The Presence of Soluble c-erbB-2 in Saliva and Serum among Women with Breast Carcinoma: A Preliminary Study

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ABSTRACT

The protein c-erbB-2, also known as Her2/neu, is a prognostic breast cancer marker assayed in tissue biopsies from women diagnosed with malignant tumors. Present studies suggest that soluble fragments of the c-erbB-2 oncogene may be released from the cell surface and become detectable in patients with carcinoma of the breast. Consequently, the purpose of this study was to assay the c-erbB-2 protein in the saliva and serum of women with and without carcinoma of the breast and to determine whether the protein possesses any diagnostic value. To determine the diagnostic utility of this oncogene, the soluble form of the c-erbB-2 protein was assayed in the saliva and serum using ELISA in three different groups of women. The three groups consisted of 57 healthy women, 41 women with benign breast lesions, and 30 women diagnosed with breast cancer. To compare the relative diagnostic utility of the c-erbB-2 protein, CA 15-3 was also measured. The CA 15-3 measurements served as a “gold standard” by which to compare the c-erbB-2 protein’s diagnostic effectiveness. We found c-erbB-2 protein in the saliva and serum of all three groups of women. The salivary and serological levels of c-erbB-2 in the cancer patients, however, were significantly higher (P < 0.001) than the salivary and serum levels of healthy controls and benign tumor patients. Additionally, the c-erbB-2 protein was found to be equal to or to surpass the ability of CA 15-3 to detect patients with carcinoma. The results of the pilot study suggest that the c-erbB-2 protein may have potential use in the initial detection and/or follow-up screening for the recurrence of breast cancer in women.

INTRODUCTION

c-erbB-2, also known as Her2/neu, is a prognostic breast cancer marker assayed in tissue biopsies from women diagnosed with malignant tumors (1, 2). c-erbB-2 protein is overexpressed in ~20–30% of malignant breast tumors and has been used in postoperative follow-up evaluation as an indicator of patient relapse (3, 4). Serological levels of c-erbB-2 among women with carcinoma of the breast have also shown that the oncprotein is elevated in 9–52% of the patients diagnosed with regional and distant metastases (1–6).

The c-erbB-2 encodes for a 185-kDa protein that has intracellular, extracellular, and transmembrane spheres of cellular activity. Additionally, c-erbB-2 has tyrosine kinase activity and is thought to be a cellular receptor (3–7). The c-erbB-2 protein also is a marker of breast cancer in tissues biopsies from women with malignant tumors (3–7). Elevated levels of this marker indicate tumor presence, aggressiveness, and a poor prognosis for patient survival. Additionally, circulating c-erbB-2 protein levels also have been detected in serum and have been found to be useful in detecting the presence of metastatic tumors (4–7). Panels designed to provide prognostic evaluation of malignant breast tissues have this marker as part of their indicators.

Recognizing the overall oncological importance of the c-erbB-2 oncogene, a pilot study was conducted and found the soluble c-erbB-2 protein present not only in serum but also in saliva. The study also suggested that the serum and saliva soluble c-erbB-2 concentrations increased in the presence of carcinoma of the breast (7). As a consequence of this finding, (7), a follow-up study was conducted to provide novel data that compared salivary and serological concentrations among women with carcinoma of the breast, those with benign breast lesions, and a healthy control group. This report describe, in detail, the findings of the follow-up study.

MATERIALS AND METHODS

Subjects. The study consisted of three groups of women. Group I was a control group. This group consisted of healthy, asymptomatic individuals from the University of Mississippi Medical Center. Health status for the control group was determined by questionnaire.

