Epithelial-Myoepithelial Carcinoma of Salivary Glands
A Clinicopathologic, DNA Flow Cytometric, and Immunohistochemical Study of Ki-67 and HER-2/neu Oncogene

KYUNG JA CHO, MD,1 ADEL K. EL-NAGGAR, MD,1 NELSON G. ORdonez, MD,1 MARIO A. LUNA, MD,1 JOHN AUSTIN, MD,2 AND JOHN G. BATSAKIS, MD1

Thirty-one salivary gland epithelial-myoepithelial carcinomas from 26 patients were studied by DNA flow cytometry, and immunostaining for Ki-67 and HER-2/neu oncogene product. The results were correlated with clinicopathologic factors and patient outcome. The tumor most commonly involved the parotid gland, and mainly affected patients in their 6th to 8th decades. The clinical course was characterized by a high incidence of local recurrence (50%) and not infrequent distant metastasis (25%). None of the patients in this cohort died of disease. DNA content analysis revealed 21 neoplasms with DNA diploidy and 5 tumors with DNA aneuploidy; all aneuploid cases were near-diploid (hyperdiploidy) and showed low proliferative activity. All aneuploid and 60% of the diploid neoplasms developed recurrences and/or metastases. Immunohistochemical analysis of Ki-67 proliferation markers also showed low overall growth fractions. Interestingly, Ki-67 immunoreactivity was largely restricted to myoepithelial cells, suggesting a central role for this cell in the development of these tumors. HER-2/neu oncogene analysis failed to demonstrate overexpression in any of the tumors examined.

This study indicates that epithelial-myoepithelial carcinoma is a low grade malignant neoplasm with a high propensity for recurrence. HER-2/neu oncogene and Ki-67 offer no additional advantages over current factors in the biologic evaluation of these neoplasms. DNA aneuploidy may allow for the identification of a subset of tumors that is more prone to recurrence and metastasis, but further studies with extended follow-up are needed. (Key words: Epithelial-myoepithelial carcinoma; Salivary gland tumors; HER-2/neu oncogene; Flow cytometry; Proliferative index; Ki-67 growth fraction) Am J Clin Pathol 1995; 103:432-437.
down and incubated in 3 mL of prewarmed pH adjusted (1.5) 5 mg/mL pepsin (Sigma Chemical Co, St. Louis, MO) for 30 minutes at 37 °C. The enzymatic digestion was stopped by adding 175 μL of 0.5 mg/mL peptatin A (Sigma Chemical Co, St. Louis, MO) after mechanical disaggregation of the tissue using syringes with 18-gauge needles. The suspensions were washed twice with DPBS, and filtered through 37 μm nylon mesh filter material (Small Parts, Miami, FL). The concentration of nuclei was measured with an automated cell counter (Coulter ZBI, Coulter Electronics, Hialeah, FL), and adjusted to 106 cells/mL. A 900 μL aliquot of the final dilute was treated with 100 μL of prewarmed 1 mg/mL RNAse A (Worthington Biochemical, Freehold, NJ) for 30 minutes at 37 °C. The suspensions were then stained with 50 μL of 0.5 mg/mL propidium iodide (Sigma Chemical, St. Louis, MO) for 30 minutes at 4 °C, and analyzed in a Coulter PROFILE flow cytometer (EPICS Division, Coulter Electronics, Hialeah, FL). In each sample, at least 5,000 cells were collected. Non-neoplastic tissue was considered to constitute the biological diploid standard and the first G0/G1 peak, accounting for at least 10% of the cells analyzed, was present. The proliferative fraction was determined as the ratio of the peak channel number of the test sample to the peak channel number of the normal diploid control. An aneuploid stemline was acknowledged when a distinct second G0/G1 peak was present. The proliferative activity was determined by staining of known positive and negative control tissue sections. For interpretation, only membrane or membrane and cytoplasmic staining were considered positive for overexpression.

**Immunohistochemistry**

Immunoperoxidase studies were performed on formalin-fixed, paraffin-embedded tissue sections using the avidin-biotin-peroxidase complex (ABC) method. The specimens were cut 3–4 μm thick, deparaffinized in xylene, and rehydrated in descending grades (100%–70%) of ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol.

To enhance the immunostaining, sections were placed in distilled water and microwave irradiated for 8 minutes using a Sharp model R9H81 microwave oven operating at a frequency of 2,450 MHz at 700 watts. After several washes in distilled water and phosphate-buffered saline, sections were incubated with a 1:10 dilution of normal horse serum to minimize background staining.

