

A Immunohistochemical Study on Cathepsin D Expression in Normal Oral Mucosa, Oral Squamous Cell Carcinoma and Verrucous Carcinoma.

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Abstract

Context:

A strong cause/effect relationship is evident between the expression levels of Cathepsin D (CD) in oral cancers and their local invasion and progression.

Aims:

The present study aims to assess the immunohistochemical expression of CD in normal oral mucosa, Verrucous Carcinoma (VC) and Oral Squamous Cell Carcinoma (OSCC).

Settings and Design : A retrospective immunohistochemical study conducted in a tertiary care dental hospital with a sample size of 30.

Materials and Methods: Immunohistochemical staining for CD was performed in 10 tissue sections each of normal mucosa, OSCC and VC. CD positivity, site of expression and intensity were analyzed semi-quantitatively under 10x and 40x magnification.

Statistical Analysis Used: Cramer's V test was done and p value is calculated.

Results:

CD expression was positive in 90% of normal oral mucosa. CD expression was mild in 33.3%, moderate in 33.3% and severe in 33.3% of well differentiated SCC, respectively. CD expression was mild in 25%, moderate in 50% and severe in 25% cases of moderately differentiated SCC respectively. All cases of poorly differentiated SCC showed severe expression of CD. CD expression was positive in all cases of VC. Increased expression of CD correlated significantly with the presence of metastasis ($p < 0.03$) and poor histologic malignancy grade ($p < 0.05$).

Conclusion:

CD was demonstrated in normal oral mucosa. There was a positive correlation between increasing grades of OSCC and increasing intensity of CD. It closely correlated with carcinoma invasion and progression. CD maybe a prognostic factor for SCC and may serve as a potential target for cancer therapy. More studies are needed to correlate CD expression in VC with its biologic behaviour.

Keywords:

Cathepsin D, Oral Squamous cell carcinoma, Verrucous carcinoma, Immunohistochemistry

Key Messages:

There was a positive correlation between increasing grades of OSCC and increasing intensity of CD. It closely correlated with carcinoma invasion and progression. CD maybe a prognostic factor for SCC and may serve as a potential target for cancer therapy.

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Introduction

Oral cancer, the sixth most common cancer worldwide has a significantly high mortality and morbidity rate. Squamous cell carcinoma (SCC), the most common malignant neoplasm of the oral cavity represents about 90% of all oral malignancies. By definition **squamous cell carcinoma is** "A malignant epithelial neoplasm exhibiting squamous differentiation as characterised by

the formation of keratin and the presence of intercellular bridge" [1]. The 5 year survival rate of patients with early stage oral squamous cell carcinoma ranges from 80 %-90 %, whereas the 5 year survival rate for advanced stage squamous cell carcinoma patients is about 40 % [2].

The major reason for an alarmingly high mortality rate in oral cancer is that about 60 % of patients present with an advanced stage of the disease at the time of diagnosis. Tumor size, histologic type and lymph node involvement are the factors which greatly influence the prognosis of oral cancer [3]. Identifying valuable prognostic markers of oral squamous cell carcinoma, are the need of the hour and they may aid in curbing the high mortality rate associated with oral cancers [4].

Oral Verrucous carcinoma, also referred to as "Ackermann's tumor" was first described by Lauren V. Ackermann in 1948. It is a low grade, well differentiated verrucous variant of oral squamous cell carcinoma and is characterized by slow exophytic growth with a local invasive pattern and a minimal to rare regional and distant metastatic potential. It clinically presents as a cauliflower – like, pebbly, mamillated warty lesions. It has a 5 year survival rate of about 50% [3,5]. Seeking reliable prognostic markers of oral verrucous carcinoma, can predict the course of the disease and the response to therapeutic intervention among affected patients [6].

