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Elevated serum levels of a c-erbB-2 oncogene product in oral squamous cell carcinoma patients

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OBJECTIVES: Amplification of the proto-oncogene c-erbB-2 (HER-2/*neu*) has been shown to be a prognostic marker in many kinds of cancer including oral squamous cell carcinoma (OSCC). In order to obtain further information on the c-erbB-2 gene product p185, it is necessary to quantify expression levels. In this study we used an enzyme-linked immunosorbent assay (ELISA) for the extracellular domain of p185 to determine whether a soluble oncoprotein fragment can be detected in the serum of OSCC patients.

METHOD: Sera from 84 OSCC patients, 51 breast cancer patients (as positive controls), and 15 healthy controls were assayed in an ELISA. To study c-erbB-2 overexpression in OSCC, and breast cancer tissue samples we used an immunohistochemical technique.

RESULTS: The mean serum value (ng/ml, mean/SD) for the normal controls was 8.46/1.29. We chose the 95% level of normal controls as a cut-off to distinguish individuals with elevated levels. The breast cancer patients' and OSCC patients' serum values were 13.83/6.82 and 13.1/4.56, respectively. Significant differences ($P < 0.0001$) were observed between normal control and OSCC, normal control and the breast cancer group. Immunohistochemically detectable p185 (intermediate to high) was noted in 30 of 61 OSCC, and 24 of 51 breast cancer patients. There was a trend of association of serum oncoprotein fragment levels with tumor stages, but not with tumor sizes, nodal stages, metastases, and oral habits including betel quid chewing, alcohol drinking and smoking in the OSCC group.

CONCLUSION: The results of the present study raise the possibility that soluble c-erbB-2 protein levels in serum is a useful parameter for monitoring the disease status as well as the effect of therapy on patients with OSCC.

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Introduction

The c-erbB-2 oncogene (HER-2/*neu*) was identified from chemical carcinogen ethyl-nitroso urea-induced neuroblastomas of the rat (1). The human homology of the rat *neu* gene is a cellular gene that presents on band q-21 of chromosome 17 and encodes a 185-kd trans membrane glycoprotein (p185) with tyrosine kinase activity and is closely related to, but distinct from human epidermal growth factor receptor (EGFR) (2). Amplification of the c-erbB-2 gene and the overexpression of the c-erbB-2 encoded protein p185 are associated with a poor prognosis in breast (3–10), ovarian (11, 12), and endometrial (13) cancers, non-small cell lung adenocarcinoma (14), and differentiated gastric adenocarcinoma (15). Over expression of c-erbB-2 oncoprotein analyzed by immunohistochemistry is significantly correlated with shorter survival of oral squamous cell carcinoma (OSCC) (16, 17). The western blotting results revealed that a relatively high level of c-erbB-2 was found in oral cancer cell lines examined as compared with other head and neck SCC (17). Similary, the human breast carcinoma cell, which expressed c-erbB-2 has been proposed to release extracellular domain of the protein (p105) into the medium (18–20).

Several studies have reported increased levels of soluble c-erbB-2 oncoprotein (p185) in the sera of certain subsets of breast cancer patients (11, 21–24). In this investigation, we report the presence of elevated levels of c-erbB-2 oncoprotein (p185) detected by enzyme-linked immunosorbent assay (ELISA) in the sera of patients with breast cancer, and OSCC.

Patients and methods

Subjects

The protocol was approved by the institutional review board and informed consent was obtained before venipuncture.

Oral squamous cell carcinoma

Serum c-erbB-2 levels were measured in 84 OSCC patients (Table 1) admitted to the Department of Oral Maxillofacial Surgery, University Hospital, Kaohsiung Medical University, Taiwan. These patients (83 male

Table 1 Clinical characteristics of 84 oral squamous cell carcinoma

Clinical parameters	*Number of patient*
Age in years	
20–29	3
30–39	15
40–49	22
50–59	28
60–69	12
70–79	4
Primary tumor site	
Buccal mucosa	32
Tongue	21
Lip	8
Gingiva	7
Hard palate	3
Retromolar area	3
Bone of maxilla	3
Floor of mouth	2
Other	5
Stage	
I	6
II	7
III	48
IV	23
Tumor size	
T1	18
T2	39
T3	12
T4	15
Nodal stage	
N0	10
N1	58
N2	15
N3	1
Metastasis	
M0	20
M1	4
Mx (undiagnosed)	60

and one female, ages ranged from 20 to 77 years, with a mean of 49.67 ± 11.35 years) with recently diagnosed but untreated cancer at the time of drawing blood were selected for inclusion in this study.

