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ORIGINAL RESEARCH

Immunohistochemical Expression of Cathepsin D in Primary and Recurrent Squamous Cell Carcinoma

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ABSTRACT

Aim: The aim of this study is to analyze and compare the immunohistochemical expression of cathepsin B in primary oral squamous cell carcinoma (OSCC) and recurrent OSCC.

Materials and methods: A total of 50 cases were studied immunohistochemically for rabbit polyclonal antihuman cathepsin D expression. A total of 10 cases of breast carcinoma were taken as positive controls. Immunohistochemical staining was performed using labeled streptavidin–biotin technique.

Results: All the 45 cases of OSCC, both primary and recurrent cases included, showed varying grades of cathepsin D immunoreactivity. Statistical significance at 5% level was observed in cathepsin D expression between the different grades of well, moderate, and poorly differentiated primary squamous cell carcinomas. In the comparison of cathepsin D staining intensity among primary squamous cell carcinomas with and without recurrence, a statistical significance between the groups was observed when the p-value was at 10%, but the same comparison was not significant when the p-value was at 5%.

Conclusion: Cathepsin D expression in primary squamous cell carcinomas with recurrences was very variable as compared with primary squamous cell carcinomas without recurrences. Comparison of cathepsin D expression in primary with their recurrent counterparts showed mostly similar intensity of expression in recurrent carcinomas, thus suggesting its limited usefulness in predicting recurrence.

Clinical significance: Although cathepsin D might have shown limited usefulness in predicting cancer recurrence, it, however, is a proven valuable tool to detect the aggressiveness of various other tumors, and if corroborated with a larger sample may hold the key to early, more effective, and more specific treatment modalities for cases of oral cancer also.

Keywords: Cathepsin D, Immunohistochemistry, Recurrent oral squamous cell carcinoma.

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INTRODUCTION

Cancer remains a major cause of worldwide deaths due to the ability of cancer cells to metastasize to form secondary tumors and also due to their propensity to recur. Oral cancer, being the sixth most common type, with a 5-year survival rate that has not improved in the past 20 to 25 years,[1] is no exception to this fact.

Oral squamous cell carcinoma, the most common form of oral malignancy,[2] accounts for almost 90 to 95% of all oral cancers.[3] It shows an increased risk of manifesting as a second or a third primary tumor of the upper aerodigestive tract very often.[4] Recurrence, which can occur either locally or at the regional lymph node or as distant recurrence, has certain prognostic factors, such as tumor dimension, margin status, grade of malignancy, perineural invasion, vascular invasion, number of positive lymph nodes, extracapsular extensions, node location, and node size governing it.[5] With this threat of recurrence always present, the clinical decision to use adjuvant therapy assumes importance. However, this being a difficult task due to the inability to detect recurrence confidently, a prognostic marker need is felt.

Of the several newer methods available to solve such problems, immunohistochemistry is a relatively simple, specific tool, while at the same time is quite sensitive. Of

the various markers used, cathepsins seem to be a group of promising prognostic markers.

Cathepsins, named in 1929 by Willstater and Baumann from the Greek word Kathepsins meaning "to digest," are a group of lysosomal enzymes involved in degradation of proteins. Normally being found in all tissues, they are traditionally referred to as proteases.[6] Their role in tumor invasion and metastasis already being known, evidence is now emerging about their role in tumor growth at both primary and metastatic sites.[7]

Cathepsin D is a ubiquitous aspartyl lysosomal endopeptidase whose function is to degrade high-molecular-weight proteins. It is also known to have a role in cancer metastasis and is postulated to initiate activation of the protease cascade involved in degradation of extracellular matrix, aiding in invasion. It has also been shown to be mitogenic for breast cancer cells.[8]

Cathepsin D has been shown to express in oral tumors,[7,9-11] squamous cell carcinoma of skin,[12] gastric cancer,[13] hepatoma,[14] normal and neoplastic thyroid tissue,[15] cutaneous malignant melanoma,[16] basal cell carcinomas,[17] and carcinomas of bronchus, colon, kidney, breast, ovary, bladder, and pancreas also.[18]

In studies conducted on breast cancer by Tandon et al[19] and Mansour et al,[20] it has been concluded that cathepsin D may be an independent predictor of early recurrence and mortality. Studies on oral cancers by Kawasaki et al[7] and Vigneswaran et al[9] have shown that increased cathepsin D expression is closely related to carcinoma invasion and metastasis.

