Appl Biochem Biotechnol (2009) 159:221–232
DOI 10.1007/s12010-008-8503-x

Proteomics and Detection of Uromodulin in First-time Renal Calculi Patients and Recurrent Renal Calculi Patients

Lau Wai-Hoe · Leong Wing-Seng · Zhari Ismail · Gam Lay-Harn

Received: 15 August 2008 / Accepted: 17 December 2008 /
Published online: 15 January 2009

Abstract Renal calculi disease or known as kidney stone disease is the most common urological disorder in both men and women, although it is more prevalent in men. The lifetime chance for an individual to develop renal calculi is ~10% whereas the risk of recurrence in a 10-year period is 74%. Therefore, a diagnostic tool for screening or detecting renal calculi is greatly needed. In this study, we analyze urinary protein profiles from patients with renal calculi for the first time (RC), healthy subjects (HS), and patients with recurrent renal calculi (RRC) to identify a biomarker for detecting the disease. Urinary proteins were isolated by salt precipitation and the proteins resolved by SDS-PAGE. Target proteins were analyzed with LC/MS/MS. Thirty-two proteins were identified from healthy subjects and patients. Uromodulin was the most abundant urinary protein in HS but was a very faint band if detected at all from those that formed renal calculi for the first time ($p < 0.05$). Yet the excreted levels of urinary uromodulin in RRC were similar to those of the HS suggesting that uromodulin is a reliable biomarker for only RC. In addition, a few immunoglobulins that were commonly found in the urine of both RC and RRC, which include Ig alpha heavy chain 1, Ig gamma-2 c region, Ig gamma-3 heavy chain disease protein, Ig heavy chain variable region, Ig heavy constant region gamma 4, and Ig heavy chain. Ig heavy chain Fab frag and antibody a5b7 chain B may serve as potential biomarkers for renal calculi disease.

Keywords Renal calculi disease · Biomarker · Proteomics · SDS-PAGE · LC/MS/MS

L. Wai-Hoe · Z. Ismail · G. Lay-Harn (✉)
School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 USM Penang, Malaysia
e-mail: layharn@usm.my

L. Wing-Seng
Lam Wah Ee Hospital, Penang, Malaysia

Introduction

Renal calculi disease affects 1–20% of the general population, with approximately 1 million people per year suffering from the disease annually. In Asia, the lifetime chance that a person be affected by renal calculi is 2–5% [1]. Formation of calculus in the kidney is a complex and multifactorial process that involves crystallization of supersaturated urine that grows into calculus on the inner surface of the kidney [2]. There are four types of calculi, i.e., calcium, struvite, uric acid, and cystines. Calcium calculus is the most common calculus composing 75% of all detected calculi [3]. The recurrence rate for calcium calculus also is higher than other types of calculi.

A number of chemical and physical factors are known to actively participate in renal calculi formation. Supersaturation of urine and the presence of urinary inhibitors and promoters for crystal aggregation are two of the main factors influencing renal calculi formation [4]. Although supersaturation of urine with calcium oxalate is common in the general population, most people are not afflicted with renal calculi. Therefore, urinary macromolecules must play a role in the formation of renal calculi [3]. The involvement of urinary macromolecules as inhibitors in calculi formation has been widely reported [3, 4]. Most urinary macromolecules are anionic with many acidic residues and often contain posttranslational modifications, such as phosphorylation and glycosylation [5]. The macromolecules exert their inhibitory effects by binding to the crystals, reducing the adhesion of crystals to renal epithelial cells [6, 7] and, therefore, preventing calculus formation.

The change in the pattern of urinary protein profiles is indicative of diseases that affect the kidney [8]. In many studies, gel electrophoresis is used to separate and map protein expression at qualitative and quantitative levels [9–11]. In this study, we used proteomics to map the urinary protein profiles of first-time renal calculi patients, recurrent renal calculi patients, and healthy subjects. The dissimilar protein bands between the patients and healthy subjects were targeted for liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis. We aimed to identify a new potential biomarker from the differentially expressed urinary proteins. Furthermore, we also evaluated the reliability of uromodulin, also known as the Tamm–Horsfall protein (THP), as a urinary protein marker for patients diagnosed with renal calculi for the first time.

Methods

Urine Specimen Collection

Midstream urine samples were collected from 30 patients with renal calculi diagnosed for the first time [RC] (19 male, 11 female), 35 patients with recurrent renal calculi [RRC] (20 male, 15 female), and 50 healthy subjects [HS] (28 male, 22 female). The urine samples were provided by the Urology Clinic, General Hospital, Pulau Pinang and Lam Wah Ee Hospital, Pulau Pinang, Malaysia. The RC and RRC were diagnosed by using radiography with Plain abdominal radiography (KUB) and/or with intravenous urolography (IVU). RC was a cohort that had not received any surgery or medication, whereas RRC had been subjected previously to calculi treatment (ranging from 10 to 2 years ago), i.e., extracorporeal shock wave lithotripsy (ESWL) and/or percutaneous nephrolithotomy (PNCL). Control subjects were asymptomatic with no history of renal calculi or any other renal related diseases, their urine

FEME tests (microscopic) show presence of no red blood cells, and included staff and students from School of Pharmaceutical Sciences, USM.

