Strong Correlation between c-erbB-2 Overexpression and Overall Survival of Patients with Oral Squamous Cell Carcinoma

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Abstract
Overexpression of c-erbB-2 (also known as HER-2/neu) has been found in many human cancers, including head and neck squamous cell carcinoma (SCC). We therefore examined the protein expression of the oncoprotein in oral SCC primary tumor samples and compared its relationship with clinical stages and survival rate. Out of 80 cases of oral SCC, high expression level (+ + or +++) of c-erbB-2 was found in 41 cases. Of the 80 cases with follow-up information, 39 were further investigated for the correlation of expression level of c-erbB-2 and survival rate. Overexpression of the oncoprotein was significantly correlated with shorter overall survival, and the patients with low and no expression of c-erbB-2 had much higher survival rates. Overexpression of c-erbB-2 was also significantly correlated with nodal stage and metastasis. We found that high expression level of c-erbB-2 was frequently detected in oral cancer cell lines but not in the other head and neck SCC cell lines. Thus, we conclude that overexpression of c-erbB-2 is a frequent event in oral SCC and is correlated with poor survival and may be used as a poor prognostic factor.

Introduction
The WHO has described oral cancer as one of the 10 most common cancers in the world (1). Oral SCC is the most common histopathological type of oral cancer, accounting for approximately 91% of all oral malignancies (1, 2). The prognosis of patients with oral SCC remains poor; the 5-year survival rate averages about 50% and has not changed much over the past 10 years (3). Therefore, there is interest in determining whether specific gene alterations occur in oral cancer and whether they are associated with disease behavior. Such studies may provide more accurate and useful prognostic markers and eventually more effective treatment.

The c-erbB-2 gene (also known as HER-2/neu) encodes a transmembrane protein of Mr 185,000 (p185), with extensive sequence homology to EGFR (4–6). Like EGFR, c-erbB-2 has intrinsic tyrosine kinase activity (7–10) and can interact with many different cellular proteins, such as src-homologous collagen, phospholipase C-γ, and GTPase-activating protein, which mediate a signal transduction pathway (11, 12). Overexpression of c-erbB-2 has been shown to increase metastatic potential by promoting the multiple adhesion and invasion steps of the metastatic cascade (13). This suggests that the c-erbB-2 gene may play an important role in carcinogenesis. c-erbB-2 overexpression has been found to correlate with poor survival of several human cancers, including breast, ovarian, lung, and stomach cancers (14–20). Although c-erbB-2 overexpression has been detected in head and neck SCC (21–23), a correlation between c-erbB-2 overexpression and survival has not been reported in oral SCC.

The purposes of this study were to examine the expression of the c-erbB-2 in oral tumor specimens as well as in head and neck SCC cell lines and to determine the relationship between the protein expression and survival in oral SCC. For the latter, we investigated retrospectively a group of patients who had been treated by surgery with or without postoperative chemotherapy, radiotherapy, or both. Our results indicate that overexpression of c-erbB-2 is significantly correlated with shorter overall survival in oral SCC.

Materials and Methods

Patient Specimens. Eighty specimens of primary oral SCC were obtained from the Department of Oral Pathology, Ninth People’s Hospital, Shanghai Second Medical University, People’s Republic of China. These patients (60 male and 20 female) underwent surgical treatment in the hospital between 1989 and 1990. Their ages ranged from 10 to 76 years (median, 54 years). The primary tumors were graded by the WHO clas-
sification of histological differentiation (24). Sixty-three cases were defined as grade I, 14 as grade II, and 3 as grade III. The tissues involved in tumor included tongue (40 cases), gum (11 cases), floor of the mouth (8 cases), palate (5 cases), cheek (10 cases), mucosa of lip (3 cases), mandible (1 case), and mucosa of mandible molar areas (2 cases). The specimens were routinely fixed in formalin, embedded in paraffin, sectioned serially every 5 µm, and stained with H&E. Accurate and detailed information on 39 of 80 patients was available. These 39 patients were therefore used to analyze the correlation between overall survival and c-erbB-2 expression. They received either chemotherapy or radiotherapy or both after their surgical treatment. Since the surgical treatment, 10 patients have died of the primary tumor and one has died of metastasis of the primary tumor to the lung.

