Overexpression of the Wilms' tumor gene *WT1* in head and neck squamous cell carcinoma

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The expression levels of the Wilms' tumor gene *WT1* were examined in 56 cases of head and neck squamous cell carcinoma (HNSCC) using quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). They included 4 cases of floor of mouth, 9 of gingiva, 25 of tongue, 10 of oropharynx, 3 of hypopharynx, and 5 larynx squamous cell carcinoma (SCC). All (100%) of 4 cases of floor of mouth, 5 (56%) of 9 gingiva, 17 (68%) of 25 tongue, 8 (80%) of 10 oropharynx, all (100%) of 3 hypopharynx, and all (100%) of 5 larynx SCC overexpressed the *WT1* gene in the range of 3.07×10^{-4}–8.60×10^{-1} levels (the *WT1* expression level in K562 leukemic cells was defined as 1.0). Thus, 42 (75%) out of 56 cases of HNSCC overexpressed the *WT1* gene. The high expression level of the *WT1* gene significantly correlated with poor histological tumor differentiation and high tumor stage of HNSCC. Immunohistochemical analysis confirmed the expression of WT1 protein in 6 cases (one floor of mouth, 2 tongue, 2 oropharynx, and one larynx SCC) with overexpression of the *WT1* gene. The direct sequencing analysis of the *WT1* genomic DNA showed no mutations in any of 10 exons of the *WT1* gene in 5 different HNSCC. These findings suggest an important role of the wild-type *WT1* gene in the tumorigenesis of HNSCC. (Cancer Sci 2003; 94: 523–529)

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 a 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]

The wild-type *WT1* gene is expressed in cancer cells derived from various kinds of cancers[10–12] and overexpressed in leukemia,[13] breast,[14, 15] lung cancer,[16] and bone and soft-tissue sarcoma.[17] Growth of *WT1*-expressing cancer cells was inhibited by treatment with *WT1* antisense oligomers.[11, 18, 19] Therefore, we had proposed that the wild-type *WT1* gene plays an oncogenic role rather than a tumor-suppressor function in the tumorigenesis of various types of cancers.[20]

Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequent cancer worldwide.[21] The overall survival rate (approximately 45%) of these cancers has only marginally improved over the last 3 decades.[22] In HNSCC, genes such as cyclin D1,[23–25] epidermal growth factor receptor,[26–28] and p16[29] have been reported to be involved in the pathogenesis of HNSCC. However, the precise mechanisms of the tumorigenesis of HNSCC remain unclear.

In the present study, we investigated the expression of the *WT1* mRNA in human HNSCC to examine the involvement of the *WT1* gene in the tumorigenesis of HNSCC, and we observed overexpression of the *WT1* gene in 42 (75%) of 56 cases of HNSCC examined.

Materials and Methods

Tissue samples. Paired HNSCC tissues and normally appearing head and neck mucosa were obtained from 56 patients. They included 4 floor of mouth, 9 gingiva, 25 tongue, 10 oropharynx, 3 hypopharynx, and 5 larynx squamous cell carcinoma (SCC). All samples were obtained with informed consent at Osaka University Hospital, Osaka University Dental Hospital and NTT West Japan Osaka Hospital. These samples were soaked in RNA later (QIAGEN, Valencia, CA) at 4°C overnight and stocked at −80°C until use. 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 sample tissues using Trizol (Invitrogen, Leek, the Netherlands) according to the manufacturer's instructions, dissolved in diethylpyrocarbonate (DEPC)-treated water, and quantified with a spectrophotometer based on the absorbance at 260 nm. 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

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Table 1. Clinical features of patients with HNSCC and *WT1* expression in cancer tissues

