Journal of
Oral Pathology & Medicine

doi: 10.1111/j.1600-0714.2012.01182.x

J Oral Pathol Med (2013) 42: 133–139

wileyonlinelibrary.com/journal/jop

Expression of Wilms' tumor 1 (WT1) in oral squamous cell carcinoma

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BACKGROUND: The product of the Wilms' tumor gene, WT1 protein, is a tumor antigen for various kinds of cancer, and WT1 peptide-based cancer immunotherapy is widely anticipated as a new possibility for cancer treatment. The aim of this study was to investigate the expression of WT1 from quantitative and morphological perspectives in oral squamous cell carcinoma (OSCC), the most widespread malignant neoplasm of the oral cavity.

METHODS: Six OSCC cell lines and tissue sections from 29 OSCC patients were analyzed. To detect WT1 expression, reverse transcription-polymerase chain reaction analysis (RT-PCR), real-time PCR, Western blots, and immunofluorescence flow cytometry for WT1 were performed on the cell lines, and immunohistochemistry and fluorescence *in situ* hybridization (FISH) were performed on the tissue sections.

RESULTS: *WT1* mRNA was found overexpressed in one of the six OSCC cell lines, with expression levels higher than that seen in human leukemia cell line (K562). Immunohistochemical analysis of tissue sections showed overexpression of WT1 protein in two patients, concentrated mainly in the cytoplasm of the outer one to three cell layers of the cancer nests. This was consistent with the expression of *WT1* mRNA observed by FISH. Meanwhile, WT1 was not detected on normal oral epithelium. WT1 protein was detected on actively proliferating cancer nests and even on elongated epithelial ridge where new droplet-cancer-nests were being formed and starting infiltration toward subepithelial layer.

CONCLUSIONS: The results suggest that WT1 plays an important role in the pathogenesis of some types of OSCC, particularly in proliferation of the cancer cells.

J Oral Pathol Med (2013) **42**: 133–139

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Accepted for publication April 24, 2012

Keywords: oral squamous cell carcinoma; overexpression; proliferation; WT1

Introduction

The Wilms' tumor 1 gene (*WT1*), a tumor-suppressor gene located at chromosome 11p13, was originally isolated and characterized as a gene responsible for Wilms' tumor, a child renal cancer (1). This gene plays an important role in kidney development, being expressed in specific tissues during nephrogenesis (2, 3), and germline mutations in the *WT1* gene are associated with abnormal urogenital development in Denys–Drash syndrome (4). In a normal body, the product of the *WT1* gene, WT1 protein, is expressed in the kidneys, gonads, uterus, decidua, skeletal muscles, ureters, arteries, mesothelium, and smooth muscle of urinary bladder (5, 6).

Meanwhile, high expression levels of the *WT1* gene were reported in leukemia cells from cancer patients (7), and an inverse correlation was found between WT1 expression levels and prognosis (7). Oji et al. (8) quantitatively examined expression of the *WT1* gene in 34 solid tumor cell lines and clearly demonstrated that this gene shows different expression levels in various cancer cell lines established from solid tumors found in stomach, colon, lung, breast, testis, ovary, uterus, thyroid, and liver. Therefore, they suggest that the *WT1* gene performs an oncogenic function not only in leukemogenesis, but also in the tumorigenesis of solid tumors, even though it was first categorized as a tumor-suppressor gene (8).

Nakatsuka et al. (9) demonstrated using immunohistochemistry that the WT1 protein is detected in many cancers including gastrointestinal and pancreatobiliary tumors, urinary and male genital tumors, breast and female genital tumors, brain tumors, soft tissue sarcoma, osteosarcoma, and malignant melanoma. Furthermore, there is increasing evidence linking the overexpression of the *WT1* gene or WT1 protein to tumorigenesis (10–17).

