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Detection of oral squamous cell carcinoma metastasis with cathepsin D: An immunohistochemical approach

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ABSTRACT

Background: The lysosomal protease cathepsin D (CD) has been associated with tumor progression in malignant tumors including oral squamous cell carcinoma (OSCC). The purpose of this study was to find out any association between the CD and lymph node metastasis and to study the correlation of CD with various clinicopathological parameters to aid in assessment of its role as a prognostic indicator.
Materials and Methods: Immunohistochemical staining was performed on 20 OSCC samples with polyclonal antibody against CD. Positive results indicative of the presence of CD were further analyzed to determine any correlation between the CD and other clinicopathological parameters. Pearson Chi-square analyses, Spearsman correlation coefficient, Mann-Whitney test, Kruskal Wallis test and student t test were used for statistical analysis ($P < 0.05$).
Results: Patients with lymph node metastasis showed statistically significant increase in CD expression ($P < 0.01$). Increasing tumor size seemed to correlate with increased CD expression ($P < 0.05$).
Conclusion: Based on its association with other clinicopathological variables, CD expression can be used for the assessment of patient survival in cases of OSCC.

Key Words: Cathepsin D, metastasis, oral squamous cell carcinoma

INTRODUCTION

Globally, oral squamous cell carcinoma (OSCC) is one of the ten most frequently diagnosed cancers. All the modern treatment modalities have been unable to modify the survival rate of OSCC. The factors that are presently considered to influence prognosis of oral cancers are tumor size, histologic type, and lymph node metastasis.[1,2]

Malignant tumor cells have a tendency to invade the surrounding matrix and penetrate the basement membrane. Extracellular matrix components comprises of collagens, glycoproteins, proteoglycans, and glycosaminoglycans. Proteases are thought to play an important role in tumor invasion and metastasis because they degrade extracellular matrices and basement membranes.[3,4]

Aberrant signaling cascades leading to selective over-expression of a number of proteinases cause the highly invasive and metastatic phenotypes of malignant cells. Such proteinases include aspartic proteases (Cathepsin D [CD]), Cysteine proteases (Cathepsin B), Matrix Metalloproteinases (MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9), serine proteinases, and urokinase-type plasminogen activator.[5]

Cathepsins are lysosomal endopeptidases, which, in normal cells, participate in the proteolysis of endocytosed proteins and proteolytic activation of secretary proteins. They are powerful inducible proteinases with broad substrate specificity; some of them may be induced by oncoproteins (ras, fos/jun), hormones, and cytokines (interleukin-1, granulocyte-macrophage colony stimulating factor). They play

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a variety of functions in intracellular processing and metabolism. Secretion and alteration in the subcellular localization of cathepsins in malignant cells is presumed to function in the digestion of the extracellular matrix components.[6-8]

CD, a lysosomal acidic protease, was first identified as a 52 kDa estrogen dependent glycoprotein in MCF-7 cells (Michigan Cancer Foundation-7 cells)). Three forms of CD include the 52 kDa procathepsin D (pCD) (enzymatically inactive), an active intermediate form (48 kDa) and mature 34 kDa and 14 kDa dimer form.[9] The mature forms of CD proteolytically degrade extracellular matrices and proteoglycans.[10]

In the last decade; however, an increasing number of studies demonstrated that enzymatic function of CD is not restricted solely to its proteolytic action, but also involves regulation of apoptosis along with a significant role in mitogenesis; numerous studies found that pCD/CD level represents an independent prognostic factor in a variety of cancers and is therefore, considered as to be a potential target of anti-cancer therapy.[11-13]

The purpose of this study was to find out any association between the CD with lymph node metastasis and to study the correlation of CD with various clinicopathological parameters to aid in assessment of its role as a prognostic indicator.