Pt ID	Location	Pathology	Age (year)	Gender	pTNM	Stage	*WT1* mRNA level	WT1 protein
1	Floor of mouth	poorly	49	M	T3N2bM0	IV	1.86×10^{-2}	n.d.
2	Floor of mouth	moderately	74	M	T1N0M0	I	6.94×10^{-3}	positive
3	Floor of mouth	moderately	51	M	T2N1M0	III	7.37×10^{-4}	n.d.
4	Floor of mouth	well	77	M	T4N0M0	IV	3.07×10^{-4}	n.d.
5	Gingiva	well	70	F	T2N0M0	II	1.40×10^{-2}	n.d.
6	Gingiva	moderately	53	M	T2N0M0	II	3.00×10^{-3}	n.d.
7	Gingiva	moderately	64	F	T2N0M0	II	6.97×10^{-4}	n.d.
8	Gingiva	moderately	53	M	T2N0M0	II	4.81×10^{-4}	n.d.
9	Gingiva	moderately	54	F	T2N0M0	II	3.19×10^{-4}	n.d.
10	Gingiva	well	41	M	T2N0M0	II	1.01×10^{-4}	n.d.
11	Gingiva	moderately	81	M	T2N0M0	II	7.36×10^{-5}	n.d.
12	Gingiva	well	45	F	T2N0M0	II	6.30×10^{-5}	n.d.
13	Gingiva	well	84	M	T3N0M0	III	5.57×10^{-5}	n.d.
14	Tongue	well	84	F	T4N1M0	IV	2.29×10^{-1}	n.d.
15	Tongue	poorly	52	F	T4N0M0	IV	1.17×10^{-2}	n.d.
16	Tongue	well	56	F	T2N0M0	II	1.10×10^{-2}	n.d.
17	Tongue	moderately	48	M	T2N0M0	II	2.45×10^{-3}	n.d.
18	Tongue	well	47	M	T3N0M0	III	2.38×10^{-3}	n.d.
19	Tongue	moderately	54	M	T3N2bM0	IV	1.91×10^{-3}	n.d.
20	Tongue	well	65	M	T2N0M0	II	1.88×10^{-3}	positive
21	Tongue	well	78	F	T2N0M0	II	1.74×10^{-3}	n.d.
22	Tongue	moderately	62	M	T1N0M0	I	1.29×10^{-3}	n.d.
23	Tongue	well	72	F	cT2N0M0	I	1.04×10^{-3}	n.d.
24	Tongue	moderately	46	M	T2N0M0	II	8.13×10^{-4}	n.d.
25	Tongue	poorly	54	F	T3N2bM0	IVA	7.50×10^{-4}	n.d.
26	Tongue	moderately	60	F	T1N0M0	I	6.14×10^{-4}	n.d.
27	Tongue	well	60	F	T2N0M0	II	4.39×10^{-4}	n.d.
28	Tongue	moderately	60	F	T1N0M0	I	4.09×10^{-4}	n.d.
29	Tongue	well	68	F	T3N2bM0	IVA	3.90×10^{-4}	n.d.
30	Tongue	moderately	50	F	T3N0M0	III	3.73×10^{-4}	n.d.
31	Tongue	moderately	63	M	T3N1M0	IV	3.33×10^{-4}	n.d.
32	Tongue	moderately	68	F	T2N0M0	II	2.64×10^{-4}	n.d.
33	Tongue	well	56	F	T2N1M0	III	2.29×10^{-4}	n.d.
34	Tongue	well	61	M	T2N0M0	II	1.98×10^{-4}	n.d.
35	Tongue	moderately	34	M	T2N0M0	II	1.92×10^{-4}	n.d.
36	Tongue	well	56	M	T2N1M0	III	1.91×10^{-4}	positive
37	Tongue	well	40	M	T3N0M0	III	1.89×10^{-4}	n.d.
38	Tongue	moderately	48	F	pT1N2bM0	IV	1.24×10^{-4}	n.d.
39	Oropharynx	moderately	63	M	T3N1M0	III	8.60×10^{-1}	n.d.
40	Oropharynx	moderately	69	M	T1N2aM0	IVA	5.00×10^{-2}	n.d.
41	Oropharynx	poorly	57	M	T4N3M0	IV	4.17×10^{-2}	n.d.
42	Oropharynx	poorly	81	F	T3N2bM0	IVA	1.40×10^{-2}	n.d.
43	Oropharynx	well	65	F	T1N0M0	I	6.36×10^{-4}	n.d.
44	Oropharynx	moderately	62	M	T2N0M0	II	4.25×10^{-4}	positive
45	Oropharynx	moderately	71	M	T3N0M0	III	4.19×10^{-4}	n.d.
46	Oropharynx	moderately	61	M	T2N2bM0	IVA	4.00×10^{-4}	positive
47	Oropharynx	moderately	60	M	T2N0M0	II	1.16×10^{-4}	n.d.
48	Oropharynx	moderately	55	M	T3N0M0	III	6.36×10^{-5}	n.d.
49	Hypopharynx	poorly	65	M	cT4N1M0	IA	4.19×10^{-1}	n.d.
50	Hypopharynx	poorly	52	M	T3N2cM0	IV	1.36×10^{-2}	n.d.
51	Hypopharynx	moderately	63	M	cT3N2bM0	IA	3.61×10^{-4}	n.d.
52	Larynx	moderately	52	M	T3N1M0	III	7.27×10^{-2}	n.d.
53	Larynx	moderately	82	M	T4N0M0	IV	5.38×10^{-3}	n.d.
54	Larynx	poorly	64	M	T3N2cM0	IVA	5.27×10^{-3}	positive
55	Larynx	moderately	72	M	T3N1M0	III	1.35×10^{-3}	n.d.
56	Larynx	poorly	57	M	T4N0M0	IVA	1.06×10^{-3}	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 as1.0. WT1 protein expression was determined by immunohistochemistry. n.d., not determined.