Urinary Protein Extraction

Urinary protein was precipitated from urine and concentrated by using a salt precipitation method [12]. One milliliter of urine was mixed with 0.27 g ammonium sulfate [$(NH_4)_2SO_4$] and vortexed for 1 min at room temperature followed by centrifugation (12,000×*g*, 20 min, 20°C). The supernatant was decanted and the gelatinous pellet collected and dissolved in TSE buffer [10 mM Tris, 1% (*w*/*v*) sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.8].

Protein Concentration Determination

The protein concentration in the urine sample was determined by using the BioRad DC protein assay according to Lowry [13]. Bovine gamma globulin (BGG) was used as a standard. The protein pellet was dissolved at 80 mL/L (original urine volume/TSE volume) in distilled water and then vortexed for 1 min. A 1:50 dilution was used for the assay and was run in duplicates. Absorbance reading (OD_{650nm}) was measured by using a microplate reader (Anthos Labtec HT2).

Sample Preparation and Electrophoresis

Protein samples were prepared for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) by adding reduced sample buffer 20% (*v*/*v*) (500 mM Tris–HCl [pH 6.8], 200 mL/L glycerol, 100 mg/L SDS, 5 mg/L bromophenol blue and 50 mL/L β-mercaptoethanol) to the protein samples. The mixture was vortexed gently and then heated to 100°C for 10 min. Six-hundred milligrams of the protein sample was separated on a 12% 16×20 cm gel at a constant voltage of 200 V. The gel was stained with Coomassie blue. Band intensities were analyzed with Quantity One software (BioRad).

Western Blotting

Western blotting was performed by using the procedure described by Towbin et al. [14]. The proteins in the gel were transferred to a piece of nitrocellulose membrane and the THP bands were detected by using anti-THP (Biomedical Technologies Inc., USA) antibodies at 1:2,000 dilution and the secondary antibody used was goat anti-rabbit IgG (H+L) horseradish peroxidase (HRP) conjugate (BioRad) at 1:1,000 dilution. The images were captured with VersaDoc Imaging System (BioRad).

In-gel Protein Digestion

The target protein bands on SDS-polyacrylamide gel were excised from the gel. In-situ protein digestion was performed as described by Gam et al. [15]. In short, the proteins were reduced, alkylated and digested with trypsin. The tryptic peptides were eluted from the gel and dried under the continuous flow of N_2 gas and resuspended in 25 μL of deionized water followed by centrifugation (5,000×*g*, 5 min, 20°C). The supernatant was transferred to a vial for LC/MS/MS analysis.

Mass Spectrometric Analysis

Five microliters of sample was loaded onto a reversed-phase column (C_{18}, 300 Å, 5 μm, 1.0 mm×150.0 mm) connected to a high-performance liquid chromatography (HPLC) system. A capillary pump was used to pump the mobile phase at a 20 μL/min flow rate; the linear gradient used was from 5% B to 95% B in 65 min. Mobile phase A was 500 μL/L formic acid in deionized water and B was 500 μL/L formic acid in acetonitrile. The HPLC was interfaced with an ion trap mass spectrometer. The dry gas temperature pressure was 300°C, dry gas flow rate was 6.0 L/min, nebulizer pressure was 30.0 psi. The above parameters were used to acquire MS data. The peptides were ionized by using electrospray soft ionization (ESI). The experimental method was made up of two scan events. The first scan event was a full scan MS and the second was the data-dependent MS/MS scan, which relied on the results of the first scan event. The most intense ion in a mass spectrum (MS) scan will automatically be isolated and excited for the tandem mass spectrum (MS/MS) scan. The parameters for the data-dependent MS/MS scan were default collision energy (voltage)=1.15 V, charge state=2, minimum threshold=1,000 counts, and the isolation width=2 *m*/*z*.

Mascot Protein Identification

The protein was identified with Mascot Protein Database Search engine. Parameters for Mascot protein search were set as follow: peptide mass tolerance was ±2 u and ±0.8 u was set for the fragment mass tolerance. Only one miscleavage was allowed. This software is available at http://www.matrixscience.com. Matches were computed by using a probability-based Mowse score as $-10\times\log(P)$, where P is the probability that the observed match was a random event. The identified proteins function, gene product and other details were determined by using SWISS-PROT (http://www.expasy.org) and Pubmed (http://www.ncbi.nlm.nih.gov).

Data Analysis

Data analysis was performed using statistical package for social science (SPSS), version 15.0. For comparisons between cohorts, Student's *t*-test was employed and the chosen level of statistical significant was $p=0.05$. All the value are presented as mean±standard error of the mean (SE).