Group II, the benign tumor group, and group III, the malignant tumor group, consisted of consecutive individuals from the surrounding community with a breast mass that were referred by a physician to the University of Mississippi Medical Center Division of Oncology for evaluation. Each patient received a thorough physical examination and was evaluated for carcinoma of the breast. Saliva and serum specimens were collected from each woman at the initial clinic appointment and prior to receiving any
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Units/mg of protein so that these findings could be compared to c-erbB-2 antigen levels were assayed using ELISA kits from Oncogene Research Products. Whole saliva was substituted in place of serum as assay specimens. The basic assay involves a colorimetric evaluation of the level of binding, which is a highly sensitive and selective detection reagent for the ligand. The CA 15-3 assay is designed to assay serum specimens. Whole saliva was substituted in place of the serum for salivary CA 15-3 determinations. CA 15-3 concentrations were expressed as units/ml.

Statistical Analysis. Statistical analyses were performed using the SPSS statistical software package (12). These data were analyzed from four different perspectives. Initially, the saliva and serum marker concentrations were summarized for each group, and descriptive analyses were conducted for the demographic and supplemental data obtained from the questionnaire. The focus was on race, medical status, tobacco use, medication usage, and menopausal status with respect to c-erbB-2 concentrations. The data were summarized by tumor type, staging, and nodal status. Because of the small number of women in the cancer group, the number of subcategories for primary tumor (T) and nodal status (N) were collapsed. The primary tumor categories were dichotomized to T1 and greater than T1, whereas nodal status was reduced to node negative and node positive, respectively.

ANOVA was used to compare the mean marker values for the three groups, focusing on the breast cancer in contrast to the non-cancer groups. Dunnett’s test was used to adjust for multiple comparisons.

Possible associations among the salivary and serum c-erbB-2 levels as well as those between c-erbB-2 and CA 15-3 concentrations in saliva and serum were assessed by Pearson’s correlation coefficient. Because the distributions of some of these concentrations were skewed, the correlates were also assessed using the square-root scale.

Finally, we conducted ROC analyses to investigate the appropriate cutoff value for each biomarker. Separately for each marker, the concentrations were recoded into dichotomous variables using various percentile cutoff values from the pooled control and cancer groups. Two-by-two tables were used to compute the sensitivity and specificity values of each biomarker for detecting disease for each cutoff value. ROC curves (sensitivity versus 1 – specificity) were constructed for c-erbB-2 and CA 15-3 concentrations in both saliva and serum. The optimum cutoff value for each marker was determined using the cutoff value that produced the largest percentage of area under its ROC curve (12–15).

RESULTS

Population Characteristics. Demographic and supplemental data obtained from the questionnaires for the three groups of women are summarized in Table 1. Frequency comparisons by race, tobacco use, medication use, and menopausal status were conducted. There were significant differences in...
race, tobacco use, and menopausal status among the three groups. More African Americans experienced carcinoma of the breast and benign tumor lesions than Caucasians. Likewise, significantly more tobacco users experienced carcinoma of the breast and benign tumor lesions than non-users. With respect to menopausal status, more perimenopausal women experienced carcinoma of the breast and benign tumor lesions than the pre- and postmenopausal women. Mean c-erbB-2 values were compared for each group according to health status (e.g., control, benign, cancer). There were no significant reportable effects on c-erbB-2 concentration attributable to these variables within each group of women (health status). Age comparisons yielded no significant group differences for c-erbB-2 values and were not linearly related to c-erbB-2 by regression modeling (Table 2).

Further analyses showed that the women with breast cancer produced detectable salivary levels of c-erbB-2 significantly higher than those produced by the benign tumor and control groups. The means, SE values, and the 95% confidence intervals for the salivary marker concentrations across the three groups were shown in Table 2 and Figures 1–3. As shown in Table 2, the mean c-erbB-2 values for the control and benign tumor groups were ~50–57% lower than the mean value for the cancer group. A strong parallel response in the corresponding serum c-erbB-2 levels was evidenced in these groups of women, with an associated range of 55–64%, although the concentrations in serum were roughly 15-fold higher than those in saliva before correcting for total protein.