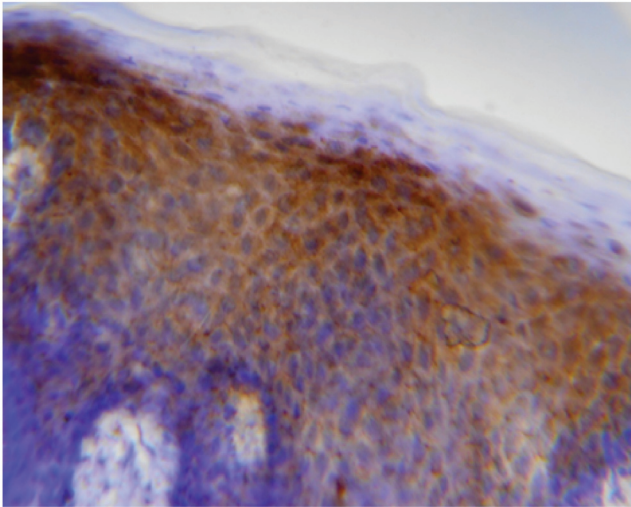
Cathepsins are endopeptidases that catalyse the degradation of proteins. The many types of Cathepsins include Cathepsin A,B,C,D,E,F,G,H,L,K,O,S,V and W. Cathepsin D is an aspartic endopeptidase. Cathepsin D is ubiquitously expressed in all mammalian normal cells and tissues (except erythrocytes). In normal cells, Cathepsin D plays an active role in the degradation of proteins and proteolytic activation of secretory proteins, growth hormones and apoptosis [7]. In cancer cells, Cathepsin D stimulates tumor cell proliferation and tumor angiogenesis. The proenzyme of Cathepsin D is hypersecreted in the tumor microenvironment and it degrades the extracellular matrix and aids in tumor invasion and metastasis [8].

Cathepsin D is overexpressed in many types of cancer including breast cancer, head and neck cancer and ovarian cancer [8]. Cathepsin D has been found to be an independent predictor of recurrence and mortality in breast cancer [4].

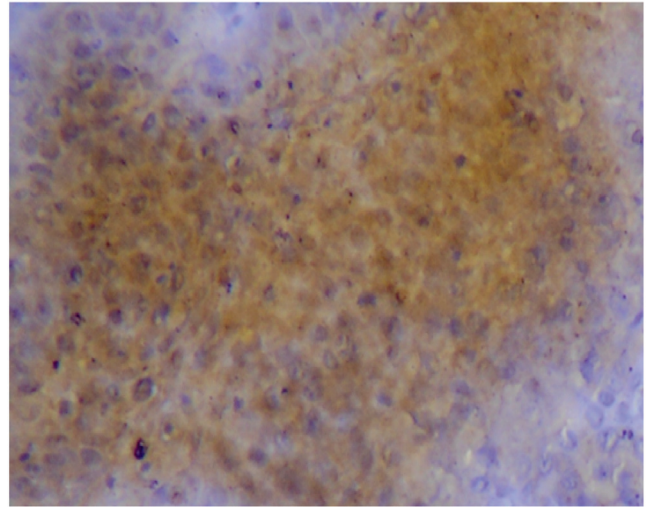
Material and Methods

The study was conducted at a tertiary care dental college and hospital in Salem in 2013. All the patients who attended the oral medicine department of *the dental college and hospital* from the period of April 2011 to

