Immuno-Histochemical Evaluation Of Cathepsin D In Malignant Salivary Gland Carcinomas

P.V. Angadi1, Y. Sivaranjini1, GS Kumar3

1Dept of Oral & Maxillofacial Pathology and Microbiology, Mamatha Dental College, Khammam, Andhra Pradesh-India.
2Dept of Oral & Maxillofacial Pathology and Microbiology, KLEVK Institute of Dental Sciences and Hospital, Belgaum, Karnataka- India.
3Dept of Oral & Maxillofacial Pathology and Microbiology, KSR Institute of Dental Sciences and Research, Tiruchengode, Tamilnadu -India

Abstract

Objective:
Cathepsin D is a lysosomal acid protease secreted in increased levels in several malignancies. However, its role in salivary gland tumors has not been studied extensively. The present study aims to assess the expression of Cathepsin D in malignant salivary gland tumors and to compare its expression in these tumors.

Study design:
A total of 30 cases of malignant salivary gland carcinomas which included 16 cases of adenoid cystic carcinoma (ACC), 9 cases of mucoepidermoid carcinoma (MEC), and 5 cases of polymorphous low grade adenocarcinoma (PLGA) were evaluated immunohistochemically using anti-Cathepsin D antibody.

Introduction
Carcinogenesis is a multistage process resulting in uncontrolled growth and differentiation in cells and tissues which undergo various changes at the genetic level to establish a malignant phenotype. These changes include self-sufficiency in growth signals, insensitivity to growth inhibitors, evasion of apoptosis, angiogenesis, and most importantly the ability to invade and metastasize. Metastasis forms the basis for the fatality, poor prognosis and difficulty in management of cancer in general.

Result:
All the cases showed positivity (100%) for Cathepsin D with intense expression noted in ACC and MEC as compared to PLGA. Comparison of these tumors revealed statistical significant difference in expression between ACC and PLGA.

Conclusion:
Intense expression of Cathepsin D in high grade carcinomas may be a marker for invasive potential and aggressive behavior.

Keywords
Cathepsin D, Adenoid cystic carcinoma, Mucoepidermoid carcinoma, Polymorphous low grade adenocarcinoma

It is a complex sequential cascade controlled by various proteases like endopeptidases, collagenases, matrixmetalloproteinases and cathepsins.(1, 2)

Cathepsins, named in 1929 by Willstatter and Baumann is derived from the Greek word Kathepsins meaning “to digest”. They are large classes of lysosomal proteases involved in protein catabolism among which cathepsin D has been studied extensively.(3, 4) It is a lysosomal acid protease secreted in small amounts in normal cells and increased levels have been reported in several malignancies.(5) Cathepsin D is known to exist in 3 forms. It is secreted as a 52 kD precursor, which is processed in the lysosomes to
intermediate active enzyme (48kD) and mature form (34kD).\textsuperscript{(6)} It is active in acidic pH and acts directly by digesting the extracellular matrix or indirectly by initiating the proteolytic cascade that is responsible for breakdown of basement membrane.\textsuperscript{(5, 6)} It also appears to be mitogenic by releasing certain growth factors and activating several growth factor receptors.\textsuperscript{(7)} Recently, a pro-angiogenic role has also been suggested.\textsuperscript{(7)}

Its over expression has been seen in breast cancer,\textsuperscript{(8,9)} prostate carcinoma,\textsuperscript{(10)} colorectal carcinoma,\textsuperscript{(11,12)} soft tissue sarcomas,\textsuperscript{(13)} lung adenocarcinoma,\textsuperscript{(14)} laryngeal cancers,\textsuperscript{(15)} thyroid carcinoma,\textsuperscript{(16,17)} oral squamous cell carcinoma,\textsuperscript{(18)} and has been correlated to poor histologic grade,\textsuperscript{(18)} advanced stage,\textsuperscript{(8)} tumor size,\textsuperscript{(15)} lymph node metastasis,\textsuperscript{(9)} high proliferation rate,\textsuperscript{(18)} tumor invasion,\textsuperscript{(13)} poor prognosis,\textsuperscript{(13)} and shorter disease free survival\textsuperscript{(18)} in various studies. Thus, Cathepsin D has been suggested to be an important marker of prognosis and indicator for tumor invasion and metastatic potential.

