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The use of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry to detect putative breast cancer markers in saliva: a feasibility study

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BACKGROUND: Technologies are now available enabling saliva to be used to diagnose disease, predict disease progression, and monitor therapeutic efficacy. This pilot study describes the use of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI) to detect putative breast cancer markers in saliva.

METHODS: Salivary specimens were analyzed as either pooled cancer saliva specimens, or individual specimens from healthy women and women diagnosed with carcinoma of the breast. The specimens were applied to a variety of protein chip arrays, washed extensively to remove unbound analytes and analyzed on a SELDI mass spectrometer.

RESULTS: The results of this initial study suggest that the WCX protein chip array prepared and washed at pH 3.5 yielded the most promising results. Additionally, the analyses revealed a number of proteins that were higher in intensity among the cancer subjects when compared with controls. These salivary proteins were present at the 18, 113, 170, 228 and 287 k*m*/*z* ranges using SELDI analyses.

CONCLUSIONS: The study suggests that saliva may be useful for high-throughput biomarker discovery.
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Introduction

Increasing interest has developed in using saliva to diagnose systemic diseases because of its simplicity in collection (1–7). The collection of saliva is relatively safe (e.g. no needle punctures), non-invasive, inexpensive to sample, and may be collected repeatedly with minimal discomfort to the patient; thereby, rendering saliva as a very desirable diagnostic medium (1–3). More importantly, saliva contains constituents that are frequently altered in the presence of systemic diseases (1–3). As a result of these significant characteristics, finding biomarkers in saliva for the detection of serious systemic illnesses, such as cancer, is on the national healthcare agenda (4–6) and is of great interest for most salivary researchers (7).

There are only a few studies in the literature concerning the use of saliva to detect malignancies remote from the oral cavity. These reports deal with the identification and quantification of cancer-related proteins in saliva that were previously discovered to be present in cancer tissue supernatants or elevated in the serum of diagnosed cancer patients. The importance of these studies, however, demonstrates the feasibility of salivary cancer diagnostics and establishes the basis for continued biomarker research (1–3).

One of these studies that serve to establish the basis for salivary cancer biomarker research comes from the research of Jenzano et al. (8–11). These investigators report the use of saliva to detect variations in kallikrein concentrations, a regulatory protease, among healthy individuals and patients with malignant breast and gastrointestinal tumors. The results of their investigations revealed higher concentrations of salivary kallikrein among patients diagnosed with malignant tumors when compared with those individuals diagnosed with benign tumors or those from a cohort of healthy subjects as measured by chromogenic tripeptide assay (CTA; 8–11).

Saliva has also been assayed for the presence of serum cancer antigens such as Cancer Antigen-125 (CA125).

Chen et al. found that saliva contained CA125, a glycoprotein complex that is an often-used serum marker for the detection and post-operative follow up of ovarian cancer. In comparing salivary CA125 concentrations among healthy women, women with benign lesions, and those with ovarian cancer, a significant elevation in CA125 concentration was found among the ovarian cancer subjects when measured by radioimmunoassay (RIA). The results also suggested that CA125 when assayed in saliva had a better diagnostic value than when assayed in serum using the same kit (12, 13).

In addition to Chen et al.'s (12, 13) findings, a subsequent study by Cornelissen et al. reported the utility of salivary CA125 for the optimization of taxol-based chemotherapy (14). Using salivary CA125 as a putative marker, the investigators were able to optimize the administration of taxol (a CA125 inhibitor) chemotherapy by monitoring the patient's circadian tumor rhythm using salivary CA125 concentrations. When the salivary CA125 concentrations were at their circadian peak value indicating when the tumor is most active, the taxol was administered for maximum effectiveness. The study suggests that by using salivary CA125 as an indicator for the timing of taxol administration the physician can optimize the efficacy of the chemotherapeutic agent.

Epidermal growth factor (EGF) is a regulatory growth factor protein responsible for tissue growth and repair in the oral cavity (15–17). The secretion of EGF levels in saliva has been reported in the literature as early as 1979 (15, 16). As EGF overexpression is thought to be implicated in tumorigenesis; it, therefore, may be useful as a tumor marker. With this in mind, a study by Navarro et al. demonstrated that EGF concentrations were higher in the saliva of women with primary breast cancer or a recurrence of breast cancer when compared to women without a malignancy (17). The highest concentrations of EGF were found in the local recurrence subgroup, suggesting a potential use for this marker in the post-operative follow up of diagnosed cancer patients (17).

