Short Communication

Activated Checkpoint Kinase 2 Expression and Risk for Oral Squamous Cell Carcinoma

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Abstract

Background: Phosphoactivation of a DNA damage response molecule checkpoint kinase 2 (pChk2) may be a marker of oral epithelial cells that have entered the precancerous and squamous cell carcinoma (SCC) stages. We explored whether there was selective expression of pChk2 in precancerous lesions but not in nonneoplastic tissue of the oral mucosa.

Experimental Design: In a retrospective cohort design, 96 biopsied clinical leukoplakias and erythroplakias with known subsequent progression to SCC were identified from 48 subjects and assigned as the cases group. Expression status of pChk2 was compared with that of the 97 leukoplakias and erythroplakias that did not progress to SCC (control groups) by immunohistochemical analysis. Included in both groups were lesions with histologically confirmed dysplasia and those that lacked histologic evidence of atypia.

Introduction

An estimated 400,000 people worldwide are newly diagnosed with oral squamous cell carcinoma (SCC) annually, which accounts for 5% of all cancers in men and 2% in women (1). Oral SCC poses a threat to public health as it is associated with a <50% mean 5-year survival rate (1). Due to the poor survival rate once malignant transformation occurs, considerable focus is now placed on early detection. During the normal cellular response to DNA damage, the checkpoint kinase 2 (Chk2) protein is phosphorylated at threonine residue 68 to generate an enzymatically active isoform of the protein, hereafter called pChk2. Remarkably, a recent study has shown that pChk2 merits further investigation as a promising biomarker that can discriminate those lesions at risk for developing SCC, regardless of histologic evidence for atypia.

Results: Subjects with pChk2-positive but histology-negative (for atypia) lesions had an 8.6 times higher risk of developing SCC compared with those with pChk2-negative and histology-negative lesions. Overall, the presence of detectable pChk2 staining was able to identify lesions at risk of developing SCC within 3 years with a sensitivity of 85.2%, specificity of 74.2%, and predictive accuracy of 78.2% (odds ratio, 19.9; 95% confidence interval, 7.3-55.5).

Conclusion: This is the first study to include histologically nonatypical cases in the analysis of a putative biomarker for oral precancerous lesions. Our data show that pChk2 merits further investigation as a promising biomarker for oral precancerous lesions and SCCs.
molecular pathways for oral carcinogenesis involves activation of the epidermal growth factor receptor, which in turn initiates the ras/mitogen-activated protein kinase and PI3K/Akt-signaling cascades (9, 10). The genetic alteration and constitutive activation of oncogenic pathways can elicit unscheduled progression into the cell cycle, leading to DNA damage checkpoint activation either directly or indirectly via formation of DNA double-strand breaks (5, 6, 8). The cellular response to DNA damage is largely coordinated by the proteins ATM and ATR, two related serine/threonine kinases that are activated early in the damage response and phosphorylate several key effectors of the response, including the Chk2 kinase (5-8). Thus, in normal cells, pChk2 can serve as an important cellular defense against malignant progression by inducing downstream signaling pathways that halt cell cycle progression, facilitate DNA repair, or promote apoptosis (7, 8).

Despite the above findings, the possible use of pChk2 expression status as an early indicator of oral cancer risk has not been previously investigated. Accurate distinction between oral premalignant and malignant lesions from clinical leuko/erythroplakias would confer opportunities for early intervention. As an initial step in the pursuit of a reliable biomarker for SCC risk assessment with clinical utility, we examined pChk2 expression Assessment.

Materials and Methods

Patients and Tissue Samples. A retrospective cohort of 145 patients with an initial biopsied oral leukoplakias or erythroplakias that resulted in a histologic diagnosis of dysplasia or other nondysplastic diagnosis, but not SCC, were included in the study. Specific histologic diagnosis for all or one “nondysplastic lesion” included epithelial hyperplasia, hyperkeratosis, mucosal inflammation, candidiasis, and oral lichen planus. The patients (n = 48) who developed SCC at a later time in the same location as the initial biopsy were stratified into the cases. For patients with multiple recurrent lesions before developing SCC (n = 26), all biopsied lesions were studied. In total, 144 lesions from 48 patients were included in the cases group. Among the patients with available follow-up information, the ones with a negative history for subsequent oral lesions during the 6-year period after the initial biopsy were selected as the controls (n = 97). The archival tissue blocks corresponding to the biopsied lesions and SCC were retrieved for immunohistochemical assays.

