IDENTIFICATION OF BIOMARKER AND DEVELOPMENT OF SCREENING METHOD FOR KIDNEY STONE DISEASE

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IDENTIFICATION OF BIOMARKER AND DEVELOPMENT OF SCREENING METHOD FOR KIDNEY STONE DISEASE

by

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ABBREVIATIONS

ACN	: Acetonitrile
BPH	: Benign prostatic hyperplasia
CID	: Collision induced dissociation
C.V.	: Coefficient of variation
DMSO	: Dimethyl sulfoxide
DTT	: 1,4-Dithioreitol
EDTA	: Ethylenedisminetetra-acetic acid
ELISA	: Enzyme-linked immunosorbent assay
ESI	: Electrospray ionization
EWSL	: Extracorporeal shock ware lithotripsy
IVU	: Intravenous urography
kDa	: kilo Dalton
KUB	: Plain abdominal radiography
m/z	: Mass to charge
MS	: Mass spectrometry
MS/MS	: Tandem mass spectrometry
MW	: Molecular weight
PAGE	: Polyacrylamide gel electrophoresis
PBS	: Phosphate buffer saline
PNCL	: Percutaneous nephrolithotomy
RP-HPLC	: Reversed phase liquid chromatography
SDS	: Sodium dodecyl sulfate

TEMED	: *N,N,N',N'*-tetramethyethylenediamine
THP	: Tamm Horsfall glycoprotein
TMB	: 3,3'5,5' Tetramethylbenzine
TSE buffer	: 10 mM Tris, 1% SDS and 1 mM EDTA at pH 8.8
TEA buffer	: 0.5% Triton X -100, 20 mM EDTA at pH 7.4
WGA	: Wheat germ agglutinin

PENGENALPASTIAN PENANDA BIOLOGI DAN PEMBANGUNAN KAEDAH PENYARINGAN UNTUK PENYAKIT BATU GINJAL

ABSTRAK

Penyakit batu ginjal adalah ganguan urologi yang paling biasa berlaku di kalangan lelaki dan wanita tetapi dengan lebih tinggi kelaziman di kalangan lelaki. Peluang masa hayat bagi seseorang mempunyai satu batu ginjal adalah lebih kurang 10% dan risiko ulangan dalam tempoh masa 10 tahun adalah 74%. Dengan itu, terdapat satu keperluan yang besar bagi membangunkan suatu kaedah penyaringan untuk pengesanan penyakit batu ginjal.

Dalam kajian ini, pendekatan proteomik digunakan untuk pengekstrakan, pengasingan dan pengenalpastian protein daripada subjek sihat, pesakit batu ginjal dan pesakit batu ginjal rekuren. Protein daripada urin diestrak dengan menggunakan teknik pemendakan garam dan estrak protein itu dilarutkan dalam penimbal pelarut. Campuran tersebut diasingkan mengikut jisim molekul masing-masing dengan menggunakan SDS-PAGE. Gel tersebut seterusnya diwarnakan dengan Coomassie Biru. Imej gel itu direkod dan dianalisis dengan menggunakan satu sistem pengimejan. Jalur-jalur sasaran protein dipotong daripada gel dan protein tersebut dicernakan oleh tripsin secara Dalam-gel. Peptida-peptida triptik seterusnya dikeluarkan daripada gel dan dianalisis dengan menggunakan LC/MS/MS, ia membolehkan penjujukan asid amino bagi peptida-peptida yang dianalisis. Spektrum produk ion yang diperolehi daripada MS/MS dicari dengan menggunakan enjin pencarian pengkalan data protein Mascot untuk pengenalpastian protein. Sejumlah dua puluh sembilan jenis protein telah dikenalpastikan daripada subjek sihat, pesakit batu ginjal dan pesakit batu ginjal rekuren. THP daripada urin dikenalpastikan sebagai satu

penanda biologi untuk pesakit batu ginjal dan telah disahkan oleh Western blotting.

Dengan mengguna THP sebagai penanda, kaedah-kaedah SDS-PAGE dan ELISA dibangunkan untuk kuantifikasi THP dalam urin. Bagi analisis SDS-PAGE, sampel urin dimendak dengan garam dan dipekat sepuluh kali. Nilai penggalan bagi perkumuhan THP dalam urin untuk SDS-PAGE adalah 1.30 mg/mmol. Walau bagaimanapun, sampel urin untuk analisis ELISA dicairkan sepuluh kali dalam penimbal sampel. ELISA yang dibangunkan mencapai kelinearan antara julat 109.33 µg/mL sehingga 945.67 µg/mL. Tambahan lagi, ketepatan asai adalah antara 98 – 101%. Kepersisan asai adalah kurang daripada 4% (C.V.) bagi kebolehulangan dan kurang daripada 5% (C.V.) bagi kebolehasilan. Kespesifikan dan kepekaan asai masing-masing adalah 86% dan 80%. Nilai penggalan bagi kepekatan THP dalam urin untuk ELISA adalah 37.00 µg/mL dan 41.20 µg/mL masing-masing bagi lelaki dan perempuan.

SDS-PAGE menunjukkan satu penggalan yang lebih jelas antara subjek sihat dan pesakit batu ginjal apabila dibandingkan dengan ELISA. SDS-PAGE adalah kaedah kuantitatif yang lebih baik untuk menentukan amaun THP dalam urin.

IDENTIFICATION OF BIOMARKER AND DEVELOPMENT OF SCREENING METHOD FOR KIDNEY STONE DISEASE

ABSTRACT

Kidney stone disease is the most common urological disorders that occurred in both men and women but with higher prevalence in men. The lifetime chance of an individual having a stone in kidney is approximately 10% and the risk of recurrence during 10 years period is 74%. Therefore, there is a great need to develop a screening method for detection of kidney stone disease.

