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Quantitative and Qualitative Differences in Protein Expression Between Papillary Thyroid Carcinoma and Normal Thyroid Tissue†

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Abstract

In order to better understand basic mechanisms of tumor development and identify potential new biomarkers, we have performed difference gel electrophoresis (DIGE) and peptide mass fingerprinting on pooled protein extracts from patients with papillary thyroid carcinoma (PTC) compared with matched normal thyroid tissue. Image analysis of DIGE gels comparing PTC and matched normal thyroid tissue protein indicated that 25% of the protein spots were differentially expressed at a 2.5-fold cutoff and 35% at two-fold. Comparison between two different pools of protein from normal thyroid tissues revealed differential protein expression of only 4% at 2.5-fold and 6% at two-fold cutoff. One hundred ninety-two protein spots were identified by MALDI-TOFMS, representing 90 distinct proteins. Excluding albumin, globins and thyroglobulin, imaging software determined 31 proteins to be differentially expressed at the two-fold (or greater) level. Individual gel comparisons (PTC vs. matched normal) from five patients established that 15/31 (48%) of these proteins exhibited statistically significant differential expression. Previously identified molecular markers in this group of proteins include cathepsin B, cytokeratin 19, and galectin-3. Novel differentially expressed proteins include S100A6, moesin, HSP70 (BiP), peroxiredoxin 2, protein phosphatase 2, selenium binding protein 1, vitamin D binding protein, and proteins involved in mitochondrial function. The use of two-dimensional gel electrophoresis (2DGE) revealed a significantly altered protein mass and/or pI in 10%–15% of proteins, suggesting alternatively spliced forms and other posttranslational modification of proteins revealed by this approach. We confirmed S100A6 as a potentially useful biomarker using immunohistochemical analysis (85% sensitivity and 69% specificity for distinguishing benign from malignant thyroid neoplasms). In summary, proteomic analysis of PTC using DIGE and mass spectrometry has confirmed several known biomarkers, uncovered novel potential biomarkers, and provided insights into global pathophysiologic changes in PTC. Many of the differences observed would not have been detected by genomic or other proteomic approaches

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Keywords

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INTRODUCTION

Thyroid neoplasms are common. Approximately 5%–10% of American adults have palpable thyroid nodules, and nearly 50% of older adults have thyroid nodules detected by ultrasonography [1]. Fortunately, the majority of these are benign, but approximately 5%–7% are malignant. Thyroid cancer can be confirmed or excluded in many patients by a fine-needle aspiration biopsy (FNAB) followed by standard cytopathologic diagnosis. However, approximately 20%–30% of patients undergoing FNAB have results that are inconclusive or indeterminate which leads to unnecessary surgical intervention in 80% of patients with benign nodules [2,3]. Approximately 20%–30% of patients with differentiated thyroid cancer have recurrent or persistent disease requiring subsequent therapy and more aggressive monitoring, and nearly 1500 people die each year from thyroid cancer [4,5]. Molecular markers capable of distinguishing benign from malignant thyroid nodules, predicting outcome or identifying molecular targets for therapy would greatly advance preoperative diagnostic decision making, perioperative staging, or provide rational strategies for specific therapy in patients with thyroid neoplasms and cancer.

Underlying the practical need to manage and treat patients with thyroid neoplasms is our desire to understand the development of thyroid neoplasms and carcinoma. The primary thyroid epithelial cell, the thyrocyte, can develop into benign thyroid neoplasms and three major types of thyroid cancer [6,7]. Papillary thyroid cancer (PTC) is the most common endocrine malignancy, and fortunately most patients diagnosed with this type of thyroid cancer have an excellent prognosis. PTC, however, often spreads via the lymphatics and many patients are plagued with persistent or recurrent lymph node disease in the neck. Follicular thyroid cancer (FTC) is more common in older individuals and is histologically distinct from PTC, although both are derived from the same cell type. FTC generally spreads via a hematogenous route and pulmonary/bone metastases are more common. Anaplastic thyroid cancer (ATC) is the least common and most aggressive type of thyroid cancer (average survival of only 4–6 months).

Over the past 10–15 years several candidate genes have been studied in the development of the different types of thyroid cancer (TSH receptor, RET/PTC, Ras, BRAF, p53, among others) [8–10]. These genes have been evaluated in thyroid cancer based on what was known for other cancers or based on what was expected from normal cell signaling (proto-oncogenes). Recent studies using gene array technology have attempted to use a hypothesis-generating approach to understand thyroid neoplasms [11–13], but these studies rely on mRNA differences which may or may not relate to significant biologic processes. mRNA differences do not necessarily reflect differences at the protein level, and these RNA-based studies fail to identify protein variants and post-translational modifications that may affect the biology of tumors.

We have employed proteomic methods to gain a better understanding of thyroid cancer and the unique alterations that are characteristic of PTC. We have identified novel differentially-expressed proteins that may provide insights into diagnosis, prognosis, and therapeutic targets for patients with thyroid neoplasms, as well as insights into the underlying pathophysiology of thyroid tumor development and progression.