**Immunohistochemical Staining.** The immunoperoxidase staining method used was modified from the avidin-biotin complex technique described by Hsu and Raine (25) and Hsu et al. (26). Briefly, tissue sections were deparaffinized and dehydrated in a graded series of alcohol. Then, they were digested in 0.05% trypsin for 15 min, blocked in 0.3% H2O2 in methanol for 15 min, and treated with 1% (v/v) normal horse serum for 30 min. Then, the slides were incubated for 3 h at room temperature with 0.003 µg/ml c-neu (Ab-3) monoclonal antibody diluted 1:3200. After extensive washing with PBS, the slides were incubated for 30 min at room temperature with biotinylated goat antimonue IgG antibody diluted 1:200 in PBS. The slides were subsequently incubated for 60 min at room temperature with avidin-biotin-peroxidase complex diluted 1:100 in PBS. The peroxidase-catalyzed product was visualized with 0.125% aminoethyl carbazole chromogen stock solution (Sigma Chemical Co.). Between steps, the slides were rinsed for 2 minutes in PBS (pH 7.6) three times. After light counterstaining with Mayer’s modified hematoxylin (Sigma Chemical Co.), the slides were dehydrated and mounted. Negative controls, in which PBS was used instead of the primary antibody, were run with each batch of staining. A previously identified strongly staining tumor tissue section was used as a positive control. Inter-assay and intra-assay consistency was maintained by including these positive and negative controls with each batch of slides stained. The prepared slides were examined by light microscopy. Those tumor cells that were immunostained with red granules were considered positive, and those cells without any immunostaining were considered negative. All the cases were reviewed independently by two pathologists. The immunoreactivity of c-erbB-2 was ranked into four groups according to percentage and staining intensity of positively stained tumor cells. Both cytoplasmic and membrane staining were observed. The cases with strong staining (>50% cells stained) were scored as high, those with moderate staining (20–50% cells stained) as intermediate, those with weak staining (up to 20% cells stained) as low, and those with no staining as negative.

**Quantitative Computerized Image Analysis.** c-erbB-2 immunostaining was objectively quantified by computerized image analysis with a SAMBA 4000 Cell Image Analysis System (Imaging Products International, Inc., Chantilly, VA), and a ×20 objective. The image analyzer was an integrated system of Windows (Microsoft, Redmond, WA)-based software for densitometric, morphometric, and red-green-blue to hue saturation intensity colorimetric analysis of cells and tissues and consisted of Nikon microscope with ×10, ×20, and ×40 objectives and a JVC 3-CCD KY-17U video camera. The SAMBA 4000 image analyzer consisted of an IBM 80-386/33-MHz clone with a VGA graphics board, 80-MB hard disc, 8 MB of RAM, Syquest 88-MB removable cartridge system, Nikon Labophot-2 microscope, Hewlett Packard PaintJet color printer, Mitsubishi color video copy processor (printer), and Polaroid freeze-frame video recorder with 35-mm film processing and instant prints capability.

The software enabled the operator, after evaluating several fields on control slides, to set a density threshold value for the best discrimination between cell nuclei and cytoplasm, stroma, or background. Similarly, a threshold for positive values was obtained by averaging the results from 10 fields of negative control tissues in which the primary antibody was replaced with an isotype-matched irrelevant antibody. The threshold value was the minimum absorbance at which the image analyzer would discriminate negatively from positively stained cytoplasm. After that value was read, background subtraction was automatically performed on every tissue after measuring an empty field on the slide. For each tissue, we measured the cytoplasmic area, MOD, labeling index (labeled nuclear area/total nuclear area measured), and quick score (labeling index × MOD). To permit simultaneous measurement of c-erbB-2 immunostaining, the samples were immunostained by avidin-biotin complex technique (25, 26), with aminoethyl carbazole chromogen as an indicator and Mayer’s hematoxylin solution as a counterstain.

**Statistical Analyses.** The χ2 test was used for analysis of contingency tables, with P < 0.05 as the criterion of statistical significance. Survival curves were calculated by the method of Kaplan and Meier, and differences between curves were analyzed by the Wilcoxon test.

**Cell Lines and Culture.** Seven head and neck SCC cell lines were obtained from the Department of Head and Neck
Surgery of the University of Texas M. D. Anderson Cancer Center. 686/LN-1 was derived from lymph node metastasis of the tongue. The other cell lines were described previously (27). The human breast cancer cell line MDA-MB-453, which has overexpression of c-erbB-2, was used as a positive control in the immunoblotting experiment. All cells were grown in DMEM/F12 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics. Cells were grown in a humidified incubator at 37°C under 5% CO₂ in air.

