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ORIGINAL PAPER

Predictive Value of Measuring p53 Labeling Index at the Invasive Front of Oral Squamous Cell Carcinomas

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Abstract Many studies have revealed the frequency of p53 abnormalities in oral cancer. However, it reports only on the relation between clinicopathological findings and p53 expression, and there is no study to examine the relation to the p53 labeling index (p53-LI). The purposes of this study were to examine the correlation between p53 labeling index (p53-LI) at the invasive front of oral squamous cell carcinomas (OSCC) and clinicopathological findings by immunohistochemical staining, and to evaluate clinical significance of measuring p53-LI at the invasive front of OSCC. Sixty-six biopsy specimens of OSCC were randomly selected. Patient age, gender, primary sites, T category, N category, degree of differentiation and mode of cancer invasion were analyzed. p53 expression did not correlate significantly with the clinical findings. However, significant differences were found between p53-LI and the degree of cell differentiation ($p<0.05$). The p53-LI of high-grade invasive tumors was significantly larger than that of low-grade invasive tumors ($p<0.05$). The overall survival rate (OS) among low-scoring p53-LI cases was 75.5% whereas that for high-scoring p53-LI cases was 40.6%. The disease-free survival rate (DFS) among low-scoring p53-LI cases was 39.5% whereas that for high-scoring p53-LI cases was 76.1%. Patients with low-scoring p53-LI had a significantly worse prognosis than those with among high-scoring p53-LI ($p<0.05$). Consequently, the measurement of p53-LI at the invasive front of OSCC is significant as one of the indicators of prognosis.

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Introduction

Carcinogenesis of the oral epithelium is complex and multi-step process [21]. Cancer cells have the characteristic of a continuous and disordered progression and the rapid progression and metastasis of cancer cells have been problematic in treatment. It is important to study the abnormalities of genes associated with the progression of cancer cells in order to elucidate the mechanism of cancer and improve clinical outcome.

Apoptosis is the death of cells through a preprogrammed mechanisms and it is the normal development of embryonic tissues as well as part of the life cycle of many adult tissue [16]. It occurs in several pathological situations in multicellular organisms and constitutes part of a common mechanism of cell replacement, tissue remodeling and the removal of damaged cells. Moreover, it has been accepted as a fundamental component in the pathogenesis of cancer and recent study has shown that the process of carcinogenesis might involve not only increased cell proliferation but also decreased cell apoptosis [12].

One of the most important genes in the regulation of apoptosis is p53 that locates on chromosome 17p. Wild-type p53 contribute to tumor suppression through at least two mechanisms in response to DNA damage, arrest of cell proliferation and induction of apoptosis [1, 8, 28]. Conversion of p53 from normal to mutant phenotype alters its histochemical characteristics, since the half-life of protein is enhanced from 6–20 min to several hours. Alteration of the p53 gene is the most commonly reported genetic abnormality in many types of cancer [10, 14] and

its mutations provide indicators of tumor progression and prognosis. Therefore, the p53 gene has been applied clinically as a target for the diagnosis and treatment of cancer [11].

In oral squamous cell carcinomas (OSCC), the degree of differentiation and the mode of cancer invasion have frequently been examined to predict progression, metastasis and prognosis [3, 18, 25]. At the invasive front the tumor frequently shows a lower degree of differentiation and higher grade of cellular dissociation than the remaining parts of the tumors. It is also important to examine the characteristics of cancer cells at the invasive front of OSCC [2].

The aims of this study were to immunohistochemically investigate the labeling index of p53 (p53-LI) in cancer cells at the invasive front of OSCC and to evaluate the correlation between p53-LI and the clinicopathological parameters.

Materials and Methods

Sixty-six biopsy specimens of OSCC were obtained from patients who received no previous treatment and had undergone surgical resection. The patients (34 males and 32 females) ranged in age from 37 to 92 years (mean age= 68 years).