Results

Figure 1 shows the urinary protein profiles from 7 HS and RC, respectively. The diagram shows the protein bands detected in the HS and RC.

A 97-kDa protein was the most intense and consistent band in all the HS but was either faint or non-detectable in the RC. Other bands detected (either from the HS or from the RC) varied in intensity amongst the subjects. A relatively intense band at 38 kDa was detected consistently in 80% of the RC, but this protein was not seen in the HS. In general, the number of consistent protein bands in the RC was lower than that in the HS.

In addition to the RC, we also determined the urinary protein profiles of RRC who had demonstrated renal calculi's clinical symptoms and had undergone ESWL and/or surgery. Analysis of urinary protein profiles for this cohort was done with emphasis given to the 97-kDa band, which was shown to be a promising protein marker for renal calculi disease.

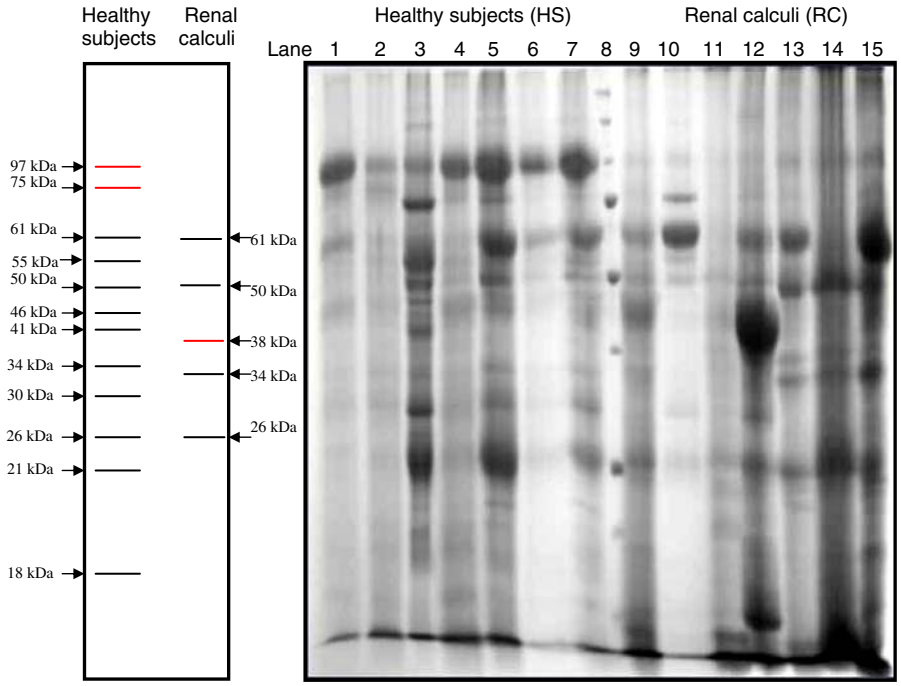

Fig. 1 Urinary protein profiles of healthy subjects (HS) and patients diagnosed with renal calculi for the first time (RC). *Lanes 1–7* HS from seven different individuals' urine samples, *lane 8* molecular weight markers, and *lanes 9–15*: RC from seven different patients' urine samples. The diagram shows the urinary protein bands consistently detected in HS and RC

Fewer protein bands were detected in the urinary protein profiles of the RRC than the HS (Fig 2). The diagram in Fig. 2 shows the protein bands detected. The 97 kDa protein band was detected consistently in 85% of the RRC, although at a lower level than that in the HS.

A protein band at 61 kDa also was an intense and consistent band in RRC. A faint band at 75 kDa was detected in 85% of the RRC, but was non-detectable in the RC.

Amongst all the bands, only bands that appeared at higher consistency (>80%) were targeted for LC/MS/MS analysis. These bands were at 97 kDa, 61 kDa, and 26 kDa for HS; 97 kDa, 50 kDa, 38 kDa, and 26 kDa for the RC, and 97 kDa, 75 kDa, 61 kDa, and 50 kDa from RRC. Although the 97-kDa protein band was neither consistent nor intense in RC, due to its potential utility, we verified its absence by mass spectrometric analysis. Figure 3 shows the mass spectrometry data for the analysis of 97 kDa protein, which was identified as THP protein.

Proteins were identified by using the Mascot protein search engine on the basis of peptide mass matching [16] with redundancy of posttranslational modification and proteolysis. Table 1 shows the urinary proteins identified in this study. Only one type of protein was found in the 97-kDa band, which was THP. We also inspected the mass spectrometry data for THP in order to determine the possibility of a false match. The identity of THP was confirmed in a Western blotting experiment in which THP-specific antibodies reacted with the 97-kDa band, indicating the band was THP. Only a single band was detected by the antibody in HS and in RRC (Fig. 4), whereas a very faint band or no band, was detected in RC.

