SPANDIDOS PUBLICATIONS

WISP-2 expression in human salivary gland tumors

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Abstract. This study was designed to disclose detailed genetic mechanisms in salivary gland tumors (SGTs) for development of novel independent marker. We constructed an in-house cDNA microarray carrying 2,201 cDNA clones derived from SGT and oral squamous cell carcinoma cDNA libraries. Four cell lines that originated from the SGT-derived cell lines were analyzed using this microarray system. The genes identified by our microarray system were further analyzed at the mRNA or protein expression level in other types of human cancer cell lines and clinical samples (ten normal salivary glands [NSGs], eleven pleomorphic adenomas, ten adenoid cystic carcinomas and three adenocarcinomas). Two up-regulated genes and six down-regulated genes were identified in common when compared with the control RNA. Of the up-regulated genes, *WISP-2*, which plays an important role in breast carcinogenesis, was selected for further analyses. We found a higher expression of the *WISP-2* gene in the SGT-derived cell lines compared with other types of human cancer cell lines. Furthermore, WISP-2 mRNA and protein expression levels in NSGs were significantly higher than those in SGTs. These results suggest that WISP-2 could be a reliable independent marker and that down-regulation or loss of the *WISP-2* gene may be associated with the development of SGTs.

Introduction

Tumors of the salivary glands account for 5% of all neoplasms of the head and neck (1,2). These tumors originate primarily in the parotid gland, occasionally in the submandibular and intraoral glands, and rarely in the sublingual gland (1,3). Salivary gland tumors (SGTs) are both benign and malignant.

The prognosis of malignant SGTs is frequently unpredictable. SGTs are slow-growing but relentless tumors, with a relatively favorable 5-year survival rate but a worse long-term outlook. Although malignant SGTs have a proclivity for invading nerves, these tumors infrequently spread via the lymphatic system. These tumors also have a protracted clinical course with local recurrences, hematogenous metastases, and a poor response to classic chemotherapeutic approaches. After surgery and radiation therapy for patients with malignant SGTs, the disease-specific survival at 15 years is approximately 40% (4).

Some markers of tumor progression, invasiveness, and prognosis are *p27 (Kip1)*, a cyclin-dependent kinase inhibitor, the oncoproteins *Bcl-2* and *Bax*, the tumor-suppressor gene product *p53*, terminal deoxynucleotidyltransferase-mediated nick end-labeling staining, and cell cycle antigen *Ki-67* (5-7). Studies of these markers in SGTs generally focused on differentiating various benign and malignant tumors. It would be helpful to discover reliable biologic markers to manage patients with malignant SGTs.

The cDNA microarray serves as a useful tool for comparing the expression levels of genes (8,9) that originate from pathologically changed tissues with those of genes that originate from normal tissues. In addition, the changes in each disease stage can be used to follow disease progression, which is useful for predicting the response to medical treatment. The emerging technology of cDNA microarray allows comparative analysis of mRNA expression of thousands of genes in parallel (10). Several studies have already demonstrated the usefulness of this technique for identifying novel cancer-related genes and classifying human cancer at the molecular level (11-13).

In this study, we first produced a specialized in-house cDNA microarray of oral squamous cell carcinomas (OSCCs) and SGTs. We have already used an in-house cDNA microarray technique to assess the gene expression profile of SGTs. Several genes identified in the present study may be involved

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in salivary gland tumorigenesis and may be potential clinical biomarkers that can improve early diagnosis and as such are potential novel therapeutic targets.

