J Oral Pathol Med (2004) 33: 595–600

www.blackwellmunksgaard.com/jopm

The expression of the c-*erb*B-2 receptor protein in glandular salivary secretions

Charles F. Streckfus[1], Lenora Bigler[1], Tracy Dellinger[2], Melinda Kuhn[1], Normand Chouinard[1], Xaoli Dai[1]

[1]*School of Dentistry, Office of Discovery, Innovation, and Graduate Studies;* [2]*School of Dentistry, Department of AEGD/GPR, University of Mississippi Medical Center, Jackson, MS, USA*

BACKGROUND: As the maintenance medium of the oral cavity, saliva is secreted from exocrine glands that include the parotid, submandibular, sublingual, and minor salivary glands. Considering that saliva is a fluid suffused with protein, it is possible that the solubilized by-products of oncogenic expression may be present in saliva. Recent studies suggest the presence of solubilized extracellular domain portion of the c-*erb*B-2 protein in serum, nipple aspirates, and saliva. As a consequence, the purpose of this study was to determine the presence and concentration of c-*erb*B-2 in major salivary gland secretions.
METHODS: Fifteen healthy women had serum, stimulated whole (SWS), parotid (SP), and submandibular/sublingual (SS) salivary secretions collected. The specimens were analyzed for c-*erb*B-2 using enzyme linked immunosorbent assays (ELISAs). Western blots using c-*erb*B-2 were also performed on these specimens.
RESULTS: The ELISAs revealed the presence of c-*erb*B-2 in SWS (24.50 Units/ml), SP (19.66 Units/ml), SS (15.59 Units/ml) and serum (1472.15 Units/ml). Western blots confirmed the presence of these 185 kDa proteins.
CONCLUSIONS: These results suggest that the protein, c-*erb*B-2, is present in relatively equal amounts in both SP and SS glandular secretions. Elevated glandular salivary c-*erb*B-2 concentrations could be useful as a preliminary, non-invasive test in clinical decision making when diagnosing salivary gland carcinomas. Additionally, this marker may have utility in distinguishing between oral lesions that are benign, pre-malignant and malignant in the oral cavity. Further research is required to determine if these findings have clinical utility.
J Oral Pathol Med (2004) **33**: 595–600

Keywords: c-*erb*B-2; HER-2/*neu*; saliva; salivary glands

Correspondence: Charles F. Streckfus, School of Dentistry, Office of Discovery, Innovation, and Graduate Studies, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216-4505, USA. Tel: +1 601 984 6013, Fax: +1 601 984 6014, E-mail: cstreckfus@sod.umsmed.edu
Accepted for publication May 21, 2004

Introduction

c-*erb*B-2, also known as HER-2/*neu*, is a member of the tyrosine protein kinase class of oncogenes. The oncogene product of this gene is a 185 kDa transmembrane glycoprotein which influences cell growth through its activation of tyrosine kinase activity. The endogenous ligand that initiates this activity has not been identified and the mechanism by which the activity is regulated is not understood (1–3); however, a point mutation in the transmembrane region or over-expression of the HER-2/*neu* gene leads to oncogenic activation. The over-expression of HER-2/*neu* has been associated with higher grade and reduced survival in carcinomas of the breast, colorectal, and head and neck tissues (3–6).

Recent studies have exhibited the existence of shed or solubilized forms of c-*erb*B-2 (4–8). The exact method for the release of soluble truncated c-*erb*B-2 (p105) and the full-length transmembrane protein (p185) is not known. It is hypothesized that soluble c-*erb*B-2 could be produced by post-translational processing of the full-length c-*erb*B-2 gene. This process may also include cell-surface proteolysis similar to that of soluble IL-2 receptor (9).