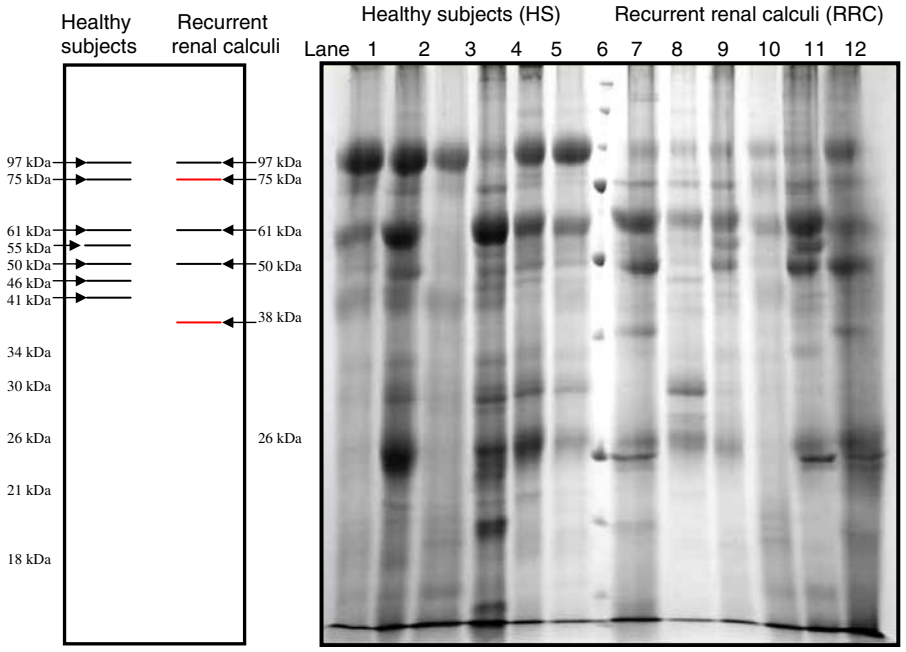

Fig. 2 Urinary protein profiles of healthy subjects (HS) and recurrent renal calculi (RRC). *Lanes 1–6* HS from six different individuals' urine samples, *lane 7* molecular weight markers and *lanes 8–13* RRC six different patients' urine samples. The diagram shows the urinary protein bands consistently detected in HS and RRC

The only protein identified in the 61-kDa band from HS was a serum albumin precursor; however, four proteins were identified in the 61-kDa band from RRC, which were serum albumin precursors, two types of immunoglobulin and a hypothetical protein. Rheumatoid factor—an IgM lambda Fab, chain L and Ig kappa V 1–5 protein, were found in the 26 kDa bands of HS and RC, respectively. Various types of immunoglobulin heavy chains were found in the 50 kDa bands of RC and RRC in addition to the alpha-1 antitrypsin precursor and several hypothetical proteins that were detected only in RRC. Immunoglobulin heavy chain V region precursors and transferrin precursors were identified from the 38 kDa and 75 kDa bands, respectively.

Table 2 shows the age range of the subjects and the average intensity of THP as measured in SDS-PAGE. The data shows that the excretion of THP in RC is significantly lower ($p<0.05$) than those in HS and RRC. However, no significant difference was detected in the excretion of THP in terms of gender and age group of the subjects in all the cohorts analyzed.

Discussion

Renal calculi disease currently is diagnosed using radiography methods, i.e., plain abdominal radiography (KUB) and/or with intravenous urography (IVU), these methods are labor intensive and time consuming. We foresee the need of developing a non-invasive screening tool by using urine specimen for detection of renal calculi disease. Therefore, a reliable urinary marker is required for the development of such tool.