MATERIALS AND METHODS

Tissue Samples

Tumor and matched normal thyroid tissue was collected from five patients undergoing surgery for PTC, and normal tissue from three patients with benign follicular adenoma (FA) was used as a normal control comparison. Specimens (100–500 mg) from each patient, verified by histopathology, were snap frozen after confirmation of tissue type. Tissue samples were collected from women aged 25–50 years with no evidence of chronic lymphocytic thyroiditis in an attempt to minimize differences due to gender, menopausal status, and autoimmune thyroid disease. Unaffected (normal) thyroid tissue was defined as tissue adjacent to the site of the lesion with no histologic signs of abnormal pathology. Normal tissue was collected from each patient undergoing surgery for PTC or benign FA so that the biologic variability in protein expression in a region proximate to pathology could be assessed. Whole-cell protein extracts were prepared from individual patients having three defined tissue types: (1) typical or ordinary PTC, (2), matched normal tissue, and (3) normal tissue from patients with benign adenomas (secondary control).

Extraction and Precipitation of Proteins

In all cases (PTC, PTC-normal, FA-normal) each tissue sample (50 mg) was homogenized in 150 mM NaCl with 50 mM Tris (pH 7.5) with 0.3% SDS and protease inhibitors (CompleteTM protease inhibitor cocktail from Roche 2× recommended concentration). The extracts were treated with 200 U/mL of DNase I and 20 U/mL of RNase A (Sigma-Aldrich, St. Louis, MO) and proteins were precipitated using a methanol/chloroform extraction protocol [14]. Dried protein pellets from the methanol/chloroform precipitation were allowed to dissolve overnight in 7 M urea, 2 M thiourea, 4% w/v CHAPS, and each was diluted to the same protein concentration after protein assay [15]. The protein assay was performed on an aliquot diluted 50-fold with water. Pools were prepared by combining equal amounts of protein from each sample.

DIGE Covalent Labeling With Cyanine Dyes (CyDyes)

A volume corresponding to 50 µg of total protein was removed from each pool (PTC, PTC-normal, FA-normal) for covalent modification with one of the two CyDyes: either Cy3 or Cy5 (Amersham Biosciences, Piscataway, NJ). The pH was adjusted by addition of pH 8.5 Tris to a final concentration of 30 mM. The CyDye (1 µL, 400 pmol/µL in anhydrous dimethylformamide (Sigma-Aldrich)) was added to the protein and the reaction allowed to proceed (30 min, 4°C in the dark). Under these conditions approximately 1% of the lysine residues of the protein are covalently conjugated to the CyDyes [16]. The reaction was then quenched by the addition of 10 nmol of lysine. After the dye reactions were complete, the labeled samples were combined (e.g., one sample labeled with Cy3 and one sample labeled with Cy5) and an additional 450 µg of unlabeled protein from each of the two samples (1000 µg total) was added to facilitate protein identifications after electrophoresis. Pharmalytes (Amersham Biosciences), DTT, and saturated aqueous bromophenol blue (1 µL) were added. The samples were brought to a final volume of 450 µL with a composition of 7 M urea, 2 M thiourea, 4% w/v CHAPS, 2% broad range Pharmalytes (3–10 NL), and 2% DTT.

Gel analysis was performed for each individual PTC/matched normal thyroid tissue using 50 µg of Cy3 and 50 µg of Cy5 labeled protein as well as 50 µg of combined (PTC and normal pool) protein for Cy2 labeling to be used as an internal standard (150 µg of total protein). The labeling for individual PTC/matched normal comparisons was performed with 200 pmol of each CyDye.

2D Gel Electrophoresis

After resuspension in the rehydration buffer, protein samples were passively rehydrated into 24-cm immobilized pH gradient strips (IPG 3–10 NL, Amersham Biosciences) for > 16 h and then focused (IPGPhor system, Amersham Biosciences) for 133 000 V-h (66 000 V-h for 150 µg of protein). Cysteine side-chains were reduced and alkylated by incubating the focused strips (10 min, room temperature) in equilibration solution (6 M urea, 100 mM Tris pH 8.8, 30% glycerol, 2% SDS, 0.25% saturated aqueous bromophenol blue) containing 0.5% DTT, followed by incubation in equilibration solution with 4.5% iodoacetamide (10 min, room temperature). Second-dimension separation was performed at 2 W/gel at 25°C (Ettan DALT12 vertical system, Amersham Biosciences) using hand-cast 12.5% gels. Voltage and current were continuously monitored throughout all runs for quality control.

Scanning of DIGE Labeled Images

Gels were scanned on a Typhoon 9400 Variable Mode Laser Imager (Amersham Biosciences) set to 100 µm of resolution. Laser and filter settings were: Cy3, excitation 532 nm, emission 580 nm, bandpass 30 nm; Cy5, excitation 633 nm, emission 670 nm, bandpass 30 nm; and Cy2, excitation 468 nm, emission 520 nm, and bandpass 40 nm. The preparative gel used for protein spot excision was stained with G-250 Coomassie Blue to assess the mass shift between CyDye labeled and unlabeled protein that occurs at low molecular weight [16,17].