The WT1 protein is a tumor antigen for these malignancies, and clinical trials of WT1 peptide-based cancer immunotherapy have been performed on various kinds of tumors (18–28), showing effective clinical responses for myelodysplastic syndrome (18–20), acute myeloid leukemia (19, 21, 22), lung cancer (19), breast cancer (19, 23), glioblastoma (24, 25), and multiple myeloma (26). Thus, WT1 peptide-based cancer immunotherapy is anticipated to have potential as a new cancer treatment. To date, there has been only one study reported(10) that investigated the expression of *WT1* gene and protein in oral squamous cell carcinoma (OSCC), the most widespread malignant neoplasm of the oral cavity. Therefore, the precise mechanisms underlying the tumorigenesis of OSCC related to WT1 remain unclear.

The aim of this study was to investigate the expression of WT1 in OSCC, both quantitatively and morphologically, to examine the feasibility of using WT1 peptide-based immunotherapy for OSCC patients.

Materials and methods

Cell lines

Six cell lines of human OSCC obtained from the Riken BioResource Center (Ibaragi, Japan) (29–32) were used in this study. Three of the six cell lines were derived from well-differentiated OSCC, while the other three were derived from poorly differentiated OSCC. Details of the cell lines are shown in Table 1. Normal human epidermal keratinocyte (NHEK) cells (KK-4001; Kurabo, Tokyo, Japan) were used as a negative control for *WT1* mRNA analyses.

RNA preparation and reverse transcription-polymerase chain reaction analysis (RT-PCR)

Total RNA was collected from confluent cultured cells using an RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) according to manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA with the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Carlsbad, CA, USA). One microlitre of cDNA solution was amplified with Amplitaq Gold PCR Master Mix (Applied Biosystems) in a volume of 12.5 μl using each set of 20-μM primers. The sequences of GAPDH and WT1 primers used are as follows.

GAPDH: forward primer, 5′-CAATGACCCCTTC ATTGACC-3′ (exon 2); reverse primer, 5′-GACAA GCTTCCCGTTCTCAG-3′ (exon 3).

WT1: forward primer, 5′ AGGGTACGAGAGCG ATAACCACAC3′ (exon 6); reverse primer, 5′ TCAG ATGCCGACCGTACAAGA3′ (exon 7).

PCRs were performed for 5 min at 95°C, followed by 30 cycles of 30 s at 95°C, 60 s at 72°C, 15 s at 72°C, and a final elongation of 15 min at 72°C using a My Cycle thermal cycler (BIO-RAD, Hercules, CA, USA).

cDNA synthesized from K562(33) (human leukemia cell line) total RNA (AM7832; Applied Biosystems) was used as the positive control (10), and cDNA synthesized from total RNA extracted from NHEK cell line was used as the negative control (34). The PCR products were then analyzed by electrophoresis on 1.8% agarose gel containing 0.1 μl Gelstar (Cambrex Bio Science, Rockland Inc., Rockland, ME, USA) per 1 ml in 0.5× TBE buffer (50 mM Tris, 50 mM boric acid, 2 mM EDTA, pH 8.0).

Real-time PCR

cDNA was analyzed quantitatively by real-time PCR using Applied Biosystems 7500 Fast real-time PCR System (Applied Biosystems). One microlitre of cDNA solution was amplified with Power SYBR Green PCR Master Mix (Applied Biosystems) in a volume of 25 μl using each set of 10-μM primers. The same primer sequences for WT1 were used for RT-PCR and real-time PCR. Each sample was tested in triplicate, and relative WT1 expression levels were evaluated using the standard curve method.

Western blots

Protein extracts from cell lines were washed twice with PBS and then lysed in RIPA buffer (50 mM Tris–HCl, pH 7.2, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Protein content of the samples was measured using the BCA reagent (Thermo Scientific, Rockford, IL, USA), and each sample containing equal amounts of protein was separated by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After being blocked with 5% non-fat dry milk in T-TBS (50 mM Tris–HCl, pH 7.2, 150 mM NaCl, and 0.1% Tween-20), the membrane was incubated with a primary anti-WT1 (1:2000, M3561; Dako, Glostrup, Denmark) antibody and also an anti-GAPDH (1:2000, IMG-5143A; Imgenex, San Diego, CA, USA) antibody as a loading control for normalization. The blots were then incubated with horseradish peroxidase-conjugated secondary antibody (sc-2954, sc-2313; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and developed using an ECL Prime detection system (RPN2109; GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions.