In this study, proteomic approach was used for extraction, separation and identification of urinary proteins from healthy subjects, stone formers and recurrent stone formers. Urinary proteins were extracted using salt precipitation technique and the protein extract was dissolved in solubilizing buffer. The mixture was separated according to their molecular weights using SDS-PAGE. The gel was then Coomassie Blue stained. The image of the gel was captured and analyzed by using an imaging system. The target protein bands were excised from the gel and proteins were digested In-gel by trypsin. The tryptic peptides were then eluted from the gel and analyzed using LC/MS/MS which allows amino acid sequencing of the analyzed peptides. The acquired MS/MS product ions spectrum was search against Mascot protein database search engine for protein identification. A total of twenty nine proteins were identified from healthy subjects, stone formers and recurrent stone formers. The urinary THP was identified as a biomarker for kidney stone disease and was confirmed by Western blotting.

Employing THP as biomarker, SDS-PAGE and ELISA methods were developed for urinary THP quantification. For SDS-PAGE analysis, the urine sample was salt precipitated and concentrated ten times. The cut-off value of THP excretion by SDS-PAGE was 1.30 mg/mmol. However, urine sample for ELISA analysis was diluted ten times in sample buffer. The developed ELISA achieves linearity within the range of 109.33 ng/mL to 945.67 ng/mL of THP. In addition, the assay accuracy was around 98 – 101%. The assay precisions were less than 4% (C.V.) for repeatability and less than 5% (C.V.) for reproducibility. The assay specificity and sensitivity were 86% and 80%, respectively. Whilst the cut-off values of THP concentration by ELISA were 37.00 µg/mL and 41.20 µg/mL for male and female, respectively.

SDS-PAGE shows a clearer cut-off between healthy subjects and stone formers as compared to ELISA. SDS-PAGE is a better method to quantify the amount of urinary THP.

CHAPTER ONE

INTRODUCTION

1.1 Kidney Stone Disease

Kidney stone disease or known as nephrolithiasis is a common problem especially in industrialised nations. It is defined as one or more stones namely aggregate of crystals mixed with protein matrices, which are formed in the kidney or in the ureter (Figure 1.1). This may cause obstruction of urine flow in the renal collecting system, ureter or urethra which causes severe pain, bleeding or local erosion of kidney tissue (Tiselius *et al.*, 2002).

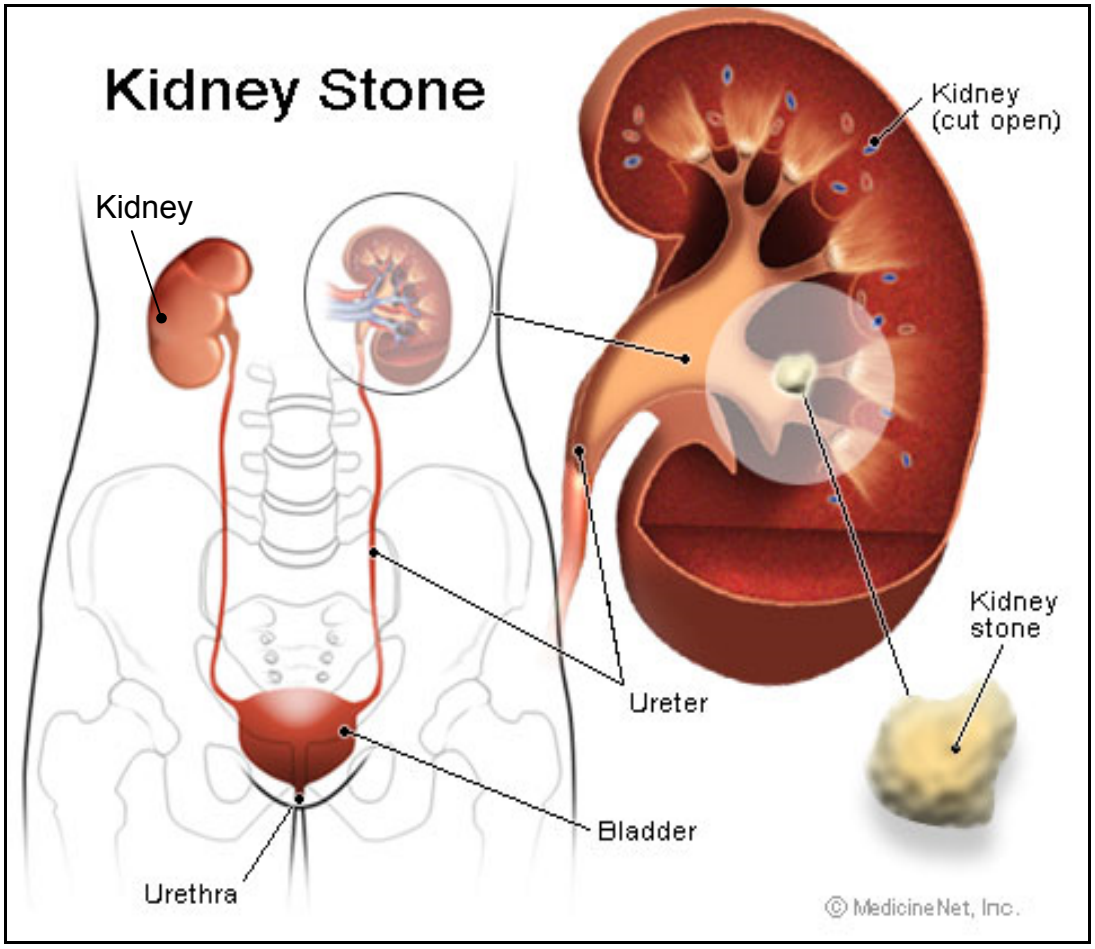

Figure 1.1: Formation of kidney stone in the kidney and ureter (MedicineNet.com, 2005).

