Tissue microarray analysis reveals the expression and prognostic significance of phosphorylated AktThr308 in oral squamous cell carcinoma

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Objectives. We aimed to investigate the association between the expression of phosphorylated AktThr308 (p-AktThr308) in oral squamous cell carcinoma (OSCC) tissues and clinicopathological parameters of OSCC patients and to verify the validity of p-AktThr308 as a prognostic biomarker.

Study design. One hundred and ninety-one patients with OSCC were recruited for the study. We tested the expression of p-AktThr308 by immunohistochemistry (IHC) with tissue microarray (TMA) and analyzed with digital pathology analysis software. The clinicopathological parameters of all patients were collected from follow-up.

Results. P-AktThr308 was detected in 95.2% of OSCC patients. The expression of p-AktThr308 was significantly correlated with local recurrence and five-year survival rate. High expression of p-AktThr308 in OSCC was associated with poor prognosis.


Oral squamous cell carcinoma (OSCC) is one of the most common malignancies, affecting about 275,000 individuals annually worldwide.1 Recently, an increase in OSCC incidence has been reported, probably due to greater general life-expectancy and increasing alcohol and tobacco consumption.2 Additionally, it has also been reported that human papillomavirus (HPV) infection might be involved in the pathogenesis of OSCC.3 OSCC has a tendency for loco-regional recurrence, second primary tumors and poor survival.4 In clinical practice, the clinical tumor-node-metastasis (TNM) classification system has been widely used for the prediction of OSCC prognosis; however, the predictive effect is poor, even for patients in the early stage (I and II) groups. Therefore, it is important to search for new biomarkers for the diagnosis and prognosis of OSCC.

The phosphorylated Akt (p-Akt) is an activated form of Akt that plays a central role in a variety of human cancers. Akt, also known as protein kinase B (PKB), is a 52 kDa serine/threonine kinase with three isoforms: Akt-1, Akt-2, and Akt-3.5 All isoforms of Akt share a high percentage of amino acid identity. Each isoform is composed of three functionally distinct regions: an N-terminal pleckstrin homology (PH) domain involved in lipid binding, a kinase domain and a C-terminal ‘tail.’ Akt activation is initiated by binding of its PH domain with 3-phosphoinositides generated by the phosphoinositides 3-kinase (PI3 K).5 After that, Akt will be translocated to the plasma membrane. However, Akt is not fully activated until it is further phosphorylated by 3-phosphoinositide-dependent kinase1 (PDK1) at Thr-308 and by PDK2 at Ser-473.6 The activation of Akt is negatively regulated by phosphatase and tensin homolog deleted on chromosome ten (PTEN), a tumor suppressor gene deleted in multiple human cancers.7 Akt activation has been implicated in a variety of biological responses including nutrient metabolism, cell growth, apoptosis, and survival. Active Akt promotes cell survival by phosphorylating and inactivating the pro-apoptotic B-cell

Statement of Clinical Relevance

Increased p-Akt expression in OSCC may indicate poor clinical outcome of OSCC, therefore, it can have significance in treatment planning.

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lymphoma-2 (Bcl-2) related protein, Bcl-2/Bcl-XL-associated death promoter (BAD), as well as affecting the transcriptional response to apoptotic stimuli and the activity of the p53 family. It has also been reported that Akt maintains cell size and survival by increasing mammalian target of rapamycin (mTOR)-dependent nutrient uptake. Moreover, p-Akt activates mTOR through direct phosphorylation and inhibition of the tuberous sclerosis 2 (TSC2) tumor suppressor. Alteration of Akt activity has been demonstrated in several human cancers including OSCC. Akt activation is associated with enhanced tumor cell invasion of pancreatic carcinoma cells and OSCC cells. Furthermore, it has been linked to aggressive clinical behavior and loss of histological features of epithelial differentiation in ovarian carcinomas. In general, activation of p-Akt regulates cell survival, proliferation, and invasion. Hence, p-Akt might be a good predictor of tumor aggressiveness.

The prognostic value of p-Akt in cancers has been reported in several previous studies, however, the sample size of these studies was relatively small. In reported in several previous studies, however, the sample size of these studies was relatively small. In order to get a better understanding of the correlation between p-Akt expression and OSCC, we examined the expression of p-Akt in OSCC patients in a larger sample size by tissue microarray (TMA). Our results showed that elevated p-Akt expression was a predictor for poor clinical outcome of OSCC. Thus, it might be a potential biomarker for the prediction of OSCC prognosis and a possible target for OSCC treatment.

