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OVEREXPRESSION OF p53, HEAT SHOCK PROTEIN (HSP) 70 AND Ki-67 IN ORAL SQUAMOUS CELL CARCINOMA

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We immunohistochemically analyzed the expression of the proteins p53, heat shock protein (HSP) 70 and Ki-67 labelling index (LI) in biopsy specimens from 70 patietns with oral squamous cell carcinoma (OSCC) and to analyze these findings in relation to the clinicopathologic parameters (CPP) and clinical course (CC) of these patients. Thirty eight (54.3%) of the 70 oral squamous cell carcinomas examined were positive for p53 protein. p53 protein expression was not correlated significantly with CPP and CC. Forty four (62.8%) of the 70 oral carcinomas were positive for HSP70. HSP70 positivity was significantly associated with a lower degree of histological differentiation ($p<0.01$), a high degree of nuclear polymorphism ($p<0.01$), and a high frequency of mitosis ($p<0.01$). Kaplan-Meier's survival curves showed significantly shorter survival for HSP70-positive (41%) than the HSP70-negative patients (78%) according to the results of the Cox-Mantel test ($p<0.01$). Ki-67 LI was significantly associated with lower degrees of histological differentiation ($p<0.01$), markedly diffuse invasion of the tumor ($p<0.01$), high degrees of nuclear polymorphism ($p<0.01$), and high frequencies of mitosis ($p<0.01$). The index was also significantly high in patients in high clinical stages of cancer ($p<0.01$) and patients with tumor metastasis to cervical lymph nodes ($p<0.01$). Coexpression of p53-HSP70 was found in 31 (81.6%) of the 38 p53 protein positive oral carcinomas. The mean value of Ki-67 LI was significantly higher in patients in whom both p53 protein and HSP70 were positive (39.1%) than in patients in whom both were negative (23.1%) ($p<0.01$). The results of this study suggest that an immunohistochemical examination for p53 protein and HSP70 and determination of the Ki-67 LI will be useful in assessing the biological malignancy level of OSCC, determining a therapeutic policy and predicting the prognosis of OSCC in a given case.

Introduction

Activation of cellular protooncogenes or inactivation of tumor suppressor genes are most probably genetic alterations, involved in this multistep process of carcinogesis. p53 mutations occur in approximately half of all human cancers[1]. The trascription factor p53 is the product of a tumor suppressor gene. The wild-type form has a half-life of 6～30 min. In contrast, proteins from mutated genes form complexes with a metabolically stable protein, that has a half-life of many hours[1]. This forms the basis for the detection of mutated p53 by immunohistochemistry, because wild-type p53 does not accumulate in high enough levels for detection by immunohistochemistry[2]. In oral cancer, as in a number of other human solid

tumors[3], p53 gene mutations have been reported to be a common implicated genetic event. By immunohistochemical detection techniques increased, p53 levels have been found in 50~60% of oral cancer[3)~8)].

Heat shock proteins (HSP) are produced after cell exposure to a variety of environmental and pathophysiological stressful conditions[9)10)]. The group of 70-kDa family (HSP70) mainly seems to protect all proteins from the damage caused various stressful stimuli and of particular interest, as those proteins may be implicated in the antigen presentation mechanism of the immune anti-tumor response by their association with antigenic peptides derived from cellular proteins[11)~15)]. It has been postulated that HSP70 are involved in protein products of the human c-*myc* oncogene and the p53 tumor suppressor gene. Furthermore, elevated HSP70 expression may be an indicator of biological stress experienced by tumor cells in some carcinomas and may also predict patients' clinical outcome[11)].

Recent development of a monoclonal antibody (MIB-1)[16)] to recombinant parts of the Ki-67 antigen suitable for immunohistochemical staining of formalin-fixed, paraffin-embedded tissue sections has provided a simple method of estimating the tumor cell proliferation fraction. Although the precise nature of Ki-67 antigen is unknown, MIB-1-positive cell count correlated with tumor-cell proliferation fraction estimated by thymidine labelling index[17)].

