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iTRAQ analysis of urinary proteins: Potential use of gelsolin and osteopontin to distinguish benign thyroid goiter from papillary thyroid carcinoma

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ABSTRACT

Background: Benign thyroid goiter (BTG) and papillary thyroid carcinoma (PTC) are often interchangeably misdiagnosed.

Methods: Pooled urine samples of patients with BTG (n = 10), patients with PTC (n = 9) and healthy controls (n = 10) were subjected to iTRAQ analysis and immunoblotting.

Results: The ITRAQ analysis of the urine samples detected 646 proteins, 18 of which showed significant altered levels ($p < 0.01$; fold-change > 1.5) between patients and controls. Whilst four urinary proteins were commonly altered in both BTG and PTC patients, 14 were unique to either BTG or PTC. Amongst these, four proteins were further chosen for validation using immunoblotting, and the enhanced levels of osteopontin in BTG patients and increased levels of a truncated gelsolin fragment in PTC patients, relative to controls, appeared to corroborate the findings of the iTRAQ analysis.

Conclusion: The data of the present study is suggestive of the potential application of urinary osteopontin and gelsolin to discriminate patients with BTG from those with PTC non-invasively. However, this needs to be further validated in studies of individual urine samples.

1. Introduction

Papillary thyroid carcinoma (PTC), which accounts up to 80–85% of cases of all thyroid cancers, is a differentiated thyroid carcinoma with features of papillary architecture, characteristic chromatin, nuclear grooving, nuclear orientation and/or presence of psammoma bodies [1,2]. At present, PTC is routinely detected via evaluation of ultrasound-guided fine needle aspiration cytology (FNAC) of patients with thyroid nodules [3,4]. However, results of FNAC often show a benign condition in > 80% of the cases [5]. Consequently, many patients appear to have undergone unnecessary biopsy procedures as well as having to bear the associated risks of complications and cost. Conversely, there have been patients who had been confirmed as benign thyroid goiter (BTG) via FNAC who have later been diagnosed as PTC, particularly in cases of tumors of > 4 cm in diameter [6]. In light of this, more definitive markers are needed to discriminate patients with BTG from those with PTC.

Compared to other biofluids, urine has several characteristics that make it a preferred choice for biomarker discovery research. Large amounts of urine may be easily and non-invasively obtained from patients. Despite having approximately 61% overlap with kidney and plasma protein content [7], urine lacks proteins of high abundance due to their renal tubular reabsorption [8]. This simplifies proteomics analysis and detection of tumor markers as high abundant proteins often mask those that are present in minimal amounts. Furthermore, studies of urinary proteins have also shown that the urinary proteome did not significantly change when urine was stored for 6 h at room temperature, 3 days at 4 °C, and several years at − 20 °C [9].

Whilst urine has been extensively mined for biomarkers of renal diseases such as diabetic nephropathy, IgA nephropathy, focal segmental glomerulonephritis, lupus nephritis, membranous nephropathy and acute kidney injury [10], relatively fewer investigations have been performed on cancer [11,12]. To the best of our knowledge, there has been no report on urine proteomics analysis of BTG and PTC patients. Therefore, the aim of the present study was to profile the urine of patients with BTG, patients with PTC and healthy control subjects using state-of-the-art isobaric tag for relative and absolute quantification (iTRAQ) technique and liquid chromatography-tandem mass

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spectrometry (LC-MS/MS), and identify proteins with altered abundance between the two groups of thyroid disorder patients relative to the control subjects. These urinary proteins may be used as biomarkers to distinguish BTG patients from those with PTC, as well as to provide a better perspective to understanding the perturbed biological processes that orchestrate PTC and BTG.

