Cathepsin expression in oral squamous cell carcinoma: Relationship with clinicopathologic factors

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Objective. Proteases are involved in the invasion and metastasis of carcinoma cells. In vivo, oral carcinoma cells easily invade the bone tissue and metastasize to the submandibular and neck lymph nodes. Cathepsin expression has been shown in some neoplastic tissues and serves as a prognostic indicator. The purpose of this study was to investigate the relationship between clinicopathohistologic grades and cathepsin expressions in oral squamous cell carcinoma and to investigate which cathepsin provides prognostic information for patients with oral carcinoma.
Study design. Immunohistochemical studies were performed on 78 carcinoma samples with monoclonal antibodies against cathepsins B, H, and L, and a polyclonal antibody against cathepsin D. Serial sections were stained by hematoxylin-eosin staining and classified by Anneroth's classification. Cathepsin B, H, L and D activities of blood serum were determined. Positive results indicative of the presence of cathepsin were investigated to determine any correlation between a particular cathepsin and histologic malignancy grades, tumor cell growth, serum cathepsin activities, and clinical factors.
Results. Cathepsins B, H, L, and D were positive in every case. Although the labeling indices for cathepsins B (CB-LI), H (CH-LI), and D (CD-LI) for the cancer cases showed significant differences from those of controls, cathepsin L (CL-LI) of cancer cases showed no difference from that of controls ($P < .05$). A close correlation was found between CD-LI and T categories of TNM classification ($P < .05$), and between CD-LI and PCNA-LI ($P < .05$). Furthermore, a close correlation was found between CD-LI and N categories in TNM classification ($P < .05$). Pathologically, a close correlation was found between CB-LI or CD-LI and the pattern and/or stage of invasion ($P < .05$).
Conclusion. Cathepsin D and B expression were closely correlated with carcinoma invasion and progression. These proteases may be useful in determining the prognoses of patients with oral carcinoma.
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During invasion and metastasis, malignant cells cross basement membranes at least 3 times: during their escape from the primary site and, subsequently, during both entry into and exit from the bloodstream. Various types of proteases produced by the tumor cells are thought to exert a major role in degrading proteinous components of the basement membrane during these processes. As support for this hypothesis, elevated levels of lysosomal proteinases, such as cathepsins B, H, L, or D have been reported in many cancer types.[1-5] These proteases are able to directly degrade laminin, collagen, or elastin in vitro and also process active precursor forms of other proteolytic enzymes.

Cathepsins are useful markers for identifying patients who are suffering from breast cancer[6,7] or colorectal cancer[8] who may be at an increased risk of recurrence, more frequent metastasis, or increased mortality rate. However, little is known about the prognostic value of cathepsins in oral carcinoma. Many clinical studies of oral carcinoma cases have reported that a histologic grading system using hematoxylin-eosin staining was useful for determining the biologic behavior of the carcinoma.

Our objective was to determine the correlation between the histopathologic and prognostic values of patients and immunohistochemical examination of cathepsins B, H, L, and D in oral squamous cell carcinoma. Furthermore, we investigated and analyzed the cathepsin activities of serum samples obtained from these patients.

MATERIAL AND METHODS

Patients

This study comprised 78 patients (53 men and 25 women) who visited the First Department of Oral and Maxillofacial Surgery of Nagasaki University Dental Hospital during the last decade (Table I). The primary sites of squamous cell carcinoma were the tongue (n = 40), the floor of the mouth (n = 18), and the gingiva (n = 20). Patients ranged in age from 34 to 85 years at diagnosis. All tumors were staged following the 1987 UICC staging system. As controls, normal oral mucosa (n = 20) were collected and analyzed.

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Histopathologic analysis

Surgical specimens were fixed with 10% formalin and embedded in paraffin. Four-μm sections were stained with hematoxylin-eosin. According to the classification system of Anneroth et al[9] (Table II), we analyzed each case by 6 parameters: degree of keratinization, nuclear polymorphism, number of mitoses for histologic grading of malignancy of tumor population, pattern of invasion, stage of invasion, and lymphoplasmocytic infiltration for histologic grading of malignancy of tumor-host relationship. Each parameter was graded as Grade 1 through 4 and scored.

