Featured Article

Serum Protein Profiles to Identify Head and Neck Cancer

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Abstract

Purpose: **New and more consistent biomarkers of head and neck squamous cell carcinoma (HNSCC) are needed to improve early detection of disease and to monitor successful patient management. The purpose of this study was to determine whether a new proteomic technology could correctly identify protein expression profiles for cancer in patient serum samples.**

Experimental Design: **Surface-enhanced laser desorption/ionization-time of flight-mass spectrometry ProteinChip system was used to screen for differentially expressed proteins in serum from 99 patients with HNSCC and 102 normal controls. Protein peak clustering and classification analyses of the surface-enhanced laser desorption/ionization spectral data were performed using the Biomarker Wizard and Biomarker Patterns software (version 3.0), respectively (Ciphergen Biosystems, Fremont, CA).**

Results: **Several proteins, with masses ranging from 2,778 to 20,800 Da, were differentially expressed between HNSCC and the healthy controls. The serum protein expression profiles were used to develop and train a classification and regression tree algorithm, which reliably achieved a sensitivity of 83.3% and a specificity of 100% in discriminating HNSCC from normal controls.**

Conclusions: **We propose that this technique has potential for the development of a screening test for the detection of HNSCC.**

Received 2/25/03; revised 8/13/03; accepted 12/8/03.

Grant support: National Cancer Institute's Early Detection Research Network Grant CA85087, the Department of Defense Grant DAMD17-02-1-0054, and the Virginia Prostate Center.

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Introduction

Head and neck squamous cell cancer (HNSCC) remains a significant disease, comprising >5% of all cancers in the United States and an even larger proportion of cancers worldwide (1). Tobacco use and excess alcohol consumption are well-established risk factors for HNSCC. Despite many advances in the treatment of HNSCC over the past 30 years, little progress has been made toward improving survival rates. Given the usual location of these tumors, they are often discovered in advanced stages. Treatment of advanced HNSCC frequently leaves patients disfigured, with debilitating side effects of radiation and chemotherapy manifested as compromised speech and swallowing and significantly diminished quality of life.

Despite increased awareness and education about the potential effects of tobacco and alcohol use, the incidence of HNSCC in the United States has changed very little. Indeed, prevention and early diagnosis are accepted as mainstays of successful HNSCC treatment. Yet, no accepted screening test exists for this cancer type. In fact, screening for HNSCC is not mentioned in the most recent screening guidelines of the American Cancer Society (2) due primarily to the lack of sufficient screening tools available to physicians. Aside from a complete head and neck physical examination and imaging studies in those patients with suspicious clinical findings or symptoms, there are no accepted methods to screen for these cancers.

The search for biomarkers predictive of HNSCC has focused largely on the detection of genetic abnormalities leading to the development of HNSCC (3, 4). Despite the identification and characterization of multiple molecular aberrations in HNSCC, available technology limits their routine clinical use, and none has been determined to enhance early detection of HNSCC. Recently, attention has focused on deciphering the HNSCC proteome in search of diagnostic biomarkers. Traditionally, proteomic research has involved two-dimensional gel electrophoresis to detect differences in protein expression in tissue and body fluid specimens between the healthy (control) group and the disease group (5, 6). Although two-dimensional gel electrophoresis has been the "gold standard" proteomic method, it has limitations in detection, is labor intensive, and is not easily applied in the clinical setting.

One of the recent technological advances in proteomics is the ProteinChip surface-enhanced laser desorption/ionization (SELDI)-time of flight-mass spectrometry (7, 8). Applications of this technology show great potential for the early detection of prostate, breast, ovarian, and bladder cancers (9–14). The objective of this study was to determine whether protein profiling using SELDI-time of flight-mass spectrometry could accurately distinguish patients with HNSCC *versus* healthy controls, potentially providing a novel screening approach for the detection of HNSCC.

