

Unveiling a novel biomarker panel for diagnosis and classification of well-differentiated thyroid carcinomas

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Abstract. Thyroid cancer is the most common human endocrine malignancy with increasing global incidence. Papillary thyroid carcinomas (PTC) and follicular thyroid carcinomas (FTC) are well-differentiated thyroid cancers (WDTC) accounting for 95% of all thyroid cancer cases, with survival rates of almost 100% when diagnosed early. Since PTC and FTC have different modes of metastasis, they require different treatment strategies. Standard diagnosis by fine needle aspiration with cytopathological examination can be inaccurate in approximately 10-30% of all cases and difficult to definitively classify as WDTC. Currently, there is no single or panel of biomarkers available for thyroid cancer diagnosis and classification. This study identified novel biomarkers for thyroid cancer diagnosis and classification using proteomics, which may be translated into a biomarker panel for clinical application. Two-dimensional SDS-PAGE and mass spectrometry were used to identify potential biomarkers in papillary and follicular thyroid carcinoma cell lines, and the biomarkers were validated in five PTC and five FTC tissues, with their adjacent normal tissues from Thai patients. Eight biomarkers could distinguish PTC from normal tissues, namely enolase 1, triose phosphate isomerase, cathepsin D, annexin A2, cofilin 1, proliferating cell nuclear antigen (PCNA), copine 1 and heat shock protein 27 kDa (HSP27). These biomarkers can also discriminate FTC from normal tissues, except for annexin A2. On the contrary, annexin A2, cofilin 1, PCNA and HSP27 can be used to classify the types of WDTC. These findings have potential for use as a novel multi-marker panel for more accurate diagnosis and classification to better guide physicians on thyroid cancer treatment. Moreover, our results suggest the

involvement of proteins in cell growth and proliferation, and the p53 pathway in the carcinogenesis of WDTC, which may lead to targeted therapy for thyroid cancer.

Introduction

Thyroid cancer is the most prevalent endocrine malignancy and the eighth most common cancer in females. Presently, thyroid cancer incidence has continuously increased worldwide (1,2). Moreover, the American Cancer Society has reported that the incidence of thyroid cancer is increasing most rapidly compared to other cancers (3). Papillary thyroid carcinomas (PTC) and follicular thyroid carcinomas (FTC) account for 95% of all thyroid cancer cases. They are clinically classified as well-differentiated thyroid carcinomas (WDTC) due to their biological behavior resembling normal follicular cells and good responsiveness to surgery and radioiodine therapy (4,5). However, PTC and FTC are usually curable when discovered at early stages, but survival rates may be reduced from 100% in stages I and II to 50% at stage IV (6). In addition, pathological divergences, such as patterns of metastasis, of PTC and FTC also have significant impact on cancer aggressiveness and treatment. PTC usually invades neighboring tissues and occasionally metastasizes to regional lymph nodes, whereas FTC often metastasizes to bone and lung. Thus the general treatment for WDTC, thyroidectomy with radioiodine therapy, may have different consequences for PTC and FTC (5). Prophylactic lymph node and central neck dissection is an example of different thyroid cancer management between PTC and FTC. Several studies showing that prophylactic lymph node and central neck dissection in PTC potentially decreases cancer recurrence, revealing microscopic lymph node metastases undetectable by other techniques and increasing disease-specific survival. In contrast, dissections are not recommended for most FTC, except in malignant lymph node cases (7,8). Therefore, FTC is considered to be more aggressive than PTC. For these reasons, early detection and classification of thyroid cancer is necessary for optimal treatment.

Neck ultrasound, laryngeal exam, thyroid function blood test, chest X-ray, thyroid scan with low-dose radioactive iodine and fine needle aspiration (FNA) biopsy are available techniques for thyroid cancer diagnosis. FNA with cytopathological

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examination is the most effective technique for thyroid cancer diagnosis and classification. However, approximately 10-30% of FNA results are misdiagnosed due to inadequate aspirated materials, cytodiagnostic errors, missed sampling and nodule compositions (9,10). In addition, detections of specific genetic alterations from FNA and thyroid cancer tissue samples have been studied for decades and some biomarkers have been identified (e.g. BRAF and RAS mutation). However, determination of disease status based on genomic analysis and gene expression data alone are limited due to translational modifications such as mRNA splicing and post-translational modifications. Thus, proteomics is an option to identify potential biomarkers for cancer diagnosis that can fulfill the discordance of genetic biomarkers and improve the effectiveness of thyroid cancer diagnosis (4,5,11,12). Several studies, including those from our group, have revealed potential protein biomarkers from thyroid tissues, e.g., galectin 3, cathepsin B, cytokeratin 19 (CK19) and e-cadherin (13-17). Unfortunately, these biomarkers do not have enough specificity to be used clinically. Galectin 3 is one of the most studied thyroid cancer biomarkers. We have shown galectin 3 to have higher expression in PTC, compared to FTC and benign tissues (15). However, it is overexpressed in other cancers such as breast cancer, lung cancer, esophageal cancer, and laryngeal cancer, making it difficult to discriminate between thyroid cancer and other cancers (15,18,19). In addition, galectin 3 is also expressed by macrophages and activated endothelial cells, which can interfere with diagnosis (18).

Current interest focuses on using a panel of multiple biomarkers for diagnosis, which should be more reliable than using single biomarkers. Several studies on thyroid cancer have revealed that panel biomarkers have higher specificities than a single marker alone (17,20,21). The specificity of using a panel of three biomarkers for detection of thyroid cancer, namely galectin 3, CK19 and monoclonal antibody against microvillus surface antigen (HBME), increased by 18, 14 and 4%, respectively, when compared to those of galectin 3, CK19 and HBME alone (21). However, there is still a need for classification of thyroid cancer for appropriate treatment, so it would be beneficial to have a panel of biomarkers for detection, as well as classification of thyroid carcinomas. In this study, we aimed to identify novel biomarkers for thyroid cancer diagnosis and classification using proteomics for improved thyroid cancer management.