**Cell Lysis and Western Blot Analysis.** The cells were grown to about 90% confluence before harvesting. They were washed twice with cold PBS and then lysed on ice with 0.5 ml of RIPA-B lysis buffer (20 mM Na₂PO₄, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% aprotinin, 1 mM phenylmethylsulfonyl}

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![Fig. 1 Immunohistochemical staining of oral SCC for c-erbB-2. A, high; B, intermediate; C, low; D, no expression. ×400.](image-url)
fluoride, 100 mM NaF, and 2 mM Na3VO4). The cells were then scraped from the tissue culture dishes, and the residual cell debris was removed by centrifugation. For immunoblotting, the samples were separated by 6% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Non-specific membrane binding was minimized with a blocking buffer (5% nonfat dry milk in Tween 20-PBS). The membrane was then incubated with primary antibodies (c-neu (Ab-3) monoclonal antibody, Oncogene Science, Inc., Uniondale, NY) followed by incubating with horseradish peroxidase-goat anti-mouse antibody (Boehringer Mannheim Corp., Indianapolis, IN).

Results

Immunohistochemical Staining of Oral SCC for c-erbB-2. The results of immunostaining of 80 primary oral SCCs for c-erbB-2 expression are shown in Table 1. There was wide variation in the level of c-erbB-2 expression in the primary oral SCC specimens. Fifteen (18.8%) of them showed high levels of cytoplasmic and membrane staining, 26 (32.5%) showed medium levels, and 39 (48.8%) showed low/no levels of immunoreactivity. A representative example is shown in Fig. 1. For c-erbB-2 expression, 54 of 63 cases of grade I disease (85.7%), 13 of 14 cases of grade II disease (92.9%) and all 3 cases of grade III disease (100%) demonstrated positive staining. Statistical analysis by the χ² test showed that histological grade was significantly associated with expression level of c-erbB-2 expression (P < 0.01; Table 1).

Correlation between c-erbB-2 Expression and Poor Survival. The significance of the expression of the oncoprotein in human oral SCC was studied retrospectively in 39 cases that had been followed up since their surgical treatment. Table 2 provides clinical data for the 39 oral SCC patients. The SAMBA 4000 cell image analysis system was used to objectively quantitate cytoplasm and membrane staining of the tumor specimens to determine the exact amount of c-erbB-2 protein expression in oral SCC. The reliability of the method has been discussed previously (28). The tumor specimens were observed under a light microscope connected with a computerized image analysis system. Ten microscopic fields from each of the specimens were analyzed. The oncprotein level was measured as immunostaining absorbency. Next, the expression levels were divided into three groups: high expression, 11 cases (28.2%); intermediate expression, 39 cases (51.28%); low expression, 20 cases (20.51%) with a MOD of 10-26; and low expression, 20 cases (20.51%) with a MOD of 26-70; intermediate expression, 8 cases (20.51%) with a MOD of 12-26; and low expression, 20 cases (51.28%) with a MOD of 0-10. There was a significant correlation between the MOD reading and visual quantification of protein expression levels in the tumor specimens (P < 0.001).

The relationship between TNM staging and c-erbB-2 expression is shown in Table 3. The expression of c-erbB-2 correlated with nodal stage, as well as metastasis, with statistical significance (P < 0.05 by the χ² test). The results suggest that c-erbB-2 expression may be associated with oral cancer metastasis, consistent with animal experiments that indicated that enhanced c-erbB-2 expression increased metastatic potential (13). Also, high expression level of the oncprotein was significantly associated with a shortened overall survival rate, and the patients with low and no expression had significantly higher survival rates, as shown in Fig. 2 (P < 0.001 by the Wilcoxon test).

Western Blot Analysis of Head and Neck SCC Cell Lines. Western blot analysis revealed that a high level of c-erbB-2 was found in all three oral cancer cell lines examined (Tu 138, 686/LN-1, and Tu 167) and that the others four lines had only low or no expression relative to the breast cancer cell line, MDA-MB-453, which is known to overexpress c-erbB-2 (Fig. 3). This result confirms the high frequency of c-erbB-2 overexpression in oral SCC and also suggests that c-erbB-2 expression may not be common in other head and neck SCCs.