Materials and Methods

Serum Samples. Human sera from head and neck cancer patients and controls were obtained through institutional review board approved protocols at Saint Louis University School of

Table 1 Group Demographics

A. Cancer samples

Subsite		Sex		Age range (yrs)	Ethnicity	
Hypopharynx	3 (3.1%)	Female	15 (15.1%)	44–85	African America	3 (3.1%)
Larynx	24 (24.2%)	Male	79 (79.8%)	35–88	Hispanic	1 (1%)
Nasopharynx	1 (1%)	Unknown	5 (5.1%)		Caucasian	91 (91.9%)
Oral cavity	24 (24.1%)				Unknown	4 (4.1%)
Oropharynx	14 (14.1%)			Avg. age (yrs)		
Sinuses	1 (1%)			65.8		
Skin	6 (6.1%)					
Unknown	26 (26.4%)					
Total	99					

B. Controls

Smokers		Sex		Age range (yr)	Ethnicity	
Yes	17 (16.7%)	Female	46 (45%)	21–68	African American	1 (1%)
No	85 (83.3%)	Male	53 (52%)	21–83	Hispanic	1 (1%)
		Unknown	3 (3%)		Caucasian	98 (96%)
				Avg. age (yrs)	Unknown	2 (2%)
Total	102			56.7		

Medicine and the Pennsylvania State University College of Medicine from 1997 to the present, with an equal distribution of cancers and controls between the sites. Cancer patients were staged according to the 1998 American Joint Commission on Cancer staging guidelines, and all stages of disease were represented in this group. The healthy control patients were predominantly nonsmokers who underwent standard blood donor screening and were noted to be disease free on complete head and neck examination. The two groups were unmatched but were comparable for sex and race. All serum was aliquoted and frozen at −80°C until thawed specifically for SELDI analysis.

SELDI Protein Profiling. Serum samples were processed for SELDI analysis as previously described using the IMAC3 ProteinChip pretreated with $CuSO_4$ (8). Briefly, 20 μl of serum is pretreated with 8 M urea, 1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid, and vortexed for 10 min at 4°C. A further dilution is made in 1 M urea, 0.125% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid and PBS. Diluted serum is then added to the ProteinChips with the aid of a bioprocessor. Each serum sample was assayed in duplicate, with duplicate samples randomly placed on different ProteinChips. ProteinChips are then incubated at room temperature followed by washes of PBS and water. Arrays were allowed to air, dry and a saturated solution of sinapinic acid in 50%(v/v) acetonitrile and 0.5% (v/v) trifluoroacetic acid was added to each spot. The protein chip arrays were analyzed using the SELDI ProteinChip System (PBS-II; Ciphergen Biosystems, Fremont, CA). Spectra were collected by the accumulation of 192 shots at laser intensity 220 in a positive mode. The protein masses were calibrated externally using purified peptide standards.

Data Analysis. Before analysis, the data were divided into two sets as follows: a training set that consisted of 75 samples from each group (normal and HNSCC) and a test set of 24 HNSCC and 27 normals. Spectra were analyzed with the Ciphergen ProteinChip software (version 3.0) and normalized using total ion current. Peak labeling and clustering were performed using Ciphergen's Biomarker Wizard tool, exported into a spreadsheet, and the intensity values for each peak were averaged for duplicate samples. This spectral data were then analyzed by the BioMarker Patterns software (Ciphergen Biosystems) to develop a classification tree.

Classification and Regression Tree (CART) Analysis. Details regarding CART and the computational algorithms incorporated within the BioMarker Patterns software program have been described elsewhere (15, 16). Briefly, classification trees split the data into two nodes, using one rule at a time in the form of a question. The splitting decisions in this case were based on the normalized intensity levels of peaks from the SELDI protein expression profile. Each peak or cluster identified from the SELDI profile is therefore a variable in the classification process. The process of splitting is continued until terminal nodes are reached, and additional splitting has no gain in data classification. Classification trees were constructed using the training set, and after V-fold cross validation, the accuracy of each classification tree was then challenged with the test set. Multiple classification trees were generated using this process, and the best performing tree was chosen for additional testing.

Statistical Analysis. Specificity was calculated as the ratio of the number of negative samples correctly classified to the total number of true negative samples. Sensitivity was calculated as the ratio of the number of correctly classified diseased samples to the total number of diseased samples. Comparison of relative peak intensity levels between groups was calculated using the Student's *t* test.