Immunohistochemical Assay to Assess pChk2 Expression Assessment. The formalin-fixed, paraffin-embedded sections (4 μm) were deparaffinized. Antigen was retrieved with 10 mmol/L citrate buffer. Slides were washed in PBS (0.05 mol/L; pH 7.5), and 5% defatted powdered milk in PBS was applied as a blocking agent. For endogenous peroxidase inactivation, 1.5% H2O2, and 0.1% NaN3 were applied. Slides were incubated overnight with primary rabbit polyclonal antibody, anti-Chk2T(1:100 dilution; Cell Signaling). The primary antiserum was then labeled with 50 μL of horseradish peroxidase–conjugated secondary antibody (Envision+R System Labeled Polymer-HRP Anti-rabbit-HRP; Dako-Cytomation), washed, counterstained with hematoxylin, dehydrated, and mounted. Proper positive and negative controls were used with each batch.

Two board-certified oral and maxillofacial pathologists examined the immunostaining results independently and blinded to H&E final diagnosis. Nuclear expression of pChk2 was assessed only in the epithelial components (see Fig. 1). For each case, the fraction of cells exhibiting nuclear immunoreactivity was recorded based on the intensity of staining, weak (+1), moderate (+2), and strong (+3). A cumulative score, a function of staining intensity and fraction of epithelial cells with positive staining, was assigned ranging from 0 (no staining) to 3 (all cells staining with +3 intensity). The background staining was recorded as +0.1 but was excluded from the final scoring. A score of ≥0.09, the 75th-percentile value for controls, was considered positive staining. The pattern of staining, sporadic (focal or scattered positivity) versus diffuse, and its distribution (limited to basal one third, extending up to two thirds, or involving the full thickness of the epithelium) were also recorded. In cases where the interpretation was discordant, a final consensus was reached based on discussion between the two pathologists.

Statistical Analysis. Descriptive analysis (t test and χ2 test) was used to compare the mean and percentage of subject’s age at initial biopsy, gender, clinicopathologic features, and the status of pChk2 expression. Category for pChk2 positive staining was based on the 75% quartile of the control group (score, ≥0.09) because of the abnormal distribution of pChk2. The predictability of pChk2 for SCC was assessed by calculating sensitivity and specificity. Unconditional logistic regression adjusting for age at initial biopsy was used to estimate odds ratios (OR) and the corresponding 95% confidence intervals (CI) for pChk2 biomarker. All statistical analyses were carried out using Statistical Analysis System 9.0 (SAS Institute).

Results

Patient Characteristics. Of the 145 total patients included in the study, 77 were male and 68 were female. Of the cases (n = 48), 22 patients had a history of only one biopsied precancerous lesion before occurrence of oral SCC at the same site (see Table 1). The remaining 26 patients had anywhere from 2 to 8 recurrent lesions at the same location before carcinoma developed at the site. The total number of lesions obtained from the 48 case patients was 144 and includes 48 SCC, 39 with histologic diagnosis of dysplasia of various grades and 57 without histologic atypia (i.e., hyperkeratosis, epithelial hyperplasia, etc.). The mean age at the initial biopsy for case patients was 70 years, and the time lapse from the initial lesions to SCC ranged from 1 month to 13 years.

Of the 97 patients in the control group, the mean age at the one-time biopsy of the nonrecurrent and nonmalignant transforming lesions was 57 years. The majority of the patients were biopsied during 1996 to 1999. Based on histology, 5 patients had a diagnosis of epithelial atypia and 92 had lesions that lacked histologic evidence of atypia (see Table 2).