**Ki-67**

Slides with the prepared sections were incubated overnight at 4 °C with the MIB-1 mouse monoclonal antibody to Ki-67 antigen (lot #0393.1; AMAC, Westbrook, ME; 1:100 dilution). The peroxidase staining procedure was done using ABC Elite kits (Vector Laboratorones Burlingame, CA). The immunostaining reaction was visualized using 0.05% 3,3′-diaminobenzidine in Tris-HCl buffer containing 0.01% hydrogen peroxide, pH 7.6. Sections were counterstained with 0.1% toluidine blue and mounted in permount. The extent of Ki-67 immunoreactivity was evaluated by determining the percentage of positively stained nuclei present in at least 2,000 tumor cells in randomly selected areas.

**HER-2/neu Oncogene**

Prepared slides were incubated for 1 hour at room temperature with mouse monoclonal antibody to c-erbB-2 oncogene product (Ab-3, Oncogene Science, Uniondale, NY; 1:100). The immunostaining was performed using ABC Elite kits and the immunoreaction visualized by using 3-amino-9-ethylcarbazole as chromogen. The specificity of the immunoreaction was verified by staining of known positive and negative control tissue sections. For interpretation, only membrane or membrane and cytoplasmic staining were considered positive for overexpression.

**Statistics**

Statistical comparisons of data were made with one-way analysis of variance and Fisher's exact test.

**RESULTS**

**Clinicopathologic Features**

The patients' age at the time of diagnosis ranged from 13 to 83 years (mean, 59.1 years). Sixteen of the 26 patients were female and 10 were male. Fifteen tumors arose in the parotid glands, three in the submandibular glands, three in the nasal cavity and paranasal sinuses, three in the pharynx and parapharyngeal space, and two were intraoral. Tumor size ranged from 1.5–8.0 cm with a mean of 3.6 cm in their largest dimension.

Among 26 patients with available clinical data, 22 patients were treated with surgery only. Four received post-operative radiotherapy. Margins of resection were free of tumor in all cases. Eleven patients developed local recurrences, and in six patients multiple recurrences were recorded. The interval between the primary diagnosis and the first recurrence ranged from 1 to 19 years (mean, 5 years). Two patients died of heart disease and breast cancer, respectively, and the others were alive with no evidence of disease after excision of the recurrent tumors. Five patients developed lung metastases 4 to 34 years (mean, 15 years) after the initial diagnosis. None of these patients died of the disease. There was no evidence of local recurrence or distant metastasis in 10 patients. However, these patients were recently accessioned with relatively short follow-up periods.

On microscopic examination, all neoplasms displayed a pushing nodular configuration with infiltrative margins. The appearance of EMC varied considerably in the proportion and arrangement of epithelial and myoepithelial cells and the amount of stroma. In three tumors, ductal formations were very prominent with surrounding thin rims of clear myoepithelial cells. The most common pattern consisted of nests of myoepithelial cells, with or without an epithelial component, arrayed in a hyaline stroma (Fig. 1). A cribriform-like arrangement of myoepithelial cells was focally seen in three tumors (Fig. 2). Five tumors lacked a hyaline stroma and were composed of tightly arranged cell nests with intervening thin fibrous septa (Fig. 3). In nine tumors with lobular growth, areas of coagulative necrosis were present. Cystic dilation of the ducts...
FIG. 1. Tumor nests composed of inner ductal and outer clear myoepithelial cells surrounded by hyaline stroma. (hematoxylin and eosin, x100).

with secretory materials, or intracystic encephaloid protrusions were observed in three and two tumors, respectively. Four tumors showed solid proliferations of myoepithelial cells with a few peripherally displaced and flattened tubular structures. Nuclear pleomorphism and high mitotic activity were not observed in any of the neoplasms in this study. Areas of focal squamous differentiation were found in three tumors. On a comparative evaluation between primary and secondary neoplasms, three of four tumors showed an increased myoepithelial population in the secondary lesions.

Flow Cytometry and Immunohistochemistry
Clinicopathologic findings and the results of DNA flow cytometry and Ki-67 immunostaining are listed in Table 1. Of the 31 tumors analyzed from the 26 patients, diploidy was manifested in 26 and aneuploidy in 5 patients. In one instance where samples from the primary and the recurrence were analyzed, the primary tumor revealed diploid pattern, whereas the recurrence showed aneuploidy. All aneuploid tumors were hypodiploid with low DI, ranging from 1.16 to 1.27 (Fig. 4). S-phase fraction of the tumors ranged from 3% to 10% with a mean of 6.7%.