Image Analysis

DeCyder software (v. 5.0, Amersham Biosciences) was used for spot detection and relative quantification of proteins based on the fluorescence images. Peak volumes were measured for each protein spot in the two fluorescent channels (Cy3 and Cy5). Volume ratios were calculated to directly compare volumes of each protein spot in the normal and PTC protein pools. Proteins to be excised and identified were selected using the DeCyder differential in-gel analysis (DIA) Module. Individual difference gel electrophoresis (DIGE) gels were matched using the biological variation analysis (BVA) Module (Amersham Biosciences). For individual patient samples, pools of all samples were combined and labeled with Cy2 which was used as an internal standard to calculate standardized volume ratios in the BVA module. Protein spot volume ratios were compared using a Student's *t*-test (two-tailed) in the BVA module. The BVA module was also used to measure protein mass and pI. Selected proteins identified in these samples (e.g., actin, alpha glucosidase, glycer-aldehyde-3-phosphate dehydrogenase (GAPDH), S100A6, and beta globin) were used to generate a standard curve (cubic spline) in the BVA module to calculate the measured mass of the other proteins. Similarly, pI was calculated by reference to a standard curve (log linear) using other selected proteins (e.g., annexin 5, lactate dehydrogenase, translation elongation factor 1, calreticulin, and manganese superoxide dismutase). Predicted protein mass and pI were derived from the SwissProt database (<http://us.expasy.org/sprot/>) using the mature protein form (chain) when available.

Spot Picking and Enzymatic Digestion

Protein spot excision and in-gel enzymatic digestion were performed automatically by the Ettan Spot Handling Workstation (Amersham Biosciences). Spots were excised from the gel with a 2.0 mm of picking head, and the excised spots transferred to 96-well plates. After the picking was completed, the plates were transferred to the digester where the plugs were washed twice with 100 µL of 50 mM NH₄HCO₃/50% methanol for 5 min, once with 100 µL of 75% acetonitrile for 10 min, and once with 100 µL of 100% acetonitrile for 10 min. The gel plugs were dried at room temperature for 50 min, and then trypsin (Promega, San Luis Obispo, CA) was added to each well (10 µL, 10 µg/µL in 20 mM NH₄HCO₃), and digestion proceeded at room temperature for 16 h.

Protein Identification by Mass Spectrometry

All digests were analyzed by MALDI-TOFMS (Voyager DE-PRO, Applied Biosystems, Foster City, CA) as described previously and spectra were collected over the range m/z 500–5000 [18]. No cleanup of digests was done. Peptide mass fingerprints were internally calibrated to monoisotopic trypsin peaks (m/z = 515.33, 842.51, 1045.56, 2211.11). Spectra were processed using ProTS Data (Efecta Technologies, Steamboat Springs, CO) to generate a peak list that was submitted to Mascot (Matrix Science, Boston, MA) for database searching. Spectral processing included defining baseline, noise, and signal-to-noise ratio as well as mono-isotopic peak selection. A signal-to-noise ratio in ProTS data of >4 was required for inclusion in the peak list. Database searches were conducted using a human subset of the non-redundant protein database (NCBI, database version 2/19/2004 with 142 337 sequences and 46 880 922 residues). Other settings in ProTS included peak amplitude set at 100, peak width 250, and chemical noise factor 1.5. Key Mascot parameters included the following: peptide error tolerance was set at ± 100 ppm, fixed modification of carbamidomethylation of cysteine side chains, and trypsin selected as the enzyme for peptide cleavage with one missed cleavage accepted. Searches were not constrained by pI or molecular weight. Minimum requirements for positive protein identification were described previously [18], and peptide and protein assignments were made according to recently published guidelines [19].

Immunohistochemistry

Archival formalin-fixed tissue blocks of thyroid surgical specimens were retrieved from the Department of Pathology at the UCDHSC. Specimens were stripped of linked patient identifiers and the protocol was performed with authorization from the Colorado Multiple Institution Review Board. The tissue blocks were sectioned at 5 μ m, deparaffinized in xylene, and rehydrated. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide. Antigen retrieval was performed in 20 mM citrate buffer (pH 6.0) for 10 min in a Decloaking Chamber (Biocare Medical, Walnut Creek, CA). Immunohistochemistry was performed using a non-biotin-based immunoperoxidase detection system (ImmPRESS, Vector Laboratories, Burlingame, CA) on a DAKO Autostainer (DAKO, Carpinteria, CA). Following incubation with the primary antibody (S100A6, monoclonal antibody, Sigma) for 60 min at a dilution of 1:1000, sections were reacted with an immunoperoxidase detection system. Localization of staining was performed with 3,3'-diaminobenzidine tetrahydrochloride (DAB+, DAKO). The slides were finally counterstained with hematoxylin (DAKO) and coverslipped using a non-aqueous mounting medium. Purified mouse IgG1 (BD Biosciences, San Diego, CA) at an equivalent dilution to the primary antibody was used as a negative control. The intensity of staining was subjectively scored as faint (1+), moderate (2+), or strong (3+). Cases with a 2+ or greater intensity staining in 10% or more of the epithelial cells were scored as positive for S100A6 expression.

