Amplification and Overexpression of HER-2/neu in Carcinomas of the Salivary Gland: Correlation with Poor Prognosis


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ABSTRACT

There are few reliable prognostic markers of biological aggressiveness for head and neck carcinomas in general. For salivary gland carcinomas, anatomic location, tumor size, histological grade, and extent of disease involvement are considered to be clinically important risk factors for recurrent disease. Molecular genetic alterations in salivary gland carcinomas have not been characterized, and tumor cell proteins have not been shown to be prognostically significant. Here a cohort of mucoepidermoid carcinomas of the major (parotid and submandibular) salivary glands are analyzed for a molecular genetic alteration, HER-2/neu gene amplification, and gene amplification and expression results are compared with long-term clinical follow-up information.

Archival tissues resected from 58 patients with mucoepidermoid carcinoma of salivary glands were evaluated for HER-2/neu gene amplification by fluorescence in situ hybridization and for gene expression by immunohistochemistry in a blinded fashion. Clinical follow-up information was compared with the results of these analyses to determine whether there were significant associations.

Overexpression, identified as membrane immunostaining by immunohistochemistry, was observed in 22 of 58 (38%) mucoepidermoid carcinomas. Gene amplification, characterized by fluorescence in situ hybridization, was observed in 12 (21%) cases. Eleven of the 12 cases with gene amplification were also immunostained for HER-2/neu. Both gene amplification (P = 0.0001, P < 0.0001) and immunostaining (P < 0.0001) were correlated with shorter disease-free interval and poorer overall patient survival, respectively. Multivariate analysis showed that HER-2/neu immunostaining and amplification were markers of poor prognosis independent of histopathological grade, tumor size, and involvement of regional lymph nodes.

HER-2/neu is amplified and/or overexpressed in approximately one-third of mucoepidermoid carcinomas of salivary glands. Amplification and/or overexpression appears to be an independent marker of poor prognosis in mucoepidermoid carcinomas of the salivary glands as it is in carcinomas of the breast, ovary, and endometrium.

INTRODUCTION

In the United States the incidence of salivary gland neoplasms is approximately 2 cases/100,000 individuals (1). This infrequent but nevertheless interesting group of tumors presents a variety of challenges to medical management. Survival of individuals with malignant salivary gland neoplasms depends on the site of origin, histological type of tumor, and extent of disease at diagnosis. However, there are no biological markers that permit a prediction of which individuals are likely to experience a relapse and die of their disease and which individuals will not. Furthermore, the long natural history of the tumors, the advanced age of many of the patients, and the limited number of cases available in most series complicate studies of salivary gland tumors. In an attempt to characterize molecular genetic alterations in these neoplasms and to identify new prognostic markers, we have analyzed a series of salivary gland carcinomas of a single histological type having long-term clinical follow-up information for alterations of the HER-2/neu proto-oncogene.

The HER-2/neu oncogene was first identified as a dominant transforming gene in chemically induced adenal neuroblastomas of neonatal mice and was referred to as neu (2, 3). Subsequently, three groups independently identified the human homologue of this gene (4–6). Sequence analysis of the gene demonstrated a close relationship to the human epidermal growth factor receptor (HER-1) or c-erbB oncogene (4, 6). Because of the similarities with HER-1, this gene was considered to code for a membrane receptor (4, 6). A family of proteins, the heregulins, have been isolated, cloned, and sequenced, and proposed as HER-2/neu ligands (7). However, heregulins have been shown to specifically bind HER-4, another related membrane protein (8). It has been suggested that the response to heregulin observed in the original publication (7) was due to heterodimerization of HER-2/neu with HER-4 in the breast cancer cell line (8). Hence, HER-2/neu currently may be considered to be an “orphan” receptor molecule.

Amplification and/or overexpression of HER-2/neu in human tumor tissue has been associated with a poor prognosis in ovarian (9), endometrial (10), and node-positive (9, 11) and node-negative (12–14) breast carcinomas. A single salivary gland carcinoma of unspecified histological type has been identified previously as showing amplification of HER-2/neu (4). Previous studies of HER-2/neu expression in salivary gland carcinomas are sparse, comprise heterogeneous subtypes, and show contradictory results (15, 16). In this study, a series of 58 mucoepidermoid carcinomas of major salivary glands have been analyzed for HER-2/neu amplification and expression, and the results are compared with long-term clinical follow-up information, the disease-free interval, and overall survival.