2. Materials and methods

2.1. Sample collection and processing

The study was conducted with approval granted by the Medical Ethics Committee of University of Malaya Medical Centre (Ethics Approval Number: 925.8) and in accordance to the declaration of Helsinki. Ten patients with BTG, nine patients with PTC and ten healthy subjects who had no previous medical history of chronic illnesses or health risks related to PTC and were not taking any forms of medication were recruited. Informed written consent was obtained from all subjects, prior to collection of samples. Urine and serum samples were collected, processed and stored as previously described [10,13]. In the case of urine samples, sodium azide (final concentration of 20 mM) was immediately added upon collection. The samples were centrifuged and the resulting supernatant was dialyzed at 4 °C against distilled water, freeze-dried and finally stored at − 20 °C. Pierce BCA protein assay kit was used to determine the concentration of urinary proteins (Thermo Fisher Scientific, Rockford, USA).

2.2. Biochemical tests

Quantitative measurements of free thyroxine (free T4) and thyroid stimulating hormone (TSH) in serum samples of the subjects were carried out according to the ADVIA Centaur Systems manufacturer's instructions (Siemens Medical Solutions Diagnostics Inc., Tarrytown, USA). Table 1 shows the demographic and clinical characteristics of BTG and PTC patients.

2.3. iTRAQ reagent-labeling and strong cation exchange fractionation

Urine samples from each group of subjects were pooled, subjected to acetone precipitation, reduced using dithiothreitol, alkylated using iodoacetamide and digested using trypsin. Approximately 100 μg of digested urinary proteins from each group were labelled with iTRAQ reagent in accordance to the manufacturer's instructions (Sciex, Framingham, USA) before being pooled. Peptides were then desalted using Strata-X 33 μm polymeric reversed phase column (Phenomenex, Torrance, USA) and dissolved in 10 mM potassium hydrogen phosphate buffer (pH 3) containing 10% acetonitrile, and separated on an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, USA) using a PolySulfoethyl column (4.6 × 100 mm, 5 μm, 300 Å) (Nest Group, Houston, USA). Peptides were finally eluted with a 0–400 mM gradient of potassium chloride. Peptide fractions were collected and desalted using Strata-X column.

Table 1
Demographics and clinical characteristics of BTG and PTC patients.

Parameters	BTG (n = 10)	PTC (n = 9)
Gender (F/M)[a]	10/0	4/5
Ethnicity (M/I/C)[b]	2/4/4	8/1/0
Age[c]	57.00 ± 5.27	41.22 ± 4.23
Free T4 (pmol/L)[c,d]	16.78 ± 0.58	16.56 ± 0.55
TSH (mU/L)[c,e]	1.20 ± 0.24	2.39 ± 0.39

[a] F/M refers to Female/Male population.
[b] M/I/C refers to Malay/Indian/Chinese ethnicity of the Malaysian subjects.
[c] Values are in mean ± standard error.
[d] Serum free T4 values between 11.5 and 22.7 pmol/L are considered normal.
[e] Serum TSH values between 0.55 and 4.78 mU/L are considered normal.

2.4. Mass spectrometry

Peptide fractions were analyzed by electrospray ionization mass spectrometry (ESI-MS) using the Shimadzu Prominence nano HPLC system (Shimadzu, Kyoto, Japan) that was linked to a 5600 TripleTOF mass spectrometer (Sciex). Peptides were injected into an Agilent Zorbax 300SB-C18, 3.5 μm (Agilent Technologies) and resolved with water/acetonitrile/0.1% formic acid (v/v) linear gradient. All samples were sent to the Proteomics International Facility, Harry Perkins Institute of Medical Research, Nedlands, Perth, Western Australia for MS analysis.

2.5. Data analysis

Spectral data were searched against the Swiss-Prot database with taxonomy set to *Homo sapiens* (Last update: April 2016, containing 20,200 sequences) using ProteinPilot™ 5.0 Software (Sciex) and parameters were set as follows: Cysteine alkylation - MMTS; Digestion - trypsin; Instrument - TripleTOF 5600; iTRAQ (peptide labelled) modification; Quantitate tab - checked; Detected protein threshold (unused ProtScore) - 2.0 (corresponds to proteins identified with > 99% confidence). Only protein that contains at least two unique peptides were considered for protein quantification. The quantitative protein ratios were normalized for any systematic error/bias. Differences were indicated when a p value was < 0.01, and only fold changes > 1.5 and < 0.75 were considered. The false discovery rate was < 0.1%, calculated by Proteomics System Performance Evaluation Pipeline feature in the ProteinPilot™ 5.0 Software using the reversed version of the protein sequences contained in the search database.