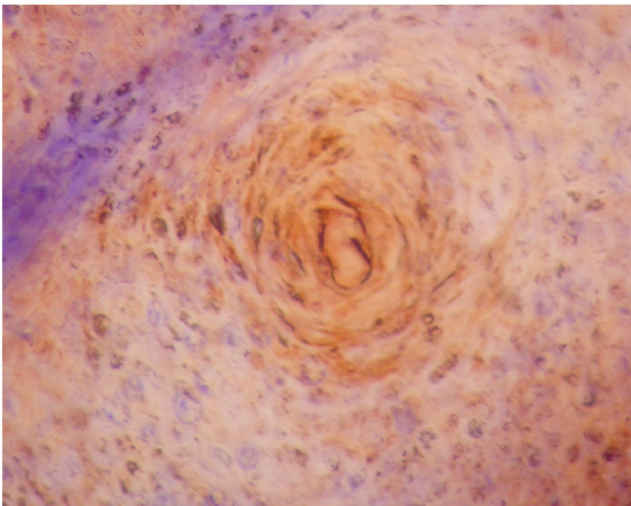
July 2013 were screened retrospectively, for the purpose of the study. Total no. of samples selected for the study include 30. **(10 normal mucosa, 10 Squamous cell carcinoma and 10 Verrucous carcinoma)** The following data was collected for each of the case: Age, gender, clinical site, TNM clinical staging (for carcinoma). Besides these, paraffin embedded tissue blocks were also retrieved from archives of the Oral & Maxillofacial Pathology department, for the above selected cases. Inclusion criteria for squamous cell carcinoma and verrucous carcinoma include excisional biopsy specimens of primary tumors in any region of the oral cavity including tongue, lips, buccal mucosa, gingiva, faucial pillars, palate. Only patients above 40 years of age were selected for the study. Exclusion criteria for squamous cell carcinoma and verrucous carcinoma include recurrent carcinomas, patients undergoing chemotherapy or radiotherapy and patients below 40 years of age. Inclusion criteria for normal oral mucosa include normal epithelium from the peripheral areas of excised non malignant specimens from the oral cavity. Both keratinized and non keratinized epithelium were included in the study. Exclusion criteria for normal oral mucosa include patients with associated systemic diseases, ulcerative or lesional mucosa and epithelium showing inflammation. Out of the 10 squamous cell carcinomas 3 were well differentiated, 4 were moderately differentiated and 3 were poorly differentiated oral squamous cell carcinoma as previously diagnosed by the Oral & Maxillofacial Pathology department. Samples were routinely fixed in 10% buffered formalin and embedded in paraffin. 3 m thick sections were made, deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by incubating in 3% H₂O₂ for 10 minutes. The tissue sections were incubated in 0.4 % casein in phosphate buffered saline for 10 minutes to block the nonspecific binding sites. Tissue sections were incubated for 1 hour with primary antibody (Mouse monoclonal antibody to Cathepsin D). The slides were incubated in post primary block and subsequently with HRP conjugated secondary antibody for 30 minutes each. Freshly prepared DAB – substrate working solution was added and incubated for 5 minutes. After thorough washing in distilled water, the slides were counter stained with Mayer's hematoxylin, dehydrated in grades of alcohol and mounted with synthetic mounting media. One section from each sample was stained with hematoxylin and eosin for corresponding histopathologic evaluation. The immunohistochemical slides were observed for positivity under 10X and 40X magnifications and recorded with a high power photomicrograph (14MP). Immunohistochemical evaluation was done by two blinded, independent observers. Presence of brown



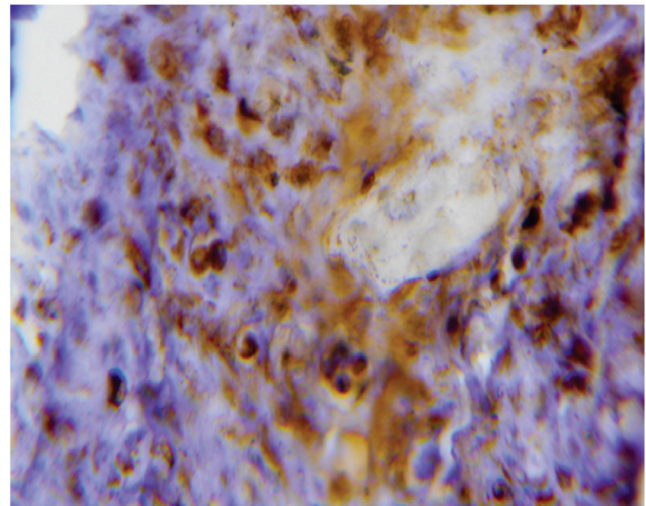
Photomicrograph 1 : 10x low power view – showing moderate cytoplasmic staining of Cathepsin D in Normal oral mucosa