Biopsy specimens were fixed in periodate–lysine–paraformaldehyde solution or 10% formalin solution, then embedded in paraffin for the preparation of serial sections (4 μm). Hematoxylin-and-eosin staining was used for histopathological examination. Immunohistochemical staining was performed by labeled streptavidin–biotin (LSAB) method. Sections were deparaffinized with xylene and rehydrated in graded ethanol, and endogenous peroxidase activity was blocked by immersion in 0.03% hydrogen peroxide (H_2O_2) for 30 min. After the sections were washed with PBS, the immunoreactivity of the target antigens was enhanced by autoclaving the sections for 15 min in 0.01 M citrate buffer (pH 6.0). The sections were rinsed with distilled water and then with PBS. Nonspecific conjugation was suppressed by utilizing 10% normal goat serum for 15 min. The sections were incubated overnight at 4°C with primary antibody against p53 (NCL-p53-DO7, Novocastra Lab. Ltd, Newcastle, UK) at a dilution of 1:200. After rinsing in PBS, the slides were incubated with biotinylated anti-rabbit immunoglobulin, and allowed to react at room temperature for 60 min. After reacting with peroxidase-conjugated streptavidin (Dako Japan, Kyoto, Japan) for 60 min, they were washed in PBS. Immunohistochemical reactions were developed with 3,3′-diaminobenzidine-tetrahydrochloride containing 0.006% hydrogen peroxide, then lightly counterstained with Mayer's hematoxylin. Negative controls were treated with all reagents except the primary antibody.

The expression of p53 was examined under a microscope at ×100 magnification. The p53-LI was determined by observing 500 cancer cell nuclei in areas of the section with highest labeling frequency, and the percentage of p53-labeled nuclei was used for analysis. The percentage of p53 expression were assessed and judged by three reviewers. Staining of less than 5% was considered negative expression.

The threshold of p53-LI was set at 25%, an approximate value to median of positive cases. p53-LI was examined in relation to clinicopathological parameters including age, gender, primary tumor site, T category (tumor size), N category (cervical lymph node metastasis), stage, degree of differentiation, mode of cancer invasion, as described by Yamamoto et al. [24] (Table 1), and recurrence.

The Mann–Whitney's U test was used for statistical analysis. Over all survival rate (OS) and disease-free survival rate (DFS) of patients were evaluated according to the Kaplan–Meier method and the differences were tested by log-rank test. Differences were considered significant at p value of <0.05.

Results

Immunohistochemical staining showed that 45 of the 66 specimens (68.5%) examined were positive for p53. The sites of expression were frequently at the invasive front of the tumors; they tended to be in the outer layer of the round-shaped tumor nests and at the invasive front of the cord-like microtumor nests (Fig. 1).

It was found that p53 expression did not significantly correlate with age, gender, primary sites, T category, N category, stage, cell differentiation, or mode of cancer invasion. There were no differences in the survival rate between p53-positive cases and p53-negative cases (data not shown).

The relationship between clinicopathological parameters and p53-LI was examined (Table 2). p53-LI did not correlate significantly with age, gender, primary sites, T category, N category, or stage. However, moderately and

Table 1 Histological grading of mode of cancer invasion

Grade	Histological characters
1	Well-defined borderline
2	Cords, less-marked borderline
3	Groups of cells, no distinct borderline
4C	Diffuse invasion, cordlike type
4D	Diffuse invasion, diffuse or widespread type

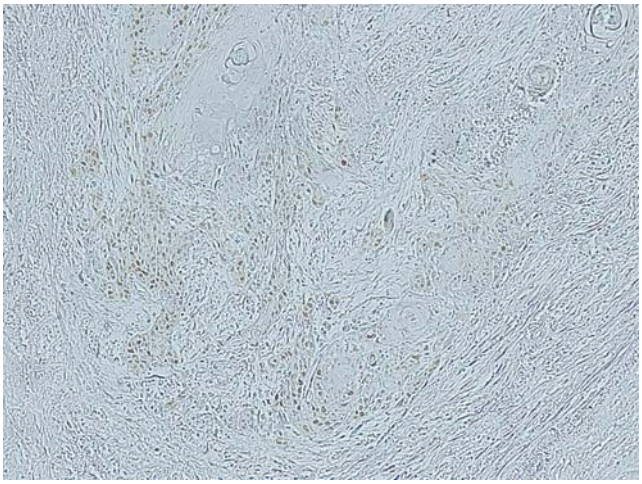

Fig. 1 Immunolocalization of p53 protein in oral squamous cell carcinomas (original magnification ×100)

poorly-differentiated OSCC specimens exhibited higher p53-LI compared with the well-differentiated OSCC specimens. Significant differences were found between p53-LI and the degree of cell differentiation ($p<0.05$). With regard to the relationship between p53-LI and the mode of cancer invasion, it was found that the index increased with increasing the invasive grade, and significant differences were found between p53-LI and the mode of cancer invasion ($p<0.05$). Relationship between recurrence of OSCC and p53-LI are shown in Table 3. The recurrence tended to occur easily to the case of high-scoring p53-LI, but not significance.