2.6. Western blotting and immunodetection

Proteins with biomarker potentials, including osteopontin, plasma protease C1 inhibitor, gelsolin and monocyte differentiation antigen CD14, were selected for validation by immunodetection as previously described [14]. Pooled urinary proteins (10 μg) were initially subjected to SDS-PAGE and subsequently transferred onto polyvinylidene difluoride membranes (PVDF; PALL Life Sciences, Port Washington, USA) at a constant voltage (100 V) for 1 h using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad, Hercules, USA). Membranes were blocked with 5% skim milk in 1 × TBS-T for 30 min and incubated overnight with primary antibodies in 1 × TBS-T at 4 °C. The membranes were washed thrice with 1 × TBS-T for 5 min and subjected to incubation for 1 h with horseradish peroxidase-conjugated secondary antibodies in 1 × TBS-T at room temperature. They were again similarly washed thrice with 1 × TBS-T and finally developed using freshly prepared 3,3′-diaminobenzidine tetrahydrochloride chromogen solution (Thermo Fisher Scientific, Rockford, USA). Images of the membranes were scanned for visual comparison using the LabScan image scanner. All primary and secondary antibodies used were obtained from Abcam (Cambridge, USA). The primary antibodies comprised monoclonal mouse antisera against gelsolin (1:5000 dilution) and monoclonal rabbit antisera against human monocyte differentiation antigen CD14 (1:5000 dilution), plasma protease C1 inhibitor (1:1000 dilution) and osteopontin (1:5000 dilution), whilst peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibodies (1:5000 dilution) were used as secondary antisera. Intensity of visualized bands was estimated using the GelAnalyzer software (www.gelanalyzer.com). Valley to valley background subtraction was performed beforehand to improve detection of the peptide bands.

3. Results

Analysis of pooled urine samples of patients with BTG (n = 10) as

Table 2
Differently expressed urinary proteins of patients with BTG and PTC.

Accession number	Protein	BTG vs Control		PTC vs Control	
		Ratio	*p* value	Ratio	*p* value
P43652	Afamin	3.5318	0.0071*	1.5417	0.1888
P01009	Alpha-1-antitrypsin	6.368	0.0186	5.6494	0.0035*
P04217	Alpha-1B-glycoprotein	3.5318	0.0062*	4.2855	0.0026*
P04083	Annexin A1	0.7447	0.3533	0.1871	0.0039*
P05090	Apolipoprotein D	1.4322	0.3754	3.8726	0.0004*
P19835	Bile salt-activated lipase	0.7047	0.2930	0.2535	0.0057*
P12830	Cadherin-1	3.3113	0.0052*	2.9107	0.0205
P00450	Ceruloplasmin	3.4356	0.0025*	1.0666	0.0863
P35555	Fibrillin-1	3.5318	0.0059*	2.3335	0.5338
P06396	Gelsolin	2.1478	0.2614	6.6681	0.0004*
P02750	Leucine-rich alpha-2-glycoprotein	7.5162	< 0.0001*	3.1046	0.0001*
P11117	Lysosomal acid phosphatase	0.3192	0.1377	0.1127	0.0005*
P08571	Monocyte differentiation antigen CD14	7.9433	0.0067*	5.2000	0.0126
P05164	Myeloperoxidase	0.1318	0.0106	0.0366	0.0003*
P10451	Osteopontin	5.5463	0.0031*	1.9588	0.7065
P05155	Plasma protease C1 inhibitor	3.6644	0.0224	5.3951	0.0012*
P05109	Protein S100-A8	0.3221	0.0033*	0.0570	0.0001*
P06702	Protein S100-A9	0.3048	0.0011*	0.0497	0.0001*

