Original Article

Proteomic Analysis of Urinary Protein Markers for Accurate Prediction of Diabetic Kidney Disorder

Samta Jain*, Anuradha Rajput, Y Kumar*, N Uppuluri*, AS Arvind**, U Tatu***

Abstract

Aim of the study : Microalbuminuria is currently the only diagnostic tool available for early diagnosis of diabetic nephropathy. The test is based on immunological detection of small quantities of albumin in the urinary samples of diabetes patients. There are several limitations of the use of microalbuminuria as an index of renal function. It is therefore desirable to identify additional protein markers that would augment prediction of diabetic nephropathy. The aim of this study is to identify urinary protein markers for specific and more accurate prediction of nephropathy in diabetes patients.

Design : 100 registered Type II diabetic patients were studied. Abundant proteins of microalbuminuria positive urinary samples of these patients were analyzed by proteomics approaches of 2-Dimentional Gel Electrophoresis (2DGE) and mass spectrometry.

Results : 2-DGE analysis of the urine sample revealed four main proteins along with albumin in these samples. These were zinc alpha-2 glycoprotein, alpha-1 acid glycoprotein, alpha -1 microglobulin and IgG as identified by Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-ToF) and by western blot.

Twenty control samples and three cases with microalbuminuria negative to positive transition does suggest the early and co-appearance of the markers with albumin. We have also analyzed full length spectrum of these samples by MALDI-ToF.

Conclusion: Our study shows the presence of additional proteins in urine samples of microalbuminuria positive diabetes patients. These proteins can be used as markers for specific and accurate clinical analysis of Diabetic nephropathy. We propose a mass spectrometry based high throughput diagnostic approach to detect these markers in the urine sample. ©

INTRODUCTION

Diabetes continues to be an important clinical problem. The number of diabetic patients is on an exponential increase worldwide. Diabetes requires long term medical attention as there are many devastating complications like renal, ocular and atherosclerosis to name a few.[1] One of the factors for mortality and morbidity associated with diabetes is diabetic nephropathy. Diabetic nephropathy is a microvascular complication associated with diabetes; causing slow deterioration of kidneys leading to end-stage renal disease (ESRD). It has a chronic and irreversible course without recovery or cure. It is well known that 40% of type I and 20% of type II diabetic subjects eventually go on to develop significant nephropathy[2] making diabetes an obvious cause for ESRD. Since the recovery from diabetic nephropathy is irreversible, an early detection is essential to prevent progression to ESRD.

Currently, the most popular method of detecting early signs of nephropathy in diabetic subjects is microalbuminuria.[3,4] This test measures small amounts of albumin excreted in the urine using ELISA or radioimmunoassay. Most commonly, a 24 hr urine sample is obtained and albumin levels of 30-300 mg per day are graded as microalbuminuria. Although the microalbuminuria test is routinely performed in diabetic patients the world over, the presence of albumin in the urine is not a very specific factor.

Albumin frequently occurs in the urine of non-diabetic subjects[5,6] and in individuals during inflammation, after exercise,[7] with hypertension,[8] and individuals with normal renal functions.[9] It arises among smokers too.[10] It is also suggested by some workers that microalbuminuria is a better marker for cardiovascular disease.[11] According to one study,[12] microalbuminuria was shown to have the predictive positive value of 43% and negative predictive value of 77% for diabetic

*Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India. **Clinigene International, Biocon, 20 km Hosur Road, Bangalore, India.
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nephropathy.

There are also reports of microalbuminuria test giving false positive results. Among patients with diabetic nephropathy lesions, the initial screening predicts normoalbuminuric level.[13] Urinary albumin levels are known to show wide day-to-day as well as lab to lab variations. Therefore, American Diabetes Association and the National Kidney Foundation insists on at least two positive tests which shows elevated levels of albumin:creatinine ratio within a time gap of 3-6 months in order to confirm the diagnosis of microalbuminuria.[9]

Given these shortcomings, there is **a** need for additional markers to augment the existing diagnostic tools for better prediction of diabetic nephropathy and its clinical management. Our study describes detection of urinary proteins, other than albumin, appearing abundantly in diabetic patients tested positive for microalbuminuria.