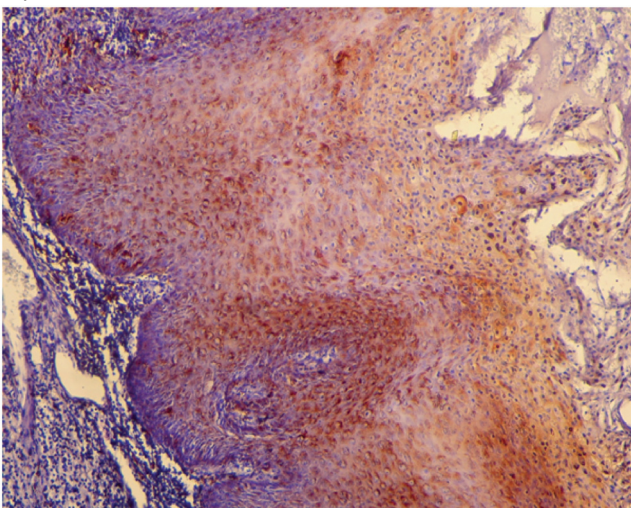
Photomicrograph 2 : 40x high power view- showing mild, cytoplasmic staining of Cathepsin D in Well differentiated Squamous cell carcinoma



Photomicrograph 3 : 40x high power view- showing moderate, cytoplasmic staining of Cathepsin D in Moderately differentiated Squamous cell carcinoma



Photomicrograph 4 : 40x high power view- showing severe, cytoplasmic staining of Cathepsin D in Poorly differentiated Squamous cell carcinoma



Photomicrograph 5 : 10x low power view- showing moderate, cytoplasmic staining of Cathepsin D in Verrucous carcinoma

coloured end product at the site of target antigen was considered as positive immunoreactivity. The expression of Cathepsin D was determined to be either positive or negative. The intensity of staining of cytoplasm in Cathepsin D positive cells was determined. The intensity was graded in all the 30 samples as follows, 0 – negative staining, 1- mild staining, 2- moderate staining, 3 - severe staining. Based on the pattern of distribution, the staining was categorized as focal and diffuse.

For squamous cell carcinomas, malignancy grading was done. Each case was graded by 1 observer in a blinded manner. Both Broder's grading system and Anneroth's etal grading system was used. The tumor cell morphology and the tumor stromal interaction were evaluated, as determined by the degree of keratinization, cellular anaplasia, mitotic rate, pattern of invasion, stage of invasion and lymphoplasmacytic infiltrate. Each of these

parameters are scored from 1 to 4. The final score for each sample is the total sum of points for each characteristic.

Dependent variable of interest was expression level of Cathepsin D. Independent variables were tumor size, nodal status, histological types of oral cancers and malignancy grading (for oral squamous cell carcinoma). The distribution of dependent variables was tested using the univariate procedure. Cramer's V test was used in analysing data. Univariate analysis was performed to assess the relationship between dependent variable and independent variables. Chi square analysis was done and p value was calculated accordingly.

Results

Immunohistochemical expression of Cathepsin D

Cathepsin D expression was positive in 9/10 (90%) cases of normal oral mucosa and 1/10 (10%) case showed a negative immunoreactivity (Table 1). Diffuse cytoplasmic staining was seen in 3/9 (33.3%) cases of normal oral mucosa and 6/9 (66.7%) cases exhibited focal cytoplasmic staining.

Among oral squamous cell carcinomas, 10/10 (100%) cases showed a positive immunoreactivity for Cathepsin D. Mild expression of Cathepsin D is seen in 1/3 (33.3%) cases, moderate expression is seen in 1/3 (33.3%) cases and severe expression is seen in 1/3 (33.3%) cases of well differentiated squamous cell carcinoma, respectively. Mild expression of Cathepsin D

is seen in 1/4 (25%) case, moderate expression is seen in 2/4 (50%) cases and severe expression is seen in 1/4 (25%) cases of moderately differentiated squamous cell carcinoma respectively. All 3/3 (100%) cases of poorly differentiated squamous cell carcinoma showed severe expression of Cathepsin D. (Table 2) Of all cases of oral squamous cell carcinoma, 2/10 (20%) cases showed mild expression, 3/10 (30%) cases showed moderate expression and 5/10 (50%) showed severe expression of Cathepsin D, respectively. All 10/10 (100%) cases of oral squamous cell carcinoma showed diffuse, cytoplasmic and membranous staining. (Table 3)

All 10/10 (100%) cases of verrucous carcinoma showed a positive immunoreactivity for Cathepsin D, of which 4/10 (40%) cases showed mild expression and 6/10 (60%) cases showed moderate expression respectively. All 10/10 (100%) cases of Verrucous carcinoma showed cytoplasmic staining. 4/10 (40%) cases of Verrucous carcinoma and 6/10 (100%) cases of Verrucous carcinoma showed diffuse staining and focal staining respectively (Table 4 and Table 5). A statistical analysis was carried out using Cramer's V test and the statistical analysis was evaluated.