The physiologic function of soluble c-*erb*B-2 is also not known. However, it is postulated that it could be (i) a carrier for the transportation, destruction, or shielding of the c-*erb*B-2 ligand, (ii) an antagonist of normal cell-surface receptor–ligand interaction, or (iii) a nonfunctional by-product of normal proteolytic action (7, 8). In support of the third postulate, a study by Zabrecky et al. (10) suggests that proteolysis is likely to be important in the normal process of receptor down regulation. Additionally, it was suggested that the remaining cell-associated cleavage product that is composed of the transmembrane and cytoplasmic domains could represent an oncogenic form of the c-*erb*B-2 receptor tyrosine kinase (10). The resulting transformation could then occur by a proportional increase in the absolute number of truncated receptors caused by the proteolysis of the overexpressed c-*erb*B-2 receptors in the tumor cell surface (7, 10).

The soluble c-*erb*B-2 receptor protein levels have been detected in serum (4–7), nipple aspirates (11), and saliva (12–14). These studies have found the presence of the soluble c-*erb*B-2 protein in these body fluids among healthy individuals and have noticed a marked increase in its concentration in the presence of patients diagnosed with a malignancy. Likewise, they have reported c-*erb*B-2 protein modulation by tumor removal, chemotherapy and tumor recurrence (7, 11, 15). Additionally, when c-*erb*B-2 over-expression has occurred, the receptors have been immunohistologically identified in tissues from carcinomas of the breast and the salivary glands (16).

As suggested by the aforementioned stimulated whole salivary (SWS) research, the detection of protein markers in saliva could be a useful tool in the detection and post-operative follow-up of specific types of cancer (12–15). As SWS is secreted from various glands in the oral cavity including the parotid gland, submandibular and sublingual glands, it would increase the understanding of the presence of c-*erb*B-2 in the oral cavity if we knew which gland(s) was (were) secreting the solubilized oncoprotein. Therefore, the purpose of this study was to determine the concentrations of the c-*erb*B-2 protein in parotid and submandibular/sublingual secretions and compare them to whole saliva and serum concentrations.

Materials and methods

Population

A randomly selected subset of 15 racially mixed women from a control group within a longitudinal study for breast cancer among women was chosen for the evaluation. The women were determined as 'healthy' by undergoing regular physical and mammography examinations and were not taking any prescribed or over-the-counter medications during the course of the study. Oral examinations prior to entry in the investigation revealed that the subjects were also orally healthy. All participants were non-tobacco users. All subjects were volunteers and understood the approved, Institutional Review Board consent form before signing the document and entering the study.

Ideally, an aged-matched panel of men should have been included in the study; but, because of the lack of availability of men during working hours and the low incidence of breast cancer among men, they are not part of the overall longitudinal study. However, it should be noted that a previous study has shown no statistical differences in stimulated whole saliva c-*erb*B-2 concentrations resulting from gender and or racial differences (13, 14).

Salivary collections

Stimulated secretions were collected in this study. It would have been of value to collect unstimulated secretions and compare the c-*erb*B-2 concentrations with their stimulated saliva counterparts; however, because of the difficulty associated with obtaining resting glandular collections and the small volumes of saliva secreted (1 ml required for the assays) in these collections, unstimulated glandular saliva specimens were not collected. Specimens were collected between 9 A.M. and 3 P.M. Blood was also drawn by a phlebotomist at the time of saliva collection.

Stimulated whole saliva collection

The method used for the collection of SWS is described by Navasesh and Christensen (17). The patient was asked to swallow any accumulated saliva and was then instructed to chew the wax at a regular rate and expectorate into a pre-weighed plastic cup. The sample was then weighed and the volume recorded. The sample was aliquotted into 1 ml cryogenic vials with the use of a micropipette and then frozen (−70°C) until further analysis. Flow rate for SWS was calculated by the total weight of saliva collection divided by 5 min and the result expressed in ml/min.