MATERIALS AND METHODS

Subjects and follow-up

This study was conducted in West China Hospital of Stomatology, Sichuan University (Chengdu, China) from 2002 to 2011. It was approved by the Committee for the Use of Human Subjects in Research, Sichuan University. Written informed consent was obtained from all subjects. A total of 191 patients with OSCC were recruited for the study, the diagnosis were all performed by senior specialists in oral surgery and oral pathology with more than 10 years’ experience. Patients were treated surgically and all surgical margins were clear of tumor cells (clear > 5 mm). None of the patients received any preoperative chemotherapy or postoperative adjuvant radiotherapy, but some patients received postoperative chemotherapy.

All patients were examined routinely every 3 months during the first year of follow-up, then every 6 months in the following 4 years and once a year thereafter. Clinical data of patients were censored at the date of last in-person visit or April 30th, 2012, irrespective of whether the patient was alive or not. Survival time was defined as the time from the study entry to death or the last follow-up visit. Essential information of patients was collected, including sex, age, smoking, and alcohol consumption, systemic diseases (including hypertension and diabetes), cell differentiation, tumor stage (small, T1/T2; large, T3/T4), lymph node metastasis, clinical TNM stage (early stage, I/II; late stage, III/IV), chemotherapy after surgical procedure, recurrence, survival time and five-year survival.

TMA design and IHC assay

A Tissue Arrayer device (Beecher Instrument, MD, USA) was used to construct TMA with cores of OSCC tissues from the 191 patients. All cases were histologically reviewed and the areas representative of histological subtype were marked in the corresponding paraffin blocks. In each case, two selected cylinders (1.5 mm in largest diameter) were removed from two different tumor loci of the same block and subsequently brought into one empty ‘recipient’ paraffin block. Thus, four different TMA blocks were constructed from formalin-fixed, paraffin-embedded blocks of 191 OSCCs.

TMA blocks were sectioned at a thickness of 4 μm and heated to 55 °C, dehydrated in xylene and graded alcohols. Antigen retrieval was performed with 0.01 M citrate buffer at pH 6.0 at 95 °C. Before staining, endogenous peroxidase was blocked with 3% hydrogen peroxide solution. Immunohistochemical staining was performed on the TMA slides using primary antibody against p-Akt (Rabbit polyclonal pan-AKT (phospho T308) antibody, abcam, USA). TMA slides were incubated with primary antibody diluted in 50 mM Tris–HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20 at 4 °C overnight, followed by incubations with the ChemMate EnVision/HRP, Rabbit/Mouse (ENV) reagent of Envision Detection Kit (Dako Corporation, Carpinteria, CA, USA) for 30 min. The reaction was visualized with the ChemMate EnVision/HRP, Rabbit/Mouse (ENV) reagent of Envision Detection Kit (Dako Corporation, Carpinteria, CA, USA). TMA slides were counterstained with Harris hematoxylin, dehydrated and mounted. Sections of formalin-fixed, paraffin-embedded human breast carcinoma samples were used as a positive control for p-Akt, and the negative control was run without addition of the primary antibody.

Semi-quantitation of IHC results

The staining images of TMA were acquired using Aperio Scanscope Virtual scanning microscopic imaging system (Aperio Scanscope FL + GL; Aperio, Vista, CA, USA). Saturation and intensity of each tissue core were measured by Positive Pixel Count Algorithm, TMALab, Genie software (Aperio), which determined the average intensity of pixels and the percentage of cells stained within the tissue region of interest. The evaluation was performed three times by three independent investigators. For quality control, 50 tissue cores were randomly
selected from the whole tissue cores, and the saturation and intensity were analyzed by two pathologists using uniform criteria. These tissue cores were evaluated over 8 visual fields at a power of ×400 under a light microscope (Olympus Optical, Tokyo, Japan). No significant differences were found between these two methods.

Staining of p-AktThr308 was scored as average intensity of all pixels ($I_{\text{avg}}$), ($I_{\text{avg}} = (I_{wp} + I_p + I_{np})/N_{wp}$; sum of intensity values for all weak-positive pixels; $I_p$, sum of intensity values for all positive pixels; $I_{np}$, sum of intensity values for all strong-positive pixels; $N_{wp}$, number of weak-positive pixels; $N_p$, number of positive pixels; $N_{np}$, number of strong-positive pixels). The staining of each tissue core was scored with 0 or 1 or 2 or 3 based on the $I_{\text{avg}}$ ($0 = I_{\text{avg}} > 196$, $1 = 182 \leq I_{\text{avg}} < 196$, $2 = 172 \leq I_{\text{avg}} < 182$, $3 = I_{\text{avg}} < 172$).