However, till date, no study seems to have been done to try and correlate the expression of cathepsin D in primary OSCC (POSCC) and their recurrent counterparts to study if possible the prognostic value of cathepsin B. Our study, therefore, is an attempt in this direction.

MATERIALS AND METHODS

Tissues

Paraffin-embedded tissues of previously diagnosed cases of POSCC and recurrent OSCC (ROSCC) from the Department of Oral Pathology and Microbiology, SDM College of Dental Sciences & Hospital, Dharwad, India, were used for this study purpose.

A total of 50 cases were studied immunohistochemically for rabbit polyclonal antihuman cathepsin D expression. A total of 30 cases of POSCC and their recurrent counterparts were first stained for studying the expression. A total of 10 cases of POSCC that had no record of recurrence on follow-up study were also assessed. A total of 10 cases of breast carcinoma were taken as positive controls.

Immunohistochemistry

Immunohistochemical staining was performed using labeled streptavidin–biotin technique. About 5 µm sections were made from formalin-fixed, paraffin-embedded tissue blocks and taken into silanized slides (SIGMA, USA). The sections were dewaxed in xylene and rehydrated in graded alcohols. Antigen retrieval was done by the pressure cooker method in a 10 mM citrate buffer (pH 6.0) for 2 minutes. Endogenous peroxidase activity was blocked by covering the tissue sections with 3% hydrogen peroxidase for 15 minutes. Then, the sections were incubated for 8 hours in a humidifying chamber with prediluted polyclonal antihuman cathepsin D antibody at 4°C.

The sections were incubated with biotinylated link (secondary) antibody for 15 minutes. This was followed by incubation with streptavidin–peroxidase for 15 minutes. The antigen–antibody reactions were visualized with the 3,3′-diaminobenzidine chromogen. Tris-buffered saline was used in place of primary antibody in negative control tissue sections. The sections were washed and then lightly counterstained with Harris's hematoxylin, dehydrated and mounted with DPX (synthetic resin made up of a mixture of distyrene (polystyrene), plasticizer (tricresyl phosphate), and xylene).

Interpretation of Staining

Presence of brown-colored end product at the site of target antigen, i.e., in the cytoplasm, was taken as being indicative of positive reactivity. The negative tissue control demonstrated absence of specific staining.

Breast carcinoma was taken as positive control, with each batch of staining, as cathepsin D was positive in both the cancer and stromal cells. Intensity of staining was graded for statistical analysis in ascending order: 0 – negative staining, 1 – weak staining, and 2 – intense staining in well, moderately, and poorly differentiated POSCC and ROSCC.

Statistical Analysis

A rank-sum two-sample test (Mann–Whitney U test) was used for the statistical interpretation of staining results in POSCC and ROSCC. A p-value <0.05 was taken as statistically significant.

RESULTS

A total of 45 biopsies, comprising 28 cases of primary squamous cell carcinomas and 17 cases of recurrent squamous cell carcinomas, were studied for the expression of the protease cathepsin D. The 28 cases of primary squamous cell carcinomas included 11 cases that showed recurrence and 17 cases that were just primaries. Of the 17 recurrent squamous cell carcinomas, 11 cases had

biopsies of the primary counterparts in the department. The remaining 6 cases were referred to the department from Cancer Hospital, Hubli; hence, their primary biopsies were not available for study (Table 1).

Of the 28 cases of primary squamous cell carcinomas studied, 15 were well-differentiated carcinomas, 8 were moderately differentiated carcinomas, and 5 were poorly differentiated carcinomas. Among the 17 cases of recurrent carcinomas, 9 were well and 8 were moderately differentiated carcinomas (Table 1).

Two other observers evaluated the staining and intensity (faculty members of the department) independently and the average of the observations was taken.

Cathepsin D staining was evaluated based on the intensity of staining of the cytoplasm and intensity of staining of positive tumor cells. The intensity was graded in all the cases from 0 to 3; 0 value was given for negative staining, 1 for mild staining, 2 for moderate staining, and 3 for intense staining.