Materials and methods

Cell culture. Human papillary thyroid carcinoma (B-CPAP) cell line and human follicular thyroid carcinoma (FTC-133) cell line were kindly provided by Professor Johan Lillehaug, University of Bergen, Norway. B-CPAP cells were cultured in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin-amphotericin-B solution. FTC-133 cells were cultured in 1:1 mixture of DMEM: Ham's F-12 media supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin-amphotericin-B solution and 1% 200 mM L-glutamine. The cells were incubated at 37°C in 5% CO₂.

Human specimens. Five human papillary carcinoma (PTC) tissues and their adjacent normal tissues, as well as five human

follicular carcinoma (FTC) tissues and their adjacent normal tissues were obtained from Phramongkutklo Medical Center, Bangkok, Thailand after approval of the research protocol (S012H) by the Institutional Review Board of the Royal Thai Army Medical Department, Thailand. Histopathology confirmed diagnoses of the tissues. Tissues were stored at -80°C until ready to be processed.

Protein extraction. Cells were harvested from culture flasks in 0.25 M sucrose with protease inhibitor cocktail (ratio of 500:1) (Sigma-Aldrich P8340, St. Louis, MO, USA) using cell scraper and then centrifuged at 500 x g at 4°C for 10 min. The cell pellets were lysed using lysis buffer containing 9 M urea, 2% CHAPS, 2% DTT, 5% ampholine (pH 3-10) and 500:1 protease inhibitor cocktail for 1 h at room temperature, sonicated and then centrifuged at 12,000 x g at 4°C for 10 min. Bradford assay was used to determine protein concentration in supernatants.

Invasion and migration assays. Cell invasion and migration assays were performed using Transwell chambers (pore size 8.0 µm, Corning). For invasion assay, the upper chambers were coated with Matrigel (30 µg protein/well). Cells (10⁴) were seeded onto the upper chamber and 600 µl medium containing 10% FBS was added to the lower chamber, and incubated at 37°C for 24 h with 5% CO₂. Non-migrating or non-invading cells on the upper chambers were removed using cotton swabs. The migrating or invading cells on the upper chamber were fixed in 25% methanol for 15 min and then stained with 0.5% crystal violet for 15 min. Excess dye was rinsed with RO water for 30 sec, swabbed and then cells dried at 70°C overnight. Photographs were taken and then dye was eluted using HCl-methanol solution (1:9 ratio) for 5 min. Absorbance was measured at 550 nm.

Two-dimensional SDS-PAGE and image analysis. Immobiline™ Drystrips (7 cm, pH 3-10 nonlinear) were rehydrated for 16 h with 150 µg protein samples. The first dimension was performed at 7,000 Vh by using Ettan IPGphor 3 (GE Healthcare Co.). For the second dimension, strips were incubated with equilibration buffer containing 1,4-dithioerythritol (DTT) for 10 min followed by incubation with equilibration buffer containing iodoacetamide (IAA) for 10 min. Proteins were separated in 12.5% SDS-PAGE gel at 10 mA/gel using PowerPac Basic™ (Bio-Rad) apparatus. Gels were stained with Coomassie blue R-250 and then scanned by using Labscan 5.0 software. Protein spots were analyzed by ImageMaster 2D Platinum 7.0 program and differential protein expression between the cell lines was measured as percent volume. Protein spots with expression >1.3-fold were selected for identification.

In-gel digestion. Selected spots were excised and then destained with 50% acetonitrile (ACN) in 0.1 M NH₄HCO₃ followed by reduction and alkylation using 10 mM DTT for 45 min at 60°C and 100 mM IAA at room temperature for 30 min in the dark, respectively. Finally, dried gels were digested with 0.01 µg of trypsin (Promega Co.) in digestion buffer at 37°C overnight and then supernatants were collected for protein identification.

Protein identification using LC-MS/MS. Trypsin-digested peptides were identified by LC-MS/MS. In brief, C₁₈ EASY-nLC™ column (Thermo Scientific, Rockford, IL, USA) was used to concentrate and desalt digested peptides. Peptides were eluted off the column by using solutions A and B which were composed of 0.1% formic acid in 97% water with 3% ACN and 0.1% formic in 97% ACN, respectively, and injected into nano ESI MS/MS (Amazon speed ETD, Bruker Co.) to generate MS/MS spectra. Parent mass peaks within the range of 50–3000 m/z were selected for MS/MS analysis and the MS/MS spectra were processed using Bruker Compass 1.4 software. Mascot search engine (www.matrixscience.com) was used to identify proteins with the following parameters: NCBI database in Homo sapiens taxonomy, enzyme used: trypsin, missed cleavage allowed: one, fixed modification: carbamidomethyl (C) and variable modification: phospho (ST) and phospho (Y), peptide tolerance: 1.2, MS/MS mass tolerance: 0.6 kDa, and peptide charges: 1+, 2+ and 3+. Identified proteins with consistent molecular weight and pI with their positions in the gels, Mascot score >25 and $p \leq 0.05$ were considered as candidate biomarkers.