Breast cancer patients

Fifty-one breast cancer patients (ages ranged from 27 to 80 years with a mean of 49.29 ± 12.15 years) admitted to the Department of Surgery, University Hospital, Kaohsiung Medical University, Taiwan were included in this investigation. All serum samples were taken after the histological diagnosis, but before initial surgery.

Control group

Reference values were based on the sera of 15 healthy subjects (10 male and five female, ages ranged from 25 to 59 years with a mean of 43.67 ± 11.3 years) who attended our clinic for routine physical examination.

Methods

Determination of serum c-erbB-2 p185 oncoprotein fragment

The amount of c-erbB-2 oncoprotein fragment was quantitated by ELISA using the human *neu* oncoprotein ELISA kit (QIA10 c-erbB-2 1c-*neu*; Rapid Format ELISA, Oncogene Research Products, Boston, MA, USA). The procedure according to the instructions of the manufacturer was followed. All measurements are means of duplicate determinations.

Immunohistochemistry

Sections were cut at 5 μ and processed for immunohistochemistry based on the labeled streptavidin-biotin peroxidase method (Dako LSAB®2 System; Dako, Carpinteria, CA, USA). Deparaffinized sections were microwaved in 0.01 mol/l citrate buffer, pH 6.0 (750 W) twice for 5 min with an interval of 1 min between cycles to check on the fluid level in the jars. After microwave irradiation the sections were allowed to cool down to room temperature (about 30 min).

Endogenous peroxidase activity was quenched by first incubating the specimens for 5 min with 3% hydrogen peroxide. Subsequentially the specimens were incubated with an appropriately characterized (1:200 with Dako Ab diluent) monoclonal antibody (Dako) against c-erbB-2 (also known as HER-2/*neu*) for 1 h, followed by sequential incubations with biotinylated link antibody and peroxidase-labeled streptavidin (20-min each) (Dako LSAB®2 System). Staining was completed after a 5-min incubation with a freshly prepared 3,3′-diaminobenzidine (DAB; Dako) substrate-chromogen solution. Slides were then counterstained with hematoxylin and coverslipped with an aqueous mounting medium.

Each set of experiments included positive and negative controls. The positive control for the c-erbB-2 visualization was an infiltrating ductal carcinoma of breast. The negative control was omission of the primary antibody substituted with diluent buffer.

Statistical analysis

The chi-square test for trend (treating concentrations as ordinal variables) was used to analyze the significance of the number of elevated serum c-erbB-2 levels in the OSCC, and breast cancer groups compared with the control group. Single item analysis (AVOVA/*t*-tests) and regression analysis were used to identify any correlation between c-erbB-2 serum levels with different clinical characteristics (tumor stage, size, nodal stage and metastasis), and personal habits (alcohol drinking, betel quid chewing, smoking). All statistical tests were conducted by the Statistical Analysis System® (SAS Institute, Cary, NC, USA), and a *P*-value of 0.05 or less was adopted as statistically significant.

Results

Serum concentration of the c-erbB-2 oncoprotein (p185) in the control group

The mean serum value for the normal controls ($n = 15$) was 8.46 ng/ml with a standard deviation of 1.29 ng/ml (Table 2).

Oral squamous cell carcinoma patients

There were 61 of 84 patients' tissue samples of sufficient size and integrity to allow for immunohistochemical

study. Thirty of these 61 cases were found to be intermediate to high immunohistochemically positive (49.2%) for the c-erbB-2-encoded protein p185 (Fig. 1). The OSCC patients' mean serum value of c-erbB-2 (p185) was 13.83 ng/ml with a standard deviation of 6.82 ng/ml (Table 2).

Breast cancer patients

The mean serum c-erbB-2 (p185) level of these 51 patients was 13.10 ng/ml with a standard deviation of 4.56 ng/ml (Table 2). Twenty-four of these 51 cases were found to be immunohistochemically positive for the c-erbB-2-encoded protein p185 (data not shown).

Statistical analysis

We chose 95% of normal controls' serum c-erbB-2 protein level as the cut-off to distinguish individuals with elevated (overexpressed) levels (Figs 2 and 3). The number of OSCC (59 of 84, 70.2%), and breast cancer (34 of 51, 66.7%) patients with an elevated serum c-erbB-2 protein level was significantly greater than that of the control group ($P < 0.005$).