Stimulated parotid gland saliva collection

The Carlson–Crittenden cup was used to collect stimulated parotid (SP) saliva from the right Stenson's duct as described by Heft and Baum (18). Collection time was 5 min. Stimulation was invoked using a 2% citric acid solution which was applied every 30 s to the lateral borders of the dorsum of the tongue. Specimens were collected into pre-weighed cryogenic vials, reweighed after collection, and were immediately frozen until assayed for c-*erb*B-2.

Stimulated submandibular/sublingual saliva collection

Collection of saliva from both the stimulated submandibular and sublingual (SS) glands was obtained by using the NIDCR collector as described by Tylenda et al. (19). SS gland saliva was collected from the orifice of Wharton's duct with the collector. The submandibular and sublingual secretions are combined due to the confluence of the orifices at the Wharton's duct (19). Wharton's duct was isolated and dried with gauze sponges prior to collection. Glandular stimulation was achieved by applying 2% citric acid at 30-s intervals to the dorsal surface of the tongue (19). Similar to SP, SS was collected into cryogenic vials for a 5-min period and then frozen.

Serum collection

One vial of blood was collected from the subjects. Each specimen was allowed to coagulate and was then centrifuged for 15 min. The serum component was separated and aliquotted into cryogenic vials and immediately frozen.

Laboratory techniques and measurements

The frozen specimens were thawed and the saliva and the serum from the blood specimens were analyzed for total protein and the c-*erb*B-2 concentrations. Saliva was substituted in place of serum as assay specimens. Specimens were assayed in triplicate.

Total protein assay

Samples of saliva were assayed for protein using the bicinchoninic acid (BCA) method (Pierce Chemical, Co.,

Rockford, IL, USA) that is a highly sensitive and selective detection reagent for the cuprous ion. This method measures protein concentrations from 0.5–20 mg/ml. In this assay, BCA serves as a chelating agent for Cu^{+1} forming a color complex in the presence of protein. Aliquots of saliva (100 µl) were placed in microtiter plates and the Pierce BCS protein assay reagent added to the wells. Samples were incubated for 30 min at 37°C and the optical density read at 562 nm in a microplate spectrophotometer. The final concentration of each substance was derived from a standard curve and data were expressed as mg/ml.

The modified BCA version of the Lowry technique has a tendency to under estimate salivary protein concentrations; however, the Lowry and this assay are used to a greater extent in the literature to measure salivary protein concentrations (20). Consequently, in order to compare these results to other studies, this protein assay technique was used.

c-erb*B-2 assay*
Serum and salivary c-*erb*B-2 protein levels were assayed using enzyme linked immunosorbent assay (ELISA) kits (Oncogene Research Products, Cambridge, MA, USA). The basic 'sandwich' ELISA assay involves a colorimetric evaluation of the level of binding which was formed with the intensity of the color formed by the enzymatic reaction being proportional to the target protein present. The absorbance was read at 490 nm in a microplate spectrophotometer and the ligand concentration calculated from a standard curve. c-*erb*B-2 data were expressed and reported as Units/ml.

SWS Western blotting
Western Blotting was performed using the Bio-Rad Mini-Protein system (Rockford, IL, USA). Briefly, the salivary specimens were run 'neat' while the serum specimens were diluted 1:200 in sample buffer. The specimens were boiled for one minute prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) which was carried out in 7.5% Tris–HCl Bio-Rad Ready Gels at 100 V for 1.5 h in SDS electrophoresis buffer. Using polyvinylidene diflouride (PVDF) for blotting, the transfer was run at 100 V for 1.5 h in transfer buffer (standard buffer plus 1% SDS). The membrane was blocked in incubation buffer (3% dry milk in TTBS) for 0.5 h with agitation. The incubation buffer was replaced with antibody specific for c-*erb*B-2 (Transduction Laboratories, San Jose, CA, USA) diluted 1:2500 in incubation buffer and incubated for 1.5 h with agitation. Membrane was then washed three times over a 15-min period in wash buffer. Fresh incubation buffer containing 1:1000 sheep mouse IgG-AP conjugate (Amdex, Piscataway, NJ, USA) was added and incubated 1.5 h with agitation. The membrane was then washed twice with wash buffer followed by a double washing in phosphate buffered saline (PBS). In order to reduce background, the membrane was incubated in 0.2% glutaraldehyde for 15 min, washed three times in PBS, and then incubated 10 min in 0.1 M Ethanolamine (pH 9) working substrate solution (Zymed BCIP/NBT Substrate Kit, San Francisco, CA, USA) and agitated until color developed (5–10 min) (21).