Materials and methods

Tissue specimens and cell lines. Tissue specimens were obtained at the time of surgical resection at Chiba University Hospital. We obtained written informed consent from all patients before the start of the study. All clinical specimens remained anonymous to ensure patient privacy. Five specimens of normal submandibular gland tissue and three of ACCs were used to construct the cDNA library. In addition, four SGT-derived cell lines, including HSG, HSG-AZA1, HSG-AZA3, and HSY were used. A neoplastic human salivary intercalated duct cell line (HSG) and its derivatives, HSG with a myoepithelial cell phenotype (HSG-AZA1) and HSG with an acinar cell phenotype (HSG-AZA3), induced by 5-aza-2'-dC treatment of HSG cells, were already reported (14). HSY was based on immunocytochemical characteristics in morphology, and it was thought that it occurred from intercalated ducts or acinus domain (15). These cell lines were cultured in Dulbecco's modified Eagle medium/F-12 HAM (DMEM/F12) (Sigma-Aldrich Co., St. Louis, MO), and incubated in a medium containing collagenase P (Boehringer Mannheim, Mannheim, Germany) 100 mU/ml (10 ml of medium total/preparation) for 2 h at 37˚C. The medium was collected, centrifuged at 500 x g for 10 min, and the cells were washed 3 times with medium. After a final washing, the cells were plated in DMEM/F12 containing 10% fetal bovine serum (Sigma) and 50 units/ml penicillin and streptomycin (Sigma) (16).

RNA preparation and construction of the cDNA library. Total RNA was extracted from the tissue specimens described previously using the Trizol reagent (Invitrogen Corp., Carlsbad, CA), according to the manufacturer's instructions. The oligo-capped cDNA library was constructed using 1000 μg of RNA derived from normal submandibular glands and ACCs (16).

DNA sequencing and bioinformatics analysis for data management. Bacterial culture and plasmid extractions were performed in a 96-well format (Multiscreen Assay Systems, Millipore, Bedford, MA). The sequencing reactions were performed in 9600 thermal reactors (Applied Biosystems, Foster City, CA) using a dye terminator cycle sequencing kit (Applied Biosystems). The reaction products were analyzed using the ABI-3700 DNA sequencer (Applied Biosystems) and partial 5'-end sequences were obtained for each clone. The cDNA clones were clustered according to their 5'-one-pass sequence using Dynaclust software (Dynacom, Chiba, Japan) with a default parameter. The nucleotide sequences were compared with the National Center for Biotechnology Information nucleotide databases using the Basic Local Alignment Search Tool (BLAST) programs (http://www.ncbi.nlm.nih.gov/BLAST/) (17).

Preparation of in-house cDNA microarray. A total of 2,016 cDNA clones were picked up from the oligo-capped cDNA library, and their 5'-end nucleotide sequences determined. Excluding overlapping clones, 778 distinct genes were selected. In addition, 1,423 nonidentical genes derived from the cDNA library of a mixture of OSCC and normal oral mucosa that had already been fixed in our laboratory was included in the production of the microarray (17). The cDNA microarrays consisting of these 2,201 cDNA clones were produced as previously described (8,18). Briefly, polymerase chain reaction (PCR)-amplified cDNA products were mixed with nitrocellulose in dimethyl sulfoxide just before printing and then spotted onto carbodiimide-coated glass slides (Nisshinbo Industries, Tokyo, Japan) using a robot SPBIO-2000 (Hitachi Software Engineering Co., Yokohama, Japan). The microarray also included cDNAs of housekeeping genes such as human ß-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), to serve as internal controls. A luciferase gene from *Photinus pyralis* was used as a negative control (17).