MATERIALS AND METHODS

Sample selection

Twenty primary OSCC specimens were retrieved from Department of Oral and Maxillofacial Pathology of a tertiary care teaching dental hospital of north India. The specimens included previously untreated, surgically resected tumors for which complete clinico-pathological data was available. After reviewing each case, selected paraffin blocks were obtained for tissue sectioning. Clinicopathological information on each case including age, sex, and tumor size, nodal status, tumor grading, and staging was collected. Out of 20 patients, 13 (65%) patients were males and 7 (35%) patients were females. Other patient and tumor characteristics are mentioned in Table 1. Grossly, identified cervical lymph nodes were examined microscopically to classify patients as "node-negative" or "node-positive."

Immunohistochemical (IHC) procedures

Paraffin-embedded sections (5 μm thickness) were deparaffinised and rehydrated, and endogenous paraffin activity was blocked with 3% H_2O_2 in methanol. For CD antibody immunostaining (Biogenex), antigen retrieval was achieved by boiling tissue specimens for 5 min at 95°C and 5 min at 98°C in antigen retrieval machine (EZ v.2.1 antigen retriever system) in citrate buffer (pH 6). Staining was performed using standard avidin-biotin method with appropriate secondary antibody. Antibody-reactive sites were visualized with the chromogen substrate diaminobenzidene tetrachloride. Breast cancer specimen was taken as a positive control. Normal mouse or rabbit serum was substituted for monoclonal or polyclonal antibodies. The sections were counterstained lightly with hematoxylin. One section from each sample was stained with hematoxylin and eosin for concurrent histopathologic evaluation.

IHC evaluation of tumor samples

A reproducible semi-quantitative assessment of IHC staining was used to evaluate the expression level of CD in tumor samples. CD expression was measured by examining 100 tumor cells in four randomly selected high power fields. The number of CD positive tumor cells was calculated along with the intensity of staining ranging from 0 to 4. Score (0-4) for CD staining in each tissue section was then calculated by multiplying and proportion of positive cells at each staining intensity by the numerical value of that intensity. The CD expression was graded from 0 to 4 and expression levels of each cathepsin in tumor samples were graded based on the total score as follows: Negative expression = score 0-0.5; low expression = score 0.6-1.0, high expression = score > 1.0.

Malignancy grading of OSCC

H and E sections were graded as well differentiated, moderately differentiated and poorly differentiated

Table 1: Patient and tumor characteristics

Sex	No. of patients
Female	7
Male	13
Age	
Mean	46.5 years
Range	25-68 years
T stage	
T1	04
T2	15
T3	01
N stage	
N1	10
N2	10
M0	20

OSCC on the basis of Anneroth classification, which included degree of keratinization, nuclear polymorphism, number of mitosis, pattern of invasion, stage of invasion and lymphoplasmocytic infiltration for histologic grading of malignancy of tumor-host relationship. In each case, the lympho-plasmocytic infiltration was graded as marked, moderate, slight or none.

Grading of OSCC

First group without lymph node metastasis included 4 (20%) cases of well-differentiated OSCCs, 3 (15%) cases of moderately differentiated and 3 (15%) cases of poorly differentiated OSCCs, and second group with lymph node metastasis included 10 (50%) cases of well-differentiated OSCCs.

Statistical analysis

The resulting data were analyzed using the SPSS (version 13.0). The distribution of dependent variables was tested using the univariate procedure. Both non-parametric and parametric statistical procedures were used in analyzing data, depending upon variable of interest (i.e., Pearson Chi-square analyses, Spearsman correlation coefficient, Mann-Whitney test, Kruskal Wallis test and student *t* test).

RESULTS

CD expression

CD staining was found to be positive in all the cases, although there was a diverse expression with intensity and number of positive cells varying from very low to very high levels. Chief pattern of the staining was paranuclear punctuate type among the differentiated cells at the center of the tumors with the undifferentiated cells at the infiltrating margins, showing diffuse cytoplasmic and cell surface staining. 6 cases (30%) of OSCC showed a low level of CD expression [Figures 1 and 2], whereas 14 (70%) cases exhibited high levels of CD expression [Figures 3 and 4].

