Serum Protein Profile Analysis Following Definitive Treatment in Patients With Head and Neck Squamous Cell Carcinoma

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Objective: To determine the sensitivity and specificity of surface-enhanced laser desorption and ionization time-of-flight mass spectrometry (SELDI-TOF-MS) assay for head and neck squamous cell carcinoma (HNSCC) disease surveillance.

Design: The SELDI-TOF-MS serum protein profiles of patients with HNSCC were analyzed to determine the sensitivity and specificity of the SELDI assay for HNSCC detection following definitive treatment.

Setting: Academic research.

Patients: Thirty-two patients with previously untreated HNSCC.

Intervention: Serum samples were collected prospectively at 3-month intervals following treatment during a 24-month follow-up period.

Main Outcome Measures: Ninety-three serum samples were analyzed.

Results: The SELDI-TOF-MS identified protein peaks in the range of 0 to 100 kDa. Classification tree analysis based on peak expression distinguished pretreatment from 6-month posttreatment samples with 75.0% sensitivity and 87.5% specificity. Samples collected at 3 months following treatment did not significantly differ from pretreatment samples. Serum samples from patients who were disease free at 6 months or longer following treatment differed from matched pretreatment samples by the overexpression of a protein peak at 6495 Da, while serum samples from patients with recurrence differed from matched pretreatment samples by the underexpression of a protein peak at 4493 Da.

Conclusions: Proteomic analysis of serum protein profiles distinguishes pretreatment and posttreatment samples from patients with HNSCC with a high degree of sensitivity and specificity. After 6 months, serum protein profiles seem to have distinct differences in peak expression based on disease status. Further investigation of the clinical usefulness of this technology in HNSCC detection and surveillance is warranted.


Research efforts directed at improving early detection of malignancy have focused on the identification of tumor biomarkers in biological fluids for use as a diagnostic or screening tool. Proteomic analysis has received increasing attention in recent years as a method of distinguishing alterations in protein expression associated with malignancy. Conventional proteomic analysis techniques use gel electrophoresis, which is time and labor intensive and has limited resolution of proteins smaller than 10 kDa or few in number. ProteinChip surface-enhanced laser desorption and ionization time-of-flight mass spectrometry (SELDI-TOF-MS) (Ciphergen Biosystems, Inc, Fremont, California) detects low-molecular-weight proteins that are affinity bound to a protein chip array with high-throughput capability and high sensitivity and may identify protein biomarkers previously unsuspected of having a causal association because of small size and number.

Findings from previous studies suggest that SELDI-TOF-MS may facilitate detection of head and neck squamous cell carcinoma (HNSCC)–associated serum tumor biomarkers. It has been previously shown that proteomic analysis of serum protein profiles by SELDI-TOF-MS distinguishes patients with HNSCC from control subjects with a high degree of sensitivity and specificity. To date, there are no published reports evaluating the efficacy of SELDI-TOF-MS for HNSCC disease surveillance following definitive treatment, to our knowledge. We sought to determine the sensitivity and specificity of serum protein profiling using...
SELDI-TOF-MS for HNSCC detection following definitive treatment and to determine if posttreatment serum protein profiles differed based on disease status.

METHODS

PATIENTS

Serum samples were collected prospectively from patients evaluated by the Head and Neck Tumor Board at the Medical College of Georgia from April 2004 to August 2005, through an institutional review board–approved protocol. Clinical data were retrospectively reviewed in compliance with the Health Insurance Portability and Accountability Act. All patients gave informed consent.

SERUM SAMPLES

Serum samples were centrifuged, and the serum was aliquoted into 500-µL aliquots and frozen at −80°C until SELDI analysis. A quality control serum was used to assure that the sample processing and SELDI instrument were performing adequately.

