Loss of PTEN Expression as a Prognostic Marker for Tongue Cancer

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Background: Abnormalities of PTEN, a candidate tumor suppressor gene located at 10q23.3, play an important role in the tumorigenesis of multiple tumor types.

Objectives: To investigate the expression of PTEN and its clinical implication in squamous cell carcinoma of the tongue.

Design: Retrospective analysis of PTEN protein expression in archived primary oral tongue tumor samples.

Setting: Academic center.

Patients and Methods: PTEN expression was determined by immunohistochemical analysis in tissue samples from 41 patients with stage II, III, and IV squamous cell carcinoma of the tongue. All the patients underwent curative surgical treatment with a median follow-up of 81 months. The Kaplan-Meier method was used for survival analysis. Multivariate analysis was performed according to the Cox proportional hazards model.

Results: Lack of staining for PTEN was demonstrated in 12 (29%) of the 41 tumors. Patients whose tumors lacked PTEN expression had a significantly shorter overall survival time ($P = .03$) and event-free survival time ($P = .01$) than those patients with positive PTEN expression. Multivariate regression analysis demonstrated that PTEN expression is an independent predictor of poor outcome when compared with tumor stage and nodal status.

Conclusions: Although genetic alterations of the PTEN gene are rare in head and neck squamous cell carcinoma, loss of PTEN is not an uncommon event in squamous cell carcinoma of the tongue. Lack of PTEN expression may be an independent prognostic indicator for clinical outcome in patients with this tumor type.


HEAD AND NECK squamous cell carcinoma (HNSCC) accounts for 3% to 5% of all malignancies in Western countries, with cancer of the oral cavity accounting for 30% of these cancers. In the United States alone, it was estimated that 30,200 new cases of oral cancer would be diagnosed in 2000, with an estimated 7800 deaths. Of these, an estimated 6900 new cases of oral tongue cancers would be diagnosed in 2000, with an estimated 1700 deaths. While for early-stage tumors excellent cure rates can usually be achieved, the 5-year survival rate for advanced-stage disease is only 40% to 60%, with little improvement during the last 2 decades. To further improve the survival rate of these patients, new biomarkers for understanding tumor biology and its prognostic value are crucial for future management.

PTEN/MMAC1/TEP1 (phosphatase and tensin homolog deleted on chromosome TEN), located at 10q23.3, is a tumor suppressor gene that encodes a dual-specificity phosphatase with lipid and protein phosphatase activity. Germline mutations of PTEN are found in patients with Cowden syndrome, a familial syndrome associated with a predisposition for multiple benign hamartomas, and malignant breast and thyroid neoplasms. Somatic mutation or deletion of PTEN has been reported in a variety of tumor types, including glioblastoma, melanoma, breast, prostate, and endometrial carcinomas. Genetic analysis of PTEN in head and neck cancers has demonstrated alterations in PTEN in 5% to 10% of tumors, suggesting that PTEN may play a role in head and neck tumorigenesis. Because alternative mechanisms may also inactivate gene function, such as promoter hypermethyl-
MATERIALS AND METHODS

STUDY POPULATION

Specimens of oral tongue SCC were obtained from archived tissue samples of surgically resected, pathologic, stage II, III, and IV tumors from 41 patients treated at The University of Texas M. D. Anderson Cancer Center, Houston, from 1991 to 1994 under a prospective oral tongue surgical pathology protocol. All patients were treated by surgery and received a median of 81 months of follow-up care after surgical treatment. Survival data were available for all patients; the minimum length of follow-up care was 10 months. The study population consisted of 25 men and 16 women. The mean age of patients was 56.2 years (SD, 11.2 years).

IMMUNOHISTOCHEMICAL STAINING FOR PTEN PROTEIN

Paraffin-embedded, 4-µm thick tissue sections from all 41 primary tumors were stained for the PTEN protein using a primary rabbit polyclonal anti-PTEN antibody (Zymed Laboratories, San Francisco, Calif.). Deparaffinization of all sections was performed through a series of xylene baths, and rehydration was performed through graded alcohols. The sections were then immersed in methanol containing 0.3% hydrogen peroxidase for 20 minutes to block the endogenous peroxidase activity and were incubated in 2.5% blocking serum to reduce nonspecific binding. Sections were incubated overnight at 4°C with primary anti-PTEN antiserum (1:100). The sections were then processed using standard avidin-biotin immunohistochemical analysis according to the manufacturer's recommendations (Vector Laboratories, Burlingame, Calif.). Diaminobenzidine was used as a chromogen, and commercial hematoxylin was used for counterstaining. Adjacent normal-appearing epithelium within the tissue sections served as a positive internal control.

