PROTEOMIC STUDIES IN ENDEMIC NEPHROPATHY

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Abstract

Endemic nephropathy (EN) is a chronic tubulointerstitial nephropathy with an early insidious and slow development into terminal renal failure. Proteomics is the systematic study of a proteome, which is the total protein content of a cell, organism or body fluids. Application of proteomic technologies in nephrology has enabled more detailed analyses of protein functions and examined their importance in various physiological and pathological states. Biomarkers with high specificity and sensitivity to early diagnosis are needed for a better understanding of the mechanisms of EN development and its consequences. Urine beta2-microglobulin (B2M) was mainly used as a tubular marker of EN but recently alpha1-microglobulin (AMBP) was proposed for the diagnosis of EN. We studied the urine proteins of 360 patients with EN, diabetic nephropathy (DN) and acute kidney injury (AKI) and the healthy population using proteomic tools. Protein maps from the urine of patients with EN showed significant differences in comparison to the healthy subjects and patients with DN and AKI. Our study highlights six proteins in urine that were differentially excreted in the urine of EN patients compared with the other groups and have potential to be markers for EN prediction. In one of our studies, using routine biomarkers, we investigated the potential of urine B2M, AMBP, albumin and total protein as diagnostic markers for EN, in comparison to glomerulonephritis, nephrosclerosis and a healthy state. Modern proteomic technologies are still robust investigation tools, but can access a vast amount of information from one set of experiments in comparison to a classic diagnostic approach.

Key words: endemic nephropathy, beta2-microglobulin, alpha1-microglobulin, urine proteomics.

Endemic nephropathy (EN) is a chronic tubulointerstitial nephropathy with an early insidious and slow development into terminal renal failure. In the fifties, it was described as a disease of the population in the alluvial plains of South East Europe, along the tributaries of the River Danube in Bosnia and Herzegovina, Bulgaria, Croatia, Romania and Serbia [1–3]. The epidemiology and the etiology of EN were widely explored [4]. The disease usually affects adults in their fourth/fifth decade with eventual end-stage renal failure in their sixth decade [4]. Recently, fewer people have been affected with EN, and at older ages, but it is still considered as a major health problem in some endemic regions [5]. An increased frequency of upper urinary tract tumours (UUT) and sporadic cases of urinary bladder tumours in the population of endemic villages was described in the first reports on EN [6]. A recent survey of UUT in the South Morava River basin and its tributaries where EN is endemic revealed an increased frequency of tumours not only of the renal pelvis and ureter but also of urinary bladder tumours [7–9].

The exact causes of the disease are not completely known. Inheritance is of importance, but the effect of environmental factors is stron-
The family character of the disease was considered one of the main diagnostic criteria of EN. However, equal rates of diseases in different ethnic groups from the same focus, the appearance of EN in migrants, equal representation of the illness among household members’ related by blood and maritally, all suggest that the role of heritage is not critical. One research has also shown the possibility of a specific chromosomal marker (3q25) in patients and subjects at high risk of the disease. Limited studies exploring different genetic factors in EN etiology were directed towards searching for variation in enzyme activity in patients and their healthy relatives, investigation of genetic heterogeneity of xenobiotic-metabolizing enzymes, transporters and the factor of fibrosis, as well as identification of structural chromosomal aberrations in blood and tumour samples from EN patients [4]. Environmental factors still play a decisive role. Protozoa, bacteria, rickettsiae and even viruses were ruled out as the possibility of the emerging disease. Special attention was, however, caused by the study of the influence of mycotoxins (ochratoxin A), Aristolochia species (aristolochic acid 1 and 2), as well as numerous pollutants present in the environment.

