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 tissues, 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 in the SGT-derived cell lines 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 outcome. 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...
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 the acinar cell phenotype (HSG-AZA3), and HSY with an 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 used to construct the cDNA library. In addition, 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 Transciptase (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 Quant-Array 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.

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 Transciptase (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 Quant-Array 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
Down-regulated genes

<table>
<thead>
<tr>
<th>Unigene number</th>
<th>Definition</th>
<th>Chromosome location</th>
<th>Relative mRNA expression ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hs. 300697</td>
<td>Immunoglobulin heavy constant mu</td>
<td>14q32.33</td>
<td>0.1</td>
</tr>
<tr>
<td>Hs. 77326</td>
<td>Insulin-like growth factor-binding protein 3</td>
<td>7p13-12</td>
<td>0.15</td>
</tr>
<tr>
<td>Hs. 401145</td>
<td>T cell receptor beta chain</td>
<td>4p11</td>
<td>0.25</td>
</tr>
<tr>
<td>Hs. 272499</td>
<td>Dehydrogenase/reductase member 2</td>
<td>14q11.12</td>
<td>0.25</td>
</tr>
<tr>
<td>Hs. 283565</td>
<td>Fos-like antigen 1</td>
<td>11q13</td>
<td>0.32</td>
</tr>
<tr>
<td>Hs. 297753</td>
<td>Human vimentin gene</td>
<td>10p13</td>
<td>0.36</td>
</tr>
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manufacturer. The primer sequences used to analyze WISP-2 mRNA expression were 5'-ATGGGCAAGGGTCAAGACCG TAGTC-3' (nucleotides 1083-1106) and 5'-GGATGAAGA GAAGGCACACAGA-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 MgCl2, 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 MgCl2, 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 anti-human 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 goat 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).
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 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
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
the inconsistency regarding the functional relevance of WISP-2 is unknown, the reduced expression of WISP-2 in the cell line (42,43). Although the mechanism by which formation, suggesting it may be a negative regulator of growth described the loss of expression of this gene after cell trans-
tumor-suppressive properties (35).

locus (34,35,40,41). In contrast, the finding that WISP-2 is underexpressed in human colon tumors suggests potential 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

architecture is similar except in their C-terminal domain, which is absent in the WISP-2 gene (35). The functional

expression of WISP-2 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

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