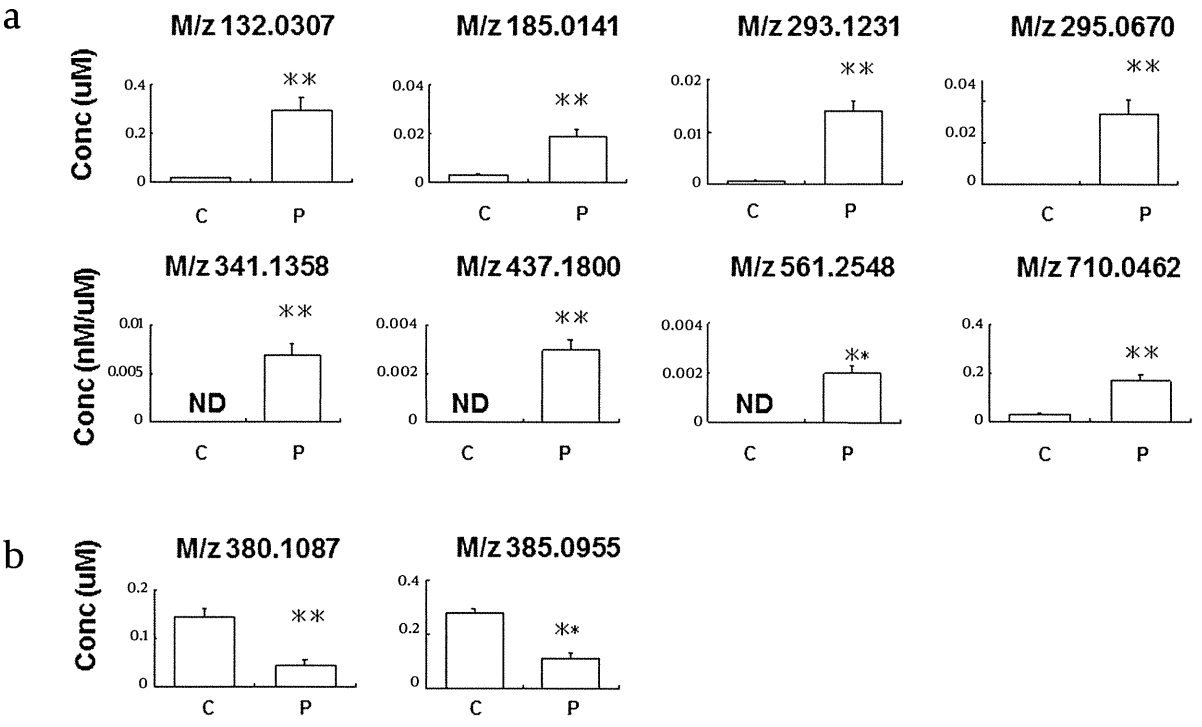

Figure 6. Changes in unidentified metabolites which were (a) increased or (b) decreased in the serum of patients with stage 1-2 CKD. C: Control, P: CKD patients. M/z: mass-to-charge ratio of the metabolite. ND: not detectable. **: $p < 0.01$ vs. controls.

decreased in the serum (from 13.8 ± 2.0 to 8.7 ± 0.8 μM, $p < 0.05$). Several changes were also found in oxidative stress-related amino acids. In particular, carnosine and hypotaurine were significantly lower in the urine of CKD patients, whereas hypotaurine and taurine were elevated in the serum.

4.3. Changes in nucleic acid metabolites in the serum and urine of patients with stage 1-2 CKD

In cation and anion analysis mode, multiple changes in nucleic acid metabolites were observed in the serum and urine of patients with early stage CKD *(Figure 4)*. In particular, hypoxanthine in the serum was markedly elevated (from 4.0 ± 0.7 to 209.8 ± 53.3 μM, $p < 0.01$), whereas adenosine was decreased in both the serum and urine of patients.

4.4. Changes in carbohydrate metabolites in the serum and urine of patients with stage 1-2 CKD

Several changes in carbohydrate metabolites were also observed in the serum and urine of patients with CKD *(Figure 5)*. Serum lactate increased from 2331 ± 422 to 12903 ± 2273 μM ($p < 0.01$), whereas urine citrate, fumarate and 3-phosphoglycerate were decreased compared to controls.

4.5. Changes in unidentified metabolites in the serum of patients with stage 1-2 CKD

The metabolome analysis revealed that serum levels of several novel unidentified metabolites were also markedly increased *(Figure 6a)* or decreased *(Figure 6b)* in the patients with CKD compared to controls.

5. Discussion

There have been several reports about serum amino acid patterns in advanced (stage 5) CKD, also known as end-stage renal disease (ESRD) (5-8). In general, the essential amino acid levels are decreased, while the nonessential amino acids are either within the normal range or increased, so the ratio of essential to nonessential amino acids is decreased in ESRD. It has been assumed that these changes are due to low protein intake, deficiency of excretory and metabolic functions of the diseased kidneys, toxic effect of uremia and, in dialysis patients, loss of protein and amino acids by the dialytic procedure (6, 7). The results of this study were compatible with the previous reports on patients with ESRD, and suggest that the changes in amino acid metabolism were already detectable at an early stage of CKD. It is interesting that these changes were seen even without marked renal insufficiency, suggesting that changes in amino acid metabolism are an early event in the course of CKD, and do not require the presence of uremia. Interestingly, not all nonessential amino acids were increased. In particular, glutamine was decreased in both the serum and urine, whereas glutamate was increased, suggesting possible changes in the conversion equilibrium of these two amino acids in these patients. We also found evidence for changes in oxidative stress in early stage CKD. In particular, the free-radical scavengers carnosine

and hypotaurine were decreased in the urine of patients, but conversely hypotaurine and taurine were increased in the serum. We speculate that these free-radical scavengers may have been decreased in the urine because of increased oxidative stress in the kidney, and this was counteracted by increases in the serum.

An important advantage of this method is that multiple metabolic pathways could be analyzed simultaneously using the three modes of electropherogram analysis. Regarding nucleic acid metabolites, serum and urine adenosine and urine guanine were decreased and serum hypoxanthine increased in the patient group, suggesting the possibility that degradation of purine nucleotide was elevated in these patients with stage 1-2 CKD. Interestingly, hypoxanthine was markedly increased in the serum of patients, to about 50 times the level of controls. Previous report in patients on dialysis showed that plasma concentrations of hypoxanthine and uric acid were increased in patients with ESRD (17). In this study hypoxanthine was already increased in patients with stage 1-2 CKD, even though serum uric acid was unchanged. These results may be important because hypoxanthine may act as a cardiotoxin (18), possibly by causing mitochondrial damage through increased oxidative stress (19). A recent report also suggested that hypoxanthine accumulation in xanthine oxidoreductase depletion mice caused progression of renal interstitial fibrosis, also by an oxidative stress-related mechanism (20). These results suggest the hypothesis that increased hypoxanthine may be one reason for the increased incidence of cardiovascular disease in patients with CKD (3, 4), as well as a potential risk factor for progression of renal disease.

Concerning carbohydrate metabolism, we found that serum lactate was increased, but other TCA cycle metabolites, such as citrate and fumarate, were decreased, suggesting that changes in glucose metabolism may also be evident from an early stage in CKD. An important advantage of metabolome analysis is the potential to identify new and unidentified metabolites which could have important pathophysiological functions. In our studies, we found that several novel unidentified metabolites were significantly increased in the serum of patients with CKD, whereas others were decreased. At present, the molecular structures of these metabolites are unknown. It is possible that these unidentified products may have novel pathophysiological functions, or may be new disease markers for renal injury. We are therefore planning further extended studies to examine these possibilities. One caveat of this study is that the patients with stage 1-2 CKD in our study were all candidates for renal biopsy, and may not be representative of the general population of stage 1-2 CKD. Thus, the possibility that these changes specifically appeared in proteinuric kidney diseases, but may not seen in early stage CKD without proteinuria, cannot be completely ruled out. Moreover, CKD of various etiologies were considered together in the patient group, because we were unable to discover a clear correlation between specific etiologies and their metabolomic profiles. Based on our current findings, further studies are warranted for comparisons between different renal diseases. In summary, the results of this study suggest that metabolic analysis may be used for detecting changes in amino acid, nucleic acid, and carbohydrate metabolites in the serum and urine of patients with early stage CKD, as well as for detecting unidentified metabolites which may have novel functions. Understanding these changes may be important for developing new strategies to prevent cardiovascular events and progression to ESRD in patients with CKD.

Financial support

None declared.

Conflict of interest

The authors declare that they have no conflicts of interests related to this study.

Acknowledgements

This study was supported by a Grant-in-Aid for JSPS Fellows (2155542) and Grants for Scientific Research (20590984, 2155542, 20680105) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, and the G-COE program 'Center for Human Metabolomic Systems Biology' from MEXT of Japan.