Statistical analysis

Continuous variables were presented as means ± standard deviations (SD). Categorical variables were presented as frequencies and percentages. Associations between the clinicopathological characteristics of the patients and p-AktThr308 expression levels were analyzed by chi-square test or unpaired Student’s t test. Univariate logistic regressions were used to evaluate the predictive effect of each factor for OSCC recurrence. All potential factors influencing the probability of recurrence were analyzed, including sex, age (<60 or ≥60), smoking, drinking, systemic diseases (including hypertension and diabetes), cell differentiation, tumor stage (small, T1/T2; large, T3/T4), lymph node metastasis, clinical TNM stage (early stage, I/II; late stage, III/IV), chemotherapy after surgical procedure and p-AktThr308 expression level. Furthermore, some factors ($P < .05$) in univariate logistic regression were included in the multivariable step-wise logistic regression model. Survival curves were determined by the Kaplan–Meier method, and the differences were compared on the basis of variables’ category with the use of the log-rank test. To determine the hazard ratio, univariate Cox proportional-hazards model was used to assess the contribution of the following factors: sex, age (<60 or ≥60), smoking, drinking, systemic diseases (including hypertension and diabetes), cell differentiation, tumor stage (small, T1/T2; large, T3/T4), lymph node metastasis, clinical TNM stage (early stage, I/II; late stage, III/IV), chemotherapy after surgical procedure and p-AktThr308 expression level. Multivariate step-wise Cox proportional-hazards model was used to evaluate the overall survival with inclusion of all significant variables ($P < .05$) identified by the univariate analysis. To determine discrimination, the sensitivity and specificity of the given data were identified as well.

### Table I. Clinicopathological parameters and characteristics by p-AktThr308 status of 191 patients with OSCC in TMA

<table>
<thead>
<tr>
<th>Variables</th>
<th>Subgroup</th>
<th>Subgroup</th>
<th>Subgroup</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td>134 (70.2)</td>
<td>63 (64.3)</td>
<td>71 (76.3)</td>
</tr>
<tr>
<td></td>
<td>57 (29.8)</td>
<td>35 (35.7)</td>
<td>22 (23.7)</td>
</tr>
<tr>
<td>Age (year)</td>
<td>&lt;60</td>
<td>≥60</td>
<td>&lt;60</td>
</tr>
<tr>
<td>Smoking</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>99 (51.8)</td>
<td>92 (48.2)</td>
<td>58 (62.4)</td>
</tr>
<tr>
<td>Drinking</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>99 (51.8)</td>
<td>92 (48.2)</td>
<td>56 (60.2)</td>
</tr>
<tr>
<td>Systemic diseases</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>62 (32.5)</td>
<td>129 (67.5)</td>
<td>30 (32.3)</td>
</tr>
<tr>
<td>Cell differentiation</td>
<td>Well</td>
<td>Moderate/poor</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>127 (66.5)</td>
<td>64 (33.5)</td>
<td>56 (60.2)</td>
</tr>
<tr>
<td>Tumor stage</td>
<td>Small (T1/T2)</td>
<td>Large (T3/T4)</td>
<td>58 (59.2)</td>
</tr>
<tr>
<td></td>
<td>106 (55.5)</td>
<td>85 (44.5)</td>
<td>51 (56.6)</td>
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<tr>
<td>Lymph node metastasis</td>
<td>Non-metastatic</td>
<td>Metastatic</td>
<td>73 (74.5)</td>
</tr>
<tr>
<td></td>
<td>128 (67.0)</td>
<td>63 (33.0)</td>
<td>59 (60.2)</td>
</tr>
<tr>
<td>Clinical TNM stage</td>
<td>Early stage (I/II)</td>
<td>Late stage (III/IV)</td>
<td>49 (50.0)</td>
</tr>
<tr>
<td></td>
<td>80 (41.9)</td>
<td>111 (58.1)</td>
<td>31 (33.3)</td>
</tr>
<tr>
<td>Chemotherapy after surgical procedure</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>100 (52.4)</td>
<td>91 (47.6)</td>
<td>48 (51.6)</td>
</tr>
<tr>
<td>Recurrence</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>43 (22.5)</td>
<td>148 (77.5)</td>
<td>30 (32.5)</td>
</tr>
<tr>
<td>Survival time (month)</td>
<td>Mean ± SD</td>
<td>37.2 ± 25.2</td>
<td>42.8 ± 26.4</td>
</tr>
<tr>
<td>Five-year survival</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>61 (31.9)</td>
<td>130 (68.1)</td>
<td>12 (12.9)</td>
</tr>
</tbody>
</table>

