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The relationship of the histologic grade at the deep invasive front and the expression of Ki-67 antigen and p53 protein in oral squamous cell carcinoma

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BACKGROUND: Although many histopathologic characteristics of oral squamous cell carcinoma (O-SCC) have been identified as prognostic factors, accurate, and unequivocal factors have not been clearly identified. The purpose of this study was to evaluate a potential association between the histologic grade of malignancy at the deep invasive front and the expression of Ki-67 antigen and p53 protein in O-SCC.

METHODS: The expression of Ki-67 antigen and p53 at the invasive tumor front area of O-SCC was examined by immunohistochemistry of archived tissue from 62 cases. The mean age of patients was 60.7 years (range: 37–89) and the male–female ratio was 1.6:1 (38 men, 24 women). There were 20, 17, 14, and 11 cases classified as stage I to stage IV, respectively. The correlation between the intensity of immunostaining for Ki-67 antigen and p53 and the histologic grade of malignancy at the deep invasive front (invasive front grade, IFG) was analyzed. The expression of Ki-67 antigen and p53 in normal oral epithelia (10 cases) was also investigated.

RESULTS: The mean Ki-67 labeling index (LI) in the O-SCC samples was 32.8 ± 12.0% ($n = 62$). The mean total score of IFG (IFG score) was 9.1 ± 2.7 points ($n = 62$). There was a significant linear correlation between the IFG score and the Ki-67 antigen ($\gamma = 0.651$, $R^2 = 0.596$, $P < 0.0001$). Of 50 tumors examined, 27 (54.0%) exhibited p53-positive nuclear immunostaining. The staining patterns for Ki-67 antigen and p53 were similar. Both Ki-67-LI and p53-positive status were significantly correlated with the IFG scores.

CONCLUSION: The findings of this study demonstrate that overexpression of Ki-67 antigen and p53 at the deep tumor invasive front of O-SCC is associated with histologic grade of malignancy.

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Keywords: deep invasive front; histologic grade of malignancy; Ki-67 antigen; oral squamous cell carcinoma; p53 protein

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Introduction

Several reports have examined the usefulness of clinical and pathologic factors for the prognosis of oral squamous cell carcinoma (O-SCC) (1–10). In particular, the histologic features of O-SCC may differ widely from area to area within the same tumor (11–14), and it is believed that the most useful prognostic information can be deduced from the invasive front of the tumors, where the deepest and presumably most aggressive cells reside (15–18). Recent seminal studies by Bryne et al. (19) suggest that the invasive tumor front is the region of the tumor with the highest prognostic utility. Similarly, we previously found that the histologic grade of malignancy at the deep invasive front (invasive front grade, IFG) had a high prognostic value for SCC of the tongue (20).

Tumor cell proliferation activity is believed to indicate the degree of aggressiveness of the tumor (21). The proliferative activity of carcinoma cells is generally considered related to the degree of malignancy of carcinoma tissue (18, 21, 22). The two most common immunohistochemical markers used to study cell proliferation are proliferating cell nuclear antigen (PCNA) and the Ki-67 antigen (18). The Ki-67 antigen is expressed in proliferating cells (in the G1, S, G2, and M phases), but not in resting cells (G0 phase) (18, 21).

The tumor suppressor gene for p53 (TP53) is located on the short arm of chromosome 17, and encodes a protein of 393 amino acids (23). Point mutations in p53 are important in the development of malignancy, and are frequently observed in carcinomas of various tissues, including O-SCC (24, 25).

Recently, immunohistochemical detection of the Ki-67 antigen and p53 protein was associated with the prognosis of human carcinoma (22, 25–28). However, few studies have described the correlation between IFG and expression of Ki-67 antigen and p53 in O-SCC (18, 21, 29, 30). This immunohistochemical study was designed to evaluate the association between IFG and expression of Ki-67 antigen and p53 in archived tissues.

Patients and methods

Tissue specimens were surgically removed from 77 patients with O-SCC at Kyushu Dental College Hospital between 1993 and 1998. Excluded from the present study were 15 patients with disseminated disease, other serious illness, or poor general condition that precluded treatment with curative intent. This study focuses on the remaining 62 patients (38 men, 24 women; mean age 60.7 years; range: 37–89) in which the biopsy specimens had confirmed tumor infiltration into the connective tissue and for which the quantity of the specimen was sufficient for malignancy grading. All patients were treated with surgical of the tumor and immediate reconstruction through the use of skin grafts, myocutaneous flaps, or free flaps. None of the patients received pre-operative treatment. Classical or modified radical neck dissection was performed in all patients with clinically positive neck nodes.