Some 43 years ago AA was suggested as the etiologic agent of EN. Ivic has found AA in flour obtained from wheat contaminated with seeds of Aristolochia clematis in the endemic region [10]. He has conducted a survey of the geographical distribution of the plant, Aristolo-

Table 1

Epidemiological, clinical and functional characteristics of EN; data according to Stefanović [4]

<table>
<thead>
<tr>
<th>Epidemiological characteristics</th>
<th>Clinical characteristics</th>
<th>Functional changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residence in an endemic settlement</td>
<td>Progressive renal insufficiency</td>
<td>Proteinuria of the tubular type</td>
</tr>
<tr>
<td>Family history of renal disease and of renal deaths</td>
<td>Anemia – normochromic or slightly hypochromic</td>
<td>Impaired urinary acidification</td>
</tr>
<tr>
<td>Family history of urothelial tumors</td>
<td>Polyuria, polydipsia, nocturia</td>
<td>Glycosuria</td>
</tr>
<tr>
<td>Occupational history of farming</td>
<td>Hypertension – rare</td>
<td>Increased uric acid excretion</td>
</tr>
<tr>
<td></td>
<td>Urothelial tumors – common</td>
<td>Renal salt wasting</td>
</tr>
<tr>
<td></td>
<td>Abnormalities on urinalysis</td>
<td>Impaired concentrating capacity</td>
</tr>
<tr>
<td></td>
<td>Small or shrunken kidneys</td>
<td>Decreased glomerular filtration rate</td>
</tr>
</tbody>
</table>

Although there are no specific criteria for the diagnosis of EN, the following epidemiological, clinical, laboratory and histological features are important in the diagnosis of EN [12]:

- epidemiological data: the disease occurs in hotspots, endemic in the rural population settlements along the rivers Kolubara, Drina, Sava, Morava, in the former Yugoslavia, Bulgaria and Istar in tributaries of the Danube in Romania; has a familiar character, and not hereditary; treatment of the sexes was approximately to the same extent, ear-
lier the disease manifested after the end of the second decade of life;

- Clinical and laboratory data: unnoticed, insidious beginning, chronic course, no edema and febrile phase, blood pressure moderately elevated in about 40–50% of cases, usually in advanced stages of the disease; laboratory findings indicate the primary tubular damage – proteinuria is sparse and tubular type (B2M, AMBP); in advanced stages of the disease anaemia is more pronounced; lethal outcome appears only after years or decades and the disease with symptoms of uraemia;

- Pathological-anatomical and pathological-histological findings: macroscopic – almost symmetrically mass reduced on both sides, microscopic – interstitial and tubular changes with small cell infiltration localized in the intestitium.

Due to the lack of markers of early diagnosis, the disease is diagnosed at the stage of chronic renal failure, where the therapy has not shown significant results. Diagnostic markers with high specificity and sensitivity for early diagnosis of diseases are needed for a better understanding of the mechanism of the development of EN and its consequences, and also an adequate implementation of effective treatment.

In the absence of an identified etiological factor, effective prevention of EN is not yet possible. Treatment of EN is similar to that of all chronic interstitial nephropathies [4].

**Proteomic research in nephrology**

Proteomics is the systematic study of a proteome, which is the total protein content of a cell, organism or body fluids (blood, urine, sweat, etc.). While the genome is considered as a static component, the proteome shows dynamic properties, where the protein profile changes depend on the presence of many extra- and intracellular stimuli. The most common proteomic methods are various mass spectrometry (MS) based techniques (matrix-assisted laser desorption/ionization (MALDI), surface-enhanced laser desorption/ionization (SELDI) and others), which are commonly combined with different protein separation methods (liquid chromatography (LC), two dimensional electrophoresis (2D-E), capillary electrophoresis (CE) and others). These measurements provide characteristically peptide spectra, which can be assigned to a specific protein after using computer programmes and online protein data base search (specific details about proteomic technologies were briefly described in the following review articles [13–15]). Application of proteomic technologies enabled more detailed analyses of the functions of proteins and examined their importance in various physiological states, in sickness and during the implementation of appropriate therapy. Clinical proteomics presents the application of proteomic analyses in order to discover diagnostic and prognostic biomarkers, to monitor the condition of patients and the efficacy of therapeutic protocols and to understand the mechanisms of the formation, development and progression of the disease. It is considered to be one of the leading areas of research nowadays [16–18]. In order to find a specific disease markers, different body fluids (urine, blood, sweat, dialisates) or even tissue samples (fresh tissue, frozen tissue, paraffin embedded blocks) could be used in experimental and clinical research, depending on the disease pathway.