Western blot analysis. Proteins were separated on 12.5% SDS-PAGE and electro-transferred onto PVDF membranes. Membranes were blocked with 5% bovine serum albumin (BSA) at 4°C, overnight and then probed with antibody against Annexin A1 (1:1000, Chemicon International Inc.), annexin A2 (1:1000, Abcam Inc.), heterogeneous nuclear ribonucleoprotein K or hnRNP K (1:500, Cell signaling Technology Inc.), 14-3-3σ (1:500, Abcam Inc.), pyruvate kinase (1:1000, Santa Cruz Biotech Inc.), enolase 1 (1:1000, Abcam Inc.), glyceraldehyde-3-phosphate dehydrogenase or GAPDH (1:1000, Abcam Inc.), triose phosphate isomerase or TPI (1:2000, Abcam Inc.), copine 1 (1:2000, Abcam Inc.), cathepsin D (1:1000, Abcam Inc.), cofilin 1 (1:500, Abcam Inc.), heat shock 27 kDa protein or HSP27 (1:10000, Abcam Inc.), β-actin (1:10000, Cell signaling Technology Inc.), proliferating cell nuclear antigen or PCNA (1:5000, Abcam Inc.) and tubulin as loading control (1:5000, Cell Signaling Technology Inc.) for 2 h at room temperature. Then, membranes were washed with TBS/T buffer (TBS, 0.1% Tween-20) and then blocked with 5% skim milk for 30 min. The membranes were incubated with the corresponding secondary antibodies for 45 min and washed again. Finally, membranes were incubated with ECL reagent for 5 min and exposed to film. Labscan 5.0 instrument was used to scan exposed film and band intensities were measured using ImageQuan TL software (GE Healthcare Co.).

Pathway analysis. The network most significantly influenced when comparing PTC and FTC cell lines was predicted by using Ingenuity Pathway Analysis (IPA®, Qiagen Redwood City, CA, USA; www.qiagen.com/ingenuity). Accession numbers and fold-changes of proteins were provided in a dataset. Criteria input in the software are as follows: Reference set, Ingenuity Knowledge Base (genes only); Relationship to consider, Direct and Indirect relationships; Networks, interaction; Data source, all; Confidence, Experimentally Observed; Species, Human; Tissues and cell lines, Other cell line; and Mutation, all.

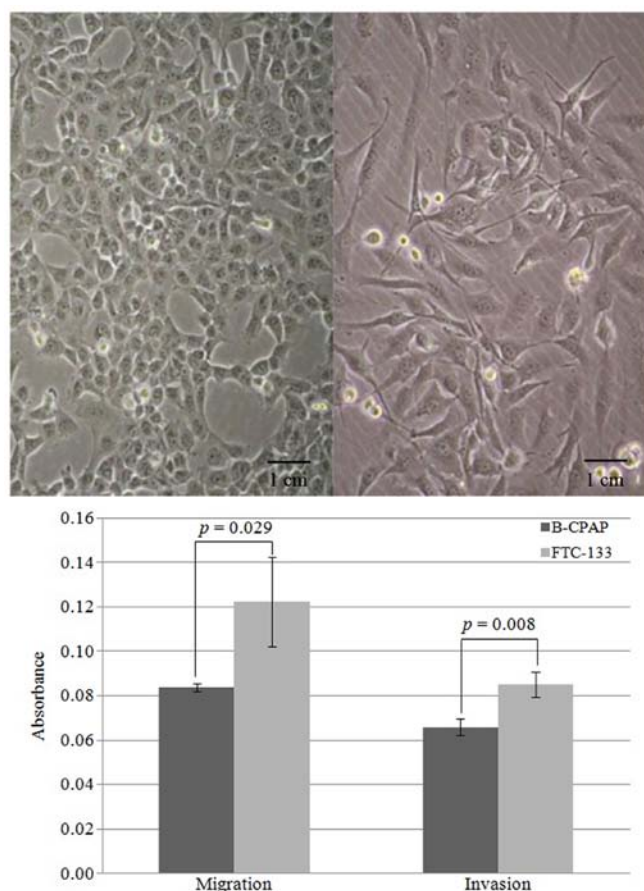


Figure 1. Characteristics of B-CPAP and FTC-133 cell lines. Cell morphologies of B-CPAP (left) and FTC-133 (right) under phase contrast microscope at x100 magnification are shown on the upper panel. Invasiveness of B-CPAP and FTC-133 cells, as determined by Transwell migration and invasion assays, are shown in the bottom panel. The results showed that FTC-133 is significantly more invasive than B-CPAP cells.

Biomarker validation. Proteins from human tissues were extracted using a hand homogenizer. In brief, 20 mg of tissue was lysed by 150 μl of RIPA buffer (150 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0 and 1 mM EDTA) containing protease inhibitor and then homogenized by hand homogenizer and incubated on ice for 1 h. Samples were centrifuged at 12,000 x g at 4°C for 10 min and the supernatants were collected. Protein concentrations in samples were determined by using Bradford assay. Then, protein markers were validated by western blot analysis as previously described.

Statistical analysis. Experimental data are presented as the mean ± SE. The statistical significances of data between samples were determined using the appropriate statistics, namely ANOVA for spot analysis and unpaired Student's t-test or paired Student's t-test for others. Data were considered significantly different at p -values <0.05.

Results

The differential diagnosis of well-differentiated thyroid carcinomas such as papillary thyroid carcinoma and follicular thyroid carcinoma requires skilled pathology examination

Table I. Identification of selected proteins by LC/MS/MS and Mascot database search.