*P < .05, unpaired student’s t test was used for hypothesis testing of the relationship between survival time and p-AktThr308. Chi-square test was used for hypothesis testing of the relationship between the other variables and p-AktThr308.

**RESULTS**

**Clinicopathological parameters of 191 patients with OSCC**

A total of 191 OSCC patients were recruited in this study, only 5 patients dropped out for private reasons. Therefore, the discontinuation rate, 2.6%, was quite low, thus having no influence on the interpretation or conclusions. This cohort of OSCC patients included 134 (70.2%) male and 57 (29.8%) female, with a median age of 60 years. The clinicopathological parameters of all the samples can be found in Table I.
Correlation between p-Akt expression and clinicopathological parameters

The expression of p-AktThr308 was scored based on the Iavg (Figure 1). The patients with score 0 (4.7%) and 1 (46.6%) were classified into subgroup 1 (low expression of p-AktThr308), the patients with score 2 (39.8%) and 3 (8.9%) were classified into subgroup 2 (high expression of p-AktThr308). The two groups were well matched to sex and age (Table I).

The increased expression of p-AktThr308 in OSCC was significantly associated with smoking (P = .005), drinking (P = .024), increased frequencies of lymph node metastasis (P = .024), high clinical TNM stage (P = .020), recurrence (P = .002), and decreased survival time (P < .001) and five-year survival rate (P < .001).

Multivariable logistic regression analysis on influencing factors for local recurrence

The potential indexes influencing the probability of recurrence included sex, age (<60 or ≥60), smoking, drinking, systemic diseases (including hypertension and diabetes), cell differentiation, tumor stage (small, T1/T2; large, T3/T4), lymph node metastasis, clinical TNM stage (early stage, I/II; late stage, III/IV), chemotherapy after surgical procedure and p-AktThr308 expression level. All these factors were included in univariate logistic regression analysis (Table II). To get a more precise combined analysis of all factors and to control for confounding factors more effectively, four factors (P < .05) from the univariate logistic regression, including cell differentiation, lymph node metastasis, clinical TNM stage (early stage, I/II; late stage, III/IV) and p-AktThr308 expression, were analyzed in the multivariable step-wise logistic regression model. The result showed that clinical TNM stage (P = .007, OR = 3.068, 95% CI: 1.354-6.948) and p-AktThr308 expression level (P = .008, OR = 2.740, 95% CI: 1.302-5.767) could significantly influence the probability of recurrence (Table III).

Survival analysis

The Kaplan–Meier survival analysis was applied to estimate cancer-specific survival rates. The results showed that the cumulative survival rate of patients with high p-AktThr308 expression was significantly lower than those with low p-AktThr308 expression (P < .001). Additionally, we performed subgroup analysis concerning cell differentiation, tumor stage, lymph node metastasis, clinical TNM stage, and recurrence. In all the subgroups, high p-AktThr308 expression was significantly associated with poor survival (data not shown). Univariate Cox proportional hazards survival analysis showed that the survival of patients was associated with several prognostic factors (Table IV). Poor prognosis was significantly associated with moderate or poor cell differentiation (P = .042), high tumor stage (P = .013), positive lymph node metastasis (P = .001), high clinical TNM stage (P < .001), recurrence (P = .030) and high p-AktThr308 expression level (P < .001). Multivariate step-wise Cox proportional hazards survival analysis confirmed the prognostic value of high clinical TNM stage (P < .001, OR = 1.947, 95% CI: 1.341-2.828) and
high p-Akt Thr308 expression level ($P < .001$, OR = 2.032, 95% CI: 1.415-2.917) (Table V).

