

Characterization of the HER-2/*neu* Oncogene by Immunohistochemical and Fluorescence *in Situ* Hybridization Analysis in Oral and Oropharyngeal Squamous Cell Carcinoma

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ABSTRACT

Purpose: The role of HER-2/*neu* in squamous cell carcinoma (SCC) of the head and neck is not well defined. The purpose of the current study is to measure the frequency of HER-2/*neu* expression, to demonstrate HER-2/*neu* gene amplification in the cases found to be positive for protein overexpression, and to investigate the prognostic significance of overexpression and/or amplification in SCC of the head and neck.

Experimental Design: A cohort of 77 patients with SCC of the oral cavity or oropharynx, with stage III or IV disease and uniformly treated with surgical resection and postoperative radiation, served as the primary patient population for the study. Of these, 56 patients had adequate follow-up and paraffin-embedded specimens available for analysis. Median follow-up was 6.1 years. Each of the paraffin-embedded specimens were immunohistochemically stained for HER-2/*neu* expression and graded for intensity of staining by a pathologist. All cases that demonstrated positive staining by immunohistochemistry were analyzed by fluorescence *in situ* hybridization (FISH) to assess HER-2/*neu* amplification status.

Results: Five-year survival for the 56 evaluable patients was 40%, with 25% experiencing local relapse, 18% regional relapse, and 25% distant relapse. The percentage of tumors staining positive for HER-2/*neu* by immunohistochemistry was 17%. There was no statistically significant correlation between HER-2/*neu* and T stage, N stage, tumor

grade, survival, or disease-free survival. HER-2/*neu* expression did correlate with vascular endothelial growth factor expression. FISH analysis revealed four cases that were amplified for HER-2/*neu*. Of note, of the 4 amplified cases, 2 suffered regional relapse, 1 suffered distant metastasis, and all 4 expired by 5 years of follow-up.

Conclusions: This is the first demonstration of HER-2/*neu* gene amplification by FISH in SCC of the head and neck. FISH validates a previously contested controversial role for HER-2/*neu* gene overexpression in SCC of the head and neck. The prognostic significance and clinical implications of HER-2/*neu* expression and amplification in head and neck cancer will require additional studies.

INTRODUCTION

The HER-2/*neu* oncogene (alternatively known as *c-erbB-2* or *neu*) encodes a M_r 185,000 glycoprotein, p185, with extracellular, transmembrane, and intracellular domains (1, 2). Both the HER-2/*neu* gene sequence, located on the short arm of chromosome 17, as well as the p185 protein share extensive sequence homology with the epidermal growth factor receptor, and the protein has been designated a member of the epidermal growth factor receptor tyrosine kinase superfamily. It is thought that overexpression of p185 leads to increased basal tyrosine kinase activity, thus transforming cells by chronically stimulating signal transduction pathways (2). Overexpression of HER-2/*neu* has been associated with advanced disease, metastasis, and poor clinical outcome in breast, ovarian, non-small cell lung, endometrial, salivary, and differentiated gastric carcinomas (1, 3-5). Less is known about tumors of nonglandular origin.

SCC² of the head and neck is a common epithelial neoplasm, and a large proportion of these are derived from the mucosa of the oral cavity and oropharynx (6). The 5-year survival rate for patients with oral and oropharyngeal SCCs has remained steadfastly at 53% (6, 7), failing to improve despite significant research exploring the pathogenesis and management of these tumors. At present, therapeutic decisions are based on clinical-pathological parameters, including age, TNM stage, and histological grade. Although useful, these factors often fail to differentiate between more and less aggressive lesions. As such,

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² The abbreviations used are: SCC, squamous cell carcinoma; VEGF, vascular endothelial growth factor; IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization; TNM, Tumor-Node-Metastasis.

it is often difficult to identify lesions more likely to recur or result in death attributable to disease. In the search for more reliable prognostic indicators, several investigators have looked at the possible relevance of biological molecular markers. Theoretically, a marker that consistently and reproducibly identified high-risk patients with aggressive disease would help select these patients for more aggressive management, thus improving disease-free survival.

Biological markers already reported as adverse prognostic indicators in SCC include p53, cyclin D1, epidermal growth factor receptor, and VEGF receptor (8). Amplification and/or overexpression of the *HER-2/neu* gene may also serve as potential prognostic markers. Studies of *HER-2/neu* in head and neck SCC are few, and results have been discordant (2, 4, 5, 9–13). The reported incidence of *HER-2/neu* overexpression has ranged from 0 to 47%. Also, most of these reports have been solely immunohistochemical studies and thus are subject to the well-known variability of IHC. A recent report by Pauletti *et al.* (14) has demonstrated the superiority of FISH analysis over IHC for assessing *HER-2/neu* alteration as a prognostic factor. The main advantage of FISH over IHC is that it bypasses the potential problem of antigenic changes secondary to the processes of tissue fixation and embedding. Also, unlike immunohistochemistry, FISH analysis provides a quantitative assessment of *HER-2/neu* gene status. A number of recent studies have looked at *HER-2/neu* gene alteration using FISH analysis in breast and other neoplasms (14–28). To the best of our knowledge, head and neck tumors have not been assessed for *HER-2/neu* gene amplification using FISH analysis.

The purpose of this study was 3-fold: (a) we wished to measure the frequency of *HER-2/neu* protein overexpression in our series of head and neck squamous cell carcinomas; (b) we wanted to ascertain *HER-2/neu* gene status by FISH analysis in tumors positive for *HER-2/neu* on immunohistochemistry; and (c) we wanted to determine whether *HER-2/neu* overexpression or amplification had prognostic implications or clinicopathological associations. In an effort to ensure population homogeneity and enhance the validity of the study, we included patients with advanced but resectable squamous cell carcinoma of the oral cavity or oropharynx treated with gross surgical resection and postoperative external beam radiation therapy. Tumors from other sites of the head and neck, or those treated with other therapeutic modalities, were excluded from the study.

