Objective: To confirm the expression of 14-3-3 sigma in oral malignant lesions and in adjacent nonmalignant oral epithelium to provide a clue to the involvement in the cell cycle progression and note any association with human papillomavirus (HPV) status. 14-3-3 Sigma plays important roles in a wide range of vital regulatory processes, including signal transduction, apoptosis, cell cycle progression, and DNA replication. 14-3-3 Sigma is an exclusive epithelial marker, and data on its expression in different malignancies are very scarce.

Design: Western blotting, immunohistochemical analysis, and polymerase chain reaction were performed.

Setting: An academic university laboratory.

Patients: Adults with known oral squamous cell carcinomas (SCCs) that were surgically resected.

Main Outcome Measures: The DNA of HPV-16 E6 was detected by polymerase chain reaction, and protein expression of 14-3-3 sigma was evaluated by Western blot and immunohistochemical analysis.

Results: The immunoreactive 14-3-3 sigma protein was detected mainly in the cytoplasm of differentiated squamous cells of oral SCC lesions as well as adjacent nonmalignant squamous mucosa. Immunoreactivity for 14-3-3 sigma was observed in 93% of SCC lesions (27 of 29), including HPV-negative cases. No significant association was observed between 14-3-3 sigma expression and clinicopathologic parameters. A statistically significant correlation was found between 14-3-3 sigma protein expression and the Ki-67 labeling index. 14-3-3 Sigma expression was correlated inversely with HPV-16 E6.

Conclusion: These findings suggest that 14-3-3 sigma may act as a negative regulator of the cell cycle progression in oral SCC.


The 14-3-3 family of proteins consists of 7 isoforms that are highly conserved over many eukaryotic organisms. Much recent interest has focused on the sigma isoform expressed in human epithelial cells (sometimes called HME-1 or stratifin). A crucial role of 14-3-3 sigma is its control of the G2 cell cycle checkpoint. At G2 phase, cdc2-cyclin B1 normally enters the nucleus to initiate mitosis. In response to DNA damage, 14-3-3 sigma is induced in a p53-dependent manner and prevents the cdc2-cyclin B1 complex from entering the nucleus. These changes provide an opportunity for DNA repair of damage before further cell cycle progression. Indeed, cells lacking 14-3-3 sigma function have impaired cell cycle control after DNA damage and increased genomic instability.

Reports that describe variability in 14-3-3 expression portray an obscure picture; elevation in 14-3-3 expression has been correlated with cancer. However, suppression in 14-3-3 expression has also been related to cancer. 14-3-3 Sigma downregulation in squamous cell carcinomas (SCCs) may be due to sufficient sigma loss to immortalize squamous epithelium. Another possibility is that silencing of sigma is not required for immortalization but occurs at a stage after immortalization. Loss at this stage may result in impaired differentiation, altered response to apoptotic stimuli, or higher levels of proliferation.

Uterine cervical cancers without 14-3-3 sigma expression were predominantly those lacking human papillomavirus (HPV) DNA. 14-3-3 Sigma methylated oral carcinomas were significantly more likely to lack DNA sequences from HPV. Patients without 14-3-3 sigma expression had significantly worse overall survival rates than patients with 14-3-3 sigma expression.
thermore, the loss of 14-3-3 sigma in ovarian cancer cases showed invasive and progressive characteristics. Our group's previous study demonstrated that patients with HPV-negative oral SCC had worse disease-specific survival than those with HPV-positive disease. This same relationship was also found in previous studies focusing on oral cancer and the oncogenic HPV types.

In the present study, we aimed to confirm the expression of 14-3-3 sigma in oral malignant lesions and in adjacent nonmalignant oral epithelium to provide a clue to the involvement in the cell cycle progression and note any association with HPV status.

METHODS

PATIENTS AND SAMPLES

The patients provided written informed consent under a protocol approved by the appropriate institutional review boards. Cancerous lesions, along with patient-matched nonmalignant tissue when available, were obtained at the time of surgery. Tissue specimens from 29 oral SCCs were retrieved from the Department of Clinical Laboratory, Hiroshima University Dental Hospital. After surgical removal, cancer tissues and nonmalignant tissues were collected into sterile tubes, immediately frozen in liquid nitrogen, and stored at -80°C until analyzed. Hematoxylin-eosin-stained frozen sections of tumor specimens were examined under light microscopy to assess the relative amounts of cancer tissue and normal tissue. Tissue blocks were carefully trimmed to remove nonmalignant tissue before extraction of DNA and protein. Histopathologic examination was performed on 10% formalin-fixed, paraffin-embedded specimens by a pathologist in the Pathology Division.

ORAL SCC

Twenty-nine patients with cancer were included in the study, 20 men and 9 women, their ages ranging from 48 to 90 years (median age, 67 years). In clinical appearance, papillomatous and leukoplakialike SCCs were present in 4 patients each. Granulomatous and ulcerative SCCs were also present in 10 patients each. Histologic grade 3 disease (poorly differentiated neoplasms) was noted in 2 of the 29 patients, while the remaining cases were grouped into a single category of grade 1/2 (well/moderately differentiated).