Immunohistochemical analysis

After deparaffinization and rehydration, sections were pretreated with 3% H_2O_2/metanol to block endogenous peroxidase activity. After treatment with normal serum for 10 minutes at room temperature to block nonspecific staining, the sections were incubated with either mouse anti-human cathepsin B, H, or L antiserum (rabbit antihuman cathepsins B, H and L, Athens Research and Technology, Athens, Greece), PC10 (mouse antihuman PCNA, DAKO, Copenhagen, Denmark) or rabbit antirat cathepsin D at 4°C over night. The antisera against cathepsin D purified from the rat spleen were obtained in rabbits, as described previously.[1,10] The IgG fractions from these antisera were prepared by passage through a column of antigen-coupled Separose 4B (Pharmacia, Uppsala, Sweden). The sections were incubated with sheep biotinylated-antimouse or rabbit (Fab)´2 for 30 minutes and with the avidin-HRP complex Regent for 20 minutes at room temperature. The color was developed with 3,3´-aminobenzidine-4HCl and 0.02% H_2O_2. The sections were then counterstained with hematoxylin. When tumor heterogeneity existed, we examined 1000 tumor cells, each at 5 microscopic views that were selected randomly.

Cathepsin serum activities

Venous blood (10 mL) of 41 patients was drawn, allowed to clot, and centrifuged at room temperature. Serum samples were stored at –70°C until analysis. Cathepsin activities were determined as described previously.[11]

Serum enzymatic activities of cathepsins B, H, and L were determined with fluorogenic substrates: benzyloxycarbonyl-arginyl-4-methyl-7-coumaryl-amide (Z-Arg-Arg-MCA), arginine-4-methyl-7-coumarylamide (Arg-MCA) and benzyloxycarbonyl-phenylalanyl-arginine-4-methyl-7-coumarylamide (Z-Phe-Arg-MCA), respectively.[12] After incubation for 10 minutes at 40°C, enzyme reactions were stopped by the addition of 0.1 M sodium acetate buffer (pH 5.0) containing 0.1 M sodium iodoacetate. The liberated product, 7-amino-4-methylcoumarin, was estimated by measurement of the fluorescence intensity at 460 nm using a Hitachi fluorescence spectrophotometer (Model F-4000 [Hitachi, Tokyo, Japan]). The excitation wavelength was 380 nm. One unit of the enzyme activity was defined as the amount of each enzyme required to release 1 μmol of 7-amino-4-methylcoumarine per minute. Cathepsin D activity was measured with 1.5% acid denatured bovine hemoglobin in 0.01 M acetate buffer at pH 3.8 as a substrate. After incubation at 40°C for 40 minutes, the reaction was stopped by trichloroacetic acid. After centrifugation at 3000 rpm for 10 minutes, the trichloroacetic acid soluble fraction was used for measurement by the Folin-Lowry reaction. The color measurement was done at 660 nm. The amount of protein was determined by the method of Lowry with bovine serum albumin as a standard.[13]

Table I. Clinical characteristics of patients with oral carcinoma

	No. of patients
Sex	
Male	53
Female	25
Age (y) 34-85	
T-stage	
T1	23
T2	32
T3	13
T4	10
N-stage	
N0	50
N1	17
N2	11
M-stage	
M0	78

Histopathologic pattern of all cases is squamous cell carcinoma. The tumor sites were the tongue (n = 40), floor of the mouth (n = 18), and gingiva (n = 20). TNM category is by UICC classification.

Statistical evaluation

We analyzed results by using the Student *t* test, Pearson's correlation coefficient, one-factor ANOVA, and Scheffé test. Survival was calculated from the day of biopsy. The survival curves for these patients were calculated by the Kaplan-Meier method. Statistical significance was calculated with the log rank test. *P* values less than .05 were considered significant.

RESULTS

Immunohistochemical stains

In our series of oral carcinoma cases, the 4 antigens cathepsin B, H, L, and D were found to be positive in every case, although in some cases at very low levels.