Table 2 Serum c-erbB-2 levels

Concentration (C; ng/ml)	*OSCC (n = 84)*	*Breast cancer (n = 56)*	*Control (n = 15)*
$C \leq 8$	13 (15.5)	3 (5.4)	5 (33.3)
$8 < C \leq 10$	8 (9.5)	8 (14.3)	7 (46.7)
$10 < C \leq 12$	18 (21.4)	16 (28.6)	3 (20.0)
$12 < C \leq 14$	14 (16.7)	9 (16.1)	0
$14 < C \leq 16$	6 (7.1)	10 (17.9)	0
$16 < C \leq 18$	9 (10.7)	2 (3.6)	0
$C > 18$	16 (19.1)	8 (14.3)	0

The figures in the brackets are percentages of test samples and controls with the given concentration of serum c-erbB-2. The mean serum c-erbB-2 levels (ng/ml, mean ± SD) were 13.83 ± 6.82, 13.1 ± 4.56 and 8.46 ± 1.29 for OSCC, breast cancer and control group, respectively.

The mean serum levels of c-erbB-2 protein in the patients' alcohol status, betel quid chewing status, smoking status, and different clinical characteristics (stage, size, nodal stage and metastasis) of OSCC are computed in Table 3. The ANOVA/*t*-test revealed that none of the differences among different categories were

