p-Akt and its relationship with clinicopathological features and survival in oral squamous cell carcinoma: an immunohistochemical study

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BACKGROUND: The aim of this study was to evaluate, through immunohistochemical reaction in samples of oral squamous cell carcinoma, the correlation between the expression status of protein kinase B (p-Akt) and patient survival as well as histological grade and some clinicopathological features.

METHODS: Samples were collected from 46 patients with oral squamous cell carcinoma. The immunohistochemical expression of p-Akt was analysed, as were clinicopathological features including the use of tobacco, tumour stage, size and infiltration of metastatic lymph nodes. The association of immunostaining with histological grade was analysed in 40 patients. The associations were examined for statistical significance using a chi-square test. Overall survival rates were estimated by the Kaplan–Meier method and compared using a log rank test (P > 0.05).

RESULTS: The results indicated a statistically significant association with p-Akt immunostaining for the variables lymph node metastasis (P = 0.006), tumour size (P = 0.044) and survival rate (P = 0.0298).

CONCLUSION: From these results, the present study suggests that high p-Akt expression found in oral squamous cell carcinoma patients may contribute to tumour growth, metastasis to regional lymph nodes and shorter survival time.

Keywords: oral cancer; p-Akt; recurrence; survival; tumour growth

Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common neoplasm in the world and accounts for 90% of malignancies that affect this region (1). Recently, there has been an increased incidence of OSCC, probably because of a generally longer life expectancy together with persistent consumption of alcohol and tobacco (2, 3). This neoplasm frequently displays metastasis to the cervical lymph nodes and a high recurrence pattern, resulting in a poor prognosis (2, 4). Despite improvements in surgical treatment, radiotherapy and chemotherapy, the 5-year survival rate remains at an unacceptable 30–50% (5, 6). Genetic and epigenetic studies can be useful for identifying molecular targets to assist in the identification of chemotherapeutics with which to improve these prognostic indices.

Protein kinase B (p-Akt) is a serine/threonine kinase that signals via phosphatidylinositol-3 kinase (PI3K) and has a key role in various cellular processes such as apoptosis, proliferation, differentiation and metabolism (7–9). It is associated with a variety of human malignancies including OSCC (2, 9–11). Studies have shown that a high expression of p-Akt in OSCC is associated with an increased frequency of metastasis, tumours in more advanced stages, recurrence and reduced survival time (1, 12–14).

Although it is known that the clinical behaviour of squamous cell carcinomas of the head and neck varies among the different sites that affects, most studies concerning this neoplasm examine the tumour as a single entity. As studies about the expression of p-Akt in cancer confined to oral cavity are limited, we conducted the present study to obtain more accurate information about the clinical importance of p-Akt overexpression in SCCs of the oral cavity.

Materials and methods

Specimens and inclusion criterion

A total of 46 paraffin-embedded biopsy specimens of OSCC from 32 (69.5%) males and 14 (30.5%) females with a mean age of 60 years (range, 27–85) were selected in the period
Patients were selected from patients (with a diagnosis confirmed by histopathology) who had primary tumours of the oral cavity with surgery as the only treatment modality. The smokers consumed at least 10 cigarettes per day. The patients were monitored for up to 62 months after the diagnosis. The mean follow-up of the patients was 28.02 months (range, 0–62). A total of 12 patients with a change in staging in the period between diagnosis and surgery were excluded. In our study, there were no cases with distant metastasis. The required data were obtained from patient records, summarised on standardised forms and stored in a database. The primary tumour was clinically staged according to the TNM classification defined by the 2002 UICC (15) and by the AJCC (16). The ethical committee of the Joao de Barros Barreto University Hospital approved this work under approval number 51641/12.

**Evaluation of histological grade and clinicopathological features**

The histological grade assessment followed the parameters of the World Health Organization (17) and was carried out by two pathologists without prior knowledge of the clinical data of the patients. Of the 46 samples, six were not included in this analysis because there was no consensus between the pathologists; thus, histopathology was graded in 40 samples.

The relationships between p-Akt protein and clinicopathological features, including the use of tobacco, size of tumour (T), infiltration of metastatic lymph nodes (N), stage and the histological grade, were assessed.