Table II. Anneroth's classification

Histologic grading of malignancy of tumor cell population				
	Points			
Morphologic parameter	*1*	*2*	*3*	*4*
Degree of keratinization	Highly keratinized (>50% of the cells)	Moderate keratinization (20%-50% of the cells)	Minimal keratinization (5%-20% of the cells)	No keratinization (0%-5% of the cells)
Nuclear polymorphism	Little nuclear polymorphism (>75% mature cells)	Moderately abundant nuclear polymorphism (50%-70% mature cells)	Abundant nuclear polymorphism (20%-50% mature cells)	Extreme nuclear polymorphism (0%-25% mature cells)
Number of mitoses/HPF	0-1	2-3	4-5	>5
Histologic grading of malignancy tumor-host relationship				
	Points			
Morphologic parameter	*1*	*2*	*3*	*4*
Pattern of invasion	Well-delineated infiltrating borders, with pus	Infiltrating, solid cords, bands and/or strands	Small groups or cords of infiltrating cells (n > 15)	Marked and widespread cellular dissociation in small groups of cells (n < 15) and/or in single cells
Stage of invasion (depth)	Carcinoma in situ and/or questionable invasion	Distinct invasion, but involving lamina propria only	Invasion below lamina propria adjacent to muscles, salivary gland tissues and periosteum	Extension and deep invasion replacing most of the stromal tissue and infiltrating jaw bone
Lymphoplasmocytic infiltration	Marked	Moderate	Slight	None

HPF, High-power field.

Positive staining was seen in the carcinoma cells, and the staining was cytoplasmic and coarsely granular (Figs 1-4).

Carcinoma cells with intense immunoreaction for cathepsin B and D were localized at the advancing margin of the carcinoma tissue, whereas cathepsins H and L were found diffusely in the tumor cell nest.

Cathepsin B labeling index (CB-LI), cathepsin H labeling index (CH-LI), cathepsin L labeling index (CL-LI), and cathepsin D labeling index (CD-LI) were determined as mean percentages of cathepsin-positive tumor cells by examining 1000 tumor cells on randomly selected microscopic views. The mean CB-LI, CH-LI, CL-LI, and CD-LI for the oral carcinoma patients were 23.3, 16.4, 4.5, and 23.6, respectively. The mean CB-LI, CH-LI, CL-LI, and CD-LI of controls were 10.1, 7.2, 4.3, and 9.1, respectively.

Although CB-LI, CH-LI, and CD-LI of carcinoma cases showed significant differences from those of control epithelial cells, CL-LI of carcinoma cases showed no difference from controls (Student t test, $P < .05$).

Cathepsin staining and clinical stages

No significant difference was found among CB-LI, CH-LI, or CL-LI of T categories; however, a close correlation was found between CD-LI and T categories (Student *t* test, $P < .05$; Table III).

The percentage of pN+ cases was 35.9% (28/78) in this study. CB-LI, CH-LI, CL-LI, and CD-LI in pN+ cases were 25.2, 16.9, 5.2, and 30.2, respectively. CB-LI, CH-LI, CL-LI, and CD-LI in pN– and N– cases were 22.3, 16.1, 4.0, and 19.8, respectively. CD-LI of lymph node–positive cases was significantly higher than that of lymph node–negative cases.

Cathepsin staining and pathologic parameters

Close correlations were observed between CB-LI and nuclear polymorphism, pattern of invasion, stage of invasion, and lymphoplasmocytic infiltration (one-factor ANOVA, the Scheffé test: $P < .05$; Tables IV and V). CD-LI had close correlations with nuclear polymorphism or patterns of invasion or stage of invasion or lymphoplasmocytic infiltration (one-factor ANOVA, the Scheffé test: $P < .05$). CH-LI and CL-LI had no relationship with all 6 parameters.

Relationship between cathepsins and PCNA

PCNA was detected as granular staining in nuclei and nucleoli. The outer regions of expanding tumor masses displayed higher levels of positivity rates than those cells more centrally positioned.

The lowest level and the highest level for PCNA was 7.3% and 60.1%, respectively. The mean PCNA-LI was 26.1.

Although there was no correlation of the localization of cathepsin D and PCNA (Fig 5), a close correlation was found between CD-LI and PCNA-LI (Pearson's correlation coefficient, Student *t* test, $P < .05$).