PATIENTS AND METHODS

Patient Selection. Criteria for patient inclusion in this study were as follows: (a) presentation to the Department of Therapeutic Radiology at Yale School of Medicine between 1981 and 1992 with a histologically confirmed diagnosis of squamous cell carcinoma in the oral cavity or oropharynx; and (b) treatment with primary surgical excision and postoperative external beam radiotherapy with curative intent. Patients were excluded from this study if they had a prior history of head and neck squamous cell carcinoma, presented with metastatic disease, or failed to receive a full course of radiation therapy. A review of radiation records identified 77 patients who met the entry criteria. Of these, 14 had incomplete follow-up, and 10 tissue samples were unavailable, leaving 56 patients for inclu-

sion in the study. Although the strict selection criteria limited the total number of available cases, it was our intent to keep the patient cohort as homogeneous as possible. We acknowledge that this results in a relatively small sample size, but we considered this to be a pilot investigation to determine the feasibility of conducting larger studies in the future.

With the approval of the appropriate Institutional Review Boards, paraffin-embedded tissue samples were obtained from the hospital archives. Covariables including demographics, staging, clinical, pathological, and treatment parameters were extracted from patient charts. Local recurrence was defined as recurrence of disease at a site within the upper aerodigestive tract anatomically contiguous with the primary tumor. Regional recurrence was considered recurrence of disease within the cervical lymphatic system. Distant recurrence was considered recurrence of disease that did not meet the definitions of local or regional recurrence and was not considered to represent a second primary tumor based on its histology and/or clinical manifestations. Forty-one (73%) patients were followed until death, and the median follow-up time of the surviving patients was 6.1 years with a minimum of 2.8 years.

All patients were treated with gross total surgical resection and postoperative external beam radiotherapy to a median dose of 60 Gy. Final surgical margins were negative in 38 (68%) patients. Patients with evidence of tumor approaching within one high power field of final surgical margins were categorized as having positive margins in accordance with the method of Beitler *et al.* (29). Forty-nine (88%) patients received a radical or modified neck dissection. Eleven (20%) patients received adjuvant chemotherapy with the hypoxic cytotoxins mitomycin C or porfiromycin as part of an institutional protocol (30, 31). Five (9%) patients received intraoperative brachytherapy. All patients were staged clinically according to the American Joint Committee on Cancer TNM classification system (32). Clinically N_0 patients were restaged for the purposes of this analysis if pathological examination of the neck dissection specimen was positive for nodal metastases.

Immunohistochemistry. Immunohistochemical analysis was performed on 5-cm-thick tissue sections prepared from formalin-fixed, paraffin-embedded archival tissue from the resected primary tumor. Sections were deparaffinized in xylene and rehydrated in graded alcohols. Endogenous peroxidases were quenched with 10% hydrogen peroxide in PBS. Antigen retrieval was conducted by heating slides in the microwave at high power for 2 min, followed by 2 min of cooling and another 2 min of heating. Slides were then blocked with 10% normal rabbit serum and incubated with the primary antibody, the murine monoclonal CB11 antibody (Novocastra, Burlingame, CA) diluted 1:40, overnight at 4°C. The biotinylated secondary rabbit antimouse antibody (Zymed, San Francisco, CA) was applied to the slides for another hour at room temperature (1:100). The streptavidin-horseradish peroxidase complex (Vectastain kit; Vector, Burlingame, CA) was then applied and visualized with diaminobenzidine. Sections from a cell block of the SK-BR-3 breast tumor cell line, known to overexpress the *HER-2/neu* oncoprotein, were used as positive controls. The primary antibody incubation was substituted with incubation with 10% normal rabbit serum, and these slides served as negative controls.

An experienced pathologist (D. C.), blinded to the clinical outcomes, examined multiple microscopic fields to score the tissue sections for tumor staining intensity (0, none; 1+, faint and focal membrane staining of <10% of tumor; 2+, moderate membrane staining of >10% of tumor; 3+ and 4+, strong and intense membrane staining of >10% of the tumor). Samples staining as moderate, strong, or intense were considered positive in the statistical analysis.

FISH. FISH analyses were performed using the PathVysion HER-2 DNA Probe kit (Vysis, Downers Grove, IL), containing two directly labeled fluorescent DNA probes specific for the HER-2/*neu* gene locus (LSI HER-2/*neu* SpectrumOrange) and the chromosome 17 centromeric α satellite DNA (CEP 17 SpectrumGreen). Paraffin sections, 5 cm thick, from the 12 cases characterized as positive for HER-2/*neu* overexpression on IHC were chosen for FISH analysis. Four cases characterized as having some membrane staining, but on <10% of the specimen (1+), were also selected for FISH analysis. Finally, three cases with no membrane staining but strong cytoplasmic staining were also included in the FISH analysis for a total of 19 cases. Sections from the paraffin-embedded blocks of the SK-BR-3 breast tumor cell line were used as positive controls. Slides were baked at 60°C overnight, deparaffinized in xylene, and rinsed in 100% ethanol. Sections were then pretreated in sodium thiocyanate (Vysis) at 80°C for 10 min, digested with pepsin for 15 min (4 mg/ml in 0.2 M HCl; Vysis), and rinsed in graded ethanol (70, 85, and 99%). Ten μ l of probe/hybridization mixture, including blocking DNA, was applied to a previously demarcated area of the specimen and contained with coverslips sealed with rubber cement. The probes and specimen were codenatured and hybridized using the Vysis Hybrite hybridization system. The Hybrite unit was programmed to allow 5 min of denaturation at 73°C, followed by overnight hybridization at 37°C. The slides were subsequently washed in 2 \times SSC/3% NP40 and counterstained with 4',6-diamidino-2-phenylindole.

HER-2/*neu* gene amplification was quantified by comparing the ratio of LSI HER-2/*neu* to CEP 17 probe signals in accordance with PathVysion HER-2 DNA Probe kit criteria. All sections were examined directly using an Olympus AX70 epifluorescence microscope equipped with narrow band pass filters. Each slide was initially scanned at low power to assess heterogeneity and identify appropriate areas of tumor tissue with clearly defined nuclei. The 60 \times objective was then used to score signals in 60 nonoverlapping tumor cell nuclei to determine the average number of HER-2/*neu* and chromosome 17 copies/cell for each tumor specimen. The ratio of these averages was used to determine the presence of HER-2/*neu* gene amplification. Specimens with a HER-2/*neu*:chromosome 17 ratio greater than two were scored as positive for HER-2/*neu* gene amplification. Specimens with an average of more than two chromosome 17 signals/cell were considered aNeusomic.