RESULTS

Sample Preparation and DIGE Analysis

Figure 1A shows a 2D gel (DIGE) comparing pooled normal thyroid protein from women with PTC ($n = 5$) and pooled normal thyroid protein from women with FA ($n = 3$). Protein spots that DeCyder determined to have $<$ two-fold difference in volume ratio between the two pools of normal thyroid tissue are indicated as yellow, those with $\geq$ two-fold higher in normal thyroid tissue protein from patients with PTC are red and for FA patients are green. Of the 1414 unique protein spots detected by DeCyder, only 89 (6.3%) were determined to have $\geq$ two-fold difference in volume ratio (23 increased and 66 decreased) by the software. This indicates a remarkably similar protein expression profile in these two pools of normal thyroid tissue from patients with different thyroid neoplasms. When the volume ratio threshold was set at 2.5-fold,

only 56 (3.9%) of the spots showed differential expression between the two pools of normal thyroid tissue.

Figure 1B shows the results comparing pooled protein from PTC tissue and pooled protein from matched normal thyroid tissue from five patients. Initial 2D image data analysis by DeCyder software detected a total of 2623 spots, and 783 were excluded by more stringent analysis criteria as either being outside the area of interest selected, extremely low intensity or dust particles (i.e., high slope) as described in the software instructions. In contrast to the normal–normal comparison (Figure 1A), DeCyder software assessed 628 (34.2%) of the remaining 1840 spots as $\geq$ two-fold different in volume ratio (128 increased and 500 decreased) between the normal and neoplastic thyroid tissue (PTC). None of these spots was the same as those of the normal–normal comparison. When the volume ratio threshold was set at 2.5-fold, 459 (25%) of the spots had differential protein expression between PTC and matched normal thyroid tissue. The 2D gel was repeated after labeling the two pools of protein with the opposite CyDye (i.e., a dye swap experiment) and the results were very similar. A linear regression analysis comparing volume ratios in the two dye swap gels showed a slope of 0.96 and $r^2 = 0.81$ (data not shown), which indicates good correlation of individual volume ratios between the gels. Figure 2 shows the pseudocolor image of a gel that represents a dye swap of the gel shown in Figure 1B with numbers labeling the spots that were picked and identified by mass spectrometry.

Protein Identification

Protein spots (289) were picked from the gel, digested with trypsin, and subjected to MALDI-TOFMS analysis. These spots were chosen for excision based on spot quality, spot volume/abundance, position on the gel, and proximity to other ‘picked’ spots (i.e., to avoid overlap). Analysis focused on 192 spots which returned valid identifications. Good quality spectra were obtained for both small (Figure 3) and large proteins (Figure 6 and 7, supplementary materials). The identification of proteins such as S100A6, a relatively small protein (10 kDa), was facilitated by careful technique, the use of robotics to prepare clean digests, and the use of advanced peak detection algorithms for extraction of peak lists. The proteins included in this analysis comprise 52% of the measurable protein content of the sample based on spot volume detection. A total of 90 distinct proteins were identified (Table in supplementary section). One hundred forty four identified spots represented 87 individual proteins when albumin, globin, and immunoglobulins were excluded. DeCyder analysis determined that 58 of these protein spots (40%) had a $\geq$ two-fold volume ratio difference between PTC and matched normal thyroid tissue. When the isoforms of thyroglobulin, a well-known marker of thyroid cancer, were excluded, 31 distinct protein spots with $\geq$ two-fold volume ratio difference were identified (Table 1). The abundance (column 3) represents the percent protein attributable to the individual spot (i.e., spot volume measured in DeCyder software) compared with the total volume of all spots (1840) detected in the gel. All abundance determinations are based on the normal thyroid tissue. The median abundance of all the detected proteins was 0.03% (range 0.003%–10.7%).

Two-dimensional gel electrophoresis (2DGE) provides experimentally determined mass and pI values for each protein (mass and pI are reported in columns 5 and 7 of Table 1). Three proteins were either above or below the proteins selected as internal mass standards, and these are reported as either $<$ or $>$ the highest/lowest standards. Of the proteins in Table 1 with a measurable mass, 23 (82%) were within 20% of their predicted molecular weight. Four proteins were larger than predicted and one was smaller (column 6, Δ mass, bold numbers). The larger than predicted proteins are likely glycosylated, while the smaller protein may be a splice variant or a cleavage product.

Individual DIGE Analysis

To determine whether the protein expression differences observed in the pooled protein comparisons (Table 1, column 4) were statistically significant, separate DIGE gels were run for each of the five patients comprising the pooled sample (Figure 2). For each subject, 50 µg of tumor protein (Cy3) and 50 µg of matched normal thyroid protein (Cy5) were run together on a single gel along with 50 µg of a pool of equal amounts of protein from all tumor and normal samples (Cy2 internal standard). The last two columns in Table 1 show the average volume ratio of each protein and the significance (*P*-value) of the expression differences. Fifteen distinct proteins reached statistical significance for differential expression based on the individual gel analyses (*italics*). Four examples of the individual gel analyses are shown in Figure 4. S100A6, HSP 70 (BiP), and peroxiredoxin 2 are all proteins with relatively high abundance (Table 1, column 3) that are significantly differentially expressed (PTC vs. normal), while one isoform of cathepsin B is not significantly different on individual gel analysis. The pooled analysis for this isoform of cathepsin B was most likely biased by the high level of expression in one normal sample with profound underexpression in the matching PTC tissue.