MATERIALS AND METHODS

Tumor Tissue. Fifty-eight mucoepidermoid carcinomas of the major salivary glands (from an equal number of patients) stored as paraffinized tissue blocks in the archives of the University of Texas M. D. Anderson Cancer Center were analyzed for HER-2/neu gene amplification and expression. The neoplasms were accessioned between 1956 and 1981. The parotid gland was the site of the carcinoma in 30 cases and submandibular gland in 6 cases. The sites of two were unknown. None were identified in the sublingual gland. Tumors ranged from 0.4 to 9.0 cm in diameter with a mean of 2.75 cm. Histopathological review confirmed that all cases were mucoepidermoid carcinomas, and they were graded as described by Batsakis and Luna (17).

Patient Information. Complete clinical follow-up information, including age, sex, date of diagnosis, stage of disease, treatment, presence of recurrent disease, date of death, and cause of death, were recorded.
in the files of the Department of Pathology, M. D. Anderson Cancer Center, University of Texas. The remaining two cases were lost to clinical follow-up.

The racial origins of 30 women and 26 men were identified as white in 43, black in 8, and “hispanic” in 5 people. Racial origins of hispanic patients were not specified. Age at the time of diagnosis ranged from 10 to 77 years with a mean of 52 years. All patients had local resection of the carcinoma, including 37 total parotidectomies, 3 subtotal parotidectomies, 10 superficial parotidectomies, and 6 submandibulectomies. Twenty-two patients were also treated with radiation therapy. One patient was treated with chemotherapy. The histopathological grade of 9 tumors was grade I; 26 tumors, grade II; and 21 tumors, grade III. The facial nerve was involved by tumor in 18 cases. At resection 24 tumors showed local invasion of surrounding tissues beyond the salivary gland. Twenty-one patients had involvement of regional lymph nodes and one patient had distant metastases at diagnosis. Clinical follow-up ranged from 4 to 351 months, with a mean of 131 months. The laboratory analyses were performed blinded to the clinical information.

Immunohistochemistry. A series of 28 antibodies have been screened previously for their ability to detect HER-2/neu by immunohistochemistry in formalin-fixed, paraffin-embedded tissue sections (18). A rabbit anti-HER-2/neu polyclonal antiserum with no cross-reactivity to EGF receptor (9) was among the most sensitive antibodies for identifying HER-2/neu receptor in paraffinized sections and was used in this study.

The immunohistochemical staining method, as described previously (14, 18, 19), involved the sequential application of three antibodies to tissue sections:

Fig. 1. Salivary gland mucoepidermoid carcinomas were grouped according to the level of HER-2/neu immunostaining into three categories illustrated by three examples. (A) Mucoepidermoid carcinoma demonstrating an absence of HER-2/neu immunostaining. (B) Mucoepidermoid carcinoma detectable to moderate membrane immunostaining of tumor cells. (C) Mucoepidermoid carcinoma demonstrating strong membrane immunostaining of tumor cells. \( \times 1600 \).
sections in posthybridization washing solution and 2 × standard saline citrate (0.1 M NaCl, 0.02 M Na citrate), the probes were detected with avidin-FITC and rhodamine-labeled anti-digoxigenin antibody. The tissue sections were washed with 1 × phosphate buffered detergent (Oncor, Inc.) buffer and the signal was amplified by successive incubations with anti-avidin antibody and FITC avidin/rhodamine-labeled anti-digoxigenin antibody. The nuclei were considered to be consistent with HER-2/neu gene amplification (9, 11, 12).

Statistical Methods. The two outcome measures of primary interest were time to recurrence and time to death. Kaplan-Meier product-limit estimates of time to recurrence and survival time were plotted (20, 21). The Cox proportional hazard model (logrank test) was used (stratified and unstratified) to test for statistical significance (22–24). Associations between variables were measured by standard methods for contingency tables (25). All P values reported are two-sided.

RESULTS

Correlation between Clinicopathological Features and Outcome. Male gender and certain pathological features including site of the primary tumor, tumor size, histological grade, local invasiveness, and involvement of regional lymph nodes were associated with an increased risk of recurrent disease and/or shorter overall survival, or both (Table 1). Tumor size was positively correlated with death from disease (P = 0.007) and with recurrent disease, although the latter was not statistically significant (P = 0.30). Twenty-three of 33 patients with tumors smaller than the mean (2.75 cm in diameter) had no evidence of disease at last follow-up, while only 8 of 23 patients with tumors larger than the mean had no evidence of disease at last follow-up (P = 0.003).