Results

Serum Samples. The experimental and control populations are characterized in Table 1, A and B, respectively. A total of 99 sera from patients with HNSCC was used in the experimental group. Tumor locations varied from many subsites (24.2% larynx, 24.1% oral cavity, 14.1% oropharynx, 3.1% hypopharynx, 1% nasopharynx, 1% sinuses, 6.1% skin, and

Table 2 Protein peaks differentially expressed in head and neck squamous cell carcinoma *versus* control serum

m/z[a]	P[b]	m/z[a]	P[b]
2778	<0.0001	7805	<0.0001
2951	<0.0001	7830	<0.0001
3772	<0.05	7920	<0.0001
3888	<0.001	7971	<0.0001
4181	<0.02	8928	<0.0001
4464	<0.0001	9094	<0.001
5064	<0.0001	9134	<0.0001
5078	<0.0001	9181	<0.0001
5242	<0.0001	9287	<0.001
5335	<0.0001	9416	<0.0001
5363	<0.001	10264	<0.05
5544	<0.01	10843	<0.05
5905	<0.0001	11722	<0.0001
5920	<0.0001	11922	<0.0001
6110	<0.0001	13350	<0.0001
7764	<0.0001	14687	<0.0001

[a] m/z mass/charge.
[b] P calculated from Student's t test.

26.4% from unrecorded subsites). Age range was 35–88 years with the mean age of 65.8 years. Male to female ratio was ~5:1.

There were a total of 102 sera from patients in the control group that was predominantly nonsmoking. Age ranged from 21 to 83 years with a mean age of 56.7 years. Male to female ratio was ~1:1. Overall, the age and ethnicity of the two groups were comparable.

Data Analysis. Peak detection using Ciphergen's ProteinChip software identified an average of 90 peaks/spectrum. Of these, 80 common peaks or clusters were generated from the training set, with masses ranging from 2,000 to 21,000 Da. Because the majority of the peaks detected were in this mass range, it was considered the most useful for protein profiling. Each cluster was determined with a mass window of 0.2% and represents one protein peak. As shown in Table 2, 32 of these peaks were found to have significant differential expression levels between the HNSCC and control sera.

CART Analysis. Using all 80 peaks, classification trees were created using the training set with V-fold cross-validation. This type of cross-validation uses random numbers to split up the data in the training set for testing each tree. On the basis of the CART analysis, no single peak was identified as having the ability to separate HNSCC sera from normal sera alone. However, the underexpression of a protein peak at 5064 Da was used in all of the classification trees as the first primary splitter. This peak was identified as being significantly differentially expressed (Table 2). Fig. 1 is a representative gelview (Fig. 1*A*) and spectra (Fig. 1*B*) showing the underexpression of this peak in the HNSCC sera when compared with the control sera. Averaged normalized intensity values for the 5064 Da peak in each sample are plotted in Fig. 2. The average expression is 3-fold lower in HNSCC sera compared with the average expression in the control sera.

Of the many classification trees generated, the most accurate tree, based on internal cross-validation results, was used for additional analysis. The CART algorithm is a supervised classification tool, meaning the disease status of the samples in the training set is known. The most accurate tree correctly classified 90.7% of the HNSCC sera in the training set (Table 3). This classification tree algorithm was then challenged with a blinded test set (in which the disease status is unrevealed) consisting of 27 sera from healthy individuals and 24 sera from patients diagnosed with HNSCC (distinct from the training set). A total