Immunohistochemistry

For validation we chose to study the most abundant protein (Table 1, column 3) with the largest volume ratio difference between PTC and matched normal thyroid tissue (Table 1, column 4 and 10), S100A6. Immunohistochemistry was performed on paraffin-embedded tissue from patients with benign and malignant thyroid tumors. Figure 5 shows immunostaining for S100A6 on three different tumor types and normal thyroid tissue. Staining intensity and distribution for the different tumor types are shown in Table 2. Any positive staining for S100A6 had an overall 95% sensitivity and 31% specificity to distinguish benign from malignant thyroid neoplasms. When the definition for positive staining was limited to more intense staining ($\geq 2+$), the overall sensitivity and specificity were 85% and 62%, respectively. The best predictive staining for S100A6 ($>2+$ intensity and $\geq 10\%$ – 50% of cells positive) had an overall sensitivity of 85% (93% for PTC and 71% for FTC) with a specificity of 69% to distinguish benign from malignant thyroid tumors. Using a chi-square (χ^2) analysis, S100A6 significantly identified malignant thyroid neoplasms.

DISCUSSION

Biomarkers are potentially useful tools for the diagnosis and prognosis of cancers and for the identification of novel therapeutic targets. Most biomarkers have been identified through a candidate approach (i.e., derived from basic biology and pathophysiology insights), but with recent advances in genomics and proteomics, biomarkers can now be identified by discovery (or hypothesis generating) strategies that are not limited by our existing knowledge of biology. Others have used genomic approaches to find biomarkers of thyroid neoplasms with some success [12,13,20–28], but there have been few attempts to use proteomics as a discovery tool in thyroid neoplasms [29,30].

An early proteomic study on PTC compared a membrane fraction between normal and PTC tissues [31]. A few protein spots appeared to be differentially expressed in PTC, but these were not identified. A more recent study included mass spectrometry and comparison of gels stained by Coomassie [30], which identified six protein spots overexpressed in PTC. Of those six spots, four were isoforms of cathepsin B, and one was ATP synthase D chain which were confirmed in our study (Table 1). The fact that only six differentially expressed proteins were identified speaks to the difficulty of making inter-gel comparisons with traditional staining methods.

In a direct comparison of genomic and proteomic approaches using mouse liver response to drug treatment, Tian et al. [32] showed that the overall patterns of mRNA and protein

expression were similar, but the differential mRNA expression could identify only 40% of the variation in protein expression, suggesting significant posttranscriptional changes unaccounted for by gene array analysis. In the present study, we report the first direct assessment of the qualitative and quantitative differences in protein expression between papillary thyroid carcinoma (PTC) and matched normal thyroid tissue, and we use our findings to demonstrate some of the unique benefits arising from a specific proteomic-based strategy.

We adopted DIGE for protein separation and peptide mass fingerprinting by MALDI-TOFMS for protein identification. DIGE reduces experimental variability and enhances quantitative precision because two samples (or sample pools) can be compared on a single 2D gel. Consequently, both major and minor differences in expression levels can be established that might be missed by other approaches [30,31]. Further, the separation and quantification steps are performed on intact proteins, and therefore important information about protein isoforms and their relative levels are available through DIGE. These data are of considerable biological relevance, but are not available through genomics or proteomic studies based on shotgun strategies. The benefits of the DIGE approach have been demonstrated in studies of lung [33] and colon cancer [34], and in work on the regulation of protein expression in a transgenic mouse model [35]. Although a powerful combination, it is important to note that our analytical strategy, like all others, only provides quantitative and qualitative information on a subset of the proteins in these tissues. The use of whole tissue can also contain non-thyocyte proteins such as albumin and globins identified in this analysis.

When analyzed by DeCyder software, DIGE gels comparing PTC and matched normal tissue show that 35% of protein spots were differentially expressed at $\geq$ two-fold. As a direct comparison, DeCyder analysis indicates only 6% of spots were differentially expressed between two protein pools of normal thyroid tissue from different groups of patients. This control for analytic and biologic variability is helpful and indicates that many of the differences can be attributed to biologic differences between PTC and normal thyroid. Of the 1840 protein spots in the PTC-normal comparison detected by DeCyder, the software determined 27% had lower expression in PTC, 8% were overexpressed and 65% were not different at the two-fold cutoff. Underexpressed protein spots were identified in PTC at a greater than 3/1 ratio compared with over-expressed proteins spots. Many of the underexpressed protein spots were found in the upper left of the gel (Figure 1B) and appear to primarily represent isoforms of thyroglobulin, although only a subset of these spots were picked and identified by mass spectrometry. Potential biomarkers in PTC were identified on pooled protein from thyroid tissue (PTC and matched normal) of five different patients with similar clinical characteristics. These data were further validated by performing individual patient gel analyses, confirming that changes observed in the pooled gel analysis were statistically significant changes for 48% of the identified proteins. These two complementary proteomic approaches refine the list of potential biomarkers to those that have the highest likelihood of success in non-proteomic validation strategies (i.e., immunohistochemistry, ELISA, etc.).