Histopathological grading was also significantly correlated with development of recurrent disease as indicated by a shorter disease-free interval (P = 0.006), and death from disease associated with a shorter overall survival (P < 0.0001). Recurrent disease was experienced by 2 of 9 patients with grade I, 10 of 26 with grade II, and 13 of 21 patients with grade III tumors. None of 9 grade I, 7 of 26 grade II, and 18 of 21 grade III carcinoma patients died of their disease (Table 1).

Presence of locally invasive disease at the time of diagnosis was significantly correlated with disease-free interval (P = 0.009) and death from disease (P = 0.02). Only 10 of 32 patients lacking locally invasive tumor at the time of diagnosis experienced recurrent tumor, while 15 of 24 with locally invasive tumor experienced recurrence. Ten of 32 patients lacking local invasion died of their disease, while 15 of 24 patients with local invasion died of their disease.

The abbreviation used is: FITC, fluorescein isothiocyanate.
Involvement of regional lymph nodes was also associated with recurrent disease ($P = 0.001$) and death from disease ($P < 0.0001$) (Table 1).

**HER-2/neu Expression.** Twenty-two of 58 (38%) mucoepidermoid carcinomas of the salivary gland showed varying degrees of immunostaining for HER-2/neu (Fig. 1; Table 2). No immunostaining was identified in the remaining 36 carcinomas. When present, immunostaining was limited to tumor cells, primarily of the cell membranes of these cells. Both the adenocarcinomatous and the squamoid components were immunostained with similar frequency and intensity in a given mucoepidermoid carcinoma. Benign salivary epithelium, either glandular or ductal, was not immunostained (Fig. 2), nor was any stromal tissue component.

**HER-2/neu Gene Amplification.** Fluorescence in situ hybridization was used to identify the HER-2/neu gene and $\alpha$ satellite DNA for chromosome 17 (centromere) in interphase tumor cell nuclei. The HER-2/neu fluorescence signals were compared with the $\alpha$ satellite DNA signals to determine a ratio for binding of the respective probes. Mucoepidermoid carcinomas, the nuclei of which contained an average of at least twice as many copies of HER-2/neu gene relative to chromosome 17 centromere were considered to have gene amplification. Amplification was found in 12 (21%) cases analyzed (Fig. 3; Table 2). Most of the carcinomas showing gene amplification had the HER-2/neu signals distributed over a geographically limited area of the nucleus. This grouping of the signals is consistent with location in homogeneous staining regions of chromosomes. Only tumor cells showed gene amplification. Normal salivary cells, fibroblasts, endothelial cells, and lymphocytes showed no amplification of HER-2/neu.

**Comparison of Gene Amplification with Expression.** Eleven of 12 tumors (93%) with gene amplification by fluorescence in situ hybridization also had membrane immunostaining for HER-2/neu protein. The single salivary gland carcinoma with gene amplification that lacked immunostaining had a HER-2/neu (chromosome 17 centromere ratio of 2.04). The tumor was from a 50-year-old male patient who died of a massive myocardial infarction after 14 months of clinical follow-up without any evidence of recurrent disease. Eleven
of the 46 (19%) carcinomas which did not show gene amplification had membrane immunostaining. Thirty-five carcinomas had neither gene amplification nor membrane immunostaining.

Comparison of Gene Amplification/Immunostaining with Clinical Data. Male gender was significantly \((P = 0.02)\) associated with salivary gland carcinomas having HER-2/neu immunostaining (Table 1). Of 30 salivary gland tumors in women, 7 were immunostained and 23 were not immunostained, whereas of 26 tumors in men, 15 were immunostained and 11 were not immunostained. Immunostaining was found more frequently in salivary gland tumors from older patients than in salivary gland tumors from younger patients (trend test, \(P = 0.04\)).

HER-2/neu immunostaining of tumors was also correlated with racial group. None of 8 black patients and none of five hispanic patients had immunostaining in their carcinomas, while 22 of 43 white patients did have HER-2/neu immunostaining in their carcinomas \((P = 0.003)\).

Immunostaining showed a trend for larger tumors to be more frequently immunostained than smaller tumors (trend test, \(P = 0.04\)). HER-2/neu immunostaining was found more frequently in tumors with higher grade (trend test, \(P = 0.0001\)) and with involved regional lymph nodes \((P = 0.001)\). Although 4 of 6 submandibular gland carcinomas were immunostained compared with only 18 of 50 parotid gland carcinomas, this difference was not statistically significant \((P = 0.31)\). Salivary gland carcinomas with locally invasive disease at the time of diagnosis were only weakly associated with an increased frequency of immunostaining \((P = 0.55; \text{Table 1})\).