Material And Methods

Clinical subjects and urine collection : We studied 100 type II diabetic patients with age ranging from 25-60yrs. All patients were characterized clinically as microalbuminuria positive with urinary albumin levels in the range of 20-100 mg / min / lit as estimated by radioimmunoassay. Twenty control samples were collected; eighteen were microalbuminuria negative from diabetic subjects, one was microalbuminuria negative from non-diabetic subject and another was microalbuminuria positive from non-diabetic subject. About 5ml of urine was collected from each patient in a clean plastic container and flash frozen.

Protein isolation and estimation : 0.7ml of urine was centrifuged at 3500rpm for 5 min at 4°C to pellet out the cells / debris if any. Proteins from 0.5 ml of the supernatant were isolated using Acetone Precipitation and estimated using Bradford's analysis. The microalbuminuria estimation method used in the study is immunoturbidometric method.

2-D gel electrophoresis : 10mg of protein from each sample was subjected to 2-D gel electrophoresis as described by using ampholines of p I range 3.5-9.5 (Amersham Pharmacia, Uppasala, Sweden). Following 2-DE, the proteins were detected by Coomassie blue staining. Gels were scanned on a laser scanner and images were analyzed using Melanie 2-D gel analysis software.

In-gel digestion : Coomassie stained spots of interest were cut out from the 2-D gel, pooled and cut further into small pieces ~2-3 mm in diameter. A blank gel piece with no protein spot was also separately processed as a control. The gel pieces were vortexed in 25mM ammonium bicarbonate in 50% Acetonitrile 3 -4 times, dried under vacuum, and incubated with 10mM DTT in 25mM ammonium bicarbonate at 56°C for 1h. Reduced cysteines were modified with 55mM Iodoacetamide in 25mM ammonium bicarbonate for 45 min at room temperature in the dark. The gel pieces were washed 3 - 4 times in 25mM ammonium bicarbonate in 50% Acetonitrile and then completely dried. Sequencing grade Trypsin, 0.1 mg / ml (Promega Scientifics) was added just enough to cover the gels and digestion carried out for 12h at 37°C. The peptides were extracted from the gels with first 0.1ml water and followed by two extractions with 0.5% TFA in 50% Acetonitrile. The peptide solution was then concentrated in a speed-vacuum and used for MALDI-TOF analysis.

MALDI-ToF and PMF : Mass spectrometry was performed on 'Ettan MALDI-ToF Pro' (Amersham Biosciences, Sweden). One volume of peptide mixture was mixed with one volume of alpha-cyano hydroxy cinnamic acid solution matrix (Ettan chemicals) and subjected to MALDI analysis. Monoisotopic masses of all peptides were measured with delayed extraction operated in the reflectron mode. All spectra were externally calibrated using peptide standard mix and 200 shots were accumulated to give an acceptable spectrum.

Protein Identification of peptide fragments was performed by using in built 'Ettan MALDI Software' with 'proteo Metrics LLC' search engine. The criterion given for search was oxidation at methionine residues, carbamidomethylation at cysteine residues, up to 1 missed cleavage was allowed and the protein was searched against the entire human database.

For the full-length analysis of the urine sample, one volume of acetone precipitated sample was mixed with two volumes of sinnamic acid matrix solution (Sigma-Aldrich) and subjected to MALDI-ToF where masses of all the proteins were measured in full-length mode. All spectra were externally calibrated with BSA (Bovine Serum Albumin). 200 shots were accumulated to give an acceptable spectrum.

Results

Microalbuminuric urinary profile by 2-D : Towards examining the abundant urinary proteins in the diabetic microalbuminuria positive patients, we performed 2 -D gel electrophoresis of the urine samples. In order to restrict the analysis to abundant proteins, the gels were stained with coomassie blue stain. Fig. 1(a) represents the 2 -D images of some of the samples. As expected, a spot corresponding to albumin (69kDa, pI 5.7) was seen among all the microalbuminuria positive samples and in addition to albumin, several other protein spots were visualized, of which three spots labeled MA1 (38kDa, pI 5.3), MA2 (33kDa, pI 5.3) and MA3 (43kDa, pI 3.5) were distinctly seen in most of the samples whereas the spot labeled MA4 did not appear frequently. The intensity of the MA1, MA2 and MA3 did not show any correlation with the intensity of albumin and these spots were found to be more prominent than albumin in a few cases. A