One measure of the robustness of any hypothesis-generating approach to biomarker discovery is its ability to detect previously identified markers. Galectin-3, cytokeratin 19, and cathepsin B are three examples of well-defined biomarkers in PTC [2,36–38]. No genomics study has identified mRNA over-expression of all three biomarkers, while only three studies report upregulation of two of these three. This may be because the cDNA arrays employed were not comprehensive [22], or it may have resulted from differences between mRNA and protein expression. Regardless, in the present proteomic study we found all three proteins, galectin-3, cytokeratin 19, and cathepsin B, to be overexpressed in PTC. Conversely, we measured no change in GAPDH levels in PTC relative to normal tissue. This is significant because this protein has been used as an internal loading control in many mRNA and protein analyses.

The proteomic approach adopted in our current study (DIGE analysis and protein identification, a two-fold differential protein expression cutoff as assessed by DeCyder analysis, statistical validation using individual DIGE analysis, and assessment of protein abundance) has allowed us to prioritize the most promising biomarkers. Using this strategy, we have identified three novel biomarkers: S100A6, peroxiredoxin 2, and HSP 70 (BiP). S100A6 is a member of the S100 protein family which is associated with a range of biological functions (e.g., enzyme regulation, cytoskeletal organization, transcriptional regulation) and with putative involvement in many diseases (e.g., Alzheimer's, Down syndrome, multiple sclerosis, cancer, ventricular hypertrophy, heart failure, rheumatoid arthritis, chronic bronchitis, cystic fibrosis, and psoriasis) [39]. Increased S100 protein expression in PTC has been reported, but there are many different isoforms of S100 that have not been fully characterized in PTC [40–43]. Our proteomic approach identified two isoforms of S100, S100A6, and S100A8. The S100A6 isoform was significantly overexpressed in PTC, but the S100A8 isoform was not differentially expressed between PTC and normal thyroid tissue (Table in supplemental data). We have further confirmed the utility of S100A6 as a biomarker using immunohistochemistry with a commercially available monoclonal antibody specific for S100A6. As part of our ongoing studies, we first reported these findings in abstract form [44]. Subsequently, another group independently confirmed that this biomarker has a reasonable sensitivity (75%) and specificity (95%) for distinguishing PTC from benign adenoma, but the sensitivity for FTC was much lower (35%) [45]. This is not unexpected since we identified S100A6 in a proteomic analysis of PTC. The sensitivity and specificity values we calculated in the current study may have been different from those reported by Ito et al. because we used a different antibody.

In addition to confirming increased expression of known biomarkers, we identified differential expression of two distinct forms (different pI) of cathepsin B. Cathepsin B is a lysosomal cysteine proteinase which is involved in the processing of thyroglobulin in the thyroid [46]. Cathepsin B is reportedly over-expressed in many tumors including thyroid cancer [30,38] and is believed to play a role in tumor invasion and metastasis [47,48]. Interestingly, one form has fivefold lower expression in PTC (although not statistically significant on individual gel analysis), while a more acidic form has 2.5–4-fold higher expression in PTC, indicating that posttranslational modification may be as important or more important in PTC than total cathepsin B content. These findings would be missed by microarray analysis and most other proteomic methods.

We have also identified a group of mitochondrial proteins with increased expression in PTC relative to control tissue. These include aconitase 2 (which is reportedly overexpressed in some cancers [49]), ATP synthase, and voltage-dependent anion channel 1 (VDAC1) which is a membrane protein (porin) increased in a malignant rat liver cell line [50]. There are also reports of overexpression of mitochondrial genes in renal and salivary oncocytomas [51], prostate carcinoma [52], and melanoma [53]. In addition, the Hurthle cell (oxyphilic/oncocytic) thyroid tumors are characterized by having a greatly increased numbers of mitochondria, partially uncoupled respiration [54], and overexpression of mitochondrial mRNA as assessed in microarray analysis [55]. In PTC, differential display PCR has also confirmed increased message level for mitochondrial genes and increased numbers of mitochondria of increased size [56]. Earlier ultrastructural studies also indicated that mitochondria in PTC appear to be increased and structurally abnormal [57–60]. We have independently confirmed these observations by demonstrating upregulation of several mitochondrial proteins in PTC in this proteomic study.

In summary, proteomic analysis of PTC has confirmed changes in several known biomarkers, identified several potential novel biomarkers, and provided insights into global pathophysiologic changes in PTC. We also identified protein isoform differences and posttranslational modifications not accessible by genomic or other proteomic approaches.

Application of this approach to other thyroid neoplasms will undoubtedly uncover new biomarkers that should improve our understanding of these different disorders as well as provide new markers for diagnosis, prognosis, and therapeutic intervention in thyroid and other cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PTC	papillary thyroid carcinoma
FTC	follicular thyroid cancer
FA	benign follicular adenoma
DIGE	difference gel electrophoresis

Figure 1.