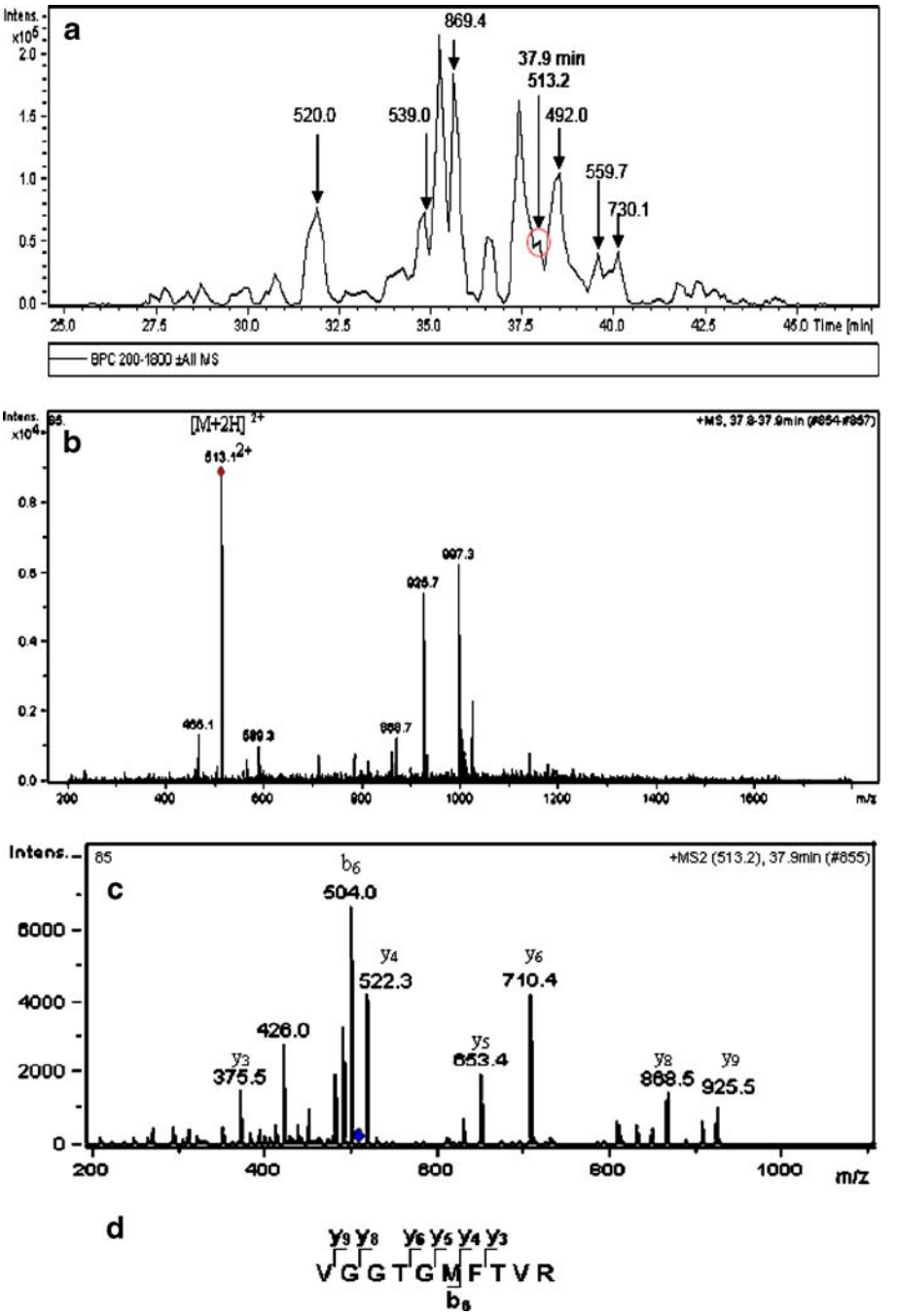

Fig. 3 Identification of THP precursor. (**a**) Base peak chromatogram. (**b**) A full scan MS spectrum. (**c**) A full scan MS/MS spectrum of 513.2 peptide ion. (**d**) The peptide amino acid sequences derived from the full scan MS/MS spectrum in (**c**)

Table 1 List of urinary protein detected; healthy subjects (HS), patients diagnosed with renal calculi for the first time (RC), recurrent renal calculi (RRC).

Healthy subjects (HS)		
Molecular weight (kDa)	SWISS-PROT accession number	Down-regulated protein
97	P07911	Uromodulin precursor (THP)
61	P02768	Serum albumin precursor
26	(1ADQL)	Rheumatoid factor—an IgM lambda Fab, chain L
Patients diagnosed with renal calculi for the first time (RC)		
Molecular weight (kDa)	SWISS-PROT accession number	Up-regulated protein
97	P07911	Uromodulin precursor (THP)
50	(CAD19027)	Sequence 31 from patent EP115804
	Q569J1	Immunoglobulin alpha heavy chain 1
	Q16167	Serum albumin fragment
	P01859	Ig gamma-2 chain C region
	P01860	Ig gamma-3 heavy chain disease proteins
	(AAR32446)	Ig heavy chain variable region
	P01861	Ig heavy chain constant region gamma 4 (fragment)
	Q9UGP3	Immunoglobulin heavy chain (fragment)
	P01825	Ig heavy chain Fab fragment, antibody a5b7, chain B
	Q6P005	Hypothetical protein
38	P01743	Ig heavy chain V region precursor
26	Q6GMW0	Ig kappa V 1-5 protein
Recurrent renal calculi (RRC)		
Molecular weight (kDa)	SWISS-PROT accession number	Up-regulated protein
97	P07911	Uromodulin precursor (THP)
75	P02787	Transferrin precursor
61	P02768	Serum albumin precursor
	P01857	Ig gamma-1 chain C region
	(1IGAA)	Ig alpha 1 chains a and b, heavy, chains c and d, light, c
	Q5TAN9	KIAA0266 protein
50	P01009	Alpha-1-antitrypsin precursor
	(AAR32446)	Ig heavy chain variable region
	(CAD19027)	Sequence 31 from patent EP115804
	Q569J1	Immunoglobulin alpha heavy chain 1
	P01859	Ig gamma-2 chain C region
	P01860	Ig gamma-3 heavy chain disease proteins
	P01743	Ig heavy chain V region precursor
	P01861	Ig heavy chain constant region gamma 4 (fragment)
	Q9UGP3	Immunoglobulin heavy chain (fragment)
	P01825	Ig heavy chain Fab fragment, antibody a5b7, chain B
	Q6MZV7	Hypothetical protein
	Q6P005	Hypothetical protein