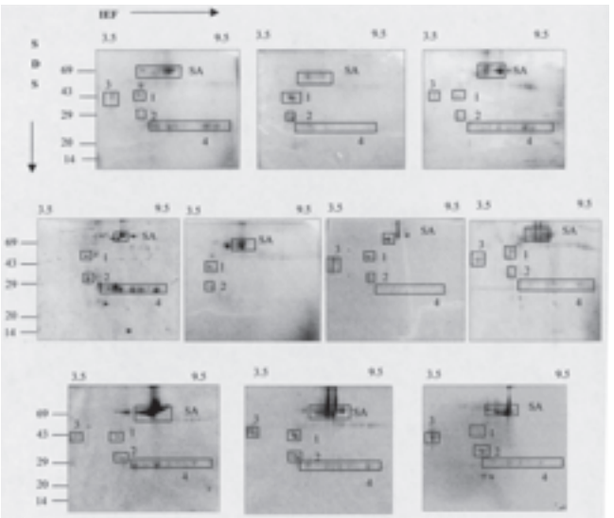

Fig. 1(a): 2-D gel profiles of microalbuminuria positive urine samples of Type II patients. The protein labelled SA is albumin, 1 is zinc alpha-2 glycoprotein (38 kDa, pI 5.3) 2 is alpha-1 microglobulin (33Kda, pI 5.3) 3 is alpha-1 acid glycoprotein (43Kda, pI 3.5), and 4 is IgG seen as a train of spots in ~25 kDa region. The respective concentration of protein is mentioned below each gel.

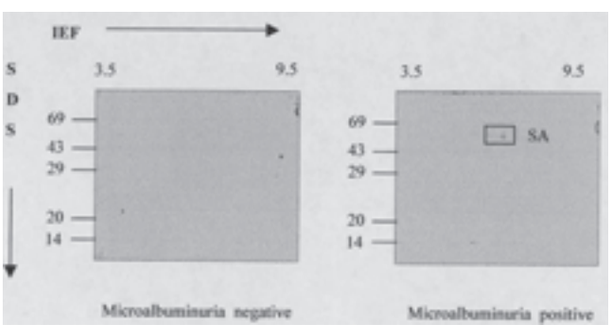

Fig. 1(b): two control 2-D gel profiles of non-diabetic samples. The left panel is microalbuminuria negative sample and right panel is microalbuminuria positive. None of the markers are visible in either case but trace amounts of albumin are seen in the positive sample.

