphism, Arg300, A→G in exon 7 was detected (data not shown).

Discussion

The *WT1* gene was originally isolated as a tumor suppressor gene responsible for Wilms' tumor, a kidney neoplasm of childhood. However, we have hypothesized that the *WT1* gene plays an oncogenic role in tumorigenesis of various types of cancers on the basis of the following findings[19]: (a) the wild-type *WT1* gene was overexpressed in leukemia,[13] breast cancer,[14, 15] lung cancer,[16] and bone and soft-tissue sarcoma,[17] (b) high expression levels of *WT1* mRNA are significantly correlated with poor prognosis in leukemia,[13] breast cancer,[15] and with high tumor-stage in testicular germ-cell tumors,[27] (c) growth of *WT1*-expressing cancer cells was inhibited by the treatment with *WT1* antisense oligomers,[11, 18, 19] (d) constitutive expression of *WT1* blocked differentiation, and instead induced proliferation in response to granulocyte colony-stimulating factor (G-CSF) in 32D cl3 myeloid progenitor cells[28] and normal myeloid progenitor cells.[29] The present study, demonstrating that the *WT1* gene was overexpressed at both mRNA and protein levels in the majority of primary thyroid cancer examined, and that the *WT1* gene overexpressed was the non-mutated, wild-type, supported our hypothesis and indicated that the wild-type *WT1* gene plays an important role in the tumorigenesis of primary thyroid cancer.

Although *WT1* mRNA expression levels in thyroid follicular adenoma were not significantly different from those in normal-appearing thyroid tissues, WT1 protein was detected in adenoma cells in the majority of thyroid follicular adenoma cases examined, whereas it was undetectable or only faintly stained in normal thyroid cells. This discrepancy may arise from low abundance of *WT1*-expressing adenoma cells in the tissue examined by RT-PCR. In this setting, the *WT1* expression levels of adenoma tissues were determined to be low, because the *WT1* mRNA from *WT1*-expressing adenoma cells was diluted by mRNA from *WT1*-non-expressing adenoma cells and normal thyroid cells contained in the specimens. We previously reported that high expression levels of the *WT1* gene in myelodysplastic syndrome were significantly correlated with disease progression to overt leukemia.[30] Similarly, an increase in WT1 protein expression in thyroid cells may reflect the disease progression from normal thyroid cells to thyroid adenoma and then to thyroid cancer. Thyroid adenoma cells are distinguished from normal thyroid cells by their ability to proliferate. The wild-type *WT1* gene might be involved in promotion of cell proliferation and play an oncogenic role in the tumorigenesis of primary thyroid cancer.

The present results strongly indicate that the wild-type *WT1* plays an important role in the tumorigenesis of primary thyroid cancer. This suggests that WT1 could be a new molecular target for treatment of thyroid cancers expressing WT1. We and others have demonstrated that WT1-specific cytotoxic T lymphocytes specifically kill *WT1*-expressing leukemia cells[31, 32] and that WT1 protein is an attractive tumor rejection antigen.[33–37] Our results presented here may provide a rationale for immunotherapy targeting WT1 protein as a new treatment strategy for thyroid cancers expressing *WT1*.

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