Among POSCC, of the 15 cases of well-differentiated squamous cell carcinoma (Table 2), three showed moderate staining, while the remaining showed only mild staining pattern (Fig. 1). In the eight cases of moderately differentiated carcinomas (Table 2), five showed moderate staining (Fig. 2), two showed mild staining, and only one case was intense staining. Among the five cases of poorly differentiated carcinomas, four were intense in their staining pattern (Fig. 3) and one was moderate to stain.

In the 17 cases of recurrent carcinomas, of the 9 cases of well-differentiated recurrent carcinomas, mild staining was seen in 5 cases (Fig. 4), moderate in 2 cases, and intense in 2 cases. The other eight cases of moderately differentiated recurrent carcinomas showed mild staining in two cases, moderate staining in five cases (Fig. 5), and intense staining pattern in one case (Fig. 6).

The rank-sum two-sample test (Mann–Whitney) between staining intensities of all the three groups of POSCC showed a statistically significant outcome

Table 1: Expression of cathepsin D in different grades of POSCC and ROSCC

Category	*Total number of cases*	*Well differentiated*	*Moderately differentiated*	*Poorly differentiated*
POSCC without recurrence	17	07	05	05
POSCC showing recurrence	11	08	03	00
ROSCC having primary record	11	06	05	00
Referred ROSCC	06	03	03	00

Table 2: Comparison of cathepsin D staining intensities between different grades of POSCC using Mann–Whitney U-test

	Intensity of staining				
Grade of POSCC	*Mild*	*Moderate*	*Intense*	*Total cases*	*p-value*
Well differentiated	12	3	0	15	<0.05
Moderately differentiated	2	5	1	8	
Well differentiated	12	3	0	15	<0.05
Poorly differentiated	0	1	4	5	
Moderately differentiated	2	5	1	8	<0.05
Poorly differentiated	0	1	4	5	

S: Significant

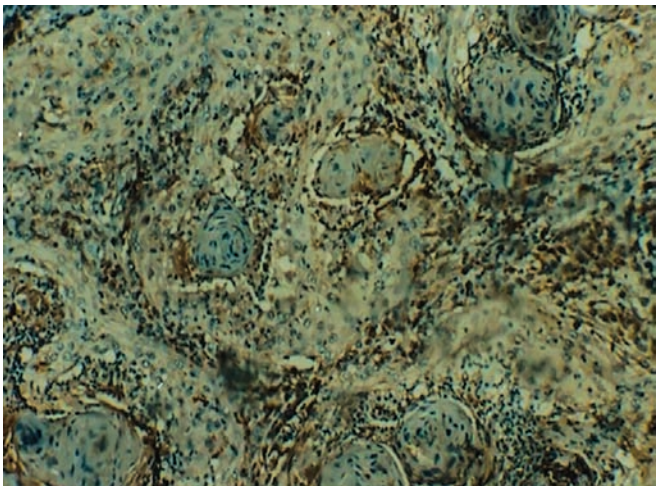

Fig. 1: Photomicrograph showing mild cathepsin D expression in well-differentiated primary squamous cell carcinoma (100×)

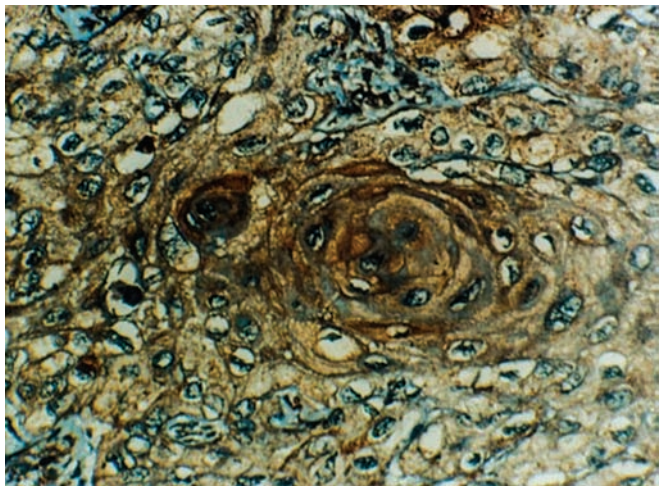

Fig. 2: Photomicrograph showing moderate cathepsin D expression in moderately differentiated primary squamous cell carcinoma (250×)