Spot ID	Accession no.	Protein name	MW/pI	Coverage	Peptide match	Mascot score	Fold change	P-value	Function
8	gil28336	β -Actin	42128/5.22	16	5	260	3.1521	0.006	Structure protein
19	gil5031635	Cofilin 1	18719/8.22	30	5	216	2.255	0.002	Invasion and metastasis
60	gil4504517	Heat shock 27 kDa protein	22826/5.98	36	8	358	3.9332	0.000	Stress response
68	gil4502101	Annexin A1	38918/6.57	4	2	76	1.6415	0.022	Cell growth and proliferation
69	gil4507645	Triosephosphate isomerase (TPI)	26938/6.45	55	12	548	1.3202	0.049	Glycolysis
95	gil4503143	Cathepsin D	45037/6.10	6	3	119	2.4007	0.000	Invasion and metastasis
115	gil31645	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	36202/8.26	30	9	311	2.2176	0.001	Glycolysis
130	gil49456555	Proliferating cell nuclear antigen (PCNA)	29029/4.57	32	9	395	1.7506	0.005	Cell growth and proliferation
195	gil4503571	Enolase 1	47481/7.01	41	19	657	1.6857	0.029	Glycolysis
228	gil35505	Pyruvate kinase (PK)	58411/7.58	30	17	714	1.7031	0.017	Glycolysis
359	gil460789	Heterogeneous nuclear ribonucleoprotein K (hnRNPk)	51325/5.13	5	2	71	Present in FTC133	0.000	Cell growth and proliferation
381	gil350610434	14-3-3 σ	26584/4.90	14	4	150	Present in BCPAP	0.000	Anti-apoptosis and drug resistance
386	gil114794644	Annexin A2	35448/8.21	33	10	470	Present in BCPAP	0.000	Invasion and metastasis
419	gil23397696	Copine 1	59649/5.52	11	7	236	Present in BCPAP	0.000	Others

from fine-needle aspiration of the thyroid nodule. Even though both types of carcinomas are treatable by thyroidectomy and iodine ablation, follicular thyroid carcinoma is considered to be more aggressive and has higher recurrence than papillary thyroid carcinomas (4,22-24). Treatment regimens using molecular-targeted chemotherapy also differ, so it is prudent to distinguish between papillary thyroid carcinoma and follicular thyroid carcinoma (25). Herein, we provide a panel of biomarkers that will not only differentiate between these two well-differentiated thyroid carcinomas but also diagnose normal from cancerous lesions.

Cell line characterization. In order to understand the differences between papillary thyroid carcinoma and follicular thyroid carcinoma, cell lines were characterized. Papillary thyroid carcinoma (B-CPAP) and follicular thyroid carcinoma (FTC-133) cell lines exhibited different morphologies and invasiveness (Fig. 1). B-CPAP cells are epithelial-like whereas FTC-133 cells appear spindle-like (Fig. 1 top panel). The doubling times for B-CPAP and FTC-133 cells are 22.8 ± 1.5 and 27.9 ± 1.2 h, respectively. Moreover, FTC-133 cells demon-

strated increased migration and invasion compared to B-CPAP cells (Fig. 1 bottom panel), consistent with the clinical observation that follicular thyroid carcinomas are more aggressive than papillary carcinomas.

Biomarker identification using proteomics. Classical proteomics using two-dimensional polyacrylamide gel electrophoresis, followed by trypsin digestion and LC/MS/MS, were performed to identify the global protein expression differences between B-CPAP and FTC-133 cells. The proteomic patterns showed one hundred and one protein spots differing in expression by >1.3 -fold, as analyzed using ANOVA and these spots were identified by mass spectrometry (data not shown). Fourteen spots appeared in only B-CPAP cells and 39 spots appeared in only FTC-133 cells. In addition, 45 spots had higher expression in B-CPAP cells and 39 spots had higher expression in FTC-133 cells when compared to one another. For B-CPAP cells, functional classification revealed the predominant role of proteins involved in cell growth and proliferation (24%), structure (14%), glycolysis (14%), anti-apoptosis and drug resistance (12%), invasion and

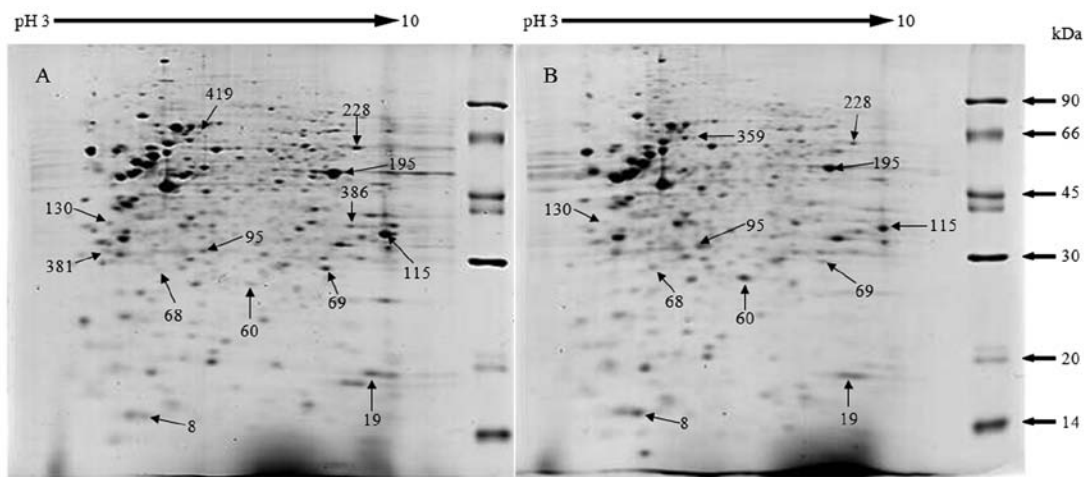


Figure 2. Representative two-dimensional SDS-PAGE of thyroid cancer cell lines. Panels A and B show 2D-gels of B-CPAP cell line and FTC-133 cell line, respectively. Crude lysates (150 μ g) were separated by using 7 cm pH 3-10 nonlinear strips and 12.5% SDS-PAGE. Proteins were stained using Coomassie blue R-250. Gels were analyzed using ImageMaster 2D Platinum 7.0 software and ANOVA for statistical analysis. Fourteen selected spots are shown, which were identified by mass spectrometry. The number of the spot corresponds to the number reported in Table I.