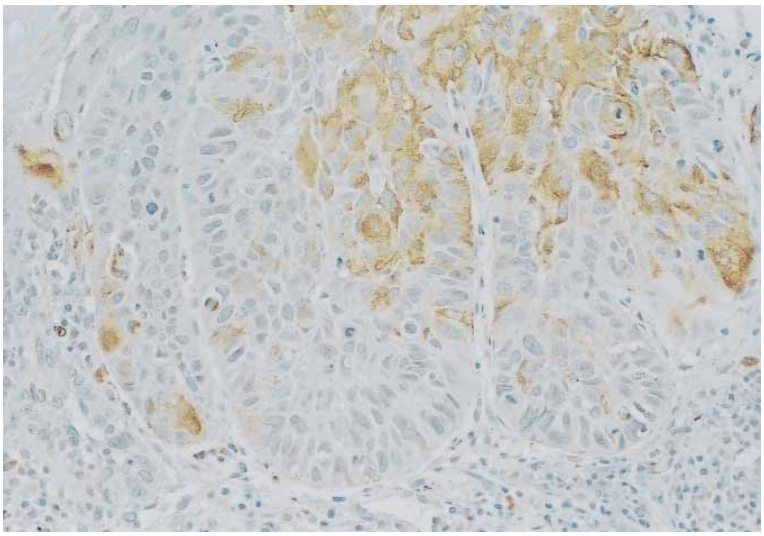

Fig 1. Immunostaining for cathepsin B. Carcinoma cells with intense immunoreaction for cathepsin B are localized at the advancing margin of the carcinoma tissue (×50).

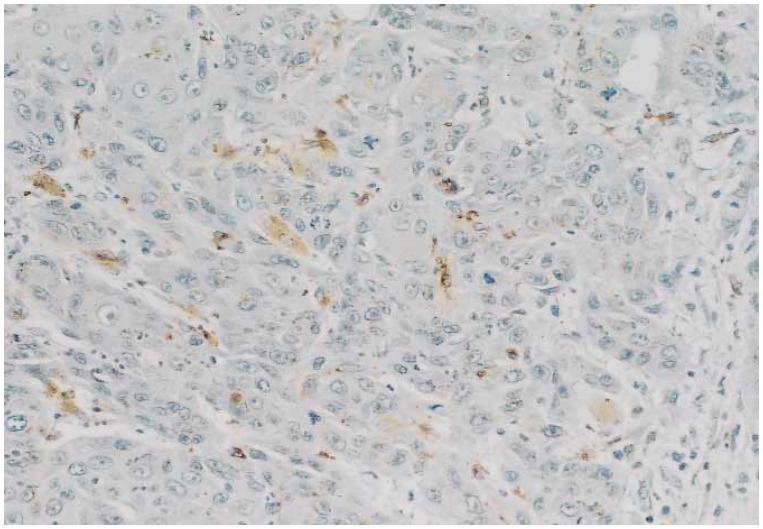

Fig 2. Immunostaining for cathepsin H (×50).

Activities of cathepsins found in patient's serum

No correlation was found between cathepsins of the carcinoma patients and controls. No correlation was found between cathepsin LI and the activities of cathepsins in the patient's serum.

Prognosis by survival time

There were statistically significant differences between CD-LI and survival time by the Kaplan-Meier method (Fig 6). There was no significant difference between the other cathepsin LI and survival time by the Kaplan-Meier method.

DISCUSSION

One of the most important factors in the prognosis of the oral cancer patient is invasion of the extracellular matrix of the oral mucosa. Tumor cell invasion involves attachment of tumor cells to the underlying basement membrane, local proteolysis, and migration of tumor cells through the proteolytically modified region. Local proteolysis is facilitated by proteases outside the tumor cell, perhaps bound to the cell surface and/or secreted from the tumor cells.[14] Recent data suggest that proteases inside the tumor also participate in local proteolysis by digesting phagocytosed extracellular matrix.[15-17]

Multiple steps are involved in cancer cell metastasis, and proteases are believed to participate in many of those steps.[18] To date, most investigators have focused on the role of proteases in the final steps of tumor