Statistical Analysis. Molecular marker status and relevant covariables were assembled in a database and analyzed using SAS User 61s Guide, Version 6.12 (SAS Institute, Cary, NC). All tests of statistical significance were two-sided. Follow-up time and time to recurrence were calculated from the date of surgery to the date of the relevant outcome. To enhance the power of statistical comparisons, the follow-

Table 1 Clinicopathological distribution and immunohistochemistry results of the cohort

	Entire cohort (%)	HER-2/ <i>neu</i> positive (%)
Total	56 (100)	9 (100)
Site		
Oral cavity	32 (57)	3 (33)
Oropharynx	24 (43)	6 (67)
T stage		
1–2	19 (34)	3 (33)
3–4	37 (66)	6 (67)
N stage		
0	10 (18)	2 (22)
1	24 (43)	2 (22)
2–3	22 (39)	5 (56)
AJCC ^a stage		
II	5 (9)	2 (44)
III	16 (28)	0
IV	35 (63)	7 (78)
Grade		
Poorly to moderately	11 (20)	1 (11)
Moderately	22 (39)	5 (56)
Moderately well	11 (20)	2 (22)
Well-differentiated	12 (21)	1 (11)
Margins		
Negative	38 (68)	6 (67)
Positive	18 (32)	3 (33)
Sex		
Male	46 (82)	7 (78)
Female	10 (18)	2 (22)
Race		
White	47 (84)	7 (78)
Black	9 (16)	2 (22)
Age		
41–56	14 (25)	3 (33)
56–59	14 (25)	4 (44)
59–66	14 (25)	0
66–79	14 (25)	2 (22)

^a AJCC, American Joint Committee on Cancer.

ing categories were collapsed: T stage 1 and 2, T stage 3 and 4, N stage 2 and 3, and tumor grade poorly and poor-to-moderately differentiated.

Bivariate analyses for the associations between clinical-pathological variables and HER-2/*neu* positivity were conducted using the χ^2 test of statistical significance. Bivariate analyses for the association between HER-2/*neu* positivity and local recurrence, regional recurrence, distant recurrence, disease-free survival, and overall survival were conducted using the Kaplan-Meier Log-Rank test. Unadjusted relative risks were calculated using a Cox proportional hazards model.

RESULTS

A total of 77 patients were eligible for the analysis, but 21 were excluded because of incomplete follow-up data and/or insufficient archival tissue, leaving 56 (73%) patients included in the analysis. Frequency statistics for clinical-pathological characteristics of the 56 patients in our cohort are presented in Table 1. Fifty-one (91%) patients presented with stage III or IV disease, with the remaining 5 patients presenting with stage II. A total of 14 (25%) patients experienced local recurrence, 10 (18%) regional recurrence, and 14 (25%) distant recurrence.

Fig. 1 A SCC case exhibiting 4+ staining on IHC.

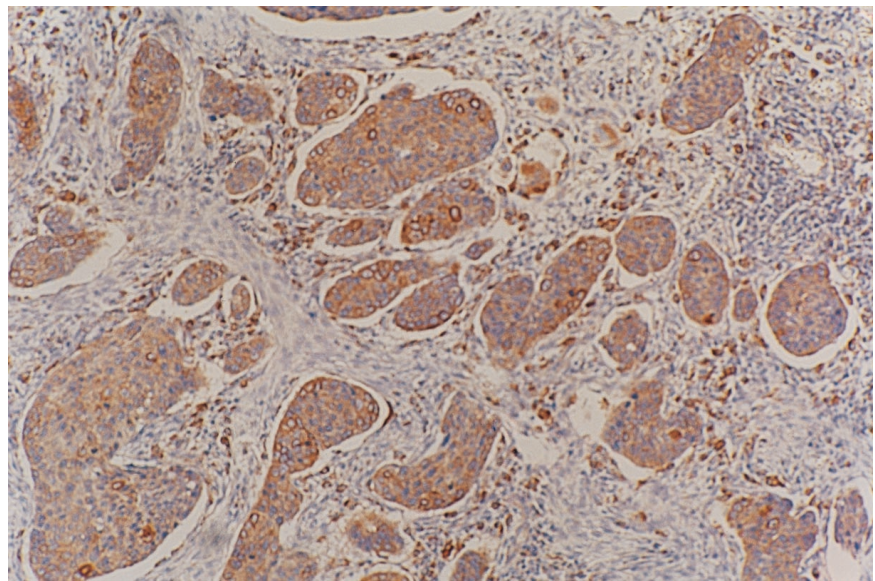


Table 2 VEGF positivity and HER-2/neu overexpression

	HER-2/neu positive (%)	HER-2/neu negative (%)
VEGF positive	7 (78)	16 (34)
VEGF negative	2 (22)	31 (66)
Totals ^a	9 (100)	47 (100)

^a $P = 0.015$.

Forty-one (73%) patients died during the follow-up period. Five-year survival for this cohort was 40% (22 patients).

Immunohistochemistry. In the 67 tumors assessed for membranous HER-2/neu immunohistochemical staining, 53 (78%) were clearly negative, 4 (6%) were 1+, 10 (15%) were 2+, 1 (2%) was 3+, and 1 (2%) was 4+. With a cutoff point for positivity between 1+ and 2+, a total of 12 (17%) tumors satisfied criteria for HER-2/neu positivity (Fig. 1). Twenty-nine (43%) tumors displayed cytoplasmic staining, but only those tumors with concomitant membranous staining were considered positive for purposes of the analysis.

Bivariate statistics for the relationships between clinical-pathological variables and HER-2/neu positivity are presented in Table 1. No significant associations were noted between HER-2/neu positivity and primary tumor site, T stage, N stage, tumor grade, margin status, sex, race, and age.

No significant associations between HER-2/neu positivity and local, regional, or distant recurrence were observed. HER-2/neu positivity also failed to correlate with disease-free survival (relative risk, 0.83; 95% confidence interval, 0.29–2.4) and overall survival (relative risk, 1.4; 95% confidence interval, 0.62–3.3).