However, there are only two studies in English language literature of Cathepsin D expression in salivary gland tumors with minimal information regarding its role in this category of human cancers.\textsuperscript{(20, 21)} Vigneswaran et al\textsuperscript{(21)} evaluated cathepsin D in selected benign and malignant salivary gland tumors while Barnes et al\textsuperscript{(20)} studied its expression only in salivary duct carcinoma. The present study aims to assess the expression of Cathepsin D in malignant salivary gland tumors i.e. varying grades of adenoid cystic carcinoma (ACC), mucoepidermoid carcinoma (MEC) and polymorphous low grade adenocarcinoma (PLGA) which exhibit diverse behavior and also to compare its expression in these tumors.

### Materials and Methods

This laboratory based study involved the use of buffered formalin fixed, paraffin embedded tissues of histopathologically diagnosed cases of malignant epithelial salivary gland tumors retrieved from the department of Oral Pathology and Microbiology. A total of 30 cases which included 16 cases of ACC, 9 cases of MEC and 5 cases of PLGA were evaluated immunohistochemically for Cathepsin D expression. The cases of adenoid cystic carcinoma were further categorized into tubular (n=2), cribriform (n=8) and solid variants (n=6). The mucoepidermoid carcinomas included revealed varying proportions of mucous, epidermoid and intermediate cells and were graded as low (n=4), intermediate (n=1) and high grade (n=4). The cases of PLGA were arising in the palate (n=4) and lip (n=1) and were characteristically low grade as described in literature.

Immunohistochemistry: 2-3 serial section of 5um thickness were made and taken on silanised slides. The sections were then deparaffinised, rehydrated through xylene and descending grades of alcohol. Antigen retrieval was carried out by heating in a pressure cooker in 10mM citrate buffer (pH-6.0) for 2x5min. The sections were then incubated after covering with 3% hydrogen peroxide for 15min to block the
peroxidase activity, following which incubation with primary anti Cathepsin D antibody (DAKO, Denmark) was carried out for 8 hours at 4°C using optimal dilution of 1:100. After further incubations with secondary Ab (45min) and Streptavidin peroxidase (30min), visualization was performed using freshly prepared DAB chromogen for 10min. The slides were then counterstained using Harri’s hematoxylin. For each batch of staining, a negative control where the primary antibody was replaced by negative control buffer and a positive control of breast carcinoma were used. Cytoplasmic staining was considered positive for Cathepsin D staining. The positive cases were further analyzed for the intensity of staining which were graded on a scale of 1-3 where 1 indicated mild staining, 2 - moderate staining and 3 – intense staining according to Kandalaft et al. Two other observers carried out all these observations in order to eliminate interobserver bias.

**Results**

Breast carcinoma which was used as positive controls exhibited Cathepsin D in the cytoplasm and negative controls did not show any stain. (Fig. 1). Focal expression was noted in the acinar and ductal cells of the normal salivary gland adjacent to the tumor tissue. All cases (100%) of salivary gland malignancies studied i.e. adenoid cystic carcinoma (Fig. 2), mucoepidermoid carcinoma (Fig. 3 A, B, C) and polymorphous low grade adenocarcinoma (Fig. 3D) exhibited Cathepsin D positivity but varied in the intensity of staining. Table I summarizes the results and the grading of stain intensity. In 16 cases of adenoid cystic carcinoma, 56% demonstrated intense staining while moderate and mild staining was observed in 38% and 6% respectively. Regarding the grades, the tubular ACC depicted mild (1) to moderate staining (2), moderate (6) to intense staining (2) was observed in cribriform variant while a predominant intense staining was seen with solid variant (n=6). Cathepsin D expression was intense in 34% cases of mucoepidermoid carcinoma while 44% were moderate and 22% exhibited mild positivity. The low grade MEC demonstrated mild (n=1) to moderate intensity,
the intermediate grade exhibited moderate (n=1) expression while all the high grade MEC expressed cathepsin D Intensely (n=4). None of the PLGA cases stained intensely and out of 5 cases, 80% (n=4) showed mild intensity and 20% (n=1) displayed moderate intensity.