The present study was undertaken to examine immunohistochemically the expression of p53 protein and HSP70 and the growth potential of tumor cells in biopsy specimens from patients with oral squamous cell carcinoma (OSCC) and to analyze these findings in relation to the clinicopathologic parameters (CPP) and clinical course (CO) of these patients.

Materials and Methods

Patients and tissue preparation

Tissue specimens were collected from 70 patients with OSCC (tongue, 50 cases; mandibular ginviva, 7 cases; buccal mocusa, 6 cases; hard palate, 3 cases; oral floor, 2 cases; maxillary gingiva, 2 cases) between June 1986 and April 1993. Twenty-six of these patients were female and 44 were male. Their ages ranged from 31 to 83 years. Surgical biopsy specimens of OSCCs were routinely fixed in formalin and embedded in paraffin. Serial sections (4 μm thick) were then prepared, mounted on aminopropyltrithoxysilame-coated slides and allowed to dry overnight. The first and last sections were stained with hematoxylin and eosin for histopathologic evaluation, and the subsequent sections were used for immunohistochemistry analysis.

Monoclonal antibodies

Monoclonal antibodies PAb1801 (Oncogene Science, Uniodale, NY), w27 (Stanta Cruz Biotechnology, Inc. Santa Cruz, Calif), and MIB-1 (Immunotech SA, Marseilles, France) were used. PAb1801 is specific for human p53 and reacts with an epitope between amino acids 32 and 79. w27 reacts with HSP72 and HSP73 of the HSP70 family. MIB-1 reacts with Ki-67 nuclear antigen, which is associated with the cell cycle (G1, S, G2. M, phases) and absent in resting (GO) cells. The dilutions used for the p53, HSP70, Ki-67 antibodies were 1 : 100, 1 : 500, and 1 : 100 respectively.

Immunohistochemcial staining

Briefly, the sections were deparaffinized by routine procedures and incubated in a microwave oven for 4 to 5 min at 90°C in citrate buffer (pH 6.0). After allowing them to cool to room temperature (RT), they were overlaid with 0.3% H_2O_2 and incubated at RT for 30 min. The sections were subsequently incubated with the primary antibody to p53, HSP70 or Ki-67 at 4°C overnight. The sections were then washed with phosphate-buffered saline (PBS), incubated with biotinylated horse anti-mouse IgG for at RT for 30 min, allowed by avidin-biotin horseradish peroxidase complex (Vector Laboratories, Burlingame Calif). The final reaction product was visualized with diaminobenzidine and the sections were counterstained

with Harri's hematoxylin. Human breast and colorectal cancers were used as positive controls. The primary antibodies were substituted with PBS in section used as negative controls.

Quantitation

Tumors were designated positive for p53 protein or Ki-67 when their cells displayed a distinct brown nuclear staining. Cytoplasmic staining without nuclear staining was discounted. Brownish nuclear and cytoplasmic staining were considered to be positive for HSP70. Coexpression of p53 protein and HSP70 occur when both nuclear staining for p53 protein and cytoplasmic staining for HSP70 were present. All immunostaining slide were analyzed and scored in a binded fashion by two different observers without knowledge of clinical data. The percentage of p53 protein, HSP70 and Ki-67 positive cells, irrespective of the staining intensity, determined by visual counting of 1000 tumor cells in multiple random fields. We determined the frequency distribution of the p53 protein and HSP70 protein labelling index and found that the distribution of frequencies differed markedly in two groups divided by the labelling rate of 20%, as shown in Fig. 3. Following this findings, the patients were classified into two groups according to the numbers of tumor cells positive for p53 protein and HSP70: (−), less than 20% positive cells; (+), more than 20% positive cells.

