Salivary duct carcinoma: immunohistochemical profile of an aggressive salivary gland tumour

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ORIGINAL ARTICLE

Background: Salivary duct carcinoma (SDC) is considered to be a distinct malignancy of the major salivary glands, because of its highly aggressive behaviour, and the high rate of recurrence, metastasis, and disease related death.

Aims: To investigate expression of the proteins involved in the retinoblastoma (pRb) and p53 pathways, which control cell cycle progression at the G1/S checkpoint, and also expression of the c-erbB-2 oncoprotein in SDCs.

Methods: Using a streptavidin–biotin method, five cases of SDC were evaluated immunohistochemically for the presence of cyclin D1, CDK4 (cyclin dependent kinase 4), p16 (CDK2A), pRb (retinoblastoma protein), E2F-1, p53, mdm2 (murine double minute 2), bcl-2, and the c-erbB-2 oncoprotein to determine whether there was a correlation between the expression of these proteins and patient outcome.

Results: All of the cases showed deregulation of the pRb and p53 pathways. Of the five patients analysed, only the patient with longterm survival (10 years) was not positive for c-erbB-2 expression.

Conclusions: c-erbB-2 overexpression was associated with a poor prognosis. Aggressive behaviour, recurrence, and metastatic potential do not appear to be related to cell cycle deregulation, but seem to be associated with the c-erbB-2 oncoprotein, which is involved in matrix degradation and proteolitic activity, in addition to increases in vessel permeability, endothelial cell growth, proliferation, migration, and differentiation. There was a correlation between c-erbB-2 oncoprotein expression and aggressive behaviour in SDCs.

MATERIALS AND METHODS

Five cases of salivary duct carcinoma were retrieved from the archives of the A. C. Camargo Cancer Hospital, São Paulo, Brazil. The cases were selected on the basis of their microscopic appearance. Sections (3 µm thick) were cut from the 10% formalin fixed, paraffin wax embedded specimens, and stained by the streptavidin–biotin method, using antibodies directed against cyclin D1 (Dako, Glostrup, Denmark), cyclin dependent kinase 4 (CDK4; Santa Cruz Biotechnology, Santa Cruz, California, USA), p16 (NeoMarkers, Fremont, California, USA), E2F-1 (Santa Cruz), retinoblastoma protein (pRb; Dako), p53 (Dako), murine double minute 2 (mdm2; Novocastra, Newcastle upon Tyne, UK), bcl-2 (Novocastra), and c-erbB-2 (Dako) (table 1). Sections were dewaxed, rehydrated in graded alcohols to distilled water, and then subjected to antigen retrieval (table 1). They were then incubated with 0.3% hydrogen peroxide in methanol (30 minutes) to quench endogenous peroxidase, followed by incubation in 1% bovine serum albumin in buffered solution for 60 minutes and with avidin–biotin complex to block biotin crossreaction. The sections were then incubated with primary antibody (table 1) and immunostained with streptavidin–biotin–peroxidase, followed by diaminobenzidine chromogen solution. All steps were followed by washes in buffered solution. Finally, sections were counterstained with Mayer’s haematoxylin and mounted in a xylene based mountant. Negative controls were incubated in buffered solution without primary antibodies. Sections from normal salivary glands were included as internal controls.

Abbreviations: CDK, cyclin dependent kinase; mdm2, murine double minute 2; pRb, retinoblastoma protein; SGT, salivary gland tumour
All immunohistochemical slides were examined for positive staining by light microscopy. We counted a minimum of 500 positive nuclei from the neoplastic cells, from five different microscopic fields at a magnification of \( \times 400 \); 500 nuclei from normal salivary gland cells were also counted as controls. The results were evaluated semiquantitatively and graded according to the following scale: cyclin D1, CDK4, and E2F-1 were considered to be overexpressed when more than 20% of the nuclei showed positivity; p16 and pRb were considered negative if all the cells showed no nuclear expression (negative nuclear expression was considered to be abnormal expression).\(^{14-16}\) When evaluating the expression of the c-erbB-2 protein, intense cell surface membranous staining in most cells was interpreted as positive. Diffuse cytoplasmic staining was ignored, although its importance remains uncertain.\(^9\)

Table 2 provides general information about the tumours.

**RESULTS**

**Clinical findings**

The patients comprised four men and one woman, with ages ranging from 50 to 67 years (median, 58.5). All tumours were located in the parotid gland. Two patients presented regional lymph node metastases at the time of diagnosis. Four patients presented facial paralysis, one with regional lymph node metastasis. There was no evidence of metastasis in three patients. The patients were initially treated with total parotidectomy. Adjuvant treatment such as neck dissection, radiotherapy, and, in some cases, chemotherapy had no effect on the outcome. Three patients underwent radiotherapy with or without chemotherapy, and one received only chemotherapy. Tumour sizes varied from 3.0 cm to 8.0 cm (median, 5.5). The outcome varied from seven months to 10 years (table 2).