DeCyder analysis of 2D gels from two pools of normal thyroid tissue (A), and pooled PTC tumor and matched normal thyroid tissue (B). Protein extracts (50 μ g each) were covalently conjugated to two different fluorescent dyes and then the extracts were pooled and separated on the same gel. The first (horizontal) dimension was a pH 3–10 non-linear focusing gradient, and the second vertical dimension was a 12.5% homogenous SDS polyacrylamide gel. Two circular self-adhesive reference markers were applied on the lateral edges of the gel and were detected by the software. These are denoted by a yellow symbol ($\oplus$). (A) Proteins from pools of non-tumor thyroid tissue from patients with benign tumors (adenoma) were conjugated to Cy3 and protein from pools of non-tumor thyroid tissue from PTC patients were conjugated to Cy5. Proteins upregulated in non-tumor tissue from PTC patients are outlined in red, downregulated are in green. A threshold level of +two-fold and two-fold, as determined by DeCyder software was applied. Yellow spots represent proteins with an equal level of

expression in both tissues ($<$ two-fold or $\geq$ two-fold) as determined by the software. Proteins pooled from non-tumor thyroid tissue from three patients with benign tumors were compared to non-tumor tissue pooled from five patients with PTC. (B) Proteins from pools of non-tumor thyroid tissue from PTC patients were conjugated to Cy3, and protein from pools of PTC tumor tissue from the same patients were conjugated to Cy5. Proteins overexpressed in PTC are shown in red, those underexpressed in green. This comparison between non-tumor tissue, and PTC from the same patient represents pools from five patients.

Figure 2.

Pseudocolor image of 2D gel comparing pooled PTC tumor and matched normal thyroid tissue. Proteins from pools of non-tumor thyroid tissue from PTC patients were conjugated to Cy5 and protein from pools of PTC tumor tissue from were conjugated to Cy3. The gel was run as described in Figure 1. Proteins assessed to be overexpressed in PTC are red, those underexpressed are in green. Yellow spots represent proteins roughly equally abundant in both tissues. Numbers correspond to identified proteins in Table 1 and supplementary data.

Figure 3.

Peptide mass fingerprint recorded for spot number 1759 (S100 calcium-binding protein A6; accession number P06703). Italicized numbers above m/z values are amino acid residue numbers, and trypsin autohydrolysis peaks (T) were used for internal calibration of the spectra. Boldfaced m/z values match peptides from this protein. The inset represents 11 peptides matched out of 34 masses submitted to the Mascot search engine (i.e., seven peptides in initial pass without oxidized M- and N-terminal acetylation being selected). Sequence coverage represented by these peptides for this relatively small protein was 65% enabling its identification by peptide mass fingerprinting. The theoretical mass (10.2 kDa) and pI (5.3) of this protein are consistent with its position on the gel (Figure 2).

Figure 4.

Individual patient analytical DIGE analysis. Analytical DIGE gels (50 μ g each PTC and matched normal) were run and analyzed for each of five individual patients. Gels were matched with the preparative gel (Figure 3) by assigning individual spots in BVA analysis. Standardized log abundance for each gel in each channel (Cy3 and Cy5) was determined by DeCyder analysis. Open squares and dashed lines represent each individual gel. Closed squares and solid lines represent the average of the five individual gels. (A) S100A6 (ave. vol. ratio 6.3, $P = 0.0014$), (B) peroxiredoxin 2 (ave. vol. ratio 0.27, $P = 0.03$), (C) HSP 70 (BiP) (ave. vol. ratio 0.26, $P = 0.0012$), (D) cathepsin B (ave. vol. ratio 0.56, $P = 0.45$).

Figure 5.

Immunohistochemical staining for S100A6. Slides were prepared as described in Figure 1. Following incubation with the primary mouse monoclonal antibody against S100A6 (1:1000 dilution) for 60 min, sections were reacted with a non-biotin-based immunoperoxidase detection system. Localization of staining was performed with DAB+. The slides were counterstained with hematoxylin. (A) PTC, (B) FTC, (C) Hurthle cell carcinoma, and (D) normal thyroid (40× objective).

Table 1
Differentially Expressed Proteins in PTC Compared with Matched Normal Thyroid Tissue