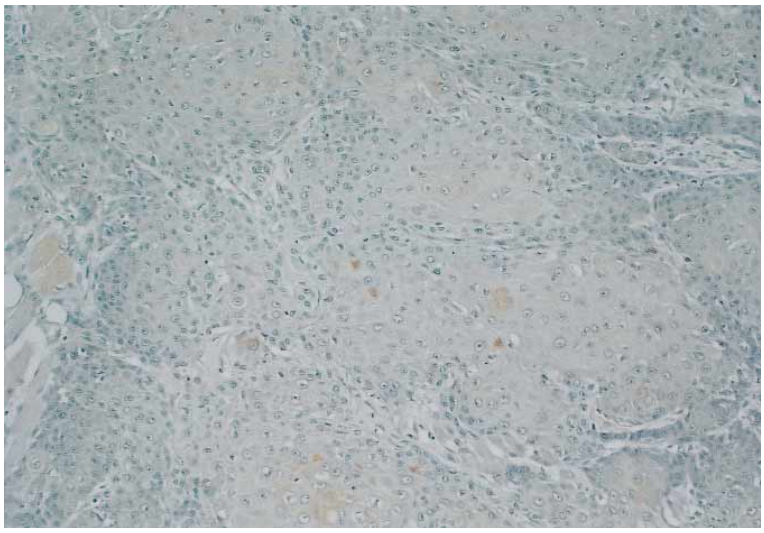

Fig 3. Immunostaining for cathepsin L (×25).

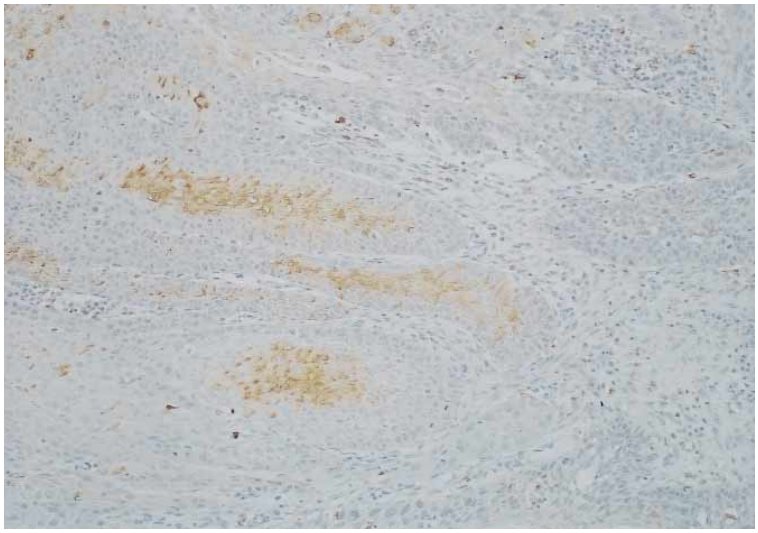

Fig 4. Immunostaining for cathepsin D. Carcinoma cells with intense immunoreaction for cathepsin D are localized diffusely in the tumor cell nest (×25).

progression: invasion and metastasis.[19] Evidence is now emerging that indicates that proteases are involved in tumor growth at both the primary and metastatic sites.[14,20]

Endopeptidases are categorized into 5 major classes: cysteine, aspartic, serine, metallo-, and threonine proteases. Cysteine proteases, in particular cathepsin B, were shown, in the early 1980s, to be associated with malignancy.[15] In vivo studies have found that there is an inverse correlation between cathepsin B staining and basement membrane staining in bladder,[21] gastric,[22] lung,[23] and colon[24] carcinomas. This would be consistent with a functional role of cathepsin B in degrading these extracellular matrix components in vivo. Cathepsin B is active against large substrates such as laminin and fibronectin under physiologic conditions of pH and temperature, and, consequently, could degrade extracellular matrix components outside the cell.