References

1. Szczech LA, Harmon W, Hostetter TH, Klotman PE, Powe NR, Sedor JR, et al. World Kidney Day 2009: problems and challenges in the emerging epidemic of kidney disease. *J Am Soc Nephrol.* 2009;**20**(3):453-5.
2. Chamney M, Pugh-Clarke K, Kafkia T. Management of co-morbid diseases in a patient with established renal failure. *J Ren Care.* 2009;**35**(3):151-8.
3. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *Am J Kidney Dis.* 2002;**39**(2 Suppl 1):S1-266.
4. Sarnak MJ, Levey AS, Schoolwerth AC, Coresh J, Culleton B, Hamm LL, et al. Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. *Circulation.* 2003;**108**(17):2154-69.
5. Alvestrand A, Bergstrom J, Furst P, Germanis G, Widstam U. Effect of essential amino acid supplementation on muscle and plasma free amino acids in chronic uremia. *Kidney Int.* 1978;**14**(4):323-9.
6. Bergstrom J, Alvestrand A, Furst P. Plasma and muscle free amino acids in maintenance hemodialysis patients without protein malnutrition. *Kidney Int.* 1990;**38**(1):108-14.
7. Canepa A, Filho JC, Gutierrez A, Carrea A, Forsberg AM, Nilsson E, et al. Free amino acids in plasma, red blood cells, polymorphonuclear leukocytes, and muscle in normal and uraemic children. *Nephrol Dial Transplant.* 2002;**17**(3):413-21.
8. Furst P. Amino acid metabolism in uremia. *J Am Coll Nutr.* 1989;**8**(4):310-23.
9. Oresic M. Metabolomics, a novel tool for studies of nutrition, metabolism and lipid dysfunction. *Nutr Metab Cardiovasc Dis.* 2009;**19**(11):816-24.
10. Spratlin JL, Serkova NJ, Eckhardt SG. Clinical applications of me-

tabolomics in oncology: a review. *Clin Cancer Res.* 2009;**15**(2):431-40.
11. Kim K, Aronov P, Zakharkin SO, Anderson D, Perroud B, Thompson IM, et al. Urine metabolomics analysis for kidney cancer detection and biomarker discovery. *Mol Cell Proteomics.* 2009;**8**(3):558-70.
12. Waterman CL, Kian-Kai C, Griffin JL. Metabolomic strategies to study lipotoxicity in cardiovascular disease. *Biochim Biophys Acta.* 2010;**1801**(3):230-4
13. Soga T, Baran R, Suematsu M, Ueno Y, Ikeda S, Sakurakawa T, et al. Differential metabolomics reveals ophthalmic acid as an oxidative stress biomarker indicating hepatic glutathione consumption. *J Biol Chem.* 2006;**281**(24):16768-76.
14. Levey AS, Eckardt KU, Tsukamoto Y, Levin A, Coresh J, Rossert J, et al. Definition and classification of chronic kidney disease: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). *Kidney Int.* 2005;**67**(6):2089-100.
15. Hirayama A, Kami K, Sugimoto M, Sugawara M, Toki N, Onozuka H, et al. Quantitative metabolome profiling of colon and stomach cancer microenvironment by capillary electrophoresis time-of-flight mass spectrometry. *Cancer Res.* 2009;**69**(11):4918-25.
16. Soga T, Igarashi K, Ito C, Mizobuchi K, Zimmermann HP, Tomita M. Metabolomic Profiling of Anionic Metabolites by Capillary Electrophoresis Mass Spectrometry. *Anal Chem.* 2009;**81**:6165-74.
17. Shahbazian H, Zand Moghadam A, Ehsanpour A, Khazaali M. Changes in plasma concentrations of hypoxanthine and uric acid before and after hemodialysis. *Iran J Kidney Dis.* 2009;**3**(3):151-5.
18. Tarantola M, Motterlini R, Beretta M, Rovida E, Samaja M. Impairment of the post-anoxic recovery of isolated rat hearts by intravascular hypoxanthine and xanthine. *Biomater Artif Cells Artif Organs.* 1990;**18**(2):309-20.
19. Oliveira PJ, Rolo AP, Monteiro P, Goncalves L, Palmeira CM, Moreno AJ. Impact of carvedilol on the mitochondrial damage induced by hypoxanthine and xantine oxidase–what role in myocardial ischemia and reperfusion? *Rev Port Cardiol.* 2002;**21**(12):1447-55.
20. Ohtsubo T, Matsumura K, Sakagami K, Fujii K, Tsuruya K, Noguchi H, et al. Xanthine oxidoreductase depletion induces renal interstitial fibrosis through aberrant lipid and purine accumulation in renal tubules. *Hypertension.* 2009;**54**(4):868-76.

Transcriptional Regulation of Organic Anion Transporting Polypeptide SLCO4C1 as a New Therapeutic Modality to Prevent Chronic Kidney Disease

TAKEHIRO SUZUKI,[1] TAKAFUMI TOYOHARA,[2] YASUTOSHI AKIYAMA,[1] YOICHI TAKEUCHI,[1] EIKAN MISHIMA,[1] CHITOSE SUZUKI,[1] SADAYOSHI ITO,[1] TOMOYOSHI SOGA,[3] TAKAAKI ABE[1,4,5]

[1]Division of Nephrology, Endocrinology, and Vascular Medicine, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan

[2]Department of Cell Growth and Differentiation, Center for iPS cell Research and Application, Kyoto University, Kyoto 606-8507, Japan

[3]Institute for Advanced Biosciences, Keio University, Tsuruoka 997-0052, Japan

[4]Department of Clinical Biology and Hormonal Regulation, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan

[5]Division of Medical Science, Tohoku University Graduate School of Biomedical Engineering, Sendai 980-8574, Japan

Received 1 March 2011; revised 26 April 2011; accepted 10 May 2011

Published online 7 June 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.22641

ABSTRACT: Uremic toxins accumulate in patients with chronic kidney diseases (CKDs) and cause further progression of renal damage and cardiovascular diseases. Recently, it was reported that some of the organic anion transporting polypeptides (OATPs) and the organic anion transporters (OATs) are involved in the renal elimination of uremic toxins. SLCO4C1 is the only OATP expressed at the basolateral side of proximal tubular cells in human kidney, and it mediates the excretion of uremic toxins. The overexpression of human SLCO4C1 in rat kidney promotes the renal excretion of uremic toxins and reduces hypertension, cardiomegaly, and renal inflammation in renal failure. Statins induce SLCO4C1 expression thorough transcriptional factor Aryl hydrocarbon receptor through binding of the xenobiotic responsive element at its promoter region. The administration of statin in a rat renal failure model facilitated the elimination of uremic toxins and mitigated organ damage. In addition, metabolomic analysis of rat renal failure models and patients with CKD by capillary electrophoresis–mass spectrometry is a useful method for identifying new uremic solutes and explores surrogate biomarkers for detecting the progression of early stage CKD. © 2011 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 100:3696–3707, 2011

Keywords: Organic anion-transporting polypeptide transporters; Organic anion transporter; ABC transporters; Membrane transport; Uremic toxin; Statin; Chronic kidney disease; Metabolome analysis; Capillary electrophoresis; Mass spectrometry

INTRODUCTION

The kidney is involved in the elimination of various intrinsic and extrinsic compounds from the body, and in the regulation of homeostasis and pharmacokinetics. Various water-soluble substances are excreted from the blood into the urine by the kidney. These renal excretory systems comprise three major components, that is, glomerular filtration, tubular secretion, and tubular reabsorption. Glomerular filtration can be partially compensated for by hemodialysis (HD), but the accumulation of uremic toxins that cannot be effectively eliminated by dialysis leads to the progression of renal damage, hypertension, and cardiovascular diseases (CVDs). The selective and specific excretion and reabsorption of various metabolic compounds and urinary-eliminated drugs and metabolites is not possible by artificial means. Many of the urinary-eliminated drugs and metabolites are water soluble and cannot easily penetrate the lipid bilayer

Correspondence to: Takaaki Abe (Telephone: +81-22-717-7163; Fax: +81-22-717-7168; E-mail: takaabe@med.tohoku.ac.jp)

Journal of Pharmaceutical Sciences, Vol. 100, 3696–3707 (2011)

of cell membranes. Cell membrane transporter proteins or ATP-dependent pump proteins are needed for selective and efficient transport in renal tubular cells.

The organic anion transporting polypeptide (OATP/SLCO/SLC22) family and the organic anion transporter (OAT/SLC21) family include cell membrane transporting proteins that carry various organic anions and neutral compounds. Organic anion transporters are expressed on both the apical and basolateral (vascular) sides of renal tubular cells and transport various compounds across the cell membrane.[1]

Recently, OATs and OATPs were reported to transport not only urinary-excreted substances such as drugs and metabolites but also uremic toxins that accumulate in patients with chronic kidney diseases (CKDs).[2–8]

Chronic kidney disease is defined as an estimated glomerular filtration rate (eGFR) less than 60 mL/min per 1.73 m^2 of body surface area.[9] The prevalence of CKD is now estimated at approximately 10% of the population, so CKD is becoming an increasingly more important public health problem. CKD is strongly associated with cardiovascular events and the prognosis.[9] With the progression of CKD, various uremic toxins accumulate, causing renal damage and hypertension worsening the prognosis.[10,11] More than 110 organic compounds have now been identified as uremic toxins.[12] Among these toxins, guanidino compounds, such as guanidino succinate (GSA) and asymmetric dimethylarginine (ADMA), increase in patients with CKD and correlate with the prognosis.[11,13] Particularly, ADMA is an inhibitor of nitric oxide synthase and is associated with hypertension, renal damage, cardiac hypertrophy, and cardiovascular events.[14,15]

The mortality from CVD in patients undergoing dialysis is markedly higher than that in the general population, with about one half of the deaths in dialysis patients are attributed to CVDs.[16] The cardinal features of uremic cardiac disease are left ventricular hypertrophy (LVH), reduced capillary density, fibrosis, and ventricular remodeling. Those cardiovascular outcomes, such as heart failure, ischemic heart disease, stroke, and peripheral vascular disease, aggravate the mortality.[16] Cardiac hypertrophy is a powerful independent predictor of the patient's survival in CKD, and the regression of LVH is associated with reduced risk in CVDs and improves the survival rate.[17]

A reduction of the accumulated uremic toxins protects against the development of hypertension, renal damage, and CVD in patients with CKD, but there is no established treatment. In addition, early detection of an impairment of renal function could enable treatment to prevent further deterioration and complications.