There was a significant association of both HER-2/neu gene amplification \((P = 0.0001)\) and immunostaining \((P = <0.0001)\) with an earlier relapse of the patients. Similarly, a significant association was observed both between HER-2/neu gene amplification \((P < 0.0001)\) and between immunostaining \((P < 0.0001)\) and shorter overall survival of the patients (Table 2; Fig. 4). Twenty-five patients died of their mucoepidermoid carcinomas, 31 had no evidence of disease identified at the last clinic visit, and 2 were lost to clinical follow-up. Of the 25 who died of disease, 19 had tumors with membrane staining and 6 had tumors with no staining. Gene amplification was identified in 11 of the 25 people who died of their disease. Of the 31 patients who were clinically disease-free at the last follow-up, 28 had tumors with no membrane staining and only 3 had membrane staining. Only 1 of the 31 disease-free individuals had gene amplification, while the other 30 disease-free patients did not have gene amplification.

Among the 34 cases with node-negative disease, HER-2/neu immunostaining was correlated with both recurrent disease \((P = 0.02)\) and death from disease \((P = 0.0013)\). Overall HER-2/neu immunostaining also proved to be an independent predictor of the clinical outcome by multivariate analysis. HER-2/neu immunostaining provided additional predictive value \((P = 0.0013)\) in the presence of
Fig. 4. Actuarial survival curves for patients with salivary mucoepidermoid carcinomas. Cases with gene amplification and positive immunostaining of tumor cells had a poorer outcome than cases lacking amplification or lacking immunostaining (see Table 2 for P values). (A) Recurrence-free interval of patients with (- - - -) and without (—) HER-2/neu gene amplification. (B) Overall survival of patients with (- - - -) and without (—) HER-2/neu gene amplification. (C) Recurrence-free interval of patients with (- - - -) and without (—) HER-2/neu immunostaining. (D) Overall survival of patients with (- - - -) and without (—) HER-2/neu immunostaining.

other significant prognostic factors such as grade, nodal status, and tumor size.

DISCUSSION

Salivary gland neoplasms are relatively uncommon and represent less than 2% of human neoplasms. The majority of salivary gland neoplasms occur in the parotid gland with tumors found less commonly in submandibular, sublingual, and other minor salivary glands. Although neoplasms are less common in the submandibular, sublingual, and minor salivary glands, a high proportion of tumors in these glands are malignant. Overall, mucoepidermoid carcinomas are the most common malignancies of the salivary glands (26). As confirmed in the current study, larger tumor size, higher histological grade, presence of locally invasive disease, and involved regional lymph nodes are pathological parameters correlated with a less favorable prognosis. Currently, however, no tumor markers that may predict aggressive biological behavior of salivary gland carcinomas have been identified. The results obtained in this study indicate that HER-2/neu is one such marker of biological aggressiveness.

Four studies of HER-2/neu immunostaining in salivary gland carcinomas have been reported (15, 16, 27, 28). One of these studies reported a correlation between clinical outcome and HER-2/neu immunostaining (16), one failed to demonstrate such an association (15), one reported findings in a rare histological type of salivary gland carcinoma resembling primary ductal carcinoma of the breast (27), and another study reported findings in both benign and malignant tumors but had only limited follow-up information (28). In the study reporting no association, 131 salivary gland tumors, encompassing 69 benign adenomas and 62 carcinomas of various types with 6 mucoepidermoid carcinomas, were analyzed (15). Only five carcinomas (one mucoepidermoid carcinoma) in this study were immunostained for HER-2/neu. Although there was no statistically significant correlation with clinical outcome, four of the five patients having salivary gland carcinomas with HER-2/neu immunostaining died of their disease, and the fifth died of a myocardial infarction. Since the CB11 mouse monoclonal antibody, an antibody known to recognize only approximately 50% of paraffin-embedded breast carcinomas with HER-2/neu overexpression (18), was used for immunohistochemistry
in this study, it is likely that the small number of immunostained cases is an underestimate of the number of cases with HER-2/neu overexpression.

In another study of 59 archival salivary gland carcinomas evaluated for HER-2/neu expression by immunohistochemistry, 19 (22%) cases were positively immunostained (16). Interestingly, the positive cases were either pure adenocarcinomas (6 of 20 cases) or adenocarcinomas occurring in a pleomorphic adenoma (7 of 15 cases). None of nine mucoepidermoid carcinomas were reported as having positive immunostaining. When the adenocarcinomas and adenocarcinomas in pleomorphic adenoma were considered as a single group, HER-2/neu immunostaining was associated with both a shorter disease-free interval and poorer overall survival. In a study of nine cases of a rare ductal type of salivary gland adenocarcinoma morphologically resembling ductal carcinoma of the breast, immunostaining for HER-2/neu was demonstrated in all cases, and eight of the nine patients died of their carcinoma (27).