Ki-67 immunostaining manifested a heterogenous staining pattern within the tumor and revealed a generally low growth rate, from 1% to 20% (mean, 5.6%). Immunoreactivity was primarily found in the myoepithelial component, more often at the periphery than in the central portion of the tumors (Figs. 5A and 5B).

Immunostaining for neu (c-erbB-2) oncoprotein showed only granular cytoplasmic staining of mild-to-moderate intensity in some tumors, and was mostly in epithelial cells. All lesions lacked membrane staining.

No apparent correlation between the biologic course and the histopathologic features, tumor size, DNA ploidy, and proliferative activity were found, although, as shown in Table 2, all five tumors with DNA aneuploidy developed recurrence or metastasis. Approximately 60% of DNA diploid neoplasms also recurred or metastasized.

DISCUSSION
Epithelial-myoepithelial carcinoma is an uncommon, low-grade salivary gland neoplasm that exhibits a dual composition of epithelial and myoepithelial cells.612-14 The tumor manifests a spectrum of cytomorphologic and structural features that may resemble other salivary gland tumors.15 Fonseca and colleagues5 described four architectural types that included solid, tubular, cribriform, and papillary.

The neoplasm most often involves the parotid gland, and less frequently, the submandibular and other minor salivary glands.35,6 In this series, the tumor primarily affected the parotid gland in older patients with the exception of one tumor in

FIG. 2. Cords of epithelial-myoepithelial cells in hyaline stroma forming a cribriform-like pattern (hematoxylin and eosin, x100).

FIG. 3. Epithelial-myoepithelial carcinoma displaying compact nests of myoepithelial and ductal cells with scanty stroma.
Epithelial–Myoepithelial Carcinoma of Salivary Glands