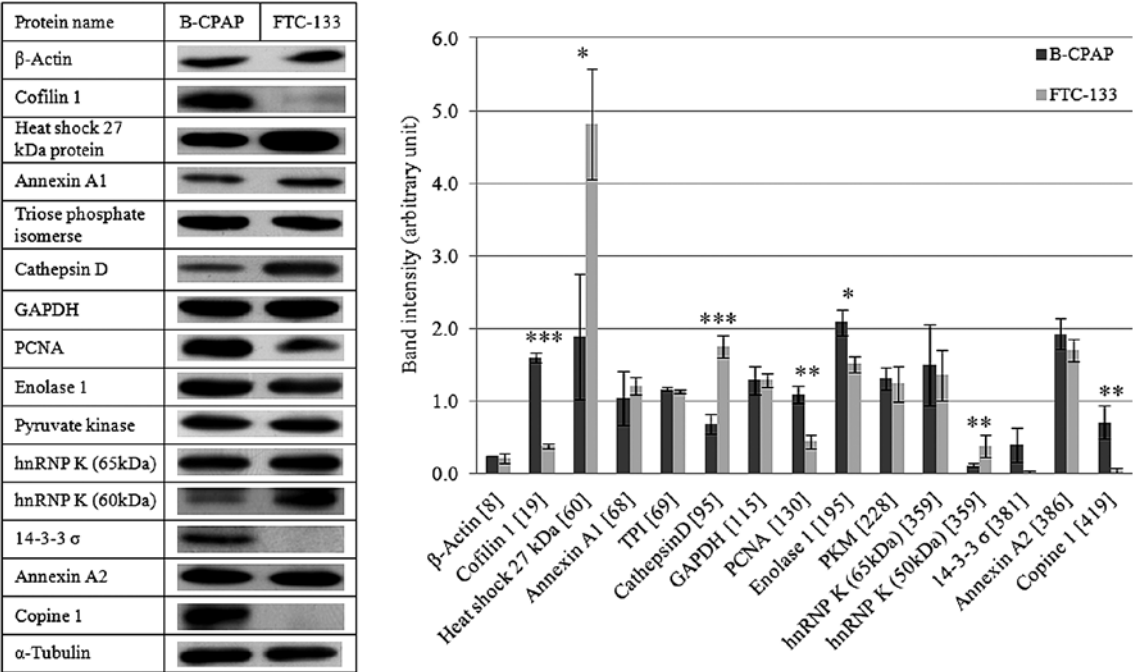


Figure 3. Western blot analyses of selected spots. Western blot bands of selected proteins are shown in the left panel. Band intensities were measured by ImageQuanTL software and normalized using tubulin control, as shown in the right panel. Cofilin 1, HSP27, cathepsin D, enolase 1, PCNA, hnRNP K and copine 1 showed significantly different expression between B-CPAP and FTC-133 cell lines. * $p<0.05$, ** $p<0.005$, and *** $p<0.001$ using Student t-test.

metastasis (10%), stress response (10%) and other functions (16%). On the other hand, proteins involved in cell growth and proliferation (30%), invasion and metastasis (12%), anti-apoptosis and drug resistance (10%), stress response (10%), glycolysis (10%) and other functions (20%) were mainly found in FTC-133 cells.

Fourteen spots were selected for further study, representing proteins covering the major functions in the two cell lines, which showed the most significant and/or high fold-changes in expression. For cell growth and proliferation, proliferating cell nuclear antigen (PCNA), heterogeneous nuclear ribonucleoprotein K (hnRNP K) and annexin A1 were selected. For

glycolysis, triose phosphate isomerase (TPI), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), enolase and pyruvate kinase were selected. For invasion and metastasis, we selected cathepsin D, annexin A2 and cofilin 1. For anti-apoptosis and drug resistance, stress response, structure, and other functions, 14-3-3 σ , heat shock 27 kDa protein (HSP27), β -actin, and copine 1 were selected, respectively.

Representative gels of lysates of B-CPAP and FTC-133 cells are shown in Fig. 2, with 14 selected spots differing between the two cell lines labeled. The identities of three spots present in only B-CPAP cells, one spot in only FTC-133 cells, six spots that increased in B-CPAP and four spots that increased