**EN urine proteomic research.** Diagnostic markers with high specificity and sensitivity for early diagnosis of diseases are needed for a better understanding of the mechanism of the development of EN and its consequences, and also for an adequate implementation of the effective treatment. Urine beta2-microglobulin (B2M) was mainly used as a tubular marker of EN but recently alpha-1-microglobulin (AMBp) was proposed for early EN diagnosis. To improve visualization/detection of proteins and identification of EN-related biomarkers in urine for an early diagnosis and in prevention, we combined two-dimensional differential in-gel electrophoresis (2D-DIGE) and MS [19]. The study involved 360 people divided into six groups: 60 healthy persons living in areas of EN (Kutleš, Leskovac and surroundings, HEN), 60 EN patients with low protein in the urine (below 150 mg/mL), 60 EN patients with high levels of protein in the urine (above 150 mg/mL), 60 patients with DN, 60 patients with prerenal AKI, and 60 healthy persons who do not live in the areas of EN (HGER) (Table 2).
Table 2

Experimental study design – number of samples per experimental group used for each method are provided; HEN – healthy individuals from EN regions, EN – endemic nephropathy patients, LP – EN patients with low proteinuria, HP – EN patients with high proteinuria, DN – patients with diabetes mellitus type 2 and diabetic nephropathy, AKI – acute kidney injury, HGER – healthy individuals from Germany

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>2D gel electrophoresis</th>
<th>2D DIGE</th>
<th>Western blot analysis</th>
<th>Dot blot analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEN = 60</td>
<td>5 pool of urine samples (10 urine samples pro pool)</td>
<td>5 pool of urine samples (10 urine samples pro pool)</td>
<td>20</td>
<td>52</td>
</tr>
<tr>
<td>EN = 60</td>
<td>5 pool of urine samples (10 urine samples pro pool)</td>
<td>5 pool of urine samples (10 urine samples pro pool)</td>
<td>20</td>
<td>LP – 29, HP – 28</td>
</tr>
<tr>
<td>DM = 60</td>
<td>5 pool of urine samples (10 urine samples pro pool)</td>
<td>5 pool of urine samples (10 urine samples pro pool)</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>ARI = 60</td>
<td>5 pool of urine samples (10 urine samples pro pool)</td>
<td>5 pool of urine samples (10 urine samples pro pool)</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>HGER = 60</td>
<td>5 pool of urine samples (10 urine samples pro pool)</td>
<td>5 pool of urine samples (10 urine samples pro pool)</td>
<td>20</td>
<td>60</td>
</tr>
</tbody>
</table>

To map the proteome of the urine pools protein precipitation followed with 2-DE analyses was performed. Delta 2D analysis of the gels revealed around 417 ± 12 and 481 ± 34 protein spots in the pH 4–7 2D gel region from urine pool samples from EN patients with microproteinuria and macroproteinuria respectively, whereas only 369 ± 11 proteins could be visualized in the pH 4–7 2D gel region from urine pool sample collected from HEN. Additionally, protein pools were formed and analysed from urine samples from patients with AKI, DN and HGER as described above. For high confidence protein identification we performed MALDI-TOF/TOF-MS/MS and Mascot data search [20]. This allowed the identification of 213 proteins from urine pools, which corresponded to a library of 61 non-redundant proteins. To identify with high reproducibility and statistical significance proteins differentially excreted in EN urine compared to the other group we performed 2D-DIGE analyses of the different pool samples and compared the resulting maps with each other. 2D-DIGE protein maps were generated in 4–7 pI range from different urine pools. As expected the resulting gels showed a large protein release in the urine of patients with kidney disease. Qualitative and quantitative analyses of the 2D-DIGE maps with Delta 2D and Prizma4 software revealed a list of 43 proteins, which were differentially excreted in the urine from EN patients compared to HEN, but among these proteins, a pattern of six different proteins identified as AMBP, APOH, B2M, LMAN2, POT1 and SOD1, were significantly (p < 0.001) released at high level in the urine from EN patients compared to the other groups. To validate the diagnostic marker identified using proteomics, the protein excretion levels in urine samples from different sample groups were quantified using WB. Our results showed that the differences in protein excretion level of the following proteins, AMBP, APOH, B2M, LMAN2, POT1 and SOD1 depend on the urine origin. The highest level of these proteins quantified by WB analysis was found in the urine of EN patients as compared to the urine from DN patients (p < 0.001) and the urine from HEN (p < 0.001). WB detection of these proteins was almost negative in urine from patients with prerenal AKI and HGER (p < 0.001). Interestingly higher levels of these proteins were also found in urine from HEN as compared to urine from patients with prerenal AKI and HGER (p < 0.001). Using the ROC curve analysis, the area under curve (AUC) and 95% confidence interval for examined urinary proteins were calculated. The resulting data showed that the identified protein markers could differentiate EN from DN, AKI, HEN and HGER with high specificity and sensitivity. To validate the usefulness of the identified EN markers for diagnosis, we performed dot blot analysis on the larger patient cohort. The statistical analysis of the dot blot data confirmed with higher significance an increased
Proteomic studies in endemic nephropathy