* Protein level was considered significantly increased if ratio was ≥ 1.5 and *p* value was < 0.01.

well as those with PTC (n = 9) and control healthy subjects (n = 10) using iTRAQ method was able to identify a total of 646 proteins with > 99% confidence. When the levels of the urinary proteins from patients with BTG and those with PTC were compared to the healthy control subjects, only 18 proteins were found to be significantly altered (Table 2). Eight urinary proteins, including leucine rich alpha-2-glycoprotein, alpha-1B glycoprotein, afamin, cadherin-1, ceruloplasmin, fibrillin-1, monocyte differentiation antigen CD14 and osteopontin were significantly increased ($p < 0.01$; fold-change ratio > 1.5) in patients with BTG, whilst the levels of S100-A9 and S100-A8 appeared significantly reduced compared to the controls (Fig. 1). However, comparing the urinary proteins of patients with PTC with those of the control subjects showed significant enhanced levels of alpha-1-antitrypsin, apolipoprotein-D, gelsolin, plasma protease C1 inhibitor, leucine rich alpha-2-glycoprotein and alpha-1B glycoprotein ($p < 0.01$; fold-change ratio > 1.5) but significant reduced levels of annexin A1, bile salt-activated lipase, lysosomal acid phosphatase, myeloperoxidase, S100-A9 and S100-A8 proteins (Fig. 2). The complete raw data for the iTRAQ proteomics analysis is available in Supplementary Table 1.

When the data was taken together, both groups of BTG and PTC patients appeared to show similar enhanced levels of leucine rich alpha-2-glycoprotein and alpha-1B glycoprotein and reduced expression of S100-A8 and S100-A9 proteins compared to the control subjects. Whilst patients with BTG demonstrated different higher levels of afamin, cadherin-1, ceruloplasmin, fibrillin-1, monocyte differentiation antigen CD14 and osteopontin, significant enhanced levels of alpha-1-antitrypsin, apolipoprotein-D, gelsolin and plasma protease C1 inhibitor and lower levels of annexin A1, bile salt-activated lipase, lysosomal acid phosphatase and myeloperoxidase appeared to be unique to patients with PTC. Among the six BTG and eight PTC proteins that were differently altered, we then selected osteopontin, plasma protease C1 inhibitor, gelsolin and monocyte differentiation antigen CD14 for validation by immunoblotting. These proteins were chosen for their characteristic features, and among those that showed highest levels of fold-change difference when compared to the control subjects.

Fig. 3 demonstrates the representative images generated from the

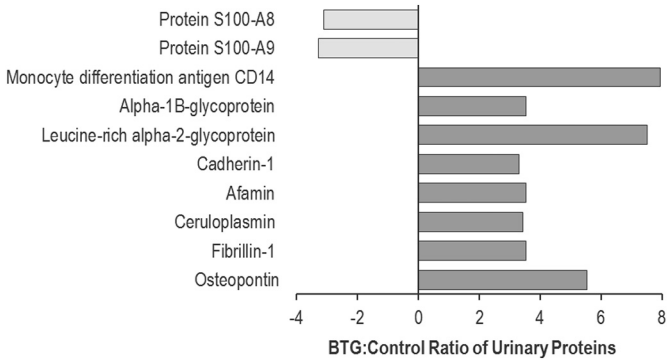

Fig. 1. Urinary proteins of significant altered levels in patients with BTG. iTRAQ-based proteomics analysis showed 10 urinary proteins that were significantly altered in levels in the urine of patients with BTG relative to control subjects. Bars on the positive side represent enhanced levels of proteins, whilst those on the negative side represent lowered levels of proteins.