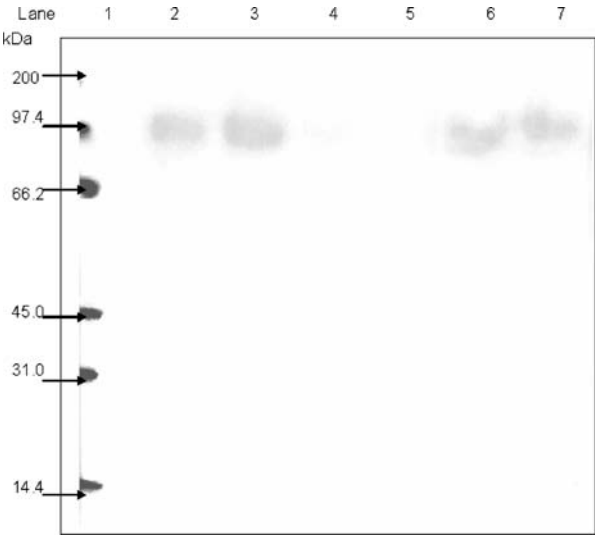

Fig. 4 Immunoblot of standard THP, healthy subject (HS), patient diagnosed with renal calculi for the first time (RC) and recurrent renal calculi (RRC). *Lane 1* prestain protein markers, *lane 2* standard THP, *lane 3* HS, *lanes 4–5* RC, *lanes 6–7* RRC

THP has been the urinary marker candidate that is widely studied in renal calculi disease. THP has shown to be both inhibitor and promoter for renal calculus formation [17, 18]. THP inhibits calculus formation by coating crystals, which thus inhibits the attachment of new crystals and prevents crystal growth and aggregation. However, self-aggregation and polymerization of THP occur in high calcium concentration, high ionic strength and low pH environment [19] that promotes calculus formation by forming a mesh to which crystals can adhere, thereby initiating crystal growth, although claims that THP does not effect calculus formation also can be found [20]. Defective THP may enhance the self-polymerization that promotes calculus formation [21].

THP excretion is reduced remarkably in patients with renal calculi [6, 22], making it a good biomarker candidate for detection of renal calculi disease. In this study, we evaluate the usefulness of THP as biomarker for diagnosis of renal calculi disease. We found that

Table 2 The age range and the intensity of THP in healthy subjects (HS), patient diagnosed with renal calculi for the first time (RC) and recurrent renal calculi (RRC).

	Number of sample	Average age (Age range)	Intensity of THP (Intensity×mm)[a]
Healthy subjects (HS)	50	34.7±2.6 (18–84)	5,269.08±314.34
Male	28	45.6±3.5 (22–84)	4,997.09±409.64
Female	22	20.7±0.4 (18–23)	5,615.25±489.08
Renal calculi (RC)	30	52.4±2.2 (28–74)	184.67±30.11*
Male	19	54.5±2.8 (36–74)	182.77±38.82*
Female	11	48.8±3.7 (28–68)	187.94±49.86*
Recurrent renal calculi (RRC)	35	51.5±2.4 (28–80)	2,454.49±244.34
Male	20	50.3±2.9 (25–70)	1,894.24±229.71
Female	15	53.1±4.2 (26–80)	3,201.48±416.03

Comparisons between groups were performed by Student's t-test. All values were presented as mean±SE

[a] As measured in SDS-PAGE

*$p<0.05$ (significant compared with HS)

THP can serve as a good marker candidate only in the RC, whereby its excretion is significantly lower ($p<0.05$) if detectable in this cohort. Nevertheless, it is not a suitable biomarker for the RRC, its excretion in this group of patients was indistinguishable from those of the HS. Thus, the usability of this protein is restricted to only the RC, whom has no previous record of calculus formation.

In the search for other biomarker candidates, we found certain urinary proteins, i.e., albumin, transferrin, and alpha-1-antitypsin precursor were excreted at higher levels in RRC, if detectable in RC. Albumin is the major urinary protein excreted in the calculi patients and it may involve in calculus formation [23], although it was reported to be a potent inhibitor to calculus formation [24]. Albumin also involved in regulating the composition of the calculus protein matrix [25], although Grover and Ryall [26] concluded that albumin influenced neither mineral deposition nor calculus formation. Transferrin was said to be involved in calculus formation as it was detected in the renal calculus protein matrix [25, 27]. In healthy subjects, 99.5% of excreted transferrin is reabsorbed and hence, very low amounts of transferrin were present in urine. Alpha-1-antitrypsin precursor acts as a co-precipitating substance in renal calculus formation [25, 28]. In our present study, we see the potential of these three proteins as biomarkers for RRC.