Protein name	Swiss-Prot acc number	Spot number (Figure 2)	Abundance (% spot vol)	Ave. vol. ratio	Measured mass (kDa)	Δ mass (kDa) ^d	Measured pI	Δ pI ^d	Mascot score	Ave. vol. ratio (indiv gels)	P value
(A) Overexpressed in PTC											
S100 calcium-binding protein A6	P06703	1759	0.20	6.5	10.2	0.0	4.9	-0.4	121	6.3	0.0014
ATP synthase, F0 complex, d	O75947	1590	0.03	2.2	25.7	7.2	5.1	-0.2	136	2.0	0.037
Moexin	P26038	697	0.02	2.0	73.2	5.5	6.6	0.5	287	2.8	0.027
Cathepsin B	P07858	1413	0.01	3.4	31.6	3.8	5.1	-0.1	105	4.8	0.0063
Aconitase 2	Q99798	551	0.01	2.3	82.6	0.2	7.5	0.6	288 ^a	5.0 ^c	0.0026
Galectin-3	P17931	1470	0.01	2.3	29.8	3.7	8.1	-0.5	95	3.1	0.0058
Voltage-dependent anion channel 1	P21796	1369	0.01	3.8	33.4	2.8	8.1	-0.5	154	2.6	0.038
Cyokeratin 19	P08727	1165	0.01	2.6	41.8	-2.3	4.8	-0.2	283	2.9	0.00038
S100 calcium-binding protein A6	P06703	1762	0.01	6.4	<10.2	ND	4.9	-0.5	129	3.6 ^c	0.15
Ferritin, light polypeptide	P02792	1613	0.01	5.1	24.7	4.8	5.5	0.0	152	5.5	0.055
Annexin 1 (lipocortin I)	P04083	1283	0.01	5.0	36.2	-2.4	6.5	-0.1	272	4.8	0.096
Annexin 1 (lipocortin I)	P04083	1280	0.04	3.9	36.2	-2.4	6.9	0.2	305	1.9 ^b	0.8
ATP synthase, F1 complex, beta	P06576	965	0.01	3.4	55.1	3.3	4.8	-0.2	206	1.9	0.14
Chaperonin	P10809	848	0.02	2.9	63.4	5.4	5.1	-0.1	267 ^a	2.9	0.2
Cyokeratin 19	P08727	1180	0.01	2.5	41.1	-3.0	4.9	-0.2	443	1.9	0.17
ATP synthase, F1 complex, beta	P06576	980	0.02	2.3	54.1	2.3	4.9	-0.1	295	2.0	0.22
Serine proteinase inhibitor, clade A	Q5U0M1	801	0.12	2.1	65.6	18.7	4.8	-0.5	183	1.9	0.57
Tu translation elongation factor	O15276	1160	0.01	2.1	41.9	-8.3	7.0	-0.2	238	1.5	0.64
Manganese SOD	Q7Z7M4	1608	0.05	2.0	25.3	1.6	6.9	0.0	71	2.3	0.1
(B) Underexpressed in PTC											
Peroxiredoxin 2	P32119	1589	0.13	-3.2	26.1	4.2	5.4	-0.2	199	-3.7	0.03
Heat shock 70 kDa protein 5, Bip	P11021	583	0.08	-2.4	78.7	8.2	4.9	-0.1	231	-3.8	0.0012
Calreticulin	P27797	869	0.04	-3.0	62.2	15.7	4.3	0.0	133	-2.6	0.0013
Vitamin D-binding protein	P02774	898	0.03	-1.9	59.4	8.2	5.6	0.4	144	-1.9	0.005
Selenium binding protein 1	Q13228	1013	0.02	-3.4	52.1	-0.2	6.3	0.1	100 ^a	-2.6	0.00097
Actin	P60709	1269	0.01	-4.3	36.6	-5.0	4.8	-0.5	97	-1.9	0.017
Protein phosphatase 2	P62714	1281	0.01	-3.6	36.3	0.7	5.0	-0.2	132	-2.6	0.002
Cathepsin B	P07858	1423	0.02	-5.1	30.9	3.1	7.6	2.3	88	-1.8	0.45
Alpha 1 type I collagen	P02452	322	0.06	-3.7	> 104	ND	9.3	0.0	84	ND	ND
Alpha 1 type I collagen	P02452	316	0.03	-3.8	> 104	ND	8.7	-0.6	86 ^a	-7.1 ^c	0.054
Phosphoglycerate kinase 1	P00558	1188	0.01	-3.1	40.4	-4.1	7.4	-0.9	138	-4.2 ^b	0.27
Sorbitol dehydrogenase	Q00796	1246	0.03	-2.5	37.6	-0.6	7.9	-0.4	135	-2.1 ^b	0.072

Proteins with statistically different expression between PTC and matched normal tissue are shown in italics.

Mass or pI differences > 20% from predicted are shown in bold.

ND, not determined.

^a MALDI-ToF identification confirmed by LC/MS/MS. Aliquots (2 μ L) were analyzed by nano LC-ESI-MS/MS on a quadrupole ion trap mass spectrometer (LCQ DECA, Thermo Finnigan) [61].

^b Spots on only four of the individual gels identified.

^c Spots on only three of the individual gels identified.

^d Δ mass and Δ pI are measured-calculated values.

Table 2
Immunohistochemical Staining of Thyroid Tumors for S100A6

		Staining intensity			
Histologic diagnosis	Number of cases	0	1	2	3
PTC	13	0 (0)	1 (8)	6 (46)	6 (46)
FTC	7	1 (14)	1 (14)	2 (29)	3 (43)
FA	13	4 (31)	4 (31)	5 (38)	0 (0)
Percentage of cells staining positive					
Histologic diagnosis	Number of cases	0%	1%–10%	10%–50%	> 50%
PTC	13	0	0	0	13 (100)
FTC	7	1 (14)	0	1 (14)	5 (71)
FA	13	4 (31)	2 (15)	0	7 (54)

Number in parentheses represent percentage of cases in each category.