Histopathological examination

Different histological parameters were evaluated and tumors were graded as follows; well differentiated, moderately differentiated and poorly differentiated depending on the degree of keratin pearl formation, keratinization and overall resemblance epithelium accord-

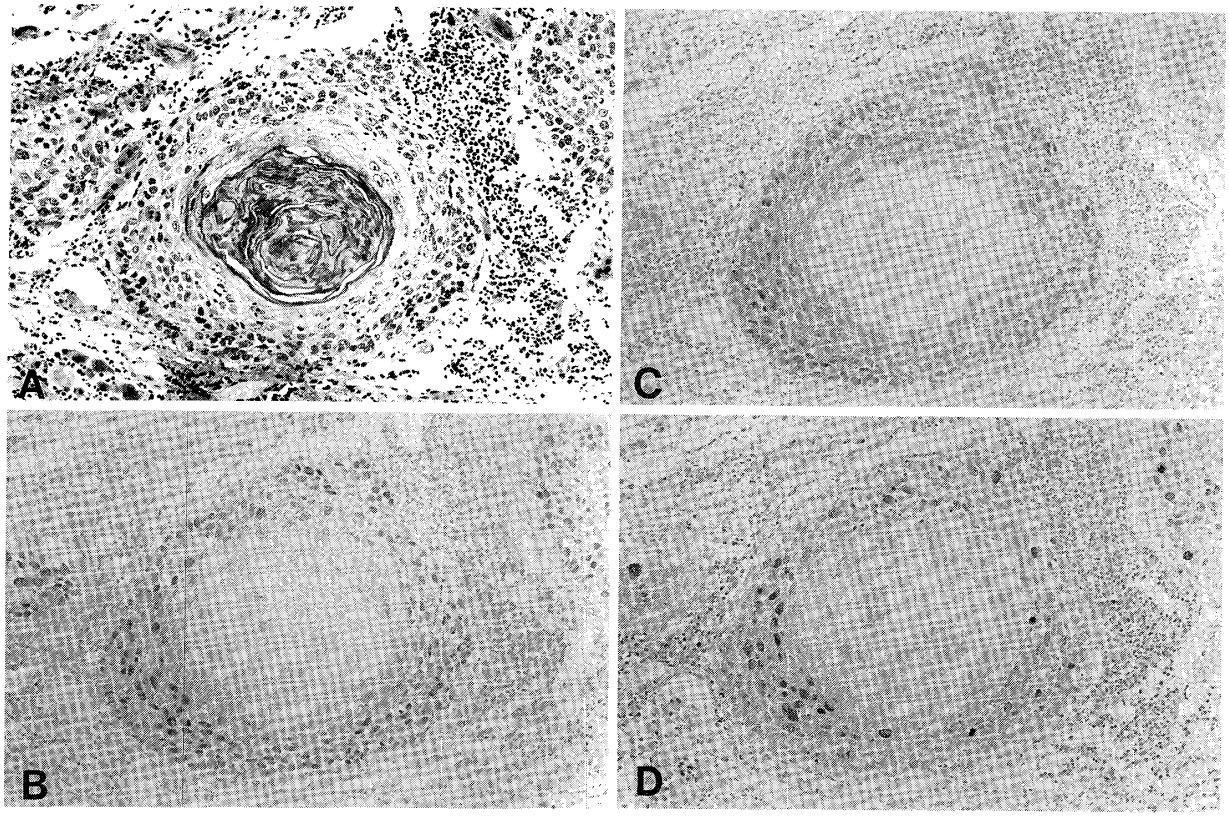


Fig. 1 Serial sections of well-differentiated oral squamous cell carcinoma
(A) Hematoxylin and eosin staining, (B) Immunohistochemical expression of p53 protein, (C) Immunohistochemical expression of HSP70, (D) Immunohistochemical expression of Ki-67. p53 protein positive cells and HSP70 positive cells are seen in the peripheral area of the tumor cell nest with light hematoxylin counterstain, paraffin-embedded material (original magnification ×200).

ing to UICC classificatin. Other parameter were assessed according to a modification of grading system (Anneroth et al[18]); pattern of invasion (1, pushing, well delineated infiltrating borders; 2, infiltrating, solid cord, bands and/or strards; 3, small groups or cords of infiltrating cells; 4, marked and widespread cellular dissociation in small groups and/or in single cells); lymphoplasmacytic infiltration (1, marked; 2, moderate; 3, slight; 4, none); nuclear polymorphism (1, little; 2, moderately; 3, abundant; 4, extreme); number of mitoses (high power field) (1, 0-1; 2, 2-3; 3, 4-5; 4, >5). Only the most invasive parts of the tumors were graded, since these parts may include the most prognostic information.