OS and DFS of all patients were 64.7% and 65.0%, respectively. The OS of patients with high-scoring p53-LI was 40.6%, while those of patients with low-scoring p53-LI was 75.5% (Fig. 2). The DFS of patients with high-scoring

Table 2 Clinicopathological parameters in relation to p53-LI

Variable	Number	p53-LI <25%, *n* (%)	p53-LI ≥ 25%, *n* (%)	*p* value
Age, years				
<65	27	21 (77.8)	6 (22.2)	0.26
≥65	37	24 (64.9)	13 (35.1)	
Gender				
Male	34	22 (64.7)	12 (35.3)	0.30
Female	30	23 (76.7)	7 (23.3)	
Primary site				
Tongue	24	16 (66.7)	8 (33.3)	0.85
Lower gingiva	16	11 (68.8)	5 (31.2)	
Upper gingiva	10	8 (80.0)	2 (20.0)	
Floor of mouth	8	5 (62.5)	3 (27.5)	
Others	6	5 (83.3)	1 (16.7)	
T category				
T1	10	8 (80.0)	2 (20.0)	0.62
T2	34	25 (73.5)	9 (26.5)	
T3	9	5 (55.6)	4 (44.4)	
T4	11	7 (63.6)	4 (36.4)	
N category				
N0	41	31 (75.6)	10 (24.4)	0.36
N1	16	9 (56.3)	7 (43.7)	
N2	7	5 (71.4)	2 (28.6)	
Stage				
S1	10	8 (80.0)	2 (20.0)	0.78
S2	23	25 (73.5)	6 (26.5)	
S3	14	5 (55.6)	5 (44.4)	
S4	17	7 (63.6)	6 (36.4)	
Cell differentiation				
Well	45	34 (75.6)	11 (24.4)	0.02
Moderate	16	11 (68.8)	5 (31.2)	
Poor	3	0 (0.0)	3(100.0)	
Mode of invasion				
1	7	7 (100.0)	0 (0.0)	0.05
2	14	11 (78.6)	3 (21.4)	
3	25	17 (68.0)	8 (32.0)	
4C	13	9 (69.2)	4 (30.8)	
4D	5	1 (20.0)	4 (80.0)	

Table 3 Relation between recurrence of OSCC and p53-LI

	p53-LI <25%, *n* (%)	p53-LI ≥ 25%, *n* (%)
Recurrence (−)	23 (51.1)	5 (26.3)
Recurrence (+)	22 (48.9)	14 (73.7)

p53-LI was 39.5%, while those of patients with low-scoring p53-LI was 76.1%. Patients with high-scoring p53-LI had a significantly worse prognosis than those with low-scoring p53-LI ($p<0.05$).

Discussion

The p53 gene functions as a growth suppressor gene and causes a reduction in cell proliferation and DNA replication. Wild-type p53 has a short half-life and was not detected immunohistochemically in any of the normal tissues in this study. By contrast, mutations of p53 result in a greatly extended protein half-life permitting immunohistochemical detection. Many studies have reported the expression of p53 protein variants and the occurrence of p53 gene abnormality in the malignant tumors of several organs, leading to the concept that the p53 protein is closely involved in the malignant transformation of cells [5, 9, 17]. Previous immunohistochemical studies on various organs found that the p53 protein expressed in 46.5% of colorectal cancers [17], 82% of lung cancers [14], 15.5% of breast cancers [5], and 62% of cervical cancers [9], indicating organ-dependent differences. Girod et al. [7] reported p53 expression in approximately half (54%) of OSCC examined. In the present study, we found that p53 expressed in 68.5% of oral squamous cell carcinomas.

Invasion and metastasis are the most crucial characteristics of malignant tumors. The Yamamoto–Kohama classification, based on the mode of OSCC invasion, is considered an important factor in determining appropriate treatment. The highly invasive grades of tumors represent highly metastasizing tumors, leading to poor prognoses [26]. Other studies have reported that the invasive front of OSCC is an important indicator for prognosis [4]. The mode of invasion is one factor used for the histopathological classification of OSCC. Therefore, we investigated p53 expression at the invasive front of oral squamous cell carcinomas. In the present study, we found that p53 showed higher expression at the invasive front of tumors. Previous studies have also found a striking accumulation of p53 positive cells at the invasive front of tumors [6, 15]. Consequently, these results prove that p53 gene mutation may arise in tumor cells at the invasive front.