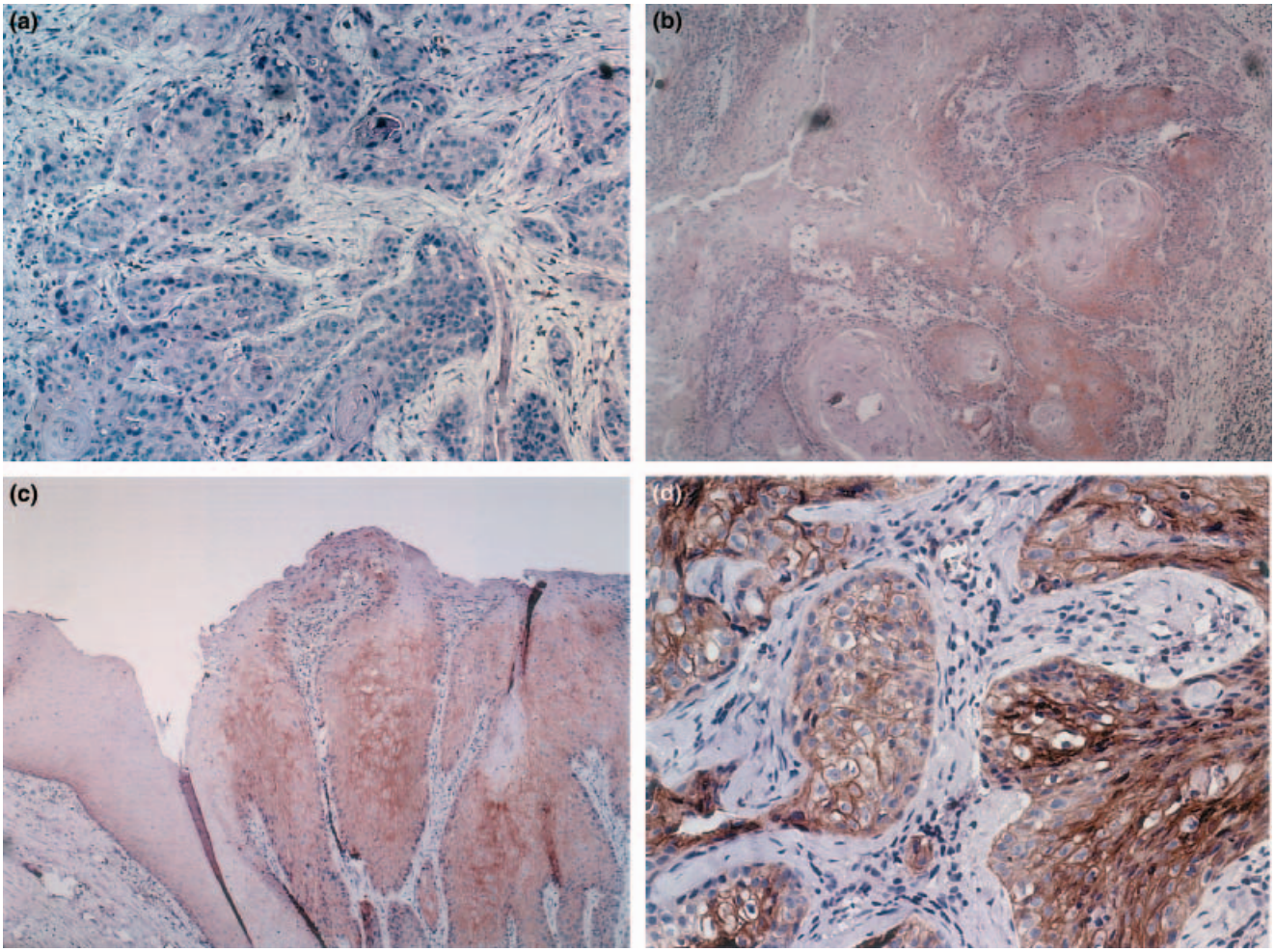

Figure 1 Representative immunohistochemical staining for the expression of HER-2/*neu* (c-erbB-2 *neu*) in oral SCC (a: no expression, ×20; b: low expression, ×10; c: intermediate expression, ×10; d: high expression, ×40).

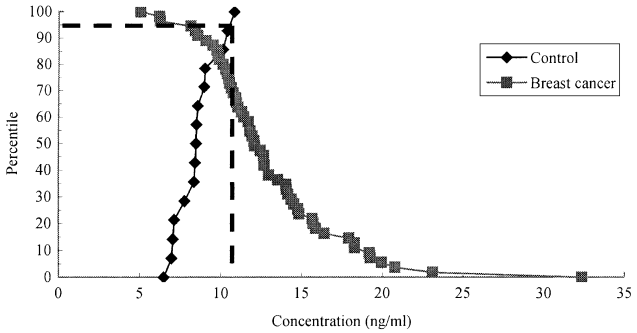

Figure 2 Percentile of serum level of breast cancer patients shows 66.7% (34 of 51) patients with an elevated (>95% cutoff value of normal control) serum c-erbB-2 protein level.

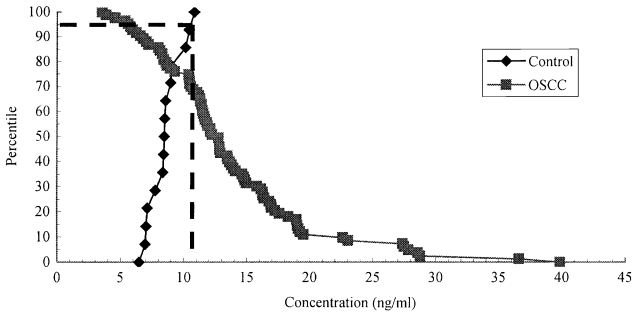

Figure 3 Percentile of serum level of oral squamous cell carcinoma patients shows 70.2% (59 of 84) patients with an elevated (>95% cutoff value of normal control) serum c-erbB-2 protein level.

shown to be statistically significant. A regression model was also used to compare these differences among the above variables. The adjusted means and 95% confidence intervals are shown in Table 3. Although the significant levels among these variables after adjusting each other were still higher than the level of 0.05, a borderline significant level of 0.0677 was found in stages. It was shown that the mean serum levels increased as the stages increased.

Discussion

In previous studies of several groups, immunohistochemically detected p185 was found to be a prognostic factor for survival time in ovarian cancer (24), OSCC (16, 17), and breast cancer (6, 25, 26). However, Khademi et al. (27) reported high percentage (76%) strong immunoreactivity of c-erbB-2 but no correlation with histological grading and nodal involvement in head and neck tumors. Nagler et al. (28) reported no correlation between c-erbB-2 staining (18% positive but significant with $P < 0.0001$) and any clinical parameters or survival in tongue SCC. The detection of a p185 oncoprotein in sera of ovarian cancer patients (24), and breast cancer patients (11, 22–24) have been reported, and might be useful as a diagnostic tool for patients with c-erbB-2-positive tumors. The present study reported an elevated serum level of a c-erbB-2 encoded p185 oncoprotein in patients with OSCC, and breast cancer. In OSCC, not

Table 3 Association between serum c-erbB-2 levels and oral habits and clinical characteristics of 84 OSCC patients