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 sequences. 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. *WT1* was detected in all of 15 normal-appearing head and neck mucosa examined at the levels ranging from 1.83×10^{-5} to 1.97×10^{-4} (mean±SD of the *WT1* expression levels: $1.15\times10^{-4}\pm9.3\times10^{-5}$) (Fig. 1). Mean+2SD of the values obtained from normal control subjects is generally used as the cut-off level.[30–32] Therefore, mean+2SD (3.01×10^{-4} level) of the *WT1* expression levels in normal-appearing head and neck mucosal tissues was set as the cut-off level for overexpression of the *WT1* gene in HNSCC tissues. According to this criterion, all of the normally appearing head and neck mucosal tissues examined were scored as negative.

Ratios of 4 different spliced forms of the *WT1* gene, 17AA(+)KTS(+) (spliced form A), 17AA(+)KTS(−) (spliced form B), 17AA(−)KTS(+) (spliced form C), and 17AA(−)KTS(−) (spliced form D) to total *WT1* transcripts were calculated by the method reported previously.[16] In short, three ratios of 17AA(+) to 17AA(−) ([A]+[B]/[C]+[D]), KTS(+) to KTS(−) ([A]+[C]/[B]+[D]), and 17AA(+)KTS(−) to 17AA(−)KTS(−) ([B]/[D]) were determined by densitometric measurement of the products of PCR performed using forward and reverse primers jumping 17AA coding sequences, forward and reverse primers jumping KTS coding sequences, and paired forward primer 5′ to 17AA coding sequences and reverse primer consisting of sequences spanning from exons 9 to 10 but lacking KTS coding sequences, respectively. These three ratios were determined as the average values of 4 independent experiments. When the ratios of 17AA(+) to 17AA(−), KTS(+) to KTS(−), and 17AA(+)KTS(−) to 17AA(−)KTS(−) are written as K_1, K_2, and K_3, respectively, the following equations hold: ([A]+[B])/([C]+[D])=K_1, ([A]+[C])/([B]+[D])=K_2, and [B]/[D]=K_3. By solving these equations, the values of [A], [B], [C], and [D] were obtained.

