Detection of Papillary Thyroid Carcinoma With Serum Protein Profile Analysis

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Objective: To determine the sensitivity and specificity of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) for papillary thyroid carcinoma (PTC) detection.

Design: The SELDI-TOF-MS protein profiles of patients with PTC, patients with benign nodular disease (BND), and healthy controls were analyzed to determine the sensitivity and specificity of SELDI-TOF-MS assay for PTC detection. Data analysis was performed to process the spectral data and classify the disease status of the patients.

Setting: Academic tertiary care hospital.

Patients: Serum samples were collected prospectively from 7 patients with PTC, 8 patients with BND, and 7 healthy control volunteers.

Intervention: All patients diagnosed as having PTC or BND underwent thyroidectomy from October 21, 2004, to January 31, 2006.

Main Outcome Measures: Twenty-two serum samples were analyzed.

Results: Most protein peaks resolved by the SELDI-TOF-MS assay were in the range of 1 to 20 kDa. Classification tree analysis based on peak expression distinguished patients with PTC from those with BND with 85.7% sensitivity and 100% specificity. Serum samples from patients with PTC differed most significantly from those of patients with BND by the underexpression of a protein peak at 11 101 Da.

Conclusions: This pilot study demonstrates that proteomic analysis of serum protein profiles distinguishes patients with PTC from patients with BND with a high degree of sensitivity and specificity. Further investigation into the clinical utility of this technology in PTC biomarker detection and surveillance is warranted.


Thyroid carcinoma is one of the most common carcinomas of the head and neck, with 33 550 new cases annually in the United States. Thyroid gland nodularity is relatively common, affecting 50% of the population in autopsy and ultrasonography surveys. The risk of malignancy in thyroid nodules is approximately 5%. Fine-needle aspiration biopsy is currently the most sensitive and specific test for distinguishing benign from malignant thyroid nodules; however, fine-needle aspiration biopsy is nondiagnostic in up to 13% of cases, ultimately requiring surgical biopsy via thyroidectomy for diagnosis in these cases. Thyroglobulin levels were shown to be a useful prognostic indicator of disease recurrence in patients with a history of well-differentiated thyroid malignancy but may be falsely elevated in patients previously treated with partial thyroidectomy and undetectable in a significant proportion of patients with residual disease or in patients with antithyroglobulin antibodies. These diagnostic limitations have driven the search for diagnostic markers to improve detection of suspected thyroid neoplasia and reduce the need for surgery.

Several potential tumor biomarkers associated with well-differentiated thyroid carcinoma have been identified, but to date, none have been shown to be consistently expressed or useful as a diagnostic, prognostic, or screening marker. We and others have previously shown that proteomic analysis of serum protein profiles by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) distinguishes patients with head and neck squamous cell carcinoma from controls with a high de-
degree of sensitivity and specificity. Rather than relying on one or several individual protein alterations, which may vary in degree of expression among patients, the pattern of protein expression encompasses the effect of a variety of alterations and appears to be specific and sensitive in identifying patients with head and neck squamous cell carcinoma. To our knowledge, no published reports have evaluated the efficacy of SELDI-TOF-MS in patients with well-differentiated thyroid carcinoma. We hypothesized that SELDI-TOF-MS may have utility as a diagnostic tool in the workup of suspected thyroid carcinoma, and sought to determine the sensitivity and specificity of serum protein profiling using SELDI-TOF-MS in distinguishing patients with well-differentiated thyroid carcinoma from healthy controls.

**METHODS**

Serum samples from patients with papillary thyroid carcinoma (PTC), patients with benign nodular disease (BND), and healthy controls were collected prospectively from patients who presented to the Department of Otolaryngology–Head and Neck Surgery at the Medical College of Georgia from October 21, 2004, to January 31, 2006. Clinical data were retrospectively reviewed in compliance with the Health Insurance Portability and Accountability Act. All patients gave informed consent.

**SERUM SAMPLES**

Samples were centrifuged, divided into 500-µL aliquots, and frozen at −80°C before SELDI-TOF-MS analysis. Accuracy of the SELDI-TOF-MS sample processing was ensured with the use of a quality control serum.

**SELDI-TOF-MS PROTEIN PROFILING**

Serum samples were processed as previously described. An automated workstation (Biomek 1000; Beckman Coulter Inc, Fullerton, California) was used to process serum samples, increasing the degree of reproducibility. The SELDI-TOF-MS analysis was performed on a copper-treated chip array (IMAC-3 ProteinChip System; Ciphergen Biosystems Inc, Fremont, California). Each serum sample was analyzed in triplicate with the random placement of each sample in a 96-well bioprocessor format. Preparation of samples for SELDI-TOF-MS analysis was completed by vortexing 20 µL of serum with 30 µL of 8M urea with 1% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid) in phosphate-buffered saline at 4°C for 10 minutes. A total of 100 µL of 1M urea with 0.125% CHAPS was then added to the serum mixture and vortexed. Phosphate-buffered saline was next added to make a 1.5 dilution of the serum mixture, which was added to the protein chip array. After 30 minutes of incubation at room temperature, the protein chips were washed with phosphate-buffered saline and dried. A total of 1 µL of saturated sinapinic acid solution in 0.5% trifluoroacetic acid and 50% acetonitrile was then applied to each array twice, allowing the array to dry between each application. The SELDI-TOF-MS instrument (Ciphergen Protein Biology System IIC; Ciphergen Biosystems Inc) was used with an autoloader to ensure high throughput. 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-one peptide molecular mass standard (Ciphergen Biosystems Inc) was used to generate a peptide standard spectrum for mass accuracy calibration.