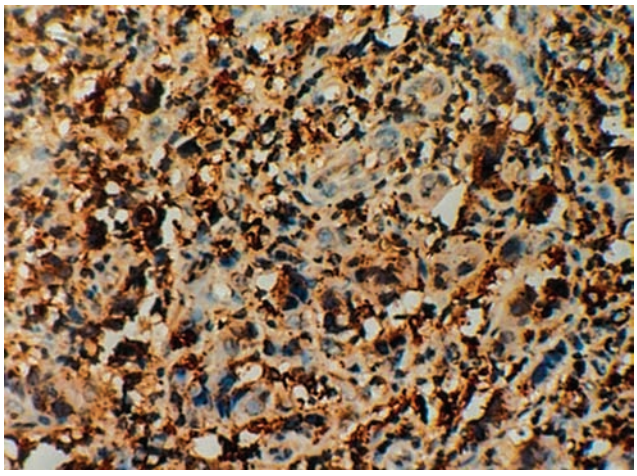

Fig. 3: Photomicrograph showing intense cathepsin D expression in poorly differentiated primary squamous cell carcinoma (250×)

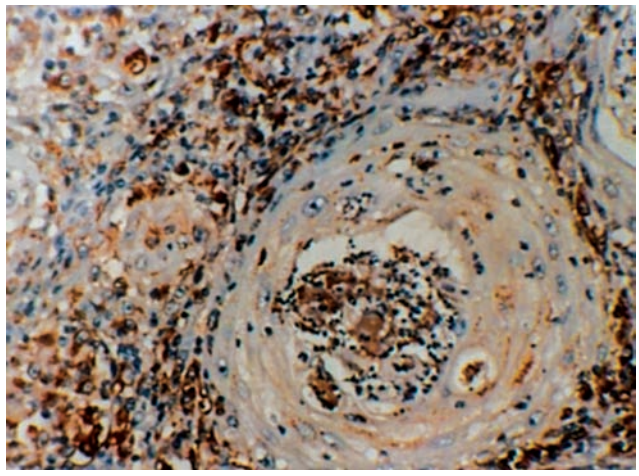

Fig. 4: Photomicrograph showing mild cathepsin D expression in well-differentiated recurrent squamous cell carcinoma (250×)

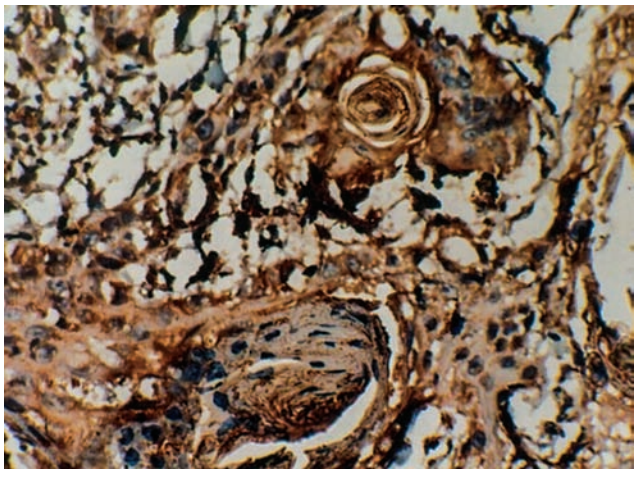

Fig. 5: Photomicrograph showing moderate cathepsin D expression in moderately differentiated recurrent squamous cell carcinoma (250×)

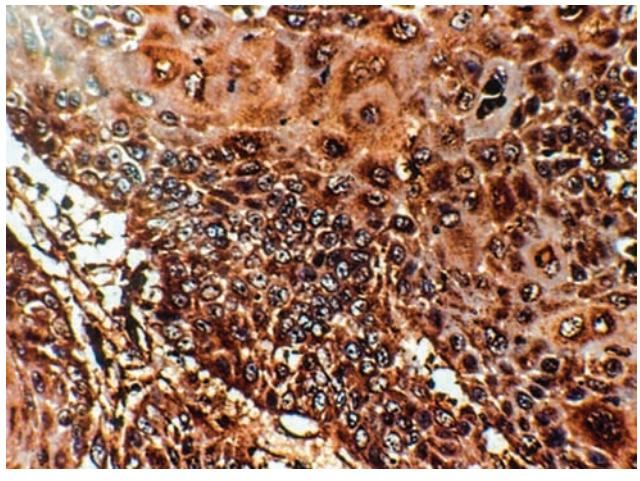

Fig. 6: Photomicrograph showing intense cathepsin D expression in poorly differentiated recurrent squamous cell carcinoma (250×)

(Table 2). However, the p value was seen to be insignificant when different grades of ROSCC were compared with each other (Table 3).