Most of the urinary proteins identified in RC and RRC were derived from immunoglobulins (Ig). These proteins were present solely in the urine of the renal calculi patients. The high level of immune-response proteins identified in renal calculi patients implies tubular injuries or damage caused by calculi lodging in the kidney. Tubular cell injuries allow crystal attachment to the cell lining [29] that leads to the excretion of large amount of IgG [30] and low MW proteins in the urine as a result of impaired reabsorption [31]. Several hypothetical proteins identified probably are immune-response proteins since they have immunoglobulin domains for antigen binding. The potential of the immunoglobulins that were common in RC and RRC but absent in HS is worth further investigation. The common immunoglobulins found in RC and RRC were Ig alpha heavy chain 1 (Q569J1), Ig gamma-2 chain C region (P01859), Ig gamma-3 heavy chain disease proteins (P01860), Ig heavy chain variable region (AAR32446), Ig heavy chain constant region gamma-4 (fragment) (P01861), Immunoglobulin heavy chain (fragment) (Q9UGP3) and Ig heavy chain Fab fragment, antibody a5b7, chain B (P01825) while Ig alpha 1 chains a and b, heavy chain c and d, light c (1IGAA) were detected only in RRC.

THP is a good biomarker for distinguishing RC from HS, but it is not a suitable biomarker for RRC. We foresee the potential of a few immunoglobulins, which were found in the urine of RC and RRC, but were absent in the urine of HS.

Acknowledgments We thank the staff and patients of the urology clinic, Penang General Hospital, Lam Wah Ee hospital and the students and staff of the School of Pharmaceutical Sciences, USM for providing the urine specimens for this study.

References

1. Barbas, C., García, A., Saavedra, L., & Muros, M. (2002). Urinary analysis of nephrolithiasis markers. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, *781*(1–2), 433–455. doi:10.1016/S1570-0232(02)00557-3.
2. Hess, B., & Kok, D. J. (1996). Nucleation, growth, and aggregation of crystals. In F. L. Coe, M. J. Favus, C. Y. Paak, J. H. Parks, & G. M. Preminger (Eds.), Kidney stones. Medical and surgical management. Philadelphia, PA, USA: Lippincott-Raven.