Streckfus et al. (18–22) and Bigler et al. (23) conducted a series of studies to assess the utility of solubilized c-*erb*B-2 as a salivary tumor marker for the detection of carcinoma of the breast (18–23). These studies demonstrated that solubilized c-*erb*B-2 is present in the saliva of both healthy and diseased individuals, but is elevated among subjects diagnosed with breast cancer. Additionally they found that salivary c-*erb*B-2 is reliable (22), and may also be used in patient post-operative follow up (23). The same researchers also found the presence of other cancer-related protein markers such as: CA15-3, EGF receptor, cathepsin-D, p53, and Waf-1 in saliva (18).

Protein analyses using surface-enhanced laser desorption/ionization

The aforementioned studies employed the use of CTA (9–11), RIA (12–14, 17), and enzyme-linked immunosorbent assay (ELISA; 18–23). These assays, albeit very accurate, require sample preparation and can only measure one specific analyte per analysis rendering protein analyses time-consuming, labor-intensive and expensive considering the numerous cancer-related proteins that appear to be present in saliva. New technologies, however, have come forth making multianalyte discovery possible. One such technology is the surface-enhanced laser desorption/ionization (SELDI; Ciphergen Biosystems, Inc., Fremont, CA, USA) mass spectrometry technology. The SELDI can rapidly perform the separation, detection, and analysis of proteins at the fentomole level directly from biologic samples (24–27).

The protein chip array is defined by multiple, addressable locations of chemically or biologically defined protein docking sites on a chip (24–27). The protein chip array miniaturizes and amplifies the ability to rapidly define complex protein compositions by simultaneously investigating a variety of purification conditions on multiple surfaces. The protein chip arrays allow the researcher to affinity capture minute quantities of proteins via specific surface chemistries. Each aluminum chip contains eight individual, chemically treated spots for sample application; this set-up facilitates simultaneous analysis of multiple samples. A colored, hydrophobic coating retains samples on the spots and simultaneously allows for quick identification of chip type. Typically, a few microliters of sample, applied on the protein chip array, yield sufficient protein for analysis with the protein chip reader. Additionally, 12 protein chip arrays aligned side-by-side create a 96-well plate template. A typical experiment, using protein chip array technology, requires 1–3 h of work at the bench followed by automated sample analysis with the protein chip reader (24–27).

Using the aforementioned SELDI technology, the authors of this study performed an exploratory study comparing protein profiles in salivary specimens from a group of breast cancer patients and known healthy controls. The objective was to determine if: (i) it was possible to protein profile salivary specimens using SELDI mass spectrometry, and (ii) these salivary protein profiles were possibly altered in the presence of carcinoma of the breast.

Methods and materials

Population

Due to the exploratory nature of this investigation, the patient panel in this study consisted of six women. Three healthy women and three cancer patients diagnosed with ductal carcinoma *in situ* of the breast and classified as stage 0 ($T_{dis}N0M0$) as defined by TNM system proposed by the American Joint Commission on Cancer Staging and End Results Reporting had their saliva assayed (28). Stage 0 subjects were selected as this represents the smallest clinically detectable tumor load providing evidence of the marker's sensitivity or threshold for tumor detection. Additionally, tumors with nodal or metastatic involvement may provide different protein profiles that would confound profiling for early tumor detection. Due to the limited resources of this feasibility study, patients with benign lesions were not assayed;

however, they will be included in future studies in order to determine the specificity of the biomarker.

The cancer subjects were otherwise medically healthy and their saliva and specimens were collected prior to treatment. After agreeing to and signing the Institutional Review Board approved consent form, saliva and serum samples and medical histories were collected. Pathology reports were received on the three cancer patients to confirm their disease status.

In order to ascertain the effects produced by individual variances, a pooled saliva specimen from cancer patients with varying cancer stages were also assayed. Saliva from these individuals was also collected under the auspices of an IRB approved protocol and consent form.

Stimulated whole saliva collection

The participants were evaluated between 8:00 AM and 5:00 PM. The study required that the individuals not eat, drink, smoke, or brush their teeth for at least 60 min prior to saliva collection. The subject first swallowed accumulated saliva in the mouth. An unflavored, unsweetened piece of chewing gum base was placed in the mouth and masticated (60 chews/min) as monitored with a metronome (29). Accumulated saliva was expectorated into a pre-weighed cup for a total of 5 min. Flow rates (ml/min) were determined gravimetrically and the physical characteristics were recorded (e.g. blood in saliva). Specimens containing tinges of blood were discarded. Prompt processing of the salivary specimens is essential when performing proteomic analyses (30). Inherent enzymatic activity in saliva should be halted at the time of collection in order to inhibit ongoing enzymatic activity which is likely to cleave proteins that are not involved with biologically relevant pathways (31). To prevent protein degradation, 1 μl/ml of saliva of protease inhibitor cocktail (Sigma Diagnostics, St Louis, MO, USA) was added to the specimens. Afterwards the specimens were immediately frozen (−70°C) until ready for analyses.