**Immunohistochemistry**

Sections of 3 μm thickness fixed in 4% formalin were dewaxed with xylene and hydrated in an ethanol series. For antigen retrieval, sections that received anti-p-Akt antibody were immersed in 10 mM monohydrated citrate buffer solution (pH 6.0) and heated in a microwave oven at 95°C for 15 min. Peroxidase activity was blocked with 6% hydrogen peroxide and methanol solution in two baths for 15 min each at room temperature. After washing with Tris buffer (pH 7.4), the slides were incubated with the primary antibody anti-p-Akt [p-Akt 1/2/3 (Ser 474): sc-135651, Santa Cruz Biotechnology, CA, USA], diluted 1:200, at 4°C overnight. The sections were subsequently exposed to avidin–biotin complex (LSAB-Kit + HRP; DakoCytomation, Carpinteria, CA, USA) and to 3, 3′-diaminobenzidine chromogen (DAB++; DakoCytomation). The sections were counterstained with Meyer’s haematoxylin, dehydrated in ethanol, cleared in xylene and mounted. Slices of prostate adenocarcinoma were used as the positive control, and the negative control was obtained by omitting the primary-specific antibody during the reaction.

Immunostained sections were examined by light microscopy at 40× magnification, and digital images were captured using an electron microscope model Eclipse Nikon Ci-POL (Nikon Metrology Europe NV, Leuven, Belgium). All sections were evaluated without knowledge of clinical status by two pathologists, under a fixed focus. The staining was considered positive when cells with brown staining were observed, indicating the presence of DAB in the immunohistochemistry reaction, and staining was considered negative for cells that did not show brown staining. Immunohistochemical staining of p-Akt was considered positive in the nucleus and/or cytoplasm of carcinoma cells.

The scoring system has previously been published in the literature (18). The analysis was based on intensity and distribution of staining. The distribution of stained cells was analysed as follows: 0 (0%), 1 (1–50%) and 2 (51–100%). The intensity of staining was rated as follows: 0 (no staining), 1 (mild staining), 2 (moderate staining) and 3 (strong staining).

The staining pattern of the specimens was defined by the sum of the values found in the distribution of data for the intensity of immunostaining, thus obtaining the final record (FR) as follows: FR0, FR2, FR3, FR4 and FR5. Using this method, FR0 and FR2 were considered negative staining, while FR3, FR4 and FR5 were considered positive staining.

**Statistical analysis**

Data were analysed using the Statistical Package for Social Sciences software for Windows, version 18.0 (SPSS Inc, Chicago, IL, USA). Associations between p-Akt expression and clinicopathological parameters were examined for statistical significance using a chi-square test. Overall survival rates were estimated by the Kaplan–Meier method and compared using a log rank test. A P value of <0.05 was considered significant.

![Figure 1](image-url)  
**Figure 1**  
Immunoreexpression of p-Akt protein in OSCC epithelium, simultaneously nuclear and cytoplasmic (×400 original magnification) (A). The immunostaining was located at the periphery and at the centre of the tumour island (B).
Results

p-Akt immunostaining

Immunohistochemistry results showed positive immunostaining for p-Akt in 78.26% (36/46) samples and negative immunostaining for p-Akt in 21.74% (10/46) samples. The pattern of p-Akt distribution was simultaneously cytoplasmic and nuclear (Fig. 1A). The immunostaining was observed in cells located at the periphery and also at the centre of the tumour island (Fig. 1B).

Clinical profiles of patients with OSCC and evaluation of histological grade

A total of 46 samples were included in the analysis. Patient characteristics are summarised in Table 1.

Moderately differentiated tumours (45%), followed by poorly differentiated (37.5%) tumours, were the most represented in our sample. Well-differentiated tumours represented only 17.5%.

The chi-square test showed a statistically significant difference for the variable lymph node metastasis \((P = 0.006)\), reflecting an association between the presence of metastasis and immunostaining of p-Akt in OSCC. The size of the tumour was also revealed to have a statistically significant association with p-Akt immunostaining \((P = 0.044)\).

No significant relationship was found between p-Akt immunostaining and smoking \((P = 0.634)\) or tumour stage status \((P = 0.190)\).

Overall survival

Overall survival was defined as the period between the date of diagnosis of the disease until the last follow-up or death. The Kaplan–Meier curve showed that the probability of survival for a patient with oral cancer after 1 month of monitoring the disease was 98.3% and that the passage of time tended to reduce this probability, as shown in Fig. 2. The probability of a patient surviving after 62 months of follow-up was 24.60%. Follow-up periods were available for all patients with OSCC.