Comparison of clinico-pathlogical and immuno-histochemical parameters among lymph node positive and negative patients

Sex of the patient and lymph node metastasis were not significantly associated. The cases with lymph node metastasis showed a significantly increased intensity of CD expression ($P < 0.05$). OSCC with lymph node metastasis showed only slight lymphoplasmocytic infiltration in a highly significant manner ($P < 0.001$). A significantly increased CD expression was observed in patients with lymph node metastasis (100% cases)

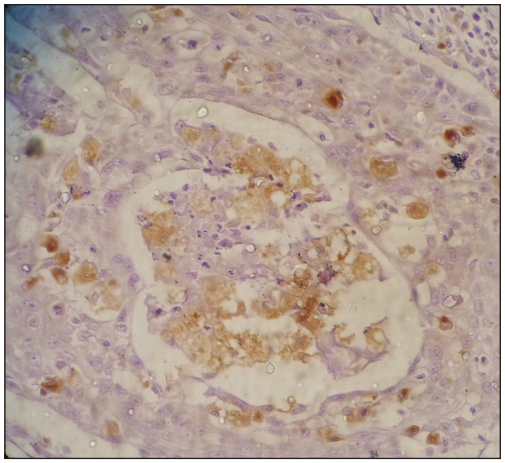

Figure 1: Photomicrograph showing low cathepsin D expression in an tumour island of OSCC without metastasis (cathepsin D immunostaining, original magnification ×40)

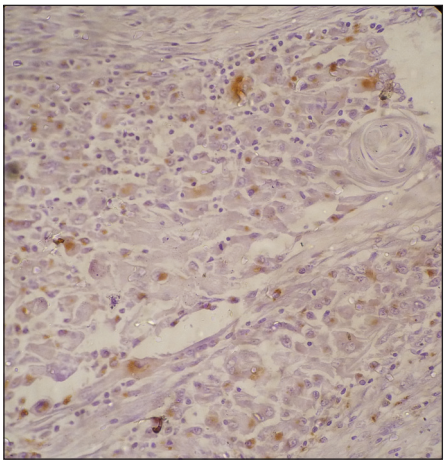

Figure 2: Photomicrograph showing extremely focal cathepsin D expression in another case of OSCC without metastasis (cathepsin D immunostaining, original magnification ×40)

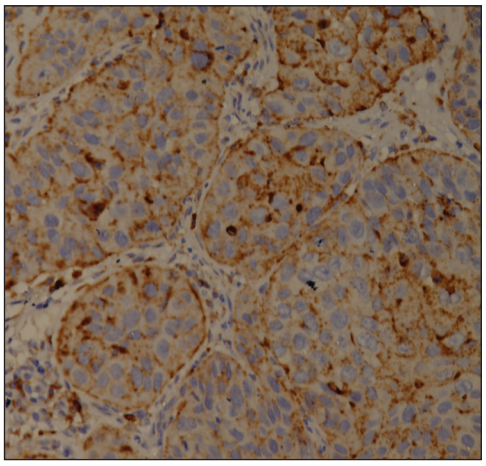

Figure 3: Photomicrograph showing a high level of cathepsin D expression in OSCC with metastasis (cathepsin D immunostaining, original magnification ×40)

compared to patients without lymph node metastasis ($P < 0.01$) [Table 2]. Although, the correlation of tumor duration and CD expression with the sex of the patient was not significant, number of CD positive cells was significantly increased in male patients without lymph node metastasis ($P < 0.05$) [Table 3].