Statistical analysis

The statistical significance of the individual findings and their associations was checked by Student's t-test and Fisher's exact probability test. Overall length of survival was estimated in univariate analyses by the method described by Kaplan-Meier and compared with the results of the Cox-Mantel test. A 1% level of significance was in all statistical calculation.

Results

Expression of p53

p53 protein was detected in the nuclei of cancer cells (Fig. 1, Fig. 2). Thiry eight (54.3%) of the 70 OSCCs examined were positive for p53 protein, and the rest were negative. p53 protein expression was not correlated significantly with CPP and CC (Table 1). With regard to their Kaplan-Meier's survival curves (Fig. 4), no statistical difference was found between patients with tumors positive for p53 protein and those with tumors negative for p53 protein.

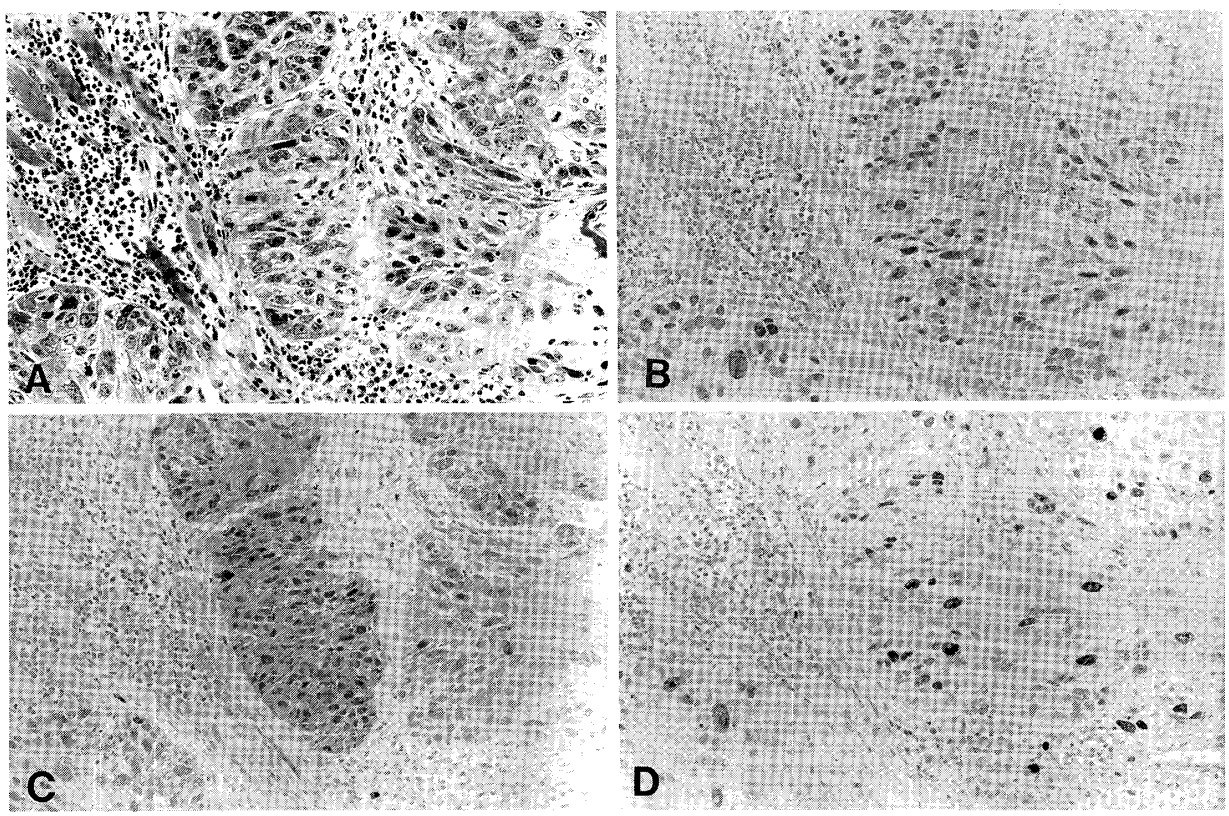


Fig. 2 Serial sections of poorly-differentiated oral squamous cell carcinoma
(A) Hematoxylin and eosin staining, (B) Immunohistochemical expression of p53 protein, (C) Immunohistochemical expression of HSP70, (D) Immunohistochemical expression of Ki-67. p53 protein positive cells and HSP70 positive cells are seen throughout the tumor cell nest with light hematoxylin counterstain, paraffin-embedded material (original magnification × 200).

Table 1 Relationships between clinicopathological parameters and expression of p53, HSP70 and Ki-67 in oral cancer

Variables			p53(+) n=38	p53(−) n=32	HSP70(+) n=44	HSP70(−) n=26	Ki-67 (mean)	LI(%) (±SD)
Differentiation	well	(n=15)	7	8	5	10 ┐	11.9	10.3 ┐
	moderate	(n=33)	17	16	22	11 ┘**	38.2	14.3 ┘*
	poor	(n=22)	14	8	17	5	52	17.7
Pattern of invasion	I, II	(n=27)	13	14	16	11	26.7	18 ┐