A significant association was found between HER-2/neu positivity and VEGF positivity [stained and reported previously on the same series (7)]. HER-2/neu positive lesions were more likely to be VEGF positive (Table 2; $P = 0.015$).

Table 3 FISH results

Case number	IHC score	HER-2/neu/CEP 17 signals	Mean CEP 17 signals/cell
1	4+	2.49	1.48
2	3+	3.15	1.56
3	2+	2.52	2.27
4	2+	1.11	1.50
5	2+	0.89	1.50
6	2+	1.16	3.87
7	2+	0.71	2.66
8	2+	0.75	1.42
9	2+	1.00	2.27
10	2+	0.93	3.39
11	1+	0.84	1.88
12	1+	0.88	2.22
13	1+	3.44	1.80
14	0	0.93	3.23
15	0	1.02	2.36
16	0	0.90	1.83

FISH. FISH analysis generated quantifiable signal in 16 of the 19 sections selected. FISH results for this cohort are shown in Table 3. Four tumors exhibited oncogene amplification using our criterion of HER-2/neu:centromere 17 signal ratio of greater than two, and two of these are shown in Fig. 2. The amplified cases were 4+, 3+, 2+, and 1+ on immunostaining. A total of seven cases were nonamplified but polysomic for chromosome 17 using our criteria of an average of more than two centromere 17 signals/cell. Four of these cases were 2+ on immunostaining, one was 1+, and two did not exhibit any membrane staining but did display intense cytoplasmic staining. These data are also presented in Table 4.

For statistical analysis, we divided our series into three groups, those tumors amplified for HER-2/neu, those nonamplified but polysomic for chromosome 17, and the rest of the cohort, including cases disomic on FISH or cases not analyzed

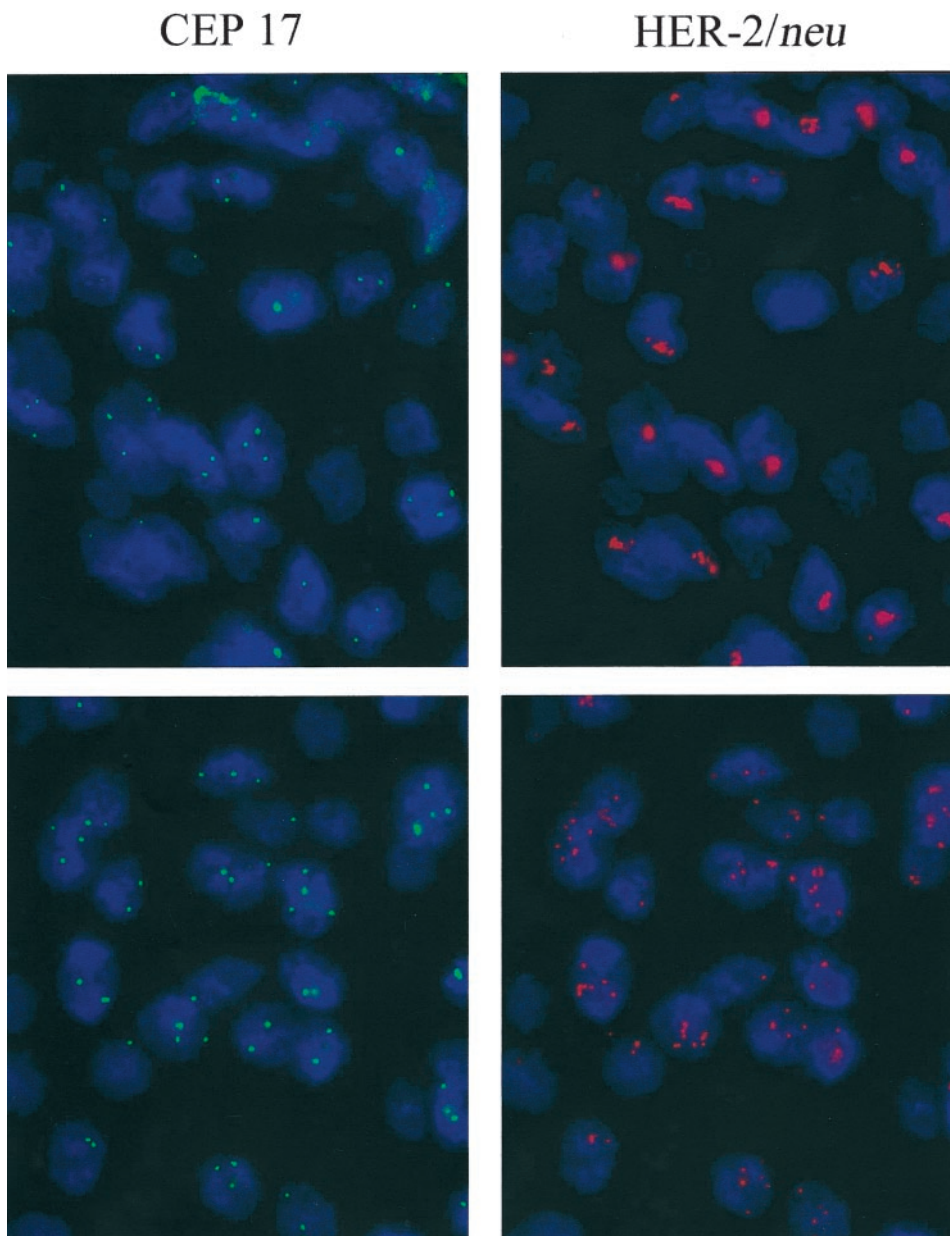


Fig. 2 FISH analysis demonstrating HER-2/neu gene amplification in two SCCs of the head and neck. Top, case 1; bottom, case 2.

by FISH, because they had a score of zero by IHC. Bivariate statistics for the relationships between clinicopathological variables and FISH status (amplified, polysomic, or disomic/not overexpressed) did not reveal any significant associations. A trend toward higher T and N stage among the amplified cases was noted. All four amplified cases presented with stage IV disease. These findings are shown in Table 5.