1.1.1 Epidemiology of Kidney Stone Disease

Kidney stone disease affects 1 - 20% of the general population. According to data provided by the U.S. National Health Interview Survey (1990 - 1992) approximately 1 million people suffered from stone disease yearly. In Asia, its lifetime incidence is 2 - 5% (Barbas *et al.*, 2002).

The incidence and prevalence of kidney stone also varies in proportion to age, race and gender. Kidney stone disease afflicts both men and women but with higher prevalence in men than in women. The lifetime chance of an individual having a stone is 10 - 15% and the peak age of onset is 20 - 30 years old (Sandhu *et al.*, 2003b). The risk of recurrence is 74% within 10 years for the first-time stone formers (Lewandowski and Rodgers, 2004) and therefore increasing the risk of permanent kidney damage despite modern techniques of stone removal.

1.1.2 Aetiology and Pathogenesis

Formation of stone in kidney is a complex and multifactorial process. It is a crystallization process taking place in supersaturated urine, where the urine substances form crystals that stick together and subsequently grow into stones on the inner surface of the kidney (Hess and Kok, 1996). There are four types of stones namely calcium stones, struvite stones, uric acid stones and cystines stones. Calcium stones are the most predominant that compose 75% of all stones (Bihl and Meyers, 2001). The recurrence of calcium stone is greater than other types of kidney stones.

A number of chemical and physical factors are known as active participants in stone formation. There are three main factors relevant to stone

formation: supersaturation of urine with stone constituents, urinary matrix as well as concentration of inhibitors and promoters of crystal aggregation (Anne and Gill, 1999). In theory, when the concentration of stone constituents (calcium, oxalate and phosphate) reached saturation state, stone formation will be induced by association of small amounts of crystalloid to form nuclei (nucleation). The nuclei grows and aggregates on the surfaces of collecting ducts and renal papillary epithelium (Bihl and Meyers, 2001; Tiselius, 1996). Renal epithelial cells specifically bind and internalize the crystal aggregates. Events that occur after crystal aggregates binding could be important in pathogenesis of stone i.e. cellular responses might be essential for the initiation of stone formation (Barbas *et al.*, 2002).

Stone formation is inhibited by substances in urine that prevent crystallization. However it will only occur once the stone salts were exceeded. Saturation of urine with calcium oxalate is common in the population; therefore the role of other factors in the formation of stone must be crucial (Bihl and Meyers, 2001). For instance, although most people will achieve urinary supersaturation at some time yet only some will form kidney stone. The role of lithogenic risk factors including promoters and inhibitors is to predispose stone formers or to protect non-stone formers. Other important factor is uric acid which can precipitate persistently in acidic urine even in the absence of hyperuriceamia (an excess of uric acid in blood) or hyperuricosuria (an excess of uric acid in urine) (Barbas *et al.*, 2002). Catalyst-like ability of uric acid can cause formation of calcium oxalate stone without being incorporated into the crystals (Bihl and Meyers, 2001; Srinivasan *et al.*, 2005). Likewise, urinary pH has an essential part in many inhibitor or promoter reactions (Tiselius, 1981).

Urine normally contains inhibitors (e.g., pyrophosphate, citrate and macromolecules) that prevent crystal growth. Macromolecular factors such as chondroitin sulphate and haparin are inhibitors to crystal aggregation (Tiselius, 1996). Therefore, insufficient quantity of inhibitors that prevent crystallization or inhibitors fails to induce the necessary chemical reactions that prevent the formation of crystals is the initial step in the development of kidney stone. A number of urinary macromolecules have been investigated as potential inhibitor, one of which is Tamm Horsfall Glycoprotein (THP) or uromodulin (Schnierle *et al.*, 1996).

1.1.3 Clinical Presentation

Kidney stone develops slowly and asymptomatic or silent until they begin to move down the urinary tract, producing either hematuria or some degree of urinary obstruction. It also causes pain which is the classic symptom associated with lodged stone (Bihl and Meyers, 2001). In acute ureteral colic, the pain is severe and often starts in the flank region and moves down to the groin. It may radiate to the groin or testes depending on the location of obstruction. In addition, nausea, vomiting, chills, fever and elevated blood pressure are common. The other classic symptoms are frequent urinate and dark urine. Complications of kidney stone are recurrence of stones, urinary tract infection, ureter obstruction, kidney damage and decrease or loss of function of the affected kidney (Sandhu *et al.*, 2003b).

1.1.4 Diagnosis

Documentation of stone characteristic is extremely important (type, size and location) for therapeutic management of kidney stone disease. Preliminary evaluation should include urinalysis, urine culture and plain film of the abdomen (Bihl and Meyers, 2001). Diagnostic imaging like Plain Abdominal Radiography (KUB), Intravenous Urography (IVU) and ultrasound are used to confirm that the symptoms are caused by stone in the case of symptomatic kidney stone disease. X-ray or ultrasound can help to locate the stone and determine its size and shape. More than two-thirds of kidney stones are radiopaque and can be seen on radiogram. Not all radiopaque stones (up to 34%) can be seen on an x-ray or ultrasound; some are too small and others may be obscured by bones or overlying bowel gas. Uric acid stones are particularly radiolucent because they do not absorb enough x-rays therefore must be detected by other means (Sandhu *et al.*, 2003b).

KUB is irreplaceable in the management of a known radiopaque ureteric calculus especially in planning extracorporeal shock ware lithotripsy (ESWL) or monitoring the progress of the stone fragments after ESWL. It is also more reliable for assessing stone status (Lingeman, 1996).