The authors opted to use stimulated whole saliva (SWS) for their diagnostic medium over glandular secretions or unstimulated whole saliva. SWS albeit may not be as pure as the glandular secretions (e.g. debris and bacterial particulates); however, it is the method of choice when searching for salivary biomarkers because of the large amount of saliva that is produced, the ease in collection, and the reproducibility of analyte analyses in SWS production (22, 29, 32, 33). More importantly, mechanical- or reflexive-stimulated saliva is not as susceptible to the influence of circadian rhythms (22, 29, 30, 32); hence, because of its logistical advantages, SWS is the more desirable method for salivary specimen collection in this study (22, 29, 32, 33).

Blood specimens were also obtained by a phlebotomist after the saliva was collected. The serum was separated from the specimen and frozen at −70°C.

SELDI protein analyses

Prior to SELDI analysis, the frozen SWS and serum samples were allowed to thaw and the SWS was clarified by centrifugation (500–1500 *g* for 20 min) to separate the saliva proper from any gross particulates that may be present in the specimens. The samples were assayed for protein using the bicinchoninic acid method (Pierce Chemical, Co., Rockford, IL, USA).

Preliminary experiments were performed using varying affinity chip arrays in order to determine which array exhibited the best performance for salivary protein profiling. These arrays (ProteinChip®, Ciphergen Biosystems Inc.) were the hydrophobic surface array (H4), the strong anion-exchange array (SAX), and the weak cation-exchange array (WCX). The H4 array is used for capturing proteins through hydrophobic interactions; whereas, the SAX array can be used to analyze molecules with a negative charge on the surface. In contrast to the SAX chip array, the WCX array used to analyze molecules with a positive charge on the surface. We also varied the binding buffer at a pH of 3.5 and at 7.5 in order to optimize chip performance. Saliva and serum from three healthy controls and three early stage cancer patients were used for these experiments. These specimens were run 'neat'. A control sample of pooled human saliva from cancer patients with varying cancer stages was also profiled and was used for data calibration between experiments. This specimen was, likewise, run 'neat'.

The aforementioned pooled specimens were also purged through fractionation columns. Fractionations columns (Ciphergen Biosystems Inc.) were used to remove possible undesirable materials (e.g. mucins) missed by centrifugation that could potentially produce 'noise' in the spectral analysis. After fractionation, the specimens were placed on the arrays and profiled. The results, however, were not productive; so much protein was lost on the columns that we abandoned the concept and performed a quick fractionation to remove mucins and other particulates and then pooled the fractionated samples.

Cell extracts (ATCC, Manassas, VA, USA) from the SKBR-3 HER-2/*neu* receptor-positive breast cancer cell line which express the oncogene product p185 (33), MFC-7 HER-2/*neu* receptor-negative breast cancer cell line and which does not express the oncogene product p185 and from the HeLa cell line, a cervical cancer cell line with fibroblastic properties were protein profiled. The SKBR-3 cell line was used as a positive control for p185 expression, the MFC-7 and HeLa cell lines as a negative control for p185 expression. As HER-2/*neu* has been identified in the extracts of the SKBR-3 cell line it can serve as an indicator with respect to the presence of high-molecular weight proteins and should appear in the protein profile. This was necessary as the SELDI unit is most effective in measuring proteomic patterns within the range of 0–20 kDa (34).

Prior to SELDI analysis, the frozen-SWS samples were thawed and clarified by centrifugation (500–1500 *g* for 20 min) in order to separate the saliva proper from any gross particulates that were present in the specimens. Samples of equal volume (5 μl) were spotted on the protein chip arrays using a randomized allocation chip scheme. All specimens were assayed in triplicate

using the PBS Model IIC mass spectrometer (Ciphergen Biosystems Inc., Fremont, CA, USA) and the time-of-flight spectra generated by averaging 190 shots in a positive mode with a laser intensity of 230, detector sensitivity of 7, and a focus lag time of 900 ns. Mass accuracy was calibrated using the 'All-In-1' peptide molecular weight standard (Ciphergen Biosystems Inc.).

Briefly, the SELDI process may be described as follows: (i) capture or 'dock' one or more proteins of interest on the protein chip array, directly from the original source material, without sample preparation and without sample 'labeling'; (ii) enhance the 'signal-to-noise' ratio by reducing chemical and biomolecular 'noise' (i.e. achieve selective retention of target on the chip by washing away undesired materials); (iii) read one or more of the target protein(s) retained by a rapid, sensitive, laser-induced process that provides direct information about the target (molecular weight); and (iv) process (characterize) the target protein(s) at any one or more locations within the addressable array directly *in situ* by engaging in one or more on-the-chip binding or modification reactions to characterize protein structure and function (24–27).