Microarray procedure. Total RNA was extracted from the four SGT-derived cell lines, HSG, HSG-AZA1, HSG-AZA3, and HSY. Universal Human Reference RNA (UHRR) (Stratagene, La Jolla, CA) was used as the control. Microarray analysis was performed as described previously (18). Briefly, Cy3-dUTP or Cy5-dUTP (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) was incorporated during reverse transcription of the 20 μg of total RNA using SuperScript II Rnase H^- Reverse Transcriptase (Invitrogen) and oligo(dT) primer (Sigma Genosys, Ishikari, Japan). In each experiment, fluorescent cDNA probes (Cy3- or Cy5-labeled) were prepared from 2 μg of the experimental mRNA sample isolated from each cell line and 2 μg of the control mRNA sample (Cy5- or Cy3-labeled). Different fluorescence-labeled probes from the cell line and the control sample were mixed and applied to the microarray, followed by incubation at 65˚C overnight in a humid atmosphere. The fluorescent images of the hybridized microarrays were scanned with a fluorescent laser confocal slide scanner (ScanArray Lite, Packard BioChip Technologies LLC, Billerica, MA). Images were analyzed with QuantArray software version 3.0 (Packard BioChip Technologies LLC), according to the manufacturer's instructions. To control for labeling differences, experiments were carried out in duplicate in which the fluorescent dyes were switched during cDNA synthesis for the SGT-derived cell lines and UHRR (Stratagene). Each pair of probes was hybridized to a separate microarray.

Analysis for mRNA and protein expression of WISP-2 in vitro. Of the up-regulated genes identified by microarray analysis, *WISP-2* was selected for further analyses, such as real-time quantitative RT-PCR (qRT-PCR) and Western blotting. Total RNAs obtained from the SGT-derived cell lines and other types of human cancer cell lines, including OSCC, hepatocarcinoma (HC), bladder cancer (BC), malignant melanoma (MM), lung cancer (LC), esophageal cancer (EC), and gastric cancer (GC) and normal fibroblast (NF) were reverse transcribed as described above. qRT-PCR was performed to evaluate expression levels of *WISP-2* mRNA in the above mentioned cell lines using a LightCycler FastStart DNA Master SYBR Green 1 kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the procedure provided by the

p-regulated and down-regulated gene in HSG, HSG-AZA1, HSG-AZA3 and HSY.

Unigene number	Definition	Chromosome location	Relative mRNA expression ratio
Up-regulated genes			
Hs. 194679	WNT1 inducible signaling pathway protein 2 (WISP-2)	20q12-q13.1	4.32
Hs. 1369	Decay accelerating factor for complement (CD55, DAF)	1q32	5.70
Down-regulated genes			
Hs. 300697	Immunoglobulin heavy constant mu	14q32.33	0.1
Hs. 77326	Insulin-like growth factor-binding protein 3	7p13-12	0.15
Hs. 401145	T cell receptor beta chain	4p11	0.25
Hs. 272499	Dehydrogenase/reductase member 2	14q11.12	0.25
Hs. 283565	Fos-like antigen 1	11q13	0.32
Hs. 297753	Human vimentin gene	10p13	0.36

manufacturer. The primer sequences used to analyze *WISP-2* mRNA expression were 5'-ATGGCAGAGGTGCAAGACC TAGTC-3' (nucleotides 1083-1106) and 5'-GGATGAAGA GAAGGCACACAGAGA-3' (nucleotides 1315-1338). The sequence of specific primers was checked before use to avoid amplification of genomic DNA or pseudogenes by the Primer3 program (available at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Amplified products were analyzed by 3% agarose gel electrophoresis to ascertain their size and purity. The PCR reactions using a LightCycler (Roche) apparatus were carried out in a final volume of 20 μl of a reaction mixture consisting of 2 μl of FirstStart DNA Master SYBR Green I mix (Roche), 3 mM $MgCl_2$, and 0.2 μl of the primers, according to the manufacturer's instructions. The reaction mixture then was loaded into glass capillary tubes and subjected to an initial denaturation at 95˚C for 10 min, followed by 45 rounds of amplification at 95˚C (10 sec) for denaturation, 61˚C (10 sec) for annealing, and 72˚C for extension, with a temperature slope of 20˚C/sec, performed in the LightCycler. The transcript amount for the *WISP-2* gene was estimated from the respective standard curves and normalized to the GAPDH transcript amount determined in corresponding samples. The statistical significance of the expression levels of *WISP-2* mRNA between two groups (SGT-derived cell lines vs. other cell lines) was calculated with the Mann-Whitney's U-test. $P<0.05$ was considered significant.