Recently, we isolated a human kidney-specific OATP, termed SLCO4C1, and functionally characterized it as a digoxin transporter.[2] The OATP family is involved in the membrane transport of bile acids, conjugated steroids, thyroid hormone, eicosanoids, peptides, cardiac glycosides (digoxin, digitoxin, and ouabain), and numerous drugs (antibiotics, hydroxymethylglutaryl-CoA reductase inhibitors/stains, and anticancer drugs).[18,19] In the kidney, SLCO4C1 might be the first step in the transport of digoxin and various compounds into the urine. We revealed that many compounds accumulate during renal failure, and that the kidney-specific OATP SLCO4C1 excretes uremic toxins resulting in reductions of the blood pressure (BP) and renal inflammation.[7] To generalize these results for clinical use, it is necessary to examine the accumulation of uremic solutes precisely. We have revealed that (1) human kidney-specific OATP SLCO4C1 is the responsible molecule for excreting uremic toxins,[2] (2) the overexpression of human SLCO4C1 in rat kidney promotes the renal excretion of uremic toxins and reduces hypertension, cardiomegaly, and inflammation in renal failure,[7] and (3) metabolomic analysis of blood and urine samples from patients with CKD can be used to evaluate each substance to detect early stage CKD.[8]

Compounds highly correlated with eGFR and whose plasma concentrations change in a manner approximated by the first-degree equation are excellent candidates for detecting CKD and identifying uremic toxins that might aggravate the kidney function in the early stage of CKD. The above-mentioned results identified a number of uremic compounds, many of which are novel, that predict worsening renal function. These compounds provide diagnostic information and may be targets for therapies designed to treat the complications of CKD.[8]

Renal transporter proteins are potential therapeutic targets for CKD to improve the prognosis of patients with damaged kidneys and related CVDs.

Molecular Entity of Renal Tubular Transporting System

Organic anion transporters are membrane proteins composed of 12 transmembrane domains, and both the *n*-terminal and *c*-terminal are located in the cytosol. Organic anion transporters are subdivided into two major gene families, that is, OAT (OAT, SLC21)[20,21] and OATP (OATP, SLC22/SLCO)[18,19] based on the amino acid sequence homology.

OATs transport relatively small organic anions (generally <400 Da) such as *p*-aminohippurate (PAH), probenecid, and fluorescein. OATPs can transport comparably bulky compounds (generally >500 Da) such as steroid hormones, thyroid hormone, and bile acids.

Both OAT and OATP families are expressed in renal tubular proximal cells that conduct the renal tubular excretion and reabsorption of various anionic compounds.[22,23] ATP-binding cassette (ABC) transporters regulate the ATP-dependent active efflux transport of many kinds of organic anions and cations. Members of the ABC transporter family, that is, multidrug resistance gene1 product (MDR1, pgp1),[24] multidrug resistance protein (MRP)2,[25] and MRP4[26] are distributed in the apical membrane of renal tubular cells and regulate tubular excretion into urine.

OATs are localized in both the apical (OAT4, URAT1) and basolateral sides (OAT1,OAT2, OAT3) of renal proximal tubular cells.[22,23] The apical side OATs are considered to control tubular reabsorption from urine into cells but also some excretion of anionic compounds into the urine. OATs localized in the basolateral membrane are thought to mainly regulate the uptake of organic anions from blood into cells as the first step of renal tubular excretion. Although several ABC transporters and OATs are expressed in human kidney proximal tubular cells,[22,23,27–30] only SLCO4C1 is exclusively expressed and localized in the basolateral side of human renal proximal tubular cells[2] (Fig. 1a).

OAT Family

In human, at least, five OATs are expressed in proximal tubules.[23,30] OAT1,[31] OAT2,[32,33] and OAT3[31,34] are expressed in the basolateral side and OAT4[35] and URAT1[36] are localized at the apical lumen. Basolateral OATs mainly uptake various organic anions from blood into tubular cells. However, the transport by OAT at the apical membrane is still controversial.[30,37] In addition, URAT1 predominantly transports uric acid from urine into tubular cells and plays a role in renal tubular uric acid reabsorption.[36,38,39]

Chronic Kidney Disease and Organic Anion Transporter

In patients with renal failure, various organic anion uremic toxins such as indoxyl sulfate (IS), 3-carboxyl-4-methyl-5-propyl-2-furanopropionic acid (CMPF), indoleacetate (IAA), and hippuric acid (HA) accumulate.[12,40]

Among them, IS is an uremic toxin derived from dietary tryptophan. Tryptophan is converted to indole by tryptophanase in intestinal bacteria such as *Escherichia coli*. Indole is absorbed into the blood stream from the intestine and subsequently undergoes oxidization and sulfate conjugation in the liver to form IS. IS is water soluble and is normally excreted by kidney into urine. In serum, around 90% of IS is bound to albumin and excreted mainly via active secretion by renal proximal tubular cells.[41]

In CKD patients, as the kidney function declines, reduced renal excretion results in elevated serum concentrations of IS. The oral adsorbent AST-120 prevents the intestinal absorption of indole, reduces the accumulation of IS, and might ameliorate nephrotoxicity.[42]

Niwa et al.[43,44] reported that administration of IS in rat renal failure models promotes the renal damage progression, and oral uremic toxin absorbents reduced the level of IS in both serum and urine. Enomoto et al.[3] also showed that IS is accumulated in proximal tubules of IS-overloaded rats with renal failure. Rat Oat1/Slc22a6 and Oat3/Slc22a8 are also expressed in the proximal tubular cells.[3]

Because the cytotoxicity of IS was enhanced by the overexpression of rat Oat1/Slc22a6 or Oat3/Slc22a8 in a cell culture system, this transporter was thought to be one of the responsible molecules for uptaking IS.[3] Deguchi et al.[6] reported that not only IS but also CMPF, IAA, and HA were taken up by both human OAT1/SLC22A6 and OAT3/SLC22A8 from blood into tubular cells. Because OATs are also expressed in bone osteoblasts, muscle cells, and the blood–brain barrier, it is possible that OATs are involved in the transport of uremic toxins and the pathogenesis of uremia in various organs.[42,45]

The expression profiles of OATs were studied in animal experimental models. The downregulation of rat Oat1/Slc22a6 and Oat3/Slc22a8 expression was reported in 5/6-nephrectomized rats as a chronic renal failure (CRF) model,[46] and in a rat ischemic–reperfusion model[47,48] and cisplatin-induced nephropathy model[49,50] as acute renal failure models. These reports suggest that the reduced expression of OATs in renal failure is one of the causes of the decreased renal excretory function.[51,52]

In human, quantitative real-time polymerase chain reaction (PCR) analyses of renal biopsy specimens from patients with kidney diseases have been reported. Indeed, OAT1/SLC22A6 and OAT3/SLC22A8 expressions were decreased in patients with CKD.[53,54] Furthermore, downregulation of OAT3/SLC22A8 was significantly related to the renal excretion of the antibiotic cefazolin, a known substrate of OAT.[54] These results suggest the importance of OATs in CKD.

In mice, Oat1/Slc22a6 and Oat3/Slc22a8 are also localized on the basolateral membrane, so they are considered to be responsible for tubular uptake in renal proximal tubules. Knockout mice of these OATs have been established. Despite the lack of morphological changes in Oat1-null and Oat3-null mice, there are considerable alterations in renal uptake and/or secretion of organic anions in these two knockout mice. In Oat1-null mice, the decreased renal secretion of PAH and reduced renal uptake of the diuretic furosemide with impaired diuretic responsiveness to this drug were reported.[55] In Oat3-knockout mice, renal uptake of taurocholate, estrone-3-sulfate, and PAH were greatly decreased in an *in vitro* study