Stenman et al. (28) reported immunohistochemical localization of HER-2/neu in frozen tissue samples of 33 benign and 19 malignant salivary gland tumors. Although membrane staining was identified in the majority of the tumors, high expression, consistent with overexpression, was observed in only 32% of the salivary gland carcinomas. Immunoblot analysis of a carcinoma, strongly positive by immunostaining, and a pleomorphic adenoma, weakly positive by immunostaining, confirmed the high levels of p185HER-2/neu protein from the carcinoma and weak, barely detectable levels of p185HER-2/neu protein from the adenoma (28). This is similar to the findings in frozen breast and ovarian carcinomas where high expression (overexpression) by immunoblot is correlated with gene amplification by Southern blot (9).

Pleomorphic adenomas of the salivary gland were investigated for HER-2/neu immunostaining in another study (29). Only cytoplasmic staining, not membrane staining, was described (29). However, HER-2/neu is known to be a membrane receptor protein and, as described above, is on epithelial membranes of pleomorphic adenomas in another study using frozen tissue and the same polyclonal HER-2/neu antibody (28). With specific antibodies, only membrane immunostaining, not cytoplasmic staining, has been correlated with HER-2/neu overexpression (9, 18). Although HER-2/neu receptor is synthesized in the cytoplasm and receptor may also be transiently in the cytoplasm during membrane internalization and receptor processing, the vast majority of receptor is expected to be on the cell membranes, suggesting that the reported cytoplasmic staining in pleomorphic adenomas is probably not related to HER-2/neu protein content.

In the current study, HER-2/neu gene amplification and immunostaining were correlated with shorter disease-free interval ($P = 0.0001$, $P < 0.0001$), as well as shorter overall survival ($P < 0.0001$, $P < 0.0001$). In addition, some clinical parameters such as male gender, non-“white” racial grouping, and pathological parameters including large tumor size, metastases to regional lymph nodes, and high grade histopathology were associated with HER-2/neu amplification and/or HER-2/neu immunostaining of tumor cells. Although the number of cases was small, restriction of the analysis to carcinomas lacking involvement of regional lymph nodes at diagnosis showed a correlation between HER-2/neu amplification/expression and both shorter disease-free interval and shorter overall survival, indicating predictive value in node-negative as well as node-positive tumors. HER-2/neu immunostaining proved to be a prognostic marker of poor clinical outcome that was independent of tumor site, tumor size, grade, and lymph node status by multivariate analysis. These results, therefore, indicate that HER-2/neu amplification/overexpression is an important prognostic marker in salivary gland mucoepidermoid carcinomas as it is in breast, endometrial, and ovarian carcinomas.

Recently, HER-2/neu immunostaining in node-positive breast cancer has been correlated with response to high dose doxorubicin chemotherapy. Those women in the Cancer and Leukemia Group B clinical trial of low and high dose chemotherapy whose breast carcinoma cells had HER-2/neu immunostaining and received high dose doxorubicin chemotherapy had a longer disease-free interval ($P < 0.001$) and a longer overall survival ($P < 0.001$) than women whose tumor lacked immunostaining but received similar therapy (30). HER-2/neu has not yet been tested as a predictor of responsiveness to doxorubicin-based chemotherapy in other cancers, but this information is of great interest.

Considerable circumstantial evidence suggests that alterations in the HER-2/neu gene may play an important role in neoplastic transformation or tumor progression. Interestingly, studies with transgenic mice expressing a c-neu transgene not only developed multifocal invasive breast cancer but also had bilateral salivary gland hyperplasia and increased c-neu expression in the salivary gland (31). No salivary gland carcinomas developed in this animal model of rapidly progressive breast cancer; however, male mice expressed higher levels of c-neu than did female mice in the salivary glands, and only male mice had bilateral hyper trophy and hyperplasia of parotid glands. The reason for this gender difference in mice is not known but is of interest, considering our observation that men had a higher frequency of increased HER-2/neu immunostaining and amplification in salivary gland carcinomas than did women. Men also had a worse prognosis than women with mucoepidermoid carcinoma of the salivary glands.

In conclusion, our findings strongly suggest that alterations in HER-2/neu are important in salivary gland mucoepidermoid carcinomas as they are in adenocarcinomas of the breast, endometrium, and ovary.

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REFERENCES

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