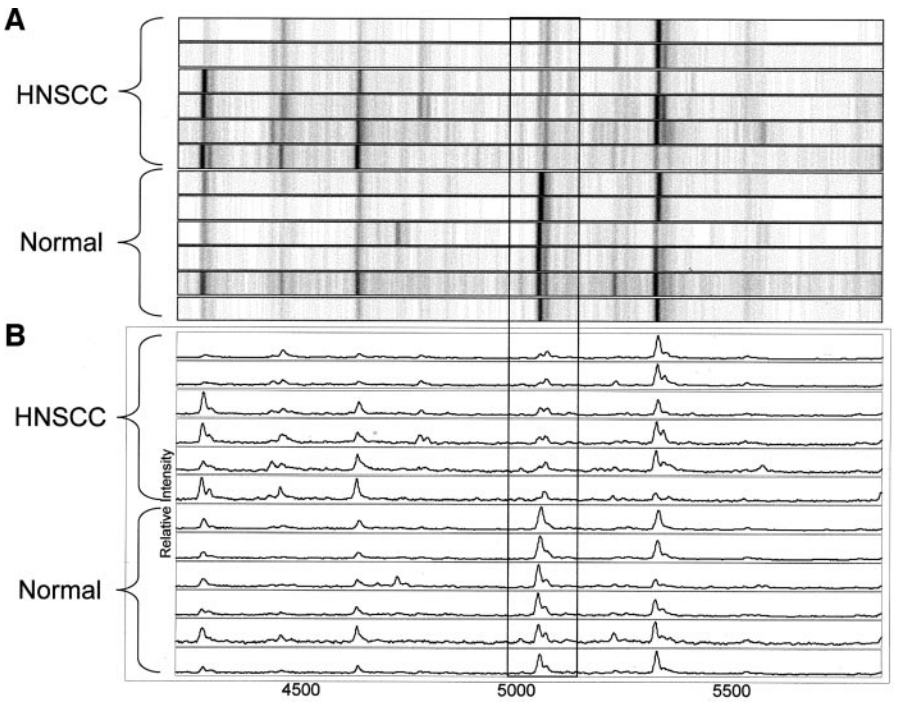

Fig. 1 Representative surface-enhanced laser desorption/ionization gelview (*A*) and spectra (*B*) from sera of six head and neck squamous cell carcinoma (HNSCC) patients compared with sera from six normal controls ranging from 4000 to 6000 m/z is shown. The *box* identifies a peak with an average mass of 5064 Da that is underexpressed in HNSCC compared with normal serum.

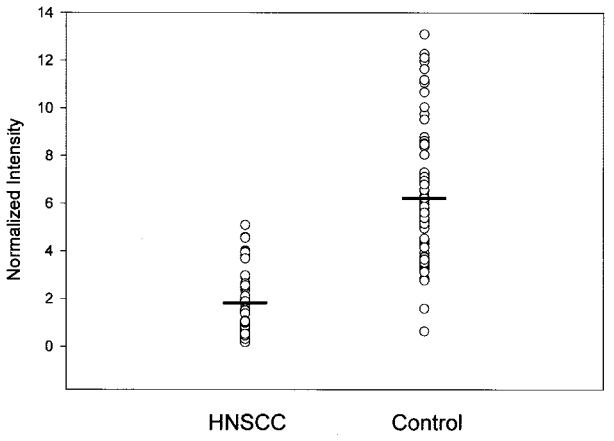

Fig. 2 Expression level of 5064 Da protein in sera of HNSCC patients compared with sera from normal controls. ———, mean normalized intensity; ○, values of individual patients.

of 100% of controls and 83.3% of HNSCC samples was correctly identified. The topology of the classification tree consisted of three primary peaks (5,064, 13,881, and 15,139 Da) and five terminal nodes (Fig. 3).

Reproducibility. We have optimized the SELDI system for high-throughput assays and have demonstrated reliable reproducibility using a pooled normal serum sample (10). The intra-assay and interassay coefficient of variance for peak masses is routinely 0.05% with normalized intensity coefficient of variance values of 15–20%.

We furthermore devised several exercises to demonstrate the reproducibility and robustness of both the protocol and the decision tree classifier. The design of this experiment was to compare a subset of the total samples to the total sample set, which were separately run and analyzed 3 months apart. Fig. 4 is an example of the reproducibility of the SELDI spectra of each of these runs. In addition, we examined the replicate agreement between each sample duplicate. In the first exercise, we asked whether the data set run 3 months earlier would retain a high correct classification rate when used to challenge the algorithm established from the total data set. The earlier data subset was correctly classified at a rate of 85% (Table 4). Because the early data set was limited to a single institution and derived from a subset of the larger data set, this comparison demonstrates the robustness of the decision tree algorithm over time and cohort heterogeneity. We next evaluated the reproducibility of the process by examining the agreement between duplicates of the same sample. In building the decision tree, the duplicates were averaged to generate a single intensity value for each peak. In this exercise, we uncoupled the duplicates and "dropped" them down the decision tree. The result was >90% agreement in final classification. This low incidence of duplicates that misclassified approaches the overall misclassification rate and therefore meets the expectation of strong duplicate agreement. As a last demonstration of our experimental reproducibility, we measured the variation in peak intensity values between duplicates at the mass positions for each primary decision peak. As shown in Table 4, the average coefficient of variance between duplicates at each peak mass was between 27 and 33% of total intensity. This value was smaller than the average variance for the decision peaks over the entire class of samples, which was 50%.

