นิพนธ์ต้นฉบับ

Urinary N-Acetyl-β-D-Glucosaminidase and Microalbumin Levels in Urine of Diabetic Patients*

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Abstract

Objective : To determine levels of N-acetyl-β-D-glucosaminidase (NAG) activity, microalbumin (MA) in second-morning urine for early detection of diabetic nephropathy.

Subjects : A hundred healthy individuals and 220 diabetic patients in which 14 of these patients, nephropathy has been developed.

Methods : Kinetic-enzymatic assay using 2-chloro-4-nitrophenyl-N-acetyl-β-D-glucosaminide (CNP-NAG) as substrate for NAG determination, Bromphenol blue (BPB) dye-binding for microalbumin. The tests were performed on automated chemistry (Abbott CCx) analyzer.

Results : Mean (Standard deviation for second-morning urinary NAG, MA in diabetics were statistically higher than in healthy individuals (25.33 ± 10.83 *vs.* 18.06 ± 4.78 U/gm creatinine, 2.78 ± 4.65 *vs.* 1.44 ± 0.51 mg/gm creatinine, respectively, $p<0.05$). Using cut-off levels receiver operating characteristic (ROC) curve, at MA 3.48 mg/gm creatinine and NAG 37.2 U/gm creatinine, sensitivity, specificity for early detection of diabetic nephropathy were 94.1%, 93.1% by using MA alone, 75.0%, 90.7%, by using NAG alone, and 100%, 98.3% when the two tests were used in combination.

Conclusion : Urinary NAG activity and MA could be useful for detection of early diabetic nephropathy which can not be detected by urinary analysis or other routine renal function tests.

Key words: Microalbumin, NAG, diabetic nephropathy

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บทคัดย่อ: ระดับ N-Acetyl-β-D-Glucosaminidase และ Microalbumin ในปัสสาวะผู้ป่วยเบาหวาน*

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วัตถุประสงค์ : เพื่อบ่งชี้การเกิดพยาธิสภาพของไตระยะเริ่มแรกในผู้ป่วยเบาหวาน โดยการตรวจวัดระดับ N-acetyl-β-D-glucosaminidase (NAG) activity, ไมโครอัลบูมิน (Microalbumin, MA) ในปัสสาวะ
ตัวอย่างที่ศึกษา : ปัสสาวะจากคนสุขภาพดี 100 คน และผู้ป่วยเบาหวาน 220 ราย ในจำนวนนี้มี 14 รายที่เป็นโรคไต
วิธีการ : ใช้หลักการ Kinetic-enzymatic assay โดยมี 2-chloro-4-nitrophenyl-N-acetyl-β-D-glucosaminide (CNP-NAG) เป็นสับสเตรทสำหรับการตรวจวัด NAG activity, การรวมตัวกับสี Bromphenol blue (BPB) สำหรับการตรวจวัดไมโครอัลบูมิน โดยใช้เครื่องทดสอบอัตโนมัติ (Abbott CCx)
ผลการศึกษา : ค่าเฉลี่ย ± ค่าเบี่ยงเบนมาตรฐานสำหรับ NAG, MA ในผู้ป่วยเบาหวานมีค่าสูงกว่ากลุ่มสุขภาพดีอย่างมีนัยสำคัญ คือ 25.33 ± 10.83 *vs.* 18.06 ± 4.78 U/gm creatinine, 2.78 ± 4.65 *vs.* 1.44 ± 0.51 mg/gm creatinine ตามลำดับ ($p < 0.05$). จากการศึกษาโดยใช้ Receiver operating characteristic (ROC) curve ได้ค่า Cut-off สำหรับ MA และ NAG เป็น 3.48 mg/gm creatinine และ 37.2 U/gm creatinine ค่าความไว ความจำเพาะต่อการบ่งชี้การตรวจพบโรคไตระยะเริ่มแรกในผู้ป่วยเบาหวาน (Diabetic nephropathy) ที่ค่า Cut-off ดังกล่าวได้เป็น 94.1%, 93.1% เมื่อใช้การตรวจวัด MA อย่างเดียว, 75.0%, 90.7% เมื่อใช้การตรวจวัด NAG อย่างเดียว และ 100%, 98.3% เมื่อใช้ทั้งสองการทดสอบร่วมกัน
สรุป : การตรวจวัดระดับ NAG activity และ MA ในปัสสาวะ สามารถเป็นตัวช่วยบ่งชี้การเกิดพยาธิสภาพของโรคไตในระยะเริ่มแรกซึ่งยังไม่สามารถตรวจพบได้ด้วยวิธีการตรวจปัสสาวะและการทดสอบสมรรถภาพไตประจำวันอื่นๆ