Later studies have shown that cathepsins B, H, and L are involved in cancer progression by either direct degradation of the extracellular matrix or by activation of other proteases, such as urokinase-type plasminogen activator.[15] This involvement could be accomplished by increased secretion of mRNA and protein levels and activity.[15]

Cathepsins L and B are secreted either as mature forms or as proforms, both of which are active.[25] Since

Table III. Relationship between cathepsins LI and clinical factors

	CB-LI	*CH-LI*	*CL-LI*	*CD-LI*
T1	24.7 ± 12.9	15.5 ± 6.8	4.0 ± 1.9	12.7 ± 5.2
T2	23.0 ± 8.6	17.0 ± 6.3	4.3 ± 1.9	21.4 ± 5.8
T3	23.3 ± 10.1	17.9 ± 7.2	5.0 ± 2.5	31.2 ± 5.0
T4	21.2 ± 6.5	14.6 ± 1.6	5.4 ± 1.9	45.6 ± 5.6
pN(–) or N(–)	22.3 ± 8.8	16.1 ± 6.6	4.0 ± 2.0	19.8 ± 10.9
pN(+)	25.2 ± 11.7	16.9 ± 5.6	5.2 ± 2.0	30.2 ± 10.5

*$P < .05$ (Scheffé test).
†$P < .05$ (Student t test).

Table IV. Cathepsin labeling indices and histologic grading of malignancy of tumor cell population of Anneroth's classification

Morphologic parameter	*CB-LI*	*CH-LI*	*CL-LI*	*CD-LI*
Degree of keratinization				
1 (n = 43)	23.7 ± 9.3	16.5 ± 6.6	4.3 ± 1.8	24.4 ± 12.1
2 (n = 26)	21.2 ± 9.3	16.5 ± 6.2	4.6 ± 2.3	21.3 ± 10.3
3 (n = 9)	28.0 ± 13.9	15.5 ± 4.7	4.9 ± 2.1	25.8 ± 14.8
4	-	-	-	-
Nuclear polymorphism				
1 (n = 37)	19.7 ± 7.5	16.9 ± 6.5	4.2 ± 2.0	20.7 ± 10.6
2 (n = 27)	24.6 ± 11.1	16.0 ± 4.8	4.9 ± 1.7	22.2 ± 9.7
3 (n = 10)	28.1 ± 9.8	15.1 ± 8.8	5.2 ± 2.9	29.7 ± 13.6
4 (n = 4)	36.7 ± 3.9	17.7 ± 6.0	2.7 ± 1.4	44.1 ± 6.6
No. of mitoses				
1 (n = 55)	22.8 ± 9.8	16.0 ± 6.2	4.6 ± 2.1	23.2 ± 11.1
2 (n = 18)	24.0 ± 10.5	17.1 ± 6.6	4.3 ± 2.2	23.4 ± 13.0
3 (n =5)	26.7 ± 11.2	18.1 ± 4.9	3.7 ± 0.8	28.1 ± 16.5
4	-	-	-	-

* P < .05 (Scheffé test).

Table V. Cathepsin labeling indices and histologic grading of malignancy tumor-host relationship of Anneroth's classification

Morphologic parameter	*CB-LI*	*CH-LI*	*CL-LI*	*CD-LI*
Pattern of invasion				
1 (n = 16)	12.3 ± 1.8	17.6 ± 7.6	5.4 ± 1.5	10.1 ± 3.0
2 (n = 33)	20.5 ± 5.7	15.8 ± 5.1	4.3 ± 2.4	22.8 ± 9.5
3 (n = 19)	29.9 ± 4.6	15.4 ± 5.3	4.2 ± 2.1	29.8 ± 8.5
4 (n = 10)	37.7 ± 10.9	18.3 ± 8.6	4.2 ± 1.1	35.6 ± 12.2
Stage of invasion				
1 (n = 23)	12.1 ± 1.8	15.7 ± 6.0	4.9 ± 2.1	12.0 ± 6.2
2 (n = 28)	22.2 ± 3.8	15.7 ± 5.3	4.4 ± 2.2	25.6 ± 8.7
3 (n = 22)	31.8 ± 4.1	17.7 ± 6.4	4.1 ± 1.9	30.6 ± 9.7
4 (n = 5)	44.2 ± 5.9	17.7 ± 10.9	4.5 ± 1.3	34.1 ± 16.2
Lymphoplasmocytic infiltration				
1 (n = 41)	18.7 ± 7.5	17.3 ± 6.1	4.8 ± 2.1	17.0 ± 7.5
2 (n = 22)	27.6 ± 10.4	14.6 ± 5.5	4.1 ± 1.5	23.6 ± 7.1
3 (n = 15)	29.6 ± 9.4	16.5 ± 7.2	4.1 ± 2.3	41.5 ± 7.6
4	-	-	-	-