After analysis, all protein spectra were compiled, and qualified mass peaks (signal-to-noise ratio >5) with mass-to-charge ratios (m/z) between 2000 and 150 000 were autodetected. Peak clusters were completed using second-pass peak selection (signal-to-noise ratio >2), within 0.3% mass window and estimated peaks were added. The peak intensities were normalized to the total ion current of m/z between 2000 and 150 000 and descriptive statistics were performed. One of the characteristics of proteomics spectra is that peaks centered at higher mass values tend to be broader and lower than low mass values (34). Such a transformation in general reduces the range of intensity data. As a result, the variance of the transformed peak intensity (across multiple samples) tends to be less volatile over the entire length of the spectrum. These analytical techniques were performed using PROTEINCHIP Software 3.0 (35).

Results

The results of the SELDI assays were encouraging. The initial experiments used for the selection of the optimum chip array and buffering pH determined that the WCX array at a pH of 3.5 demonstrated the best performance in profiling salivary proteins. Figure 1 is a representative illustration of one of the samples comparing the results between using a binding buffer at a pH of 3.5 and at a pH of 7.5. As illustrated in Fig. 1 low-molecular weight spectrum, there appears to be less noise in the spectrum by using a binding buffer with a lower pH. Additionally, in the high-molecular weight range, more mass peaks were obtained using the binding buffer at a pH of 3.5. Likewise for serum, Fig. 2 is a representative comparison of spectra from cancer subjects and health controls at the 25 000 and 35 000 m/z ranges on the WCX arrays at a binding buffer pH of 3.5 and 7.5. Similar to saliva, the WCX array at 3.5 produced the optimal results.

Figure 3 is a representative WCX spectrum from the saliva specimens between the 0 and 200 km/z range. As illustrated in Fig. 3, there are mass peaks with pronounced intensities in the 10–14, 21–25, 53–56, 65 and 78 km/z ranges. These mass peaks, in part, are consistent with salivary proteins known to have approximately the same mass (36–38) such as: cystatins (10–14 kDa), immunoglobulin light chain fragments (20–37 kDa), α-amylase (55–57 kDa), albumin (69 kDa), lactoferrin proteins (72 kDa), and/or peroxidase (72–78 kDa).

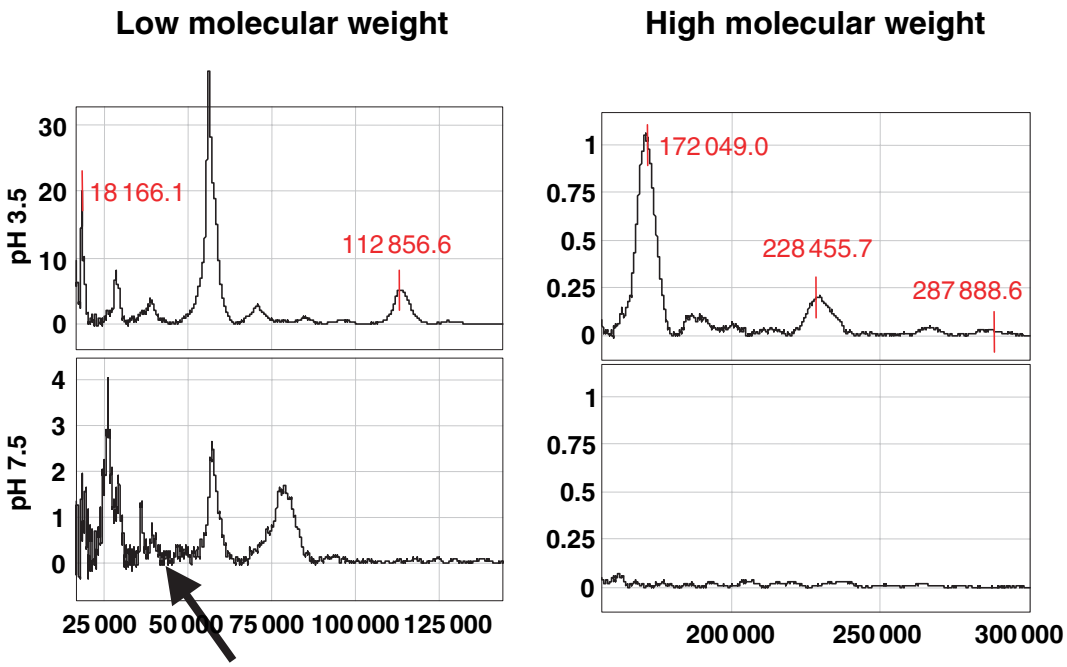

Figure 1 Comparison of salivary spectra obtained at a pH of 3.5 and 7.5. Arrow indicates noise created by using a binding buffer at a pH of 7.5.