Statistical analysis
Statistical analyses were performed using the SPSS® statistical software package (22). Graphic illustrations and descriptive analyses were performed on all dependent variables (e.g. flow rates, etc.) according to the independent variable specimen source (e.g. serum, saliva, etc.). Spearman correlations were also performed comparing glandular c-*erb*B-2 concentrations to whole saliva and salivary secretions to serum.

Results

Fifteen women between the ages of 22 and 57 with an overall mean age of 31.1 years participated in the study. SWS and SS were collected on all 15 subjects; however, four subjects refused to have their blood drawn and one subject, after repeated attempts to draw blood, was unable to produce a specimen. Because of the difficulty in obtaining SP specimens using the Carlson–Crittenden cup, the investigators were only able to obtain SP on nine of the selected participants.

The mean averages for SWS, SP, and SS flow rates are illustrated in Table 1 and Fig. 1. These findings are consistent for those exhibited by healthy individuals in other studies (17–19). Total salivary protein concentrations are also shown in Table 1 and Fig. 2. These results are, likewise, consistent with those from healthy individuals (23, 24). Salivary c-*erb*B-2 concentrations (Units/ml) are shown in Table 1 and are illustrated in Fig. 3. Salivary c-*erb*B-2 concentrations were detected in the major salivary glands and were less than c-*erb*B-2 concentrations assayed in whole saliva. SP levels were relatively equivalent to SS c-*erb*B-2 concentrations. The presence of c-*erb*B-2 is also supported by the Western blots shown in Fig. 4.

c-*erb*B-2 concentrations were greater in serum than in any of the salivary secretions. The SWS and serum c-*erb*B-2 concentrations were in the same order of magnitude as reported for other healthy individuals using the same kit (5, 12–15). As also reported (10), the presence of c-*erb*B-2 in serum is also present in the Western blots as shown in Fig. 5.

Table 1 Descriptive statistics for c-*erb*B-2 concentrations in stimulated whole and glandular saliva and serum

Source	*n*	*Flow rates (ml/min)*	*c-erbB-2 (Units/ml)*	*Total protein (µg/ml)*
Stimulated whole saliva	15	1.81 (±0.26)	24.50 (±6.40)	1.15 (±0.16)
Stimulated submandibular/ sublingual gland saliva	15	0.61 (±0.06)	15.59 (±3.55)	0.69 (±0.10)
Stimulated parotid gland saliva	9	0.43 (±0.06)	19.66 (±5.11)	0.70 (±0.16)
Serum	10	NA	2465.15 (±180.78)	49.49 (±0.88)

The values are mean (±standard error).

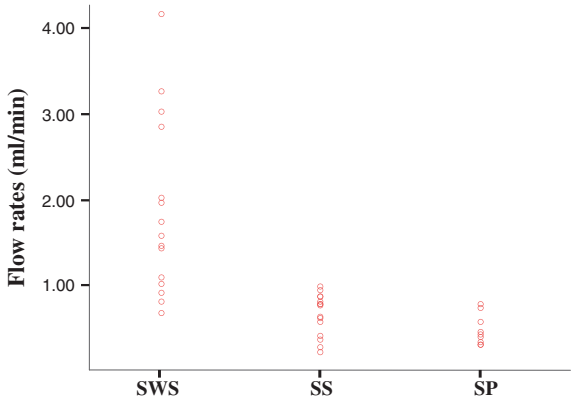

Figure 1 Scatter plots representing salivary flow rates data from the stimulated whole saliva (SWS), stimulated submandibular/sublingual gland (SS), and stimulated parotid gland (SP) specimen groups.