Correlation of CD expression with other clinico-pathological variables

No significant association was observed between sex of the patient and other variables such as tumor duration, CD expression, and the number of CD positive cells in any case [Table 3]. The intensity of CD staining was not significantly related to duration of the tumor and number of CD positive cells in all the cases [Table 4]. No significant relation could be observed between size of the tumors and other parameters including CD expression and number of CD positive cells in cases without lymph node metastasis. However, increasing CD expression was significantly associated with tumor size in cases with lymph node metastasis [Table 5]. Furthermore, tumor size did not correlate to the CD intensity, and the number of CD positive cells in SCC [Tables 5 and 6]. The intensity of CD staining did not correlate with the increasing tumor grading in any case [Table 7]. The number of CD positive cells and CD expression did not significantly rise with increasing tumor staging [Table 8]. Although, the correlation between CD expression and intensity of expression to tumor grading was

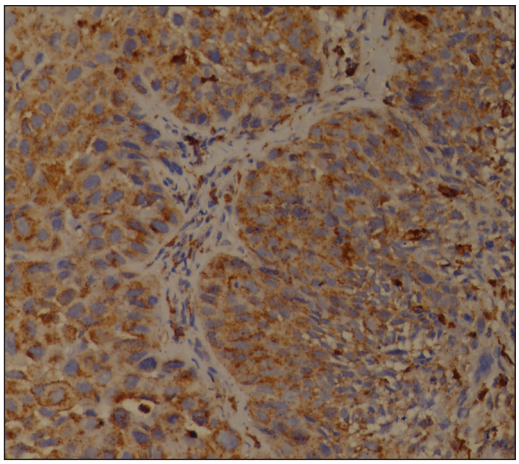

Figure 4 : Photomicrograph showing a high level and intensity of cathepsin D expression in another case of OSCC with metastasis (cathepsin D immunostaining, original magnification ×40)

Table 2: Qualitative analysis of clinical parameters in SCC

Parameters	SCC without lymph node metastasis ($n = 10$) (%)	SCC with lymph node metastasis ($n = 10$) (%)	*P* value (<0.05)
Sex			
Male	7 (70)	6 (60)	1.00 (non-significant)
Female	3 (30)	4 (40)	
Intensity			
1	0 (0)	0 (0)	0.02 (significant)
2	5 (50)	1 (10)	
3	5 (50)	4 (40)	
4	0 (0)	5 (50)	
Lymphoplasmocytic Infiltration			
Marked	7 (70)	0 (0)	0.003 (highly significant)
Slight	3 (30)	10 (100)	
Tumor size			
T1	2 (20)	2 (20)	1.0 (non-significant)
T2	8 (80)	8 (80)	
Tumor stage			
Stage I	2 (20)	0 (0)	0.01 (significant)
Stage II	8 (80)	3 (30)	
Stage III	0 (0)	3 (30)	
Stage IV	0 (0)	4 (40)	
Cathepsin D expression			
High	4 (40)	10 (100)	0.01 (significant)
Low	6 (60)	0 (0)	

Table 3: Correlation of sex with duration cathepsin D expression and number of cathepsin D + cells

SCC (squamous cell carcinoma)	Duration (mean rank)	Cathepsin D expression (mean rank)	Number of cathepsin D + cells (mean rank)
SCC without lymph node metastasis			
Sex			
Male	5.86	5.43	6.79
Female	4.67	5.67	2.50
P value	0.554 (non-significant)	0.91 (non-significant)	0.04 (significant)
SCC with lymph node metastasis			
Sex			
Male	5.67	6.6	6.67
Female	5.25	3.7	3.75
P value	0.82 (non-significant)	0.13 (non-significant)	0.13 (non-significant)

Table 4: Correlation of staining intensity with duration, number of cathepsin D + cells and age

SCC (squamous cell carcinoma)	Duration (mean rank)	Number of cathepsin D + cells (mean rank)	Age (mean rank)
SCC without lymph node metastasis			
Staining intensity			
2	5.10	6.20	6.6
3	5.90	4.80	4.4
P value	0.66 (non-significant)	0.46 (non-significant)	0.24 (non-significant)
SCC with lymph node metastasis			
Staining intensity			
3	5.63	4.5	5.0
4	4.5	5.4	5.0
P value	0.52 (non-significant)	0.62 (non-significant)	1.00 (non-significant)