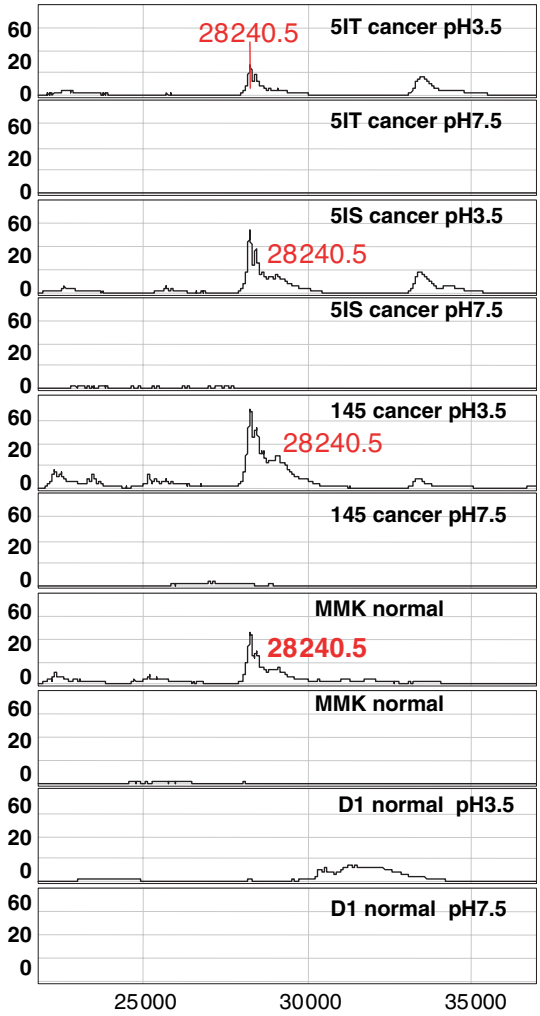

Figure 2 Comparison of serum spectra obtained at a pH of 3.5 and 7.5.

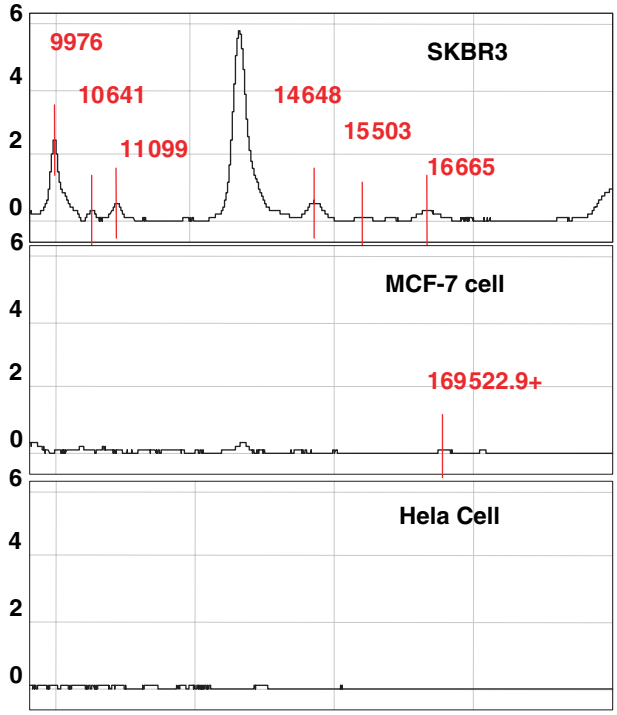

Figure 4 Spectra of SKBR-3, MCF-7, and HeLa cell culture extracts.

The results of the cell extract spectra are illustrated in Fig. 4. There are several peaks demonstrating moderate intensity in the 100 to 175 km/z range in the SKBR-3 cell line extract that may represent portions of the p185 protein. There is a low-intensity mass peak at the 17.6 km/z present in the MCF-7 cell extract and the appearance of no mass peaks in the HeLa cell extracts.

Figure 5 illustrates the spectra of proteins in the 100–200 km/z for the cell extracts, the pooled saliva, the individual's saliva, and serum cancer specimens. As