	III, IV	(n=43)	25	18	28	15	41.2	20.2 ┘*
Lymphplasmacytic infiltration	I, II	(n=35)	18	17	18	17	29.1	16.2
	III, IV	(n=35)	20	15	26	9	36.8	20.8
Nuclear polymorphism	I, II	(n=30)	16	15	13	18 ┐	16.9	16.6 ┐
	III, IV	(n=40)	22	18	31	9 ┘**	47.9	15.1 ┘*
Number of mitoses	I, II	(n=33)	17	16	14	18 ┐	16.9	12.5 ┐
	III, IV	(n=37)	21	14	30	7 ┘**	50.4	14.7 ┘*
Clinical stage	I, II	(n=43)	24	19	29	14	32.3	18.1 ┐
	III, IV	(n=27)	14	13	15	12	45.6	20.3 ┘*
Cervical lymph node metastasis	positive	(n=38)	23	15	27	11	46.1	19.1 ┐
	negative	(n=32)	15	17	17	15	26	15.9 ┘*

*p<0.01 (paired Student's t-test), **p<0.01 (Fisher's exact probability test).

Expression of HSP70

HSP70 was observed in the nuclei and cytoplasm of cancer cells (Fig. 1, Fig. 2). Forty four (62.8%) of the 70 OSCCs were positive for HSP70; and the rest were negative for HSP70. With respect to the CPP and CC (Table 1), HSP70 positive was significantly associated with a lower degree of histological differention ($p<0.01$), a high degree of nuclear polymorphism ($p<0.01$), and a high frequency of mitosis ($p<0.01$). Kaplan-Meier's survival curves (Fig. 4) showed significantly shorter survival for HSP70-positive (41%) than the HSP70-negative patients (78%) according to the results of the Cox-Mantel test ($p<0.01$).

Expression of Ki-67

Ki-67 was observed in the nuclei of the cancer cells (Fig. 1, Fig. 2). The Ki-67 LI ranged from 5 to 83% (mean 36.7%). According to the CPP and CC (Table 1), markedly diffuse invasion of the tumor ($p<0.01$), high degrees of nuclear polymorphism ($p<0.01$), and high frequencies of mitosis ($p<0.01$). The index was also significantly high in patients in high clinical stages of cancer ($p<0.01$) and patients with tumor metastasis to cervical lymph nodes ($p<0.01$).