 DOI 10.1002/jps

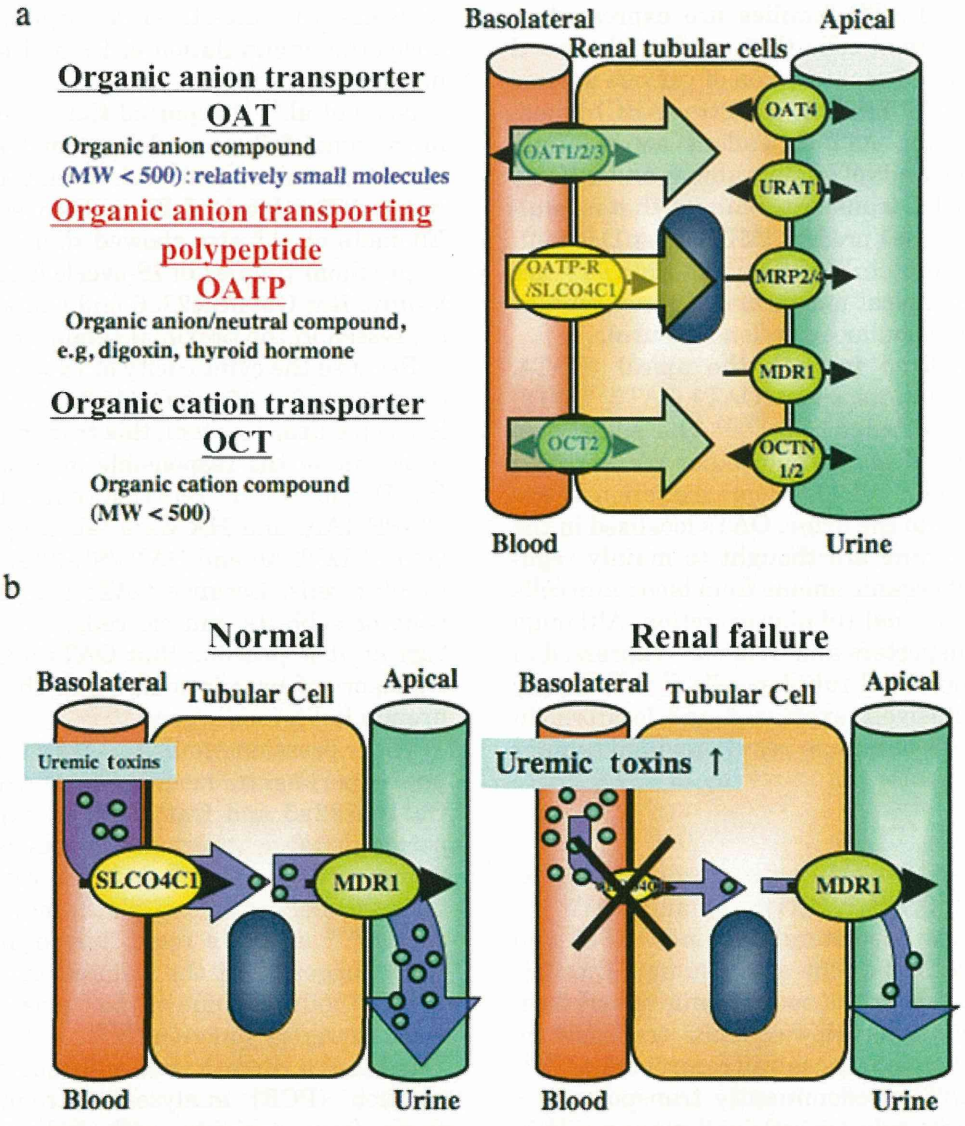

Figure 1. The molecular transporting system of the renal proximal tubular cell. (a) The molecular process of transporting membrane proteins in human proximal tubular cells. The expression patterns are those of OAT(OAT1, OAT2, OAT3), OATP(SLCO4C1) at the basolateral side, and OAT(OAT4, URAT1), and ABC transporters (MDR1, MRP2, MRP4) at the apical side. (b) SLCO4C1 (basolateral) and MDR1 (apical) expression levels in normal and impaired renal tubular cells. In normal kidney, basolateral SLCO4C1 and apical MDR1 work in concert to excrete drugs and uremic toxins from blood into urine. In renal failure states, the expression level of basolateral SLCO4C1, but not MDR1, is downregulated and renal excretory functions decrease.

(i.e., renal slice).[56] Recently, an *in vivo* study of Oat3-konckout mice showed that although renal secretion of PAH is not changed, penicillin G and estrone-3-sulfate plasma clearance were reduced.[57] Another study reported impaired clearance of methotrexate (MTX) in Oat3-knockout mice.[58] However, although the renal clearance of a prototypical anionic compound, for example, PAH, or established substrates transported by OATs such as penicillin G, estrone-3-sulfate, and MTX are significantly changed in Oat-knockout animals, so far there has yet been no report of plasma concentration or renal clearance changes in representative uremic toxins such as IS, CMPF, IAA, and HA. The redundancy of Oat expression (both Oat1 and Oat3 expressed at basolateral side of proximal tubules) and some overlap of the substrate specificity (IS is transpoted not only by Oat1 but also by Oat3) might abrogate the effect of a single-Oat gene deletion. Moreover, as several Oatps are also expressed in mice kidney and some of them are distributed in proximal tubules[23,59] (Table 1), the possible compensated renal elimination of uremic toxins by these Oatps

Table 1. Organic Anion Transporter Polypeptides

Protein Name	Gene Symbol	Novel Protein Name	Novel gene Symbol	Expression
Human OATP				
PGT	*SLC21A2*	OATP2A1	*SLCO2A1*	Widely
OATP-A	*SLC21A3*	OATP1A2	*SLCO1A2*	Brain
LST-1/OATP-C/OATP2	*SLC21A6*	OATP1B1	*SLCO1B1*	Liver only
LST-2/OATP8	*SLC21A8*	OATP1B3	*SLCO1B3*	Liver only
OATP-B/MOAT1	*SLC21A9*	OATP2B1	*SLCO2B1*	Widely
OATP-D	*SLC21A11*	OATP3A1	*SLCO3A1*	Widely
OATP-E	*SLC21A12*	OATP4A1	*SLCO4A1*	Widely
OATP-F	*SLC21A14*	OATP1C1	*SLCO1C1*	Brain, testis
OATP-J/OATP-RP4	*SLC21A15*	OATP5A1	*SLCO5A1*	Breast
GST/OATP-1	*SLC21A19*	OATP6A1	*SLCO6A1*	Tesits
OATP-R	*SLC21A20*	OATP4C1	*SLCO4C1*	Kidney
Rat oatp				
oatp1	*slc2la1*	Oatp1a1	*Slco1a1*	Kiver, kidney
rPGT	*slc2la2*	Oatp2a1	*Slco2a1*	Widely
OAT-K1 OAT-K2	*slc2la4*	Oatp1a3-v1 Oatp1a3-v2	*Slco1a3*	Kidney
oatp2	*slc2la5*	Oatp1a4	*Slco1a4*	Retina, liver, brain
oatp3	*slc2la7*	Oatp1a5	*Slco1a5*	Retina, brain, liver, kidney
oatp4/risz-1	*slc2la9*	Oatp1b2	*Slco1b2*	Liver only
moat1/oatp-B	*slc2la10*	Oatp2b1	*Slco2b1*	Widely
oatp-D	*slc2la11*	Oatp3a1	*Slco5a1*	Widely
oatp-E	*slc2la12*	Oatp4a1	*Slco4a1*	Widely
oatp5	*slc2la13*	Oatp1a6	*Slco1a6*	Kidney
oatp14	*slc2la14*	Oatp1c1	*Slco1c1*	Brain
rGST-1/oatp16	*slc2la16*	Oatp6b1	*Slco6b1*	Tesits
rGST-2/oatp18	*slc2la18*	Oatp6c1	*Slco6c1*	Tesits
oatp-R	*slc2la20*	Oatp4c1	*Slco4c1*	Kidney

Nomenclature of the organic anion transporter polypeptide family.

cannot be excluded. To clarify the contribution of OATs to the renal excretion of various uremic toxins, further experiments (i.e. double knockout or conditional knockout of Oat1 and Oat3) should be necessary.

OATP Family

Organic anion transporting polypeptide was first isolated as a transporting membrane molecule in the liver, which uptakes bile acids from blood into hepatocytes.[18] So far, 11 human clones and 14 rat clones of OATPs that form the OATP/SLC22/SLCO gene family have been identified.[59] The expression of OATPs is distributed throughout various organs such as the central nervous system, endocrine system (thyroid, pituitary), placenta, reproductive organs (testis, ovary), liver, kidney, and intestine (Table 1). The OATP family is involved in the membrane transport of bile acids, conjugated steroids, thyroid hormone, eicosanoids, peptides, cardiac glycosides (digoxin, digitoxin, ouabain), and numerous drugs.[18,60] OATPs are crucial transporting proteins that regulate the uptake, excretion, and metabolism of various hormones and metabolites. OATPs also determine the drug availability of organs, their elimination, and the pharmacokinetics.[18,59,60]

SLCO4C1, OATP in Kidney

In the kidney, various water-soluble and protein-binding compounds are mainly excreted from blood through renal tubular cells. Several ABC transporters, MRP2, MRP4, and MDR1, are reported to be expressed in the apical membrane of proximal tubular cells and are thought to mediate the tubular secretion of renal-excreted drugs such as digoxin, MTX, and irinotecan.[24–26,29] On the contrary, such drugs have to transverse the basolateral membrane of tubular cells and need membrane transporting proteins. OATPs have thus been regarded as a first step molecule in the transport of digoxin and various compounds into the urine.

We have recently identified a kidney-specific OATP, human SLCO4C1/OATP4C1/OATP-R, and its rat homologue Slco4c1/Oatp4c1/oatp-R.[2] SLCO4C1 is the only OATP expressed in human kidney and is localized at the basolateral membrane of proximal tubular cells. SLCO4C1 transports thyroid hormone, digoxin, an endogenous digoxin-like compound (ouabain), and MTX.[2,7] In a rat renal failure model, renal tubular basolateral rat Slco4c1 expression was decreased.[2] On the contrary, the expression level of MDR1, a member of the ABC transporter family that mediates the tubular secretion of digoxin at the apical membrane of proximal tubular cells, was not changed[24] (Fig. 1b). This reduction of SLCO4C1 in the proximal tubules