Table 1 Summary of oral squamous cell carcinoma cell lines based on the original reports of their establishment

References	*Cell line*	*Differentiation*	*Character*	*Origin*	*Gender*	*Age*	*Medium*
(29)	SAS	Poor	–	Tongue	Female	69	RPMI1640 + 10% FBS
(30)	HSC-3	Poor	Invasive	Lymph node metastasis from tongue	Male	63	MEM + 10% FBS
(31)	HO-1-u-1	Poor	–	Oral floor	Male	72	RPMI1640 + 10% FBS
(32)	Ca9 22	Well	Invasive	Gingiva	Male	43	MEM + 10% FBS
(30)	HSC-2	Well	Pressure	Lymph node metastasis from oral floor	Male	69	MEM + 10% FBS
(30)	HSC-4	Well	Pressure	Lymph node metastasis from tongue	Male	63	RPMI1640 + 10% FBS

Immunofluorescence flow cytometry
The OSCC cell lines in which overexpression of *WT1* mRNA was detected by PCR were analyzed for WT1 protein using immunofluorescence flow cytometry (FACS). Single-cell suspension was obtained following trypsinization of the cultured cells. Intra stain (M3561; Dako) was used for fixation (1% formaldehyde) and permeabilization. After blocking the cells with 1 mg/ml of heat-aggregated human IgG (Sigma-Aldrich) for 30 min at 4°C, they were incubated with mouse anti-human WT1 monoclonal antibody (M3561; Dako), and the bound antibody was detected by fluorescein-conjugated $F(ab')_2$ of rabbit anti-mouse Ig (F0313; Dako). Normal rabbit serum was used as a negative control for the primary antibody. Finally, the cells were analyzed using the Beckman Coulter Epics XL flow cytometer and EXPO32 ADC software (Beckman Coulter, Brea, CA, USA).

Tissue samples
A total of 29 cases of OSCC diagnosed between 2008 and 2010 were selected from the paraffin-embedded tissue archives of our university hospital.

The study was approved by the research ethics committee of the School of Dentistry, Iwate Medical University in Morioka, Japan.

Immunohistochemistry
Formalin-fixed, paraffin-embedded tissue sections 4 μm thick were used for histopathological analyses. For antigen retrieval, these sections were placed in a microwave for 10 min in 10 mM citrate buffer (pH 6.0). After endogenous peroxidase activity in the sections was blocked with 0.3% H_2O_2 solution, they were incubated with mouse anti-human WT1 monoclonal antibody (1:100, M3561; Dako) at room temperature for 1 h. They were then processed with Dako EnVision+ Dual Link System-HRP (K4063; Dako) for 45 min, and immunoreactive WT1 protein was visualized with 3,3′-diaminobenzidine using DakoCytomation Liquid DAB+ Substrate Chromogen System (K3467; Dako).

In situ *hybridization*
A 50 base-pair DNA probe (5′-FITC-AG-GGTACGAGAGCGATAACCACACAACGCC-CATCCTCTGCGGAGCCCAAT-3′) was designed to be complementary to *WT1* mRNA based on gene data from GenBank (accession no. BC032861.2.) and labeled with fluorescein isothiocyanate (FITC) (Takara Bio, Shiga, Japan). *In situ* hybridization was performed using an ISHR Starting Kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Briefly, deparaffinized and heat-pre-treated 4-μm-thick tissue sections were digested with 5 μg/ml proteinase K, treated with 0.17% acetic anhydrate, and then subjected to treatment with pre-hybridization solution containing 50% formamide and 2× standard saline citrate (SSC) for 30 min at 42°C. Hybridization was carried out by adding 1 μg/ml diluted probe in hybridization buffer and the samples were incubated for 16 h at 42°C. After treating with washing solution (50% formamide and 2× SSC) and ribonuclease, sections were evaluated under C1si True Spectral Imaging Confocal Microscope System (Nikon, Tokyo, Japan). Sense probe hybridization was used as a control for background levels.