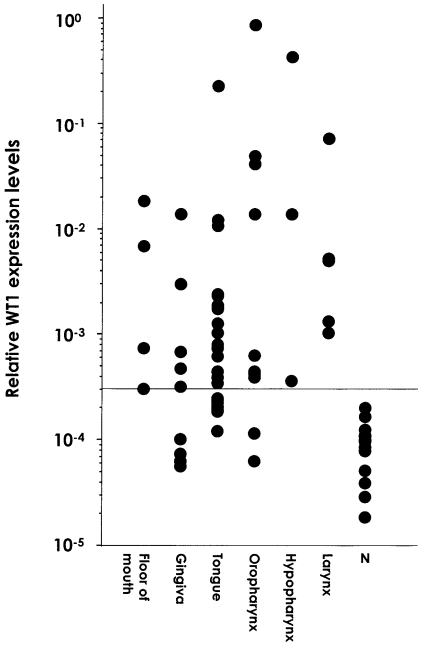

Fig. 1. Overexpression of the *WT1* gene in HNSCC. Relative *WT1* expression levels in HNSCC and normal-appearing head and neck mucosa tissues were examined by a quantitative real-time RT-PCR method. WT1 expression level in leukemia cell line K562 was defined as 1.0. N, normal-appearing head and neck mucosa tissues. A horizontal line at the 3.01×10^{-4} level indicates the cut-off level that discriminates between normal and abnormal *WT1* expression.

Sequencing analysis. Genomic DNA was isolated from the frozen HNSCC tissues with a standard technique, dissolved in distilled water, and quantified with a spectrophotometer according to 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 μ*M* of each dNTP, 1.25 U of Ex*Taq* polymerase (TaKaRa, Shiga), 0.5 μ*M* forward and reverse primers[16, 33] (Table 2) in a total volume of 50 μl. For amplification of exon 1 of the *WT1* gene that 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 μ*M* of each dNTP, 1.25 U of PLATINUM Pfx DNA polymerase (Invitrogen), and 0.5 μ*M* forward (A-1) and reverse (AA-2) primers (Table 2) in a total volume of 50 μl. PCR amplification was carried out using a 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 out from the gel, and purified using a Qiaquick gel extraction kit (QIAGEN). 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

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

Exon	Primer	
	Name	Sequence
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.[33]

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 methylgreen.

Statistical analysis. Statistical analysis to examine correlations between *WT1* expression levels in HNSCC and the clinical parameters was performed using the unpaired *t* test or one-way factorial analysis of variance (ANOVA). Fisher's protected least significant difference (PLSD) was used as a post hoc test.

Results

***WT1* gene overexpression in HNSCC.** *WT1* gene expression levels in HNSCC were examined by means of quantitative real-time RT-PCR. As shown in Table 1 and Fig. 1, all (100%) of 4 floor of mouth SCC overexpressed the *WT1* gene at levels ranging from 3.07×10^{-4} to 1.86×10^{-2}. Five (56%) of 9 gingiva SCC overexpressed the *WT1* gene at levels ranging from 3.19×10^{-4} to 1.40×10^{-2}. Seventeen (68%) of 25 tongue SCC overexpressed the *WT1* gene at levels ranging from 3.33×10^{-4} to 2.29×10^{-2}. Eight (80%) of 10 oropharynx SCC overexpressed the *WT1* gene at levels ranging from 4.00×10^{-4} to 8.60×10^{-1}. All (100%) of 3 hypopharynx SCC overexpressed the *WT1* gene at the levels ranging from 3.61×10^{-4} to 4.19×10^{-1}. All (100%) of 5 larynx SCC overexpressed the *WT1* gene at levels ranging from 1.06×10^{-3} to 7.27×10^{-2}. Thus, the *WT1* gene was overexpressed in 42 (75%) of 56 cases of HNSCC examined.