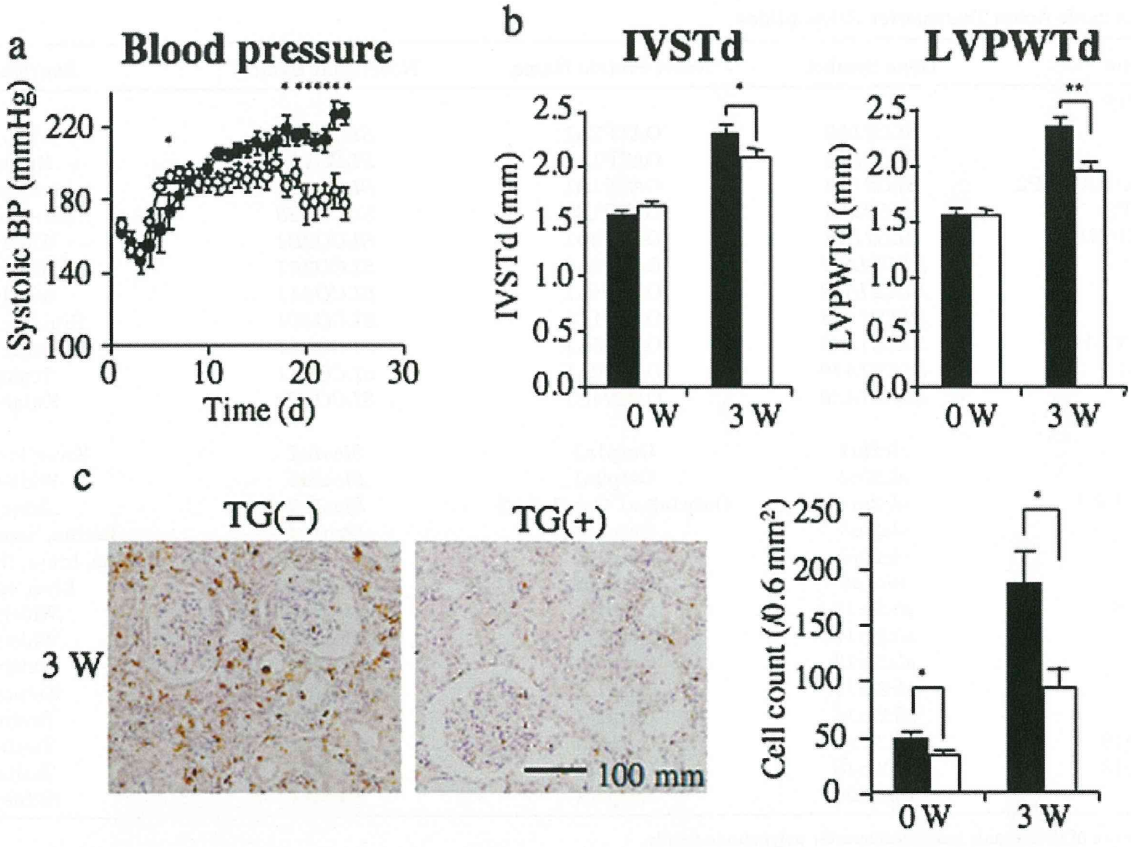

Figure 2. Hypertension, cardiomegaly, and renal inflammation are ameliorated in 5/6-nephrectomized (Nx) human SLCO4C1-proximal tubular cell-specific overexpressing transgenic (TG+) rats compared with TG(−) Nx littermates. (a) Blood pressure TG(−) Nx rats(filled circle) and TG(+) Nx rats(open circle). $^{*}p < 0.05$ versus TG(−) Nx rats. (b) Interventricular septum thickness during diastole (IVSTd) and left ventricular posterior wall thickness during diastole (LVPWTd) were measured by echocardiogram before and 3 weeks after 5/6 nephrectomy. TG(−) Nx rats (filled bar) and TG(+) Nx rats (open bar). $^{*}p < 0.05$, $^{**}p < 0.01$. (c) CD68 staining in the rat kidney before and 3 weeks after 5/6 nephrectomy. CD68-positive cell number counts were performed before and 3 weeks after 5/6 nephrectomy. $^{*}p < 0.05$ compared with TG(−) rat.

may be one of the mechanisms of the impaired urinary excretion of digoxin and drugs in renal failure[2,7] (Fig. 1b). Furthermore, in human, SLCO4C1 is the only OATP in the kidney, whereas several oatps exist at the basolateral and apical membrane of the proximal tubules in rodent kidney[2,7,23,30,60] (Table 1). In rat kidney, Oatp1a1/Slco1a1/oatp1, Oatp1a3-v1,Oatp1a3-v2/Slco1a3/OAT-K1,OAT-K2, Oatp1a7/Slco1a7/oatp3, Oatp1a6/Slco1a6/oatp5, and Oatp4c1/Slco4c1/oatp-R are expressed[23,59] (Table 1). At least, Oatp1a1, Oatp1a3-v1, and Oatp1a3-v2 are reported to be localized on the apical membrane of rat proximal tubules, whereas Oatp4c1 is localized on the basolateral membrane of rat proximal tubules.[23]

However, except for Oatp4c1/Slco4c1, so far no human ortholog of other rodent Oatps have yet been identified[23,59] (Table 1). This species diversity of the OATP family subtypes and their multiple locations in the proximal tubules make it difficult to extrapolate from experimental studies in rodents to human. The redundancy of multiple clones expression in rat kidney and some overlapping transporting properties (i.e., some organic compounds such as thyroid hormones, statins, and conjugated steroids are transported both by Oatp1a1 and Oatp1a7) make it difficult to investigate the contributions of those Oatps within the proximal tubules cells or whole kidney excretion.[18,59,60] To overcome these issues, we generated a transgenic (TG) rat harboring human SLCO4C1 in rat kidney and clarified the physiological and pathophysiological roles of human SLCO4C1.[7]

When the renal mass was reduced by 5/6 nephrectomy (Nx), the BP was significantly decreased in SLCO4C1-transgenic [TG(+)] rat compared with TG(−) littermates (Fig. 2a). In 5/6 nephrectomized SLCO4C1-transgenic [TG(+)Nx] rats, cardiac hypertrophy was also significantly reduced[7] (Fig. 2b).

In CKD patients, renal inflammation is also a risk factor for renal damage, morbidity, and mortality.[61] Immunohistochemically, mononuclear cell infiltration

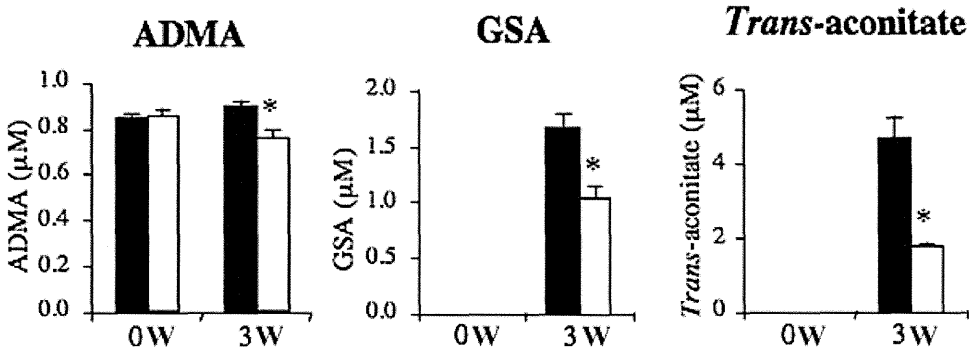

Figure 3. Metabolomic analysis of uremic toxins in rats with CRF. Metabolome analysis and characterization of uremic toxins. The plasma concentration of ADMA, GSA, and *trans*-aconitate before and 3 weeks after 5/6 nephrectomy. TG(−) Nx rats (filled bar) and TG(+) Nx rats(open bar). $^*p < 0.05$.

was strongly detected in TG(−)Nx rat kidneys by the macrophage marker, CD68. On the contrary, TG(+)Nx kidneys demonstrated less infiltration of macrophages (Fig. 2c). These data indicate that the expression of human SLCO4C1 in rat kidneys ameliorated not only the hypertension but also the inflammation in renal failure.

Metabolomic Analysis of Kidney-Specific Human SLCO4C1-TG Rat

A vicious cycle of progressive chronic renal damage occurs in patients with CKD, but there has been no specific therapeutic strategy for renal disease.

The accumulation of toxic uremic solutes, so called "uremic toxins," promotes renal damage, the progression of atherosclerosis, and evokes hypertension and the deterioration of cardiomyopathy (uremic cardiomyopathy) and other CVDs.[12,40] The mortality of patients with CKD, especially end-stage renal disease (ESRD) or those undergoing HD therapy, is very high.

Some of the causes of the poor prognosis and outcomes are derived from the malignant cycle of renal damage and the accumulation of uremic toxins.

Our assumption is that a method to reduce the serum level of uremic toxins and prevent their accumulation in organs could be a beneficial and specific therapeutic modality for CKD that would alleviate the progressive renal damage and reduce the mortality and morbidity of patients with CKD.[7–17] AST-120 is an oral sorbent that absorbs indole in the intestine and reduces the serum IS. AST-120 is administered in CKD patients and ameliorates the progression of CKD,[62] but the removal of uremic toxins is restricted mainly to IS,[63] whereas the accumulation of other harmful uremic toxins is thought to promote renal damage.

Hemodialysis also partially reduces some uremic toxins in patients with CRF, but the reduction of such toxins is transient and followed by an elevation to the previous level by the next HD, whereas other uremic solutes that are not effectively removed by HD still exist aggravating renal damage and CVDs in CRF patients.

Recently, we revealed that many uremic toxins accumulate in renal failure using capillary electrophoresis–mass spectrometry (CE–MS),[64] and that the kidney-specific OAT SLCO4C1 was involved in the excretion of uremic toxins resulting in reductions of the blood pressure and renal inflammation.[7,8] With the progression of CKD, various uremic toxins accumulate, subsequently causing renal damage and hypertension, known as the "malignant cycle."[12,40] Accordingly, the reduction of uremic toxins can help protect against renal damage and decrease the progression to ESRD and the need for HD. Renal transporter proteins are potential therapeutic targets for CKD to improve the prognosis of patients with damaged kidneys and some related CVDs.

To understand the mechanism by which SLCO4Cl exerted antihypertensive and anti-inflammation effects, a comprehensive quantitative metabolomic analysis was performed. Blood and urine specimens were measured by CE–MS and high performance liquid chromatography (HPLC). Although the plasma concentration of ADMA, GSA and *trans*-aconitate were significantly increased 3 weeks after the Nx, the increments were significantly decreased in TG(+)Nx rats compared with TG(−)Nx rats (Fig. 3). These data suggest that the excretion of uremic toxins was increased in TG(+) rats.

In CKD patients, the accumulation of uremic toxins causes difficulty in controlling BP, impairs renal function, and worsens the prognosis.[9] Among these toxins, the guanidino compounds GSA and ADMA are increased in CKD patients and correlate with the prognosis.[10–13] It is well known that the accumulation of guanidino compounds (including ADMA and GSA) and several uremic toxins generate oxidative stress causing further renal damage in CKD patients.[65–68]

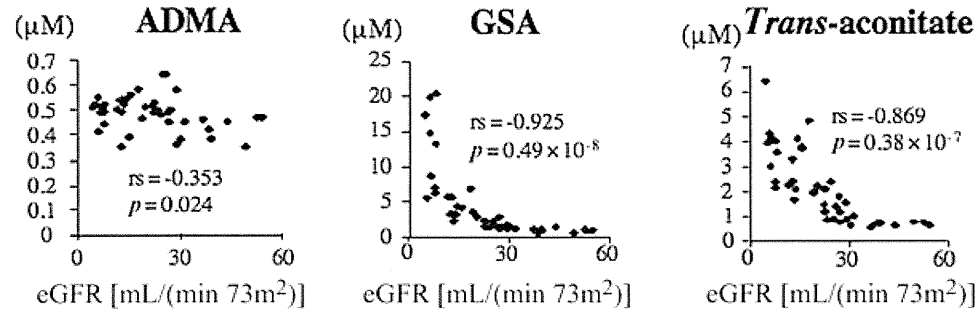

Figure 4. Metabolomic analysis of uremic toxins in patients with CKD. Relation between uremic toxins and eGFR as well as plasma creatinine in 41 CKD patients. Correlation between eGFR and the plasma ADMA, GSA, and trans-aconitate in CKD patients.