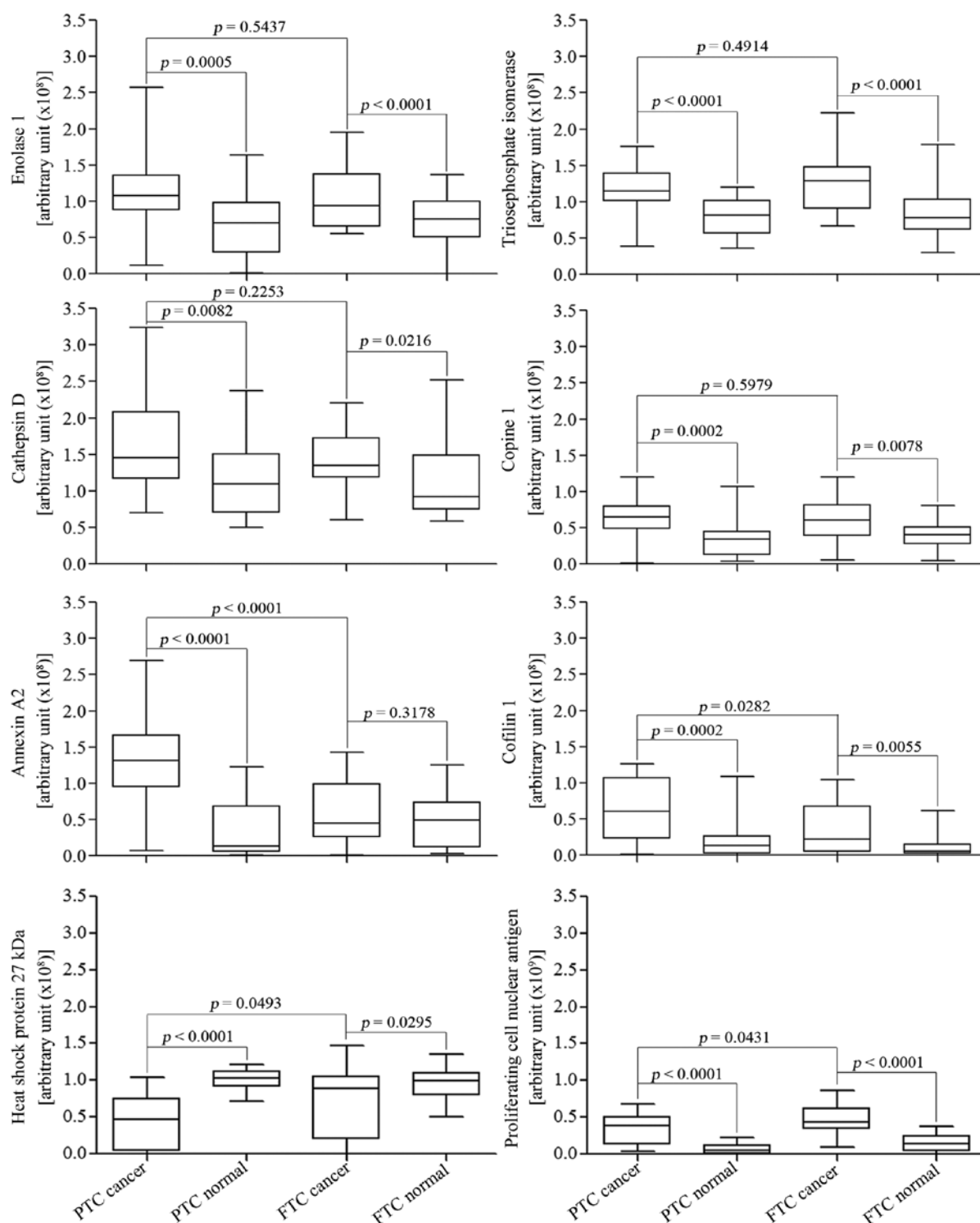


Figure 5. Validation of potential biomarkers for differentiating and classifying PTC, FTC and normal thyroid tissues using western blots. Immunoblots of 5 PTC and 5 FTC tissues with their normal adjacent tissues were performed in triplicate, and band intensities were measured using ImageQuantTL software. Out of the fourteen proteins selected from cell lines, eight proteins showed significant differences in expression when comparing between cancer types, and between cancer and normal tissues, using Student's t-test and paired t-test, respectively. The eight proteins are enolase 1, triose phosphate isomerase (TPI), cathepsin D, cofilin 1, PCNA, copine 1, annexin A2, and heat shock 27 kDa protein (HSP27). Among them, annexin A2 and cofilin 1 are significantly upregulated and HSP27 and PCNA are significantly downregulated in PTC when compared to FTC. All eight can differentiate between PTC and normal tissues, whereas all but cathepsin D 34 kDa and annexin A2 can be used to differentiate between FTC and normal tissues.

interactions with tumor protein p53 (p53), extracellular signal-regulated kinase 1/2 (ERK1/2) and interleukin-6 (IL-6), which are proteins regulating cell growth and proliferation. The expression of p53 is significantly higher in FTC-133 by

at least 3-fold than in B-CPAP, but there were no statistically significant differences in IL-6 and ERK1/2 expression, as determined by Western blots (data not shown). Overexpression of p53 in lymph node metastasis of papillary carcinomas has

Table II. Clinical pathology of human thyroid cancer tissues.

Case	Gender	Age	Diagnosis
PTC1	Male	73	Papillary thyroid carcinoma, both lobes
PTC2	Female	80	Papillary thyroid carcinoma, follicular variant
PTC3	Male	70	Papillary thyroid carcinoma at right lobe
PTC4	Male	23	Papillary thyroid carcinoma at left lobe
PTC5	Male	33	Papillary thyroid carcinoma
FTC1	Male	51	Follicular thyroid carcinoma at left lobe
FTC2	Female	48	Follicular thyroid carcinoma at left lobe
FTC3	Female	36	Follicular thyroid carcinoma at right lobe
FTC4	Female	51	Follicular thyroid carcinoma at right lobe
FTC5	Male	26	Follicular thyroid carcinoma

been reported using immunohistochemistry, suggesting that p53 is associated with cancer aggressiveness (26). This is consistent with our observations that higher overexpression of p53 is found in the more invasive FTC-133 cells rather than in B-CPAP cells, from both pathway analysis validation and invasion assay. Thus, IPA revealed p53 to be a potential marker with clinical relevance to determine aggressiveness of thyroid cancers.

Biomarker validation. In order to assess the potential use of the fourteen identified proteins from our proteomic study as biomarkers, the expression levels of these proteins were evaluated in human thyroid cancer tissues and their adjacent normal tissues. Table II lists clinical pathology information of five PTC and five FTC tissues from surgical dissection. Western blot analysis revealed seven proteins, namely enolase 1, TPI, cathepsin D, copine 1, annexin A2, PCNA and cofilin 1, to be significantly increased in cancerous tissues, whereas HSP27 showed decreased expression in cancerous tissues when compared to their normal adjacent tissues. All eight proteins can distinguish between PTC and normal tissues. However, all proteins, except for annexin A2, can distinguish between FTC and normal tissues. Four proteins have the potential to be used as biomarkers for classifying thyroid cancer, since annexin A2 and cofilin 1 were significantly upregulated, and HSP27 and PCNA were significantly downregulated in PTC tissues when compared to FTC tissues (Fig. 5).