TABLE 1. CLINICOPATHOLOGIC, FLOW CYTOMETRIC, AND IMMUNOHISTOCHEMICAL DATA

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Site</th>
<th>DI</th>
<th>SPF (%)</th>
<th>Ki-67 (%)</th>
<th>Rec (years)</th>
<th>Meta (years)</th>
<th>Follow-up (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57</td>
<td>M</td>
<td>Parotid</td>
<td>1.00</td>
<td>5</td>
<td>1.0</td>
<td>Yes (19)</td>
<td>No</td>
<td>NED (20)</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
<td>M</td>
<td>Parotid</td>
<td>1.22</td>
<td>11</td>
<td>1.8</td>
<td>No</td>
<td>Yes (14)</td>
<td>NED (35)</td>
</tr>
<tr>
<td>3</td>
<td>13</td>
<td>M</td>
<td>Parotid</td>
<td>1.18</td>
<td>14</td>
<td>3.2</td>
<td>Yes (5)</td>
<td>No</td>
<td>NED (24)</td>
</tr>
<tr>
<td>4</td>
<td>67</td>
<td>F</td>
<td>Parotid</td>
<td>1.00</td>
<td>4</td>
<td>4.8</td>
<td>Yes (3, 4)</td>
<td>No</td>
<td>DOC (13)</td>
</tr>
<tr>
<td>5</td>
<td>55</td>
<td>F</td>
<td>Palate</td>
<td>1.00</td>
<td>6</td>
<td>9.4</td>
<td>Yes (1, 10)</td>
<td>No</td>
<td>NED (18)</td>
</tr>
<tr>
<td>6</td>
<td>27</td>
<td>M</td>
<td>Parotid</td>
<td>1.00</td>
<td>15</td>
<td>5.6</td>
<td>Yes (1, 15, 18)</td>
<td>No</td>
<td>NED (26)</td>
</tr>
<tr>
<td>7</td>
<td>31</td>
<td>M</td>
<td>Parotid</td>
<td>1.00</td>
<td>4</td>
<td>8.9</td>
<td>No</td>
<td>No</td>
<td>NED (3.5)</td>
</tr>
<tr>
<td>8</td>
<td>46</td>
<td>F</td>
<td>Maxilla</td>
<td>1.00</td>
<td>3</td>
<td>3.6</td>
<td>No</td>
<td>No</td>
<td>NED (4)</td>
</tr>
<tr>
<td>9</td>
<td>75</td>
<td>F</td>
<td>Parotid</td>
<td>1.00</td>
<td>4</td>
<td>5.2</td>
<td>No</td>
<td>Yes (4)</td>
<td>NED (4)</td>
</tr>
<tr>
<td>10</td>
<td>79</td>
<td>F</td>
<td>Parotid</td>
<td>1.00</td>
<td>3</td>
<td>4.0</td>
<td>No</td>
<td>No</td>
<td>NED (2)</td>
</tr>
<tr>
<td>11</td>
<td>69</td>
<td>F</td>
<td>Parotid</td>
<td>1.00</td>
<td>4</td>
<td>2.2</td>
<td>No</td>
<td>No</td>
<td>NED (6)</td>
</tr>
<tr>
<td>12</td>
<td>68</td>
<td>M</td>
<td>Parotid</td>
<td>1.00</td>
<td>5</td>
<td>4.8</td>
<td>No</td>
<td>No</td>
<td>NED (1.5)</td>
</tr>
<tr>
<td>13</td>
<td>47</td>
<td>F</td>
<td>Oral</td>
<td>1.00</td>
<td>3</td>
<td>2.6</td>
<td>Yes (4)</td>
<td>No</td>
<td>NED (4)</td>
</tr>
<tr>
<td>14</td>
<td>50</td>
<td>M</td>
<td>Parotid</td>
<td>1.00</td>
<td>5</td>
<td>5.7</td>
<td>No</td>
<td>Yes (7)</td>
<td>NED (8)</td>
</tr>
<tr>
<td>15</td>
<td>69</td>
<td>F</td>
<td>Parotid</td>
<td>1.00</td>
<td>8</td>
<td>2.3</td>
<td>No</td>
<td>No</td>
<td>NED (0.4)</td>
</tr>
<tr>
<td>16</td>
<td>69</td>
<td>F</td>
<td>Parotid</td>
<td>1.00</td>
<td>4</td>
<td>13.9</td>
<td>No</td>
<td>No</td>
<td>NED (1.0)</td>
</tr>
<tr>
<td>17</td>
<td>77</td>
<td>F</td>
<td>Submandibular</td>
<td>1.00</td>
<td>3</td>
<td>17.2</td>
<td>No</td>
<td>No</td>
<td>NED (1.5)</td>
</tr>
<tr>
<td>18</td>
<td>56</td>
<td>F</td>
<td>Pharynx</td>
<td>1.20</td>
<td>7</td>
<td>6.4</td>
<td>Yes (7, 8)</td>
<td>No</td>
<td>DOC (13)</td>
</tr>
<tr>
<td>19</td>
<td>54</td>
<td>M</td>
<td>Maxilla</td>
<td>1.00</td>
<td>3</td>
<td>1.0</td>
<td>No</td>
<td>Yes (11)</td>
<td>AWD (15)</td>
</tr>
<tr>
<td>20</td>
<td>59</td>
<td>F</td>
<td>Pharynx</td>
<td>1.00</td>
<td>4</td>
<td>5.1</td>
<td>Yes (4, 7)</td>
<td>No</td>
<td>NED (8)</td>
</tr>
<tr>
<td>21</td>
<td>59</td>
<td>F</td>
<td>Paranasal sinus</td>
<td>1.00</td>
<td>5</td>
<td>4.8</td>
<td>No</td>
<td>No</td>
<td>NED (2.8)</td>
</tr>
<tr>
<td>22</td>
<td>67</td>
<td>M</td>
<td>Submandibular</td>
<td>1.00</td>
<td>5</td>
<td>1.0</td>
<td>Yes (3, 4)</td>
<td>No</td>
<td>NED (4)</td>
</tr>
<tr>
<td>23</td>
<td>80</td>
<td>F</td>
<td>Parapharynx</td>
<td>1.00</td>
<td>6</td>
<td>4.9</td>
<td>No</td>
<td>No</td>
<td>NED (0.5)</td>
</tr>
<tr>
<td>24</td>
<td>83</td>
<td>M</td>
<td>Parotid</td>
<td>1.00</td>
<td>6</td>
<td>19.7</td>
<td>No</td>
<td>No</td>
<td>NED (0.3)</td>
</tr>
<tr>
<td>25</td>
<td>72</td>
<td>F</td>
<td>Parotid</td>
<td>1.27</td>
<td>6</td>
<td>3.8</td>
<td>Yes (3)</td>
<td>No</td>
<td>NED (4)</td>
</tr>
<tr>
<td>26</td>
<td>72</td>
<td>F</td>
<td>Submandibular</td>
<td>1.00</td>
<td>8</td>
<td>3.8</td>
<td>Yes (10)</td>
<td>No</td>
<td>AWD</td>
</tr>
</tbody>
</table>