We also evaluated the protein expression status by Western blot analysis. Briefly, SGT-derived cell lines and SCC-derived cell lines were lysed in lysis buffer [10 mM Tris base (pH 8.0), 400 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, 100 mM phenylmethylsulfonyl fluoride, and 0.01% protease inhibitor cocktail (Sigma)] at 4˚C for 10 min. Cell extracts were centrifuged for 15 min at 4˚C. The protein concentration was determined in the supernatant. Protein extracts were electrophoresed on 11% sodium dodecyl sulfate-polyacrylamide electrophoresis gels, transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA), and blocked for 1 h at room temperature in 5% skim milk. Immunoblot PVDF membranes were washed with 0.1% Tween-20 in TBS (TBS-T) 5 times, and 2 μg/ml affinity-purified goat antihuman WISP-2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added directly to the TBS-T solution for 2 h at room temperature. PVDF membranes were washed again and incubated with a 1:10000 of horseradish peroxidase-conjugated antigoat IgG Ab (Santa Cruz Biotechnology) as a secondary antibody for 20 min at room temperature. Finally, membranes were incubated with ECL+ horseradish peroxidase substrate solution included in the ECL+ kit (Amersham), and immunoblotting was visualized by exposing the membrane to Hyperfilm (Amersham) (16,19).

Analysis for mRNA and protein expression of WISP-2 in vivo. The mRNA and protein expression status of WISP-2 in clinical samples were examined. Surgical specimens of five SGTs including three ACCs and two ACs, and five NSGs were selected for qRT-PCR analysis. Total RNAs were isolated from them using Trizol reagent (Invitrogen). The statistical significance of the expression levels of *WISP-2* mRNA between two groups was calculated with the Mann-Whitney's U-test. $P<0.05$ was considered significant.

Immunohistochemical staining was performed on 10 NSGs and 24 SGTs (11 PAs, 10 ACCs and 3 ACs). Briefly, the 4-μm paraffin-embedded specimens were deparaffinized and hydrated. The slides were treated with endogenous peroxidase in 0.3% hydrogen peroxide for 30 min, and the sections were blocked for 2 h at room temperature with 1.5% blocking serum (Santa Cruz Biotechnology) in phosphate-buffered saline (PBS) before reaction with anti-human WISP-2 polyclonal antibody (Santa Cruz Biotechnology) at a dilution of 1:100. The sections then were incubated with primary antibody and nonimmune control antibody for 3 h at room temperature in a moist chamber. After incubation, the samples were incubated with biotinylated antigoat IgG and peroxidase-conjugated streptavidin. The peroxidase reaction was performed using a 3,3'-diaminobenzidine tetrahydrochloride (Dako Japan Inc., Kyoto, Japan). Then the slides were counterstained with hematoxylin, dehydrated in graded ethanol, cleaned in xylene, and mounted. As a negative control, duplicate sections were immunostained without exposure to primary antibodies (16,19).

Table II. Primer pairs for RT-PCR analysis.

Gene name	Forward primer	Reverse primer	Size
WISP-2	5'-ATGGCAGAGGTGCAAGACCTAGTC-3'	5'-TCTCTGTGTGCCTTCTCTTCATCC-3'	258
GAPDH	5'-CATCTCTGCCCCCTCTGCTGA-3'	5'-GGATGACCTTGCCCACAGCCT-3'	305