Table 4 compares cathepsin D-staining patterns in different grades of POSCC that showed recurrence (11 cases) with those primary carcinomas that did not show any recurrence (17 cases). In the 11 cases of recurrent carcinomas, 8 cases showed mild staining intensity and 3 cases showed moderate staining intensity. Among the 17 nonrecurrent cases, 6 cases each showed mild and moderate staining patterns, while 5 cases showed an intense staining pattern. Among these two groups, however, no statistical significance was noted at 5% level of significance, but when the same two groups were

Table 3: Comparison of cathepsin D staining intensities between different grades of recurrent squamous cell carcinoma using Mann–Whitney U test

	Intensity of staining				
Grade of ROSCC	*Mild*	*Moderate*	*Intense*	*Total cases*	*p-value*
Well differentiated	5	2	2	9	>0.05
Moderately differentiated	2	5	1	8	

S: Significant

Table 4: Comparison of cathepsin D staining intensities between POSCC with recurrence and with no recurrence

	Intensity of staining				
Grade of carcinoma	*Mild*	*Moderate*	*Intense*	*Total cases*	*p-value*
POSCC with recurrence	8	3	0	11	>0.05
POSCC with no recurrence	6	6	5	17	<0.10

S: Significant

Table 5: Comparison of expression of cathepsin D in recurrent and nonrecurrent carcinomas

	Intensity of staining				
Case status	*Mild*	*Moderate*	*Intense*	*Total cases*	*p-value*
Recurrent cases	7	7	3	17	<0.05
Nonrecurrent cases	6	6	5	17	

S: Significant

compared again at 10% level of significance, a statistically significant association was observed.

In our study, we also compared the expression of cathepsin D staining between all the recurrent and nonrecurrent carcinomas as a whole (Table 5). Of the 17 recurrent cases, 7 were mild staining, 7 were moderate staining, and 3 cases showed intense staining. Of the 17 nonrecurrent carcinoma cases, 6 each were mild and moderate staining and 5 cases were intense staining. There was no statistical significance between the two groups of recurrent and nonrecurrent carcinomas.

DISCUSSION

Cancer has been one of the most dangerous and common causes of morbidity worldwide due to its nature to metastasize and form secondary tumors and even recur. The process of metastasis is a multistep process and involves several tumor cell–host and cell–matrix interactions.[8]

The process of local metastasis involves attachment of tumor cells to underlying basement membrane, local proteolysis, and migration of cells through the proteolytically modified region.[21] It is well known that proteolytic degradation of extracellular matrix plays a crucial role in cancer invasion.[22,23] For distant metastasis, the tumor cell requires entry into blood circulation (intravasation) near the site of primary tumor and exit at a secondary site (extravasation). Both these processes require degradation of the basement membrane, which is brought about by a concerted effort by various proteases,[8] which include type IV collagenases, heparanases, and cathepsins.[6]

Cathepsins are a large family of ubiquitous lysosomal enzymes involved in protein degradation[6] known to catalyze the hydrolysis of a variety of protein substrates and are involved in pathologic processes like tumor invasion and metastasis.[24]

Cathepsin D, an aspartic endopeptidase,[6] is a glycoprotein in nature[24] and is capable of cleaving laminin, fibronectin, and proteoglycans.[25]

Cathepsin D is present in almost all mammalian tissues like macrophages, epithelium, spleen, liver, lungs, brain, lymph nodes, stomach, and kidney.[18] Enhanced expression of cathepsin D in human neoplasms, such as carcinoma of the lung, stomach, colon, kidney, breast, ovary, bladder, pancreas, liver, and neural carcinomas and melanomas suggests its role in invasion and metastasis and in prognosis of breast, endometrial, and gastric carcinoma.[18]

In our study, the role of cathepsin D in POSCC and ROSCC has been evaluated in a total of 45 biopsies, comprising 28 primary squamous cell carcinomas and 17 recurrent squamous cell carcinomas.

The 28 cases of primary squamous cell carcinoma studied included 11 cases with recurrence and 17 cases without recurrence. Cathepsin D expression was evaluated on a scale of 0 to 3, with 0 implying negative staining and 1, 2, 3 being indicative of progressive increase in staining intensity respectively.