Discussion

We previously demonstrated that there was a significant association between c-erbB-2 overexpression and oral cancer progression (23). In the study reported here, we used a different cohort to further investigate the relationship between c-erbB-2 expression and patient survival, histological grade, and TNM stage. The c-erbB-2 gene product was expressed in 86.4% of the
Table 3  Relationship between TNM staging and c-erbB-2 expression in 39 oral SCCs

<table>
<thead>
<tr>
<th>TNM</th>
<th>No. of cases (%)</th>
<th>Level of expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>High</td>
</tr>
<tr>
<td>T1</td>
<td>21 (53.85)</td>
<td>4 (10.26)</td>
</tr>
<tr>
<td>T2</td>
<td>13 (33.33)</td>
<td>4 (10.26)</td>
</tr>
<tr>
<td>T3</td>
<td>5 (12.82)</td>
<td>3 (7.69)</td>
</tr>
<tr>
<td>N0</td>
<td>28 (71.79)</td>
<td>5 (12.82)</td>
</tr>
<tr>
<td>N1</td>
<td>2 (5.13)</td>
<td>2 (5.13)</td>
</tr>
<tr>
<td>N2</td>
<td>2 (5.1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>N3</td>
<td>7 (17.95)</td>
<td>4 (10.26)</td>
</tr>
<tr>
<td>M0</td>
<td>28 (71.79)</td>
<td>5 (12.82)</td>
</tr>
<tr>
<td>M1</td>
<td>11 (28.21)</td>
<td>6 (15.38)</td>
</tr>
<tr>
<td>Total</td>
<td>39 (100)</td>
<td>11 (28.21)</td>
</tr>
</tbody>
</table>

* No. of cases (%).
* P values were calculated by the χ² test.

Fig. 2  Correlation between c-erbB-2 expression and survival rate. Survival curves were calculated by the method of Kaplan and Meier. P values: high versus low, P < 0.001; high versus intermediate, P < 0.125; high versus low/none, P < 0.028; high versus intermediate, P < 0.015.

80 oral SCC specimens examined. c-erbB-2 overexpression may, therefore, be a characteristic of oral SCC. In this study, c-erbB-2 gene expression was significantly correlated with poor survival (P < 0.001). Amplification of the EGFR gene has been reported in both cell culture and fresh tissue samples (29–31). A significant correlation between EGFR levels and tumor size and stage was also reported (31). Because the EGFR family, including c-erbB-2, c-erbB-3, and c-erbB-4, is known to form homodimers and heterodimers, which may trigger different signal pathways, it will be interesting to examine EGFR, c-erbB-3, or c-erbB-4 expression and to analyze whether a subset of homodimers or heterodimers might be correlated to survival or other clinicopathological parameters of oral SCC.

In the study reported here, we observed both cytoplasmic and membrane staining of c-erbB-2 protein in our tumor specimens. We obtained similar results by using another antibody (NCL-CB11, Vector Laboratories, Inc.) specific to the c-erbB-2 protein (data not shown). The validity of cytoplasmic staining of c-erbB-2 protein has been questioned. Some researchers have demonstrated c-erbB-2 cytoplasmic staining in breast tumors that correlates with the c-erbB-2 amplification (32). On the other hand, some argue that c-erbB-2 cytoplasmic staining could be an artifact or nonspecific (33–35). However, several reports indicated that EGFR and c-erbB-2 can be detected in the nucleus (36–38). Furthermore, c-erbB-3 was reported to have a secreted form, as well as being a transmembrane tyrosine kinase receptor (39). These findings strongly suggest that these members of the EGFR family not only function as membrane-associated receptors but are also capable of performing other unknown functions inside or even outside the cell. In addition, multiple antibodies gave the same results; therefore, we believe that c-erbB-2 cytoplasmic staining of tumor specimens is not nonspecific.

It was previously demonstrated that overexpression of c-erbB-2 could be detected in head and neck SCC. However, the expression of c-erbB-2 did not correlate to survival (21, 22). These earlier reports have included tumor specimens from other head and neck regions, such as the larynx and pharynx, as well as the oral cavity. From our Western blotting results, we found that high expression of c-erbB-2 was found only in the oral SCC (Fig. 3). These results suggest that c-erbB-2 overexpression may be a characteristic of oral SCC but not of the other head and neck SCCs. This may explain why the earlier reports did not show the correlation of c-erbB-2 expression level and survival. In our study, there are five metastatic patients in the low/none c-erbB-2-expressing group having a long (5-year) survival. Overexpression of c-erbB-2 in non-small cell lung carcinoma has been shown to induce resistance to chemotherapeutic agents...
(40). Low c-erbB-2 expression levels may contribute to longer survival rate in those cases because they may be more sensitive to postsurgery chemotherapy. In conclusion, c-erbB-2 expression is common in oral SCC, and high expression level of the protein is strongly correlated with poor survival.

Acknowledgments

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