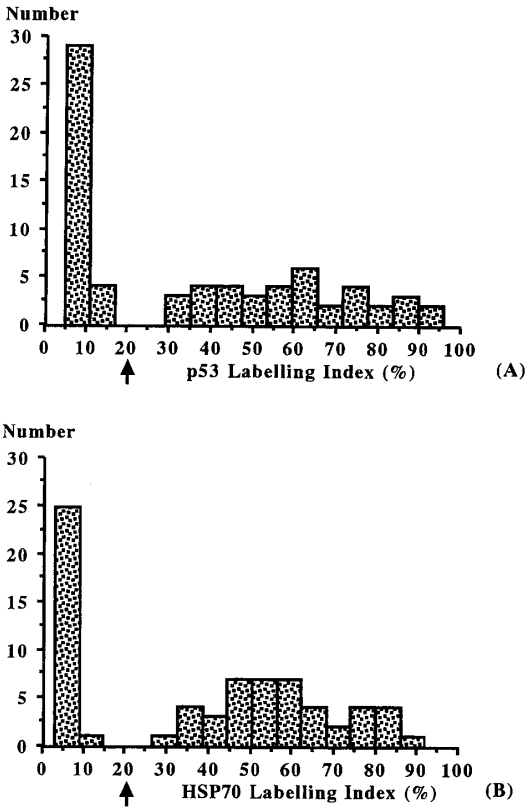
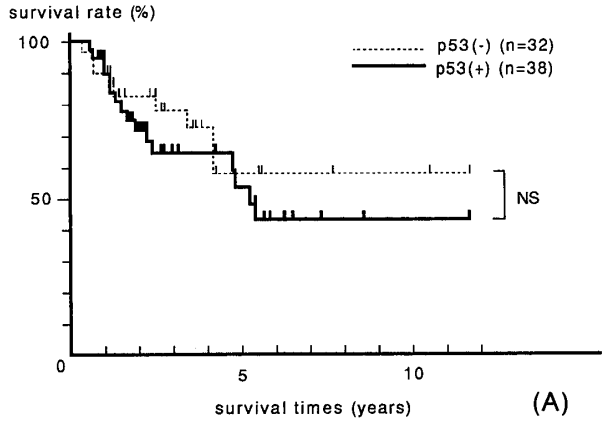


Fig. 3 Histogram of p53 and HSP70 labelling index
(A) Histogram of p53 labelling index
Arrow indicates cut-off point (20%).
(B) Histogram of HSP70 labelling index
Arrow indicates cut-off point (20%).



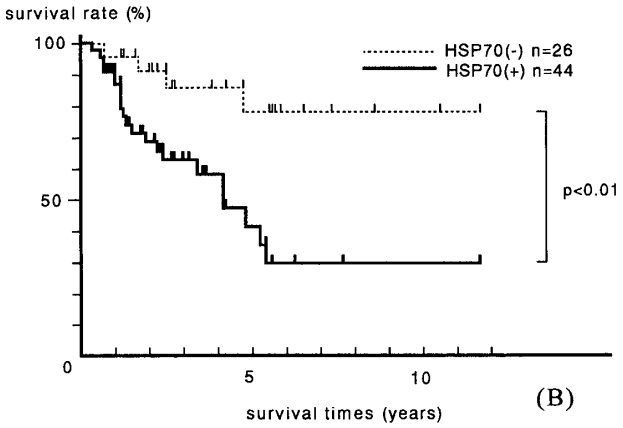


Fig. 4 Kaplan-Meier survival curves of patients with oral squamous cell carcinoma according to p53 protein and HSP70 expression
$p<0.01$ (Cox-Mantel test)

Relationship between p53-HSP70 coexpression and Ki-67 immunoreactivity

The mean value of Ki-67 LI for p53 protein positive cases was 37.7% which was higher than the 28.2% of p53 protein negative cases. There was no significant correlations between Ki-67 index and p53 protein expression (Table 2). The mean value of Ki-67 LI for HSP70 positive tumors was 43.4% which was significantly higher than the 25.2% of the negative ($p<0.01$) (Table 2). Coexpression of p53-HSP70 was found in 31 (81.6%) of the 38 p53 positive OSCCs. The mean value of Ki-67 LI was significantly higher in patients in whom both p53 protein and HSP70 were positive (40.8%) than in patients in whom both were negative (23.1%) ($p<0.01$) (Table 2).