Expression of WT1 protein in HNSCC. Expression of WT1 protein was then examined by immunohistochemistry in 6 cases (patients 2, 20, 36, 44, 46, and 54) with overexpression of the *WT1* gene. In all of these 6 cases, WT1 protein was dominantly detected in the nuclei of carcinoma cells, but not in the neighboring, normal stromal cells. In normal head and neck mucosal tissues, WT1 protein was detected in basal and prickle cells, but the intensity of staining was weaker compared to that in carcinoma cells. These results showed that the *WT1* gene was overexpressed at the protein level in HNSCC cells. Representative results were shown in Fig. 2.

Correlation of *WT1* expression levels with clinicopathological parameters. Whether or not *WT1* expression levels in HNSCC correlate with clinicopathological parameters was statistically analyzed. High *WT1* expression levels in HNSCC significantly correlated with poor histological tumor differentiation (Table 3

Table 3. Correlations between *WT1* mRNA expression levels and clinicopathological characteristics in HNSCC

Clinicopathological characteristics	Total	*P* value
All cases	56	
Gender		
Male	36	0.6637[a]
Female	20	
Age		
<65 years	38	0.1896[a]
≥65 years	18	
Tumor differentiation		
Well	18	0.007[b]
Moderate	29	
Poor	9	
Tumor location		
Floor of mouth	4	0.0848[b]
Gingiva	9	
Tongue	25	
Oropharynx	10	
Hypopharynx	3	
Larynx	5	
T stage		
pT1	7	0.0067[b]
pT2	18	
pT3	24	
pT4	7	
N stage		
pN0	35	0.0015[a]
pN1–N3	21	
Clinical stage		
I	8	0.1018[b]
II	19	
III	12	
IV	17	

Statistical analysis was performed by unpaired *t* test (a) or ANOVA (b).

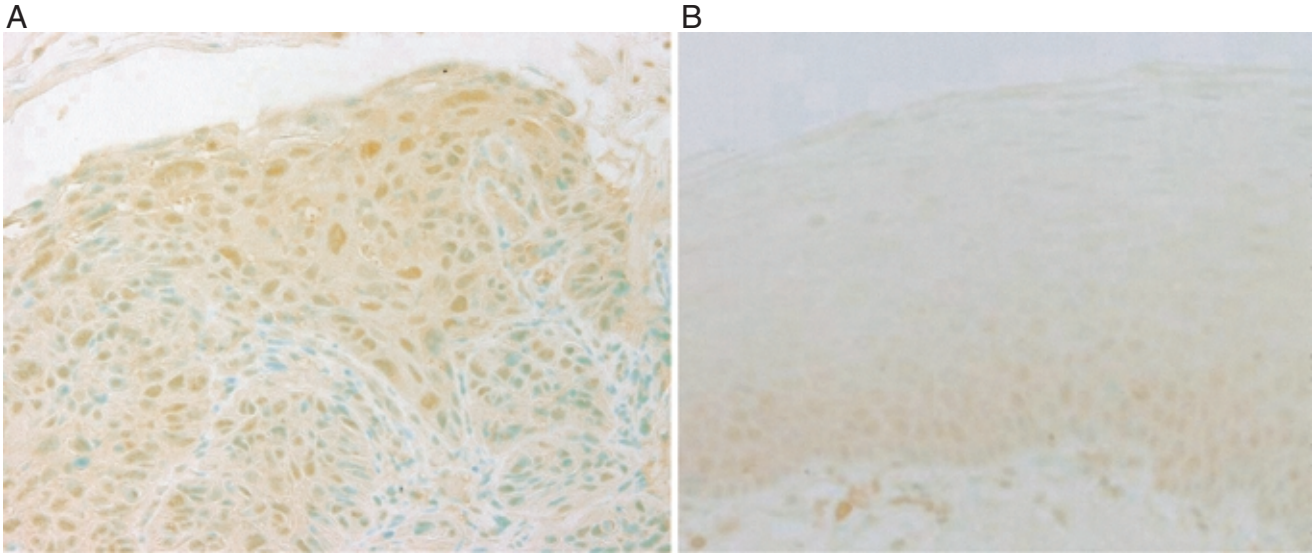

Fig. 2. WT1 protein expression in tongue SCC. A section from tongue SCC tissue of patient 20, stained with an anti-WT1 antibody; WT1 protein appears as brown in carcinoma cells but not in neighboring, normal stromal cells (A). WT1 protein was also detected in normal cells; however, only basal and prickle cells were stained and the intensity of staining was weaker compared to that in carcinoma cells (B).