คำหัส: ไมโครอัลบูมิน, NAG, Diabetic nephropathy

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Introduction

Diabetic nephropathy is one of complications causing morbidity and mortality in diabetic patients characterized by the early appearance of hypertrophy of both glomerular and tubular elements, the subsequent development of thickened glomerular and tubular basement membranes associated with enhanced glomerular permeability to albumin. The cumulative incidence of nephropathy is 30% to 50% in insulin-dependent diabetes mellitus (IDDM) and 10-15% in non-insulin dependent diabetes mellitus (NIDDM). Prevalence of renal complications found in Thai diabetics is 8.6 - 17.1%.[1] Known risk factors for the development of diabetic nephropathy are duration of diabetics, systemic hypertension, renal hemodynamics especially an increased glomerular filtration rate (GFR), poor glycemic control, genetic predisposition, *etc.*

The hallmark of overt diabetic nephropathy is 'Albustix' positive proteinuria (Clinical proteinuria) which is invariably associated with long duration of diabetes, systemic hypertension, elevated serum creatinine and retinopathy. The earliest functional abnormality in the diabetic kidney disease is renal hypertrophy associated with a raised glomerular filtration rate which appears soon after diagnosis and is related to poor glycemic control. Associated changes may result in disruption of the protein cross-linkages that make the membrane an effective filter leading to progressive leak of protein into urine. The earliest evidence of this is 'microalbuminuria' which in turn, after some years, progress to intermittent albuminuria followed by persistent proteinuria. [2]

'Microalbuminuria' has been defined as an increased excretion of albumin above the reference range for healthy non-diabetic subjects but is undetectable by Albustix test. Urinary albumin excretion rate persists between 20 and 200 μg/min in an overnight or 24-hour sample on at least positive in 2 of 3 occasions within a period of 6 months. An early detection of microalbuminuria is essentially attempted to determine in routine laboratory for preventing nephropathy and monitoring diabetes management.[3] Various techniques have been used for measuring microalbumin in urine including radial immunodiffusion,[4] immunoturbidimetric,[5] radioimmunoassays,[6] enzyme immunoassays,[7,8,9] latex agglutination,[10] dye-binding methods.[11,12]

N-acetyl-β-D-glucosaminidase (EC 3.2.1.30; NAG) is responsible for the degradation of mucopolysaccharides and glycoproteins. Urinary NAG has its origin largely in the epithelial cells of the proximal tubule which contain a particularly large number of lysosomes.[3] Due to high molecular weight, NAG in serum can not penetrate into the glomerular filtrate if the glomerular membrane is intact. Under normal circumstances the low level of NAG in the urine repre-

sents leakage as part of exocytosis and pinocytotic activity of the tubular epithelial cells. An elevated urinary NAG can occur as a result of increased metabolic activity due to seriously increased glomerular filtration accompanied with decrease in reabsorption, increased excretion of exogenous substances which are attacked by lysosomes or destruction of cells in the proximal tubule.

An increase in NAG excretion in urine must invariably be interpreted as a sign of proximal cell dysfunction.[14] The first occurrence of elevated NAG activity is usually a sign of reversible process. It has been proposed that urinary NAG assays might have considerable potential for investigating and detecting early diabetic nephropathy.[15] Several trials investigating patients with type I and type II diabetes mellitus have noted that the NAG activity was raised in urine, and some evidences suggested that this increase occurred prior to microalbuminuria.[16]

Determination of microalbumin in urine is assumed to be a predictor of clinical glomerulopathy while NAG informs about the renal tubular dysfunction. In order to establish a sensitive regimen for the clinical monitoring of renal involvement in diabetics, thereby, combining the advantages of quantitative analysis of urinary microalbumin and also the measurement of NAG, a lysosomal enzyme localized in the proximal tubules, should be served as parameter for the assessment of both glomerular and tubular damages.

This paper aims to verify the two methods of urinary microalbumin and NAG activity for detection of renal disease as early as possible and establish a reference value of both recommended methods.

Materials and Methods

One hundred samples of second-voided morning urine without preservative from healthy individuals aged 35-50 years. All specimens are Albustix negative, no history of diabetes mellitus or renal diseases. Urine specimen were centrifuged and the supernates were kept at -20 °C for analysis compared to 220 diabetics urine from Diabetics Control Clinic, Buddhachinnaraj hospital. Based on standard routine urinalysis, 14 of the patient group were classified to be diabetic nephropathy which proteinuria and casts were presented.

Microalbumin determination was performed based on the dye-binding property of microalbumin with bromphenol blue (BPB)[12] and enzymatic activity of NAG was done using a new synthetic substrate, CNP-NAG.[17]

Results

Mean±SD of NAG activity and microalbumin in urine of diabetic patients were significantly higher than those from healthy adults (25.33±0.83 *vs.* 18.06±4.78 U/gm. crea-

tinine and 2.78±4.65 *vs.* 1.44±0.51 mg/gm. creatinine, $p<0.05$), respectively. Correlation coefficient (r) of microalbumin in urine and NAG/gm. creatinine in diabetics was 0.48 ($p<0.05$) and increased to be 0.78 ($p<0.05$) in diabetic with nephropathy.

Using the cut-off level at mean ± 3SD, 2.97 mg/gm. creatinine of microalbumin and 32.40 U/gm. creatinine for NAG activity for suspection of diabetic nephropathy, we found that NAG activity of healthy control were all negative and only 3 had higher microalbumin values than the cut-off level. With receiver operating characteristic (ROC) curve, the cut-off level of microalbumin and NAG were at 3.49 mg/gm. creatinine and 37.2 U/gm. creatinine, respectively.