Table 2 Relationships between Ki-67 immunoactivity and p53, HSP70 expression

Variables		Ki-67 (mean)	LI(%) (±SD)	
p53(+)	(n=38)	37.7	17.5	
p53(−)	(n=32)	28.2	15.2	
HSP70(+)	(n=44)	43.4	18.7	*
HSP70(−)	(n=26)	25.2	18.7	*
p53(+), HSP70(+)	(n=31)	40.8	17.2	*
p53(−), HSP70(−)	(n=19)	23.1	17.2	*

*$p<0.01$ (paired Student's t-test).

Discussion

The present study was undertaken to examine immunohistochemically the expression of p53 protein and heat shock protein (HSP) 70 and growth potential of tumor cells in biopsy specimens from patients with oral squamous cell carcinoma (OSCC) and to analyze these findings in relation to the clinicopathologic parameters (CPP) and clinical course (CC) of these patients. Abnormalities in p53 anti-oncogene have been detected at high incidence in cells of many cancers such as lung[19], breast[20] and colorectal[21] cancers. Abnormalities of p53 and some other genes are thought to be involved in the onset and progression of cancer. A mutant p53 protein, encoded by an abnormal p53 gene, has a more stable structure and a longer half-life than does wild-type p53 protein. For this reason, the mutant p53 protein can be detected by an immunohistochemical check for overexpression of p53 protein. One possible reason for the higher structural stability of the mutant p53 protein is the fact that the mutant protein is associated with HSP70[22][23]. In past immunohistochemical studies of p53 protein in cases of OSCC, the incidence of p53 protein was often between 50 and 60%[3][5]~[8]. Regarding the relationship between p53 protein expression and CPP and CC, some investigators reported that p53 protein expression was seen more frequently in poorly-differentiated OSCC than in well-differentiated OSCC[6][7], while others have reported that the prognosis was poorer in p53 protein-positive patients than in p53

protein-negative patients[24] or that CPP and CC had no signficicant correlation with p53 protein expression[3)5)8]. In the present study, p53 protein expression was not correlated significantly with CPP or CC. However, since the type of primary antibody, the method of immobilization and the criteria used for a evaluating chromatic responses of p53 protein differed among different immunohistochemical studies, it is difficult to directly compare the results of these studies. Bearing this in mind, we determined the frequency distribution of the p53 protein labelling index and found that the distribution of frequencies differed markedly in two regions divided by the labelling rate of 20%. Following this finding, we divided patients into a p53 protein-positive group and a p53 protein-negative group, using the labeling index of 20% as a cut-off level.

Prior to the present paper, the only report concerning immunohistochemical examination of HSP70 in cases of OSCC was published by Sugreman[25]. They reported that HSP70 expression was seen more frequently in patients with lower degrees of histological differentiation. In the present study, we carried out a comprehensive analysis of immunohistochemical detection of HSP70 in relation to the course of CPP and CC in patients with OSCC. No such analysis had been made prior to the present study. Meanwhile, Ciocca et al.[11] quantified the HSP70 content of breast cancer, using Western blotting, and found that the prognosis was poorer in patients with HSP70-rich breast cancers. In the present study of OSCC patients, the incidence of HSP70 expression was high in cancers in which cell growth potentials were high, such as cancers with a low degree of histological differentiation, a high degree of nuclear atypism and a high frequency of mitosis. The five-year survival rate was significantly lower in HSP70-positive patients than in HSP70-negative patients.

Regarding the relationship between the Ki-67 labelling index (Ki-67 LI) and CPP and CC in OSCC patients, the present study revealed that the Ki-67 LI was significantly higher in patients with high cell growth potentials, i.e., patients with low degrees of histological differentiation, markedly diffuse invasion of the tumor, high degrees of nuclear atypism or high frequencies of mitosis. The index was also significantly high in patients in high clinical stages of cancer and patients with tumor metastasis to cervical lymph nodes. Komiyama[26], who studied the relationship between the Ki-67 LI and CPP and CC in patients with tongue cancer, also reported results similar to those of the present study, saying that the index was significantly high in patients with aggressive histologic type and those with metastasis to cervical lymph nodes. These results suggest that numerical, objective analysis of the tumor growth potentials using the Ki-67 LI provides a useful indicator for selecting a therapy.