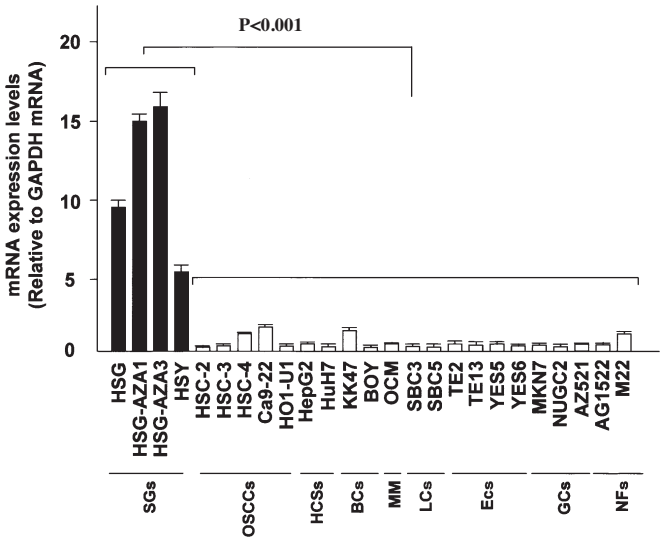

Figure 1. Quantification of mRNA levels in SGT-derived cell lines by qRT-PCR. The *WISP-2* expression levels in SGT-derived cell lines are significantly higher than in other types of human cancer cell lines [OSCC, hepatocarcinoma (HC), bladder cancer (BC), malignant melanoma (MM), lung cancer (LC), esophageal cancer (EC) and gastric cancer (GC) (P<0.001)]. P<0.05 is considered significant. The data are expressed as the mean ± SD.

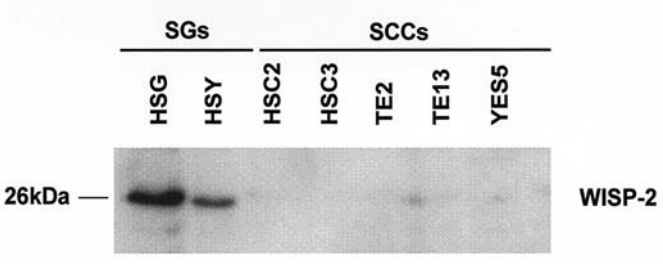

Figure 2. Representative results of expression of WISP-2 protein in SGT-derived cell lines. To investigate WISP-2 protein expression in HSG and HSY, we performed Western blot analysis. The size of the WISP-2 protein band is 26 kDa.

Results

Construction of the cDNA library and in-house cDNA microarray. We constructed a full-length-enriched cDNA library using the oligo-capping method (18) with RNA from clinical samples as described previously. We first determined the 5'-end sequence of 2,016 cDNA clones from the library and the nucleotide sequences were compared with the GenBank nucleotide databases using BLAST. To evaluate the cDNA

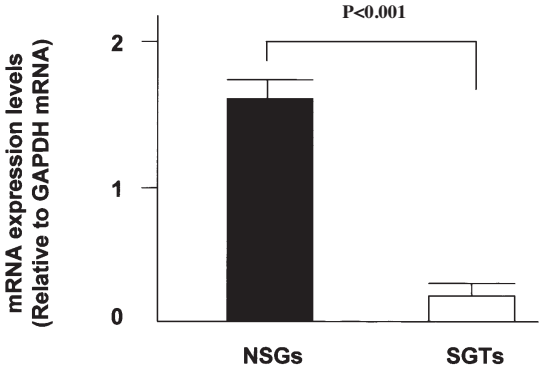

Figure 3. Comparison of *WISP-2* mRNA expression levels between NSGs and SGTs using qRT-PCR. The ratios (*WISP-2*/GAPDH) for NSGs (n=5) and SGTs (n=5) ranged from 0.43 to 2.41 (median, 1.18) and from 0.01 to 0.31 (median, 0.07). The *WISP-2* expression levels in NSGs were significantly higher than those in SGTs (P=0.009). A probability of P<0.05 is considered significant.

library, the nucleotide sequences of 96 randomly chosen clones were investigated. The results showed that 65% of the cDNA clones were full length. The average length of the cDNA insert was approximately 1.6 kilobase pairs. The 778 distinct clones were selected from the sequence information. In addition, 1,423 nonidentical genes derived from the cDNA library of a mixture of OSCC and normal oral mucosa that had already been fixed in our laboratory was included in the production of the microarray. The cDNA microarrays consisting of these 2,201 cDNA clones were produced as previously described. The in-house cDNA microarray reflects characteristics of SGTs, NSGs, OSCC, and oral mucosa.