IVU remains the first-line investigation for suspected ureteric calculus. The radiographic findings are both simple to interpret and highly accurate in diagnosing renal obstruction. For stones in the pelvicalyceal system, its size and local anatomy can clearly defined. Although there is a risk of allergy and contrast nephropathy, IVU remains the gold standard for such identification (Bihl and Meyers, 2001). Ultrasound also used in management of stone, which can

indicate whether a stone is in the kidney or ureter and degree of any obstruction. Stone size can be measured directly on ultrasound (Sandhu *et al.*, 2003b).

Biochemical test such as blood and urine can identify the presence of infection and establish the stone's chemical composition. The presence of urinary crystals or minute amounts of blood (microhematuria) in microscopic examination, either finding provides strong evidence of kidney stones (Parks and Coe, 1997).

Furthermore, for recurrence kidney stones including any with a radiologic evidence of new stone formation, an increase in size of a pre-existing stone or passage of stone in the past year. However, most of the kidney stone disease is idiopathic, indicating the absence of any identifiable clinical cause of the disease and without discernible biochemical and/or anatomical abnormality (Lewandowski and Rodgers, 2004).

1.1.5 Management of Kidney Stone Disease

Management of kidney stones depends on the clinical presentation, stone location and size (Bihl and Meyers, 2001). With an acute presentation, the presence of complication such as secondary infection or renal impairment may necessitate immediate intervention. While, uncomplicated cases can be managed conservatively with adequate fluids and analgesia. In both cases, if the stone does not pass spontaneously, definitive stone treatment is required and is often performed as delayed and elective procedure (Sandhu *et al.*, 2003a). Blockage of urine flow requires emergency drainage that can be achieved by placement of retrograde stent when the kidney stone fails to pass.

The advances in urological techniques have drastically altered management of patients with symptomatic kidney stones disease who require treatment. Most symptomatic upper urinary tract stones are small and occur in normal kidneys and pass spontaneously if it is less than 5 mm in diameter. However, up to 80% of stones are greater than 5 mm in diameter and are needed to be removed by surgery or treated with ESWL (Lingeman, 1996). The percutaneous nephrolithotomy (PNCL) (Sandhu *et al.*, 2003a) approach is best suited for removal of large and complex stones, whereas ESWL is a non-surgical technique that uses high energy shock wave to break the large stones into small fragments that will pass through urinary system (Erhard *et al.*, 1996).

1.2 Organic Matrix of Kidney Stones

Kidney stones compose of an outer crystalline phase which mostly contains urate, calcium, oxalate and phosphates. The inner core of the stone contains organic stone matrix, which derived from a variety of sources including macromolecules of kidney and serum origin, exfoliated epithelial cells, cells originating in blood and urinary proteins (Khan and Hackett, 1993).

One of the main theories concerning stone formation in the kidney is matrix theory (Van Aswegen and Du Plessis, 1991). According to the matrix theory, urinary proteins such as uromucoid promote precipitation of calcium oxalate and/or calcium phosphate crystals. Urinary proteins may act as binding surface for inorganic ions and thus provide a framework for deposition of stone salts (calcium, oxalate, phosphate and etc). Thus, the concentration of urinary proteins may play an integral role in stone formation.

Organic matrix of kidney stone has long been investigated yet its functional significant relationship to the kidney stone development remains obscure. A better understanding of the components of stone matrix and their interaction on the molecular level seems mandatory in order to gain better insight of stone formation. The effects of specific urinary macromolecules on calcium oxalate crystallization have been evaluated by Rose and Sulaiman (1982) and Drach *et al*. (1982). The authors explained that mucoprotein and mucopolysaccharides may act as promoters for stone formation and as enhancers for nucleation because of their ability to bind calcium. The organic matrix of the kidney stone accounts for 2 to 3% of total stone weight as reported by Sugimoto *et al*. (1985). Some matrix components play significant role in stone formation while others do not. The organic matrix of kidney stone mainly contains macromolecules derived from urine, which may serve as inhibitors to the formation of stone. There are variety of urinary macromolecules presence in urine and organic matrix of stone. The macromolecules reported were nephrocalcin (14 kDa), fibronectin (230 kDa), prothrombin fragment 1 (32 kDa), osteopontin (67 kDa) and THP (95 kDa). Thus, urinary macromolecules were suggested to be the major component of the organic matrix of kidney stones (Govindaraj and Selvam, 2002). Nevertheless, most of the stone proteins have not been fully characterized, which is due to the difficulties in isolating them in their native form. Although various methods including gel permeation chromatography and high performance liquid chromatography for the separation of proteins from organic stone matrix have been carried out, these methods could not completely isolate the proteins. Consequently, a complete profile of renal stone proteins is not available to date.

1.3 Urinary Proteins of Kidney Stone Disease

Human urine is formed in kidney via ultrafiltration from the plasma to eliminate waste products such as urea and metabolites. Components in the ultrafiltrate are selectively reabsorbed result in less than 1% of ultrafiltrate is excreted as urine. Urine production under normal physiologic conditions is 1 - 2 L/day however it also depends on fluid intake (Adachi *et al.*, 2006).

The glomerular filtrate has a similar composition of plasma except that it is almost free of proteins. Almost all the protein on the glomerular filtrate is reabsorbed and catabolized by proximal convoluted tubular cells, which result in the excretion of urinary protein less than 150 mg/24 hour (Adachi *et al.*, 2006; Christensen and Gburek, 2004). Thus, the amount of protein presence in urine is very low.