Discussion

Detection of head and neck cancer at early disease stages is paramount to successful clinical therapy. Yet, early-stage head and neck cancer lacks specific symptoms or biomarkers that accurately and reliably distinguish patients with HNSCC from normal controls. A number of studies have described limited success in identifying HNSCC-associated protein, DNA, and RNA biomarkers that potentially could aid the early diagnosis and prognosis of HNSCC. Reverse transcription-PCR was used to detect metastasis-associated cytokeratin 19-positive tumor cells in sera from a small number of patients with nasopharyngeal carcinoma. However, several longitudinal blood samples were required to reach a sensitivity of 83.3% (five of six patients; Ref. 17). An analysis of serum concentrations of multiple biological markers, including basic fibroblast growth factor, vascular endothelial growth factor, and matrix metalloproteinase-2 by ELISA in 26 HNSCC patients after primary chemoradiation therapy, showed that only increased basic fibroblast growth factor concentrations correlated with earlier loss of locoregional control (18). Other studies have investigated several conventional serological markers in 26 HNSCC patients and found none to be of statistical significance (19). Antibodies to p53 tumor suppressor protein were detected in the sera of 25% of 271 patients with oral SCC (20) and at a low percentage in saliva from HNSCC patients (21, 22). Nucleic acid-based microsatellite analysis and tumor-specific aberrant promoter methylation have been used as markers to detect tumor-specific alterations in serum and saliva of patients with HNSCC (23–27). These approaches are often subjective, can be technically challenging, and require a panel of microsatellite markers or selected genes. In general, nucleic acid-based methods for detection of cancer have been assessed with a limited number of samples and will require additional trials to confirm these early results.

Despite the identification and extensive study of several potential tumor markers, none has been found to have clinical use as a diagnostic marker or screening tool for HNSCC. It seems probable that given the complexity of the genetic and molecular alterations that occur in HNSCC cells, the expression pattern of these complex changes may hold more vital information in screening, diagnosis, and prognosis than the individual molecular changes themselves. Protein expression profiling was

Table 3 Classification tree analysis of the head and neck squamous cell carcinoma (HNSCC) training and test sets[a]

	Group[b]	Percentage correct	Percentage misclassified
Training set	Normal (n = 75)	88.0% (66/75)	12.0% (9/75)
	HNSCC (n = 75)	90.7% (68/75)	9.3% (7/75)
Test set	Normal (n = 27)	100% (27/27)	0.0% (0/27)
	HNSCC (n = 24)	83.3% (20/24)	16.7% (4/24)

[a] Peaks used in tree: m/z 5,064, 13,881, 15,131.
[b] Serum from control and HNSCC patients.