SELDI PROTEIN PROFILING

The SELDI-TOF-MS was performed on serum samples to identify protein peaks in the range of 0 to 100 kDa. Serum samples were processed robotically using an automated workstation (Biomek 1000; Beckman Coulter, Inc, Fullerton, California) to increase the degree of reproducibility. A copper-treated chip array (IMAC-3; Ciphergen Biosystems, Inc) was used for SELDI analysis as previously described with modification. Triplicate runs were performed for each serum sample with the random placement of each sample in a 96-well bioprocessor format. Briefly, serum samples were prepared for SELDI analysis by vortexing 20 µL of serum with 30 µL of 8M urea with 1% CHAP (3-[(3-cholamidopropyl) dimethylammonium]-1-propanesulfonic acid) in phosphate-buffered saline (PBS) at 4°C for 10 minutes. Next, 100 µL of 1M urea with 0.125% CHAP was added to the serum mixture and vortexed briefly; PBS was added to make a 1:5 dilution of the serum mixture, which was then added to the ProteinChip array. After 30 minutes' incubation at room temperature, the protein chips were washed with PBS and air dried. Next, 1 µL of saturated sinapinic acid solution in 0.5% trifluoroacetic acid and 50% acetonitrile was applied to each array twice, allowing the array to dry between each application. The SELDI instrument (Ciphergen Protein Biology System IIC, Ciphergen Biosystems, Inc) was used with an autoloader, which increases the high throughput tremendously. The protein chips were assayed with a laser intensity of 180 and a sensitivity of 8. A total of 192 shots were collected and averaged for each sample. The all-in-1 peptide molecular mass standard (Ciphergen Biosystems, Inc) was used to generate a peptide standard spectrum for mass accuracy calibration.
SELDI-TOF-MS ANALYSIS

All triplicate spectra were compiled after the completion of the SELDI assay. Mass calibration was performed using the all-in-1 peptide standard spectrum. The default background subtraction was applied, and the peak intensities were normalized using the total ion current from a mass charge of 1000 to 100 000 Da. A biomarker detection software package (Ciphergen Biomarker Wizards, Ciphergen Biosystems, Inc) was used to autodetect protein peaks (Figure 1). Protein peaks were selected based on a first pass of signal-noise ratio of 3 and a minimum peak threshold of 20% of all spectra. This process was completed with a second pass of peak selection at 0.2% of the mass window, and the estimated peaks were added. These selected protein peaks were averaged as clusters and were exported to a commercially available software package (Biomarker Patterns, Ciphergen Biosystems, Inc) for further classification analysis.

CLASSIFICATION AND REGRESSION TREE ANALYSIS

Classification and regression tree analysis (CART) was performed using the common protein peaks identified by SELDI as previously described. A decision classification tree algorithm was generated based on the identification of protein peaks differentially expressed between HNSCC and control samples. CART splits these data into 2 groups or nodes by sequentially separating samples by rules based on the presence or absence of a peak until terminal nodes are reached. An optimal classification tree is then built, and 10-fold cross-validation is used to estimate the error rate of this tree. Ten-fold cross-validation randomly splits these data into 10 partitions, 9 of which are used for training; the remaining data set is used as a pseudotest sample to validate and test the classification tree. This validation process is performed 10 times. This technique allows use of the full complement of patients to build a classification tree with predictive value when the sample size is small. The $P$ value of each cluster was calculated using nonparametric analysis, which indicates the discriminate power of each cluster between groups.

RESULTS

PATIENT CHARACTERISTICS

Serum samples were collected longitudinally from 32 patients with HNSCC. Samples (mean, 3 [range, 2-6] samples per patient) were collected at 3-month intervals following treatment during a 24-month follow-up period. Ninety-three serum samples were analyzed. The mean age of patients with HNSCC was 58.9 years (age range, 39-85 years). The oral cavity was the most common primary site (13 patients), followed by the larynx (12 patients), oropharynx (5 patients), hypopharynx (1 patient), and nasopharynx (1 patient) (Table). Most patients (65.6%; 21 patients) had HNSCC stage III or IV disease. Patients with HNSCC were equally distributed with respect to primary tumor size. Surgical treatment was used in 20 patients (62.5%), and radiation therapy was used in conjunction with surgery or chemotherapy in 25 patients (78.1%). The mean follow-up was 17.8 months (range, 10-33 months). Recurrent disease developed in 10 patients (31.3%) following definitive treatment.