Immunohistochemical result of HSP70 was positive in 31 of the 38 cases with p53 protein positive patients. So, we concluded that the mutant p53 protein might be correlated with overexpression of HSP70. In fact, in other cancer patients[21)27)13], similar result has been reported in literature. In OSCC patients of Stage I and II, p53 protein was positive in 55.8% and HSP70 was positive in 67.4%. If results are combined with the finding that p53 protein and HSP70 were detected immunohistochemically in precancerous lesions of the oral cavity, it seems likely that the expression of p53 protein and HSP70 takes place at relatively early stages of the development of OSCC[28]. Regarding the relationship between tumor cell growth and synchronous expression of p53 protein and HSP70, we found that the Ki-67 LI tended to be higher in p53 protein-positive patients than in negative ones, and that this index was significantly higher in HSP70 positive-patients than in negative-patients. We also noted that the Ki-67 LI was significantly higher in patients in whom both p53 protein and HSP70 were positive than in patients in whom both were negative. The immunohistochemical finding of p53 and HSP70 expression in OSCC

patients probably indicates that normal p53 protein has undergone variation and lost its function of regulating its proper cell cycle, and that the half-life of p53 protein is prolonged by the mutation of this protein, resulting in promotion of carcinogenesis and acceleration of tumor cell growth.

The results of this study suggest that an immunohistochemical examination for p53 protein and HSP70 and determination of the Ki-67 LI will be useful in assessing the biological malignancy level of OSCC, determining a therapeutic policy and predicting the prognosis of OSCC in a given case.

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口腔扁平上皮癌におけるp53蛋白，熱ショック蛋白（HSP）70，Ki-67の発現に関する免疫組織化学的検討

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口腔扁平上皮癌患者70例の生体標本において，p53蛋白，熱ショック蛋白（HSP）70の発現と，腫瘍細胞の増殖能をKi-67標識率を用いて免疫組織化学的に検索し，臨床病理学的因子，臨床経過との関連を検討した．p53癌抑制遺伝子異常は多くの臓器の癌細胞に高頻度に検出され，癌化およびその進展に他の遺伝子異常とともに寄与していると考えられている．またその産物である変異型p53蛋白は野生型p53蛋白に比べ構造が安定化し半減期が延長するため，過剰発現として免疫組織化学的に検出が可能である．この構造安定化の原因として変異型p53蛋白がHSP70と会合することが考えられている．

p53蛋白の過剰発現は54.2%に認めたが，臨床病理学的因子，臨床経過との有意な相関は認めなかった．HSP70の発現は低分化なもの，核異型の高度なもの，核分裂像の多いものなど細胞増殖が活発な細胞で高頻度に認め，5年生存率はHSP70陽性群が陰性群に比べて有意に低下していた．細胞増殖能（Ki-67標識率）との関連性では低分化のもの，核異型が高度なもの，核分裂の多いものなどの細胞増殖が活発な細胞で，また臨床病期の進行した症例，頚部リンパ節転移を認めた症例でもKi-67標識率が有意に高値を示した．

次にp53蛋白とHSP70の相関を検討すると，p53蛋白染色陽性の38例中31例がHSP70染色も陽性であり，変異p53蛋白とHSP70との相関が認められると考えた．p53蛋白，HSP70の同時発現と細胞増殖との関連では，p53蛋白，HSP70の同時陽性群では陰性群に比べKi-67標識率が有意に高値を示した．

本研究の結果からp53蛋白，HSP70の免疫組織化学的発現とKi-67標識率を検索することは，口腔扁平上皮癌の生物的悪性度を知るうえで有用であり，さらに治療方針の決定や予後推定に際しての指標となり得ることが示唆された．