excretion of these six proteins in urine samples from EN LP and EN HP patients than the other groups (AKI, DN, HGER) \( (p < 0.001) \). It was also noted that these proteins were present in significantly higher concentration in urine from HEN than in urine from HGER. The data confirmed a clear discrimination of patients with EN LP and EN HP from AKI and DN patients and healthy control HGER. APOH, POT1 and LMAN2 from urine samples from HEN in comparison to urine samples from EN LP were almost in the same range, without significant difference (Figure 1).

Figure 1 – I: 2-D DIGE digital image of urine proteome from EN: the 213 identified proteins corresponded to a library of 61 non-redundant proteins, which were labelled on the gel image, each protein was presented with its gene name. The proteins were separated over a pI range 4–7 by IEF and then on a 12% SDS PAGE. The first dimension separation of proteins in 2D electrophoresis involves the separation of proteins based on their isoelectric points (IEF) (passing through the set pH gradient of IPG strips with the help of electricity of the proteins which are arranged within a tape). The second dimension involves the separation of proteins based on their molecular weight and speed of the electric field. 2-DE of samples was carried out by an adapted protocol; II: Graphics represent separation of the 2D-DIGE detection channels and enlargement of the gel regions of interest showing the quantification of protein found to be differentially excreted in urine from HEN, EN-L, EN-H, DN, AKI and HGER. On the y-axis the spot-volume-percentage is given and the x-axis shows the corresponding pools where the protein spot was analysed. Labelling of the graphics corresponds to the gene names listed on the gel. Some of proteins represented in this figure fulfil the criteria of potential biomarker for EN: AMBP, APOH, B2M, LMAN2, POT-1 and SOD1. Statistical analyses were performed by Prism 4 software \( (**p < 0.01, ***p < 0.001) \). Biomarkers of tubular injury, \( \alpha_1 \)-microglobulin (AMBP) (A), \( \beta_2 \)-microglobulin (B2M) (C), were found to be significantly excreted in urine from EN patients. Further examination of differences in the excretion of protein in the urine of respondents in other groups, shows four additional different proteins identified as \( \beta_2 \)-glycoprotein 1 (APOH) (B), mannose-binding lectin 2 (LMAN2) (D), protective protein telomeres 1 (POT1) (E) and superoxide dismutase [Cu-Zn] (SOD1) (F), with significantly \( (p < 0.001) \) higher concentrations in the urine of EN patients in comparison to other experimental groups.
Comparative analysis with data from earlier studies performed on urine with 2-DE revealed that 25–31 of 61 identified proteins were already reported in the context of diabetic nephropathy [21–23]. Whereas several proteins presented in this study, especially mannose-binding lectin 2 (LMAN2), protection of telomeres protein 1 (POT-1) and superoxide dismutase [Cu-Zn] (SOD1), seem to be characteristic of EN and were not described in other kidney diseases. Interestingly higher levels of these proteins were also found in urine from HEN as compared to urine from HGER.