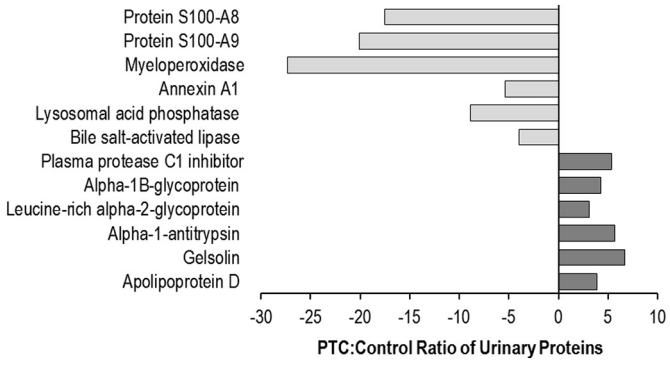

Fig. 2. Urinary proteins of significant altered levels in patients with PTC. iTRAQ-based proteomics analysis showed 12 urinary proteins that were significantly altered in levels in the urine of patients with PTC relative to control subjects. Bars on the positive side represent enhanced levels of proteins, whilst those on the negative side represent lowered levels of proteins.

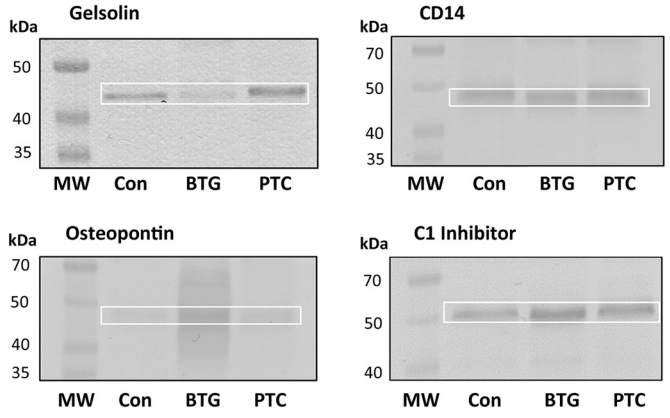

Fig. 3. Immunoblotting of urinary proteins of interest. Four proteins with biomarker potentials were selected for validation by immunodetection. Pooled urinary proteins were initially subjected to SDS-PAGE and transferred electrophoretically onto PVDF membranes. Immunodetection was performed using monoclonal mouse antisera against gelsolin and monoclonal rabbit antisera against human monocyte differentiation antigen CD14, plasma protease C1 inhibitor and osteopontin, whilst peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibodies were used as secondary antisera.

immunoblotting experiments. Whilst the results obtained for osteopontin and gelsolin appeared to corroborate with the findings of iTRAQ-based quantitative proteomics analysis, the plasma protease C1 inhibitor bands were significantly higher in both BTG and PTC patients relative to the control subjects. The bands of osteopontin appeared

Table 3
Putative sequences of 45 kDa gelsolin peptides.

Peptide sequence	Amino acid
AILTAQLDEELGGTPVQSR	507–525
ILTAQLDEELGGTPVQSR	508–525
AQLDEELGGTPVQSR	511–525
AGALNSNDAFVLK	585–596
TPSAAYLWVG	598–607
TPSAAYLWVGTGASEAEK	598–615
WVGTGASEAEK	605–615
TGAQELLR	616–623
AQPVQVAEGSEPDGFWEALGGK	627–648
DSQEEEKTEALTSAK	714–728
YIETDPANR	730–738

intensely stained in patients with BTG, relative to the control subjects as well as patients with PTC, whilst patients with PTC demonstrated intense ~45 kDa gelsolin bands when compared to the control subjects and patients with BTG. When analyzed by densitometry, the intensity ratios for BTG:control bands for osteopontin and PTC:control bands for gelsolin were both > 2.0, whilst ratios of PTC:control bands for osteopontin and BTG:control bands for gelsolin were both < 2.0. On the contrary, the levels of monocyte differentiation antigen CD14 seemed to be comparable between the three different groups of BTG patients, PTC patients and control healthy subjects, and no apparent difference can be detected in the intensities of the plasma protease C1 inhibitor between BTG and PTC patients compared to the control subjects. In the case of gelsolin, analysis of the MSMS spectra using protein pilot software 1.0 version 1 further showed 11 peptide sequences that matched the C-terminus region of the protein (Table 3).