In particular, ADMA, an inhibitor of nitric oxide synthase, is implicated in hypertension, renal damage, cardiac hypertrophy, and cardiovascular events.[14,15]

Trans-aconitate is a competitive inhibitor of aconitase.[69] Aconitase is a key enzyme that catalyzes citrate to isocitrate via *cis*-aconitate in the TCA cycle and the accumulation of *trans*-aconitate inhibits the TCA cycle and respiration in tissues.[69] However, the existence of *trans*-aconitate in mammals, its biological effects, and precise role in renal failure have not been clarified. When *trans*-aconitate was administered to rats intraperitoneally, the BP of the injected rats was immediately elevated compared with control.[7] In addition, *trans*-aconitate significantly induced super oxide production in human kidney proximal tubule cells.[7]

Metabolomic Analysis of CKD Patients

To further confirm that not only ADMA and GSA, but also *trans*-aconitate, exist in humans and that their concentrations are increased in accordance with the CKD progression, CE–MS analysis of 41 CKD patients at various stages was performed. The plasma level of *trans*-aconitate was significantly correlated with the increase of plasma creatinine and inversely correlated with the eGFR similar to ADMA and GSA (Fig. 4).[7,8] Because the plasma level of *trans*-aconitate in non-CKD patients is quite low, these data suggest that *trans*-aconitate could serve as a biomarker for predicting the onset of renal damage, and that theelimination of *trans*-aconitate could have a beneficial effect in CKD.

Functional Analysis of SLCO4C1 Promoter and Exploration of Compounds that Enhance SLCO4C1 Expression

Thus, drugs that upregulate SLCO4C1 in the kidney may facilitate the excretion of uremic toxins and reduce renal inflammation decelerating the progression of renal damage and entry of HD. To address this issue, we isolated the promoter region of human SLCO4C1. We identified xenobiotic responsive element (XRE) motifs containing the core sequence 5′-CACGC-3′ at position 126. That sequence is generally recognized by Aryl hydrocarbon receptor (AhR) and AhR nuclear translocator heterodimer,[70] although the flanking sequences are not typical compared with the cyp1a1 XRE motif.[71,72] AhR binds "classical" ligands of such environmental pollutants as halogenated aromatic hydrocarbons [e.g., dioxin, benzopyrene, 3-methylcholanthrene (3-MC)] [73] (Fig. 5a).

Human SLCO4C1 promoter activity was increased by 3-MC. As AhR can also bind to a structurally divergent range of chemicals,[73] we next screened various compounds. Interestingly, the HMG-CoA reductase inhibitors (statin), fluvastatin (2.3-fold at 10 μM), and pravastatin (1.3-fold at 30 μM) upregulated the SLCO4C1 promoter activity (Fig. 5b). Deletion experiments showed that all constructs exerted potent promoter activation but removal of the XRE core segment or mutation in the XRE core motifs abolished the response to fluvastatin.[7] Various clinically available statins, simvastatin, lovastatin, cerivastatin, itavastatin, mevastatin, atorvastatin, rosuvastatin, and pitavastatin upregulate SLCO4C1 transcription.[7] The binding was further characterized by chromatin immunoprecipitation (ChIP) assay. Application of the antibody against AhR resulted in a positive band for both 3-MC and fluvastatin.[7] These data suggested that statins regulate SLCO4C1 transcription through the AhR–XRE system. In human kidney proximal cells, the application of fluvastatin and pravastatin significantly enhanced SLCO4C1 mRNA expression.[7] The uptake of thyroid hormone, T3, a representative substrate of SLCO4C1, was also significantly facilitated by fluvastatin and pravastatin, suggesting potentiation of the SLCO4C1 function in the proximal tubules.[7]

We next examined the effects of pravastatin *in vivo*. Pravastatin was administered to 5/6-nephrectomized (Nx) Wistar rats and the renal tubular function was examined. After the administration of pravastatin, BP was not changed but the mRNA level of

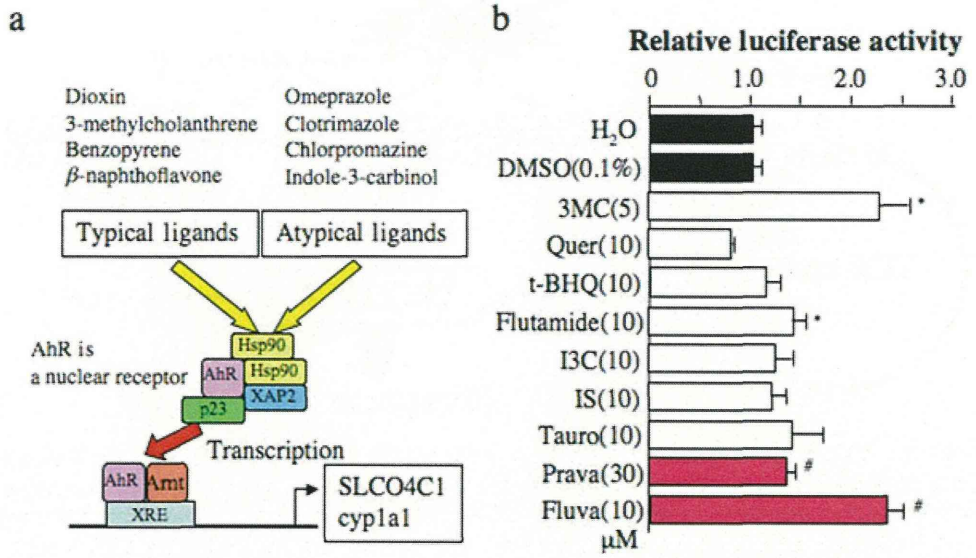

Figure 5. Transcriptional analysis of SLCO4C1 promoter and ligand screening. (a) Typical and atypical ligands of aryl hydrocarbon receptor (AhR) and schematic illustration of AhR–XRE axis of transcriptional regulation. (b) Enhancement of promoter activity of human SLCO4C1 with various compounds (concentration as indicated, μM). Quer, quercetin; t-BHQ, *tert*-butylhydroquinone; I3C, indole-3-carbinole; IS, indoxyl sulfate; Tauro, taurocholic acid; Prava, pravastatin; Fluva, fluvastatin. $^{*}p < 0.05$ compared with dimethyl sulfoxide (DMSO), $^{**}p < 0.05$ compared with H_2O.

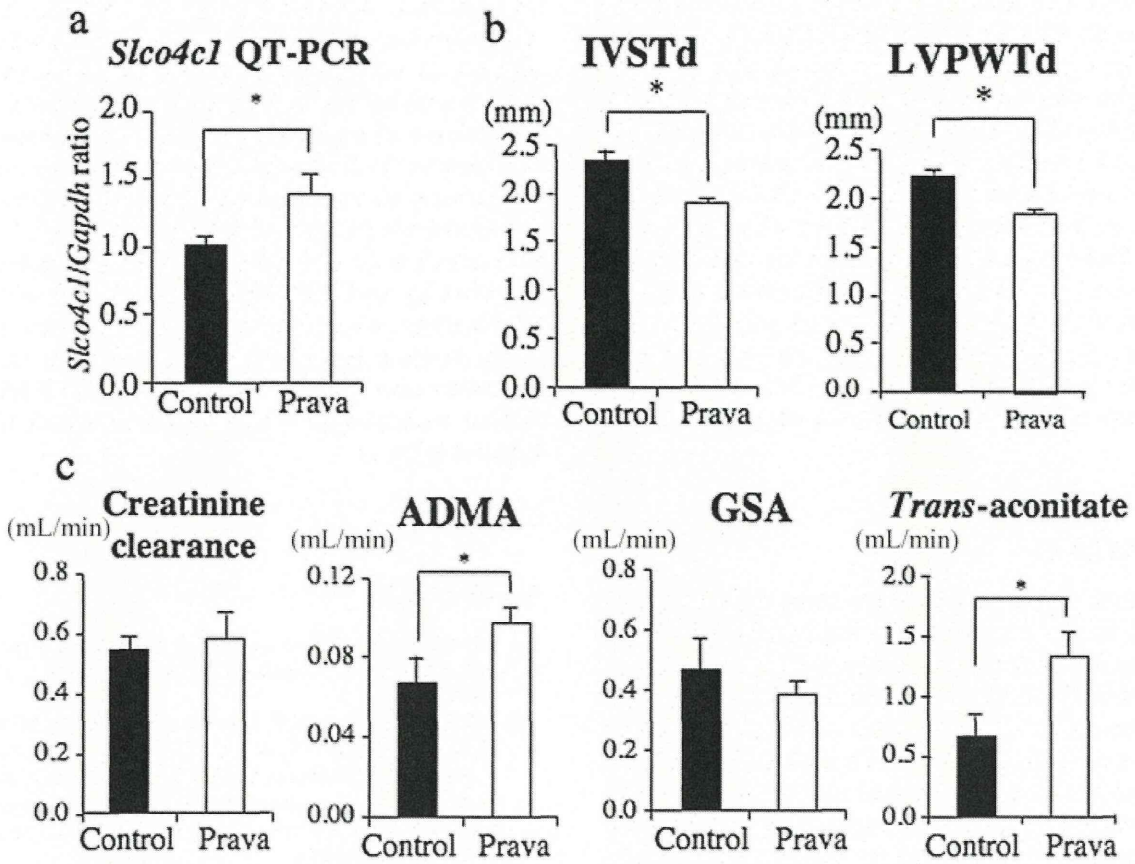

Figure 6. Effects of statins on SLCO4C1 expression and function *in vitro* and *in vivo*. (a) The mRNA expression of rat slco4c1 in the kidney after pravastatin administration. (b) IVSTd and LVPWTd before and after 5/6 nephrectomy. $^{*}P<0.05$. (c) Renal clearance of creatinine, ADMA, *trans*-aconitate, and GSA 3 weeks after 5/6 nephrectomy.