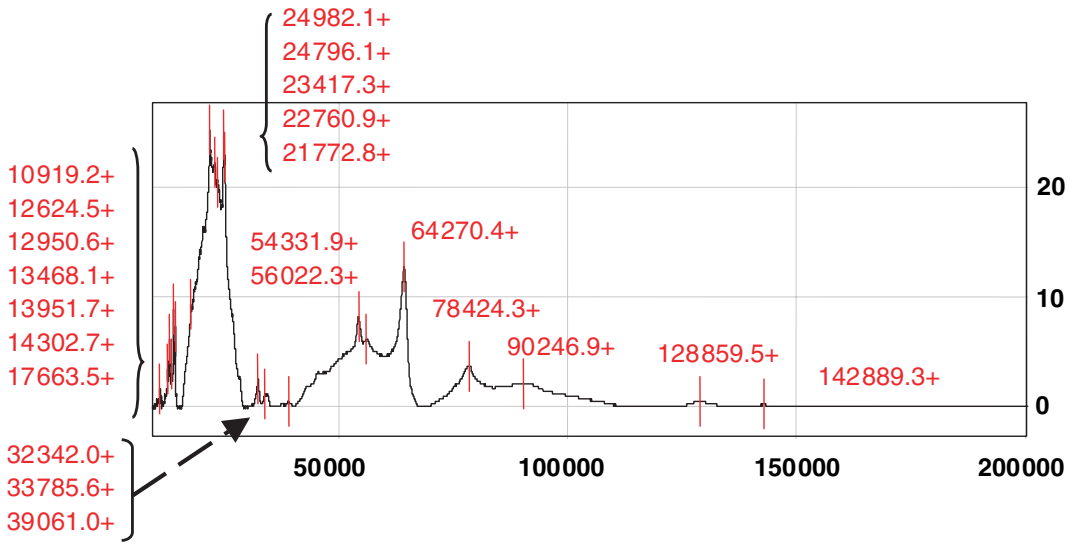

Figure 3 Representative spectra of known salivary constituents in the 50 000–200 000 m/z range.

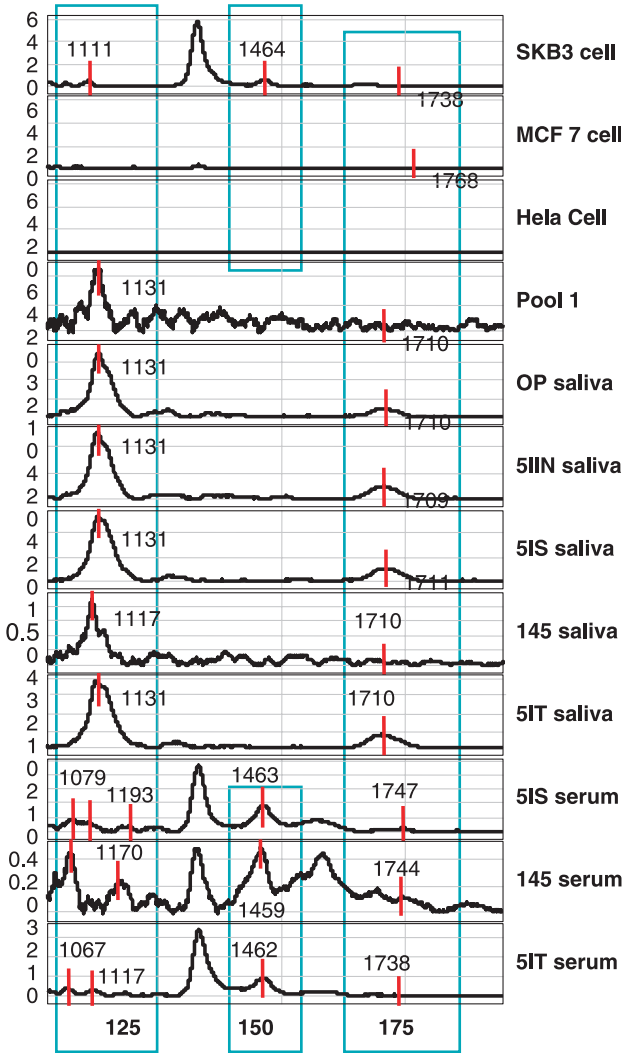

Figure 5 The spectra of proteins in the 100–200 km/z for the cell extracts, the pooled saliva, the individual's saliva, and serum cancer specimens.

Fig. 5 illustrates, the SKBR-3 cell extract, saliva and serum spectra commonly have mass peaks in the 110–113 km/z and the 170–175 km/z ranges. The MCF-3 compares with the SKBR-3 cell extract, and the saliva and serum specimens with one mass peak in the 170 km/z range. The HeLa cell extract profile did not compare with either the MCF-3, SKBR-3 cell extracts, or the saliva and serum specimens profiles. It is worth mentioning that the SKBR-3 cell extract and serum demonstrated mass peaks at the 145 000 m/z range. This protein was not present in the saliva specimens. Spectral profiles were similar in the healthy controls, but with lower intensity values.