**Statistical Analyses**

Rank sum two sample Mann-Whitney U test was used for comparison and correlation between various salivary gland tumors studied.

### Table 1: Cathepsin D positive cases in different groups of carcinomas and their intensity grading

<table>
<thead>
<tr>
<th>Groups of cases</th>
<th>Total cases</th>
<th>Positive cases</th>
<th>Percentage</th>
<th>Intensity of staining (1)</th>
<th>(2)</th>
<th>(3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC</td>
<td>16</td>
<td>16</td>
<td>100%</td>
<td>1</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>MEC</td>
<td>9</td>
<td>9</td>
<td>100%</td>
<td>2</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>PLGA</td>
<td>5</td>
<td>5</td>
<td>100%</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2: Statistical analysis using Mann Whitney U test in various groups of carcinomas

<table>
<thead>
<tr>
<th>Group</th>
<th>Total cases</th>
<th>Positive Staining</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC</td>
<td>16</td>
<td>(1) 1  6  9</td>
<td>0.2573(NS)</td>
</tr>
<tr>
<td>MEC</td>
<td>9</td>
<td>(2) 2  4  3</td>
<td></td>
</tr>
<tr>
<td>ACC</td>
<td>16</td>
<td>(3) 1  6  9</td>
<td>0.0057(S)</td>
</tr>
<tr>
<td>PLGA</td>
<td>5</td>
<td>1  4  3</td>
<td>0.0619(NS)</td>
</tr>
<tr>
<td>MEC</td>
<td>17</td>
<td>2  4  3</td>
<td></td>
</tr>
<tr>
<td>PLGA</td>
<td>9</td>
<td>4  1  0</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations:

ACC-Adenoid Cystic Carcinoma
MEC-Mucoepidermoid carcinoma
PLGA-Polymorphous low grade adenocarcinoma

**Discussion**

Cathepsin D is an aspartic endopeptidase active at an acidic pH and was first reported by Westely and Rochefort (1979) in breast cancer.
Cathepsin D In Salivary Gland Malignancy, Punnya.V.A, et. al.

Its expression is induced by several growth factors like ILF, FGF etc. and it is considered to be mitogenic,(6) increases cell proliferation by activating growth factors or by interacting with growth factor receptors (EGFR, ILGF-1). Recently, a role in inhibiting apoptosis and angiogenesis has also been considered.(6) Since it is known to accumulate in a greater proportion in malignant cells, Cathepsin D can facilitate tumor growth and metastasis by aiding in degradation of extracellular matrix, increased proliferation, tumor neovascularisation and activation of other growth factors and proteases (1,4,5,6,22,23).

Salivary gland carcinomas which account for 5-7% of all head and neck cancers represent histologically most heterogeneous group of tumors. Malignant salivary gland tumors especially show greatest diversity in their cellular composition and morphological pattern. The frequency of the different histologies varies depending on whether the cancer arises in the major (parotid, submandibular) or minor (mucosal) glands, but overall, MEC, ACC, and adenocarcinoma together represent the majority of all salivary gland malignancies.(24) The current diagnosis and classification of salivary gland carcinomas is mainly based on the neoplastic phenotype and architectural arrangement of neoplastic cells. Diagnostic difficulties are often encountered in differentiating certain malignant tumors like PLGA and ACC. (24) Furthermore, salivary gland carcinomas of various histologic types lack histomorphologic criteria by which local aggressive or metastatic behavior can be predicted. Since significant differences are noted among different histologic types of salivary gland carcinomas in their biologic behavior, use of an immunomarker that could predict this would aid in prognostic and therapeutic assessment. (25)

Studies of Cathepsin D in salivary gland tumors are limited. (20,21) The present study evaluated the expression of Cathepsin D in malignant salivary gland tumors like adenoid cystic carcinoma (16), mucoepidermoid carcinoma (9) and polymorphous low grade adenocarcinoma (5). The pattern and intensity of expression was almost uniform in all the staining batches, which composed of a positive control, a negative control and various malignant tumors, suggesting that the immunohistochemical procedure utilized is standardized and hence the results can be considered reliable.