GENOMIC DNA EXTRACTION AND POLYMERASE CHAIN REACTION

We extracted genomic DNA from oral tissues using a genomic DNA purification kit to detect HPV-16 E6 DNA. All samples were analyzed for the presence of HPV-16 E6 by polymerase chain reaction (PCR) as described previously.

IMMUNOHISTOCHEMICAL ANALYSIS

Sections were deparaffinized, and peroxidase was quenched with methanol and 3% hydrogen peroxide for 15 minutes. Thereafter, slides were placed in antigen unmasking buffer, pH 6.0, and then underwent microwave treatment (three 4-minute treatments). The primary reaction with mouse monoclonal anti-14-3-3 sigma antibody (clone 1433S01; Neo Markers, Fremont, California) and MIB-1 (Zymed Laboratories Inc, San Francisco, California) for Ki-67 was applied for 60 minutes at room temperature. Following washing with Tris-buffered saline (TBS), sections were incubated with a secondary goat antirabbit antibody (Medical and Biological Laboratories, Nagoya, Japan) for 60 minutes and washed in TBS. The color was developed by 5-minute incubation with 3,3-diaminobenzidine tetrahydrochloride solution, and sections were weakly counterstained with hematoxylin.

Human normal skin with strong cytoplasmic 14-3-3 sigma expression was used as the positive control. Normal mouse IgG was substituted for the primary antibody as the negative control, at a concentration at which immunostaining of control slides gave faint staining. The percentage of tumor cells with cytoplasmic 14-3-3 sigma reactivity was recorded after inspection of all fields in the tissue sample. The percentage of positive cells was recorded in each individual field, and the final score for each case was the median value obtained. We classified the cases into 4 categories according to their positive cell rate as follows: --, negative; +, weak staining in any tumor cells or moderate staining in 1 of 10 tumor cells; ++, moderate or strong staining in fewer than 1 of 3 tumor cells; and ++++, strong staining in more than 1 of 3 tumor cells. For Ki-67 labeling index, the number of positively staining nuclei was counted in 1000 tumor cells at high magnification and then expressed as a percentage of the total number of positive cells. All immunostaining was interpreted independently by 2 different observers who were blind to genetic abnormalities.

STATISTICAL ANALYSIS

Significant differences were analyzed with the Fisher exact test. P values less than .05 were regarded as statistically significant.

RESULTS

DETECTION OF HPV-16 E6

The DNA of HPV-16 E6 was detected in 11 of 29 oral SCC lesions (approximately 38%) (Table 1). It was relatively common in SCC of the floor of the mouth (3 of 5; 60%), buccal mucosa (2 of 4; 50%), gingiva (3 of 8; 37%), and the tongue (2 of 9; 22%). By clinical appearance, HPV-16 E6 DNA was relatively common in papillomatous (3 of 4; 75%) and granulomatous (4 of 10; 40%) oral SCCs, but it was less common in ulcerative (3 of 10; 30%) and leukoplakialike (1 of 4; 25%) SCCs.

IMMUNOHISTOCHEMICAL ANALYSIS FOR 14-3-3 SIGMA

Immunohistochemical results are summarized in Table 1. Representative results of immunohistochemical analysis are shown in Figure 1. Immunoreactivity (either ++ or ++++) for 14-3-3 sigma was observed in the cytoplasm of 51% of oral SCCs, including those testing negative for HPV-16 E6. The immunoreactivity in the neoplastic squamous epithelium was generally more intense than that in the nonneoplastic squamous epithelium adjacent to the lesions. Immunoreactivity of 14-3-3 sigma protein was mainly expressed in the cytoplasm of differentiated squamous cells of oral SCCs and in nonneoplastic squamous cells. However, the distribution pattern of immunopositive cells was slightly different between them. In nonneoplastic squamous epithelium, positive cells were mainly observed in granular and spinous layers (Figure 1), but they were hardly seen in the basal layers. In oral SCC,
immunopositivity was generally observed in differentiated tumor cells away from tumor-mesenchymal border (Figure 1). In some cases, immunopositivity was observed even at the border.

The distribution of negative, weak, moderate, and strong staining for 14-3-3 sigma in oral SCCs was 7% (2 of 29), 41% (12 of 29), 45% (13 of 29), and 7% (2 of 29), respectively. An even more dramatic increase in 14-3-3 sigma immunoreactivity was observed in HPV-positive oral SCCs. In those specimens, 82% of carcinoma cells expressed moderate or strong 14-3-3 sigma immunoreactivity (n=9). Two of fourteen 14-3-3 sigma–weak or

<table>
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<tr>
<th>Case No./Sex/Age, y</th>
<th>Site</th>
<th>Clinical Appearance</th>
<th>Differentiated</th>
<th>14-3-3 Sigma Findings</th>
<th>HPV-16 E6 Findings</th>
<th>Ki-67, %a</th>
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<td>+</td>
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<td>+</td>
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<td>Well</td>
<td>+++</td>
<td>–</td>
<td>25</td>
</tr>
</tbody>
</table>

Abbreviations: –, negative; +, weak staining in any tumor cells or moderate staining in 1 of 10 tumor cells; ++, moderate or strong staining in fewer than 1 of 3 tumor cells; ++++, strong staining in more than 1 of 3 tumor cells.