Based on the clinical diagnosis rendered at the time of biopsy, ~50% were described as leukoplakias and 10%...
as mixed erythroleukoplasias (data not shown). The remaining 40% had more specific diagnosis, such as lichen planus, candidiasis, dysplasia, or carcinoma, which may have presented as leukoplasias, erythroplasias, or erythroleukoplasias. Only one case was described clinically as an erythroplakia. It is well-accepted that the erythroplasias and mixed erythroleukoplasias are associated with worse histologic grade then leukoplasia.

Figure 1. Photomicrographs of representative pChk2 immunostaining. A. Nuclear staining (brown) of the surface epithelium demonstrating considerable variation in the intensity of the stain within a given field. A diffuse staining pattern is observed (as opposed to sporadic and scattered positivity) involving full thickness of the epithelium. Magnification, ×100. B. A mitotic figure staining dark brown +3 intensity (short arrow), the golden brown staining (two arrowheads) is of +2 intensity, the lighter but discernable brown staining (two longer arrows) is of +1 intensity, and the faint brownish color (circle) was considered as background staining. A complete absence of brown stain (rectangle) was interpreted as negative staining. Magnification, ×400. The fraction of cells exhibiting nuclear staining of various intensity (0, +1, +2, and +3) was estimated and added together to formulate the cumulative score. For example, in the field shown ~1% has +3 intensity, 15% with +2, and 30% with +1 intensity, which will yield (1% × 3) + (15% × 2) + (30% × 1) = 0.63 cumulative score. Each slide was independently reviewed thrice and then the results between two pathologists compared to assure consistency.
Such correlation between the clinical presentation and the histologic grade could not be evaluated due to incompletely available clinical information.

**pChk2 Expression and SCC Risk.** A significant high level of pChk2 expression (defined as score ≥0.09) was detected in 91.7% of SCC and in 76.0% of oral lesions that later progressed to SCC (see Table 2). The precancerous lesion (those that later progress to carcinoma that may or may not exhibit histologic atypia) patients with positive pChk2 status were at 10.5 times risk for developing SCC (age-adjusted OR, 10.5; 95% CI, 4.8-22.6) compared with negative pChk2 patients. The OR was considerably higher for histology-confirmed dysplasia lesions in the case group (OR, 14.3; 95% CI, 4.9-41.5).

**pChk2 Expression and Histologic Diagnosis.** Of the 57 lesions in the cases with ‘nondysplasia’ histologic diagnosis, pChk2 immunoreactivity was observed in 71.9%. In comparison, of the 92 nondysplasia lesions among controls, only 25.0% had positive pChk2 staining. Of the five subjects in the control group that had histologic atypia, two were positive for pChk2. Overall, pChk2-positive but histology-negative (for atypia) lesions had an 8.6 times risk of developing SCC at the same site compared with pChk2-negative and histology-negative lesions (age-adjusted OR, 8.6; 95% CI, 3.7-19.8).

**Discussion**

Identification of precancerous oral lesions at risk for malignant transformation currently follows a two-step standard procedure. It first requires clinical detection of leukoplakia and erythroplakia. The second step is to perform microscopic analysis of the biopsied leuko/erythroplakia. Only those with histology-confirmed dysplasias are considered to be precancerous (11). This standard guideline based on histomorphology has obvious limitations. As evidenced in the concept of field cancerization, molecular alterations precede morphologic changes in the epithelial lesions during the initial period.
of oral carcinogenesis (12). Accumulating genetic and epigenetic changes then result in histologically identifiable cellular atypia. Additional mutations of critical genes lead to malignant transformation (9). Therefore, the precancerous lesion may or may not exhibit histologically detectable morphologic alterations necessary for a diagnosis of atypia or dysplasia at the time of a biopsy. Furthermore, each precancerous lesion has varying time lapse until progression to SCC. From our cases group, the shortest time interval for occurrence of SCC from a nondysplastic lesion was within few months, which was similar to that observed for some of the dysplastic lesions. Hence the diagnosis of “precancerous lesion” should not be delayed until there is histomorphologic change. Molecular biomarkers that can reliably identify precancerous cells regardless of the presence of histologic evidence of atypia would contribute to cancer prevention by means of enhanced detection and subsequent treatment.