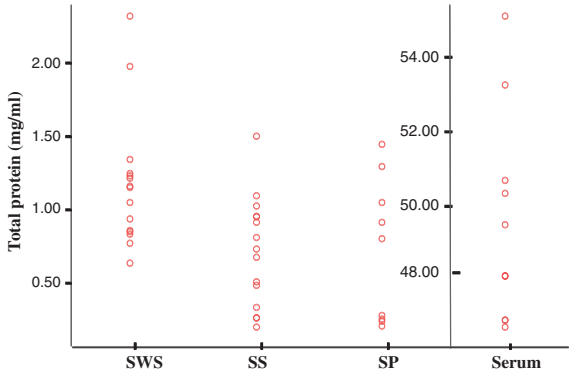

Figure 2 Scatter plots representing salivary total protein data from the stimulated whole saliva (SWS), stimulated submandibular/sublingual gland (SS), and stimulated parotid gland (SP) specimen groups.

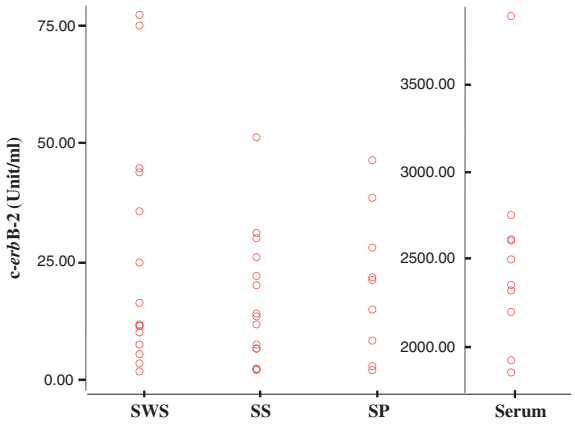

Figure 3 Scatter plots representing salivary c-*erb*B-2 data from the stimulated whole saliva (SWS), stimulated submandibular/sublingual gland (SS), and stimulated parotid gland (SP) specimen groups.

There were no significant correlations among the specimen sources, i.e. SP with SS glandular c-*erb*B-2 concentrations or the glandular concentrations with serum or SWS c-*erb*B-2 concentrations. There were also no correlations within specimen source. That is to say that c-*erb*B-2 concentrations were not correlated with total protein, or in the case of salivary specimens, flow rates.

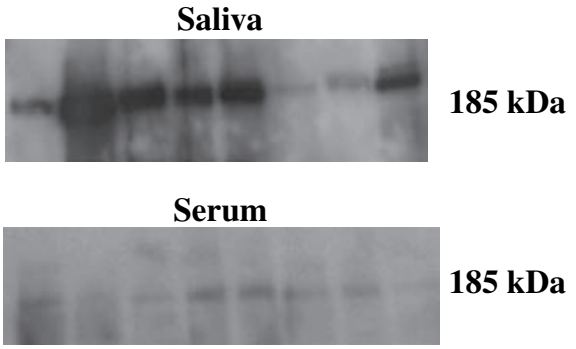

Figure 4 Western blots comparing stimulated whole saliva bands with serum bands at the 185 kDa molecular weight level.

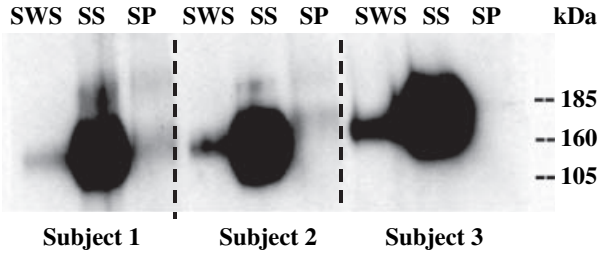

Figure 5 Western blots comparing stimulated whole saliva (SWS), stimulated submandibular/sublingual gland (SS), and stimulated parotid gland (SP) saliva specimen bands at the 185 kDa molecular weight level.