*$P < .01$ (Scheffé test).

mannose-6-phosphate receptors are the main receptors responsible for the trafficking and targeting of lysosomal enzymes, alterations in this mechanism were initially suggested to be the major source of enhanced cathepsin secretion. However, in addition to this mechanism, other membrane-associated receptors were found to bind procathepsins, suggesting that mannose-6-receptor–independent pathways exist.[15] This probably accounts for the membrane-bound cathepsin B observed in certain tumors. Such binding could

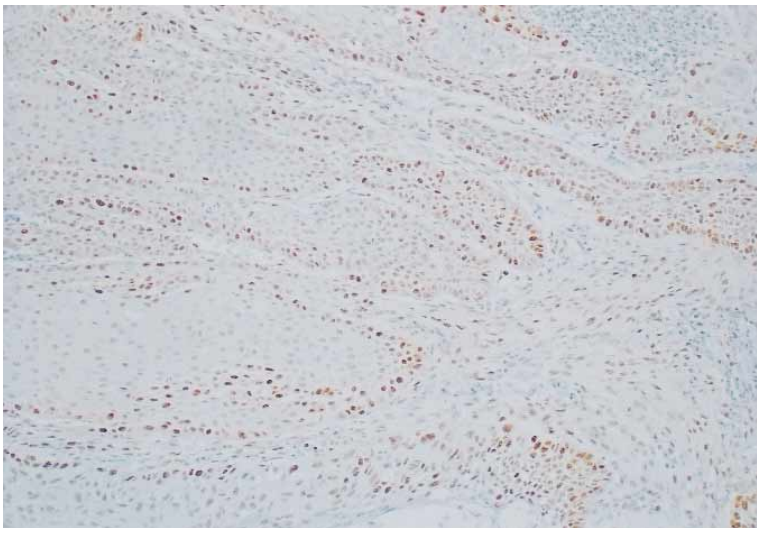

Fig 5. Immunostaining for proliferating cell nuclear antigen. Carcinoma cells with intense immunoreaction for proliferating cell nuclear antigen are localized at the advancing margin of the carcinoma tissue (×25).