ANALYSIS OF PRETREATMENT AND POSTTREATMENT SAMPLES

Peak detection software (Ciphergen Biomarker Wizards) was used for peak detection in this study. After analyzing 350 spectra, it resolved 392 clusters in the range of 2 to 100 kDa. The $P$ value was calculated for each cluster to determine the importance of each cluster in comparison of the patients with HNSCC and healthy control subjects. These clusters were used in the subsequent classification analysis. Commercially available software was used for the classification analysis (Ciphergen Biomarker Pattern Software, Ciphergen Biosystems, Inc). This is a tool for tree-structured data analysis that is derived from CART. CART analysis is a nonparametric regression method based on the recursive partitioning method. The classification analysis results in a decision tree based on statistically significant differences in peak intensity that correctly classifies samples based on the variable under study, as previously described.

The small sample size precluded separation of training and blinded test sets. The whole sample set was used as a training set, and then a 10-fold validation was performed on samples as a test set. This 10-fold validation test correctly classified patients by treatment status (pretreatment or posttreatment) with 71.8% sensitivity and 65.6% specificity (Figure 2). Compared with pretreat-
Serum proteomic analysis in patients with HNSCC using SELDI-TOF-MS following treatment seems to be useful in identifying differences in the serum proteome based on response to treatment after 6 months. Proteomic differences were not apparent in samples obtained less than 6 months following treatment, suggesting that changes in the serum proteome that distinguish responders from nonresponders are not readily apparent immediately after treatment. Serum samples from patients who were disease free at 6 months following treatment demonstrated overexpression of a protein peak at 6495 Da, while samples from patients with recurrent disease showed statistically significant underexpression of a protein peak at 4493 Da. These distinct differences in peak expression based on disease status suggest that these low-molecular-weight proteins potentially contain disease-specific information and that changes in expression patterns may be disease specific.9

**COMMENT**

Figure 2. Diagram of biomarker pattern analysis in the classification of head and neck squamous cell carcinoma by treatment status as pretreatment (in blue) and posttreatment (in red). Each node represents a splitting rule where the samples are split into 2 daughter nodes. Each node also displays the peak mass (M), the cutoff intensity level (I), the number of samples, and the composition of the samples. Each terminal node is classified as pretreatment (pre) or posttreatment (post) based on the majority population in that terminal node.

Figure 3. Representative spectra of matched pretreatment (left column) and 6-month posttreatment (right column) samples from 4 disease-free patients with head and neck squamous cell carcinoma. Serum samples from patients who were disease free at 6 months or longer following treatment differed from matched pretreatment samples by the overexpression of a protein peak at 6495 Da (red tick).

RESPONSE TO TREATMENT

Posttreatment samples were further classified as disease free or recurrence on the basis of response to definitive treatment. Samples collected at 3 months following treatment did not statistically significantly differ from pretreatment samples by disease status. Serum samples from patients who were disease free at 6 months or longer following treatment differed from matched pretreatment samples by the overexpression of a protein peak at 6495 Da (Figure 3), while serum samples from patients with recurrent disease differed from matched pretreatment samples by the underexpression of a protein peak at 4493 Da (Figure 4).
Previous proteomic efforts in HNSCC have focused on detection of tumor-associated serum biomarkers in patients with untreated disease compared with controls through pattern recognition associated with the disease state rather than peptide identification.2-5 The large amounts of data generated by mass spectrometry allow identification of discriminatory patterns of protein expression between cancer and control specimens. The identification of differentially expressed protein peaks by SELDI-TOF-MS is useful for group classification and has the potential to identify protein biomarkers of small size and number.2-5,10-12 Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) resolves proteins larger than 20 kDa, and 2-dimensional gel electrophoresis is labor and time intensive, can be difficult to reproduce, and has limited resolution of proteins with molecular weights less than 10 kDa. SELDI-TOF-MS allows identification of low-molecular-weight proteins less than 20 kDa and yields a rich number of small proteins whose profiles have been used to classify the presence or absence of disease from different tumor types with high sensitivity and specificity.2,5,6,10-12 However, to our knowledge, the disease specificity of differentially expressed serum proteomic patterns has not been established,13 reproducibility varies based on methodological differences between investigative groups, and no putative biomarker corresponding with MS-based protein profiles in patients with HNSCC has been identified to date.