DOI 10.1002/jps

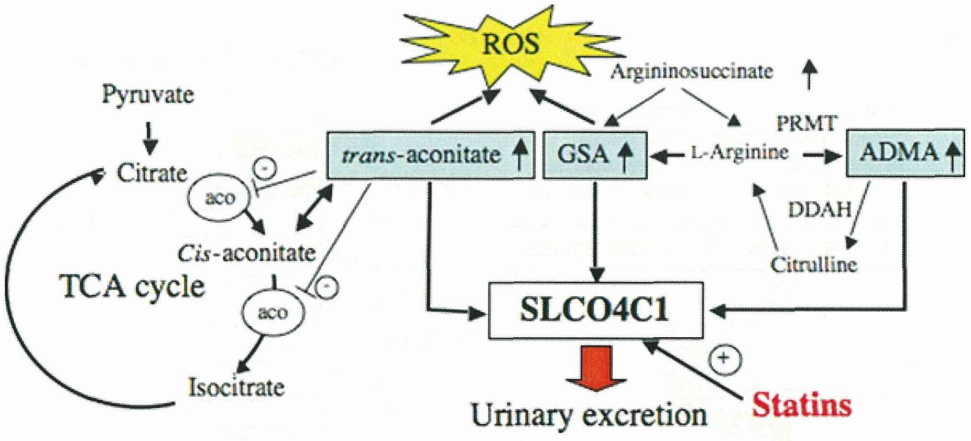

Figure 7. Uremic toxins and SLCO4C1 transporter in renal failure. ADMA is formed by protein ariginine *N*-methyltransferase (PRMT) from arginine and degrades to citrulline by dimethylarginine dimethylaminohydrolase (DDAH). Note that SLCO4C1 facilitates the excretion of GSA, ADMA, and *trans*-aconitate and that statins increase the expression and the function of SLCO4C1 resulting in reductions of the uremic toxins and BP. *Trans*-aconitase inhibits aconitase activity and induces reactive oxygen species (ROS). Aco, aconitase.

rat slco4c1 was significantly increased in the kidney (Fig. 6a) Under this condition, the ADMA and *trans*-aconitate clearance was significantly increased in the pravastatin-treated Nx rats without changing Ccr, although the change in GSA clearance was not statistically significant (Fig. 6c). In addition, cardiac hypertrophy was also decreased in the pravastatin-treated group (Fig. 6b). It is suggested that statins function as a nuclear receptor ligand that recruits the AhR–XRE system and upregulates SLCO4C1 transcription to facilitate the excretion of uremic toxins like a transgene phenotype. Because significantly increased levels of GSA and ADMA were reported in patients with autosomal dominant polycystic kidney disease (ADPKD),[13] our data also support this clinical study and will be a new clue for increasing the protection against renal damage in ADPKD patients.

CONCLUSIONS

Organic anion transporters have been vigorously investigated as key molecules in the regulation of the renal excretion of intrinsic compounds and the pharmacokinetics. Recently, a new finding that OATs and OATPs also carry uremic toxins and regulate renal excretion was reported.[2–7] OATs and OATPs might be involved in the emergence of uremic toxicity and the progression of renal damage. The OAT/SLC22 family mediates the uptake of uremic toxins into various organs and might cause "uremia" aggravating end-organ damage. SLCO4C1 is the only OATP/SLC21/SLCO expressed in human kidney, and it plays a critical role in the renal elimination of urinary-excreted drugs and uremic toxins. An enhancement of the urinary excretion of uremic toxins and the amelioration of end-organ damage were also observed in proximal tubular cell-specific human SLCO4C1-overexpressing TG rat as well as statin-induced SLCO4C1 upregulation rat renal failure models. Further examination in clinical trials is needed to verify the effects of increasing uremic toxin transporter in patients with CKD. Metabolomic analysis enables the comprehensive assessment of known and newly identified uremic toxins in CKD patients, and this method is promising for the exploration of surrogate biomarkers of early renal damage and CVDs. A new therapeutic strategy to regulate the expression and function of renal uremic toxin transporters for the elimination of toxins and use the reduction of candidate uremic toxins as surrogate biomarkers in renal therapy is needed (Fig. 7).

REFERENCES

1. Pritchard JB, Miller DS. 1993. Mechanisms mediating renal secretion of organic anions and cations. Physiol Rev 73:765–796.
2. Mikkaichi T, Suzuki T, Onogawa T, Tanemto M, Mizutamari H, Okada M, Chaki T, Masuda T, Toki T, Eto N, Abe M, Satoh F, Unno M, Hishinuma T, Inui K, Ito S, Goto J, Abe T. 2004. Isolation and characterization of a digoxin transporter and its rat homologue expressed in the kidney. Proc Natl Acad Sci USA 101:3569–3574.
3. Enomoto A, Takeda M, Tojo A, Sekine T, Cha SH, Khamdang S, Takayama F, Aoyama I, Nakamura S, Enou H, Niwa T. 2002. Roles of organic anion transporters in the tubular transport of indoxyl sulfate and the induction of its nephrotoxicity. J Am Soc Nephrol 13:1711–1720.

4. Enomoto A, Takeda M, Taki K, Takayama F, Noshiro R, Niwa T, Endou H. 2003. Interactions of human organic anion as well as cation transporters with indoxyl sulfate. Eur J Phramacol. 466:3–20.
5. Deguch T, Ohtsuki S, Otagiri M, Takanaga H, Asaba H, Mori S, Terasaki T. 2002. Major role of organic anion transporter 3 in the transport of indoxyl sulfate in the kidney. Kidney Int 61:1760–1768.
6. Deguchi T, Kusuhara H, Takadate A, Endou H, Otagiri M, Sugiyama Y. 2004. Characterization of uremic toxin transport by organic anion transporters in the kidney. Kidney Int 65:162–174.
7. Toyohara T, Suzuki T, Morimoto R, Akiyama Y, Souma T, Shiwaku HO, Takeuchi Y, Mishima E, Abe M, Tanemoto M, Masuda S, Kawano H, Hamemura K, Nakayama M, Sato H, Mikkaichi T, Yamaguchi H, Fukui S, Fukumoto Y, Shimokawa H, Inui K, Terasaki T, Goto J, Ito S, Hishinuma T, Rubera I, Tauc M, Fujii-Kuriyama Y, Yabuuchi H, Moriyama Y, Soga T, Abe T. 2009. SLCO4C1 transporter eliminates uremic toxins and attenuates hypertension and renal inflammation. J Am Soc Nephrol 20:2546–2555.
8. Toyohara T, Akiyama Y, Suzuki T, Takeuchi Y, Mishima E, Tanemoto M, Momose A, Toki N, Sato H, Nakayama M, Hozawa A, Ito S, Soga T, Abe T. 2010. Metabolomic profiling of uremic solutes in CKD patients. Hypertens Res 33:944–952.
9. Go AS, Chertow GM, Fan D, McCulloch CE, Hsu CY. 2004. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. N Engl J Med 351:1296–1305.
10. Zoccali C. 2008. Asymmetric dimethylarginine: A novel marker of risk and a potential target for therapy in chronic kidney disease. Curr Opin Nephrol Hypertens 17:609–615.
11. Marescau B, Nagels G, Possemiers I, De Broe ME, Becaus I, Billiouw JM, Lornoy W, De Deyn PP. 1997. Guanidino compounds in serum and urine of nondialyzed patients with chronic renal insufficiency. Metabolism 46:1024–1031.
12. Vanholder R, Van Laecke S, Glorieux G. 2008. What is new in uremic toxicity? Periatr Nephrol 23:1211–1221.
13. Torremans A, Marescau B, Kranzlin B, Gretz N, Billiouw JM, Vanholder R, De Smet R, Bouwman K, Brouns R, De Deyn PP. 2006. Biochemical validation of a rat model for polycystic kidney disease: Comparison of guanidino compound profile with the human condition. Kidney Int 69:2003–2012.
14. Zoccali C, Mallamaci F, Maas R, Benedetto FA, Tripepi G, Malatino LS, Catallotti A, Bellanuova I, Böger R; CREED Investigators. 2002. Left ventricular hypertrophy, cardiac remodeling and asymmetric dimethylarginine (ADMA) in hemodialysis patients. Kidney Int 62:339–345.
15. Fliser D, Kronenberg F, Kielstein JT, Morath C, Bode-Böger SM, Haller H, Ritz E. 2005. Asymmetric dimethylarginine and progression of chronic kidney disease: The mild to moderate kidney disease study. J Am Soc Nephrol 16:2456–2461.
16. Parfrey PS, Foley RN. 1999. The clinical epidemiology of cardiac disease in chronic renal failure. J Am Soc Nephrol 10:1606–1615.
17. London GM, Pannier B, Guerin AP, Blacher J, Marchais SJ, Darne B, Metivier F, Adda H, Safar ME. 2001. Alterations of left ventricular hypertrophy in and survival of patients receiving hemodialysis- follow-up of an interventional study. J Am Soc Nephrol 12:2759–2767.
18. Mikkaichi T, Suzuki T, Tanemoto M, Ito S, Abe T. 2004. The organic anion transporter (OATP) family. Drug Metab Pharmacokinet 19:171–179.
19. Hagenbuch B, Meier PJ. 2004. Organic anion transporting polypeptides of the OATP-SLC21 family-phylogenetic classification as OATP-SLCO superfamily, new nomenclature and molecular functional properties. Pflugers Arch 447:653–665.
20. Koepsell H, Endou H. 2004. The SLC22 drug transporter family. Pflugers Arch 447:666–676.
21. Rizwan AN, Burckhardt G. 2007. Organic anion transporters of the SLC22 family—Biopharmaceutical, physiological, and pathological roles. Pharm Res 24:450–470.
22. Inui K, Masuda S, Saito H. 2000. Cellular and molecular aspects of drug transport in the kidney. Kidney Int 58:944–958.
23. Sekine T, Miyazaki H, Endou H. 2006. Molecular physiology of renal organic anion transporters. Am J Physiol Renal Physiol 290:F251–F261.
24. Laouari D, Yang R, Veau C, Blanke I, Friedlander G. 2001. Two apical multidrug transporters, P-gp and MRP2, are differently altered in chronic renal failure. Am J Physiol Renal Pysiol 280:F636–F645.
25. Smeets PH, van Aubel RA, Wouterse AC, Van Den Heuvel JJ, Russel FG. 2004. Contribution of multidrug resistance protein 2 (MRP2/ABCC2) to the renal excretion of *p*-aminohippurate (PAH) and identification of MRP4 (ABCC4) as a novel PAH transporter. J Am Soc Nephrol 15:2828–2835.
26. van Aubel RA, Smeets PH, Peters JG, Bindels RJ, Russel FG. 2002. The MRP4(ABCC4) gene encodes a novel apical organic anion transporter in human kidney proximal tubules—Putative efflux pump for urinary cAMP and cGMP. J Am Soc Nephrol 13:595–603.
27. Sweet DH, Bush KT, Nigam SK. 2001. The organic anion transporter family—From physiology to ontogeny and the clinic. Am J Physiol Renal Physiol 281:F197–F205.
28. Wright SH, Dantzler WH. 2004. Molecular and cellular physiology of renal organic cation and anion transport. Physiol Rev 84:987–1049.
29. van de Water FM, Masereeuw R, Russel FG. 2005. Function and regulation of multidrug resistance proteins (MRPs) in the renal elimination of organic anions. Drug Metab Rev 37:443–471.
30. Anzai N, Kanai Y, Endou H. 2006. Organic anion transporter family—Current knowledge. J Phramcol Sci 100:411–426.
31. Motohashi H, Sakurai Y, Saito H, Masuda S, Urakami Y, Goto M, Fukatsu A, Ogawa O, Inui K. 2002. Gene expression levels and immunolocalization of organic ion transporters in the human kidney. J Am Soc Nephrol 13:866–874.
32. Sekine T, Cha SH, Tsuda M, Apiwattanakul N, Nakajima N, Kanai Y, Endou H. 1998. Identification of multispecific organic anion transporter 2 expressed predominantly in the liver. FEBS Lett 429:179–181.
33. Enomoto A, Takeda M, Shimoda M, Narikawa S, Kobayashi Y, Kobayashi Y, Kobayashi Y, Yamamoto T, Sekine T, Cha SH, Niwa T, Endou H. 2002. Interaction of human organic anion transporters 2 and 4 with organic anion transport inhibitors. J Pharmacol Exp Ther 301:797–802.
34. Kusuhara H, Sekine T, Utsunomiya-Tate N, Tsuda M, Kojima R, Cha SH, Sugiyama Y, Kanai Y, Endou H. 1999. Molecular cloning and characterization of a new multispecific organic anion transporter from rat brain. J Biol Chem 274:13675–13690.
35. Cha SH, Sekine T, Kusuhara H. 2000. Molecular cloning and characterization of multispecific organic anion transporter 4 expressed in the placenta. J Biol Chem 275:4507–4512
36. Enomoto A, Kimura H, Chairoungdua A, Shigeta Y, Jutabha P, Cha SH, Hosoyamada M, Takeda M, Sekine T, Igarashi T, Matsui H, Kikuchi Y, Oda T, Ichiba K, Hosoya T, Shimokata K, Niwa T, Kanai Y, Endou H. 2002. Molecular identification of a renal urate–anion exchanger that regulates blood urate levels. Nature 417:447–452.
37. Ekarantanawong S, Anzai N, Jutbaha P, Miyazaki H, Noshiro R, Takeda M, Kanai Y, Sophasan S, Endou H. 2004. Human organic anion transporter 4 is a renal apical organic anion/decarboylate exchanger in the proximal tubules. J Phramacol Sci 94:297–304.
38. Enomoto A, Endou H. 2005. Roles of organic anion transporters (OATs) and a urate transporter (URAT1) in the pathophysiology of human disease. Clin Exp Nephrol 9:195–205.