Correlation of Cathepsin D expression with clinical and pathological features

Increased Cathepsin D expression (Score >2) correlated with increased stage of oral cancers (pT_3/pT_4) when compared to pT_1 and pT_2 tumors. p value was non-significant ($p > 0.05$) ($p=0.2192$). Increased Cathepsin D expression (score >2) was observed in oral cancers with

Table 1: Immunohistochemical Expression Status of Cathepsin D in Oral Squamous Cell Carcinoma, Verrucous Carcinoma and Normal Oral Mucosa

Expression status	Oral Squamous cell Carcinoma		Verrucous Carcinoma		Normal mucosa	
	No.	%	No.	%	No.	%
Present	10	100.0	10	100.0	9	90.0
Absent	-	-	-	-	1	10.0

Table 2: Association between the Oral Squamous Cell Carcinoma grades and intensity of Immunohistochemical expression of Cathepsin D

SCC grade	Mild		Moderate		Severe	
	No.	%	No.	%	No.	%
Well differentiated	1	33.3	1	33.3	1	33.3
Moderately differentiated	1	25.0	2	50.0	1	25.0
Poorly differentiated	-	-	-	-	3	100

Cramer's V test value=0.476, $p=0.063$ (Significant)

Table 3: Intensity of Immunohistochemical expression of Cathepsin D In Oral Squamous Cell Carcinoma, Verrucous Carcinoma and Normal Oral Mucosa.

Site	Oral Squamous cell Carcinoma		Verrucous Carcinoma		Normal mucosa	
	No.	%	No.	%	No.	%
Cytoplasmic	0	-	10	100.0	9	100
Cytoplasmic & membranous	10	100	-	-	-	-

Cramer's V test value: 0.606, p value = 0.00042, p value is significant

Table 4: Site of Appearance of Immunohistochemical Expression of Cathepsin D in Oral Squamous Cell Carcinoma, Verrucous Carcinoma and Normal Oral Mucosa.

Site	Oral Squamous cell Carcinoma		Verrucous Carcinoma		Normal mucosa	
	No.	%	No.	%	No.	%
Cytoplasmic	0	-	10	100.0	9	100
Cytoplasmic & membranous	10	100	-	-	-	-

Table 5: Pattern of Immunohistochemical expression of Cathepsin D in Oral Squamous Cell Carcinoma, Verrucous Carcinoma and Normal Oral Mucosa.

Pattern of expression	Oral Squamous cell Carcinoma		Verrucous Carcinoma		Normal mucosa	
	No.	%	No	%	No	%
Diffuse	10	100	4	40.0	3	33.3
Focal	0		6	60.0	6	66.7

Cramer's V test value : 0.612, p=0.004, p value is significant

Table 6: Association between Cathepsin D levels and Clinicopathologic parameters

Clinicopathologic Parameters	Cathepsin D score			P value
	1	2	3	
1. Tumor size				0.2192
PT1-pT2 (n=12)	6	3	3	
PT3-pT4 (n=8)	1	3	4	
2. Node involvement				0.02812
PN0 (n=4)	3	1	0	
PN1/pN2 (n=16)	2	6	8	
3. Tumor Subtype				0.03567
SCC (n=10)	2	3	5	
VC(n=10)	4	6	0	
4. Tumor cell grade For SCC (n=10) (Range = 4-10)				0.04043
Grades 4 -5 (n=1)	1	0	0	
Grades 6-7 (n=1)	1	0	0	
Grades 8-10 (n=8)	0	4	4	
5. Tumor stromal interface grade for SCC (n=10) (Range = 5-9)				0.03685
Grades 5-6 (n=2)	2	0	0	
Grades 7-8 (n=3)	0	1	2	
Grade 9 (n=5)	0	1	4	