Bivariate analysis for the relationship between FISH status and outcome variables, both recurrence and survival, also failed to reveal any significant associations. Of the four amplified cases, two cases suffered a regional recurrence and one case suffered a distant recurrence, for a total of three events among the four cases. There were no recurrences among the amplified cases. Table 5 displays the clinical course and out-

come of the four amplified cases. Five-year survival for the disomic/not overexpressed cohort, the polysomic, and the amplified cases was 43, 50, and 0%, respectively. Mean survival time for the disomic/not overexpressed cases was 5.8 years; mean survival time for the amplified cases was 2.2 years. The *P* for these differences in overall survival was 0.15.

Polysomic cases were collapsed with both amplified cases and the rest of the cohort for statistical analysis but failed to reveal any significant associations as part of either subset.

DISCUSSION

Previous reports on HER-2/neu protein overexpression in head and neck SCC have been variable. Craven *et al.* (9)

Table 4 HER-2/*neu* gene status and clinicopathological and outcome variables

	Amplified (%)	Polysomic (%)	Remaining cohort (%)
Total	4 (100)	5 (100)	47 (100)
T stage			
1-2	1 (25)	3 (60)	15 (32)
3-4	3 (75)	2 (40)	32 (68)
N stage			
0	0	1 (20)	9 (19)
1	1 (25)	2 (40)	21 (45)
2-3	3 (75)	2 (40)	17 (36)
AJCC ^a stage			
II	0	1 (20)	4 (9)
III	0	2 (40)	14 (30)
IV	4 (100)	2 (40)	29 (62)
Overall survival (%)			
5 yr	0	40	43
10 yr	0	40	28
Mean survival time (yr)	2.2	3.1	5.8

^a AJCC, American Joint Committee on Cancer.

demonstrated HER-2/*neu* overexpression in 46% of 93 tumors by IHC but did not find overexpression to correlate with clinical parameters. Ibrahim *et al.* (10) analyzed 16 fresh-frozen SCC specimens to immunohistochemical analysis. They reported weak to intense mixed membranous/cytoplasmic staining in 14 (88%) of these. More interestingly, they also reported an increase in tumor stage with increasing intensity of HER-2/*neu* staining. Hou *et al.* (5) examined 86 human specimens of oral mucosa representing an entire range of differentiation, from normal to frank carcinoma. They reported increasingly frequent and more intense staining from normal to hyperplastic to dysplastic and lastly to carcinomatous mucosa. All 21 cases of SCC were positive, but the authors did not differentiate between membranous and cytoplasmic staining. In contrast to these studies, a number of other reports have not found HER-2/*neu* overexpression in this tumor type. Field *et al.* (4) looked at 75 SCC, using four different antibodies, but could not demonstrate any membranous staining at all. They did report 60% cytoplasmic staining. Kearsely *et al.* (11) could not demonstrate unequivocal membrane staining on IHC or HER-2/*neu* gene amplification on Southern blot analysis in any of 46 head and neck SCC cases. Two studies by Riviere *et al.* (12, 13) failed to demonstrate enhanced HER-2/*neu* transcription on Northern blot or enhanced protein expression on IHC in their series of head and neck SCCs.

A comprehensive evaluation of HER-2/*neu* in head and neck SCC was conducted by Beckhardt *et al.* (2). The authors reported that 6 of 38 (16%) head and neck SCC tissue sections showed high HER-2/*neu* oncoprotein expression, whereas another 12 (31%) showed intermediate staining. Although the study did not assess these same sections for gene amplification, the authors conducted Southern, Northern, and Western blot analysis as well as IHC on 11 head and neck SCC cell lines. Two cell lines (18%) exhibited HER-2/*neu* gene amplification, mRNA overexpression, and protein overexpression, whereas another cell line exhibited mRNA and protein overexpression in the absence of gene amplification.

In our study, the CB11 antibody was used for immunohis-

tochemical analysis. This antibody is specific for the internal domain of the p185 protein and has been shown to be highly specific (100%) but not very sensitive (51%; Refs. 16, 33). Although our criteria for positivity on immunostaining was at least 10% of cells displaying membrane positivity, others have suggested that any membranous staining with this antibody should be considered positive, because of its lower sensitivity (16). Using our criteria, a total of 12 of 67 cases (18%) analyzed by IHC were positive. If we were to include the cases characterized as 1+ (<10% membrane staining), a total of 16 (24%) of 67 cases were positive. Although no clinicopathological or outcome correlations with protein overexpression were detected in the 56 patients for whom both immunohistochemical and adequate clinical data were available, one must bear in mind the small size of this and other similar studies when compared with the significantly larger studies looking at HER-2/*neu* in the breast. Still, it is worth noting that the 5- and 10-year survival rates for HER-2/*neu*-negative patients were 44 and 25%, respectively, compared with 17 and 0% for HER-2/*neu*-positive patients. These findings have not been displayed in survival curves because of the nonsignificant *P* and wide confidence intervals involved. Nonetheless, a larger study may show this trend to be truly significant. Although we acknowledge the limitations in interpretation of the clinical implications of these data, it is clear that further studies along this avenue of investigation are warranted.

A significant number of tumors in our series displayed cytoplasmic staining (43%). HER-2/*neu* cytoplasmic staining has been reported by numerous investigators, in both breast and head and neck tumors, and its interpretation has been debated. Cytoplasmic staining may represent a technical artifact generated by a cross-reactive antibody or by antigen retrieval or may be attributable to a cross-reaction with mitochondrial protein (2, 4). Alternatively, it may represent true protein overexpression, but with an alteration in the processing or stability of p185, thus interfering with its subcellular localization (2, 4). An earlier study by Gusterson *et al.* (34) demonstrated a correlation between cytoplasmic staining and gene amplification in breast carcinomas. The report by Field *et al.* (4) cited earlier showed 60% cytoplasmic staining in 75 head and neck SCCs, and the authors were able to demonstrate that cytoplasmic staining was specific by effectively blocking cytoplasmic staining by preabsorption of the primary antibodies with appropriate blocking peptides. Still, one must remember that immunopositivity attributable to a cross-reacting antigen other than the HER-2/*neu* protein would also be blocked by these peptides. Thus, membrane staining has become the standard criterion for positivity in breast lesions, and we used this criterion in the present series as well.