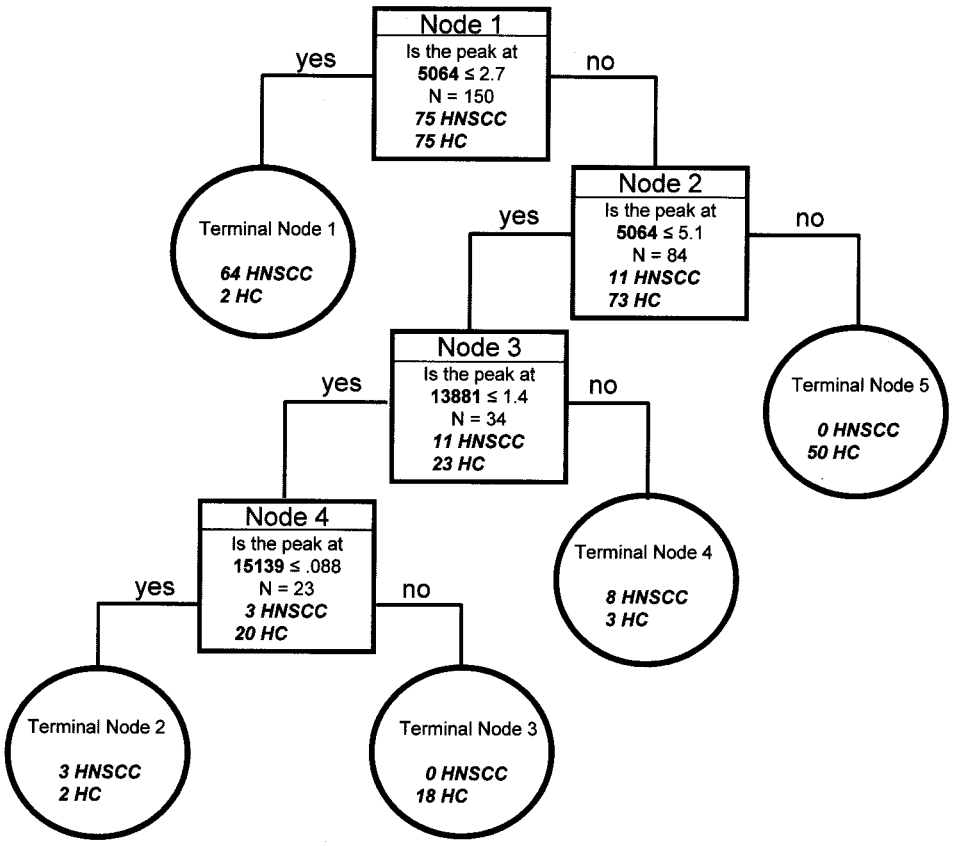

Fig. 3 Diagram of classification tree. HC, healthy control patients. HNSCC, head and neck squamous cell cancer patients. The *squares* are the primary nodes, and the *circles* indicate terminal nodes. The mass value in the root nodes is followed by ≤ the intensity value.

used previously to detect a protein of 8670 Da using a hydrophobic surface (H4) in tumor extracts of five of six HNSCC cases but not in matched normal tissue lysates (28). In a study of two matched HNSCC cell lines derived from either the primary tumor or lymph node metastasis, the SELDI ProteinChip H4 was used to identify the up-regulation of two membrane-associated proteins (annexin I and annexin II) and glycolytic protein enolase-α as well as the down-regulation of calumenin precursor in the metastatic cell line (29). To date, SELDI ProteinChip technology has not been reported as a tool to interrogate serum from HNSCC patients compared with normal controls for protein fingerprints of HNSCC.

No standard screening tool is available for HNSCC patients. Patients are most often diagnosed in the late stages of disease because of the location of the tumors and because early symptoms often mimic and are treated as benign processes such as viral upper respiratory infections. Continued efforts to identify protein profiles or patterns that differentiate cancer from noncancer could lead to earlier detection and development of diagnostic tests for HNSCC. Using SELDI-time of flight-mass spectrometry techniques, we achieved 100% specificity and 83.3% sensitivity for detection of HNSCC rapidly and reproducibly. This yielded a positive predictive value of 100%. By using the Surveillance, Epidemiology, and End Results database (30) and the most recent United States Census information, a conservative estimate of HNSCC prevalence in the United States can be approximated as 1:1000. This prevalence probably varies by region of the country and is likely higher worldwide. Assuming this prevalence, an adequate screening tool for HNSCC must have a positive predictive value of 100% to be effective. Screening tools with less than perfect specificity are unlikely to be useful in this population. The algorithm presented in this study currently provides a sufficient positive predictive value. However, it is clear that the technology must be additionally tested to ensure the high specificity persists as the test sets increase in size. If validated with more samples, in a planned multi-institutional investigation, this approach may provide an innovative test of significant benefit for clinicians treating HNSCC.