4. Discussion

To the best of our knowledge, there has been no previous report of urine proteomics analysis of patients with thyroid disorders. In this study, 18 out of 646 urinary proteins that were detected by iTRAQ technique were shown to be significantly altered in pooled urine samples of patients with BTG and PTC, relative to control healthy subjects. Whilst four of the urinary proteins were commonly altered in both groups of patients with BTG and PTC, the levels of 14 others appeared unique to either patients with BTG or those with PTC. These included significant enhanced levels of afamin, cadherin-1, ceruloplasmin, fibrillin-1, monocyte differentiation antigen CD14 and osteopontin in patients with BTG and significant higher levels of alpha-1-antitrypsin, apolipoprotein-D, gelsolin and plasma protease C1 inhibitor, and significant lower levels of annexin A1, bile salt-activated lipase, lysosomal acid phosphatase and myeloperoxidase in patients with PTC. When four of the urinary proteins among those that showed highest fold changes were subjected to immunoblotting, similar enhanced levels of osteopontin were detected in patients with BTG whilst patients with PTC demonstrated similar increased levels of gelsolin. However, the levels of plasma protease C1 inhibitor and monocyte differentiation antigen CD14 did not appear to corroborate the findings of the iTRAQ analysis.

Osteopontin, also known as urinary stone protein, is a 45 kDa matricellular glycoprotein involved in biomineralization and cell adhesion [15]. Elevated levels of osteopontin have been previously associated with many cancers including breast, ovarian, endometrial, cervical, prostate, renal, oral, esophageal, colorectal cancer, gastric, pancreatic, liver, hepatocellular, lung, head and neck, skin and thyroid cancer, and the protein appears to modulate tumorigenesis and metastasis [16]. In the case of PTC, treatment of cancer cells with recombinant exogenous osteopontin has been shown to stimulate Matrigel invasion and activate the ERK and V-AKT murine thymoma viral oncogene homolog 1/protein kinase B signaling pathways, hence suggesting its potential use as a diagnostic and prognostic histological cancer biomarker [17]. To the best of our knowledge, this is the first report on the enhanced abundance of osteopontin in the urine of BTG patients compared to PTC patients and control subjects.

Gelsolin, an 85 kDa multifunctional actin-modulating protein [18], plays a major role in cilium biogenesis and/or degradation [19]. Mutation of the gelsolin gene is known to cause amyloidosis [20], and altered expression of the protein has been implicated in a number of pathological disorders including pulmonary and Alzheimer's disease as well as in cancer [21]. Overexpression of plasma and tissue gelsolin has been previously detected in patients with cervical cancer [22], whilst its reduced expression has been reported in tissues of colorectal [23], ovarian [24], breast [25], gastric [26], pancreatic [27] and bladder cancer [28] patients. In addition, a biphasic expression of gelsolin has also been reported in relation to progression of oral carcinoma [29,30]. In the majority of these studies, gelsolin has been proposed to act as a marker for cancer progression and/or prognosis. In the present study, increased levels of what appeared to be a truncated form of gelsolin were detected in PTC patients' urine compared to patients with BTG as well as control subjects. The speculation on the fragment of gelsolin was made based on the 45 kDa band that was detected using antisera against gelsolin and the MS/MS-derived amino acid sequences that showed extensive homology to the actin-binding carboxyl-terminal half of the 85 kDa protein.

When taken together, the data of the present study is suggestive of the potential application of urinary osteopontin and gelsolin to complementarily discriminate patients with BTG from those with PTC non-invasively. With the identification of these potential urinary biomarkers, a validation study of individual urine samples that are clinically representative of the BTG and PTC patient populations using high-throughput methodologies like ELISA ought to be followed-up. This is very necessary as the iTRAQ and immunoblotting results of the present study were generated using pooled urine samples from limited numbers of patients and healthy control subjects.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2018.01.008.

Declaration of interests

The authors have declared no conflict of interest.

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