Barnes L(20) studied Cathepsin D expression in salivary duct carcinoma (SDC) and showed 42% positivity. However, this is not comparable as SDC is not included in our study. Vigneswaran et al(21) studied Cathepsin D levels in benign and malignant salivary gland tumors and observed higher expression in carcinomas as compared to benign tumors. Among the malignant tumors in their study, adenoid cystic carcinoma (100%), mucoepidermoid carcinoma (100%), poorly differentiated adenocarcinoma NOS (100%) and carcinoma ex pleomorphic adenoma (100%) showed increased expression which was suggested to indicate poor prognosis. The PLGA and acinic cell carcinoma which are generally graded as low grade malignancies exhibited negativity in their study. These findings are consistent with our study where intense expression was noted in ACC and MEC whereas predominantly mild expression was noted in PLGA. Levin et al also reported an intense (2+) expression of cathepsin D (100%) in 18 cases of ACC studied. (26) Furthermore, in our study the Cathepsin D expression seemed to correlate with the histologic grade. The cribriform and tubular variants of ACC demonstrated mild to moderate expression while the solid variant showed intense expression and low as well as intermediate grades of MEC showed mild expression in comparison to high grade tumors. This expression correlates well with the reported aggressive behavior of solid ACC and high grade MEC. Additionally, though PLGA resemble ACC, they exhibit a very low proliferative index (27) and several molecular studies have demonstrated them to be a low grade malignancy as compared to ACC. (28, 29) In agreement to their behavior, they predominantly depicted a mild Cathepsin D expression.

Interestingly, in the comparison of staining intensities among the various tumors studied, a statistical significant difference was noted on comparison of adenoid cystic carcinoma and polymorphous low grade adenocarcinoma (PLGA). This is in accordance with the reports by
Vigneswaran et al.(21) who also showed significant difference in cathepsin D expression in these two entities. This difference could be attributed to the increased expression noted in the cribriform and solid variants of ACC. However, comparison of intensity of Cathepsin D expression between ACC and MEC and MEC and PLGA showed no statistical significance. This could be due to inclusion of varying grades of these tumors. Survey of literature does not reveal any such comparison of Cathepsin D expression between the different groups of salivary gland tumors. The increased expression noted in high grade MEC and solid ACC may contribute to their aggressive behavior, which could be attributed to the proteolytic role of cathepsin D resulting in extracellular matrix degradation thus aiding in invasion and metastasis. Additionally, cathepsin D is known to be mitogenic and activates certain oncogenes (ex. cmyc). It also stimulates growth factors with interaction with growth factor receptors like EGFR, IGFR etc. and stimulates angiogenesis.(6,7,12) This is corroborated by several studies demonstrating increased growth factors, oncogenes and angiogenesis in malignant salivary gland carcinomas.(30-33) Future studies with related growth factors, angiogenic markers and oncogenes along with Cathepsin D as well as inclusion of prolonged follow-up could reveal the true significance of Cathepsin D as a marker of aggressiveness and prognosis.

Conclusion

Prominent up regulation of Cathepsin D was observed in all the malignant salivary gland tumors studied. The frequent intense expression observed in high grade malignancies in our study may suggests that Cathepsin D may be a marker for biologic aggressiveness in salivary gland malignancies. Assessment of Cathepsin D in salivary gland tumors may aid to identify patients with higher risk of developing metastasis. However, more studies and larger sample size, diverse tumors with adequate follow-up need to be undertaken to determine its true prognostic potential.

References