and Fig 3A, P=0.007, ANOVA), high T stage (Table 3 and Fig 3B, P=0.0067, ANOVA), and high N stage (Table 3 and Fig 3C, P=0.0015, unpaired t test). On the other hand, no significant correlations were observed between *WT1* expression levels and age, gender, tumor location, or clinical stage (Table 3 and Fig. 3D).

Ratios of the *WT1* spliced forms in HNSCC. The ratios of 17AA(+) to 17AA(−), KTS(+) to KTS(−), and 17AA(+)KTS(−) to 17AA(−)KTS(−) were first determined in 5 cases of HNSCC (patients 5, 14, 24, 40, and 52), and then the ratios of 4 spliced forms of *WT1* transcripts were calculated. As shown in Fig. 4, 17AA(+)KTS(+) spliced form (spliced form A) was dominant in all of the 5 HNSCC examined.

Absence of mutations in the *WT1* gene in HNSCC. To determine whether or not the *WT1* gene overexpressed in these HNSCC had mutations, the *WT1* genomic DNA from 5 cases (patients 1, 14, 15, 16, and 42) was PCR-amplified and examined by direct sequencing. The sequencing analysis demonstrated the absence of mutations in all of 10 exons of the *WT1* gene in the 5 different cases of HNSCC. Two different single nucleotide polymor-