The concentration of urinary macromolecules that may serve as inhibitors or promoters is one of the main factors for formation of stone. The involvement of urinary macromolecules (urinary proteins) as inhibitors to the formation of stone was reported by researchers. Most of the urinary macromolecules are anionic with many acidic residues and frequently contain posttranslational modifications such as phosphorylation and glycosylation (Lafitte *et al.*, 2002). They exert inhibitory effects by binding to crystals and consequently inhibit the adhesion of crystals to renal epithelial cell. Amongst the urinary macromolecules reported are nephrocalcin (14 kDa), an acidic protein of tubular cells origin. Nephrocalcin in the kidney stone patients' urine is lacking of gamma-carboxyglutamic acid (GLA) residues which reduce its ability to inhibit crystals nucleation and calcium aggregation (Nakagawa *et al.*, 1987).

Fibronectin (230 kDa): it is a multifunctional alpha 2-glycoprotein that distributed throughout the extracellular matrix and body fluids. Recent studies have demonstrated that it is excreted from tubular cells and its secretion can be stimulated by calcium oxalate crystals (Tsujihata *et al.*, 2000). It may inhibit calcium oxalate crystals aggregation and their attachment to tubular cells (Tsujihata *et al.*, 2000).

Prothombin fragment 1 or crystal matrix protein (32 kDa): It is a peptide generated from sequential cleavage of prothrombin. There is an increasing evidence reported that urinary prothrombin fragment 1 plays a contrasting roles involved in early stage of stone formation as both the active participant and the inhibitor of calcium oxalate nucleation, growth and aggregation (Grover and Ryall, 1999).

Osteopontin or uropontin (67 kDa): In a crystal matrix, osteopontin has several biological functions including involvement in biomineralization and stone formation (Denhardt and Guo, 1993). Amino acid sequence of osteopontin contains a high proportion of aspartic acid residues, which binds calcium and is strongly associated with calcium oxalate crystals. Therefore, osteopontin has been suggested to be potent inhibitor to calcium oxalate crystallization and may also involve in crystal retention of crystallization in the kidneys (Fouad *et al.*, 1998).

Tamm Horsfall glycoprotein (THP), (85 kDa): THP may play an important role in stone formation and has great potential to serve as biomarker for detection of kidney stone disease (Schnierle *et al.*, 1996).

1.4 Tamm Horsfall Glycoprotein (THP)

In 1950, Tamm and Horsfall discovered a glycoprotein in urine, which was named as Tamm Horsfall Glycoprotein (THP). THP is the most abundant glycoprotein present in the urine of healthy subjects (Tamm and Horsfall, 1950). THP is secreted by the thick ascending limb of the loop of Henle (Gokhale *et al.*, 1996; Muchmore and Decker, 1985). THP inserted into luminal cell surface by the glycosyl-phosphatidylinositol (GPI)-anchor and then excreted in urine at a rate of 50 - 100 mg/day (Cavallone *et al.*, 2001; Kumar and Muchmore, 1990). Thus, THP is a glycosylphosphatidylinositol (GPI)-anchored protein.

Muchmore and Decker (1985) identified a 85 kDa uromodulin in the urine of pregnant women. Uromodulin is commonly known as THP (Pennica *et al.*, 1987). The author explained that its molecular mass, abundance in urine and characteristic resembled with THP (Muchmore and Decker, 1985). In addition, uromodulin was reported to possess the same amino acids structure of THP (Pennica *et al.*, 1987). However, its carbohydrate contents especially mannose chains and physiological function are slightly different from THP (Devuyst *et al.*, 2005).

1.4.1 THP Structure

THP is a monomeric glycoprotein of approximately 85 kDa but has a strong tendency to form macroaggregates of several million Daltons. It has 639 amino acids and 48 cysteine residues. In addition, it contains about 30% carbohydrate and heavily glycosylated by polyantennary sialated N-linked glycans (Fletcher *et al.*, 1970; Pennica *et al.*, 1987). The sialic acid may play an

important role to maintain THP function in kidney stones formation (Knörle *et al.*, 1994).

1.4.2 Physiological Function

The physiological function of THP still remains unclear. It has been suggested that it may play an important role in maintaining water permeability of the thick ascending limb of the loop of Henle due to its gel forming properties (Menozzi *et al.*, 2002). It may also act as an adhesion molecule and involve in stone formation where it is present in the core of the stones (Tamm and Horsfall, 1950). It may also protect urinary system from uropathogens by inhibiting bacterial adherence. As explained by Pak *et al*. (2001), THP exerts a protective role against *Escherichia coli* colonization by competing for glycans carried by membrane glycoproteins of the urinary tract, e.g., uroplakin. According to this hypothesis, the infectious disease in urinary tract could have acted as selective agents for the large release of urinary THP from the kidney. Thus, THP is essential for the normal physiological function of kidneys.

1.4.3 The Role of THP Glycomoiety in Renal Disease

THP glycomoiety has been indicated to be responsible for the binding with adhesins of pathogenic strains of *Escherichia coli* (Pak *et al.*, 2001), whereby prevent an individual from infectious disease. In addition, THP glycomoiety has been proposed to be involved in the renal diseases. There are differences in the carbohydrate composition of the THP isolated from renal disease patients and from the healthy subjects (Olczak *et al.*, 1999a), where THP isolated from the patients has alternation of its oligosaccharide chains that

affects its biological activity. Olczak *et al.* (1999b) described that THP of renal disease patients reacted more weakly with lectins, which is specific for terminal oligosaccharides. In addition, THP has been reported to be responsible for pathological conditions such as tubulointerstitial nephritis, recurrent calcium oxalate stone and nephropathy due to its ability to precipitate with other proteins (Olczak *et al.*, 1999a; Olczak *et al.*, 1999b).