DI = DNA index; SPF = S-phase fraction; Rec = recurrence; Meta = metastases; NED = alive with no evidence of disease; DOC = died of other causes; AWD = alive with disease; NA = not available.

a 13-year-old boy, There has been only one previous report of EMC in a patient in the pediatric age.15

Overall, the tumors in this series followed an indolent and protracted course that was nonetheless punctuated by recurrence and metastases. In this study, 50% of the patients developed a recurrence, and 25% experienced a metastasis. This is in agreement with previous reports.3 A characteristic biologic feature of most of the EMCs in this cohort was the long interval between diagnosis and recurrence (mean, 5 years) and metastasis (mean, 15 years). However, the incidence of recurrence and metastasis of EMC have varied widely.5-6,12,13,17 Studies showing high mortality (40%)5,16 of patients with these tumors are in conflict with our findings and those of others.5,6 This may be related to the inclusion of other salivary gland tumors with epithelial-myoeipithelial features.

DNA ploidy has been shown to be a valuable prognostic indicator in various types of neoplasms, including salivary gland tumors.15-21 Although some studies of EMC have shown a correlation between solid type, high proliferating cell nuclear antigen (PCNA) positivity, and DNA aneuploidy, and an unfavorable outcome,5,16 others have contradicted these findings.12 To our knowledge, a flow cytometric study of EMC has not been previously performed. The low incidence of aneuploidy (20%)5 previously performed. The low incidence of aneuploidy (20%)5-6,12,13,17 Studies showing high mortality (40%)5,16 of patients with these tumors are in conflict with our findings and those of others.5,6 This may be related to the inclusion of other salivary gland tumors with epithelial-myoeipithelial features.

DNA ploidy has been shown to be a valuable prognostic indicator in various types of neoplasms, including salivary gland tumors.15-21 Although some studies of EMC have shown a correlation between solid type, high proliferating cell nuclear antigen (PCNA) positivity, and DNA aneuploidy, and an unfavorable outcome,5,16 others have contradicted these findings.12 To our knowledge, a flow cytometric study of EMC has not been previously performed. The low incidence of aneuploidy (20%)5 and the near-diploid aneuploidy seen our series are consistent with the low-grade malignant nature of this disease. The infrequent DNA aneuploidy in these tumors has also been demonstrated by cytophotometric and image analysis studies.5,3

Ki-67, a monoclonal antibody that reacts with a nuclear antigen present in S, G2, and M phases,5,16 but not in G0, has been shown to correlate with clinicopathologic factors and patient survival in several neoplastic conditions including salivary gland neoplasms.15,22 In a recent study of EMC, it was shown that PCNA values were higher in the solid than the tubular-cribiform type, and that they were associated with a high recurrence rate.16 These findings show no significant correlation...
between Ki-67 score and histologic subtypes. It is interesting, however, that the Ki-67 reactivity was observed mostly in the myoepithelial cells. This indicates that the proliferative element in the progression of EMC is likely the myoepithelial cells. Such findings mirror those of PCNA immunostaining by others. In this study, the proliferative activity measured by Ki-67 immunostaining or flow cytometry, showed no significant correlation with the clinical course of the tumor.

HER-2/neu oncogene is a dominant transforming gene that shares considerable sequence homology with epidermal growth factor receptor. Amplification or overexpression of HER-2/neu in tumor tissue has been associated with poor prognosis in several tumor entities. Studies of this oncogene in salivary gland tumors are relatively few and inconclusive. These results indicate a lack of HER-2/neu overexpression in these neoplasms. In fact, the immunoreactivity was mainly granular and strictly cytoplasmic.

Although cytoplasmic staining for HER-2/neu oncoprotein has been described in various tissues, it is currently considered an artifact and only membrane and/or cytoplasmic staining represent significant staining. De Potter related cytoplasmic immunoreactivity to a cytoplasmic protein with a molecular weight of 155 kD, which could be a HER-2/neu-like cross-reacting protein or an alternative product of HER-2/neu oncogene. Corbett and colleagues, however, suggested that this might represent a precursor form of the oncogene. In salivary gland tumors, cytoplasmic HER-2/neu immunoreactivity without membrane staining has been observed primarily in benign tumors.

This study reaffirms the low-grade biologic course of EMC and also indicates that histopathologic features do not correlate with prognosis. Because all tumors with DNA aneuploidy in this study pursued an unfavorable course, it is possible that this feature may identify a subset of patients with potentially aggressive EMC. However, further studies of a larger cohort with extended clinical follow-up are needed to expand and confirm our observation.

**REFERENCES**