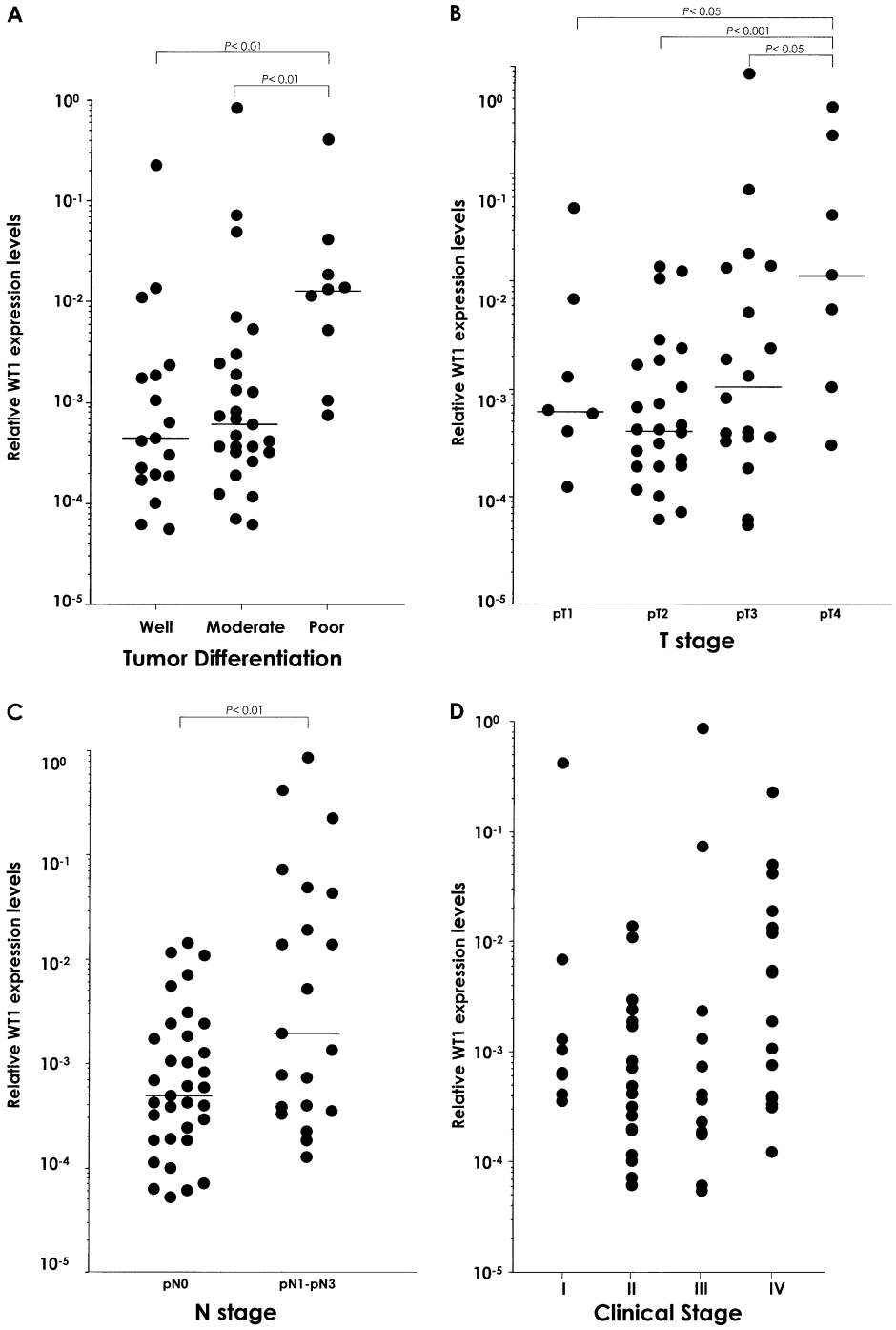

Fig. 3. Correlation between *WT1* mRNA expression levels and clinical parameters of HNSCC. A: Correlation between *WT1* mRNA expression levels and histological tumor differentiation. B: Correlation between *WT1* mRNA expression levels and T stage of HNSCC. C: Correlation between *WT1* mRNA expression levels and N stage of HNSCC. Horizontal lines indicate the median *WT1* mRNA expression level in each group.

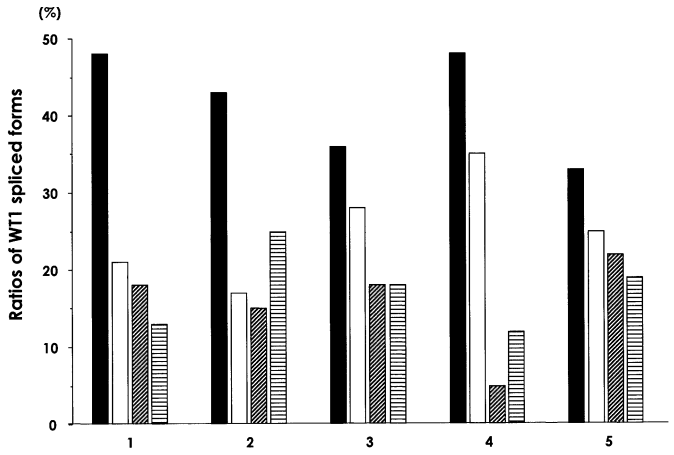

Fig. 4. Ratios of the 4 spliced forms in *WT1* transcripts of HNSCC. Ratios of the spliced forms were determined as described in "Materials and Methods." Numbers 1, 2, 3, 4, and 5 represent WT1 transcripts of patients 5, 14, 24, 40, and 52, respectively. Solid bar, 17AA(+)KTS(+); open bar, 17AA(+)KTS(−); hatched bar, 17AA(−)KTS(+); horizontally lined bar, 17AA(−)KTS(−).