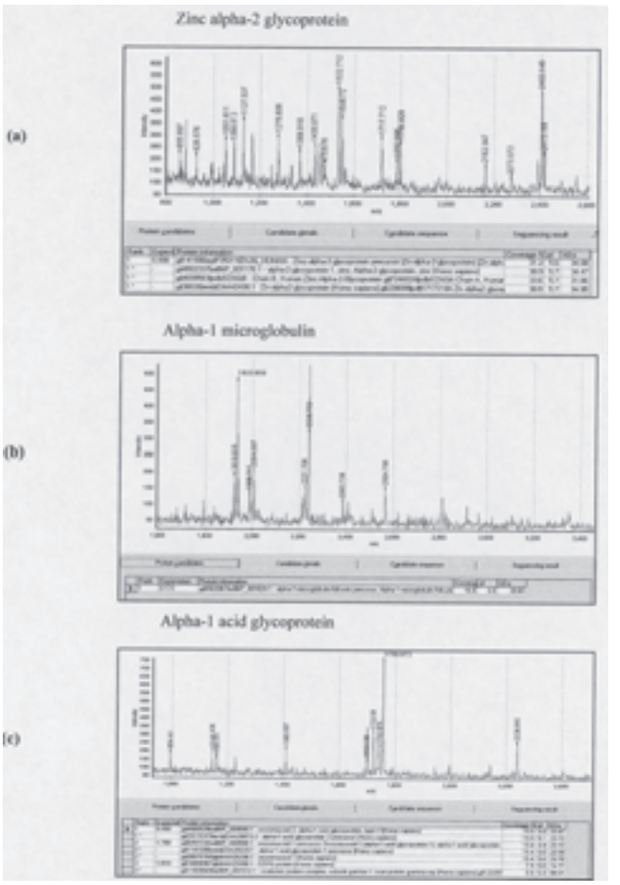

Fig. 2: The MALDI-ToF result of the three proteins subjected to in-gel Tryptic digestion from 2-D coomassie stained gels. The Protein Mass Fingerprint result is included below the respective spectrum. Lane a shows the spectrum of spot no.1 (see Fig. 1 a) and is identified as zinc alpha-2 glycoprotein with an Expectation score of 0.00. Lane b shows the spectrum of spot no.2 (see Fig, 1 a), which is identified as alpha-1 microglobulin with an Expectation score of 0.172. Lane c shows the spectrum of spot no.3 (see Fig. 1 a), which is identified as alpha-1 acid glycoprotein with an Expectation score of 0.498.

combination of albumin and the markers were observed in 86.6% of gels analyzed. MA1 was more frequently seen (80%) than MA2 and MA3 in the samples studied. Table 1 illustrates the clinical characteristics of the study subjects shown in Fig. 1(a) and Fig. 4.

As a control, we analyzed two non-diabetes samples and eighteen microalbuminuria negative samples from diabetic subjects. Fig. 1(b) shows coomassie stained 2DE images of non diabetes microalbuminuria positive in the top right panel and non diabetes microalbuminuria negative sample in the top left panel. In both the cases, none of the markers (MA1, MA2 or MA3) were seen; only albumin was seen in the positive sample. This confirms the specificity of the markers to diabetic subjects and the fact that albumin appears in a nondiabetic sample reflects the non-specificity of albumin which has been reported previously. Among the 18 microalbuminuria negative samples from diabetic subjects analyzed by mass spectrometry approach, the spectrum of eight samples show albumin along with other markers, although albumin was not picked up by dipstick method. Other samples show the presence of additional markers as given in Table 2.

Identification of Proteins by MALDI-ToF : To identify the proteins representing MA1, MA2 and MA3, all the three spots were excised from coomassie stained 2-D gels, subjected to In-gel tryptic digestion and analyzed by MALDI-ToF. MA1 was identified as Zinc alpha -2 glycoprotein with theoretical pI of 5.6 and 34kDa, MA2 as Alpha -1 microglobulin (39.89kDa, pI 6.0) and MA3 as Alpha -1 acid glycoprotein (23.5kDa, pI 5.0). MA4 that consists of trail of spots with mobility in the basic region of the gel is likely to be IgG light chain that is confirmed by immunoblotting.

Fig. 2 shows the mass spectra of all the three proteins along with their Peptide mass fingerprint analysis. A significant identification hit ($p<0.5$) was established in all the cases. The observed pI and molecular weight of

Table 1 : The table shows clinical data of the subjects studied

Sl.No	Age (yrs)	Diabetic Duration (yrs)	HbA_1c	Chol	Lipids Tri	HDL	LDL	Fasting blood Sugar	Diabetic Retinopathy	UTI	Drug ACE Inhibitors
1	56	24	8.4	197.0	160.0	43.0	122.0	239	—	neg	Nuril 5 mg
2	55	4	6.7	178.0	274.0	—	—	—	—	neg	
3	51	21	12.6	262.0	260.0	62.0	148.0	171	Mild Non Proliferative	neg	
4	19	58	8.7	126.0	92.0	40.0	67.8	97	Mild Non Proliferative	neg	
5	49	11	6.7	225.0	268.0	32.0	139.4	137	No Evidence	neg	
5	52	19	8.5	272.0	331.0	27.0	178.8	147	Mild Non Proliferative	neg	
6	39	5	6.2	199.0	129.0	—	—	—	—	neg	
7	40	10	7.0	201.0	209.0	—	—	—	—	neg	
8	57	15	6.8	215.0	147.0	51.0	—	103	Mild Non Proliferative	neg	
Case1 (visit 1)	58	19	11.0	182.0	121.0	29.0	128.8	137	Mild Non Proliferative	neg	
Case1 (visit 4)			8.5	111.0	60.0	32.0	67.0	124	Mild Non Proliferative	neg	Nruil 5 mg
Case1 (visit 5)			7.5	143.0	100.0	32.0	91.0	139	Mild Non Proliferative	neg	
Case1 (visit 7)			8.7	126.0	92.0	40.0	67.6	97	Mild Non Proliferative	neg	
Case2 (visit 1)	62	17	9.4	177.0	312.0	25.0	89.6	160	No Evidence	neg	
Case2 (visit 5)			7.6	205.0	222.0	29.0	131.6	135	No Evidence	neg	
Case 2 (visit 6)			7.9	203.0	241.0	31.0	123.8	146	No Evidence	neg	
Case 2 (visit 7)			7.8	214.0	228.0	35.0	133.4	113	No Evidence	neg	
Case 3 (visit 1)	49	11	6.5	193.0	295.0	26.0	108.0	139	No Evidence	neg	
Case 3 (visit 3)			6.4	197.0	322.0	26.0	106.6	152	No Evidence	neg	
Case 3 (visit 5)			7.2	230.0	389.0	32.0	120.2	157	No Evidence	neg	
Case 3 (visit 7)			6.7	225.0	268.0	32.0	139.4	137	No Evidence	neg	