An interesting and significant association (*P* = 0.015) was found between HER-2/*neu* membrane positivity and VEGF protein levels. VEGF staining was conducted on the same series of SCC at our institution and reported previously (7). In that study, VEGF-positive tumors were more likely to recur locally and distantly, and VEGF positivity was a strong independent predictor of poor disease-free and overall survival in a multivariate model. Interestingly, although HER-2/*neu* positivity was significantly associated with VEGF positivity in this study, the clinical associations did not hold true, perhaps because of the small

Table 5 Clinical course and outcome of FISH amplified cases

	Documented follow-up time (mo)	Local recurrence (rectime ^a months)	Regional recurrence (rectime months)	Distant recurrence (rectime months)	Survival time (mo)	Cause of death
Case 1	12	None documented	None documented	None documented	56	Unknown
Case 2	17	None documented	Yes (10)	None documented	17	Disease
Case 3	24	None documented	Yes (18)	None documented	24	Disease
Case 4	9	None documented	None documented	Yes (3)	9	Disease

^a rectime, time until recurrence.

study size. Recent data have indicated that overexpression and activation of the HER-2/*neu* protein leads to up-regulation of VEGF expression, thus promoting tumor angiogenesis (35–38). Our correlation of HER-2/*neu* overexpression with VEGF also indicates a potential relationship between these two markers, which warrants further investigation.

FISH analysis was conducted to elucidate the HER-2/*neu* gene status in those cases judged positive on IHC. Both FISH and IHC are important tools for tissue-based detection of HER-2/*neu* alteration. However, IHC is essentially qualitative, and its utility is further compromised by variable antibody sensitivities, nonstandardized staining protocols, and nonuniform criteria for positivity. More importantly, IHC is subject to the antigenic changes engendered by tissue fixation and paraffin embedding. FISH is a quantitative technique which essentially circumvents many of these problems, but with the drawback of being a longer and more expensive procedure.

To the best of our knowledge, this is the first time HER-2/*neu* gene amplification has been reported in SCC by FISH analysis. Our criterion for gene amplification was a HER-2/*neu*: centromere 17 signal ratio of >2, which corrects for polysomy-related increases in gene copy (14, 16, 18, 21). Using this criterion, four of the 16 cases analyzed by FISH were found to be HER-2/*neu* amplified. As shown in other breast cancer studies, the cases with the highest IHC scores (4+ and 3+) had gene amplification (15, 19). Because FISH analysis was done only on cases characterized as positive on IHC, it is impossible to make any judgement on the actual proportion of amplified cases in the entire series. In a recent study by Pauletti *et al.* (14), 43% of breast tumors amplified by FISH analysis were negative on immunohistochemistry. This is most likely attributable to the loss of antigenicity resulting from formalin fixation and paraffin embedding.

In our series, of the 13 cases demonstrating any membrane staining, 4 (31%) were amplified, 5 (38%) were nonamplified but polysomic, and 4 (31%) were disomic. Our criterion for polysomy was a mean of >2 chromosome 17 signals/cell. This was based on the fact that in sections of diploid tissue, the mean chromosome 17 signals/cell range between 1.5 and 2 because of nuclear truncation from sectioning.³ Of the 5 cases with chromosome 17 polysomy, 4 were characterized as 2+ on IHC, and 1 was characterized as 1+. As noted by others, polysomy may account for low-level HER-2/*neu* protein overexpression (16,

17). In a report by Gancberg *et al.* (16), >40% of a cohort of breast tumors was found to be polysomic for chromosome 17.

Four cases had some membrane staining but had no gene alteration. These cases may represent true single-copy overexpression or may be the result of a technical artifact. The phenomenon of single gene copy overexpression has been reported frequently and is usually cited as representing between 5 and 10% of all overexpressing breast tumors (1, 15, 20). Transcriptional or posttranscriptional activation is thought to be the mechanism of overexpression in these cases. In the study by Pauletti *et al.* (14) cited earlier, 7% of FISH-negative tumors were positive for HER-2/*neu* overexpression. Although the proportion in our study (31%) is much higher than that reported in breast lesions, it must be remembered that this is a different tumor type. Studies comparing IHC and FISH in endometrial cancer have shown high rates of protein immunopositivity in the absence of gene amplification (25, 26). Also, two studies conducted on prostate carcinoma did not demonstrate a statistically significant concordance between HER-2/*neu* gene amplification by FISH and protein overexpression by IHC (23, 24). Similarly, high rates of nonamplification overexpression of HER-2/*neu* have been reported in ductal carcinoma *in situ* (15) and apocrine adenosis of the breast (28). A clearer picture of the various mechanisms of HER-2/*neu* overexpression in head and neck SCC will have to await the results of a larger study.

We would again emphasize and acknowledge the limitations of our small sample size in determining prognostic significance and clinical implications of these data. Although there were no statistically significant clinicopathological or outcome associations with HER-2/*neu* amplification, there were some trends worth mentioning. Three of the four amplified cases (75%) had T3–4 and N2–3 disease at presentation compared with 67% (32 of 48) and 35% (17 of 48) for the nonamplified cases. All four cases (100%) presented with stage IV disease, as compared with 60% among the nonamplified cases. Overall survival was shortened in amplified cases, with a mean survival of 2.2 years, as compared with 5.8 years for the nonamplified cases.

Although there were two regional and one distant failure in the amplified group, there were no local failures. It is possible that this may be attributable to abbreviated survival times insufficient for the detection of local recurrences in this group. Another possibility is that HER-2/*neu* overexpression may confer increased sensitivity to radiation. This interesting prospect may be important in that HER-2/*neu* overexpression would have a greater impact in patients treated by both surgery and radiation, as in our cohort, rather than by surgery alone. Although our

³ Vysis, Inc., personal communication.

findings were not statistically significant, we feel the results of this preliminary study justify a larger, more extensive study to assess HER-2/*neu* alteration as a significant prognostic predictor in this tumor type.