There was a statistically significant association between survival rate and p-Akt immunostaining \((P = 0.0298)\) (Fig. 3).

Discussion

Serine/threonine protein kinase Akt, a downstream target of phosphatidylinositol 3-kinase (PI3K), has been considered an important oncogene responsible for the development of a

Table 1

<table>
<thead>
<tr>
<th>Clinicopathological features</th>
<th>p-Akt</th>
<th>(p)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N = 46)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\leq 40) years</td>
<td>12</td>
<td>26.09</td>
</tr>
<tr>
<td>(&gt;40) years</td>
<td>34</td>
<td>73.91</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>3</td>
<td>6.52</td>
</tr>
<tr>
<td>Male</td>
<td>31</td>
<td>67.40</td>
</tr>
<tr>
<td>Smoking</td>
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<td></td>
</tr>
<tr>
<td>No</td>
<td>3</td>
<td>6.52</td>
</tr>
<tr>
<td>Yes</td>
<td>31</td>
<td>67.40</td>
</tr>
<tr>
<td>Alcohol intake</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
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<td>4.35</td>
</tr>
<tr>
<td>Yes</td>
<td>34</td>
<td>73.91</td>
</tr>
<tr>
<td>Primary site</td>
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<td></td>
</tr>
<tr>
<td>Tongue/Floor of the mouth</td>
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<td>17.39</td>
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<tr>
<td>Other</td>
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<td>21.74</td>
</tr>
<tr>
<td>Size tumour (T)</td>
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</tr>
<tr>
<td>1 or 2</td>
<td>2</td>
<td>4.34</td>
</tr>
<tr>
<td>3 or 4</td>
<td>10</td>
<td>21.74</td>
</tr>
<tr>
<td>Lymph node metastasis (N)</td>
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<td></td>
</tr>
<tr>
<td>0 or 1</td>
<td>6</td>
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<tr>
<td>2 or 3</td>
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<td>6.52</td>
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<tr>
<td>Stage</td>
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<tr>
<td>I or II</td>
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</tr>
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<td>III or IV</td>
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<tr>
<td>Histological grade of p-Akt</td>
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<tr>
<td>Well differentiated</td>
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<tr>
<td>Moderate</td>
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<td>42.11</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>7</td>
<td>36.84</td>
</tr>
</tbody>
</table>

Chi-square.

*To calculate the histological grade of p-Akt, we considered \(N = 40\).
range of malignancies (19, 20), and it has been proven that p-Akt is overexpressed in OSCC (1, 21, 22). This study sought to evaluate the immunohistochemical expression of the protein p-Akt in samples of OSCC and to correlate the expression status with survival, histological grade and some clinicopathological features of patients.

Metastasis to regional lymph nodes represents the major cause of mortality in patients with head and neck squamous cell carcinoma (23), suggesting that inhibition of metastasis would probably improve the prognosis for these patients. Our results have revealed that metastasis to lymph nodes is statistically associated with the expression of p-AKT in OSCC \( (P = 0.006) \). Li et al. (2) in 2013, using tissue microarray analysis to verify the validity of p-Akt\(^{Thr308} \) as a prognostic biomarker in OSCC, demonstrated that increased expression of p-Akt\(^{Thr308} \) in OSCC was significantly associated with an increased frequency of lymph node metastasis \( (P = 0.024) \), corroborating our findings.

The diversity of processes by which cells develop their most aggressive and invasive phenotype requires a knowledge of the different protein pathways. There are a range of proteases that control the degradation and remodelling of the extracellular matrix (ECM) leading to tumour invasion and proliferation (23). Matrix metalloproteinases 2 (MMP-2) and 9 (MMP-9) are the most extensively studied proteins of the MMP family as a result of their high correlation with invasion and migration in cancer. The PI3K/AKT signalling pathway is involved as an upstream trigger of MMP-2 and MMP-9 regulation (23, 24). Previous studies have demonstrated that Akt increases the production of proteases when it is activated and that when there is suppression of Akt, the expression of MMP-2 and MMP-9 is inhibited, resulting in a reduction in invasion and metastasis in OSCC (23, 24). Moreover, high levels of MMP-2 and MMP-9 have been reported in samples of OSCC (25) associated with poor prognosis (including the development of lymph node metastases and poor survival) (26, 27).