The tumors were located in the tongue (23 cases), in the maxillary gingiva (19 cases), in the mandibular gingiva (13 cases), in the oral floor (four cases), and in the buccal mucosa (three cases). There were 20, 17, 14, and 11 cases classified as stage I to stage IV, respectively.

Histopathologic evaluation

Paraffin-embedded specimens were retrieved from the archives, and 4–6 μm serial sections were cut. Alternate sections were stained with hematoxylin and eosin (H & E). Two pathologists and two oral surgeons, who were blind to clinical data, reviewed all pathologic specimens. The initial biopsy for histologic diagnosis was performed so that the excised specimen included a portion that was as deep as possible with respect to the border between tumor and normal tissue, and contained a representative portion sufficient to determine the histologic grade of malignancy.

The histologic grade of malignancy at the deep invasive front was determined by the method of Bryne et al. (19). For each tumor, the degree of keratinization, nuclear polymorphism, pattern of invasion, and host response (degree of leukocyte infiltration) were graded and given scores between 1 and 4, which were summed to yield the total IFG score. Tumor depth was measured from the surface of the normal mucosa to the deepest portion of the tumor.

Immunohistochemical studies

Tissue sections were deparaffinized and incubated in methanol with 3% hydrogen peroxidase for 5 min to eliminate endogenous peroxidase activity. Antigen retrieval was conducted by autoclaving the sections used for Ki-67 antigen and p53 immunostaining at 121°C in 0.01 M citrate buffer (pH 6.0) for 10 min. The sections were treated with normal goat or rabbit serum for 15 min to block non-specific binding, and then incubated overnight at 4°C with the primary antibodies. The following monoclonal antibodies were used: anti-Ki-67 antigen (MIB-1, diluted 1:200; Immunotech, Paris, France) (29, 30) and anti-p53 (DO-7, diluted 1:100; Dako, Denmark) (31, 32). The EnVision plus kit (Dako, Tokyo, Japan) was used for application of the secondary antibody, according to the manufacturer's instructions, and the reaction products were visualized by immersing the sections for 3–10 min in 0.03% diaminobenzidine (DAB) solution containing 2 mM hydrogen peroxide. The sections were then briefly counterstained with Mayer's hematoxylin, dehydrated, and mounted. Sections from breast cancer with known Ki-67 or p53 overexpression were used as positive control. The negative control was done by omission of the each primary antibody.

The Ki-67-positive cells were quantified by light microscopy at 200× magnification. Positively stained cells in the invasive front area and in the normal epithelium were counted in at least 10 fields for each slide. Cells that lacked a clear nucleus were excluded and a minimum of 1000 cells was counted in each section. The percentage of positive cells was then calculated to obtain the labeling index (LI). The pattern of p53 immunostaining in the invasive front area was also assessed and classified as negative (−) when $<10\%$ of the cells were reactive for p53 and positive (+) when more than 10% of the cells were reactive for p53 (25).

Statistical analysis

All data were tabulated and statistical tests were performed using the STATVIEW software package (SAS Institute, Cary, NC, USA). The correlation between Ki-67-LI and IFG scores was analyzed by the Spearman rank correlation test. The correlation between the IFG scores and the pattern of p53 immunostaining, or Ki-67-LI were evaluated with the Mann–Whitney *U*-test. The results were considered significant when the *P*-value was <0.05.