In summary, our report unequivocally demonstrates, for the first time, the concurrent alteration of the HER-2/*neu* oncogene at the level of protein overexpression and gene amplification in a subset oral cavity/oropharynx SCC. We think that this preliminary report also demonstrates some interesting trends that, if confirmed in larger sample sizes, may have clinical implications. The current controversy and confusion surrounding the role of this oncogene in SCC of the head and neck is reminiscent of the early uncertainty regarding its now well-established role in breast cancer. We feel it is premature to conclude that HER-2/*neu* alterations may have prognostic significance, but that it is also too early to dismiss that possibility without a larger, perhaps multicenter study.

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REFERENCES

- Slamon, D. J., Godolphin, W., Jones, L. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., and Ullrich, A. Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. *Science (Wash. DC)*, *244*: 707–712, 1989.
- Beckhardt, R. N., Kiyokawa, N., Xi, L., Liu, T. J., Hung, M. C., el-Naggar, A. K., Zhang, H. Z., and Clayman, G. L. HER-2/*neu* oncogene characterization in head and neck squamous cell carcinoma. *Arch. Otolaryngol. Head Neck Surg.*, *121*: 1265–1270, 1995.
- Press, M. F., Pike, M. C., Hung, G., Zhou, J. Y., Ma, Y., George, J., Dietz-Band, J., James, W., and Slamon, D. J. Amplification and overexpression of HER-2/*neu* in carcinomas of the salivary gland: correlation with poor prognosis. *Cancer Res.*, *4*: 5675–5682, 1994.
- Field, J. K., Spandidos, D. A., and Yiagnisis, M. c-erbB-2 expression in squamous cell carcinoma of the head and neck. *Anticancer Res.*, *12*: 613–620, 1992.
- Hou, L., Shi, D., and Tu, S. Oral cancer progression and c-erbB-2/*neu* proto-oncogene expression. *Cancer Lett.*, *65*: 215–220, 1992.
- Landis, S. H., Murray, T., Bolden, S., and Wingo PA. Cancer Statistics, 1999. *CA Cancer J. Clin.*, *49*: 8–31, 1999.
- Smith, B. D., Smith, G. L., Carter, D., Sasaki, C. T., and Haffty, B. G. Prognostic significance of vascular endothelial growth factor protein levels in oral and oropharyngeal squamous cell carcinoma. *J. Clin. Oncol.*, *18*: 2046–2052, 2000.
- Smith, B. D., and Haffty, B. G. Molecular markers as prognostic factors for local recurrence and radioresistance in head and neck squamous cell carcinoma. *Radiat. Oncol. Investig.*, *7*: 125–144, 1999.
- Craven, J. M., Pavelic, Z. P., Stambrook, P. J., Pavelic, L., Gapany, M., Kelley, D. J., Gapany, S., and Gluckman, J. L. Expression of c-erbB-2 gene in human head and neck carcinoma. *Anticancer Res.*, *12*: 2273–2276, 1992.
- Ibrahim, S. O., Vasstrand, E. N., Liavaag, P. G., Johannessen, A. C., and Lillehaug, J. R. Expression of c-erbB proto-oncogene family members in squamous cell carcinoma of the head and neck. *Anticancer Res.*, *17*: 4539–4546, 1997.
- Kearsely, J. H., Leonard, J. H., Walsh, M. D., and Wright, G. R. A comparison of epidermal growth factor receptor (EGFR) and c-erbB-2 oncogene expression in head and neck squamous cell carcinoma. *Pathology*, *23*: 189–194, 1991.
- Riviere, A., Becker, J., and Loning, T. Comparative investigation of c-erbB2/*neu* expression in head and neck tumors and mammary cancer. *Cancer*, *67*: 2142–2149, 1991.
- Riviere, A., Wilckens, C., and Loning, T. Expression of c-erbB2 and c-myc in squamous epithelia and squamous cell carcinoma of the head and neck and the lower female genital tract. *J. Oral Pathol. Med.*, *19*: 408–413, 1990.
- Pauletti, G., Dandekar, S., Rong, H., Ramos, L., Peng, H., Seshadri, R., and Slamon, D. J. Assessment of methods for tissue-based detection of the HER-2/*neu* alteration in human breast cancer: a direct comparison of fluorescence *in situ* hybridization and immunohistochemistry. *J. Clin. Oncol.*, *18*: 3651–3664, 2000.
- Jimenez, R. E., Wallis, T., Tabasczka, P., Reuter, V. E., Epstein, J. I., Folpe, A. L., de Peralta-Venturina, M., Tamboli, P., Ansell, I. D., Grignon, D. J., Young, R. H., and Amin, M. B. Determination of Her-2/*neu* status in breast carcinoma: comparative analysis of immunohistochemistry and fluorescent *in situ* hybridization. *Mod. Pathol.*, *13*: 37–45, 2000.
- Gancberg, D., Lespagnard, L., Rouas, G., Paesmans, M., Piccart, M., Di Leo, A., Nogaret, J. M., Hertens, D., Verhest, A., and Larsimont, D. Sensitivity of HER-2/*neu* antibodies in archival tissue samples of invasive breast carcinomas. *Am. J. Clin. Pathol.*, *113*: 675–682, 2000.
- Jacobs, T. W., Gown, A. M., Yaziji, H., Barnes, M. J., and Schnitt, S. J. Comparison of fluorescence *in situ* hybridization and immunohistochemistry for the evaluation of HER-2/*neu* in breast cancer. *J. Clin. Oncol.*, *17*: 1974–1982, 1999.
- Press, M. F., Bernstein, L., Thomas, P. A., Meisner, L. F., Zhou, J. Y., Ma, Y., Hung, G., Robinson, R. A., Harris, C., El-Naggar, A., Slamon, D. J., Phillips, R. N., Ross, J. S., Wolman, S. R., and Flom, K. J. HER-2/*neu* gene amplification characterized by fluorescence *in situ* hybridization: poor prognosis in node-negative breast carcinomas. *J. Clin. Oncol.*, *18*: 2894–2904, 1997.
- Hoang, M. P., Sahin, A. A., Ordonez, N. G., and Sneige, N. HER-2/*neu* gene amplification compared with HER-2/*neu* protein overexpression and interobserver reproducibility in invasive breast carcinoma. *Am. J. Clin. Pathol.*, *113*: 852–859, 2000.
- Ratcliffe, N., Wells, W., Wheeler, K., and Memoli, V. The combination of *in situ* hybridization and immunohistochemical analysis: an evaluation of HER-2/*neu* expression in paraffin-embedded breast carcinomas and adjacent normal-appearing breast epithelium. *Mod. Pathol.*, *10*: 1247–1252, 1997.
- Pauletti, G., Godolphin, W., Press, M. F., and Slamon, D. J. Detection and quantitation of HER-2/*neu* gene amplification in human breast cancer archival material using fluorescence *in situ* hybridization. *Oncogene*, *13*: 63–72, 1996.
- Persons, D. L., Borelli, K. A., and Hsu, P. H. Quantitation of HER-2/*neu* and c-myc gene amplification in breast carcinoma using fluorescence *in situ* hybridization. *Mod. Pathol.*, *10*: 720–727, 1997.
- Ross, J. S., Sheehan, C. E., Hayner-Buchan, A. M., Ambros, R. A., Kallakury, B. V., Kaufman, R. P., Jr., Fisher, H. A., Rifkin, M. D., and Muraca, P. J. Prognostic significance of HER-2/*neu* gene amplification status by fluorescence *in situ* hybridization of prostate carcinoma. *Cancer (Phila.)*, *79*: 2162–2170, 1997.
- Ross, J. S., Sheehan, C., Hayner-Buchan, A. M., Ambros, R. A., Kallakury, B. V., Kaufman, R., Fisher, H. A., and Muraca, P. J. HER-2/*neu* gene amplification status in prostate cancer by fluorescence *in situ* hybridization. *Hum. Pathol.*, *28*: 827–833, 1997.
- Saffari, B., Jones, L. A., El-Naggar, A., Felix, J. C., George, J., and Press, M. F. Amplification and overexpression of HER-2/*neu* (c-erbB2) in endometrial cancers. Correlation with overall survival. *Cancer Res.*, *55*: 5693–5698, 1995.
- Riben, M. W., Malfetano, J. H., Nazeer, T., Muraca, P. J., Ambros, R. A., Ross, J. S., *et al.* Identification of HER-2/*neu* oncogene amplification by fluorescence *in situ* hybridization in stage I endometrial carcinoma. *Mod. Pathol.*, *10*: 823–831, 1997.
- Ishikawa, T., Kobayashi, M., Mai, M., Suzuki, T., and Ooi, A. Amplification of the c-erbB-2 (HER-2/*neu*) gene in gastric cancer cells. Detection by fluorescence *in situ* hybridization. *Am. J. Pathol.*, *151*: 761–768, 1997.