lymph node metastasis. (pN_1/pN_2). P value was significant ($p < 0.05$) ($p = 0.02812$). For each case of squamous cell carcinoma, histological malignancy grades for tumor cell morphology and tumor stromal interface was assessed independently. Malignancy grades for tumor cell morphology ranged from 4-10. Malignancy grades for tumor stromal interface ranged from 5-9. A significant positive correlation was observed ($p < 0.05$) ($p = 0.04043$) between increased expression of Cathepsin D and malignancy grade for tumor cell morphology. A significant positive correlation was also observed ($p < 0.05$) ($p = 0.03685$) between increased Cathepsin D expression and malignancy grade for tumor stromal interface (Table 6).

Discussion

The aim of this study was to assess the immunohistochemical expression of Cathepsin D in normal oral mucosa, oral squamous cell carcinoma and verrucous carcinoma and to study the probable role of Cathepsin D in the biological behaviour of normal oral mucosa, oral squamous cell carcinoma and verrucous carcinoma.

Cathepsin D in Normal Oral Mucosa

In the present study, Cathepsin D is present in 9/10 (90%) cases of normal oral mucosa. All the 9 cases of normal oral mucosa showed mild expression of Cathepsin D. 3/10 (33.3%) cases of normal oral mucosa showed diffuse staining and 6/10 (66.7%) of normal oral mucosa showed focal staining. Cathepsin D is an ubiquitous enzyme present in normal mammalian tissues [7] like keratinocytes, epithelium, spleen, liver, lungs, brain, lymph nodes, stomach and kidney [4]. Cathepsin D is an aspartic protease that requires an acidic pH to be active. The human Cathepsin D gene is located on chromosome 11p15 [8,9]. In mammalian cells, Cathepsin D exists in 3 forms namely 1) Procathepsin D; 2) Intermediate form; and 3) Mature form [9,10]. Functions of Cathepsin D include intracellular degradation of proteins, proteolytic activation of enzymatic precursors, polypeptide hormones, growth factors and apoptosis [11].

Cytoplasmic and cell surface expression of Cathepsin D may be due to the change in cellular location from lysosomes to plasma membrane [7,12].

In the present study, Cathepsin D expression was positive in 9/10 (90%) of cases of normal oral mucosa. All the positive cases showed cytoplasmic staining. This explains the fact that Cathepsin D is ubiquitously present in normal keratinocytes in the oral epithelium. The cytoplasmic staining may be due to the subcellular

localization of Cathepsin D from the lysosomes to the plasma membrane.

Cathepsin D in Oral Cancer

In the current study, severe expression of Cathepsin D is seen in poorly differentiated squamous cell carcinoma. This is similar to a study by Loncaric et al, where poorly differentiated squamous cell carcinoma showed severe expression of Cathepsin D [13].

Vigneswaran N et.al in his study, concluded that Cathepsin D is one of the key enzymes involved in local invasion and metastasis of oral cancer. Cathepsin D is described as a potential prognostic marker and a target for therapeutic intervention. Tumor cells positive for Cathepsin D showed diffuse cell surface and cytoplasmic immunostaining [3]. This is similar to the current study where 10/10 (100%) cases of oral squamous cell carcinoma showed diffuse cytoplasmic and membranous staining.

Cathepsin D expression was significantly increased in patients with lymph node metastasis [14]. Cathepsin D is described to be a potential prediction of cervical lymph node metastasis in head and neck squamous cell carcinoma [15]. In the present study, high levels of Cathepsin D expression ($p < 0.05$) was observed in lymph node positive oral carcinomas ($pN1/pN2$). This is similar to the study by Vigneswaran et al [3]. Similar results are seen in a study by Osama M. Ghazi et al, where Cathepsin D expression correlated significantly with tumor site, tumor stage, lymphovascular invasion, perineural invasion and depth of invasion [7].

In the current study, significant statistical correlation of Cathepsin D expression with four important clinicopathological parameters are seen, namely, node involvement, tumor subtype, tumor cell grade and tumor stromal interface grade in oral squamous cell carcinomas. Of these tumor subtype and lymph node metastasis considerably influence the prognosis of oral cancer [14].