Table 5: Correlation of tumor size with cathepsin D expression and number of cathepsin D + cells

SCC (squamous cell carcinoma)	Cathepsin D expression (mean rank)	Number of cathepsin D + cells (mean rank)
SCC without lymph node metastasis		
Tumor size		
T1	14	10.50
T2	41	44.50
P value	0.53 (non-significant)	0.88 (non-significant)
SCC with lymph node metastasis		
Tumor size		
T1	9	8.5
T2	4.6	4.75
P value	0.08 (significant)	0.17 (non-significant)

Table 6: Correlation of tumor size with intensity in SCC

SCC (squamouscell carcinoma)	Tumor size	Intensity			*P* value
		2	3	4	
SCC without lymph node metastasis	T1	1 (10)	1 (10)		1 (non-significant)
	T2	4 (40)	4 (40)		
SCC with lymph node metastasis	T1	0 (0)	1 (10)	1 (10)	0.86 (non-significant)
	T2	1 (10)	3 (30)	4 (40)	

found to be insignificant, number of CD positive cells correlated significantly with increasing tumor grade in squamous cell carcinoma (SCC) without lymph node metastasis ($P < 0.05$) [Table 9]. Lymphoplasmocytic infiltration did not correlate with CD positive cells, CD expression, and staining intensity in cases without lymph node metastasis [Table 10]. No correlation was found between lymphoplasmocytic infiltration and CD staining intensity [Table 11].

DISCUSSION

The goal of this study was to determine whether high levels of CD expression in surgical specimens of oral carcinomas are associated with increased propensity for local invasive growth and lymph node metastasis. Oral carcinomas with high levels of CD expression showed intense cytoplasmic and cell surface staining of tumor cells, most often concentrated at the invasive front of tumors.

In our study, high levels of CD expression was observed in oral carcinomas with regional lymph node metastasis (N1/N2) compared with node-negative tumors (No) ($P < 0.05$). Moreover, we observed that CD expression also correlated with tumor size in OSCC with lymph node metastasis [Table 5]. Though, CD expression did not correlate with the stage of the tumor, but the CD positive cells correlated with the tumor grade [Table 9].

Spyratos *et al.*[14] stressed on the role of CD as a prognostic marker for the disease-free interval in patients with breast cancer and emphasized that CD was a more effective prognostic indicator than lymph node metastasis, in the case of lymph node negative patients. The relationship between CD expression and poor survival has been known to differ among lymph node positive and negative patients.[15,16]

In a study by Henry *et al.*, high levels of CD have been associated with good outcome in breast cancer,[17] whereas high levels of CD have been found

Table 7: Correlation of staining intensity with staging of SCC

SCC (squamous cell carcinoma)	Intensity	Stage I (%)	Stage II (%)	Stage III (%)	Stage IV (%)	*P* value
SCC without lymph node metastasis	2	1 (10)	4 (40)			1 (non-significant)
	3	1 (10)	4 (40)			
SCC with lymph node metastasis	2		0 (0)	0 (0)	1 (10)	0.11 (non-significant)
	3		0 (0)	1 (10)	3 (30)	
	4		3 (30)	2 (20)	6 (60)	

Table 8: Correlation of staging with the number of cathepsin D + cells and cathepsin D expression

Staging	Number of cathepsin D + cells (mean rank)	Cathepsin D expression (mean rank)
SCC without lymph node metastasis		
Stage I	5.25	7.00
Stage II	5.56	5.13
P value	0.84 (non-significant)	SCC with lymph node metastasis
SCC with lymph node metastasis		
Stage II	6.67	5.67
Stage III	6.00	6.3
Stage IV	4.25	4.7
P value	0.55 (non-significant)	0.78 (non-significant)

Table 9: Correlation of histopathological diagnosis with the number of cathepsin D + cells and cathepsin D expression