Results

Antigen Ki-67 immunoreactive cells were found in all 62 tumors examined. The intensity of the nuclear immunoreactivity for Ki-67 varied from weak to strong. The nuclei of tumor cells were considered positive for Ki-67 when they appeared either clearly light brown or brown under light microscopy. Of 50 tumors examined for the pattern of p53 expression, 27 (54.0%) exhibited p53-positive granular or reticular nuclear immunostaining. The p53-positive cells were observed predominantly in the invasive tumor front areas and not in the central

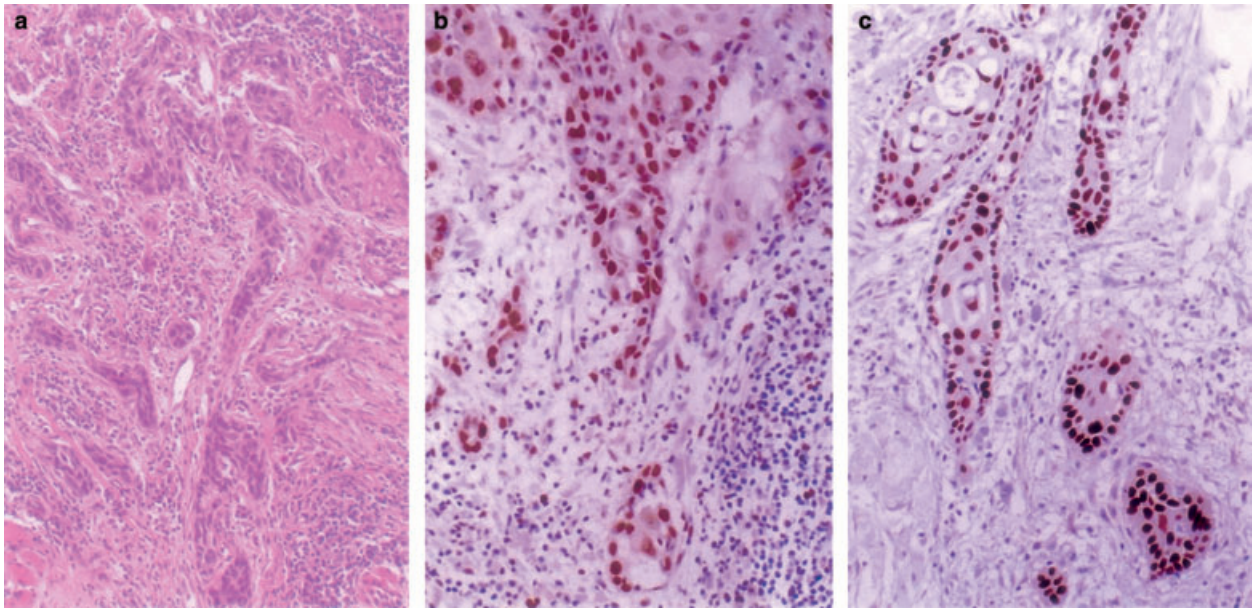

Figure 1 Immunohistochemical localization at the deep invasive front of oral squamous cell carcinoma. (a) Hematoxylin and eosin stain (original magnification: ×110). (b) The proliferating cells are immunostained with the Ki-67 antigen (original magnification: ×150). (c) Photomicrograph of nest of invading cells that are demonstrating strong nuclear staining for p53 protein (original magnification: ×150).

areas of the tumors. Microscopically, the patterns of immunostaining for Ki-67 and p53 appeared to be similar (Fig. 1).

The mean Ki-67-LI in O-SCC tissue specimens was 32.8 ± 12.0% ($n = 62$). The Ki-67-LI level was significantly higher in O-SCC than in normal squamous epithelium (3.9 ± 2.1%, $n = 10$).

The mean total IFG score was 9.1 ± 2.7 points ($n = 62$). There was a linear correlation between the IFG score and the Ki-67-LI ($\gamma = 0.651$, $R^2 = 0.596$, $P < 0.0001$; Fig. 2). After the selection of cut-off levels, single-parameter analysis using the log-rank test revealed significant results for the IFG scores. Figure 3 shows the relationship between disease-free survival (times/rates) and IFG scores. Patients with IFG scores of <10 points had significantly better prognoses than did those with IFG scores of more than 10 points.

The analysis of the markers of cell proliferation revealed that the mean Ki-67-LI increased with increasing IFG values (Fig. 4). The mean Ki-67-LI was significantly higher in specimens with IFG scores of ≥10 points (40.1 ± 9.7%; $n = 31$) than in specimens IFG scores of <10 points (25.6 ± 9.5%; $n = 31$; $P < 0.0001$). Likewise, the mean IFG score of the p53-positive cases was 10.1 ± 2.5 points ($n = 27$), which was significantly higher than that of the p53-negative cases (8.0 ± 2.1 points; $n = 23$; Fig. 5, $P < 0.0054$).