the three proteins are in good agreement with their localization on the 2-D gels of human plasma (Swiss 2-D PAGE at http://kr.expasy.org/ch2d/). The incongruency between the theoretical and observed MW and pI of these proteins can be associated with the posttranslational modifications.

To illustrate the consistency of the data from proteomic analysis with those acquired from conventional immunoblotting technique, we performed 2-D Western blot analysis. The proteins were transferred from 2-D to nitrocellulose membrane. Western blotting was then performed using specific antibodies to human IgG, human ZAG and human A1AG.

As shown in Fig. 3, left panel; a train of basic protein spots corresponds to antibody heavy chain (55kDa) and light chain (25kDa), a 38kDa spot corresponding to ZAG and 33kDa acidic spot corresponding to A1AG can be seen upon Western blotting. The coomassie stained gel showing the total protein profile is also shown in the right panel of the Figure. The protein spot seen upon western blot is consistent with identifications by proteomic analysis.

Presence of additional urinary markers may precede the appearance of Albumin : To analyze time of appearance of the three markers in correlation with albumin, three cases were investigated with microalbuminuria negative to positive transition. The 3 selected cases had type II diabetes and were scheduled on a routine physical examination. In the course of this examination, the dipstick test gave negative result initially for first few sittings and eventually the same person tested positive during further sittings. Urine samples were collected

Table 2 : The microalbuminuria negative diabetic subjects taken as control are listed in the left column. The right column lists the proteins identified by mass spectrometry method in the corresponding samples.

Sample no.	Proteins detected in the sample
control 1	IgG, MA2
control 2	IgG
control 3	IgG, SA
control 4	
control 5	IgG, SA, MA1
control 6	IgG, SA, MA1, MA3
control 7	IgG, SA
control 8	IgG, SA, MA2
control 9	IgG, SA, MA1, MA2
control 10	IgG
control 11	IgG
control 12	
control 13	IgG, SA
control 14	IgG
control 15	IgG, MA1,MA3
control 16	IgG
control 17	IgG
control 18	IgG, SA, MA1

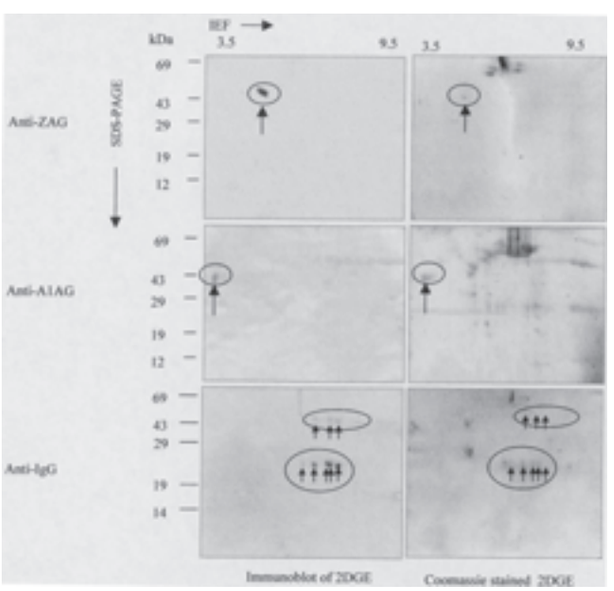

Fig. 3: Right panel shows Immunoblots of zinc alpha -2 glycoprotein, alpha -1 acid glycoprotein and IgG using the human antibodies Anti-ZAG, Anti-A1AG and Anti-IgG respectively .We see the presence of specific bands for the respective proteins. Right panel is the equivalent total profile from coomassie stained2-D gels.