			Single item analysis			*Regression analysis*		
Item	*Category*	*Sample size*	*Mean*[a]	*SD*	*P-value*[b]	*Adjusted mean*	*95% CI*[c] *(lower, upper)*	*P-value*[d]
AC	Yes	60	14.36	7.37	0.7696	11.07	6.04, 16.11	0.8779
	No	19	13.83	4.72		11.38	6.35, 16.41	
BQ	Yes	70	14.61	7.11	0.1781	13.46	8.65, 18.27	0.1427
	No	9	11.35	2.26		8.99	2.85, 15.13	
CS	Yes	68	14.21	7.00	0.9407	10.38	5.60, 15.15	0.5435
	No	11	14.38	5.71		12.08	6.13, 18.03	
S	I and II	8	12.87	7.18	0.7548	6.75	0.97, 14.47	0.0677
	III	45	14.11	6.40		10.62	4.32, 16.92	
	IV	26	14.88	7.54		16.31	10.87, 21.75	
T	T1	18	15.82	8.57	0.5450	14.10	8.96, 19.25	0.1621
	T2	35	13.93	5.45		11.96	7.18, 16.74	
	T3	12	14.89	9.01		12.20	6.02, 18.38	
	T4	14	12.40	5.26		6.64	−0.19, 13.47	
N	N0	14	15.35	9.17	0.7988	13.98	7.47, 20.49	0.3331
	N1	50	14.03	6.47		11.11	5.34, 16.89	
	N2 or N3	15	13.89	5.65		8.58	2.62, 14.55	
M	0	26	14.76	5.51	0.7481	12.37	8.16, 16.59	0.8478
	I	2	11.14	3.54		9.24	1.62, 20.09	
	X	51	14.09	7.50		12.07	8.79, 15.35	
Total		79	14.24	6.80				

[a]Serum c-erbB-2 concentration (ng/ml).
[b]ANOVA/*t*-test.
[c]95% confidence interval.
[d]Regression after adjusted by other items.
Oral habits: AC, alcohol consumption; BQ, betel quid chewing; CS, cigarette smoking.
Clinical characteristics: S, stage; T, tumor size; N, nodal stage; M, metastasis.
0, no metastasis; I, positive metastasis; X, undiagnosed.

all cases with elevated serum levels of c-erbB-2 had overexpressed c-erbB-2 p185 detected by immunohistochemical method. In this content, Slamon et al. indicated that fixation of the tumor decreases the sensitivity of the immunohistochemical detection of p185 as compared with fresh frozen specimens (5). However, a histopathologically diagnosed area might not represent the total picture of the distribution of soluble p185. Therefore, the lack of immunohistochemical staining may underestimate the levels of expression in OSCC. This could be one reason why serum levels are elevated in cases with weak or negative immunohistochemistry. Tsuji et al. (29) reported that 63.3% (38 of 60) of OSCC patients' sera showed elevated levels of squamous cell carcinoma-related antigen (SCC-Ag), but some patients in advanced stages showed a sub cutoff value in spite of them having large tumors, i.e. the size of the tumor does not correlate with serum SCC-Ag levels. The intensity of SCC-positive cells (by immunohistological stain) within an OSCC (29), or other TA-4 (30) and CEA (31) in tumors of other organs also showed no relationship to the serum SCC-Ag levels. Our current study also showed that c-erbB-2 has a similar phenomenon with the SCC-Ag. This might suggest that serum c-erbB-2 as well as serum SCC-Ag levels were not solely attributable to the antigen content of the tumor.

The case-control studies of oral cancer in Taiwan indicate a significant association between tobacco-free betel quid chewing and oral cancer (32–34). In 1995, Ko's study found that betel quid chewing was the strongest etiology of oral cancer, and showed an interaction with smoking and drinking in Taiwan (34). Although the present study showed that the mean age of our OSCC patients of betel quid chewers was significantly younger than non-chewers (48.5 years vs. 59.9 years, $P = 0.004$), the serum levels of c-erbB-2 oncoprotein correlated with neither the patients' betel quid chewing, drinking or smoking habit nor the tumor size, nodal stage or metastasis. However, there was a borderline significant correlation of the serum levels of the c-erbB-2 with the tumor stages.

There is a great need for more definitive serum markers for use in the detection, management, and response to treatment in OSCC. As other studies in the biological role of c-erbB-2 and the results of this study which showed 70.2% OSCC patients with elevated ($>95\%$ cutoff value of normal control) serum levels of c-erbB-2, it is suggested that the full and exact significance of serum c-erbB-2 levels deserve continued study in evaluating therapeutic effects and in monitoring the disease status of OSCC.

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