DISCUSSION

P-Akt is a protein, which is involved in the regulation of several aspects of cell function. It has been proven that p-Akt is overexpressed in OSCC.13,15,20 The downstream target of p-Akt, phosphorylated mTOR, appears to be a reliable biomarker in human head and neck cancer.21 Additionally, Forkhead box O3a (FOXO3a) activation, which is suppressed by p-Akt, has also been reported to be a potent therapeutic strategy for OSCC.22 Both Thr308 and Ser473 are the main phosphorylation sites of Akt, and are crucial for the formation of p-Akt. In vivo, phosphorylation of Thr308 is not dependent on phosphorylation of Ser473 or vice versa.23 However, the regulatory mechanisms and physiological importance of each phosphorylation site remain unclear. P-AktSer473 is dispensable for Akt downstream targets, TSC2 and
glycogen synthase kinase 3 (GSK3), and the target of rapamycin complex 1 (TORC1) effectors, S6 ribosomal kinase (S6 K) and 4E-binding protein 1 (4E-BP1). But these targets have been implicated in mediating a variety of biological responses in human cancer. P-AktThr308 may play an important role in the conversion of a potentially malignant oral lesion to carcinoma. It has been reported that p-AktThr308 predicts poor prognosis in acute myeloid leukemia. Although a small sample study (84 cases) has shown that p-AktThr308 predicts poor clinical outcome in OSCC through traditional IHC, the correlation between p-AktThr308 and prognosis in OSCC has not been reported. In this study, TMAs were constructed from 191 OSCC tissues, and detailed clinicopathological parameters were collected from regular follow-up. P-AktThr308 expression was detected in 95.2% of OSCCs. Our results showed that p-AktThr308 expression was significantly associated with smoking and alcohol consumption, lymph node metastasis, TNM stage, recurrence, survival times, and five-year survival rate. This was different from a previous study, in which p-AktThr308 was analyzed and was only associated with lymph node metastasis and TNM stage.13

Local recurrence is one of the main causes of treatment failure of OSCC, and contributes significantly to the relatively low survival rates of this neoplasm. The recurrence rate of OSCC varies from 18% to 76% in patients undergoing standard treatment. Our study showed that the elevated p-AktThr308 expression had a significant association with the probability of recurrence in OSCC. Furthermore, survival analysis showed that increased p-AktThr308 expression was significantly associated with lower survival. Various biological activities of p-Akt, such as enhancement of proliferation, invasiveness, and anti-apoptosis, may cause micro-invasiveness at the resected margin and promote the invasion and recurrence of OSCC. Thus, high p-AktThr308 expression in an OSCC patient may predict high probability of recurrence and shorter survival time. Multivariate logistic regression analysis and survival analysis showed that p-AktThr308 and clinical TNM stage were independent prognostic indicators. TNM stage is a conventional, useful index for OSCC prognosis in clinical practice. As noted above, p-AktThr308 showed potential as an excellent predictive biomarker for OSCC prognosis. Furthermore, when the sensitivity and specificity were assessed, p-AktThr308 showed good discrimination as a predictor for OSCC clinical outcome. Thus, p-AktThr308 may be a potential biomarker for OSCC classification. If p-AktThr308 could be used together with clinical TNM stage, it may help identify individuals with a poor prognosis.

In conclusion, increased p-AktThr308 expression was an excellent predictor of poor clinical outcome in OSCC. Therefore, it could represent a potential biomarker for the prediction of prognosis, and potentially be a target for OSCC treatment.

We are grateful to our colleagues at the Departments of Pathology for their valuable technical assistance.

REFERENCES

### Table V. Multivariate analysis of survival of 191 patients with OSCC according to the Cox proportional-hazards model

<table>
<thead>
<tr>
<th>Variables</th>
<th>β</th>
<th>SE</th>
<th>Wald χ²</th>
<th>P value</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical TNM stage (early stage/late stage)</td>
<td>0.666</td>
<td>0.190</td>
<td>12.245</td>
<td>.000</td>
<td>1.947</td>
<td>1.341-2.828</td>
</tr>
<tr>
<td>p-AktThr308 expression (low/high)</td>
<td>0.709</td>
<td>0.184</td>
<td>14.772</td>
<td>.000</td>
<td>2.032</td>
<td>1.415-2.917</td>
</tr>
</tbody>
</table>

OR, odds ratio; CI, confidence interval; Early stage, I/II; Late stage, III/IV.

Fig. 2. ROC curves of p-AktThr308 and clinical TNM stage. The ROC area for p-AktThr308 alone was 0.71 and for clinical TNM stage alone was 0.67, indicating excellent discrimination of p-Akt compared with clinical TNM stage.


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