Results

Expression of WT1 in OSCC cell lines
Expression of *WT1* mRNA was examined in six OSCC cell lines; histologically, three of them were established from poorly differentiated OSCC, and the other three were established from well-differentiated OSCC. RT-PCR revealed the expression of *WT1* mRNA in Ho-1-u-1 (Fig. 1A), which was established from poorly differentiated squamous cell carcinoma (SCC) of oral floor, but was not detected in the other five OSCC cell lines or the NHEK.

WT1 expression levels in the OSCC cell lines were quantitatively evaluated by means of real-time PCR and compared to those levels in K562 (defined as 1.0) (10). Expression levels of *WT1* mRNA in Ho-1-u-1 were 4.6 times higher than that of K562 (Fig. 1B), while expression levels in the other four OSCC cell lines were extremely low (range, 0.30×10^{-2}–0.76×10^{-1}), similar to levels seen in NHEK (0.30×10^{-1}). The HSC-3 showed no expression of *WT1* mRNA.

When expression of WT1 protein in OSCC cell lines was examined by Western blot, all samples were negative for the protein (Fig. 2). Expression level of WT1 protein in Ho-1-u-1 by FACS was found to be the same as the negative control (data not shown).

Histopathological analyses
A summary of patient details and results of immunohistochemical analyses is shown in Table 2. Ten patients were women and 19 were men. The median age was 76 years (range of 36–90), and the mean age was 70 years old. Expression of the WT1 protein was clearly shown in two (6.9%) of the 29 patients. One was from well-differentiated OSCC of the tongue, and the other

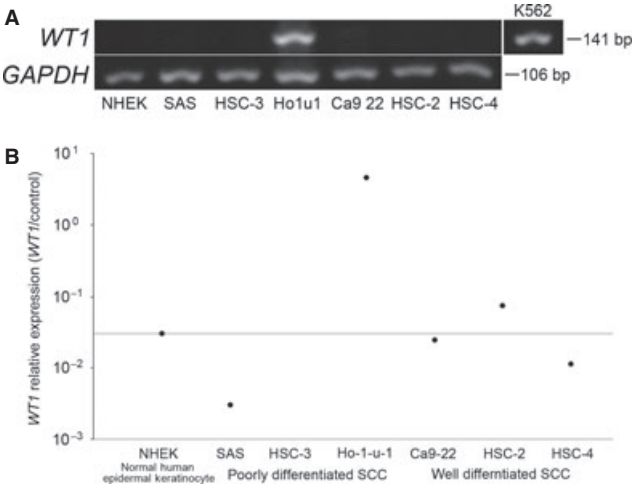

Figure 1 *WT1* mRNA expression in six oral squamous cell carcinoma cell lines. (A) *WT1* mRNA was highly expressed in Ho-1-u-1, and (B) its expression level was higher than in K562. The HSC-3 showed no expression of *WT1* mRNA.

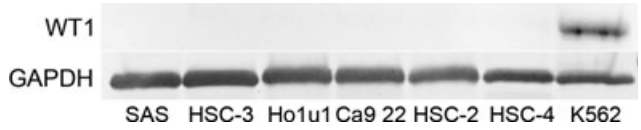

Figure 2 WT1 protein was not detected in any of the oral squamous cell carcinoma cell lines.

was from well-differentiated OSCC of the maxillary gingiva. Immunohistochemistry and *in situ* hybridization showed no expression of WT1 protein and *WT1* mRNA, respectively, in the intact epithelial area (Fig. 3A–C). WT1 protein expression was concentrated in the cytoplasm of the outer one to three cell layers (basal layer) of the infiltrating cancer nests, consistent

Table 2 Summary of clinical features of patients with oral squamous cell carcinoma and results of immunohistochemistry for WT1 protein