SCC (squamous cell carcinoma)	Well- differentiated SCC	Moderately differentiated SCC	Poorly differentiated SCC	*P* value
SCC without lymph node metastasis				
Number of cathepsin D+cells (mean rank)	3.30	6.25	8.67	0.04 (significant)
Cathepsin D expression (mean rank)	4.7	7.00	5.83	0.63 (non-significant)
SCC without lymph node metastasis				
2 (%)	2 (20)	1 (10)	2 (20)	0.77 (non-significant)
3 (%)	3 (30)	1 (10)	1 (10)	

Table 10: Correlation of lymphoplasmocytic infiltration with number of cathepsin D + cells and cathepsin D expression

Lymphoplasmocytic infiltration	Number of cathepsin D + cells (mean rank)	Cathepsin D expression (mean rank)
Marked	5.57	6.6
Slight	5.33	2.83
P value	0.91 (non-significant)	0.064 (non-significant)

Table 11: Correlation of lymphoplasmocytic intensity with staining intensity

Lymphoplasmocytic infiltration	Cathepsin D staining intensity		*P* value
	2	3	
Marked (%)	2 (20)	5 (50)	0.17 (non-significant)
Slight (%)	3 (30)	0 (0)	

No correlations possible in SCC with lymph node metastasis as all cases were of slight lymphoplasmocytic intensity; SCC: squamous cell carcinoma

both to correlate with aggressive disease and to show no relationship with patient outcome in other studies in node negative patients.[18,19]

Some of these contradictory findings could be attributed to different methodology used for pCD/CD quantification, subjectivity, different types of tissue, that is fresh versus formalin fixed and paraffin embedded, patients and diagnosis selection, and the length of follow-up period.[8,20,21]

A study by Vigneswaran *et al*.[22] in oral cancer patients correlated increased expression of CD with the presence of metastasis, poor histologic malignancy grade and high proliferation rate. High levels of CD expresssion have been observed in oral carcinomas with the regional lymph node metastasis compared to node negative tumors.[7,8] Increased serum levels of CD has been noted in patients with widespread metastatic carcinoma.[23,24]

Kawasaki *et al*.[25] also found that higher CD immunostaining was related to invasion, cell proliferation and to expression of growth factor receptors in OSCC. They also observed that CD had a significant correlation with stage of invasion and lymphoplasmocytic infiltration.

Maurizi *et al*.[26] found a higher risk of metastatic disease and poor outcome in laryngeal cancer patients with higher expression of CD. A study by Ikeguchi *et al*.[27] in 154 esophageal SCC patients found that high CD expression was associated with a poor prognosis.

Earlier clinical studies have found pCD/CD related to metastasis-free survival and disease-free survival in breast cancer patient.[14,28] Since then, numerous

clinical studies have been conducted to find an association between pCD/CD level and tumor size, tumor grade, tumor aggressiveness, incidence of metastasis, prognosis, and a degree of chemoresistance in a variety of solid tumors including head and neck tumors,[20,29,30] but some of them have given acutely contradictory results. Moreover, studies dealing with pCD/CD diagnostic and prognostic value in cancer are complicated by the fact that there are several forms of CD in a cell at the same time pCD, intermediate enzymatically active CD and mature heavy and light chain CD. Presently some of the researches are also looking for the expression of CD in dysplastic cases, and that could serve as an early attempt for cancer prevention.[31]

CONCLUSION

Patients with lymph node metastasis showed an increase in CD expression, which was statistically significant. Increasing tumor size seemed to correlate with increased CD expression. There was a statistically significant association between increasing number of CD positive cells and increasing grades of OSCC in cases without lymph node metastasis. Furthermore, there was the presence of only slight lymphoplasmocytic infiltration in cases with lymph node metastasis. Thus, based on the active potential of CD in regulating the prognosis of OSCC the design and synthesis of specific CD inhibitors can have significant research and therapeutical consequences. However, standardization of measurement techniques for CD and its other forms is recommended for further clinical usefulness in the field of oncology.

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