during all the sittings. From the first case, two negative and two positive samples from sitting no. 1,4,5,7 respectively were analyzed; from case 2, one negative and three positive samples with sitting no. 1,5,6,7 respectively and from case 3, one negative and three positive samples, sitting no. 1,3,5,7 respectively were analyzed by 2-DGE.

Fig. 4 shows the 2-D of sittingwise analysis as the sample progresses from negative to positive, the visit number and the date of visit is shown in the figure. Case 1, in the first visit which was tested to be dipstick negative shows absence of albumin as expected but interestingly shows presence of zinc alpha-2 glycoprotein, alpha -1 acid glycoprotein and alpha-1 microglobulin. The fourth visit of the patient again tested to be dipstick negative, expressed same pattern as first visit. The fifth and seventh visit samples which were dipstick positive, has albumin as well as the three markers, these are spots with good intensity. Early appearance of the three proteins than albumin may convey specificity of these proteins to microalbuminuric patients.

In Case 2 with dipstick negative result in first visit, we could view trace amounts of albumin but good intensity spots of the three markers. As the case turned dipstick positive in the fifth and remained positive in sixth and seventh visit, albumin as well as MA1, 2 and 3 were observed with good intensity.

In case 3 however, the first dipstick negative sample itself showed very trace amounts of albumin but the three markers appeared in the proceeding dipstick positive sample of sitting number three. The pattern remained similar during the fifth and seventh sitting.

The precedence of the three proteins to albumin in the above cases was detected by commassie staining. This signifies the abundance of these proteins suggesting their use as even earlier markers of diabetic kidney disease than diabetic nephropathy.

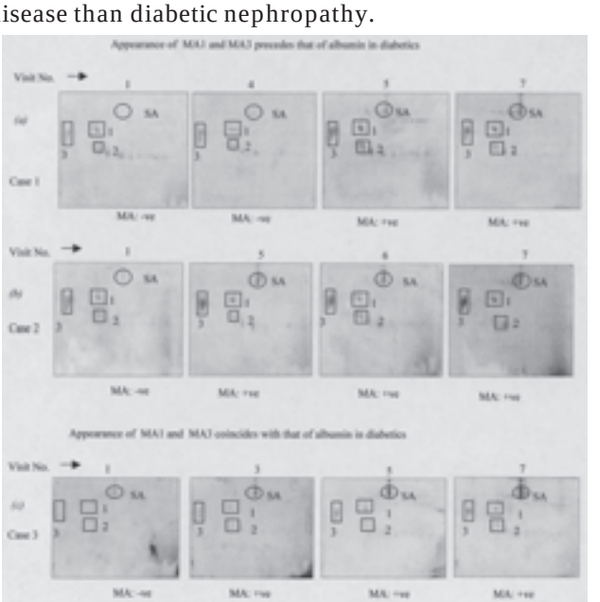

Fig. 4: The 2-D gels of 3 diabetic cases at different visits are seen here. The visit number of the sample is mentioned above the gel. 4(a) shows the presence of zinc alpha-2 glycoprotein (1), alpha-1 acid glycoprotein (3) and alpha-1 microglobulin (2) in microalbuminuria negative sample before the appearance of albumin (SA) in visit no 1 and 4. Similar pattern is seen in 4(b) for visit no.1. As the patient turns microalbuminaria positive in further visits in 4(a) and (b), all the proteins are visualised. 4(c) shows the co-appearance of the three proteins with albumin in the microalbuminuria positive sample for visit no. 2, 5, and 7. These proteins were not detected in negative sample (visit no.1).