Discussion

In this study, we identified novel potential biomarkers for thyroid cancer diagnosis and classification. Novel options for

thyroid cancer diagnosis are necessary because of the 30% inconclusive diagnosis from fine needle aspirated biopsies and the increasing incidence of thyroid incidentalomas (10). Moreover, there is increasing evidence on the impact of prophylactic lymph node and central neck dissection on PTC, revealing potential benefits of classifying thyroid cancer in the disease management (7,8). It is widely accepted that biomarkers are useful tools for detecting cancer, monitoring disease progression and possibly identifying novel therapeutic targets. Although thyroid cancer biomarkers have been studied for several decades, only few biomarkers for thyroid cancer classification are available, such as *BRAF* mutation and *RET/PTC* rearrangement in PTC, and *RAS* mutation and *PPAR γ* rearrangement in FTC (13,27). However, it is difficult to determine point mutations in genes or gene rearrangements, and these genomic biomarkers may not be correlated with actual clinical pathology (13). Thus, using protein biomarkers is a better option for thyroid cancer diagnosis and classification.

In this study, proteomic analysis was performed on thyroid cancer cell lines, B-CPAP and FTC-133, to represent papillary and follicular carcinomas, respectively. Out of one hundred and one proteins with differential expression, 14 potential biomarkers were identified from the cell line study. Validation of these proteins on thyroid cancer tissues from Thai patients revealed that expression of enolase 1, TPI, cathepsin D, copine 1, annexin A2, cofilin 1, PCNA and HSP27 are significantly different between normal and cancer tissues. Moreover, four proteins namely annexin A2, cofilin 1, PCNA and HSP27 showed significantly different expression between PTC and FTC tissues.

Among the eight newly discovered biomarkers from our study, two are glycolytic enzymes, namely enolase 1 and triose phosphate isomerase, which are overexpressed in thyroid cancer. The overexpression of glycolytic enzymes supports anaerobic proliferation (Warburg effect) and this phenomenon has been widely accepted as a major source of ATP production in cancer cells (28,29). TPI is a glycolytic enzyme that converts dihydroxyacetone phosphate to glyceraldehyde-3-phosphate (28). Some studies have reported that TPI has potential as a biomarker for lung squamous cell carcinoma and pancreatic cancer (30,31). Enolase 1 commonly exists in the cytoplasm of cells and acts as a glycolytic enzyme that converts phospho-D-glycerate to phosphoenolpyruvate (28). The expression of enolase 1 is upregulated in various types of cancers, e.g., breast and cervical cancers (29). In contrast, the expression of enolase 1 is downregulated in non-small cell lung cancer, and is associated with poor survival rates. Enolase 1 may be alternatively translated to maltose binding protein 1 (MBP1) and then acts as a MYC inhibitor, inducing cell death and suppressing cell growth (32). Enolase 1 is also associated with cancer invasion and metastasis by acting as a plasminogen-binding receptor. When enolase 1 integrates into the cell membrane, it promotes plasmin activity leading to cancer cell invasion and metastasis by degrading the extracellular matrix (33).

Cathepsin D is a member of the cathepsin protease family found in lysosomes and phagosomes that is activated at acidic pH (34). Members of the family, namely cathepsins B, D and L, have been reported to cleave thyroglobulin in the thyroid resulting in the release of thyroid hormones,

triiodothyronine and thyroxine (16,35). Cathepsins B and L are cysteine proteases that are more effective in proteolytic cleavage of thyroglobulin in thyroid lysosomes but are less stable than cathepsin D, an aspartic protease (35). Cathepsin D is overexpressed in breast cancer and proposed to be involved in cancer metastasis, cell proliferation and tumor angiogenesis, and fibroblast proliferation, thus being a marker for aggressiveness of breast cancer (34). Kraimps *et al* reported the overexpression of cathepsin D in thyroid neoplastic tissues and proposed cathepsin D to be a prognostic marker for poor survival of advanced stage thyroid cancer, consistent with our results that cathepsin D expression was upregulated in thyroid carcinomas compared to normal tissues (36). Previously, our group demonstrated cathepsin B overexpression in malignant thyroid tissues when compared to follicular adenomas (15,16). However, when comparing the two cell lines, cathepsin B did not show differential expression by at least 1.3-fold and was thus not selected for the tissue validation in this study. Using immunoblotting, we did detect the presence of the active form and heavy chain of cathepsin B, including their isoforms, at different expression levels between the two cell lines, without affecting the overall protein expression (data not shown). This suggests the involvement of post-translational modifications in cathepsin B and we plan to investigate the role of these modifications in thyroid carcinogenesis.

Annexin A2 is a calcium-dependent, anionic phospholipid binding protein that exists in monomeric and heterotetrameric forms. The monomeric annexin A2 is located in the cytoplasm, whereas the heterotetramer is located in the plasma membrane. However, annexin A2 is more strongly expressed in the cell membrane rather than the cytoplasm (37,38). The overexpression of annexin A2 has been found in several cancers including gastric carcinoma, breast cancer, colorectal cancer, pancreatic cancer, prostate cancer, high-grade gliomas and kidney cancer (37,38). Moreover, the overexpression of annexin A2 has been shown to affect biological processes such as proliferation, apoptosis, invasion and metastasis. Membrane annexin A2 acts as a receptor or a binding protein for several types of proteases, e.g., cathepsin B, plasminogen and tissue plasminogen activator, and extracellular matrix proteins. Therefore, the expression of annexin A2 on the cell membrane is associated with cancer cell invasion, lymph node metastasis and poor prognosis (37-39).