* For Ki-67 labeling indexing, the number of positively staining nuclei was counted in 1000 tumor cells at high magnification and then expressed as a percentage of the total number of positive cells.

Figure 1. Immunohistochemical expression of 14-3-3 sigma in oral cancer (hematoxylin). A, Adjacent nonneoplastic epithelial cells stained weakly in granular and spinous layers (original magnification ×10). B, The immunoreactive 14-3-3 sigma protein was mainly expressed in cytoplasm of differentiated squamous cells of oral cancer cells (original magnification ×20).

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14-3-3 sigma–negative cases tested positive for HPV-16 E6 DNA, while the others tested negative for HPV \( (P = .03) \) (Table 2).

**THE CORRELATION BETWEEN 14-3-3 SIGMA EXPRESSION AND CELLULAR PROLIFERATION**

We evaluated the correlation of 14-3-3 sigma expression with a cellular proliferation marker, the Ki-67 labeling index. We found statistically significant correlation between 14-3-3 sigma protein expression and Ki-67 labeling index in oral cancer \( (P = .002) \) (Table 2 and Figure 2).

**COMMENT**

It remains unclear how regulation of 14-3-3 sigma contributes to the development of neoplasia and whether the loss of 14-3-3 sigma expression directly correlates with malignant transformation. Given 14-3-3 sigma’s negative role in cell growth and positive role in potentiating p53 activity, it is conceivable that 14-3-3 sigma plays an important role in controlling cancer formation. Indeed, overexpression of 14-3-3 sigma can antagonize oncogene-mediated cell growth and transformation in breast cancer cell lines. Conversely, experimental downregulation of 14-3-3 sigma allows primary human epithelial cells to grow indefinitely, suggesting that a decrease in 14-3-3 sigma expression may contribute to tumor formation by promoting cellular immortalization. Importantly, 14-3-3 sigma is downregulated in several types of cancer, including breast cancer, ovarian cancer, and gastric cancer. Usually, the downregulation of 14-3-3 sigma is the result of epigenetic silencing by CpG methylation rather than genetic alteration (CpG indicates a cytosine and guanine separated by a phosphate, which links the 2 nucleosides together in DNA). Using microarray technology, Villaret et al demonstrated that 14-3-3 sigma messenger RNA was significantly overexpressed in primary head and neck SCC compared with normal tissues. In oral tissues, our study clearly revealed that SCCs showed stronger 14-3-3 sigma protein expression than the adjacent nonmalignant squamous mucosa. Although the number of oral SCC lesions examined was small, dysplasias later converting to oral SCCs showed stronger immunoreactivity than dysplasias not converting to oral SCCs in our Western blot and immunohistochemical results (data not shown).

An additional unresolved question is how 14-3-3 expression alternates with development and differentiation. 14-3-3 Sigma expression is significantly associated with the Ki-67 labeling index in gastric cancer. In our
study, overexpression of 14-3-3 sigma markedly decreased the Ki-67-positive cell number. Absence of 14-3-3 sigma was significantly correlated with a high Ki-67 labeling index. Collectively, these results suggest that 14-3-3 sigma overexpression can prevent cell proliferation.

Human papillomavirus type 16 E6 was detected in 38% of the SCC lesions in our series (n = 11), comparable with the findings of our group's previous large studies, and most of the HPV-negative SCCs showed weak or undetectable expression of 14-3-3 sigma (12 of 14). 14-3-3 Sigma is induced by p53 in response to DNA damage and mediates a G2 checkpoint. Such a mechanistic model might imply that downregulation of 14-3-3 sigma would not usually occur in cancers with a p53 mutation or in human HPV-associated carcinomas because p53 is targeted by HPV-encoded E6. 14-3-3 Sigma expression was correlated inversely with HPV. Our results are very similar to those seen by Sano et al. In conclusion, we showed that 14-3-3 sigma expression increases in oral SCC compared with the nonmalignant oral mucosa, and it may act as a negative regulator of cell cycle progression.

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Author Contributions: Drs Bhawal and Sugiyama had full access to all the data in the study, take responsibility for the integrity of the data and the accuracy of the data analysis, and contributed equally to this work. Study concept and design: Bhawal, Sugiyama, and Nomura. Acquisition of data: Bhawal. Analysis and interpretation of data: Sugiyama, Kuniyasu, and Tsukinoki. Drafting of the manuscript: Bhawal. Critical revision of the manuscript for important intellectual content: Bhawal, Sugiyama, Nomura, Kuniyasu, and Tsukinoki. Statistical analysis: Bhawal. Obtained funding: Sugiyama. Administrative, technical, and material support: Sugiyama, Kuniyasu, and Tsukinoki. Study supervision: Sugiyama and Nomura.

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