The majority of the benign tumors were fibroadenoma or fibrocystic tumors. There was little difference between salivary c-erbB-2 concentrations found in fibroadenoma and fibrocystic tumors among women who had these benign tumors. There were two women who presented with fluid-filled cysts and two with benign calcifications. Both the serum and saliva c-erbB-2 values for the fluid-filled cysts were statistically lower than those for the fibroadenoma and fibrocystic groups. Again, the responses in serum were similar to those in saliva for these two groups of benign tumors, although the rank ordering of the observed average concentrations reversed between the fibroadenoma and fibrocystic groups.

The vast majority of tumors in the cancer group were infiltrating ductal carcinomas (n = 19). There was one woman who had an infiltrating lobular carcinoma, three who had a ductal carcinoma, and seven who had miscellaneous breast malignancies. The mean salivary and serum c-erbB-2 concentrations for these groups were all substantially higher than those observed for the benign tumors.

With respect to the staging of the cancer tumors, there was one Stage 0 (T0N0M0) patient, six stage I (T1N0M0), eight stage II A (T2N0M0), and three stage II B. The IIB group was composed of one T2N1M0 and two T3N0M0 patients. There were two stage III A patients (one T2N2M0 and one T3N0M0) and three stage IIIB (one T3N2M0 and two T4N0M0). Seven patients had not been staged at the time of this report.

Seven subjects with carcinoma of the breast were node positive, and 16 were node negative. All individuals diagnosed with cancer were without evidence of distant metastases. The subcategories for primary tumor were collapsed into T1 and greater than T1 and node-positive and node-negative groups (Table 3). These analyses showed no differences with respect to tumor size for c-erbB-2 saliva and serum concentrations, but there was an elevated c-erbB-2 concentration difference between node-positive and node-negative individuals regardless of diagnostic medium (Table 3).

**Group Comparisons.** The second level of analyses was to compare group means for the women with carcinoma of the breast, women with benign lesions, and the healthy control group. One-way ANOVA was performed across the three categories of women for salivary c-erbB-2 and was found to be significant at F = 13.83 (p < 0.0001). Dunnett’s C post hoc analysis exhibited a significant difference between the cancer group and the benign tumor and control groups at P < 0.001.

A similar result was demonstrated for serum c-erbB-2 across the three groups of women. The overall ANOVA was significant at F = 19.95 (P < 0.0001), with the post hoc analyses significant at the P < 0.001 level (cancer > both non-cancer groups).

With respect to CA 15-3, the overall ANOVA was significant at F = 5.94 (P < 0.04), with the post hoc analyses significant at P < 0.05 (cancer > nonhealthy control group). Similarly, the results for serum c-erbB-2 across the three groups of women were significant at F = 20.96 (P < 0.0001), with the post hoc analyses significant at P < 0.001 (cancer > healthy control group).

Data for the salivary and serum c-erbB-2 levels corrected for total protein concentrations exhibited the same results as the uncorrected data. The overall ANOVA for salivary c-erbB-2 was significant at F = 13.80 (P < 0.0001), with the post hoc analyses significant at p < 0.001 (cancer > both non-cancer groups). The results for serum c-erbB-2 across the three groups of women were significant at F = 14.45 (P < 0.0001), with the post hoc analyses significant at P < 0.001 (cancer > both non-cancer groups).

**Marker Associations and Diagnostic Characteristics.** The correlations coefficients revealed a significant moderate association between serum and salivary c-erbB-2 at r = 0.51 (P < 0.0001). There was a significant but weaker association...
between serum c-erbB-2 and serum CA 15-3 concentrations at $r = 0.40$ ($P < 0.001$). With respect to serum c-erbB-2 concentrations corrected for total protein and their association with CA 15-3, the results exhibited a significant but relatively weak association ($r = 0.36; P, 0.001$). A similar relationship was found between salivary c-erbB-2 concentrations corrected for total protein and serum c-erbB-2 concentrations corrected for total protein at $r = 0.39$ ($P < 0.001$).