Case	*Location*	*Degree of differentiation*	*Age*	*Gender*	*Excision*	*WT1 protein*
1	Buccal mucosa	Well	90	Female	Biopsy	–
2	Buccal mucosa	Well	82	Female	Biopsy	–
3	Oral floor	Moderate	53	Male	Biopsy	–
4	Oral floor	Well	65	Male	Biopsy	–
5	Oral floor	Well	84	Male	Biopsy	–
6	Maxillary gingiva	Moderate	84	Male	Biopsy	–
7	Maxillary gingiva	Well	78	Male	Biopsy	+
8	Mandibular gingiva	Well	76	Female	Biopsy	–
9	Mandibular gingiva	Well	42	Male	Biopsy	–
10	Mandibular gingiva	Well	83	Female	Biopsy	–
11	Mandibular gingiva	Poor	80	Male	Biopsy	–
12	Mandibular gingiva	Well	71	Female	Biopsy	–
13	Tongue	Well	78	Male	Biopsy	+
14	Tongue	Moderate	62	Male	Operation	–
15	Tongue	Well	65	Male	Biopsy	–
16	Tongue	Well	72	Male	Biopsy	–
17	Tongue	Well	82	Female	Operation	–
18	Tongue	Well	53	Female	Biopsy	–
19	Tongue	Well	71	Male	Biopsy	–
20	Tongue	Well	36	Male	Biopsy	–
21	Tongue	Well	76	Male	Biopsy	–
22	Tongue	Well	77	Female	Biopsy	–
23	Tongue	Well	58	Male	Biopsy	–
24	Tongue	Well	83	Female	Biopsy	–
25	Tongue	Well	52	Male	Biopsy	–
26	Tongue	Well	54	Male	Biopsy	–
27	Lower lip	Well	78	Female	Biopsy	–
28	Lower lip	Well	70	Male	Biopsy	–
29	Angle of mouth	Well	86	Male	Operation	–

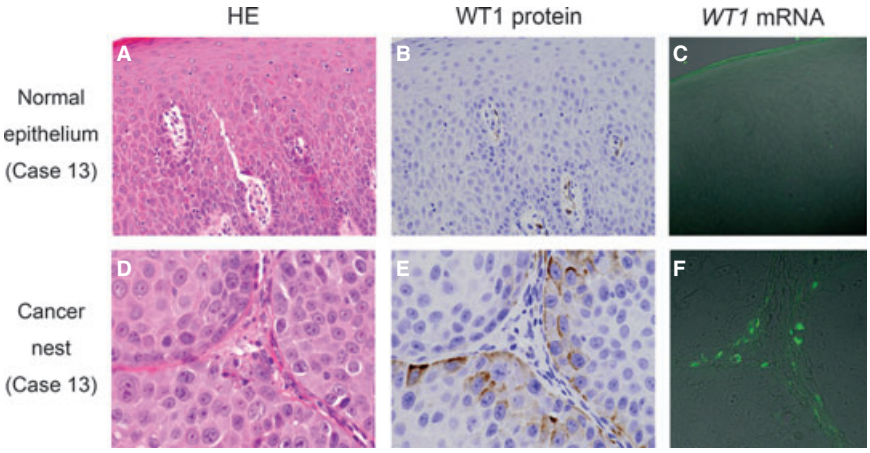

Figure 3 Detection of WT1 protein by immunohistochemistry and of *WT1* mRNA by FISH in oral squamous cell carcinoma tissue sections of case 13. (A) HE staining of normal epithelium (×200). (B) Normal epithelium was negative for WT1 protein (×200). (C) *WT1* mRNA was not detected in normal epithelium (×200). (D) HE staining of cancer nests in subepithelial layer (×400). (E) Cytoplasm of cells within basal layer of cancer nests was positive for WT1 protein (×400). (F) *WT1* mRNA was detected in basal layer of cancer nests (×400), consistent with expression of WT1 protein.

with the expression of *WT1* mRNA observed in the serial tissue sections (Fig. 3D–F).