Discussion

The authors could not find comparable studies in the literature. Consequently, the results of this investigation will serve as a reference for future studies.

The results of the study suggest that levels of c-*erb*B-2 are greater in serum than in whole saliva or the glandular secretions. Table 1 also suggests that the c-*erb*B-2 in SWS is greater in concentration than the glandular secretions; however, as illustrated in Figure 1, there are two-outliers in the SWS data around the 75 Units/ml level and when these were deleted from the data the new mean was 16.71 ($\pm$4.14) Units/ml which approximates the glandular values. Likewise the SP and SS secretions were comparable. Studies using unstimulated flow rates and varying saliva stimulants (e.g. mechanical, acid, salts, etc.) with a cohort of men are required to further elucidate these initial findings.

Western blots (Fig. 4) performed by the investigators exhibited heterogeneous bands at approximately 185 kDa for solublized c-*erb*B-2 in both the serum and saliva of healthy individuals (13). This added evidence, as determined by antibodies associated with the c-*erb*B-2 protein, suggests that the protein present in saliva is possibly c-*erb*B-2; however, without the confirmation of protein sequencing of these salivary proteins, we can not be certain that this is truly the full (p185), unaltered c-*erb*B-2 receptor protein. Likewise, the Western blots for the glandular secretions show the presence of the c-*erb*B-2 protein (Fig. 5) in these fluids. It should be noted in Figure 5 that the bands are not heterogeneous

with respect to intensity as witnessed in Figure 4 (i.e. SS > SWS > SP). An explanation for this is probably due to the fact that the investigators ran these specimens 'neat'. Future studies should use specimen amounts corrected by total protein using a total protein assay that is biochemically more rigorous than the BCA technique (20). Despite this 'shortcoming', the blots do indicate, albeit qualitatively, the presence of the c-erbB-2 protein in glandular secretions using a technique other than the ELISA method.

Recent findings using surface enhanced laser desorption ionization time-of-flight mass spectrometry proteomic analyses (25) also suggest the presence of this 185 kDa protein in saliva adding additional evidence for its presence; however, assuming that the salivary protein is the c-*erb*B-2 protein, the following question still arises: how does this very large protein (185 kDa) enter into the salivary secretions? There are several possible explanations for the presence of the c-*erb*B-2 protein in saliva. The elevated serum concentration gradient of c-*erb*B-2 may passively diffuse this protein into the lumen of the acinar complex. Considering that c-*erb*B-2 is a 185 kDa molecule with respect to size, it is very unlikely to passively diffuse into salivary secretions. It is more conceivable, however, that its presence in saliva is because of 'leakage' resulting from hydrostatic pressure, which widens the space between the tight junctions of the acinar epithelium allowing the molecule to enter the saliva. This may be possible, but an animal study using a sustained c-*erb*B-2 delivery system impregnated in the peritoneum in rats exhibited classic 'dose–response' curves when c-*erb*B-2 was assayed in saliva over time (26). This evidence taken with the repeatable clinical presence (14) of this protein in saliva suggests that the mechanism by which c-*erb*B-2 enters the saliva is probably not by leakage.

The other possible explanation is that the c-*erb*B-2 protein is secreted into the saliva by active transport. The c-*erb*B-2 protein has been shown to be present on the membrane of the ductal epithelium of salivary gland tissues (16). We also know from the literature that these receptors are present on the salivary ductal cells and in the event of c-*erb*B-2 over-expression, these receptors can be identified through immunohistological staining (16) similar to ductal carcinoma of the breast. The protein is present in healthy tissue and its function is for the regulation of normal cell growth. It is plausible that c-erbB-2 may be secreted into saliva as consequence of localized regulatory function in the oral cavity via signal transduction similar to the proposed explanation of c-*erb*B-2 protein in nipple aspirates (11). However, like saliva, the role of c-*erb*B-2 protein in nipple aspirates is also not understood.