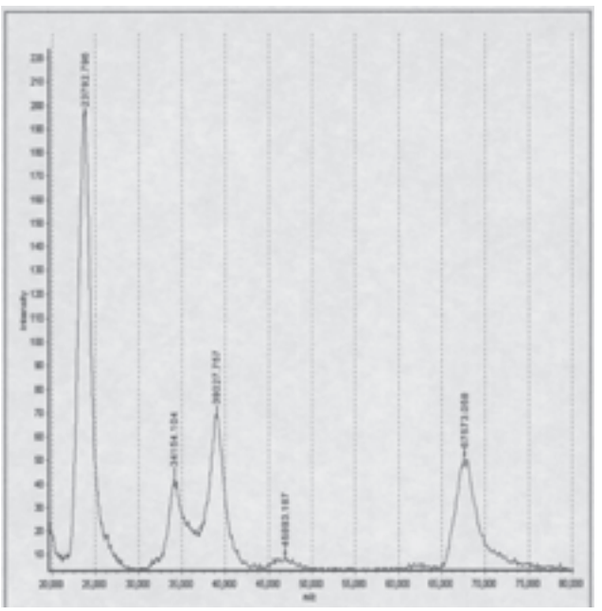

Fig. 5: The MALDI-ToF spectra of the sample subjected to linear mode gives 5 peaks of masses 23, 34, 39, 46 and 67 which corresponds to the masses of IgG, alpha-1 microglobulin, zinc alpha-2 glycoprotein, alpha -1 acid glycoprotein, and Albumin respectively.

A mass spectrometry based diagnostic approach : To employ a simple approach for detection of the proteins present in the urine sample, we subjected acetone precipitated urine samples (~100) directly to mass spectrometer and ran it in the Linear mode of the instrument.

Five peaks were observed consistently in most of the microalbuminuria positive samples (Fig. 5) with masses of 23Kda, 33Kda, 38Kda, 43Kda and 66Kda. These masses correspond to IgG, MA1, MA2, MA3 and Albumin respectively and can be co-related with the 2-D profiles of the samples from which they were taken. Two control samples were taken from non-diabetic patients, both of which did not show the spectra with any mass.

Our analysis of the urine samples using mass spectrometry approach does suggest a possible use of this approach for easy and fast diagnosis of diabetes kidney disease.

Discussion

The earliest clinical evidence currently used for the prediction of diabetic nephropathy is the 'microalbuminuria test' which has several confounding issues associated with it such as exercise, urinary tract infection, acute illness and cardiac failure. Moreover, since urinary albumin level can fluctuate widely, its specificity for diabetic nephropathy has been questioned. Towards augmenting the existing clinical tests with a more specific test, we studied 3 abundant proteins that were consistently observed in the microalbuminuric urine samples. While the urinary appearance of alpha-1 acid glycoprotein and alpha-1 microglobulin has been correlated with renal damage, this is the first report showing their presence in diabetic patients without an overt renal dysfunction.

In our study to characterize the abundant proteins in the urine of diabetic patients, we employed a proteomic approach; using 2-DGE we mapped the protein profile of microalbuminuria patients and using mass spectrometry, we identified three abundant proteins in these samples which are zinc alpha-2 microglobulin, alpha -1 microglobulin and alpha-1 acid glycoprotein, along with albumin.

We observed the presence of zinc alpha-2 glycoprotein, alpha-1 acid glycoprotein and alpha-1 microglobulin in ~100 urine samples collected from type II patients and confirmed their identification by MALDI-ToF and Western blot.

Our control study included two non-diabetic samples, of which one was microalbuminuric positive (false positive) and other was negative. Absence of all the 3 markers in both the control ensures their specificity. We further extended our experiments to those samples that tested negative for 'dipstick' and eventually turned positive in follow up cases of diabetic patients. Interestingly, we detected the appearance of all three markers in the negative samples that preceded the appearance of albumin. This early and specific manifestation of the proteins implies them to be good and early predictors of diabetic nephropathy.