**SELDI-TOF-MS ANALYSIS**

Before protein peak analysis, the all-in-one peptide standard spectrum was used for mass calibration, 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. Triplicate spectra collected from SELDI-TOF-MS analysis were then analyzed for protein peaks using the biomarker detection software package (Ciphergen Biomarker Wizards; Ciphergen Biosystems Inc). The peaks were selected based on a first pass of a signal-to-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 exported to a commercially available software package (Biomarker Patterns; Ciphergen Biosystems Inc) for further classification analysis.

### CLASSIFICATION AND REGRESSION TREE ANALYSIS

Details of classification and regression tree analysis with common protein peaks have been previously described. A classification tree algorithm was based on the identification of protein peaks differentially expressed among the PTC, BND, and control samples. The classification tree splits data into 2 groups by separating samples based on the presence or absence and the expression level of a protein peak. The regression tree analysis is continued until further splitting has no gain in data classification. This is called a learning or training data set. Once the optimal classification tree is built, 10-fold cross-validation is used to test the classification accuracy of the tree, which serves as a test data set. Cross-validation involves the random splitting of data into 10 separate partitions, with 1 set used as a test sample to validate and test the classification tree. Validation is completed 10 times, allowing for predictive value of the classification tree with small sample size. The P value of each cluster was calculated using the nonparametric analysis, which indicates the discriminate power of each cluster among the groups.

### RESULTS

**PATIENT CHARACTERISTICS**

Serum samples from 7 patients with PTC, 8 patients with BND, and 7 healthy volunteers (controls) were analyzed. Healthy volunteers were age and sex matched before SELDI-TOF-MS analysis. All patients diagnosed as having PTC or BND underwent thyroidectomy from October 21, 2004, to January 31, 2006. Of the 7 patients diagnosed as having PTC, all were women, with a mean age of 48 years (age range, 33-60 years). All of the patients diagnosed as having BND were women, with a mean age of 50 years (age range, 33-59 years). One of the patients with BND had a smoking history; none of the patients with PTC had a smoking history.

### DETECTION OF PTC AND BND

The protein peaks resolved by the SELDI-TOF-MS assay were in the range of 0 to 100 kDa. After background subtraction, mass calibration, and normalization, more than 500 protein peaks were identified. Most protein peaks identified and used in the data analysis were in the range of 1 to 20 kDa.
The classification tree analysis generated an optimal tree that separated the 2 groups with the best classification rate, and then a 10-fold validation procedure was performed 10 times to validate the optimal tree. This procedure served as a test data set because of the limitation of a small sample size.

Figure 1 shows the optimal classification tree that resulted from the training set analysis with an underexpressed protein peak with a mass of 11 101 Da, which separated the PTC and BND samples with 100% sensitivity and specificity (Table). The subsequent 10-fold cross-validation analysis (test set) distinguished patients with PTC from those with BND with 85.7% sensitivity and 100% specificity (Table). As shown in the original spectral data, this 11 101-Da protein peak was present in both sample groups with differential expression (Figure 2).

Compared with healthy controls, SELDI-TOF-MS was able to correctly classify patients with BND with 100% sensitivity and specificity in the training set and with 85.7% sensitivity and 87.5% specificity in the test set, as well as distinguish patients with PTC from controls with 100% sensitivity and specificity in the training set and 71.4% sensitivity and specificity in the test set (Table). Patients with BND differed from healthy controls most significantly by the overexpression of a protein peak at 2391 Da (Figure 3). An underexpressed protein peak at 2528 Da most significantly separated patients with PTC from healthy controls (Figure 4).

![Figure 1](image1.png)

**Figure 1.** Diagram of biomarker pattern analysis in the classification of benign nodular disease (BND) in red and papillary thyroid carcinoma (PTC) in blue. 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 (n), and the composition of the samples. Each terminal node is classified as either BND or PTC based on the majority population in that terminal node.

<table>
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<th>Specificity, %</th>
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</table>

**Table. Sensitivity and Specificity of Comparisons Among Groups**

Abbreviations: BND, benign nodular disease; PTC, papillary thyroid carcinoma.

The classification tree analysis generated an optimal tree that separated the 2 groups with the best classification rate, and then a 10-fold validation procedure was performed 10 times to validate the optimal tree. This procedure served as a test data set because of the limitation of a small sample size. **Figure 1** shows the optimal classification tree that resulted from the training set analysis with an underexpressed protein peak with a mass of 11 101 Da, which separated the PTC and BND samples with 100% sensitivity and specificity (Table). As shown in the original spectral data, this 11 101-Da protein peak was present in both sample groups with differential expression (**Figure 2**).