Comparisons were made of the salivary protein spectra produced by healthy controls and the cancer subjects. The salivary comparisons revealed six peaks, which had higher intensity levels among the cancer cohort when compared with the saliva of the healthy control group. The mass peaks with the greater intensity levels were at 18, 113, 170, 228, and 287 km/z. Figure 6 is representative comparisons of spectra from cancer subjects and health controls at the 113 and 170 km/z ranges. The bar graphs in the figure gives a descriptive comparison of the relative intensity values of the proteins in each group and suggest that there may be more of the 113 and 170 km/z protein in the saliva of cancer subjects in comparison with healthy controls. The sample sizes were too small to statistically compare the data in order to arrive at any definitive conclusions.

Discussion

The results of the feasibility study suggest that protein profiling instruments such as SELDI could be applied to salivary biomarker discovery. Additionally, the findings of this study appear to satisfy its objectives. The objectives were to see if: (i) it was possible to assay salivary specimens using SELDI mass spectrometry, and (ii) salivary protein profiles are altered in the presence of carcinoma of the breast. Based on the evidence provided in the Results section, the investigation suggests that saliva did not require special sample preparation and that the WCX at a pH of 3.5 exhibited the best analytical performance (Figs 1–3).

In an attempt to use SELDI to identify salivary biomarkers, comparisons were made between the breast cancer subjects and healthy controls and yielded five mass peaks with the greater intensity levels at 18, 113, 170, 228, and 287 km/z. We do not know the identity of these ion signals; however, there are possible candidate proteins for these peaks, which have been detected in saliva and have been observed to increase in the presence of carcinoma of the breast. Table 1 provides a list of these proteins. The proteins identified by Western blot were determined commercially by the authors using the Pharmingen PowerBlot (BD Biosciences, San Jose, CA, USA) technique (39). The other proteins have been detected in saliva by using ELISA (19, 31). The proteins bearing an asterisk represent carbohydrate cancer antigens such as CA125, CEA, GICA, etc., which have been identified by other investigators (40–44). The column on the right indicates the increase in signal produced in the presence of breast cancer.

Using the 113 km/z mass peak as an example, Fig. 6 illustrates the analysis of proteins in the molecular weight range of 100–150 km/z using the WCX chip array. As illustrated by that figure there is an increase in the quantity of proteins in the range of 100–150 km/z in the saliva among cancer patients when compared with controls, especially at the molecular weight of approximately 113 kDa. As Table 1 suggests, one possible candidate, which approximates that molecular weight is the extracellular domain (ECD) of c-*erb*B-2 receptor which is approximately 105–115 kDa based on two-dimensional (2D) and Western blot gel analyses of serum and cell supernatants (43). Additionally in Fig. 5, there are several proteins in the SKBR-3 cell extract between 100 and 115 km/z, which have been reported by Zabrecky et al. (33) as being ECD of p185/*neu* (43). If it is the ECD of c-*erb*B-2 receptor, this would be suppor-