28. Selim, A. A., El-Ayat, G., and Wells, C. A. c-erbB2 oncoprotein expression, gene amplification, and chromosome 17 a*Neusomy* in apocrine adenosis of the breast. *J. Pathol.*, *191*: 138–142, 2000.
29. Beitler, J. J., Smith, R. V., Silver, C. E., Quish, A., Deore, S. M., Mullokandov, E., Fontenla, D. P., Wadler, S., Hayes, M. K., and Vikram, B. Close or positive margins after surgical resection for the head and neck cancer patient: the addition of brachytherapy improves local control. *Int. J. Radiat. Oncol. Biol. Phys.*, *40*: 313–317, 1998.
30. Haffty, B., Son, Y. H., Papac, R., Sasaki, C. T., Weissberg, J. B., Fischer, D., Rockwell, S., Sartorelli, A. C., and Fischer, J. J. Chemotherapy as an adjunct to radiation in the treatment of squamous cell carcinoma of the head and neck: results of the Yale mitomycin randomized trials. *J. Clin. Oncol.*, *15*: 268–276, 1997.
31. Haffty, B. G., Son, Y. H., Wilson, L. D., Papac, R., Fischer, D., Rockwell, S., Sartorelli, A. C., Ross, D., Sasaki, C. T., and Fischer, J. J. Bioreductive alkylating agent porfiromycin in combination with radiation therapy for the management of squamous cell carcinoma of the head and neck. *Radiat. Oncol. Investig.*, *5*: 235–245, 1997.
32. American Joint Committee on Cancer. *Manual for Staging Cancer*, Ed. 4. Philadelphia: J. B. Lippincott, 1992.
33. Press, M. F., Hung, G., Godolphin, W., and Slamon, D. J. Sensitivity of HER-2/*neu* antibodies in archival tissue samples: potential source of error in immunohistochemical studies of oncogene expression. *Cancer Res.*, *54*: 2771–2777, 1994.
34. Gusterson, B. A., Machin, L. G., Gullick, W. J., Venter, D. J., Powles, T. J., Elliott, C., Ashley, S., Tidy, A., and Harrison, S. Immunohistochemical localization of c-erbB-2 in human breast carcinomas. *Mol. Cell Probes*, *2*: 383–391, 1988.
35. Yen, L., You, X. L., Al Moustafa, A. E., Batist, G., Hynes, N. E., Mader, S., Meloche, S., and Alaoui-Jamali, M. A. Heregulin selectively upregulates vascular endothelial growth factor secretion in cancer cells and stimulates angiogenesis. *Oncogene*, *19*: 3460–3469, 2000.
36. Petit, A. M., Rak, J., Hung, M. C., Rockwell, P., Goldstein, N., Fendly, B., Kerbel, R. S., *et al.* Neutralizing antibodies against epidermal growth factor and ErbB2/*neu* receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells *in vitro* and *in vivo*: angiogenic implications for signal transduction therapy of solid tumors. *Am. J. Pathol.*, *151*: 1523–1530, 1997.
37. O-charoenrat, P., Rhys-Evans, P., Modjtahedi, H., and Eccles, S. A. Vascular endothelial growth factor family members are differentially regulated by c-erbB signaling in head and neck squamous cell carcinoma cells. *Clin. Exp. Metastasis*, *18*: 155–161, 2000.
38. Eccles, S. A. Cell biology of lymphatic metastasis. The potential role of c-erbB oncogene signalling. *Recent Results Cancer Res.*, *157*: 41–54, 2000.

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