Proteases are described to be involved in multistep carcinogenesis and cancer progression by altering the tumor microenvironment. They cleave cell to cell adhesions and extracellular matrix and cause tumor growth and progression [7]. They also regulate tumor progression by processing of kinases, chemokines and growth factors [16]. In the present study, a semiquantitative grading technique was used. We assessed the expression levels of Cathepsin D in increasing grades of oral cancer, which closely related to their biological behaviour. Increased Cathepsin D expression ($p < 0.05$) significantly correlated with high malignancy grade for tumor cell morphology. Similarly,

high levels of Cathepsin D expression ($p < 0.05$) significantly correlated with tumor stromal interface grade for squamous cell carcinoma. This is similar to the study by Vigneswaran N et al, where a significant positive correlation existed between Cathepsin D expression and histology malignancy grade for tumor stromal interface [3]. Cathepsin D expression was more in carcinomas with a higher grade stromal invasive pattern characterized by muscular/bone invasion with minimal or no lymphoplasmacytic immune response. Cathepsin D levels are found to be elevated in thyroid carcinomas, squamous cell carcinoma, renal cell carcinoma, glioma, brain tumors, laryngeal carcinoma and ovarian carcinoma [9].

Cathepsin D orchestrates tumor invasion and the dissemination of tumor cells through its proteolytic function [17]. Cathepsin D secretion and overexpression is associated with metastasis in human cancers. Cathepsin D is seen in human cancer and it plays a critical biological role by inducing the breakdown of the tumor basement membrane, by its proteolytic action and thus, favours tumor invasion. In human cancer, Cathepsin D induces tumor invasion and progression by altering the tumor microenvironment, by actively controlling tumor cell differentiation, proliferation, cell migration, apoptosis, tumor angiogenesis, inflammation and tissue remodelling [18]. Cathepsin D is functionally similar to the rein of the RAS (Renin Angiotensin System), which plays an active role in cancer angiogenesis and cell proliferation, thereby promoting cancer growth and metastasis [19]. Cathepsin D promotes tumor invasion and plays a vital role in oral squamous cell carcinoma progression [20].

In the present study, Cathepsin D expression is present in 10/10 (100%) cases of verrucous carcinoma. 4/10 (40%) cases of verrucous carcinoma showed mild expression. 6/10 (60%) cases of verrucous carcinoma showed moderate expression of Cathepsin D. All the cases showed cytoplasmic staining. The findings in our study was similar to the study by Vigneswaran N et al [3] in which expression of Cathepsin D is either negative or low to moderate expression in a paranuclear granular distribution in verrucous carcinoma. In the present study, 4/10 (40%) cases of verrucous carcinoma showed diffuse staining and 6/10 (60 %) cases showed focal staining. The absence of severe expression of Cathepsin D in verrucous carcinoma, relates closely with their biologic behaviour.

Conclusion

In **conclusion**, the results of the study show that Cathepsin D in normal epithelium stained as a fine granular pattern in the basal layer than in other layers.

Cathepsin D is an ubiquitous enzyme and it has been demonstrated in normal oral mucosa. In squamous cell carcinoma cases, Cathepsin D expression showed cytoplasmic and membranous staining. Activated Cathepsin D may be involved in the invasion and metastasis of Squamous cell carcinoma. There is a positive correlation between increasing grades of OSCC and increasing intensity of Cathepsin D in the cases of OSCC. Substantial overexpression of Cathepsin D was found in poorly differentiated OSCC. Cathepsin D expression were closely correlated with carcinoma invasion and progression. It might be useful in determining the prognosis of patients with oral carcinoma. Cathepsin D maybe a prognostic factor for SCC and may serve as a potential target for cancer therapy. However, more studies are needed to study the correlation of Cathepsin D expression in verrucous carcinoma with its biologic behaviour.

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Ethics:	There is no ethical violation as it is based on voluntary anonymous interviews
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Guarantor:	Dr. S. Marytresa Jeyapriya will act as guarantor of this article on behalf of all co-authors.

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