Another mechanism associated with metastasis is the epithelial–mesenchymal transition (EMT) process that causes loss of epithelial cell–cell adhesions. A characteristic feature of loss of epithelial cell adhesion is a reduction in E-cadherin expression (28). Luo et al. (29) in 2014, studying the association of E-cadherin expression with the prognosis of OSCC, demonstrated that patients with reduced expression of E-cadherin appear to have a poorer overall survival compared with those with normal or higher expression of E-cadherin. Furthermore, Grille et al. in 2003, investigating the role of AKT in the biology of human squamous cell carcinoma lines, showed that AKT activation causes EMT, characterised by downregulation of numerous epithelial cell-specific proteins, including E-cadherin and β-catenin, and upregulation of the mesenchymal cell-specific protein vimentin. They concluded that an important consequence of the AKT activation often detected in human carcinomas is the acquisition of an invasive phenotype (30), corroborating our findings.

Therefore, we suggest that the p-Akt pathway operates in metastasis (through its actions in EMT associated with E-cadherin) and in tumour invasion (through association with MMP-2 and MMP-9).

There was also a statistically significant association \( (P = 0.004) \) between the expression of p-Akt and tumour size. When activated, p-Akt is important for cell survival because it promotes cell cycle progression and inhibits apoptosis (31–33). This relationship has been proved in studies of inhibitors of the p-Akt pathway, which found significant tumour growth inhibition associated with decreased levels of p-Akt (5, 32, 34–37). One of the cellular mechanisms through which the p-Akt pathway acts that may explain its relationship with tumour size is the progression of cell proliferation. Among the proteins involved in cell proliferation, related to the mechanisms of action of p-Akt, we can cite p21 and p27. Transition of cells through the G1/S checkpoint is controlled by pRb (retinoblastoma) protein, which suppresses the transcription of a battery of genes required for the G1/S traverse. The CDKs (cyclin-dependent kinases) can phosphorylate pRb protein and promote cell cycle progression. The function of the CDKs is regulated by a family of inhibitory proteins (notably p21 and p27). In cells stimulated to proliferate, p21 and p27 move from the nucleus to the cytoplasm, where they are phosphorylated by p-Akt. Consequently, these proteins are no longer able to promote cell cycle control, being accumulated in the cytoplasm (7, 38).

Our study also revealed a statistically significant inverse relationship between the presence of p-Akt and survival of patients, suggesting that this protein may be an important prognostic factor. Other studies have found similar results. Ziwe et al. in 2007, studying the prognostic significance of p-Akt activation in a cohort of patients with OSCC, found that cases with low p-AKT expression exhibit a higher probability of survival (44.9% vs. 27.9%, \( P = 0.05 \)) (13). These results corroborate the study of Li et al. (2) of 2013, which showed that increased p-Akt\(^{Thr308} \) expression was significantly associated with lower survival.

When activated AKT inhibits apoptosis and promotes cell survival by phosphorylating various substrates (39), these include members of the mdm2 family. Phosphorylation of mdm2 results in the translocation of this protein from the cytoplasm to the nucleus, reducing the levels of p53 (an important tumour suppressor playing a central role in a complex network of molecular interactions). The degradation of p53 has been implicated in the aetiology of OSCC (39–43). Sam et al. (40), in 2012, reported that mdm2 variants were present in OSCC samples at a high frequency and were significantly associated with OSCC development. This suggests that mdm2 variants may play an important role in oral carcinogenesis. Besides mdm2 protein, other proteins with anti-apoptotic activity have their actions mediated by p-Akt, such as NF-Kb, Fas, Bcl-2 and Bcl-xL (44).

The diversity of the interaction pathways of the p-Akt protein (and its association with proteins that control the activities of apoptosis, proliferation and invasiveness) may explain the association found in this study between p-Akt immunostaining and patient survival.

In conclusion, because the p-Akt pathway controls a variety of critical cellular pathways during the carcinogenic process, including those leading to increased cell proliferation (8, 9), enhanced tumour cell invasion (1, 45), cell metabolism (10) and angiogenesis (8, 9), high p-Akt expression in an OSCC patient may predict a high probability of recurrence, tumour growth and shorter
survival time. Therefore, the inhibition of this protein could bring vital therapeutic and preventive benefits for the treatment of OSCC. Genetic and epigenetic investigations are now necessary to corroborate the results obtained in the current study.

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**Acknowledgements**

The authors acknowledge the Pará State Research Foundation (FAPESPA) for financial support and scholarship grants.

**Conflicts of interest**

The authors state that they have no potential conflict of interest.