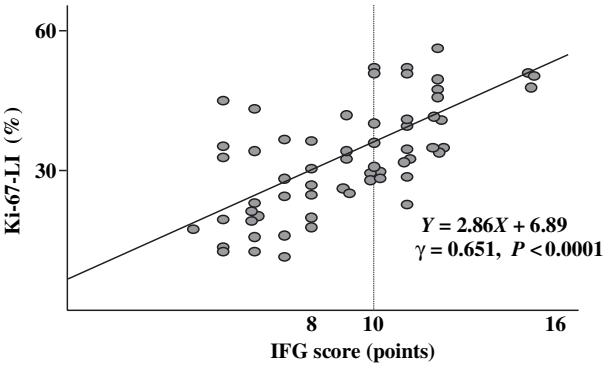

Figure 2 Correlation between Ki-67 labeling index (Ki-67-LI) and invasive front grading score.

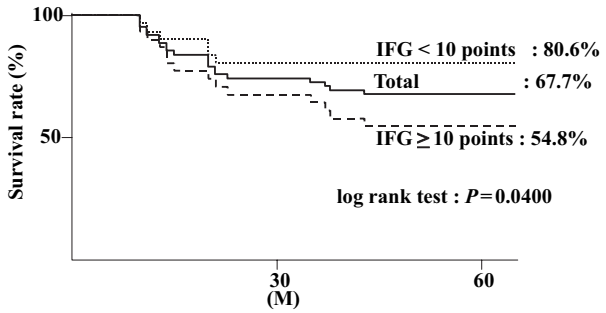

Figure 3 Disease-free survival curves in relation to total invasive front grading score.

Discussion

Studies (6, 7, 33–40) on the histologic grading of malignancy of SCC in the head and neck region have been reported as Broders' classification system (41) based on the ratio of differentiated cells was first published. A two-factor grading system comprising the degree of cellular differentiation and the depth of tumor growth was subsequently introduced and has generally been accepted. However, Broders' classification (41) of SCC according to the differentiation or maturation of

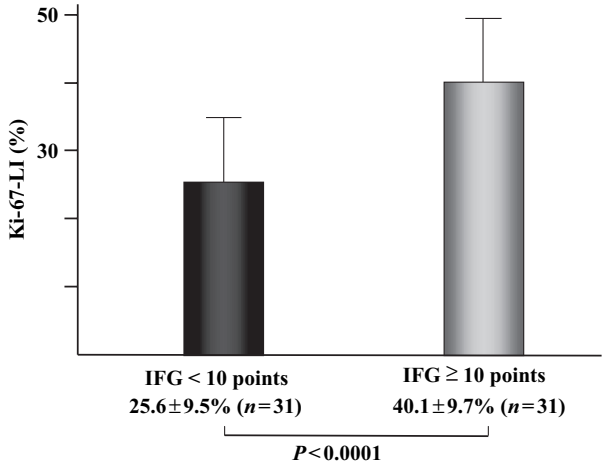

Figure 4 Correlation between Ki-67 labeling index (Ki-67-LI) and invasive front grading score.

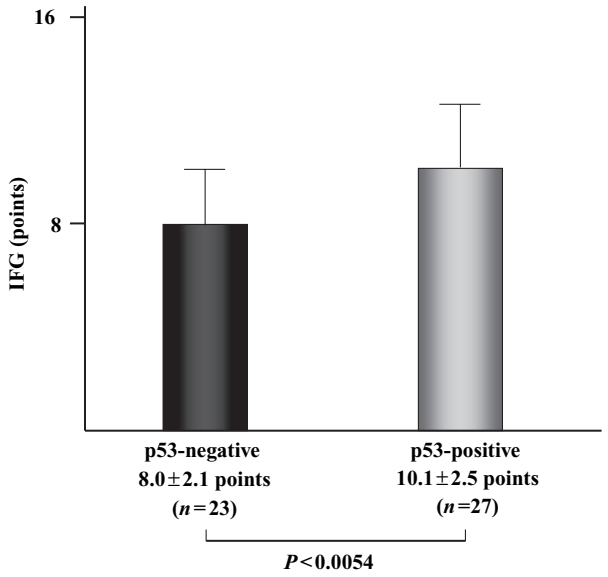

Figure 5 Correlation between immunohistochemical staining of p53 protein and invasive front grading score.