**Histopathological findings**

Histologically, the tumours showed intraductal and invasive components. The intraductal component is described as a papillary or solid growth pattern, often with central necrosis. The invasive pattern is characterised by small glands and cords associated with a pronounced desmoplastic stroma (figs 1–3). This histology has a striking resemblance to breast carcinoma of the ductal type, in which intraductal growth is marked by the presence of normal myoepithelial cells confining the tumour growth within the duct.\(^{13}\)

Normal salivary glands showed no cyclin D1 or CDK4 expression. p16 protein expression was weak in duct cells and negative in acinic cells. All normal salivary glands showed strong positivity for pRb and moderate expression of E2F-1.

**Immunohistochemical findings**

Overexpression of cyclin D1 was seen in three cases. CDK4 was overexpressed in four cases, three of which also overexpressed cyclin D1. The p16 protein showed loss of expression in two cases, whereas pRb showed no expression in one case. In three cases, the transcription factor E2F-1 was overexpressed and in two other cases it was moderately expressed. p53 protein was overexpressed in two of the five cases by more than 50%. The mdm2 oncoprotein showed

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**Table 1** Panel of antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Source</th>
<th>Antigen retrieval</th>
<th>Dilution</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin D1</td>
<td>DCS-6</td>
<td>Dako</td>
<td>WB</td>
<td>1/100</td>
<td>60 minutes</td>
</tr>
<tr>
<td>CDK4</td>
<td>C-22</td>
<td>Santa Cruz</td>
<td>–</td>
<td>1:30</td>
<td>60 minutes</td>
</tr>
<tr>
<td>p16</td>
<td>Ab-4 JC2</td>
<td>Neomarkers</td>
<td>WB</td>
<td>1:75</td>
<td>60 minutes</td>
</tr>
<tr>
<td>pRb</td>
<td>RB-1</td>
<td>Dako</td>
<td>WB</td>
<td>1:1000</td>
<td>45 minutes</td>
</tr>
<tr>
<td>E2F-1</td>
<td>KH95</td>
<td>Santa Cruz</td>
<td>MW</td>
<td>1:1500</td>
<td>45 minutes</td>
</tr>
<tr>
<td>p53</td>
<td>DO-7</td>
<td>Dako</td>
<td>WB</td>
<td>1:75</td>
<td>40 minutes</td>
</tr>
<tr>
<td>mdm2</td>
<td>1B10</td>
<td>Novocastra</td>
<td>–</td>
<td>1:50</td>
<td>40 minutes</td>
</tr>
<tr>
<td>c-erbB-2</td>
<td>Polyclonal</td>
<td>Dako</td>
<td>WB</td>
<td>1:1000</td>
<td>40 minutes</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>bcl-2/100/ D5</td>
<td>Novocastra</td>
<td>MW/ EDTA</td>
<td>1:150</td>
<td>40 minutes</td>
</tr>
</tbody>
</table>

CDK4, cyclin dependent kinase 4; mdm2, murine double minute 2; MW, citric acid in microwave; pRb, retinoblastoma protein; WB, citric acid in water bath.

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**Figure 1** Solid pattern showing oval cells with a prominent nucleolus.

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**Table 2** Clinical findings in five cases of salivary duct carcinoma

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Clinical features</th>
<th>Length of history</th>
<th>Treatment</th>
<th>Follow up</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>67</td>
<td>M</td>
<td>Parotid (7 cm, RLNM, P)</td>
<td>3 years</td>
<td>TP, ND, R, C</td>
<td>LR, 10 months</td>
<td>Death with disease after 1 year, DOD</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>M</td>
<td>Parotid (3 cm, RLNM, P)</td>
<td>2 months</td>
<td>TP, R</td>
<td>DM, 5 months</td>
<td>Death with disease after 7 months, DOD</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>F</td>
<td>Parotid (4.5 cm, NM)</td>
<td>4 months</td>
<td>TP</td>
<td>CM, 7 years</td>
<td>Death with disease after 10 years, DOD</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>M</td>
<td>Parotid (8 cm, NM)</td>
<td>45 days</td>
<td>TP, ND, R</td>
<td>LR, 1 year</td>
<td>Death with disease after 1 year, 4 months, DOD</td>
</tr>
<tr>
<td>5</td>
<td>58</td>
<td>M</td>
<td>Parotid (3.3 cm, P)</td>
<td>6 months</td>
<td>TP, ND, C</td>
<td>DM, 7 months</td>
<td>Death with disease after 10 months, DOD</td>
</tr>
</tbody>
</table>

Clinical staging was performed from data available in medical records. C, chemotherapy; CM, cervical metastasis; DM, distant metastasis; DOD, died of disease; F, female; LR, local recurrence; M, male; ND, neck dissection; NM, no evidence of metastasis; P, facial paralysis; R, radiotherapy; RLNM, regional lymph node metastasis; TP, total parotidectomy.
positivity in two cases. One case in which p53 was overexpressed also showed mdm2 overexpression (table 3).