We can only speculate as to how and why the c-*erb*B-2 protein is present in saliva. Further research is required to determine the exact method by which c-*erb*B-2 and the proteins associated with its molecular pathway (12, 27) enters the saliva. We need to understand its role in oral health and disease and the effects of systemic illnesses on c-*erb*B-2 protein production (28). Research is needed to identify saliva c-*erb*B-2 amino acid sequences and compare them with those of serum and nipple aspirates in both healthy and diseased states (28).

The data presented in this manuscript suggest the presence of c-*erb*B-2 in both stimulated whole and glandular salivary secretions. This knowledge could be of utility in diagnosing salivary gland carcinomas (29, 30). Elevated glandular salivary c-*erb*B-2 concentrations could be used as a preliminary step in clinical decision-making prior to the implementation of more invasive techniques for the diagnosis of salivary gland tumors. Additionally, this marker may have utility in distinguishing between oral lesions that are benign, premalignant, and malignant in the oral cavity. Further research is required to determine if these findings have clinical utility.

References

1. Simon MA. Receptor tyrosine kinases: specific outcomes from general signals. *Cell* 2000; **103**: 13–5.
2. Ouyang X, Gulliford T, Doherty A, et al. Detection of ErbB-2 oversignaling in a majority of breast cancers with phosphorylation-state-specific antibodies. *Lancet* 1999; **353**: 1591.
3. Chen IH, Chang JT, Liao CT, et al. Prognostic significance of EGFR and Her-2 in oral cavity cancer in betel quid prevalent area. *Brit J Can* 2003; **89**: 681–6.
4. Semba K, Kamata N, Toyoshima K, et al. A v-*erb*B-related protooncogene, c-*erb*B-2, is distinct from the c-*erb*B-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland carcinoma. *Proc Natl Acad Sci* 1985; **82**: 6497–501.
5. Breuer B, Smith S, Thor A, et al. ErbB-2 protein in sera and tumors of breast cancer patients. *Breast Cancer Res Treat* 1998; **49**: 261–70.
6. Krainer M, Brodowicz T, Zeillenger R, et al. Tissue expression and serum levels of HER-2/neu in patients with breast cancer. *Oncology* 1997; **54**: 475–81.
7. Volas GH, Leitzel K, Teramoto Y, et al. Serial serum c-*erb*B-2 levels in patients with breast carcinoma. *Cancer* 1996; **78**: 267–72.
8. Lin YJ, Clinton GM. A soluble protein related to the Her-2 proto-oncogene product is released from human breast carcinoma cells. *Oncogene* 1991; **6**: 639–43.
9. Rubin LA, Nelson DL. The soluble IL-2 receptor: biology, function and clinical application. *Ann Intern Med* 1990; **113**: 619–27.
10. Zabrecky JR, Lam T, Mckensie SJ, et al. The extracellular domain of p185/neu is released from the surface of human breast carcinoma cells, SK-BR-3. *J Biol Chem* 1991; **266**: 1716–20.
11. Kuerer HM, Thompson PA, Krishnamurthy S, et al. High and differential Expression of Her2/neu Extracellular domain in bilateral ductal fluids from women with unilateral invasive breast cancer. *Clin Can Res* 2003; **9**: 601–5.
12. Streckfus CF, Bigler L, Tucci M, Thigpen JT. The Presence of CA 15–3, c-*erb*B-2, EGFR, cathepsin-D, and p53 in saliva among women with breast carcinoma. *Cancer Invest* 2000; **18**: 101–9.
13. Streckfus CF, Bigler L, Dellinger TD, et al. The presence of c-*erb*B-2, and CA 15–3 in saliva and serum among women with breast carcinoma: a preliminary study. *Clin Can Res* 2000; **6**: 2363–70.
14. Streckfus CF, Bigler L, Dellinger TD, et al. Reliability assessment of soluble c-*erb*B-2 concentrations in the saliva

of healthy women and men. *Oral Surg Oral Med Oral Path* 2001; **91**: 174–80.
15. Bigler LG, Streckfus CF, Copeland L, et al. The potential use of saliva to detect recurrence of disease in women with breast carcinoma. *J Oral Pathol Med* 2002; **31**: 421–31.
16. Wick MR, Ockner DM, Mills SE, et al. Homologous carcinomas of the breasts, skin, and salivary glands: a histologic and immunohistochemical comparison of ductal mammary carcinoma, ductal sweat gland carcinoma and salivary duct carcinoma. *Am J Clin Pathol* 1998; **109**: 75–84.
17. Navasesh M, Christensen C. A comparison of whole mouth resting and stimulated salivary measurements. *J Dent Res* 1982; **61**: 1158–62.
18. Heft MW, Baum BJ. Unstimulated and stimulated parotid salivary flow rate in different age groups. *J Dent Res* 1984; **63**: 1182–5.
19. Tylenda CA, Ship J, Fox PC, et al. Evaluation of submandibular salivary flow rates in different age groups. *J Dent Res* 1988; **67**: 225–8.
20. Jenzano JW, Hogan SL, Noyes CM, et al. Comparison of five techniques for the determination of protein content in mixed human saliva. *Anal Biochem* 1986; **159**: 370–6.
21. Legendre N, Matsudaira P. Purification of proteins and peptides by SDS-PAGE. In: Matsudaira P, ed. *A Practical Guide to Protein and Peptide Purification for Microsequencing*. San Diego, CA: Academic Press, Inc., 1989; 52–8.
22. SPSS. SPSS for Windows, Release 10.0. Chicago, IL: SPSS, 1999.
23. Dawes C. The effects of flow rate and duration of stimulation on the concentrations of protein and the main electrolytes in human parotid saliva. *Arch Oral Biol* 1969; **14**: 277.
24. Dawes C. The effects of flow rate and duration of stimulation on the concentrations of protein and the main electrolytes in human submandibular saliva. *Arch Oral Biol* 1974; **19**: 887.
25. Streckfus CF, Bigler L. The use of salivary protein markers for the detection and post-operative monitoring of cancer patients: an eight-year study. *J Adv Dent Res* 2004; in press.
26. Brinkley J, Copeland L, Streckfus C, Tucci M, Benguzzi H. Sustained delivery of HER-2/NEU antibody by TCPL delivery device using adult male rats as a model. *Biomed Sci Inst* 2003; **39**: 324–8.
27. Bigler LG, Streckfus CF. A unique protein screening analysis of stimulated whole saliva from normal and breast cancer patients. *Preclinica* 2004; **2**: 52–6.
28. Kurachi H, Okamoto S, Oka T. Evidence for the involvement of the submandibular gland epidermal growth factor in mouse mammary tumorgenesis. *Proc Natl Acad Sci USA* 1985; **82**: 5940–3.
29. Nagler R, Kerner H, Ben-Eliezer S, et al. The prognostic role of apoptotic, Bcl-2, c-*erb*B-2, and p53 markers in salivary malignancies. *Oncology* 2003; **64**: 389–98.
30. Nagler R, Kerner H, Laufer D, et al. Squamous cell carcinoma of the tongue: the prevalence and prognostic roles of p53, Bcl-2, c-*erb*B-2 and apoptotic rate as related to clinical and pathological characteristics in a retrospective study. *Cancer Lett* 2002; **186**: 137–50.

Acknowledgment

This project was supported by the 1 R55 DE/OD12414-01 James A. Shannon Director's Award NIDCR/OD/NIH.