A potential criticism of this research is that HNSCC is most often related to tobacco and alcohol use, yet our control sera were from predominantly normal individuals without those risk factors. Indeed, our control population intentionally consists of a broad spectrum of patients more closely representing the targeted population to be screened. Additional and specifically dedicated studies are needed to distinguish between cancer patients and those at high risk alone. These studies are currently underway. A screening tool testing these two groups may possibly be successful with lower sensitivity and specificity, given the higher rate of HNSCC in smoking populations in comparison to the entire population as a whole.

Our approach to decision algorithm development has distinct advantages toward uncovering peak mass because this is determined up front and maintained throughout the process.

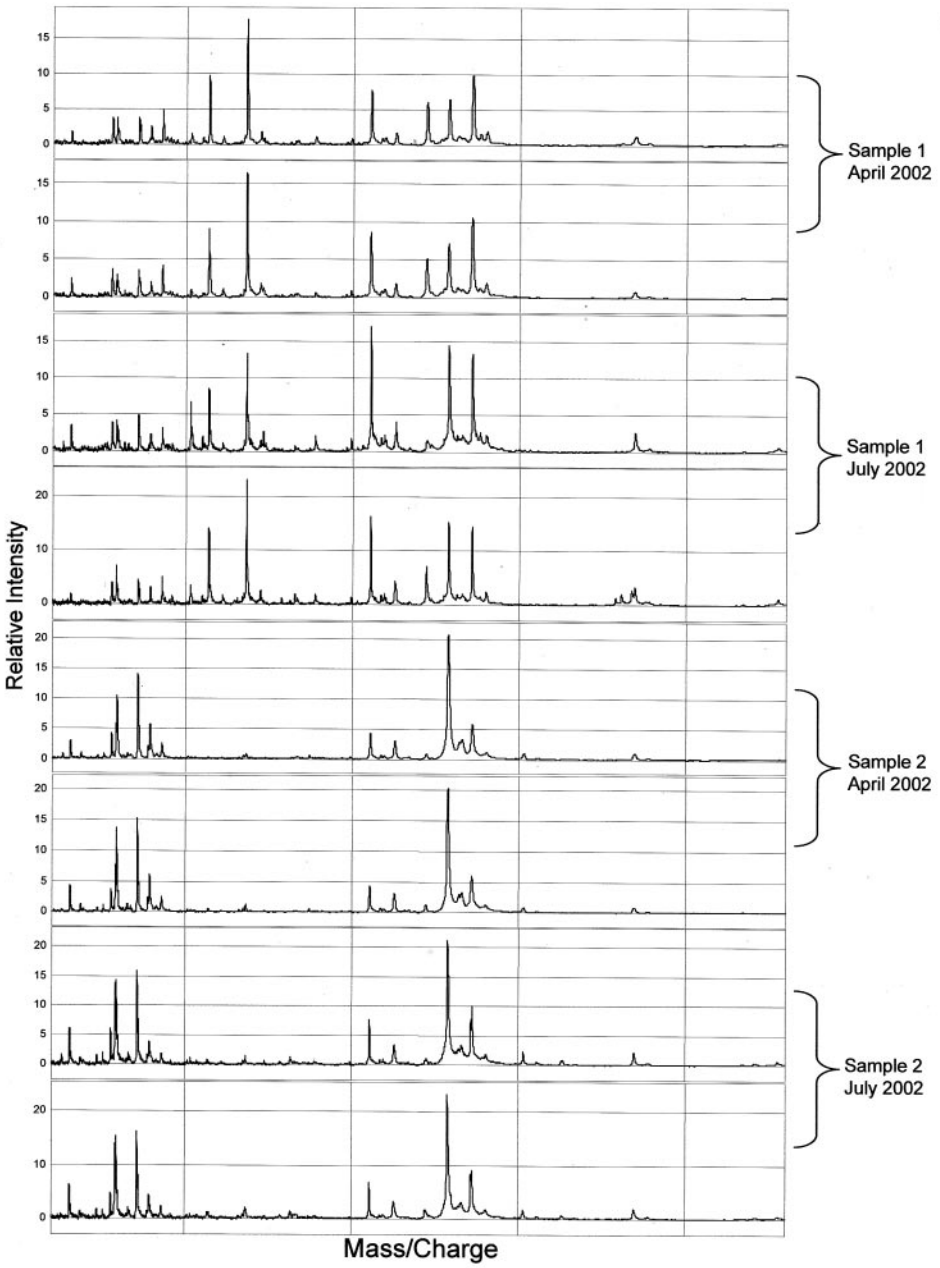

Fig. 4 Representative surface-enhanced laser desorption/ionization spectra of two different serum samples assayed three months apart using the IMAC ProteinChip.