the tumor cell population alone remains of limited value regarding both the prognosis and choice of treatment. Recent evidence suggests that cells present at the invasive tumor front of carcinomas have different molecular characteristics when compared with those in the superficial areas of the tumor, making the invasive front the most important area of the tumor for determination of the prognosis (15–17). A multiple factor histologic grading system of the invasive front of tumors of the head and neck was first described by Bryne et al. (19): it consisted of the pattern of invasion, the degree of keratinization, nuclear polymorphism, and the host response. The authors reported that a strong correlation was observed between the total malignancy grading scores and the prognosis in glotic carcinoma. However, few studies have used multivariate analysis to evaluate the value of histologic malignancy scores of the invasive front for prediction of overall prognosis and survival rates in O-SCC (38, 42–44). Bryne et al. (19) and Kearsley et al. (45) reported that strong correlations were observed between total malignancy grading scores based on several pathologic parameters and the prognosis in O-SCC. Furthermore, Kurokawa et al. (20) reported that elevated IFG scores were highly prognostic in cases of SCC of the tongue. These findings suggest that cells present at the invasive tumor front of the carcinoma have different molecular characteristics when compared with those in superficial areas of the tumor and that IFG scoring usefully evaluates the most important area of the tumor for prognostic purposes.

Tumor cell proliferation activity is believed to indicate the degree of aggressiveness of the tumor (21). The proliferative activity of carcinoma cells is generally considered related to the degree of malignancy of carcinoma tissue (18, 21, 22). As relationship between cell proliferation and cell migration, Natarajan et al. (46) reported that the expression of the cell cycling regulating protein p16 was associated with some premalignant lesions and occurred consistently in areas of microinvasion and at superficial margins of invasive SCC. Moreover, Bartkova et al. (47) reported that the lack of cell proliferation in migrating oral epithelia during the wound healing process might reflect combination of changes in several cell cycle regulatory proteins. Recently, immunohistochemical detection of the Ki-67 antigen and p53 protein was associated with the tumor cell proliferation and the prognosis of O-SCC (22, 25–28).

The Ki-67 antigen is expressed in proliferating cells (in the G1, S, G2, and M phases), but not in resting cells (G0 phase) (18, 21). Therefore, this antigen is a proliferation marker, the appearance of which correlates with the presence and severity of the malignancy. Although the level of cell proliferation has been shown to be higher in O-SCC than in normal tissues, few studies have described the correlation between IFG and markers of cell proliferation (18, 21, 29, 30). Tumuluri et al. (21) reported that cell proliferation (as measured by expression of the Ki-67 antigen) at the invasive tumor front had a strong positive correlation with the histologic grade of malignancy in human O-SCC. Moreover, Piffko et al. (29) reported that invasive tumor front of an O-SCC is composed of tumor subpopulations with higher proliferative activity. In the present study, Ki-67-LI values significantly correlated with IFG scores. This result suggests that higher Ki-67-LI values may indicate biologic malignancy.

The p53 gene encodes a 53-kDa nuclear phosphoprotein, which controls cellular proliferation and transformation (23). Mutations in and overexpression of p53 are common in SCC of the head and neck (24, 25). Overexpression of p53 is reported in approximately 50% of O-SCC cases (25). In the present study, overexpression of p53 was found in 27 of 50 (54.0%) cases of O-SCC. However, Piffko et al. (30) reported that p53 alterations apparently do not represent a molecular basis for the biologic significance of the invasive tumor front. On the contrary, the results of our previous study

(13) on O-SCC showed a correlation between the expression of Ki-67 and overexpression of p53. Those findings also suggested that expression of Ki-67 and p53 correlated with histologic grade of malignancy in O-SCC tumors. In the present study, the staining patterns of Ki-67 and p53 were similar. Both the Ki-67-LI and the p53-positive cases were significantly correlated with the cell cycle. Furthermore, we suggest that high values of Ki-67-LI and p53-positive status may be related in turn to the progression, metastatic spread, and histologic grade of malignancy in tumors.

In conclusion, we have shown that overexpression of Ki-67 and p53 in the tissue at the deep invasive tumor front is associated with histologic grade of malignancy in O-SCC.

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