Cofilin 1 is a small ubiquitous protein (~19 kDa) that regulates actin polymerization and depolymerization through different phosphorylation patterns. Actin dynamics is related to cell motility, which is an essential mechanism for cancer invasion and metastasis (40,41). The overexpression of cofilin 1 has been reported in various cancers, e.g., ovarian cancer and oral squamous cellular carcinoma (OSCC) (40-42). In papillary thyroid carcinomas, the overexpression of cofilin 1 has been reported in fine needle biopsy samples (14). Moreover, high phosphorylation levels of cofilin 1 have been reported in melanoma, and breast and prostate cancer. The upregulation of both expression and phosphorylation of cofilin 1 is associated with cancer invasion, metastasis and poor prognosis (40-42).

Proliferating cell nuclear antigen (PCNA) or cyclin is a well-known cell cycle marker that promotes DNA replication by acting as a cofactor for DNA polymerase δ , a major eukaryotic DNA polymerase (43). The expression of PCNA

in non-proliferating cells is usually low, but it is elevated in cells during S-phase or when cells have DNA damage (43). Therefore, PCNA is overexpressed in many cancers, such as thyroid cancer, breast cancer, pancreatic cancer and astrocytomas (43,44). PCNA has been reported to be involved in the molecular carcinogenesis of papillary thyroid carcinoma and the overexpression of PCNA was correlated with increased malignancy of thyroid cancers (44,45). Our results, showing that PCNA expression is higher in the more invasive FTC than in PTC, are consistent with these findings.

Copine 1 is a member of a novel family of ubiquitous calcium dependent, membrane-binding proteins. These proteins are highly conserved and widely expressed in eukaryotes. The biological function of copine 1 is poorly understood (46,47). However, several studies have reported that copine 1 has more than 20 target binding protein partners, such as tyrosine/threonine kinase/extracellular signal-regulated necrosis factor- α (TNF- α) and nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) (46,48). Therefore, copine 1 may be involved in various biological processes, e.g., inflammation, apoptosis, autophagy, growth control, mitosis, gene transcription, exocytosis and cytoskeleton organization (46-48). This is the first report to identify the upregulation of copine 1 expression in thyroid cancer, which warrants further studies.

Heat shock protein 27 kDa (HSP27) is a member of the heat shock protein family. The heat shock protein family is a group of cellular protective molecules that is activated under stress conditions such as heat and irradiation. HSP27 is inducible and is an ATP-independent chaperone activated when in the oligomeric form. Oligomerization of HSP27 is promoted by several stresses such as heat and cell-cell contact (49,50). Clinically, HSP27 is overexpressed in many cancers such as breast cancer, bladder cancer, ovarian cancer, osteosarcoma, endometrial cancer and leukemia (50,51). On the contrary, our group has reported HSP27 downregulation in malignant thyroid tumors as compared to benign tumors, consistent with our current findings that HSP27 exhibited decreases in expression in PTC and FTC compared to their adjacent normal tissues (16). Of note, HSP27 restrains cellular apoptosis and necrosis, resulting in the promotion of cancer cell survival and resistance to chemotherapy (50). Anoikis resistance is a hallmark of cancer metastasis that prevents cancer cell death under detachment (52). Approximately 20% of FTC cases usually metastasize to the bone and lung through the blood circulatory system, i.e., FTC is resistant to anoikis (4,23). However, the mechanism of anoikis resistance in thyroid cancer, especially in FTC, remains to be elucidated. We observed the overexpression of HSP27 in FTC rather than in PTC, suggesting that HSP27 may be involved in the mechanism of anoikis resistance in FTC, thus supporting the idea that FTC is more invasive than PTC.

Our results showed that four out of eight potential biomarkers (enolase 1, annexin A2, cofilin 1 and cathepsin D) are associated with invasion and metastasis in various cancers. Therefore, we hypothesize that the mechanism of thyroid cancer invasion may be initiated by overexpression of annexin A2 and enolase 1. These two proteins can intercalate into the plasma membrane of cancer cells, act as cathepsin D or plasminogen receptor and then promote the degradation of extracellular matrix to allow for translocation of cancer cells.

Consequently, cofilin 1 induces actin dynamics that initiates cancer cell motility and then promotes invasion and metastasis. Moreover, annexin A2 and cofilin 1 are significantly upregulated in PTC compared to FTC, thus, these two proteins may be involved in different modes of metastasis in PTC and FTC. Future studies on the role of annexin A2 and cofilin 1 in metastasis may improve our knowledge on the mechanisms involved and reveal specific molecular targets for the treatment of PTC.

In conclusion, we have discovered eight potential biomarkers for thyroid cancer diagnosis and four possible biomarkers that differentiate between PTC and FTC. The eight biomarkers are enolase 1, TPI, cathepsin D, cofilin 1, copine 1, annexin A2, PCNA and HSP27. All eight can be used to differentiate between PTC and normal tissues, while enolase 1, TPI, cathepsin D, cofilin 1, copine 1, PCNA and HSP27 can be used to differentiate between FTC and normal tissues. For classification of well-differentiated thyroid carcinomas, annexin A2, cofilin 1, PCNA and HSP27 can be used. Moreover, p53 may be an additional biomarker to indicate the aggressiveness of thyroid carcinomas.

We hope that a multi-marker panel using these potential biomarkers will provide for better diagnosis for early detection and classification of thyroid cancer. Validation of this panel using a greater number of samples, samples from less invasive procedures and/or from various ethnic populations will need to be conducted in order to confirm the usefulness of this panel for clinical diagnosis. Further experiments are needed to develop diagnostic assays based on this panel to improve sensitivity for detection for use in a clinical setting. Moreover, our results suggest new molecular insights on thyroid cancer metastasis, especially for PTC, and thyroid cancer anoikis resistance in FTC, which can be novel potential targets for thyroid cancer treatment.

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