Expression of cathepsin D was also evaluated in different grades of primary and recurrent squamous cell carcinoma. The grading system, which was employed here, was the one, i.e., routinely used, i.e., Broder's grading system. The well-differentiated carcinomas were mild staining predominantly (71%), majority of moderately differentiated carcinomas were moderately staining (62.5%), while 80% of all poorly differentiated OSCCs exhibited an intense staining pattern (80%).

Cathepsin D expression was found to be variable in primary carcinoma with or without recurrence. However, the staining intensities of all the three groups of POSCC showed a statistically significant relation (Table 2), which correlates with decreased staining intensity of cathepsin D in POSCC, which does not show any signs of recurrence.

In recurrent carcinomas, expression of cathepsin D showed more moderate and intense staining patterns in moderately differentiated lesions when compared with well-differentiated carcinomas (Table 3). Our observations show that the expression of cathepsin D can be correlated with Broder's grading, in that its expression was found to be more in the poorer grade of carcinoma, be it a primary or recurrent carcinoma.

It has been shown that cathepsin D has a relation with prognostic parameters like advanced tumor stage,[9] poor malignancy grade,[9,26] development of clinical metastasis,[7,27] depth of tumor invasion,[28,29] and was also reported to have increased intensity of expression in the advancing front of the lesion.[13,30] These observations which link increased cathepsin D expression to parameters reflective of a poorer grade of carcinoma were similar to our findings.

When a comparison of cathepsin D staining patterns in different grades of POSCC that showed recurrence (11 cases) with those primary carcinomas that did not show any recurrence (17 cases) was made, no statistical

significance was noted at 5% level of significance, but when the same two groups were compared again at 10% level of significance, a statistically significant association was observed.

Comparing the intensity of expression of cathepsin D between primary and all recurrent carcinomas (Table 5), its expression was generally seen to be similar in both primary and recurrent carcinoma (except in that of intense staining category).

These findings in both the above categories, though not significant, may find consolation in the fact that it has to be corroborated in relation to a larger sample size and only then should a definite conclusion be drawn. Furthermore, this variability of staining intensity in cathepsin D expression noted in the different groups of carcinomas can be explained to the possible variability in assessment, which can still be subjective, though care was taken to reduce this subjectivity by having the intensity evaluated by three independent observers.

Although studies appear to have not been done on recurrent carcinomas, our results indicate a similar pattern of cathepsin D expression in recurrent carcinomas as that seen in primary squamous cell carcinomas. This, however, needs to be substantiated with further research.

Literature search using Medline and other search engines showed only four specific studies [7,9,10,31] of cathepsin D in OSCCs, all of them correlating its expression to various prognostic factors. No study was obtained specifically on ROSCC. Therefore, our results in this category of ROSCCs and among primary carcinomas that showed recurrence at a later stage cannot be compared. However, the available literature, which clearly indicates that cathepsin D expression is an indicator of prognosis, could well be applied to recurrent cases and in primary carcinoma, which showed recurrence later.

However, these findings need to be correlated with more of such specific studies of cathepsin D expression in larger sample sizes of primary squamous cell carcinomas with their recurrent counterparts.

CONCLUSION

In this study, increased expression of cathepsin D in relation to decrease in differentiation in POSCC was partially observed. A similar pattern was observed in recurrent squamous cell carcinoma. Cathepsin D expression in primary squamous cell carcinomas with recurrences was quite variable as compared with primary squamous cell carcinomas without recurrences.

Comparison of cathepsin D expression in primary with their recurrent counterparts showed similar or increased intensity of expression in recurrent carcinomas, thus suggesting its limited usefulness in predicting recurrence. Our findings in recurrent carcinoma appear not to be reported earlier. However, the findings in this study in POSCC and ROSCC need to be corroborated from larger samples of primary squamous cell carcinoma and their recurrent counterparts.

CLINICAL SIGNIFICANCE

Although cathepsin D might have shown limited usefulness in predicting cancer recurrence, its therapeutic potential must not be overlooked. With a larger sample size and increased control over other variables, it can prove to be a valuable tool to detect the aggressiveness of a given tumor and may also hold the key to finding early, more effective, and specific treatment modalities for cases of oral cancer.

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