The comparison of ROC curves (fourth level) produced a cutoff value of 110 units/ml for salivary and 2000 units/ml for serum c-erbB-2 concentrations (Table 4 and Figs. 1A and 2A). A comparison of ROC curves was also performed on salivary and serum c-erbB-2 concentrations corrected for total protein. These values were 100 and 50 units/ml for salivary and serum c-erbB-2 concentrations, respectively (Table 4 and Figs. 1B and 2B). Salivary CA 15-3 determinations yielded a 4.0 units/ml cutoff value (Table 4 and Fig. 3A). The literature reports varying cutoff values for serum CA 15-3 levels. The manufacturer recommends 15 units/ml ($6.5$ units; Ref. 16). Many studies report 22–24 units/ml (17), whereas others report 40 units/ml (18–20). Our result for the cutoff value for serum CA 15-3 was 20 units/ml (Table 4 and Figs. 1B and 2B).

With the aforementioned cutoff values, salivary and serum c-erbB-2 concentrations were able to detect 87% and 94% of the subjects with cancer, respectively (Table 4). The salivary and serum c-erbB-2 concentrations corrected for total protein detected 77% and 84% of the subjects. This compares with 62 and 75% for the salivary and serum CA 15-3 marker. CA 15-3 levels were able to detect 65% of the malignant lesions (Table 4 and Figs. 4 and 5).

**DISCUSSION**

It is difficult to compare the results of these findings with those in the literature. Studies involving the c-erbB-2 oncoprotein vary in the types of populations studied with respect to staging, tumor type, nodal involvement, and the presence of metastases (5). Additionally, a variety of analytical techniques have been used to study the c-erbB-2 oncoprotein in both tissue and serum (2). With respect to serum, most studies have used enzyme-based immunoassays (2, 5). These techniques have varied with respect to the sensitivities of the assays and the use of either monoclonal or polyclonal antibodies (17). Some kits...
used in the literature have since been discontinued and are no longer available to researchers. Consequently, we will attempt to compare these findings with studies using similar, sample sizes, staging, and assay technology.

The results of this study suggest elevated salivary and serum c-erbB-2 levels among women with carcinoma of the breast (Table 1 and Figs. 1–3). With respect to elevated serum c-erbB-2 levels among breast cancer patients, the findings of this study agree with others found in the literature, particularly those evaluating nonmetastatic cancer (21–25). There is only one report in the literature concerning elevated salivary c-erbB-2 concentrations among women with breast cancer, and that was a preliminary study performed by the authors of this investigation (7). This earlier study used an EIA (Triton Co.) assay for determining salivary and serum c-erbB-2 concentrations. The results of that study also revealed significantly higher salivary c-erbB-2 concentrations among women with carcinoma of the breast. The assay used in the present study, using the same identical specimens from the first study, appears to be 5-fold more sensitive than the original assay.

Benign and malignant tumor comparisons yielded some potentially useful information. Subjects with fibroadenomas and fibrocystic lesions produced similar salivary and serum c-erbB-2 concentrations (Table 2). For serum, this finding compares with results obtained by Breuer et al. (5). Subjects diagnosed with infiltrating ductal carcinoma dominated the cancer population in this study. Consequently, comparisons among the various types of malignant breast lesions were not made. These analyses warrant further study, which should also include an additional group of non-breast tumors (e.g., ovarian, colon).

Further analyses of the primary tumor data revealed no substantial salivary or serum c-erbB-2 concentration differences for groups T_1 and greater than T_1 (Table 3). This observation
does agree with the findings of Watanabe et al. (25) and Kynast et al. (26). The finding suggests that c-erbB-2 receptor status may be more indicative of tumor aggressiveness than tumor volume. Further investigations comparing salivary and serum c-erbB-2 concentrations with c-erbB-2 receptor status and tumor volume are warranted.

With respect to nodal status, c-erbB-2 levels in node-positive patients were found to be elevated compared with the node-negative subjects (Table 2). This finding was in agreement with the findings reported by Krainer et al. (6).