cDNA microarray analysis. To identify the genes associated with SGTs, we analyzed the gene profiles of four SGT-derived cell lines (HSG, HSG-AZA1, HSG-AZA3, and HSY) using our in-house cDNA microarray, which contains 2,201 independent cDNA clones. The microarray was subsequently hybridized with cDNA probes labeled with fluorochromes. Eight individual hybridization experiments were performed, and fluorescent dyes for the probes were exchanged in half of the experiments. Microarray analysis showed that two genes were commonly up-regulated 2-fold or more and six genes were commonly down-regulated 0.5-fold or less in these SGT-derived cell lines, compared to the status of the UHRR control genes (Stratagene). Among them, we identified eight genes that were expressed in common (Table I).

Analyses of mRNA and protein expression of WISP-2 in vitro. The qRT-PCR analysis data were matched to mRNA

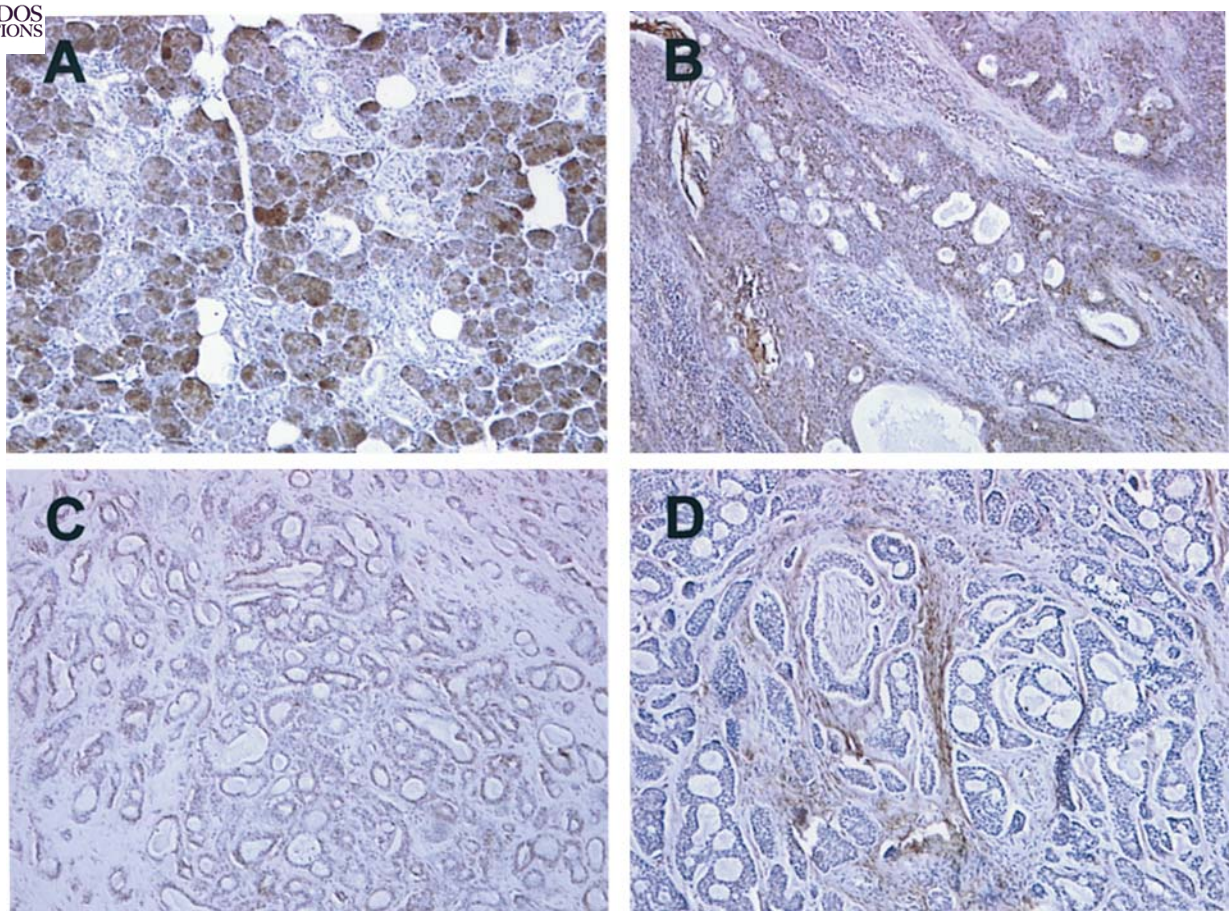

Figure 4. Immunohistochemical staining of WISP-2. (A) NSGs show strong immunoreaction of the acinar cell. (B) A moderate staining for WISP-2 is evident in a PA case. (C) A significant lower WISP-2 expression is seen in an AC case. (D) Negative staining for WISP-2 in tumor cells of ACC. Original magnification x200.

expression studied in the microarray analysis. WISP-2 and GAPDH primer pair and the RT-PCR conditions are summarized in Table II. The mRNA expression of *WISP-2* was significantly reduced in other types of human cancer cell lines (HC, BC, MM, LC, EC, and GC) and NF. The mRNA expression levels were normalized to GAPDH. Fig. 1 shows a significant increase in the expression of *WISP-2* in all SGT-derived cell lines examined compared with other cancer cell lines used as controls. The *WISP-2* expression levels in SGT-derived cell lines were significantly higher than other cancer cell lines ($P<0.001$).