Representative areas of each tissue section were selected, and cells were counted in at least 4 fields (original magnification ×200). Immunohistochemical staining was classified into 3 groups as previously reported: increased or equal staining intensity compared with the corresponding normal tissue, decreased staining intensity, and absence of staining. All slides were evaluated and scored independently by 2 investigators (J.I.L. and J.-C.S.) who were blinded to the clinical information pertaining to the subjects.

STATISTICAL ANALYSIS

Overall survival and event-free survival curves were estimated by the Kaplan-Meier method, and the resulting curves were compared using the log-rank test. Event-free survival time was calculated from the date of surgery to relapse or death. Two-sided Fisher exact test or the χ² test was used to analyze the association between 2 categorical variables. P<.05 was considered statistically significant. Multivariate analysis was performed according to the Cox proportional hazards model.

RESULTS

In the areas of normal squamous epithelium, staining for the PTEN protein was present and served as a positive internal control. The staining pattern was more prominent in the stratum spinosum and in areas of keratin differentiation, with minimal or no staining in the basal and parabasal cells (Figure 1A). The staining was cytoplasmic. The intensity of staining in the normal-appearing epithelium could be classified as moderate to strong.

Most specimens had areas of normal epithelium adjacent to carcinoma, with the normal epithelium having positive expression and the carcinoma having variable expression levels. PTEN staining among the tumor specimens was negative, diffusely weak, or a heterogeneous pattern of variable intensity. Among the heterogeneously stained specimens, staining was prominent in the well-differentiated areas.

According to our scoring criteria, loss of PTEN expression was noted in 12 (29%) of the 41 SCC tongue specimens. Weak expression was seen in 25 (61%) of the tumors, and strongly positive expression was seen in 4 (10%) of the tumors. The Table shows the relationships between the expression of PTEN and the clinicopathologic factors. The frequency of PTEN expression did not differ significantly by sex. A precise evaluation of tobacco and alcohol use was not available in a subset of our patients; therefore, analysis of PTEN expression in relation to tobacco use or alcohol consumption was not performed. There was no significant relationship between PTEN expression and the histologic grade of the tumor, the clinical stage of the disease, or nodal involvement. Positive PTEN expression was more likely to be present in patients younger than 50 years (P=.03). There was, however, no significant correlation with age and overall survival or event-free survival.
We also investigated the relationship between PTEN expression and patient’s survival. Figure 2A shows a comparison of the Kaplan-Meier overall survival curves, which demonstrates that patients whose tumors were PTEN negative had a significantly shorter survival time than those patients whose tumors were PTEN positive ($P = .03$ by log-rank test). At 5 years, only 4 (33%) of 12 patients whose tumors had negative PTEN expression were alive compared with 19 (65%) of the 29 patients whose tumors had positive PTEN expression. When event-free survival time was analyzed, patients with negative PTEN expression also showed a significantly worse prognosis than did patients with positive PTEN expression ($P = .01$). Of the 12 patients whose tumors were PTEN negative, 9 (75%) had recurrence, metastases, or death at follow-up, whereas 12 (41%) of the 29 patients whose tumors were PTEN positive had recurrence, metastases, or death ($P = .01$) (Figure 2B). As expected, clinical stage (II vs III and IV) and nodal status (N0 vs N1-3) were statistically associated with poor outcome (data not shown). These well-established clinical markers were then compared with PTEN expression status using multivariate analysis. Given the moderate patient sample size and number of events in this study, multivariate analysis could only be performed on 2 covariates at a time. When PTEN status and stage of disease were analyzed in relation to event-free survival time, PTEN continued to be a statistically significant marker ($P = .03$). When the same analysis was performed with PTEN status and nodal status in the model, PTEN remained statistically significant ($P = .02$).

**COMMENT**

PTEN is a tumor suppressor gene that encodes a dual-specificity lipid and protein phosphatase enzyme. Its major substrate is phosphatidylinositol-3,4,5-triphosphate (PIP-3), a direct product of phosphoinositol-3-kinase activity. This substrate mediates growth factor–induced activation of intracellular signaling, notably through serine-threonine kinase Akt (also known as Akt1, RAC1, or PKB), which promotes cell survival and proliferation. In actively proliferating cells with elevated PIP-3 levels, the Akt complex is activated through phosphorylation, and the role of PTEN is to maintain low levels of PIP-3. This is supported by studies that show that induction of apoptosis by low levels of PIP-3 and phosphorylated Akt has been associated with high levels of PTEN. Conversely, loss of PTEN expression results in increased Akt activity and continued cell survival and cell proliferation. In glioma, breast, and prostate cell lines, PTEN has been shown to mediate G1 cell-cycle arrest and/or apoptosis through the suppression of the phosphoinositol-3-kinase-Akt pathway. PTEN, therefore, seems to play an im-