39. Anzai N, Kanai Y, Endou H. 2007. New insights into renal transport of urate. Curr Opin Rheumatol 19:151–157.
40. Vanholder R, Smmet RD, Glorieux G, Argilés A, Baurmeister U, Brunet P, Clark W, Gohen G, De Deyn PP, Deppisch R, Descamps-Latscha B, Henle T, Jörres A, Lemke HD, Massy ZA, Passlick-Deetjen J, Rodriguez M, Stegmayr B, Stenvinkel P, Tetta C, Wanner C, Zidek W;European Uremic Toxin Work Group (EUTox). 2003. Review on uremic toxins—Classification, concentration, and interindividual variability. Kidney Int 63:1934–1943.
41. Niwa T. 2001. Indoxyl sulfate . In Textbook of nephrology; Massry SG, Glassock RJ, Eds. 4th ed. Philadelphia: Williams and Willkins, pp 1269–1272.
42. Niwa T. 2010. Indoxyl sulfate is a nephrovascular toxin. J Ren Nutr 20:S2–S6.
43. Niwa T, Ise M. 1994. Indoxyl sulfate, a circulating uremic toxin, stimulates the progression of glomerular sclerosis. J Lab Clin Med 124:96–104.
44. Miyazaki T, Aoyama I, Ise M, Seo H, Niwa T. 2000. An oral sorbent reduces overload of indoxyl sulfate and gene expression of TGF-β1 in ureic rat kidneys. Nephrol Dial Transplant 15:1773–1781.
45. Enomoto A, Niwa T. 2007. Roles of organic anion transporters in the progression of chronic renal failure. Therapeu Apheresis Dial 11:S27–S31.
46. Deguchi T. Takemoto M, Uehara N, Lindup WE, Suenaga A, Otagiri M. 2005. Renal clearance of endogenous hippurate correlates with expression levels of renal organic anion transporters in uremic rats. J Pharamacol Exp Ther 14:932–938.
47. Matsuzaki T, Watanabe H, Yoshitome K, Morisaki T, Hamada A, Nonoguchi H, Konda Y, Tomita K, Inui K, Saito H. 2007. Down regulation of organic anion transporters in rat kidney under ischemia reperfusion -induced acute renal failure. Kidney Int 71:539–547.
48. Schneider R, Sauvant C, Betz B, Otremba M, Fischer D, Holzinger H, Wanner C, Galle J, Gekle M. 2007. Downregulation of organic anion transporters OAT1 and OAT3 correlates with impaired secretion of para-aminohippurate after ischemic acute renal failure in rats. Am J Physiol Renal Physiol 292:F1599–F1605.
49. Morisaki T Matsuzaki T, yokoo K, Kusumoto M, Iwata K, Hamada A, Saito H. 2008. Regulation of renal organic ion transporters in cisplatin-induced acute kidney injury and uremia in rats. Pharm Res 25:2526–2533.
50. Aleksunes LM, Augstine LM, Scheffer GL, Cherrington NJ, Manautou JE. 2008. Renal xenobiotic transporters are differentially expressed in mice following cisplatin treatment. Toxicology 250:82–88.
51. Sun H, Frasseto L, Benet LZ. 2006. Effects of renal failure on drug transport and metabolism. Pharmacol Ther 109:1–11
52. Saito H. 2010. Pathophysiological regulation of renal SLC22A organic ion transporters in acute kidney injury—Pharmacological and toxicological implications. Pharmacol Ther 125:79–91.
53. Sakurai Y, Motohashi H, Ueo H, Masuda S, Saito H, Okuda M, Mori M, Matsuura M, Doi T, Fukatsu A, Ogawa O, Inui K. 2004. Expression levels of renal organic anion transporters (OATs) and their correlation with anionic drug excretion in patients with renal diseases. Pharm Res 21:61–67.
54. Sakurai Y, Motohashi H, Ogasawara K, Terada T, Masuda S, Katsura T, Mori N, Matsuura M, Doi T, Fukatsu A, Inui K. 2005. Pharmacokinetic siginificance of renal OAT3(SLC22A8) for anionic drug elimination in patients with mesangial proliferative glomerulonephritis. Pharm Res 22:2016–2022.
55. Eraly SA, Vallon V, Vaughn DA, Gangoiti JA, Richter K, Nagle M, Monte JC, Rieg T, Truong DM, Long JM, Barshop BA, Kaler G, Nigam SK. 2006. Decreased renal organic anion secretion and plasma accumulation of endogenous organic anions in OAT1 knock-out mice. J Biol Chem 281:5072–5083.
56. Sweet DH, Miller DS, Pritchard JB, Fujiwara Y, Beier DR, Nigam SK. 2002. Impaired organic anion transport in kidney and choroid plexus of organic anion transporter 3 (Oat3 (Slc22a8)) knockout mice. J Biol Chem 277:26934–26943.
57. Vanwert AL, Bailey RM, Sweet DH. 2007. Organic anion transporter 3 (Oat3,Slc22a8) knockout mice exhibit altered clearance and distribution of penicillin G. Am J Physiol Renal Physiol 293:F1332–F1341
58. VanWert AL, Sweet DH. 2008. Impaired clearance of methotrexate in organic anion transporter 3 (Slc22a8) knockout mice—A gender specific impact of reduced folates. Pharm Res 25:453–462
59. Suzuki T, Abe T. 2008. Thyroid hormone transporters in the brain. Cereberum 7:75–83.
60. Abe T, Suzuki T, Unno M, Tokui T, Sadayoshi I. 2002. Thyroid hormone transporters: Recent advances. Trends Endocrinol Metab 13:215–220.
61. Silverstein DM. 2009. Inflammation in chronic kidney disease: Role in the progression of renal and cardiovascular disease. Pediatr Nephrol 24:1445–1452.
62. Sanaka T, Akizawa T, Koide K, Koshikawa S. 2004. