

Salivary Biomarkers for the Detection of Malignant Tumors That are Remote from the Oral Cavity

Lenora R. Bigler, PhD, Charles F. Streckfus, DDS, MA*,
William P. Dubinsky, PhD

KEYWORDS

- Saliva • Breast cancer • Mass spectrometry • Diagnosis
- Biomarkers • Proteins • Cell signaling and pathways

Saliva is a complex and dynamic biologic fluid, which over the years has been recognized for the numerous functions it performs in the oral cavity. Modern technology, however, has unveiled a plethora of compounds never before detected in saliva (eg, drugs, pollutants, hormones; but also biomarkers of bacterial, viral, and systemic disease). Consequently, the increased scientific inquiry into salivary biomarkers has led to a more intense analysis of how saliva is collected, stored, and assayed and increased its potential use as a diagnostic medium in cancer research.¹

In a recent review of the use of saliva as research material, Schipper and colleagues² discussed how over the past 50 years, the pace of salivary research has accelerated with the advent of new techniques that illuminate the biochemical and physicochemical properties of saliva. The field of salivary research is rapidly advancing because of the use of novel approaches that include metabolomics, genomics, proteomics, and bioinformatics.

BLOOD VERSUS SALIVA AS A DIAGNOSTIC MEDIA

Blood has traditionally been the media of choice for the medical community and has, as a diagnostic media, served physicians very well over the decades. There may be some inherent disadvantages with blood, however, in biomarker discovery. Blood is basically in a closed loop (ie, the cardiovascular system), which contains numerous cells coursing through various organs that potentially can alter proteins that can exist within the circuit for a period of days or weeks. In comparison, the saliva is a

Department of Diagnostic Sciences, University of Texas Dental Branch at Houston, 6516 M.D. Anderson Boulevard, Room 4.133f, Houston, TX 77030, USA

* Corresponding author.

E-mail address: charles.streckfus@uth.tmc.edu (C.F. Streckfus).

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“real-time” fluid. The salivary glands are exocrine glands that produce protein profiles indicative of the individual’s status at the moment of collection. This potentially may be an advantage when seeking biomarkers for various diseases.

ANALYTICAL ADVANTAGES OF SALIVA

Saliva as a diagnostic fluid has significant biochemical and logistical advantages when compared with blood. Biochemically, saliva is a clear liquid with an average protein concentration of 1.5 to 2 mg/mL. As a consequence of this low protein concentration, it was once assumed that this was a major drawback for using saliva as a diagnostic fluid; however, current ultrasensitive analyte detection techniques have eliminated this barrier. Saliva specimen preparation is simple, involving centrifugation before storage and the addition of a cocktail of protease inhibitors to reduce protein degradation for long-term storage.³

Blood is a far more complex medium. A decision has to be made as to whether to use serum or plasma. Serum has a total protein concentration of approximately 60 to 80 mg/mL. Because serum possesses more proteins than saliva, assaying trace amounts of factors (eg, oncogenes) may result in a greater risk of nonspecific interference and a greater chance for hydrostatic (and other) interactions between the factors and the abundant serum proteins. Serum also possesses numerous carrier proteins (eg, albumin), which must either be removed or treated before being assayed for protein content. Additionally, it was demonstrated that clotting removes many background proteins that may be altered in the presence of disease. It was established that enzymatic activity continues during this process, which may cleave many relevant pathway-related proteins.⁴⁻⁶

It would be ideal if all enzymatic activity in serum ceased at the time of collection; however, proteomic analyses of serum has shown that this is not the case. As a consequence, plasma is also being explored as a diagnostic fluid. The main consideration in using plasma is the selection of a proper anticoagulant.⁴⁻⁶ Heparin, for example, can be used as an anticlotting agent; however, current research has found that heparin has a relatively short half-life (3–4 hours) and can produce products of coagulation that are abundantly comparable with those assayed in serum. Based on these observations, it is recommended that blood specimens be collected with ethylenediaminetetraacetic acid.

COLLECTION ADVANTAGES OF SALIVA

From a logistical perspective, the collection of saliva is safe (eg, no needle punctures), noninvasive, and relatively simple, and may be collected repeatedly without discomfort to the patient.³ Consequently, it may be then possible to develop a simplified method for home-testing, testing in a health fair setting, or in dental clinics where individuals are available for periodic oral examinations. This diagnostic potential could reach many individuals who for personal, logistical, or economical reasons lack access to preventive care.³

Blood is more complicated to collect. It requires highly trained personnel to collect it and, if collected incorrectly, can lead to misinterpretations, which can result in patient mismanagement.⁴⁻⁶ Blood specimens need to be collected in a specific sequence and under-filling tubes with additives; if not this may possibly alter protein analyses. Additionally, if specimens are collected during hospital or clinical settings, there may be a lapse of time from venipuncture to laboratory processing.⁷

PROTEOMIC ANALYSES OF SALIVA

From a biochemical point of view, proteins are the most important constituents of saliva. Currently, state-of-the-art proteomic methods can be applied to the analysis of salivary peptides and proteins.

Recently, surface-enhanced laser desorption and ionization time-of-flight (SELDI-TOF) protein chips combines matrix-assisted laser desorption and ionization TOF mass spectrometry (MS) to surface chromatography.¹ This technology uses sample chips that display various kinds of chemically enriched and active surfaces that bind protein molecules based on established principles, such as ion exchange chromatography, metal ion affinity, and hydrophobic affinity. The technique enables rapid and high-throughput detection of critical proteins and peptides directly from crude mixtures without labor-intensive preprocessing. Furthermore, SELDI-TOF-MS is sensitive and requires only small amounts of sample compared with other proteomic techniques.^{1,8,9}

Studies by Schipper and colleagues⁸ have demonstrated that SELDI-TOF-MS could serve as an alternative, rapid, and high throughput proteomic approach to profile salivary proteins and peptides. Chips with anionic (CM10) treated surfaces were effective in binding salivary proteins in the range from 6000 to 16,000 m/z. The cationic (Q10) and hydrophobic (H4) chips were found to be most valuable for the characterization of saliva peptides and proteins in the low molecular (1000–6000 m/z) range. The metal affinity binding (IMAC-Cu) chip produced the highest number of peaks in a wide molecular range. SELDI technology enables selective protein retention on protein chip array surfaces by means of distinct chromatographic surfaces. Protein chip arrays are available with a variety of chromatographic surfaces including reverse phase, cation exchange, anion exchange, and immobilized metal affinity surfaces. Other arrays are available for covalent coupling of antibodies, DNA, RNA, receptors, or other “bait” molecules onto the array surface.^{1,8,9}

Crude biologic samples can be applied directly to the protein chip arrays. After a short incubation period, unbound proteins are washed off the surface of the array. Only proteins that interact with the array chemical surface are retained for further analysis. Protein chip arrays are analyzed in the protein chip reader. The process involves laser desorption and ionization of proteins from the array surface, and detection by TOF-MS. The protein chip reader is a laser desorption and ionization TOF mass spectrometer that uses state-of-the-art ion optic and laser optic technology. The laser optics maximize ion extraction efficiency over the greatest possible sample area, and increase analytical sensitivity and reproducibility.^{1,8,9}

The reader's ion optics incorporate a four-stage, time-lag focusing ion lens assembly that provides precise, accurate molecular weight determination with excellent mass sensitivity. Protein chip systems produce data compatible with all major protein databases and for applications requiring high sensitivity. Proteins can be quantified by plotting peak intensity values against peptide or protein standard quantities. The operating mechanism of ionic exchange protein chip arrays is the reversible binding of charged molecules to the surface, and the property of a peptide-protein that governs its binding is its net surface charge.^{1,8,9} Because surface charge is the result of weak acidic and basic amino acids within the protein, binding of the protein to the array is highly pH dependent. Streckfus and colleagues⁹ performed an analysis of a large number of saliva samples from women with and without breast cancer, which identified five mass peaks that were increased more than twofold in the cancer patients.

More recently, this same group has performed MS studies using saliva,^{10,11} which when combined with previous work, corroborates earlier findings regarding the use

of saliva as a medium for biomarkers of cancer.¹² In addition, two-dimensional gel electrophoresis and two-dimensional liquid chromatography (LC) coupled with tryptic digest analysis by MS have been demonstrated to be powerful proteomic tools for the global analysis of salivary proteins. The wide range of the molecular weights of salivary proteins necessitates additional methodologies, however (eg, high performance liquid chromatography [HPLC]-MS), particularly in the analysis of small proteins. The analysis is performed on an Applied Biosystems (Foster City, California) QStar XL LC tandem MS mass spectrometer equipped with an LC packings HPLC for capillary chromatography. The HPLC is coupled to the mass spectrometer by a nanospray electrospray ionization (ESI) head for maximal sensitivity.¹³⁻¹⁵ The advantage of tandem MS combined with LC is enhanced sensitivity and the peptide separations afforded by chromatography. Even in complex protein mixtures, tandem MS data can be used to sequence and identify peptides either by peptide similarity or sequence analysis with a high degree of confidence.¹³⁻¹⁷ This technique, albeit not a high-throughput technique, is far superior to the SELDI approach. The SELDI is challenged because of its small laser and has difficulty in spectral repeatability.

Several studies by Hu and colleagues¹⁸ have used two-dimensional gel electrophoresis to separate the protein components followed by MS subsequently to identify the peptides produced from in-gel digests of the proteins of interest (two-dimensional MS). With this approach more than 300 proteins were identified in whole saliva. Although two-dimensional MS is a very powerful approach to protein separation, it has limitations when dealing with small-molecular-weight proteins, highly acidic or basic proteins, very hydrophobic proteins, or proteins in low abundance. In addition, the technique requires a relatively large amount of sample, is labor-intensive, and high gel-to-gel reproducibility is hard to achieve.

An alternative approach is the combination of LC as the separation step, with the mass spectrometer (LC-MS). Using this approach, more than 1000 peptides and proteins were identified including most known salivary proteins and serum proteins. This technique has the disadvantage that it is still labor intensive, has limited throughput, and provides little information about the relative abundance of the detected proteins. The initial salivary proteome profile of Hu and colleagues¹⁹ was greatly expanded by combining LC-MS and two-dimensional MS, identifying more than 1050 proteins in whole saliva. LC methods coupled to MS or tandem MS are particularly suitable for separation and identification of low-molecular-weight components and peptides. These methods have allowed the detection of peptides in the range of 1 to 6 kd, many of which have important biologic functions (eg, histatins, cystatins, defensins, statherins, proline-rich proteins).

Along these lines, a consortium of three research groups lead by Denny and colleagues²⁰ cataloged the proteins in human saliva collected as the ductal secretions: 1166 identifications, 914 in parotid and 917 in submandibular-sublingual saliva, were made. The results showed that a high proportion of proteins that are found in plasma or tears are also present in saliva along with unique components. The proteins identified are involved in numerous molecular processes ranging from structural functions to enzymatic-catalytic activities. As expected, most mapped to the extracellular and secretory compartments. An immunoblot approach was used to validate the presence in saliva of a subset of the proteins identified by MS approaches. These experiments focused on novel constituents and proteins for which the peptide evidence was relatively weak.

Ultimately, information derived from the work reported here and related published studies can be used to translate blood-based clinical laboratory tests into a format that uses saliva. Additionally, a catalog of the salivary proteome of healthy individuals

allows future analyses of salivary samples from individuals with oral and systemic diseases, with the goal of identifying biomarkers with diagnostic or prognostic value for these conditions; another possibility is the discovery of therapeutic targets.

The main goal to search for the complete protein salivary profile is its use as a diagnostic tool to monitor the physiologic, health, or disease status of individuals. With the advent of protein microarrays, spectra from a significant number of samples can be obtained and compared in a relatively short time with very little sample preparation or sophisticated chromatography.

Along these lines, cancer proteomics encompasses the identification and quantitative analysis of biomarkers, which are subsequently objectively measured indicators that characterize the state of health of the biologic system being analyzed. Because over 95% of all drug targets for varying carcinomas are proteins, the major goal of biomarker discovery or protein profiling is to identify disease-specific proteins and peptides from the proteome of biologic samples. Research has primarily focused on lower-molecular-weight proteins circulating within easily extractable body fluids, such as blood, urine, sputum, and saliva. The discovery of breast cancer markers in saliva has offered renewed interest in the potential use of saliva as a diagnostic fluid.

ISOTOPIC LABELING AND LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROSCOPY

Isotopic labeling of protein mixtures has proved to be a useful technique for the analysis of relative expression levels of proteins in complex protein mixtures, such as saliva. There are two methods that are based on isotopically labeled protein modifying reagents to label or tag proteins in the mixtures: the iCAT and the iTRAQ techniques.^{13–17}

The general approach for both techniques is to label two to four different saliva samples with agents that are chemically identical, but have different atomic masses. Any chemically based purification technique does not distinguish between the two; however, in the mass spectrometer they can be distinguished by their difference in atomic mass. Because they are chemically identical, they ionize with the same efficiency in the mass spectrometer permitting an estimate of their relative concentrations based on the relative peak intensities. iCAT procedure uses cysteine-specific labels that include a biotin moiety in their structure. An avidin binding step enables a high degree of enrichment of iCAT-labeled peptides.

The alternative to the iCAT procedure is the iTRAQ reagents, which are amino reactive compounds.^{11,13–17} The real advantage is that the tag remains intact through TOF MS analysis; in the tandem MS spectrum for each peptide there is a fingerprint indicating the amount of that peptide from each of the different protein pools. Because virtually all of the peptides in a mixture are labeled by the reaction, numerous proteins in complex mixtures are identified and can be compared for their relative concentrations in each mixture; in these complex mixtures there is a high degree of confidence in the identification because of the large number of peptides that can be used for protein identification.^{11,15–19}

The authors used IL-LC tandem MS technique to protein profile saliva for novel cancer-related biomarkers.^{11,19} To identify potential salivary protein markers for the detection of breast cancer the following were used: pooled (N = 10) saliva specimens from healthy subjects; pooled (N = 10) saliva specimens from benign tumor patients (fibroadenomas); pooled (N = 10) saliva specimens from stage 0 cancer subjects; and pooled (N = 10) saliva specimens from stage I breast cancer subjects. An internal standard was created by pooling 10 specimens randomly selected from the pooled population. The analytical matrix is shown in **Fig. 1**.

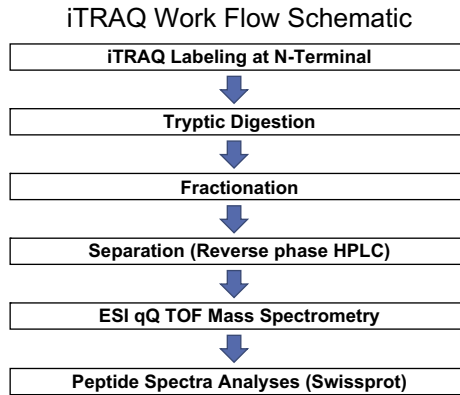


Fig. 1. Steps for the iTRAQ proteomic procedure. ESI, electrospray ionization; HPLC, high performance liquid chromatography; TOF, time of flight.

The saliva samples were thawed and immediately centrifuged to remove insoluble materials. The supernatant was assayed for protein (Bio-Rad, San Diego, California) and an aliquot containing 100 μ g of each specimen was precipitated with six volumes of -20°C acetone.

The precipitate was resuspended and treated according to the manufacturer's instructions. The treatment included blocking cysteine residues with methylmethane thiosulphate (MMTS) and trypsin digestion (**Fig. 2**). The peptides generated during the digestion were labeled with specifically coded iTRAQ reagents. The labeled peptides from each of the saliva samples were then combined and partially purified by a combination of cation exchange chromatography and desalting on a reverse phase column. The desalted and concentrated peptide mixtures were analyzed by MS.

Before MS an aliquot of the peptide mixture was separated by HPLC on a C18 75- μ \times 10-cm reverse phase capillary column (Vydac 218MS3.07510). A linear gradient of 2% to 50% acetonitrile in 0.1% formic acid, over 180 minutes followed by 40 minutes

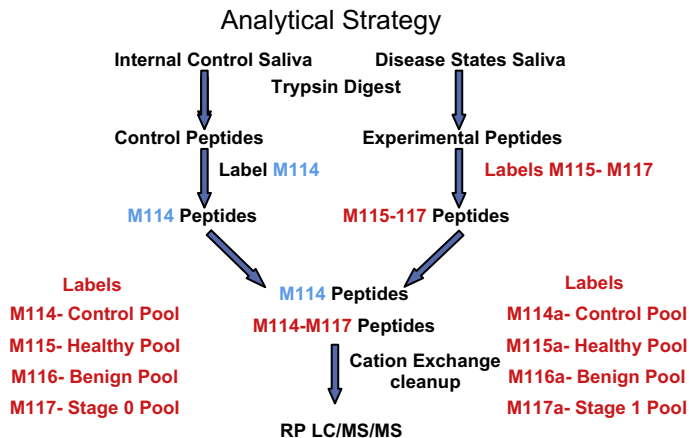


Fig. 2. Analytical comparisons associated with the iTRAQ procedure.

wash with 2% acetonitrile was used to elute the peptides directly into the mass spectrometer for tandem MS analysis. The QSTAR operates in an information-dependent acquisition mode, which detects peptides by TOF-MS and then fragments them by collision-induced dissociation in the tandem MS mode. The accumulated tandem MS spectra were analyzed by ProQuant (Applied Biosystems).

Table 1 shows the results of the experiment using pooled saliva samples. Overall 70 proteins were identified at greater than 99% confidence level (two or more peptides sequenced at >99% confidence interval) and 209 proteins at greater than 95% confidence level (at least one peptide sequenced at >99% confidence interval). These findings are slightly higher than Wilmarth and coworkers'¹³ findings using a two-dimensional LC technique with LC tandem MS.

The internal standards and their resultant protein profiles were compared and produced similar results. Likewise, the healthy and benign subject spectra between the two runs were also comparable demonstrating reliable and reproducible data for additional spectral comparisons across the two individual runs. As illustrated in **Table 2**, the healthy subjects were labeled with a 115 marker, the benign subjects with a 116 marker, and the cancer groups with the 117 marker. Comparisons are listed in **Table 2**. A list of candidate up- and down-regulated proteins is listed in **Tables 3** and **4**.

The proteins were entered into Ingenuity software, pathway analysis software application that enables researchers to model, analyze, and understand the complex biologic and chemical systems at the core of cancer research. The results of the analyses are in **Figs. 3** and **4**. **Fig. 3** shows an association with the epidermal growth factor (epidermal growth factor [EGF]) pathway, which is at the heart of carcinogenesis, whereas **Fig. 4** suggests associations with the ubiquitin pathway. As illustrated, the proteins are associated with varying pathways that are common to both the ductal epithelia of the breast and the ductal epithelia of the salivary glands. Additionally, these same proteins have been found to be either up- or down-regulated in MCF-7 and SKBR-3 cancer cell lines. Interestingly, results identified proteins that are both up- or down-regulated, have varying cellular functions, and have been validated in cell studies to be altered in the presence of carcinoma of the breast.¹¹

THE SALIVARY GLAND MODEL

The ideas outlined previously lead to the question as to what the possible mechanisms of action are in relation to models involving salivary diagnostics and breast cancer development. First, one should examine the similarities that salivary and breast tissue, and fluids from each, have in common. Pia-Foshini and colleagues²¹ describe how breast glands and salivary glands are both tubuloacinar exocrine glands sharing similar morphologic features; consequently, it is reasonable to expect similarities in pathologic processes. They differ in incidence and clinical behavior, however,

Confidence	Proteins	Before Grouping	Peptides	Spectra	% Total Spectra
>99	70	966	478	1099	73.6
>95	209	1674	626	1329	89
>66	351	1978	772	1491	99.9
As shown: >95	209	1674	626	1329	89

Comparison	Up-Regulated	Down-Regulated	Total Markers
Healthy versus benign	19	10	29
Healthy versus stage 0	15	15	30
Healthy versus stage I	9	17	26
Benign versus cancers	9	6	15

depending on whether they are primary in breast or the salivary glands. Salivary gland-like tumors of the breast are of two types: myoepithelial differentiation and those devoid of myoepithelial differentiation. Myoepithelial differentiated tumors comprise a spectrum of lesions from pleomorphic adenoma to low-grade adenosquamous carcinoma, whereas nonmyoepithelial differentiated tumors are rare acinic cell carcinomas. Nicol and Iskandar²² describe lobular carcinoma of the breast metastatic to the oral cavity mimicking polymorphous low-grade adenocarcinoma of the minor salivary glands.

Accession	Protein Name	Ratio	P Value
Q9DCT1	Aldo-keto reductase	1.3874	0.0264
P04083	Annexin A1	3.0606	0.0001
P23280	Carbonic anhydrase VI	1.5160	0.0003
P01040	Cystatin A	2.0057	0.0014
P01036	Cystatin SA-III	1.2030	0.0115
Q7NXI3	Heat shock 70kDa protein	1.2732	0.0039
Q01469	Epidermal fatty acid-binding protein	2.0963	0.0362
Q6LAF3	Histone H4	2.4094	0.0059
P01857	Ig gamma-1 chain C region	1.4396	0.0034
P13646	Cytokeratin 13	6.5643	0.0001
P19013	Cytokeratin 4	6.4958	0.0019
P48666	Cytokeratin 6C	4.4113	0.0001
P01871	Ig mu chain C region	1.5134	0.0011
Q9HC84	Mucin 5B	1.6771	0.0001
P05164	Myeloperoxidase precursor	2.7188	0.0005
P31151	S100 calcium-binding protein	2.0519	0.0001
P05109	Calgranulin A	2.1848	0.0001
P06702	Calgranulin B	1.8686	0.0001
Q9UBC9	Cornifin beta	1.8252	0.0001
P02788	Lactoferrin	1.5766	0.0001
P00760	Cationic trypsin precursor	1.1572	0.0144
P68197	Ubiquitin	1.5533	0.0138

Accession	Protein Name	Ratio	P Value
P10981	Actin-87E	0.7657	0.0107
Q8N4F0	Bactericidal/permeability-increasing protein-like 1	0.7953	0.0004
Q9GQM9	Cytochrome P450	0.7277	0.0001
P04264	Cytokeratin 1	0.6106	0.0001
P01034	Cystatin C	0.7201	0.0187
P28325	Cystatin D precursor	0.6856	0.0010
Q715P7	Fatty acid synthase	0.0311	0.0500
P00738	Haptoglobin	0.8266	0.0023
P22079	Lactoperoxidase	0.8247	0.0388
Q96DR5	Lipocalin	0.6144	0.0001
P79180	Lysozyme C	0.5309	0.0031
P07737	Profilin-1	0.6752	0.0135
P02768	Serum albumin precursor	0.7336	0.0001
P02787	Transferrin	0.7192	0.0001
P25311	Zinc-alpha-2-glycoprotein	0.8454	0.0009

Furthermore, Bretschneider and colleagues²³ report studies that suggest breast cancer and salivary gland tumors share an expression pattern unique to these two tissues (eg, BASE and SBEM). BASE encodes a putative secreted protein that is restricted to breast cancer cells and salivary gland. It seems to be estrogen receptor alpha dependent in its expression and may offer clues to how multiple disease states at distant locations can share similar mechanisms of expression and regulation.

With these studies in mind, Miksicek and colleagues²⁴ suggest that improving the diagnosis and clinical management of breast cancer requires access to a wider range of biomarkers able to reflect the molecular phenotype of breast tissue. A special mRNA identified only in mammary and salivary glands is termed “small breast epithelial mucin.” Secreted proteins that contain internally repeated densely glycosylated neutral core motifs have a well-established link to cancer as illustrated by the MUC1 gene product. This study identifies a novel breast and salivary gland-specific, mucin-like gene that is strongly expressed in normal and tumor human mammary epithelium. Furthermore, Amundadottir and colleagues²⁵ report a basic science study using transgenics that demonstrates cooperation between oncogenic proteins *c-myc* and transforming growth factor- α in mouse mammary and salivary gland tumorigenesis. The protein product of *c-myc* is responsible for transcriptional activation of cells and transforming growth factor- α is part of the EGF family and activates the EGF receptor, whose overexpression is associated with poor prognosis and high degree of invasiveness in breast cancer.

Noble and colleagues²⁶ published a pilot study that examined the feasibility of nipple aspiration to distinguish women with breast cancer from healthy women using surface-enhanced laser desorption ionization TOF-MS. Nipple aspiration fluid was collected from each breast in 21 women newly diagnosed with unilateral breast cancer and 44 healthy women. No differences were found when proteomic profiles of nipple aspiration fluid from the cancer-bearing breast and the contralateral noncancerous

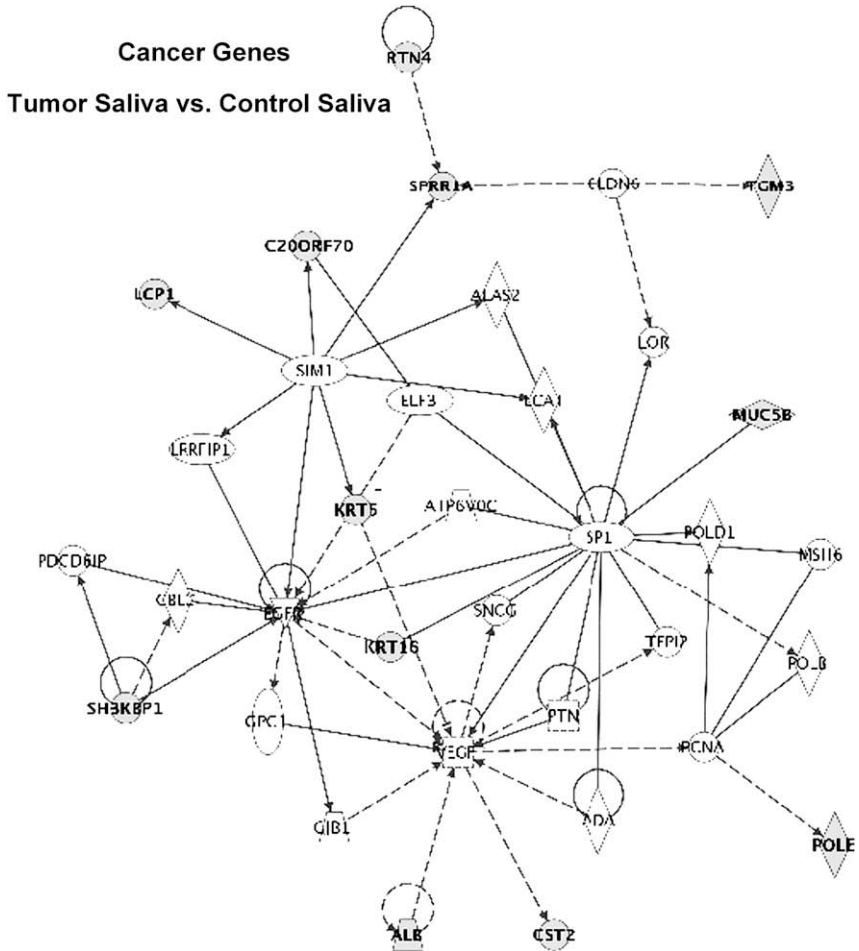


Fig. 3. Up-regulated growth pathways associated with the salivary protein profile in the presence of carcinoma of the breast.

breast were compared. In contrast, nine protein peaks were significantly different between the cancer-bearing breast compared with healthy women and 10 peaks were significantly different between the contralateral healthy breast and healthy women ($P < .05$). These data suggest that invasive breast cancer may result in a field change across both breasts and that proteomic profiling of nipple aspiration fluid may have more value in breast cancer risk assessment than as a diagnostic or screening tool.

In addition, Medrinos and colleagues²⁷ described a study that focused on an innovative technique that couples breast ductal lavage with surface-enhanced laser desorption and ionization TOF-MS to yield a highly sensitive and specific method of breast carcinoma detection. The study group included 16 women who had unilateral, biopsy-proved breast carcinoma. Studying paired ductal lavage specimens from each woman (the breast with and the breast without carcinoma); a cytologic investigation was performed on the cells present in the ductal lavage samples; and the protein content of the ductal lavage fluid was analyzed with the SELDI-TOF-MS technique

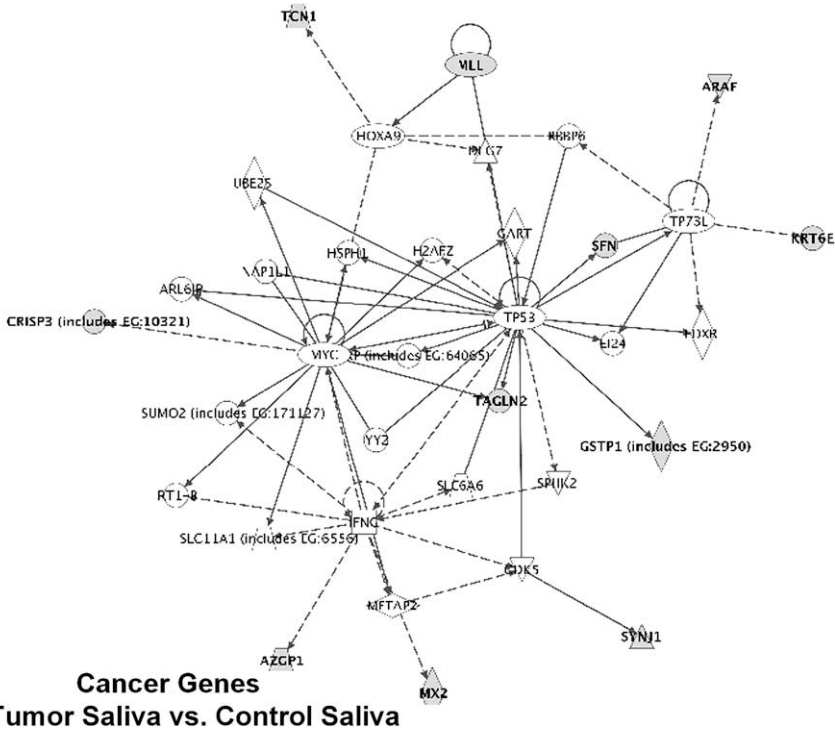


Fig. 4. Down-regulated apoptotic pathways associated with the salivary protein profile in the presence of carcinoma of the breast.

using the strong anionic exchange chip surface. Only five (31%) of 16 ductal lavage specimens from breasts with biopsy-proved carcinoma contained malignant cells, whereas the remaining samples contained only histiocytes and clusters of benign ductal epithelium. In contrast, 12 (75%) of 16 ductal lavage specimens from breasts that contained carcinoma had a different protein peak pattern compared with the paired ductal lavage specimen from the same patient's contralateral, uninvolved breast. This finding was independent of the presence of neoplastic cells in the lavage fluid. In addition, specific protein peaks, which may represent potential biomarkers, were identified in the ductal lavage fluids from breasts with carcinoma. Some of these peaks were conserved between different patients. The study suggests cross-talk from the diseased breast to the healthy breast, which also produced an altered nipple aspiration fluid protein profile.

Finally, there are several hypothetical mechanisms that may explain the presence of novel biomarkers in saliva. Because there are numerous putative salivary proteins that are possibly altered in the presence of disease, the authors focus the discussion on salivary EGF, EGFR, and HER-2/*neu* because these proteins have been investigated and are molecularly associated with one another.¹

EGFR (erbB-1) and HER-2/*neu* (erbB-2) are members of the ErbB tyrosine kinase receptor family comprising four related receptors (erbB-1, erbB-2, erbB-3, erbB-4). Collectively, they dimerize on ligand stimulation (EGF) and transduce their signals by subsequent autophosphorylation that catalyzes the receptor cytoplasmic tyrosine kinase activity, which results in the recruitment of an array of downstream signaling

cascades. The type and amplitude of activated downstream activity is related to the type of receptor expressed by a cell.

Hynes and Lane²⁸ report that none of the EGF family of peptides bind ERBB2; however, MUC4, a member of the mucin family, acts as an intramembrane modulator of ERBB2 activity. Despite having no soluble ligand, ERBB2 is important because it is the preferred heterodimerization partner of the other ligand-bound family members. Activated ERBBs stimulate many intracellular signaling pathways and, despite extensive overlap in the molecules that are recruited to the different active receptors, different ERBBs preferentially modulate certain signaling pathways, because of the ability of individual ERBBs to bind specific effector proteins. Amplification of *ERBB2* leading to overexpression of the receptor, originally detected in a subset of breast tumors, occurs in other human cancers, such as ovarian, gastric, and salivary cancers.²⁸ Intriguingly, mutations in the kinase domain of *ERBB2* have been identified in a small number of non-small cell lung cancers.²⁸ The impact of these mutations on ERBB2 activity remains to be explored. Publications describing the crystal structure of the EGFR, ERBB2, and ERBB3 ectodomains²⁸ have led to new insights into some intriguing questions concerning the process of ligand-induced receptor dimerization and biologic activity of ERBB2-targeted antibodies.

The structure of ERBB2's extracellular region is radically different from the others. ERBB2 has a fixed conformation that resembles the ligand-activated state: the domain II to IV interaction is absent and the dimerization loop in domain II is exposed.^{28,29} This structure is consistent with the data that indicate that ERBB2 is the preferred partner for the other activated ERBBs, because it is permanently poised for interaction with another ligand-bound receptor. Furthermore, this structure explains why no soluble EGF-related ligand has been found. It predicts that ERBB2 possesses a unique subdomain I to III interaction that makes ligand binding impossible because the site is buried and not accessible for interaction. To date, although HER-2/*neu* does not have a specific, known ligand, EGF, transforming growth factor- α , and the neuregulins are a few of the ligands that activate the erbB-1, erbB-3, and erbB-4 receptors. These receptors are present on both the ductal epithelium of breast and the ductal epithelium of the salivary glands and in the case of EGFR and HER-2/*neu* they can both be overexpressed in the presence of carcinoma respective to their tissues. The receptors can also be identified by using immunohistochemistry and have been clearly shown on both types of tissues. Along these lines, Hynes and Lane²⁸ and Lee-Hoeflich and colleagues²⁹ examined the central role for HER3 in HER2-amplified breast cancer with regard to targeted therapy regimens. They report using receptor knockdown by a small interfering RNA technology in cell lines to demonstrate that EGFR and HER3 each form heterodimers with HER2 and that inhibition of HER2 expression could be achieved by dual HER2/EGFR small molecule inhibitors.

It should be noted that breast cancer is not the only malignancy involving HER2 expression. Wang and colleagues³⁰ used tissue microarrays and immunohistochemical analysis to study protein expression of genes in the erb-b signaling pathway (erb-b1; erb-b2; phosphoinositide-3-kinase, catalytic, α polypeptide [PIK3CA]; phosphatase and tensin homolog [PTEN]; phosphorylated AKT [p-AKT]; and phosphorylated extracellular signal-regulated kinase [p-ERK]) in 118 advanced ovarian carcinomas and related expression to clinicopathologic features and survival. High protein expression was seen in 15.3% of cases for erb-b2, 44.1% for erb-b1, 43.2% for PIK3CA, 51.6% for p-AKT, and 28% for p-ERK. Low protein levels of PTEN were seen in 41.5% of the cases and tended to be more common in well-differentiated tumors. In multivariate analysis, only high expression of both erb-b1

and erb-b2 was an independent factor in progression-free and disease-specific survival ($P = .009$, hazard ratio = 2.46; $P = .002$, hazard ratio = 3.023, respectively). The PI3 K/AKT and RAS/MEK/ERK pathways seem to be activated in some cases of advanced ovarian carcinomas, although PIK3CA, p-AKT, p-ERK, and PTEN do not seem to be independent prognostic markers in this group of patients. By using tissue microarrays and immunohistochemical assessment, high expression of both erb-b1 and erb-b2 was an independent prognostic factor in advanced ovarian carcinomas. High activation of PI3 K/AKT and RAS/MEK/ERK pathways was detected in advanced ovarian carcinomas. PIK3CA, p-AKT, p-ERK, and PTEN did not seem to be independent prognostic markers, however, in this group of patients.

Using EGF, EGFR, and HER-2/*neu* as a molecular model, the authors postulate that in the presence of disease (eg, carcinoma of the breast) there is an overabundance of protein resulting from the rapid growth of the malignancy, which in turn produces a humoral response in the salivary glands. This response results in altered salivary protein concentrations. Another possible explanation is active transport of the proteins of interest. It is plausible that these proteins are secreted into saliva as consequence of localized regulatory function in the oral cavity by signal transduction similar to the proposed explanation of HER-2/*neu* protein in nipple aspirates. These “loop” mechanisms, in health, seem to be in equilibrium both intercellularly and extracellularly with each pathway fulfilling the resultant phenotypic process of growth, proliferation, and differentiation.

Yet another possible mechanism of action is passive diffusion of proteins from the serum to the saliva across the cell membranes. Considering the general molecular size of the aforementioned proteins, it is very unlikely proteins of their magnitude would passively diffuse into salivary secretions.

This leaves active transport of proteins from serum to saliva and whether HER-2/*neu* in saliva could have used a similar mechanism as endosomal sorting complex required for transport is an intriguing possibility. Trajkovic and coworkers³¹ established a pathway in intraendosomal membrane transport and exosome formation that required the sphingolipid ceramide. Lysosomal degradation of proteins following endocytosis is preceded by the incorporation of the proteins into intraluminal vesicles of multivesicular endosomes and delivered to lysosomes for digestion. Alternatively, the multivesicular endosomes can directly fuse with the plasma membrane, releasing the intraluminal vesicles to the extracellular environment as exosomes and thereby functioning as intercellular signaling. These studies indicate that proteolipid protein-containing exosomes and epidermal growth factor receptor-containing intraluminal vesicles can be formed within the same endosome.³¹ Salivary HER2 and other oncogenic proteins could feasibly use a similar route, but no studies have been conducted to date. Currently, relatively little is known about the signals and mechanisms that initiate protein secretion of these nonoral related proteins. Until these and other questions are answered, one can only postulate as to how and why these large proteins are secreted into the oral cavity.

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