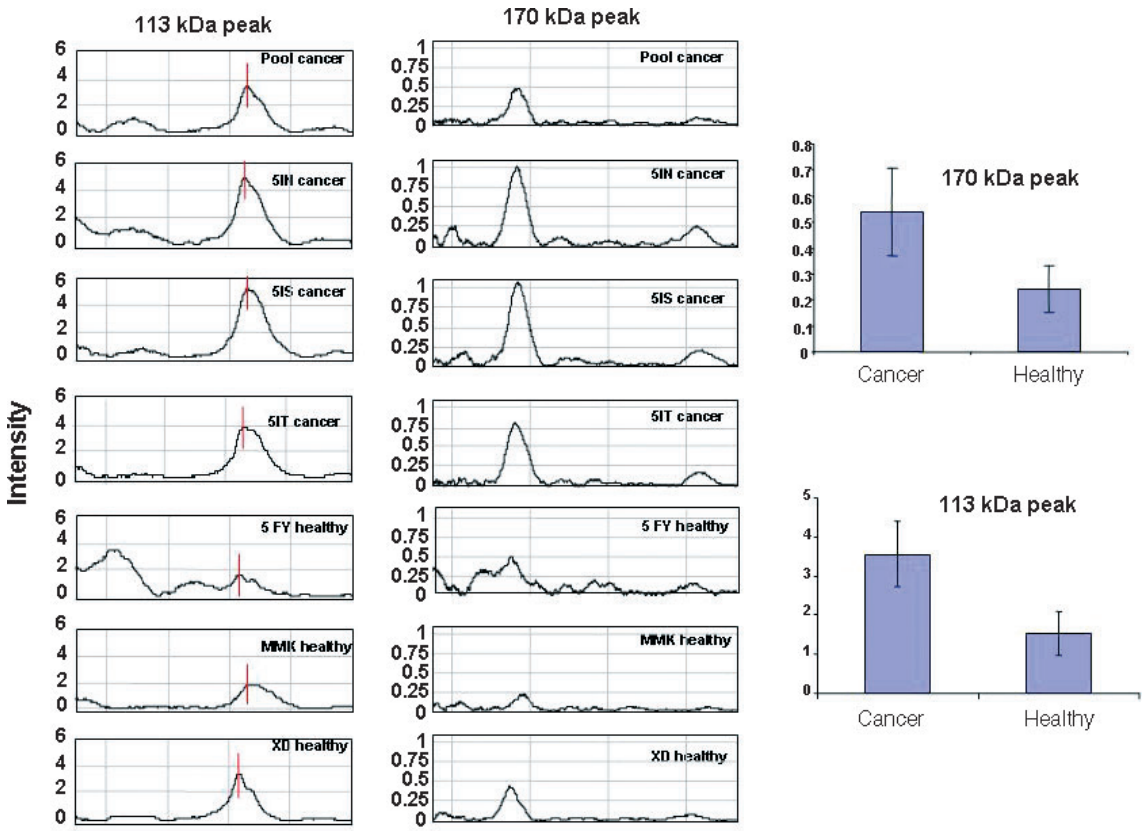

Figure 6 The spectra of the 113 and 170 kDa peaks in saliva. The left panel represents the spectral views; right panel shows the bar graphs the mean values and the standard deviations of the log-normalized intensities for the cancer subjects and the healthy individuals.

Table 1 Protein candidates for the mass peaks identified in the SELDI analyses

SELDI peak (m/z)	*Candidate protein*	*MW (kDa)*	*Western blot*	*ELISA*	*Fold change*
18	Spot 14	17	✔	–	↑2.0×
	Nm23	19	✔	–	↑2.7×
	P21	20–21	–	✔	↑2.0×
113	c-*erb*B-2 (ecd)	105–110	✔	✔	↑2.0×
	Rb protein	110–114	✔	–	↑3.8×
170	EGFr	170	✔	✔	↑4.6×
	nNOS/NOS-1	172	✔	–	↑6.7×
	c-*erb*B-2 (fragment)	185	✔	✔	↑2.0×
228	CA Mucins*	300–450	–	✔	–
287	CA Mucins*	300–450	–	✔	–

*CA mucins represent carbohydrate cancer antigens such as CA125 which have been identified by other investigators.
ELISA, enzyme-linked immunosorbent assay; SELDI, surface-enhanced laser desorption/ionization.

ted by the findings of Streckfus and Bigler using ELISA assays which produced similar results in both serum and saliva (18, 19).

As illustrated in Fig. 6 there is a spectra of proteins in the 125–250 *m/z* molecular weight range bound to the WCX biochip. The full-length c-*erb*B-2 (based on gel analysis of serum and cell supernatants) is estimated to be 185 kDa. Interestingly, a protein cluster in the range of 170 k*m/z* was more prevalent in cancer patient saliva samples (pooled saliva, 5IN, 5IS, and 5IT) than normal (5FY, MMK, and XD). Also of note, one of the donors in the normal group (XD) is known by ELISA to have quite high c-*erb*B-2 levels (C. F. Streckfus and L. R. Bigler, unpublished data) and stands out from the other two normal donors in this analysis. Similarly to the 105 k*m/z* peak, this has also been identified in SKBR-3 extracts (43) and in saliva by Western blot and ELISA analyses (18, 21).

Despite these encouraging results, however, these identifications raise two major concerns. The first is which of these ion signals represent the full-length protein and the second is the utility of these ion signals as salivary biomarkers? As this is a 'top–down' analysis it cannot be determined with any certainty from the

spectral data that these are full-length proteins or are cleaved peptides resulting from processes inherent to saliva (30). In order to identify these ion signals and thereby confirms the presence of these proteins; direct peptide sequencing will be required. Once the peptides are identified they will require validation using Western blot and/or ELISA. Once validated, then the salivary biomarker can be diagnostically evaluated among individuals with carcinoma and healthy individuals. Before diagnostic evaluation, however, the question should be ask as to whether or not there is a physiologic basis for the marker being present in saliva and is the protein modulated in the presence or absence of the disease state?

In conclusion, the results of this study suggest that SELDI mass spectrometry may be a very useful tool in the development of salivary biomarkers. Further research is required using larger sample sizes with benign tumors and other malignancies, other protein chip arrays, and by employing methods to remove the abundant proteins such as albumin, α-amylase, and antibody fragments from saliva to further reveal low-abundance proteins. Considering that proteomic profiling studies by Hu et al. (40), Huang (41), and Vitorino et al. (42) using 2D gels and mass spectrometry have demonstrated the presence of 309 salivary proteins in whole saliva, the use of SELDI can potentially be used to narrow the protein spectral profile to meaningful mass segments suitable for further validation. This in turn can facilitate the process for salivary biomarker exploration.

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