3. Bihl, G., & Meyers, A. (2001). Recurrent renal stone disease—advances in pathogenesis and clinical management. *Lancet*, *358*, 651–656. doi:10.1016/S0140-6736(01)05782-8.
4. Anne, L. T., & Gill, R. (1999). Isoelectric focusing of native urinary uromodulin (Tamm–Horsfall protein) shows no physicochemical differences between stone formers and non-stones formers. *Urological Research*, *27*, 250–254. doi:10.1007/s002400050118.
5. Lafitte, D., Dussol, B., Andersen, S., Vazi, A., Dupuy, P., Jensen, L. N., Berland, Y., & Verdier, J. M. (2002). Optimized preparation of urine sample from two-dimensional electrophoresis and initial application to patient samples. *Clinical Biochemistry*, *35*, 581–589. doi:10.1016/S0009-9120(02)00362-4.
6. Tiselius, H. G. (Ed.).(1996) The patient with renal stone disease. Dallas: Millet.
7. Trewick, A. I., & Rumsby, G. (1999). Isoelectric focusing of native urinary uromodulin (Tamm–Horsfall protein) shows no physicochemical differences between stone formers and non-stone formers. *Urological Research*, *27*, 250–254. doi:10.1007/s002400050118.
8. Pieper, R., Gatin, C. L., McGrath, A. M., Makusky, A. J., Mondal, M., Seonarain, M., Field, E., Schatz, C. R., Estock, A. M., Ahmed, N., Anderson, N. G., & Steiner, S. (2004). Characterization of the human urinary proteome: a method for high-resolution display of urinary proteins on two-dimensional electrophoresis gels with a yield of nearly 1,400 distinct protein spots. *Proteomics*, *4*, 1159–1174. doi:10.1002/pmic.200300661.
9. Smith, G., Barratt, D., Rowlinson, R., Nickson, J., & Tonge, R. (2005). Development of a high-throughput method for preparing human urine for two-dimensional electrophoresis. *Proteomics*, *5*, 2315–2318. doi:10.1002/pmic.200401267.
10. Kshirsagar, B., & Wiggins, R. C. (1986). A map of urine proteins based on one-dimensional SDS-polyacrylamide gel electrophoresis and Western blotting using one microliter of unconcentrated urine. *Clinica Chimica Acta*, *15*, 13–22. doi:10.1016/0009-8981(86)90111-7.
11. Graves, P. R., & Haystead, T. A. J. (2002). Molecular biologist's guide to proteomics. *Microbiology and Molecular Biology Reviews*, *66*, 39–63. doi:10.1128/MMBR.66.1.39-63.2002.
12. Doonan, S. (1996). Protein purification protocols. In S. Doonan (Ed.), Bulk purification by fractional precipitation (pp. 135–150). Totawa, New Jersey: Methods in molecular biology.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *The Journal of Biological Chemistry*, *193*, 265–275.
14. Towbin, H., Staehelin, T., & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America*, *76*, 4350–4354. doi:10.1073/pnas.76.9.4350.
15. Gam, L.-H., Leow, C.-H., Man, C.-N., Gooi, B.-H., & Manjit, S. (2006). Analysis of differentially expressed proteins in cancerous and normal colonic tissues. *World Journal of Gastroenterology*, *12*(31), 4973–4983.
16. Baldwin, M. A. (2004). Protein identification by mass spectrometry. *Molecular & Cellular Proteomics*, *3*, 1–9. doi:10.1074/mcp.R300012-MCP200.
17. Sikri, K. L., Foster, C. L., MacHugh, N., & Marshall, R. D. (1981). Localisation of Tamm-Horsfall glycoprotein in the human kidney using immuno-fluorescence and immuno-electron microscopical techniques. *Journal of Anatomy*, *132*, 597.
18. Hess, B., Nakagawa, Y., & Coe, F. (1989). Inhibition of calcium oxalate monohydrate crystal aggregation by urine proteins. *The American Journal of Physiology*, *257*, F99–F106.
19. Stevenson, F. K., & Kent, P. W. (1970). Subunits of Tamm–Horsfall glycoprotein. *The Biochemical Journal*, *116*, 791–791.
20. Sophasan, S., Chatasingh, S., Thanaphaichitr, P., & Dhanamitta, S. (1980). Tamm–Horsfall mucoprotein in urine of potential bladder stone formers. *The Journal of Urology*, *124*, 522–524.
21. Hess, B., Nakagawa, Y., Perks, J. H., & Coe, F. L. (1991). Molecular abnormality of Tamm–Horsfall glycoprotein in calcium oxalate nephrolithiasis. *The American Journal of Physiology*, *260*, F569–F578.
22. Bichler, K., Mittermuller, B., Strohmaier, W. L., Feil, G., & Eipper, E. (1999). Excretion of Tamm–Horsfall protein in patients with uric acid stones. *Urologia Internationalis*, *62*, 87–92. doi:10.1159/000030364.
23. Pourmand, D., Nasseh, H., Sarrafnejad, A., Mojtahedi, , Mehrsai, A., Hamidi-Alamdari, D., & Nourijelyani, K. (2006). Comparison of urinary protein in calcium stone formers and healthy individuals: a case-control study. *Urologia Internationalis*, *76*, 163–168. doi:10.1159/000090882.
24. Chen, W.-C., Lin, H.-S., Chen, H.-Y., Shih, C.-H., & Li, C.-W. (2001). Effects of Tamm–Horsfall protein and albumin on calcium oxalate crystallization and importance of sialic acids. *Molecular Urology*, *5*, 1–5. doi:10.1089/109153601750124186.
25. Dussol, B., Geider, S., Lilova, A., Leonetti, F., Dupuy, P., Daudon, M., Berland, Y., Dagorn, J. C., & Verdier, J. M. (1995). Analysis of the soluble organic matrix of five morphologically different kidney

stone. Evidence for a specific role of albumin in the constitution of the stone protein matrix. *Urological Research*, *23*, 45–51. doi:10.1007/BF00298850.
26. Grover, P. K., & Ryall, R. L. (1999). Inhibition of calcium oxalate crystal growth and aggregation by prothrombin and its fragments *in vitro*: relationship between protein structure and inhibitory activity. *European Journal of Biochemistry*, *263*, 50–56. doi:10.1046/j.1432-1327.1999.00448.x.
27. Fraij, B. M. (1989). Separation and identification of urinary protein and stone-matrix proteins by mini-slab sodium dodecyl sulfate–polyacrylamide gel electrophoresis. *Clinical Chemistry*, *35*, 658–662.
28. Umekawa, T., Kohri, K., Amasaki, N., Yamate, T., Yoshida, K., Yamamoto, K., Suzuki, Y., Sinohara, H., & Kurita, T. (1993). Sequencing of a urinary stone protein, identical to alpha-one antitrypsin, which lacks 22 amino acids. *Biochemical and Biophysical Research Communications*, *193*, 1049–1053. doi:10.1006/bbrc.1993.1731.
29. Tiselius, H.-G., Ackermann, D., Hess, B., & Boevé, E. (2002). Stone disease: diagnosis and medical management. *European Urology*, *41*, 1–11. doi:10.1016/S0302-2838(02)00045-3.
30. Bakoush, O., Grubb, A., Rippet, B., & Tencer, J. (2001). Urine excretion of protein HC in proteinuric glomerular diseases correlates to urine IgG but not to albuminuria. *Kidney International*, *60*, 1904–1909. doi:10.1046/j.1523-1755.2001.00018.x.
31. Bazzi, C. (2003). Composition of proteinuria in primary glomerulonephritides: association with tubulo-interstitial damage, outcome and response to therapy. *Giornale Italiano di Nefrologia*, *20*(4), 346–355.