In OSCC, lack of correlation between immunohistochemically detectable p53 protein accumulation and grade of differentiation has been reported [20, 22]. The result of the present study showed that the correlation between the expression of p53 protein and clinicopathological findings was not significant. However, moderately- and poorly-differentiated OSCC specimens exhibited higher p53-LI compared with well-differentiated OSCC specimens. p53-LI was significantly correlated with cell differentiation. Moreover, a higher invasion grade of OSCC was associated with a higher p53-LI. p53-LI was significantly correlated with the mode of invasion. It appears that p53 mutation is closely linked to OSCC malignancy and it may be difficult for high-grade invasive cancer cells to enter growth arrest or apoptosis. It is also possible that p53 mutation plays an important role in cancer invasion.

The presence or absence of lymph node metastasis or recurrence is significantly associated with the survival of patients with OSCC. Osaki et al. [13] reported that the expression of p53 protein was correlated with lymph node metastasis. In addition, Shin et al. [19] reported that the expression of p53 protein in primary head and neck squamous cell carcinoma was significantly both tumor recurrence and second primary tumors. In the present study, p53-LI was not significantly correlated with lymph node metastasis or recurrence; however p53-LI tended to be higher for patients with lymph node metastasis or recurrence.

The present study showed that OS and DFS of patients with high-scoring p53-LI were 40.6% and 39.5%, and that

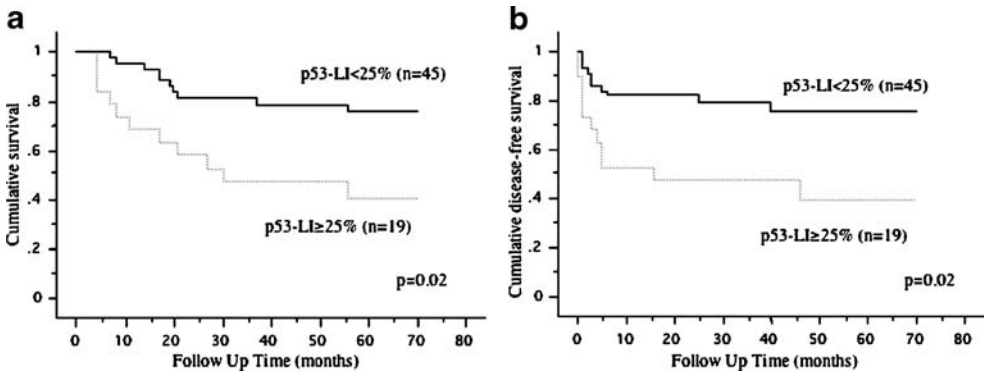

Fig. 2 Kaplan–Meier survival estimates for overall survival (**a**) and disease-free survival (**b**) by p53-LI

of the patients with low-scoring p53-LI was 75.5% and 76.1%. This data showed a significant difference between patients with high p53-LI values and those with low p53-LI values ($p<0.05$). Consequently, we presumed that the measuring of p53-LI might be a powerful aid in evaluating the prognosis of patients with OSCC.

Though we occasionally do neoadjuvant therapy, we meet the case that effect is insufficient and tumor progresses. Several authors have reported that certain specific p53 mutations may result in poor responsiveness to adjuvant radiotherapy or chemotherapy. Yamazaki et al. [27] have reported that a DNA contact mutation of p53 could be a useful marker to predict the radioresistance of OSCC. Wamakulasuriya et al. [23] have reported that p53 and P-glycoprotein co-expression predicts the biological behavior or the outcome following chemotherapy or radiotherapy in advanced head and neck cancer. We assumed that excision without neoadjuvant therapy is necessary in tumors with p53 overexpression and improvement of the prognosis can be hoped for.

In conclusion, significant relationship is shown not between p53 expression and clinicopathological parameters. However significant relationship is shown between the ratio of p53-LI and grade of cell differentiation and survival rates. Therefore, measurement of the labeling index of p53 protein at the invasive front of OSCC is important in the examination of tumor cell characteristics and the prediction of prognosis.

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