1.4.4 The Role of THP on Stone Formation

In relation to kidney stone disease, THP has been shown to be present in the core of calcium oxalate kidney stone as reported by Grant *et al*. (1973). However, it is not clear whether it is a passive support or an active participant in stone formation. The findings of crystallization assays that developed with the aim of elucidating the role of THP on stone production are controversial. Scurr and Robertson (1986) explained that THP might acts as an inhibitor or promoter of stone formation as previously proposed by Rose *et al*. (1982). However, Sophasan *et al.* (1980) claimed that THP has no effect in stone formation.

THP was implicated as both an inhibitor and promoter of stone formation (Hess *et al.*, 1989; Sikri *et al.*, 1981). Its inhibitory properties arose by coating crystals, retarding the attachment of new crystals and thus preventing crystals growth and aggregation. However, self-aggregation and polymerisation of THP is caused by high calcium concentration, high ionic strength and low pH (Stevenson and Kent, 1970), which allow the protein to act as a promoter by forming a mesh to which crystals adhere and thus initiate crystal growth .

Hess *et al.* (1989) described that THP is the major inhibitor of calcium oxalate crystal aggregation in the urine of healthy subjects. However, stone formers excreted defective urinary THP that diminish its inhibitory effect on stone formation (Hess *et al.*, 1989). Taking into account those observations, it has been suggested that not only defective THP but a decreased THP excretion may facilitate the development of kidney stones. Nevertheless, the molecular basis of the urinary THP abnormality in stone formers needs further elucidation.

In the studies of Knörle *et al*. (1994), the authors reported that THP from the healthy subjects was more glycosylated with sialic acid than the recurrent stone formers. The terminal sialic acid is essential for the inhibitor function of THP in healthy subjects, however this function was diminished in the recurrent stone formers owing to their THP lack of sialic acid (Knörle *et al.*, 1994). In 2001, Chen *et al*. demonstrated that THP exhibits inhibitory effect on stone formation. Thereafter, Carvalho *et al.* (2002) agreed that the inhibitory effect of THP is related to sialic acid. Therefore, carbohydrate structure of THP is the major feature that may regulate kidney stone formation.

1.4.5 Assays Used for THP Measurement

In accordance with these preliminary studies, THP evidently is a good candidate that may act as biomarker for detection of kidney stone disease. Therefore, researchers have been ventured variety of highly sensitive and specific assays to quantify THP, which was considered as a potential biomarker for kidney stone disease. There are large numbers of assays developed. Amongst these are electroimmunodiffusion, gel electrophoresis, radioimmunoassay and enzyme-linked immunosorbent assay (ELISA).

In a study, Boyce and Swanson (1955) showed that the amount of urinary THP excreted was constant in the healthy subjects, however it was increased in the stone formers (Boyce and Swanson, 1955). Bichler *et al.* (1975) explained that the Boyce and Swanson's method using centrifuged and precipitated urine for THP measurement may lead to less reliable results. This is because aggregated and non-aggregated forms of THP have different solubility in salts solution and their proportions in urine are unknown (Bichler *et al.*, 1975).

As a consequent, the electroimmunodiffusion was developed by Bichler *et al*. (1975) and Samuell (1979) for THP measurement. Both of the authors reported that no significant difference between healthy subjects and stone formers. The average THP excretion in urine was 40 - 50 mg/day (Bichler *et al.*, 1975; Samuell, 1979). However, Bichler *et al.* (1999) described that there are significantly decrease of THP excretion in uric acid stones patients, staghorn calculi or renal tubular acidosis. The authors explained that damage of distal and proximal tubular epithelial cells in some of the stone formers who prone to develop uric acid stones.

A rapid and specific radioimmunoassay was developed by Hunt *et al.* (1985) for THP quantification. The authors reported that there were no differences between THP concentration in fresh and frozen urine samples after dialysis. However, freezing of dialysed urine before analysis can alter the protein structure and make the THP concentration more variable.

The THP quantification has been limited by the laborious and relatively insensitive assay. Pretreatment of urine samples were included before assay such as dialysis, gel filtration, ultracentrifugation, freezing, incubation with detergents and adjustment of pH (Dawnay *et al.*, 1982).

Therefore, Dawnay *et al.* (1982) investigated factors that affecting THP quantification in order to improve the developed radioimmunoassay. The THP concentration was affected by osmolality, THP concentration and pH of the urine. Consequently, the 24 hours urine sample was extensively diluted in water (100 dilutions) before assay. The author found that THP level was not affected by the variation in THP concentration and pH of urine. This indicated that soluble form of THP was attained (Dawnay *et al.*, 1982).

Lynn *et al.* (1982) determined the amount of urinary THP excretion by using radioimmunoassay. The authors claimed that the amount of THP excreted was not influenced by exercise, age, diuresis and amounts of calcium or sodium excreted (Lynn *et al.*, 1982).

Romero *et al.* (1997) developed an ELISA to quantify urinary THP concentration. The urine sample was frozen at -20^{o}C before assay and series of diluted urine sample were incubated overnight in the assay. The authors found that THP excretion in the recurrent stone formers was significantly decreased when comparing to healthy subjects.

Currently, there is a commercially available ELISA kit (Syn^{elisa} THP assay). The ELISA kit uses 24 hour urine sample for THP excretion measurement. Using this kit, Ganter *et al.* (1999) investigated the THP excretion and its correlation to citrate in urine of stone formers. Both are potent factors in the kidney stone forming process. THP excretion in stone formers was significantly lower as compared to healthy subjects (Ganter *et al.*, 1999). The authors found that THP excretion was positively correlated to citrate. Thus, the decrease of THP in stone formers may indicate tubular dysfunction (Lynn and Marshall, 1984).