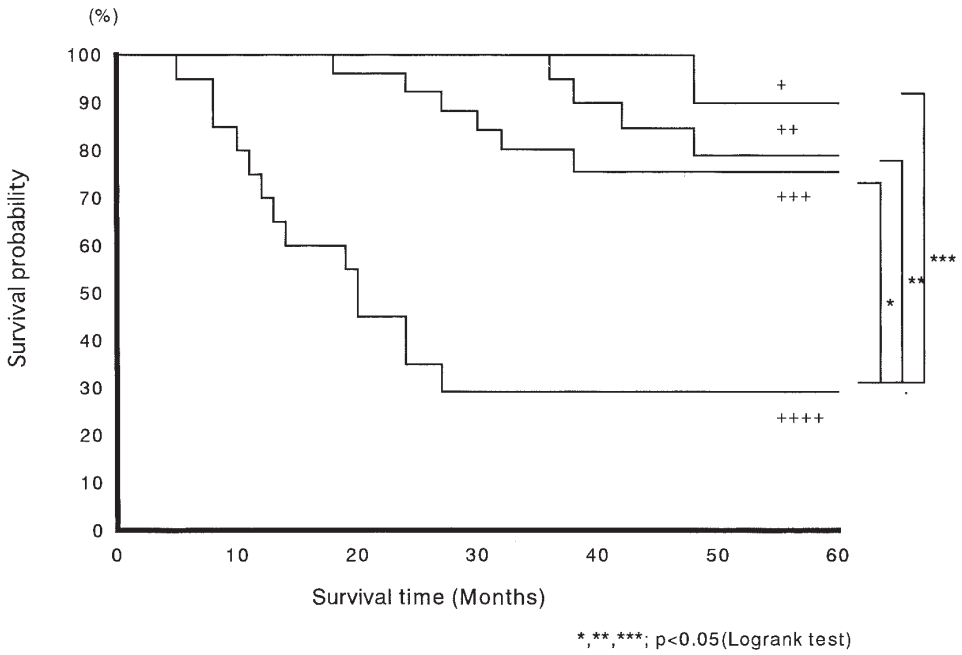

Fig 6. Survival rate calculated by measures of the Kaplan-Meier method. High CD-LI group of oral carcinoma patients reveals statistically higher survival time than the low CD-LI group. +, CD-LI < 10; ++, 10 ≤CDLI < 20; +++, 20 ≤CDLI < 30; ++++, ≥CD-LI 30.

substantially stabilize cathepsin B, which otherwise exhibits similar properties to the normal enzyme.[26] Another possible mechanism for increased cathepsin secretion is alternative splicing, as observed for cathepsins B and L.[27] Cathepsin B mRNAs lacking exons 2 and/or 3 were observed as the main forms in a number of tumors. These deletions result in highly elevated translation, which could be up to 8-fold that of the full-length mRNA.[15] The translation product was functionally active, but lacked part of the progression, which could also explain the increased size and stability of such secreted forms.[28]

Intracellular degradation of extracellular matrix also seems to be important for tumor cell invasion. Coopman et al[29] have shown a correlation between the invasive ability of different cancer cell lines and their ability to phagocytize the extracellular matrix. Another study found that large acidic vesicles, identified as

heterophagosomes,[30] are more frequently present in breast cancer cells that have migrated through Matrigel.[31] These large, acidic vesicles can phagocytize extracellular matrix and digest this material within the heterophagosome. Heterophagosomes contain mature cathepsin D and have been seen in human breast cancer cells in vivo.[30,31]

Cathepsin D overexpression in some cancers, particularly breast cancer, is not only correlated with but also facilitates the development of clinical metastasis as demonstrated in a rat tumor cell system by transfecting human cathepsin D gene.[7]

In the head and neck region, Zeillinger et al indicated that quantification of cathepsin D in the head and neck squamous cell carcinoma tissue was significantly higher than normal tissue and was independent from other pathohistologic markers.[32] Most studies indicate that cathepsin D overexpression decreases the relapse-free and overall survival by stimulating the growth of micrometastasis at distant sites, rather than by stimulating local invasion by digestion of basement membrane.

In our series, patients with cancer had higher proportions of cells expressing cathepsins B, H, and D than did control subjects. This result may suggest that these cathepsins interact with the biological activity of the cancer cells. Cathepsins B and D had particularly close correlations with the pattern and stage of invasion by Anneroth's classification system ($P < .05$), suggesting that cathepsins B and D are involved in the invasion of tumor cells. Although a sufficiently acidic pH (<5.5) is required to activate cathepsin D, cathepsin B can work at neutral pH, which may suggest that cathepsin B mainly degrades the basement membrane at the local sites.

Expression of PCNA, which reveals tumor cell growth, showed a close correlation with the expression of cathepsin D ($P < .05$). Strong expression of cathepsin D was related to cell proliferation and to expression of growth-factor receptors. Cathepsin D has interactions with growth factors, such as bFGF, EGF, and IGF-1.[33] These results may suggest that cathepsin D correlates with tumor cell growth, rather than local invasion.

Moreover, the high CD-LI group (CD-LI 30) showed a shorter survival time than the low CD-LI group (CD-LI <30). The same tendency was observed in CB-LI. Thus, the prognostic value of cathepsin D and B in oral carcinoma was suggested. It may help to define new therapies of cancer targeted at cathepsin B and D overexpression and at inhibiting the development of micrometastasis into clinical metastasis.

In summary, the present report describes cathepsin expression in oral squamous cell carcinoma samples and their relationships with clinical and pathologic parameters. Cathepsins B and D were correlated with invasion and tumor cell growth of oral carcinoma. It is suggested that these proteases have a potential to improve the prognosis of patients with oral carcinoma.

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