phisms (SNP) were detected in the *WT1* gene. Pro42, C→T in exon 1 and Arg300, A→G in exon 7 were detected in 2 (patients 1 and14) of the 5 cases of HNSCC. In another case (patient 16), a polymorphism, Pro42, C→T in exon 1 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 had proposed that the *WT1* gene plays an oncogenic role in tumorigenesis of various types of cancers on the basis of the following findings[20]: (a) the wild-type *WT1* gene was overexpressed in leukemia,[13] breast,[14, 15] lung cancer,[16] and bone and soft-tissue sarcoma,[17] (b) high expression levels of *WT1* mRNA significantly correlated with poor prognosis in leukemia[13] and breast cancer,[15] and with high tumor-stage in testicular germ-cell tumors,[34] (c) growth of *WT1*-expressing cancer cells was inhibited by 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[35] and normal myeloid progenitor cells.[36] In the present study, we demonstrated that the *WT1* gene was overexpressed at the mRNA level in the majority of the HNSCC examined and that the *WT1* gene overexpressed was the non-mutated, wild-type. Overexpression of the *WT1* gene in HNSCC was confirmed immunohistochemically at the protein level. Furthermore, it was demonstrated that the high expression levels of *WT1* mRNA significantly correlated with poor histological tumor differentiation. The findings mentioned above and the results presented here may indicate that the non-mutated, wild-type *WT1* gene is involved in blocking cell differentiation, and plays an important role in the tumorigenesis of HNSCC.

The expression levels of the *WT1* gene in HNSCC were widely distributed. As we mentioned, high *WT1* mRNA expression significantly correlated with poor differentiation of HNSCC. Poor differentiation of tumor cells is usually linked to aggressive phenotypes of cancer cells, such as rapid growth and resistance to chemotherapy. Furthermore, high expression levels of the *WT1* gene also correlated with high tumor stages (T and N stages) of HNSCC. Therefore, it is reasonable to consider that *WT1* expression level is a novel prognostic factor in HNSCC. To address this issue, we are planning to examine *WT1* expression levels in a large number of HNSCC and to assess the correlation between *WT1* expression level and prognosis.

The *WT1* gene is alternatively spliced at two sites (17AA and KTS) and yields 4 spliced forms, each of which is suggested to have different functions.[37–39] We examined the ratios of the 4 *WT1* spliced forms expressed in 5 cases of HNSCC and found that 17AA(+)KTS(+)*WT1* spliced form was dominantly expressed among them. We have demonstrated that constitutive expression of 17AA(+)KTS(+)*WT1* spliced form promoted growth and inhibited differentiation in murine myeloid progenitor 32D cl3 cells[35] and murine normal myeloid progenitor cells,[36] and the growth inhibition was restored by treatment with WT1 antisense oligomers in K562 leukemia[18] and AZ-521 gastric cancer cells.[11] Therefore, 17AA(+)KTS(+)*WT1* spliced form might play an important role in the tumorigenesis of HNSCC.

In the present study, expression of the *WT1* gene was examined by both quantitative RT-PCR and immunohistochemistry in 6 different cases. In 5 of these 6 cases, overexpression of the *WT1* gene was demonstrated at both the mRNA and protein levels. However, in the remaining one case (patient 36), the expression level of *WT1* mRNA in HNSCC tissue was below the cut-off level, while WT1 protein was immunohistochemically detected in HNSCC cells. This discrepancy may arise from low abundance of *WT1*-expressing HNSCC cells in the tissue examined by RT-PCR. In this setting, the *WT1* expression level of HNSCC tissue was determined as low, because the *WT1* mRNA from HNSCC cells was diluted by mRNA from normal mucosal cells. We are planning to examine the expression of the *WT1* gene by both RT-PCR and immunohistochemistry in a large number of HNSCC cases to determine which of RT-PCR or immunohistochemistry is better for the clinical diagnosis of WT1 expression in HNSCC.

The present results strongly indicate that the wild-type *WT1* gene plays an important role in the tumorigenesis of HNSCC. Thus, WT1 could be a new molecular target for treatment of HNSCC expressing WT1. We and others have demonstrated that WT1-specific cytotoxic T lymphocytes specifically killed WT1-expressing leukemia cells[40, 41] and suggested that WT1 protein is an attractive tumor rejection antigen.[42–46] Our results presented here may provide a rationale for immunotherapy targeting WT1 protein as a new treatment strategy for HNSCC expressing WT1.

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