However, there are certain concerns to be addressed when applying any binary recursive partitioning approach (decision tree) as is used by CART (31). Development of decision trees involves nonparametric choosing of variables as decision splitters forming the branches of a tree. The perfect tree (the one that fits the data best) is of course a very large tree with many branches that most certainly "overfits" the data. Generally, these trees have branch numbers that approach the sample size and are not usually robust to subsequent data. However, CART implements several extra steps to safeguard against this tendency. The first is called pruning; basically, branches are removed and the cost of the removal determined and this value used to establish a minimal tree size. Second, the tree is subjected to a 20-fold cross-validation that separates the data into partitions that are individually evaluated against the remaining data set. This process replaces the need for an independent test group to choose the best tree. Thus, the optimal tree that does not overfit the data are chosen. We then add a blinded test set of data held out of the training as a final evaluation of the chosen tree. Our data represents a tree with four nodes that was chosen through this multistep process. Therefore, it is unlikely that the algorithm has overfitted the data.

Many protein peaks were found to be differentially expressed with high statistical significance in HNSCC compared

Table 4 Reproducibility[a]

% correct average	% correct duplicates	% correct early subset
Classification		
97%	90.80%	85.00%
m/z	m/z	m/z
5,064	13,881	15,139
Variation in intensity of primary splitters[b]		
33%	27%	30%

[a] Reproducibility defined by combining the spectra between controls and cases.

[b] Average CV calculated by determining the variation between duplicate spectra.

with control sera (Table 2). It is notable that not all of these significant peaks are used in the classification tree algorithms. Unlike some statistical tools that optimize for single variables that can act as a predictor, the CART approach examines combinations of variables. The classification algorithm is able to examine a number of different variables at once, looking for a combination of peak expression that gives the best classification. Furthermore, a peak without a significant *P* when testing between groups may in fact be important for the classification algorithm. Likewise, a significant *P* may be achieved when testing for a group mean difference for a single protein peak and such a single peak may not be very useful for decision tree building. For instance, two of the peaks used in the best performing classification tree shown in Fig. 3 (13,881 and 15,139 Da) were individually not expressed differentially between the two groups of sera. However, they were significant to the classification tree to delineate subsets of groups that had been stratified by the significant peak at 5064 Da. The combination that gave maximum sensitivity/specificity in this study for differentiating HNSCC from the noncancer groups used the patterns of several different masses. One of these masses, the 5064 Da peak, underexpressed in HNSCC, was found in every classification tree generated with this set of sera and is one example of how SELDI technology may aid both the discovery of new biological markers for HNSCC as well as provide analysis of differences in protein expression patterns. Because it appears to be a critical protein for differentiating HNSCC from noncancer controls, work is in progress to identify and characterize this potential biomarker.

Potential areas for improvement to this approach for HNSCC will arise from studies that include high-risk groups as well as the analysis of serum SELDI profiles before, during, and after definitive treatment of HNSCC to determine whether this technique can be equally useful for monitoring patients for persistent or recurrent disease. In addition, the evaluation of other chip surfaces to improve testing accuracy and to increase the chance of discovery of potential biomarkers may also serve to improve the overall process.

In summary, SELDI protein expression profiling of sera from normal healthy individuals and from those with HNSCC resulted in specific profile patterns that identified HNSCC with 83.3% sensitivity and 100% specificity. The high sensitivity and specificity achieved in this study using SELDI-time of flight-mass spectrometry techniques coupled with a learning classification algorithm identified protein patterns in serum that distinguished healthy controls from HNSCC patients. To our knowledge, this is the first study to demonstrate the use of serum protein profiling to detect HNSCC. Additional investigation is warranted to evaluate SELDI protein profiling as an assay for early detection, diagnosis, and prognosis of HNSCC and to realize the potential for this process to result in more accurate screening, diagnosis, and treatment of HNSCC patients.

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