Compared with healthy controls, SELDI-TOF-MS was able to correctly classify patients with BND with 100% sensitivity and specificity in the training set and with 85.7% sensitivity and 87.5% specificity in the test set, as well as distinguish patients with PTC from controls with 100% sensitivity and specificity in the training set and 71.4% sensitivity and specificity in the test set (Table). Patients with BND differed from healthy controls most significantly by the overexpression of a protein peak at 2391 Da (**Figure 3**). An underexpressed protein peak at 2528 Da most significantly separated patients with PTC from healthy controls (**Figure 4**).

**COMMENT**

Serum proteomic analysis in patients with PTC and those with BND using SELDI-TOF-MS appears to have utility in identifying differences in the serum proteome based on dis-
ease status in this pilot study. Serum protein profiling using SELDI-TOF-MS identified a primary discriminant protein peak at 11 kDa that separated patients with PTC from those with BND with 87.5% sensitivity and 100% specificity. The distinct differences in peak expression based on disease status suggest that these low-molecular-weight proteins potentially contain disease-specific information, and changes in expression patterns may be disease specific.9

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has traditionally been used to identify differences in protein expression in serum, saliva, or tissue specimens, with identified proteins subsequently excised from the gel and subjected to peptide mapping analysis by MS for the identification of proteins.6 This technique is labor and time intensive, can be difficult to reproduce, and has limited resolution of proteins with molecular weights less than 10 000 Da. The 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 a variety of tumor types with high sensitivity and specificity.6,7,10-13 However, the disease specificity of differentially expressed serum proteomic patterns has not been established,14 and identification of putative biomarkers corresponding to MS-based protein profiles remains an area of active investigation.

A variety of thyroid carcinoma biomarker candidates have been proposed based on proteomic analysis of cancer and control surgical specimens. Several genes were found to be differentially expressed in thyroid cancer specimens compared with control samples from patients with benign goiters or normal thyroid glands that result in significant changes in protein expression.7 Well-characterized genes that have been validated in more than 1 follow-up study and confirmed at both the RNA and protein levels include MET, TFF3, SERPINA1, TIMP1, FN1, and TPO.3 In particular, MET protein expression may have both diagnostic and prognostic significance in patients with PTC. At the protein level, several of the S-100 proteins have been associated with increased metastasis, decreased survival, and tumor progression. One of the members of the S-100-alpha family, S-100-A4, is overexpressed in PTC and has been associated with increased invasion and metastasis.15-17 This protein is secreted in low levels by normal fibroblasts and is secreted in higher levels when fibroblasts are cocultured with tumor cells.18,19 Endothelin 1 is another candidate biomarker that is suspected to play an important role in PTC invasion, metastasis, and tumor progression.20-22 It is unknown if alterations in the amounts of these putative biomarkers result in measurable differences in serum protein profiles.

It has recently been suggested that low-molecular-weight proteins in a patient’s serum represent products of enzymatic breakdown generated after blood collection.6,23,24 Such protein fragments may result from the activity of disease-specific proteinases that arise from the tumor itself or within the tumor microenvironment. 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 Thus, 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. Identification of the peptide sequences of the low-molecular-weight proteins identified in this study by 2D-PAGE is required to determine if there is a relationship between putative proposed biomarkers and the serum protein alterations identified by SELDI-TOF-MS or if detectable differences are a result of the activity of disease-specific proteinases. The results of this pilot study suggest that SELDI-TOF-MS may have utility as a diagnostic tool in the workup of suspected well-differentiated thyroid carcinoma. Furthermore, this technology may allow identification of specific tumor-associated protein biomarkers through identification of the peptide sequences of these differentially expressed small-molecular-weight proteins, as well as identification of larger mass proteins by 2D-PAGE, and is in progress. Identification of differentially expressed proteins may shed light on possible mechanisms for the differences observed and improve our understanding of the molecular and genetic basis of thyroid cancer.

The results of this pilot study demonstrate that proteomic analysis of serum protein profiles distinguishes patients with PTC from patients with BND with a high degree of sensitivity and specificity. Serum protein profiles appear to have distinct differences in peak expression based on disease status, with underexpression of an 11 101-Da protein peak in patients with PTC compared with patients with BND. These distinct differences in peak expression based on disease status suggest that these low-molecular-weight proteins potentially contain disease-specific information and have potential for use as a screening test for occult PTC in patients with nodular thyroid disease. Further investigation of the clinical utility of this technology in PTC detection and surveillance is warranted.

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Author Contributions: Drs Moretz and Adam had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Moretz, Gourin, Terris, Weinberger, and Adam. Acquisition of data: Moretz, Gourin, Xia, Liu, Chin, and Adam. Analysis and interpretation of data: Moretz, Gourin, and Adam. Drafting of the manuscript: Moretz, Gourin, Xia, Liu, Weinberger, and Adam. Critical revision of the manuscript for important intellectual content: Gourin, Terris, and Adam. Statistical analysis: Moretz and Gourin. Obtained funding: Adam. Administrative, technical, and material support: Gourin, Terris, Xia, Liu, Weinberger, and Chin. Study supervision: Gourin, Terris, and Adam.

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REFERENCES