As expected, AMBP and B2M were found in larger amounts in the urine of EN patients compared with controls (HEN and HGER) and patients with AKI. In our previous study [24], using demonstrated urine biomarkers for kidney disease, we investigated the potential of urine B2M, AMBP, albumin and total protein as diagnostic markers for EN, in comparison to glomerulonephritis (GN), nephrosclerosis (NS) and healthy state. In EN patients AUC for urine B2M (0.828) and AMBP (0.782) was higher than for urine albumin (0.740), moreover in GN patients AUC for urine protein (0.854) and albumin (0.872) was significantly higher than that for the two low molecular weight proteins. AUC for all four urinary markers in NS patients was significantly lower than in EN patients, ranging between 0.500 and 0.595. Comparing EN to healthy controls, B2M had higher sensitivity and specificity at the cut-off levels (p < 0.001) than AMBP (p < 0.05). Comparing EN to GN, B2M was the best marker. Although B2M, AMBP and albumin are known to be markers for EN, they can hardly differentiate EN from HEN as this group also present these markers. Moreover the three markers are also known to be highly secreted in other kidney diseases e.g. DN and allograft rejection [25–29] and are not specific EN markers.

However, alteration in AMBP and B2M concentrations has been observed in patients with different diseases, including DN and EN [17, 29]. APOH is structurally related to the regulators of the complement activation family. Previously, it was suggested that it had a potential to be used as a marker of tubular injury [30]. Several reports indicate that APOH is the antigen of antiphospholipid antibodies and also behaves as an acute-phase reactant [31]. POT1 is a single-stranded telomeric DNA binding protein with a critical role in ensuring chromosome stability and preventing uncontrolled cell division and cancer development [32]. SOD1 is a copper and zinc-containing homodimer that is found almost exclusively in intracellular cytoplasmic space. Its appearance in urine samples points to cell damage and acute injury [33]. LMAN2 was found to be an important component of epithelial cells due to its role in transportation of large molecules through the cell surface or within the cell [34]. Its high expression in skeletal muscle and kidney was reported [35], suggesting its important role in IgA nephropathy, diabetic nephropathy, ischemic tubular injury and renal transplantation [36].

Conclusions

Application of proteomics technologies will provide insight into the functional significance of protein(s) in various physiological situations, disease states and therapy approaches in nephrology. Our study highlights six proteins in urine that were differentially excreted in the urine of EN patients compared with the other groups and have the potential to be markers for EN prediction. Modern proteomic technologies are still robust investigation tools, but can access a vast amount of information from one set of experiments in comparison to the classic diagnostic approach. Future development in proteomics could underline its advantages.

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Резиме

ПРОТЕОМСКИ СТУДИИ ВО ЕНДЕМСКАТА НЕФРОПАТИЈА

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Ендемска нефронатиска (ЕН) е хронична тубулонинтерстицијална нефронатиска со ран подмолен и бавен развој до терминална ренална инсуфициенција. Протеомика е систематско проучување на протеомот, кој е вкупна содржина на протеини на клетката, организмот или телесните течности. Примената на протеомските технологии во нефрологијата овозможи подетални анализи на протеинските функции и ја испита нивната важност во различни физиолошки и паатолошки состојби. Биомаркерите со висока специфичност и сензитивност за рана дијагноза се потребни за подобро разбиране на механизмот на развој на ЕН и нејзините последици. Уринарниот beta2-микроглобулин (B2M), главно, се користи како тубуларен маркер на ЕН, но неодамна alpha1-микроглобулин (AMBP) беше предложен за дијагноза на ЕН. Ги проучуваме протеините во урината на 360 пациенти со ЕН, дијабетична нефронатиска (ДН) и акутно бубрежно оштетување (АКИ) и здравата популација со користење протеомски алатки. Мапите на протеини од урината на пациентите со ЕН покажаа значителни разлики во споредба со здравите лица и пациентите со ДН и АКИ. Нашата студија нагласува шест протеини во урината што беа различно излажувани во урината на пациентите со ЕН во споредба со другите групи и што имаат потенцијал да бидат маркери за дијагноза на ЕН. Во една од нашите студии, со користење рутински биомаркер, го испитуваме потенцијалот на уринарниот B2M, AMBP, албумин и вкупните протеини како дијагностички маркери за ЕН, во споредба со гомерулонефритисот, нефростенозата и здрави луѓе. Модерните протеомски технологии се уште се робусни алатки за испитување, но може да пристапат до огромно количество информации од еден збир на експерименти во споредба со класичноти дијагностички пристап.

Ключни зборови: ендемска нефронатиска, beta2-микроглобулин, alpha1-микроглобулин, уринарна протеомика.