To investigate WISP-2 protein expression in SGT-derived cell lines and SCC-derived cell lines, we performed Western blot analysis. Fig. 2 shows representative results of Western blot analysis for WISP-2 protein expression. The size of the band was 26 kDa. A significant increase in WISP-2 expression was observed in the SGT-derived cell lines examined compared with the SCC-derived cell lines. Taken together, mRNA and protein analyses indicated that both transcription and translation products of this molecule were highly expressed in SGT-derived cell lines.

Analyses of mRNA and protein expression of WISP-2 in vivo. To examine WISP-2 mRNA expression *in vivo*, we analyzed WISP-2 mRNA expression levels in five NSGs and five SGTs using qRT-PCR. The ratios (*WISP-2*/GAPDH) for NSGs and SGTs ranged from 0.43 to 2.41 (median, 1.18) and from 0.01 to 0.31 (median, 0.07, Fig. 3). The *WISP-2* expression levels in NSGs were significantly higher than those in SGTs ($P=0.009$, Fig. 3).

To investigate the distribution of WISP-2 protein expression *in vivo*, we performed immunohistochemical staining of the protein in 34 clinical samples, including 24 SGTs (11 PAs, 10 ACCs and 3 ACs) and 10 NSGs. NSGs revealed strong immunoreaction of the acinar cell (Fig. 4A). A moderate staining for WISP-2 was evident in a PA case (Fig. 4B). A significant lower WISP-2 expression was seen in an AC case (Fig. 4C). Negative staining for WISP-2 in tumor cells of ACC was observed (Fig. 4D). No staining was observed in a control section in which primary antibody was replaced by 1% bovine serum albumin in PBS (data not shown).