Alpha-1 microglobulin has been studied as a marker for renal tubular dysfunction before. Due to its stability at low pH, its use has been suggested in screening for tubular abnormalities. Increased excretion of Alpha-1 microglobulin was found in early course while albumin excretion was still in normal range in the urine of type I patients.[14] Non-correlation of albuminuria with Alpha-1 microglobulin excretion in Type I/II may indicate whether the kidney impairment is severe or diffuse.[15] Alpha-1 microglobulin is related to duration, severity and control of type II diabetic subjects and is useful for early detection of nephropathy in diabetic subjects.[16]

Alpha-1 acid glycoprotein is a well-established marker for inflammation. It is found to predict mortality in Type II patients in a study[17] and its use as supplementary marker for development of vascular complications in Type II diabetic subjects has been reported.[18]

One of the markers, Zinc alpha-2 glycoprotein, was found in most of the samples. It is a single polypeptide chain with molecular weight range from 36-41 kDa and is found to be 38kDa in our study. ZAG is a significant factor involved in cachexia where its production increases 10 fold due to production by tumors. Northern blots show this gene to be expressed in liver, kidney, breast, prostate, pancreas and various tumors.[19] In a

recent study, the mRNA levels of ZAG, with a missense mutation, was found to increase in kidneys and liver of KK/Ta mice, the strain of mice that serves as a model to study type II diabetes.[20] Obesity is a major risk factor for the development of type II diabetes and ZAG is suggested as a possible candidate gene for regulation of body weight. ZAG is found to lower blood sugar and its lypolysis activity.[21] could possibly explain its appearance that precedes albumin in our study. ZAG has also been detected in the vitreous fluid of diabetic patients with retinopathy, which may suggest increased level of this protein in the body fluids of diabetic patients.

In an earlier study we examined urinary protein profiles of patients diagnosed with nephrotic syndrome as well as patients with end-stage renal failure[22] and observed distinct proteome of microalbuminuria positive samples from these renal complications. This confers the specificity of the marker proteins MA1, MA2 and MA3 to diabetic nephropathy. The correlation of these markers with the location and precise stage of renal injury remains to be studied.

Over the last few years there has been immense advancement in biomarker discovery and diagnosis. The use of mass spectrometry has been explored in the field of clinical diagnosis for it being a robust, sensitive and high throughput technique.[23] Mass spectrometry is being used in cancer diagnostics world wide[24, 25] and holds promise to be a useful technique in disease diagnosis. The MALDI-ToF analysis of the urine samples in our study gives a good interpretation of multiple biomarkers. Despite the limitation of it being an expensive technique, it offers high throughput and rapid analysis and has potential to be used as a diagnostic tool to analyze various kidney disorders.

With a proteomic based study on the microalbuminuria positive urine samples, we observed and identified three supporting markers that can be used for a precise examination of diabetic nephropathy. We propose a mass spectrometry based high throughput test that is a faster and easier approach to examine the presence of these proteins from the urine sample to get a reliable outcome.

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Announcement

Dr. PJ Mehta Young Scientist Award

Hypertension Society of India is being giving the Young Scientist Award for the Best paper presentation at the National Conference of Hypertension. Papers are invited from young research workers (below 35 years of age) who have done original research work in the field of hypertension and related subjects. these papers will be judged by a panel of referees. The finalists will be required to present their papers during 14th National Conference of Hypertension, on November 11th-13th, 2005 at Manipal, under the auspices of Hypertension Society of India. From these will be selected the recipient of the **Dr. PJ Mehta Young Scientist Award**. The research worker who submits his paper must attach a certificate to indicate his date of birth. the presenter selected for the award will be given 2nd Class AC train fare to and from their hometown. Please send 5 copies of the full maniscript along with the abstract to **Dr BR Bansode, Secretary General, HSI, Dr. Babasaheb Ambedkar Memorial Hospital, Central Railway, Byculla, Mumbai 400 027. Last date of receipt of manuscript : 15th August, 2005.**

Announcement

The executive committee of **Association of Physicians of India, Gwalior Chapter** for year 2005 is as follows:

Founder Chairman	:	Ajai Shanker
Chairman	:	NN Laha
Vice-Chairman	:	PC Mathur, KP Bhatnagar
Secretary	:	S Gajendragadkar
Jt. Secretary	:	Sushma Trikha
Treasurer	:	Archana Kansal
Executive Members	:	HS Bansal, JP Sharma, Lalit Mehta, R Gangil, Mrs. P Gopal, Rajeev Agrawal