Along the elongated epithelial ridge where droplet-cancer-nests were infiltrating toward the subepithelial layer, the cells equivalent to the basal layer of the newly formed cancer nests were positive for the WT1 protein (Fig. 4A,B). We also saw strong WT1 protein expression in the actively proliferating and infiltrating cancer nests (Fig. 4C,D).

There were no correlations found between the overexpression of WT1 and the histologic differentiation types of OSCC or affected tissue types.

Discussion

SCC is the primary tumor type in the head and neck region. The latest advances in the treatment of oral cancer have not significantly enhanced survival, and only about half of all individuals diagnosed with head and neck cancer will survive 5 years (35). Recently, WT1 peptide-based cancer immunotherapy has emerged as a possible new cancer treatment, and over the last 8 years, clinical trials of WT1 peptide-based cancer immunotherapy have shown effective clinical responses for specific kinds of cancers (18–26). Oka et al. (19) performed a phase I clinical trial for a WT1-based vaccine to patients with different cancers and confirmed that, except for a local inflammatory response with erythema at the WT1 vaccine injection sites, there were no observable damages to normal organs. Morita et al. (24) evaluated the toxicity of the weekly treatment schedule of WT1 injections in patients with solid malignancies and concluded that WT1 peptide-based immunotherapy is acceptable for patients. Further understanding of the role of WT1 on OSCC, however, will facilitate the development of new treatment strategies.

In this study, *WT1* mRNA expression in OSCC cell lines was examined quantitatively using real-time PCR. Overexpression of the *WT1* mRNA was demonstrated in one of six cell lines (Ho-1-u-1), with expression levels higher than that of K562. In this cell line, *WT1* mRNA appears to function as an oncogene, rather than a tumor-suppressor gene (8). In contrast, in the remaining five cell lines, expression levels of *WT1* mRNA were as low as that seen in NHEK or were not even observed (HSC-3). Furthermore, the Western blots and FACS experiments did not detect WT1 protein in any of the OSCC cell lines. These results suggest that although *WT1* mRNA can be transcribed in the cell lines, its translation to WT1 protein could be interfered by some factors or by rapid proteolysis. In tissue section samples from patients, *WT1* mRNA was not detected morphologically in intact epithelial layers by *in situ* hybridization. However, NHEK cell lines express low levels of *WT1* mRNA. This discrepancy between results of expression levels of *WT1* mRNA in intact epithelium and the cell lines could be due to differences in sensitivity of detecting methods.

Oji et al. (10) examined the expression levels of *WT1* mRNA in tissue samples obtained from patients diagnosed with head and neck SCC using real-time PCR and found that all four cases of mouth floor, five of nine cases of gingiva, and 17 of 25 cases of tongue SCC displayed overexpression of the *WT1* mRNA (68.4%). The positive rates of *WT1* mRNA were higher than those found in our study. In their study, the cut off levels of WT1 expression levels in normal-appearing mucosal tissues of patients were set at mean + 2 SD; however, endothelial cells of blood capillaries, muscle cells, and myoepithelial cells in the sample tissues also express *WT1* mRNA and protein, and blood capillaries proliferate greater in tumor stroma than in intact mucosa.