Glauser *et al.* (2000) also used the commercial ELISA kits to investigate urinary excretion of THP and to identify possible determinants of urinary THP excretion in stone formers and healthy subjects. In both of the healthy subjects and stone formers, urinary THP excretion was related to body size, renal function and urinary citrate excretion whereas dietary habits do not affect THP excretion (Glauser *et al.*, 2000). THP excretion in uric acid stone formers and staghorn calculi were found lower than healthy subjects, In addition, the decrease of THP excretion in uric acid stone formers was reported by Bichler *et al.* (1999).

The major obstacle for accurate measurement of THP is aggregation of THP that exists in various polymeric forms in urine. Hence, Kobayashi and Fukuoka (2001) have tried to resolve the problem by solubilizing urinary THP using Triton X 100, EDTA and alkaline pH instead of conventional buffer such as phosphate buffer saline (PBS) prior to ELISA analysis. The authors showed that the combination of Triton X 100, EDTA and alkaline pH is the best solubilizing buffer for THP.

Although immunoassay is selective and sensitive, it relies on the availability of specific polyclonal or monoclonal antibodies. Therefore, a lectin affinity bioassay without using antibodies was developed by Topcu (2002) to quantify the cytolytic activity of THP. The author employed lectin in the assay; lectin was suggested to have strong interaction toward THP and therefore the binding is proportional to the amount of THP involved in cytolytic activity. In view of this finding, the specific interaction of lectin and THP indicating lectin was used as capture material for development of ELISA.

1.4.6 Interaction of Lectin with THP

Lectins are glycoprotein of 60,000 - 100,000 Da molecular weights, which are known for their ability to recognize and bind certain types of carbohydrate residues. Most lectins are multimeric consist of non-covalently associated subunits. A lectin may contain two or more of the same subunit or different subunits (Pisztai, 1991).

In addition, lectins are classified as unique group of proteins that attributed with specific carbohydrate binding sites. Thus, interaction of each lectins toward a particular carbohydrate structure is very specific, even oligosaccharides with identical sugar compositions can be distinguished (Boyd, 1962; Pisztai, 1991).

Lectin determinants in the carbohydrate moieties of glycoproteins have specific ligands for lectin binding. The reactivity of lectin is represented by binding power of the combination of two individual sugars (Wu, 2003). The interaction between a lectin and its receptor may vary greatly as a result of small changes in the carbohydrate structure of the receptor (Boyd, 1962). Thus, carbohydrate moieties of lectin are responsible for many biological functions.

Wu *et al.* (1995a) reported that THP in either native or its desialylated (asialo) contain important receptors for *Triticum vulgaris* (WGA). The sialic acid and its configuration impose on the oligosaccharide chains is of major important for the interaction with WGA. However, the interaction was lost after removal of sialic acid (Wu *et al.*, 1995a; Wu *et al.*, 1995b). Sherblom *et al.* (1988) explained that the strong interaction between THP and WGA is mediated by carbohydrate residues. Abbondanza *et al.* (1980) reported that the interaction between lectin

and THP is due to the carbohydrate specificity of lectin and the high affinity of oligosaccharide chains of THP for the lectin.

The interaction of WGA to lectin receptor of THP is very specific and unique as if antigen-antibody or enzyme substrate interactions. Due to this reason, the WGA can be used as reagents for characterization of THP. Therefore, WGA can be used as capture material for development of an ELLSA to quantify urinary THP level.

1.5 Urinary Proteome

Urinary proteome is a term that represents the full protein profile of urine from the healthy individual. The content of urinary proteome contains not only plasma proteins but also kidney proteins (Pieper *et al.*, 2004; Pisitkun *et al.*, 2004). Thus, urine can be very useful in clinical diagnostics as well as a potential source of biomarkers (Thongboonkerd, 2004). Urine is excreted daily from human body and therefore it can be obtained in large quantity. Although some of the biomarker present at low concentration in urine, they may be enriched in order to achieve the measurable concentration. Another advantage of using urine for diagnosis is it allows continual collection of test sample over lengthy time periods (Adachi *et al.*, 2006; Smith *et al.*, 2005).

Urinary proteome provides sufficient information about the disease process affecting the entire renal tubules (Pieper *et al.*, 2004). The pattern of urinary protein changes as a result of diseases particularly those affecting the kidney. In general, normal urinary proteins reflect a normal tubular physiology, however information of changes in urinary proteins excretion by various interventions is essential to provide better understanding about the tubular and

glomerular responses to physiological stimuli (Thongboonkerd, 2004). In order to discover novel disease biomarker of urinary proteome in human urine, both qualitative and quantitative analysis of a large number of patients and healthy subjects' urine are needed (Pieper *et al.*, 2004).

Previously, the combination of SDS-PAGE and Western blotting were employed by using unconcentrated urine to map urinary protein of renal disease (Kshirsagar and Wiggins, 1986). The author found that many proteins in urine migrate with similar molecular weights and some proteins are not detected by silver stain. In addition, the specific identification of individual proteins using Western blotting is not feasible and limited by the availability of antibodies (Kshirsagar and Wiggins, 1986).

Recently, the advancement of mass spectrometry may be useful in complementing gel electrophoresis approach for identification of urinary proteins (Baldwin, 2004). Thus, the complete urinary proteome may be achieved. The mass spectrometry even is a powerful tool to discover biomarker of renal disease such as kidney stone. The state-of-the-art technologies integrated with protein enrichment technique, protein separation method and mass spectrometry (MS) should be conducted for biomarker discovery and protein analysis (Adachi *et al.*, 2006). There are various protein enrichment techniques that can be used to concentrate urinary proteins. In many other studies, gel electrophoresis (SDS-PAGE or 2-D gel electrophoresis) was employed to separate and map protein expression at qualitative and quantitative level (Kshirsagar and Wiggins, 1986; Smith *et al.*, 2005). Using this method, protein expression of the sample obtained from healthy and diseased individuals can be compared. The appearance and disappearance of spots or

bands can provide information about differential protein expression, while the intensity of those spots or bands provides quantitative information about protein expression (Graves and Haystead, 2002). Thus, gel electrophoresis remains an essential component of proteomics. Furthermore, liquid chromatography mass spectrometry (LC/MS/MS) and/or Western blotting can be used to identify and characterize the urinary proteome (Thongboonkerd, 2004).