Four of the five cases of SDC showed positive membranous staining for c-erbB-2; in one case, c-erbB-2 expression was seen in more than 50% of the cells, and in another all of the cells were positive (fig 4). One tumour did not express c-erbB-2. None of the cases showed positivity for the Bcl-2 protein (table 3).

DISCUSSION
Most reports indicate that SDC is an aggressive tumour with the most dismal short term prognosis of all salivary gland tumours.2–4 17 Thus, we investigated the relative expression of the cell cycle regulators in the pRb pathway and other biomarkers, such as p53, mdm2, c-erbB-2, and bcl-2, in an attempt to understand the biological behaviour of this tumour.

Four of the patients studied had a short follow up (up to a year), but one showed longterm survival (10 years). When we compared all the markers between the tumours, we found a major difference in c-erbB-2 expression. The tumour in the patient with longterm survival show a lack of c-erbB-2 expression, whereas all four patients with short survival overexpressed c-erbB-2. All cases showed Rb pathway alteration, and three cases showed abnormal expression of p53 and/or mdm2, indicating cell cycle deregulation. These alterations may be involved in the oncogenesis and progression of SDC, but the aggressive behaviour and metastasis cannot be the result of this misregulation, because matrix degradation and proteolitic activity, in addition to increases in vessel permeability, endothelial cell growth, proliferation, and migration are characteristic functions of c-erbB-2.

The overexpression of cyclin D1 seen in three of the five cases may be involved in progression and more aggressive tumour behaviour, as has been shown in several human cancers.16–18–22 These observations suggest that cyclin D1 alterations might play a role in cancer aetiology, although its overexpression is not sufficient for cell transformation, with the synergistic effects of other protooncogenes being required, as pointed out by Si and Liu.21

CDK4 acts as a protooncogene when complexed with cyclin D1.21 23–25 The overexpression of CDK4, but not cyclin D1, was seen in one patient. It is possible that c-Myc could replace cyclin D, because CDK4 is a target of c-Myc.21 23–27 One possible cause could be its relation to APC—other studies had provided evidence of a role for β-catenin/Tcf-4 in the control of c-Myc transcription.27

We observed loss of p16 in two cases. p16

Table 3  Outcome and immunohistochemical protein expression in five cases of salivary duct carcinoma

<table>
<thead>
<tr>
<th>Case</th>
<th>Outcome</th>
<th>Cyclin D1</th>
<th>CDK4</th>
<th>p16</th>
<th>pRb</th>
<th>E2F-1</th>
<th>p53</th>
<th>mdm2</th>
<th>c-erbB-2</th>
<th>Bcl-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DOD, 1 year</td>
<td>28.0</td>
<td>43.2</td>
<td>0.0</td>
<td>49.4</td>
<td>28.8</td>
<td>70.4</td>
<td>78.8</td>
<td>54.6</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>DOD, 7 months</td>
<td>0.0</td>
<td>0.0</td>
<td>23.2</td>
<td>49.4</td>
<td>23.4</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>3</td>
<td>DOD, 10 years</td>
<td>32.8</td>
<td>21.2</td>
<td>16.89</td>
<td>0.0</td>
<td>8.60</td>
<td>82.8</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>DOD, 1 year/4 months</td>
<td>29.8</td>
<td>20.2</td>
<td>0.0</td>
<td>51.0</td>
<td>15.6</td>
<td>0.0</td>
<td>0.0</td>
<td>12.4</td>
<td>0.0</td>
</tr>
<tr>
<td>5</td>
<td>DOD, 10 months</td>
<td>0.0</td>
<td>62.8</td>
<td>62.0</td>
<td>31.6</td>
<td>394</td>
<td>0.0</td>
<td>76.6</td>
<td>100.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

CDK4, cyclin dependent kinase 4; DOD, died of disease; mdm2, murine double minute 2; pRb, retinoblastoma protein.
c-erbB-2 oncoprotein expression and aggressive behaviour in salivary duct carcinomas. The tumour cells show diffuse membranous immunoreactivity.

Figure 4  c-erbB-2 immunohistochemical staining of an invasive salivary duct carcinoma. The tumour cells show diffuse membranous immunoreactivity.

D1/CDK4 overexpression, and pRb functions are progressively and cooperatively inactivated by multiple cyclin–CDK complexes during the G1 and S phases.

"All of the patients who overexpressed c-erbB-2 died of their disease in less than one and a half years, but the c-erbB-2 negative patient was still alive after 10 years"
REFERENCES