Discussion

The prognoses of malignant SGTs are frequently unpredictable. These tumors are slow growing but relentless, with a relatively favorable 5-year survival rate but a worse long-term outlook (20). It would be helpful to discover reliable biologic makers to manage patients with malignant SGTs. cDNA microarray systems have been used to identify genes in malignant tumors, such as malignant lymphoma (21), hepatocellular carcinoma (22,23), breast (24,25), prostate (26), colorectal

(27), esophageal (28,29), gastric (30), and head and neck cancer (17,31,32), and ovarian carcinoma (33). We produced an in-house cDNA microarray that contains 1,423 cDNA clones derived from the OSCC cDNA library and 778 cDNA clones derived from the SGT cDNA library. To identify the genes associated with the development of SGT, the in-house cDNA microarray was used to analyze gene expression profiles of four SGT-derived cell lines (HSG, HSG-AZA1, HSG-AZA3, and HSY). Two up-regulated genes and six down-regulated genes were identified in common when compared with the UHRR (Stratagene). Of the up-regulated genes, *WISP-2*, which plays an important role in breast carcinogenesis, was selected for further analyses by qRT-PCR, and Western blotting. Consequently, we found higher expression of the *WISP-2* gene in the SGT-derived cell lines compared to other types of human cancer cell lines derived from OSCC, HC, BC, MM, LC, EC, and GC. WISP-2 mRNA and protein expression levels in NSGs were significantly higher than those in SGTs.

WISP-2 is a member of the connective tissue growth factor/cysteine-rich 61/neuroblastoma-overexpressed (CCN) family and is coming under increasing scrutiny in cancer research (34). Two family members (*WISP-1* and *WISP-3*), closely related to the *WISP-2* gene, were identified and described in humans (35). Nucleotide and protein sequence alignment studies have demonstrated a 30-40% sequence homology within the *WISP* genes (*WISP-1*, *WISP-2*, and *WISP-3*). Their modular architecture is similar except in their C-terminal domain, which is absent in the *WISP-2* gene (35). The functional significance of this newly identified molecule has not yet been established. Increasing evidence suggests that WISP-2 proteins may play an important role in the evolution of various cancers (35), and the finding that expression of WISP-2 can be modulated by 17ß-estradiol (Banerjee SK, 5th World Congress on Advances in Oncology and 3rd International Symposium on Molecular Medicine, Athens, Greece, 2000), a natural estrogen that modulates breast cancer cell proliferation (36) and that is associated with several types of human and animal cancers (37-39), further enhances interest in mitogenic and/or carcinogenic potential of the molecule. The gene for human *WISP-2* was localized to chromosome 20q12-20q13, at a region frequently amplified and associated with poor prognosis in node-negative breast and colon cancer, suggesting the presence of one or more oncogenes at this locus (34,35,40,41). In contrast, the finding that WISP-2 is underexpressed in human colon tumors suggests potential tumor-suppressive properties (35).

A recent report on *rCop-1*, the rat orthologue of *WISP-2*, described the loss of expression of this gene after cell trans-formation, suggesting it may be a negative regulator of growth in the cell line (42,43). Although the mechanism by which *WISP-2* RNA expression is down-regulated during malignant trans-formation is unknown, the reduced expression of *WISP-2* in colon tumors and cell lines suggests that it may function as a tumor suppressor (35). Thus, the status of *WISP-2* is likely to differ from tumor to tumor. To resolve the inconsistency regarding the functional relevance of WISP-2, its expression was evaluated in SGT. Considering the possible complexity of WISP-2 function in different tumors, it seems logical that it may not be a universal positive regulator of cell proliferation. Further detailed studies are required to resolve this issue.

In conclusion, we constructed an in-house cDNA micro-array carrying 2,201 cDNA clones derived from oligo-capped SGT and OSCC cDNA libraries. Using the microarray, the most overexpressed gene in SGT-derived cell lines relative to the control was *WISP-2* together with certain genes that are selectively overexpressed in SGT-derived cell lines relative to other types of carcinoma cell lines. In addition, WISP-2 was differentially expressed in NSGs and SGTs. The expression of WISP-2 was significantly higher in NSGs compared to SGTs. Therefore, we suggest that WISP-2 is a reliable independent marker and that down-regulation or loss of the *WISP-2* gene may be associated with the development of SGTs.

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