It has recently been suggested that low-molecular-weight proteins in a patient’s serum represent products of enzymatic breakdown generated after blood collection.5,14,17 Such protein fragments may result from the activity of disease-specific proteinases that arise from the tumor or within the microenvironment of the tumor. As a result, low-molecular-weight proteins serve as an “indirect snapshot” of the enzymatic activity of tumor cells and may serve as surrogate markers for detection and classification of disease.9 The increased expression of a low-molecular-weight protein by disease-free patients may represent a by-product of an increase in protein kinase inhibitor activity, while underexpression of these is associated with loss of control of the cell cycle and increased tumor cell proliferation through increased protein kinase activity. Therefore, the results of SELDI-TOF-MS analysis in this study may directly reflect differences in enzymatic activity based on disease status, mirroring changes in enzymatic activity that occur as a result of the presence or absence of disease. The comparison of pretreatment samples with matched posttreatment samples from patients based on disease status may more accurately identify proteomic differences associated with the disease state by reducing the amount of background noise or nonspecific epiphenomena (phenotypic variability) introduced when samples from patients with cancer are compared with healthy control samples.

Alternately, proteomic differences could reflect differences in risk factors or treatment effects. Healthy smokers have been shown to exhibit distinct differences in protein profiles that distinguish these from nonsmoking healthy control subjects and patients with HNSCC.7 The small number of patients who did not smoke in this study did not allow for stratification of specimens by risk factors, and alcohol abuse was not specifically examined for this small cohort. Most patients (78.1%) received radiation therapy as part of their treatment, which could potentially induce treatment-specific proteomic changes related to inflammation. However, changes specifically due to treatment could be expected to diminish with time, and in fact proteomic differences between responders and nonresponders based on disease status did not emerge until 6 months following the completion of treatment. This pilot study was designed to answer the question of whether proteomic differences exist between responders and nonresponders. Further studies with larger numbers of patients are required to allow stratification by risk factors and treatment modalities to determine if proteomic differences are truly disease specific, which may also be illuminated by identification of differentially expressed peptides that may shed light on possible mechanisms for the differences observed.

Various HNSCC biomarker candidates have been proposed based on proteomic analysis of cancer and con-
trol surgical specimens. C-reactive protein, creatine kinase, galecin-7, heat shock proteins 27 and 60, glutathione S-transferase, cytokeratin 6β, and others have been found to be up-regulated in oral carcinoma, while annexin 1, heat shock protein 20, and myosin show decreased expression in oral carcinoma.\textsuperscript{16-18} The molecular weights of these putative biomarkers are larger than 10 kDa and do not correlate with the mass of the differentially expressed proteins between responders and non-responders in this study, supporting the hypothesis that the low-molecular-weight proteins identified may represent products of the activity of disease-specific proteinases. Identification of the peptide sequences of these differentially expressed small-molecular-weight proteins, as well as identification of larger mass proteins by 2-dimensional gel electrophoresis, is required to test this hypothesis and is in progress.

CONCLUSIONS

Proteomic analysis of serum protein profiles distinguishes pretreatment and posttreatment samples from patients with HNSCC with a high degree of sensitivity and specificity. Differences in the serum proteome that distinguish responders from nonresponders were undetectable in the first 6 months following treatment. After 6 months, serum protein profiles seem to have distinct differences in peak expression based on disease status, with overexpression of a 6495-Da protein peak in disease-free patients and underexpression of a 4493-Da protein peak in patients with recurrence. These distinct differences in peak expression based on disease status suggest that these low-molecular-weight proteins potentially contain disease-specific information. Further investigation of the clinical usefulness of this technology in HNSCC detection and surveillance is warranted.

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