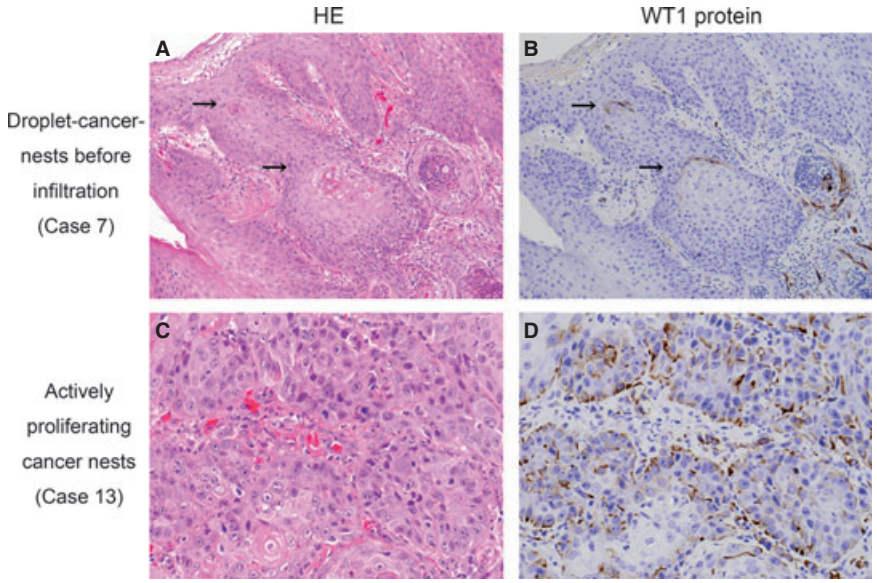

Figure 4 Detection of WT1 protein in actively proliferating cancer nests and along elongated epithelial ridge that form droplet-cancer-nests. (A, B) WT1 protein appeared along the elongated epithelial ridge during the transition to cancerous phases in case 7. WT1 positive cells were always found in basal layer (arrow) of newly forming cancer nests. (C, D) Basal layer of actively proliferating cancer nests showed strong positive signal for WT1 protein in case 13 (A; HE staining, ×200, B; WT1, ×200).

In our study, the frequency of the WT1 protein overexpression in OSCC tissue samples was 6.9%. Oji el al. (10) reported that high expression levels of the *WT1* gene showed significant correlation with poor histologic tumor differentiation and advanced tumor stage of head and neck SCC. Of the three OSCC cell lines that we examined that were established from poorly differentiated types, only one cell line showed overexpression of *WT1* mRNA. In addition, the two cases that showed positive immunohistochemical stainings were both cell lines established from well-differentiated types and affected tissues of the tongue and maxillary gingiva.

Histologically, expression of *WT1* mRNA and WT1 protein were concentrated in the outer one to three cell layer of infiltrating cancer nests, whereas the normal epithelium of the same tissue sections did not express *WT1* mRNA or WT1 protein.

Normal oral epithelium maintains its structure by a continuous renewal of cells, in which cells produced by mitotic divisions in the lower two to three cell layers of the basal layer migrate to the surface to replace those that are shed (36). Generally, this phenomenon can also be seen in the cancer nests of well-differentiated OSCC, where the basal layer of cancer nests is similar to the basal layer of normal epithelium in which cells proliferate. In this study, although expression of the WT1 protein and *WT1* mRNA was not identified in the basal layer of normal epithelium, they were both identified in the basal layer of infiltrating and proliferating cancer nests of the same tissue specimen. This suggests that WT1 is also important to neoplastic proliferation of cancer cells.

Amassing more data from OSCC cases displaying overexpression of WT1 is necessary to determine the epidemiologic characters and correlations that exist between WT1 and OSCC types. We demonstrated here the overexpression of WT1 in OSCC, and based on observations of the morphological features of tissue specimens, WT1 appears to play an important role in the pathogenesis of some types of OSCC, particularly in proliferation of the cancer cells. Two separate phase I clinical studies by Tsuboi et al. (23) and Oka et al. (19) using WT1 peptide-based cancer immunotherapy on patients with cancers of same histologic types as OSCC (i.e., squamous cell carcinoma) and that also express WT1 proteins both demonstrated a decrease in tumor marker expression and were considered to be effective. Thus, our findings and the phase I clinical study reports by Tuboi et al. (23) and Oka et al. (19) suggest that, although pathogenesis of OSCC is diverse, WT1 peptide-based immunotherapy could have potential as a new treatment option for OSCC that overexpress the WT1 protein.

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Acknowledgements

This study was supported, in part, by Grants-in-Aid for the Open Research Project (2007–2011) and Grant-in-Aid for Strategic Medical Science Research Center (2010–2014) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflicts of interest

None declared.