The investigators found a moderate association ($r = 0.51$; $P < 0.0001$) at the individual level between salivary and serum concentrations of c-erbB-2. The unexplained variability may be attributed to the “pooling” of the various types of individuals across the three groups of women and the fact that the investigators did not discern the exact mechanism by which the c-erbB-2 protein migrates from the tumor site and enters the oral cavity (diffusion, leakage, active transport). The process by which c-erbB-2 protein becomes solubilized is also not fully understood and may account for a portion of the unexplained variability observed in these studies.
variability. Further investigation, underway at present in our laboratory, is exploring this line of inquiry. The association between salivary and serum c-erbB-2 concentrations corrected for total protein concentrations was $r = 0.39 \ (P < 0.001)$.

A relationship between serum c-erbB-2 concentrations and serum CA 15-3 levels was found ($r = 0.40; \ P < 0.001$). This correlation was in agreement with the results reported by Krainer et al. ($r = 0.396; \ P < 0.002; \text{Ref. 6}$). When the serum c-erbB-2 concentrations were corrected for total protein concentrations, the association between serum c-erbB-2 concentrations and serum CA 15-3 levels was $r = 0.36 \ (P > 0.001)$.

Additionally, the results also suggest that salivary and serum c-erbB-2 levels may be equivalent to salivary and serum CA 15-3 levels as diagnostic markers (Table 4 and Figs. 4 and 5). This is also supported by the findings of Krainer et al. (6) and Narita et al. (21). The salivary and serum c-erbB-2 concentrations were able to detect 87 and 94% of the subjects with cancer, respectively. The salivary and serum c-erbB-2 concentrations corrected for total protein detected 77 and 84% of the subjects. This compares to 62 and 75% for the salivary and serum CA 15-3 marker. CA 15-3 levels were able to detect 75% of the malignant lesions at a 20 units/ml cutoff value. The manufacturer recommended a 15 units/ml cutoff value, and indeed the sensitivity did increase to 97%; however, when this adjustment was made, a sharp decline in specificity (35%) resulted, as predicted by Stenman and Heikkinen (17). Conversely, when the cutoff value was increased to 40 units/ml, the ability of the assay to detect cancer decreased to <30%. This is in agreement with the findings of Stenman and Heikkenen (17) and Safi et al. (18). Serum c-erbB-2 levels, whether corrected for total protein or not, retained a margin of specificity at the 60% level for sensitivities >90%.

Information from the health questionnaire concerning age, race, tobacco usage, presence of systemic disorders, use of prescription medications, and menopausal status was also analyzed. These analyses confirmed the results from prior reports published by us that these variables have no effect on salivary and serum c-erbB-2 concentrations (27, 28). Additionally, the findings for age by Watanabe et al. (25), and for tobacco usage and menopausal status by Breuer et al. (5) are supported by other studies; however, our study disagrees with Breuer et al. (5), who observed age-related influences on marker concentrations. Breuer et al. (5) reported that among postmenopausal women, age was significantly related to c-erbB-2 levels.

Overall, the results of this study suggest that c-erbB-2 may have the potential for use in initial detection (in combination with mammography and physical examination) and/or follow-up screening for the detection of breast cancer in women. The diagnosis of breast cancer at an earlier stage allows the physician and patient more choices in selecting various treatment options and also could benefit the management of breast cancer in women.

Because of its ease of collection, a saliva-based test could be a cost-effective adjunct diagnostic tool and/or an useful instrument in the postoperative management of cancer patients (7, 9). With respect to the postoperative management of cancer patients, it could potentially be used for monitoring the effectiveness of chemotherapy or for recurrence after therapy has concluded.

The results of this study are exploratory; however, what this study appears to indicate is the diagnostic potential of c-erbB-2 and the potential of saliva as an adjunct diagnostic fluid. Further research with respect to the utility of this marker is clearly warranted.
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REFERENCES


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