Determination of urinary MA alone gave 100% sensitivity and 89.7% specificity for diabetic nephropathy while NAG activity alone gave 75.0% sensitivity and 81.9% specificity, respectively and combination of both tests, the sensitivity and specificity were 100% and 98.3% as shown in Table 1, 2.

Table 1 Sensitivity, specificity of urinary microalbumin and NAG activity using cut-off level at mean + 3SD

	MA	NAG	NAG & MA
Sensitivity (%)	100.0	75.0	100.0
Specificity (%)	89.7	81.9	95.5
False positive (%)	9.5	16.8	3.6
False negative (%)	0.0	1.8	0.0

Table 2 Sensitivity, specificity of urinary microalbumin and NAG activity in diabetics using ROC curve for cut-off level determination.

	MA	NAG	NAG & MA
Sensitivity (%)	94.1	75.0	100.0
Specificity (%)	93.1	90.7	98.3
False positive (%)	6.4	8.6	1.4
False negative (%)	0.5	2.2	0.0

Discussion

Proteinuria in renal disease may result from glomerular and/or tubular functions. Increased glomerular permeability increased the urinary excretion of proteins such as albumin, transferrin and the acute-phase reactants *e.g.* alpha-1 antitrypsin and beta-acid glycoprotein[18] while urinary NAG activity is an extremely sensitive index of renal parenchymal damage.

Many laboratories still use reagent strips for semiquantitative determinations of urinary albumin which did not detect microalbuminuria or monitor small change in albumin concentrations. The BPB method presented here is a rapid direct quantification of urinary albumin over a wide concentration ranges without effecting of urinary pH variation, uric acid, creatinine, calcium and bilirubin at abnormally high concentrations.[11]

Pongsomboon S, 1992[12] used BPB reagent for determination of microalbuminuria which could not be detected by Albustix. The method showed within-run precision range between 3.3-4.8% whereas between-run precision was between 7.4-9.5 %. The color production was completed within 1 minute and stable for at least 30 minutes. Chanarat N and Suksriwong S, 1990[19] also reported that the determination of microalbumin by bromphenol blue (BPB) reaction, the precision of the method by optimal condition variance was between 2.0-3.0%, routine condition variance was 5.5-8.7 %. Linearity of the reaction was at least 100 mg/dL and sensitivity was at least 1 mg/dL. Accuracy of the method by % recovery evaluation was 102.3% .

Albumin in urine is stable and the samples can be stored at 4-20 °C for 10 days without preservatives. Five freeze-thaw cycles showed no change in albumin concentration compared with the original values in fresh samples.[20] Centrifuged samples stored for 1 week at 4 °C or 1 month at -20°C showed no difference in concentration even when visible precipitates were present.[21]

Microalbuminuria is assumed to be an early predictor of clinical nephropathy in diabetes mellitus. However, it only indicates glomerular involvement and does not provide information about the renal tubular function which may also be impaired in diabetes mellitus. The measurement of NAG, a lysosomal enzyme localized in the proximal tubules, served as an additional parameter for the assessment of tubular damage.[15] From our results, testing of urinary microalbumin alone the sensitivity is 94.1%, specificity 93.1%, false positive 6.4% and false negative 0.5%. Urinary NAG activity alone gives the sensitivity of 75.0%, specificity 90.7%, false positive 8.6% and false negative 2.2%. Combination of the two tests, the sensitivity raised up to 100%, specificity 98.3%, false positive decreased to 1.4% and false negative 0.0%. We also found that the positive predictive value at different prevalence 5.0, 10.0, 15.0% will increase from 72.5, 87.5 and 89.8%, respectively. Powell SC, 1983[22]

reported that urinary NAG was not only increased in primary but also in secondary renal disorders such as light chain disease and myoglobinuria. The increase of NAG in urine is not entirely specific for renal damage, but also can be observed in other disorders such as pancreatitis, ovarian cancer, *etc.*

In diabetics without nephropathy, the correlation of NAG excretion to microalbumin is only 0.48 ($n=200$, $p<0.05$) while in patients with nephropathy, it raises up to 0.78 which could be explained that the glomerular changes occur early in diabetes. At the beginning of abnormality the tubular cells still can reabsorb the increased albumin loaded, resulting in normal albumin excretion rate but increased lysosomal activity and NAG excretion. The next stage is simultaneously characterized by an increase in both NAG and albumin and this indicates that proximal cells reabsorption capacity has been exceeded. Further increase in NAG excretion will accompany a loss in the functional capacity of the cell loading to structural breakdown which ultimately results in cell necrosis.

In conclusion, microalbuminuria, NAG activity and glycemic control have well analytical characteristics, sensitivity and specificity suitable for the assessment in clinical laboratories and suggest that the quantitation of both glomerular and tubular proteinuria provides a sensitive and cost-effective tools for the non-invasive renal screening in patients with diabetes mellitus.

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