Spahr *et al.* (2001) applied gel electrophoresis and LC/MS/MS to identify proteins from unfractionated urine of healthy subjects. The authors stated that construction of urinary proteome map may provide useful information regarding differentially expressed proteins between the healthy and disease state. Hence, the urinary proteins pattern either in healthy subjects or patients should be consistent and comparative. In addition, Lafitte *et al.* (2002) described that the pattern of urinary proteins are specific either in healthy subjects or renal disease patients. Pisitkun *et al.* (2004) reported analysis of the urinary proteome of healthy subjects identified several proteins known to be involved in renal and systemic disease.

The database of urinary proteome is gradually expanded by the progressive investigation and studies. The goals are to build fundamental knowledge of renal physiology to define the pathophysiology of renal diseases and to identify novel biomarkers or new therapeutic targets. Thus, identification of an unique protein that is present either in healthy subjects or patients may a good candidate of biomarker (Pieper *et al.*, 2004).

1.5.1 Biomarker

Biomarker or biological marker is defined as a characteristic that is measured and evaluated objectively as an indicator of normal biological processes, pathological processes or pharmacologic responses to therapeutic intervention (Biomarker Definition Working Group, 2001).

Biological fluids were suggested to reflect the ensemble of tissues present within a patient. The discovery of biomarker in biological fluids which is disease specific becomes a great challenge. In view of the fact that biological fluids do not have a corresponding genome or transcriptome that permits gene expression to be measured, DNA- or RNA-based diagnostics are not applicable to biological fluids (Anderson and Anderson, 2002). Therefore, proteomics approach is one of the few options for identifying biomarker in biological fluids. In some cases, the biomarkers of interest can be present in low concentration levels and masked by a high abundance protein or cover a wide range of chemical space, making the challenge to be great and critical (Drexler *et al.*, 2004). However, proteomics analysis has greatly increased our knowledge on the protein content of clinical important biological fluids.

1.5.2 Discovery of Biomarker Using Proteomics Analysis

Proteomics analysis of biological fluids is commonly applied to distinguish the diversity protein content in sample obtained from patients with specific disease and healthy subjects (Issaq *et al.*, 2003). Initially, protein profile of healthy and specific disease individuals is compared to identify unique proteins which either presence in healthy or diseased individual. Subsequently, the proteins are digested by protease and then subjected to mass spectrometry

analysis. The mass spectrometry analysis can be categorized into biomarker discovery and proteomics diagnostic approach (Issaq *et al.*, 2003). In the biomarker discovery approach, liquid chromatography is coupled with tandem mass spectrometry (MS/MS) to identify peptides that are unique or highly abundant in samples obtained from patients with specific disease state compared with healthy subjects. This approach generates copious of data and identify hundreds of proteins. However, it is very time consuming and hence limited number of comparative samples can be analyzed (Issaq *et al.*, 2003).

In the proteomics diagnostic approach, samples from healthy and disease affected individuals are applied to proteins chips that are modified with specific chromatographic resin. Subsequently, mass spectra of the bound proteins obtained and differences in peak intensities between sample sets are ascertained by bioinformatics algorithms (Petricoin *et al.*, 2002). Most of the proteomics diagnostic approach does not reply on the actual identification of the specific protein(s) within the peak through which the diagnosis is determined, although identification of the selected peak can be pursued. This method becomes very popular due to its high throughput nature (Issaq *et al.*, 2003).

1.5.3 Identification and Validation of Biomarker

The identification of a target protein is performed by comparative searches using protein databases or *de novo* sequencing. For a biomarker to be effective, it is necessary to accurately measure the small changes in intensities of the biomarker (most of the time at low level) in a complex matrix repeatedly on a large number of samples. Hence, the possible deviation of the results due to sample handling and the analytical technique itself need to be

kept at a minimum by developing a highly sensitive and quantitative analytical assay (Drexler *et al.*, 2004). Therefore, appropriate sample preparation techniques (e.g., extraction, isolation and purification) and a suitable mass analyzer should be applied for monitoring of the biomarker.

Three important steps was suggested as a general guideline for comprehensive analysis of biomarker discovery: (1) protein isolation to remove abundant proteins such as albumin and immunoglobulin without exclude other proteins that may alter the whole urinary proteome, (2) protein separation to reduce the complexity of the protein mixture and (3) peptide sequencing with high accuracy and rapid scanning (Drexler *et al.*, 2004).

Recent advancement in mass spectrometry integrated with gel electrophoresis, LC and/or multidimensional liquid chromatography (LC/LC) strategies have led to remarkable improvement in the ability for disease biomarker discovery. Thus, the combined techniques were used to identify and subsequently validate biomarker in the biological fluids.

Quantitative protein expression profiling is a vital part in the proteomic analysis of healthy versus disease state. This will be the best option for protein quantification particularly in gel electrophoresis (SDS-PAGE or 2-D gel electrophoresis) where multiple proteins are present in one spot or band. Since tracking the protein responsible for differential staining is difficult, therefore changes in the protein expression level can be measured directly from mass spectrometry of a peptide ion derived from the protein (Graves and Haystead, 2002).

Typically, LC/MS/MS is performed to identify proteins and peptides sequence (Coon *et al.*, 2005). The difference in intensity abundance of the