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**Figure 1.** Immunohistochemical staining patterns of PTEN in squamous cell carcinoma of the tongue (primary rabbit polyclonal anti-PTEN antibody and hematoxylin counterstaining, original magnification ×200). A, Normal stratified squamous epithelium with prominent PTEN staining in the stratum spinosum and stratum superficiale and negative PTEN staining in the underlying carcinoma cells. B, Heterogeneous staining pattern with positive PTEN staining in the well-differentiated areas. C, Well-differentiated tumor with negative PTEN staining. D, Well-differentiated tumor with strongly positive PTEN staining.
important role in the modulation of cell cycle progression and/or apoptosis.

Recently, frequent genetic alterations and loss of expression of the PTEN gene were demonstrated in several malignant neoplasms.\(^4\) The studies of PTEN in HNSCC have focused exclusively on searching for mutations or deletions of the gene, with little emphasis on abnormalities at the protein level. In HNSCC, homozygous deletions of PTEN were reported in 2 of 19 patients, and mutations were detected in 1 of 19 patients.\(^5\) Two subsequent studies, however, have failed to demonstrate homozygous deletion or mutation of PTEN in the HNSCC samples studied, in both cell lines and primary tumors.\(^13,14\) Using immunohistochemical analysis of SCC of the tongue from 41 patients, we show that the rate of PTEN inactivation at the protein level may be more frequent than that identified at the genetic level. The loss of PTEN expression in 29% of patients with SCC of the tongue in this study suggests that abrogations of PTEN function may occur through multiple mechanisms. Loss of PTEN expression may be explained by decreased protein synthesis, elevated protein degradation or turnover, or other posttranslational modifications. Another possible mechanism is the epigenetic inactivation of the gene through hypermethylation of the promoter region.\(^15,16\) Indeed, inactivation of other tumor suppressor genes by methylation has been previously reported in HNSCC.\(^17,18\)

Regarding the 41 specimens of invasive SCC of the tongue, we demonstrated that loss of PTEN expression is not a rare event, since it occurred in 12 (41%) patients. The survival time analysis revealed a significant correlation between loss of PTEN expression and overall survival time \((P = .03)\) and event-free survival \((P = .01)\). When compared with other well-established clinical prognostic factors, such as nodal involvement or stage of disease, using multivariate analysis for event-free survival, the prognostic value of PTEN was retained. This suggests that the tumors with loss of PTEN may reflect a more aggressive biological behavior and that PTEN may serve

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### Table 1: PTEN Status and Clinicopathologic Characteristics of Tumor Samples From 41 Patients With Squamous Cell Carcinoma of the Tongue

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total (N = 41)</th>
<th>PTEN Negative (n = 12)</th>
<th>PTEN Positive (n = 29)</th>
<th>P Value</th>
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<tbody>
<tr>
<td>Age, y †</td>
<td></td>
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<td></td>
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<tr>
<td>≤50</td>
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<td>13 (93)</td>
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<td>11 (41)</td>
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<tr>
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<td>16</td>
<td>7 (44)</td>
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<td>23</td>
<td>5 (22)</td>
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<td>≥2</td>
<td>17</td>
<td>6 (35)</td>
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<td>4 (17)</td>
<td>19 (83)</td>
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</table>

*Data are number (percentage) of patients.
†Mean age of patients was 56.2 years.
‡One tumor specimen was of the basaloid type and, therefore, its histologic grade was not included in the analysis.

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**Figure 2.** A, Overall survival curves of patients with squamous cell carcinoma of the tongue according to PTEN expression. B, Event-free survival curves of patients with squamous cell carcinoma of the tongue according to PTEN expression.
as a potential new prognostic marker and interventional tool in the management of SCC of the tongue. The loss of PTEN in multiple tumor types has been linked to advanced disease, and a correlation between loss of PTEN and poor patient outcome has been reported in gliomas. To our knowledge, this is the first report that correlates loss of PTEN expression with poor patient outcome in HNSCC. The fact that all of the patients had tongue cancer and were treated at a single institution with lengthy follow-up care after surgery increased the statistical power of our study.

In summary, we have demonstrated that loss of PTEN is not a rare event in SCC of the tongue and bears an independent prognostic value, therefore suggesting that PTEN plays an important role in tongue tumorigenesis and progression of disease.

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