Our data show that the pChk2 biomarker can discriminate those lesions at risk for developing SCC within three years, regardless of histologic evidence of atypia, with a sensitivity of 85.2% and specificity of 74.2%. This is the first study that includes cases composed of histologically nonatypical cells in the analysis of a molecular biomarker for identification of oral precancerous lesions. It is also the first to provide evidence for activation of pChk2 during oral carcinogenesis. Phosphoactivation of the Chk2 kinase occurs at early stages of premalignancy in a broad spectrum of human cancers (5, 6). As such, expression of the pChk2 is thought to denote the onset of carcinogenesis (5-7), and various studies now support the value of pChk2 as an accurate biomarker of premalignant and malignant cells. For example, both Bartkova et al. (5) and Gorgoulis et al. (6) used immunoassays to show pChk2 expression in premalignant lesions of the lung, bladder, breast, and colon. Although the highest levels of pChk2 were found in premalignant cells, pChk2 expression persists at a slightly decreased intensity after transformation into invasive cancer. Thus, the selective expression of pChk2 in premalignant and malignant lesions and its ability to exclude benign tissues in lung, skin, and colon are well documented. The etiologic factors and oncogenic induction pathways implicated in oral SCC seem to converge at the step of DNA damage and pChk2-mediated DNA damage response. This partially explains the high sensitivity and OR observed in our study.

The data presented in this report are limited by the small sample size and availability of biopsy samples and follow-up information. In addition, control subjects were significantly younger than those who progressed to SCC. Our data, nevertheless, provide evidence that pChk2 may be a promising biomarker meriting further study in a larger cohort. Accordingly, a prospective clinical study is planned to obtain exfoliative oral epithelial smears for pChk2 immunostaining, which may provide a noninvasive and reliable clinical modality for detection of precancerous lesions and SCC.

Acknowledgments
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References

Table 3. Mean years to SCC progression, sensitivity, specificity, accuracy, OR, and median immunostain score for precancerous lesions and SCC

<table>
<thead>
<tr>
<th>Category</th>
<th>Mean years to SCC (SD)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Accuracy (%)</th>
<th>OR (95% CI)</th>
<th>Median immunostain score (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No histologic atypia</td>
<td>3.0 (2.8)</td>
<td>71.9</td>
<td>74.2</td>
<td>73.4</td>
<td>8.6 (3.7-19.8)</td>
<td>0.39 (0.36)</td>
</tr>
<tr>
<td>Atypia/dysplasia</td>
<td>2.9 (2.7)</td>
<td>82.1</td>
<td>74.2</td>
<td>76.5</td>
<td>14.3 (4.9-41.5)</td>
<td>0.55 (0.45)</td>
</tr>
<tr>
<td>Precancerous lesion (combined above two rows)</td>
<td>2.9</td>
<td>76.0</td>
<td>74.2</td>
<td>75.1</td>
<td>10.5 (4.8-22.6)</td>
<td>—</td>
</tr>
<tr>
<td>SCC</td>
<td>—</td>
<td>91.7</td>
<td>74.2</td>
<td>80.0</td>
<td>29.8 (9.4-94.6)</td>
<td>0.54 (0.40)</td>
</tr>
<tr>
<td>Precancerous lesions progressing to SCC</td>
<td>—</td>
<td>64.3</td>
<td>74.2</td>
<td>71.2</td>
<td>5.8 (2.4-13.8)</td>
<td>—</td>
</tr>
<tr>
<td>In &lt;3 y</td>
<td>—</td>
<td>85.2</td>
<td>74.2</td>
<td>78.2</td>
<td>19.9 (7.3-55.5)</td>
<td>—</td>
</tr>
<tr>
<td>After &gt;3 y</td>
<td>—</td>
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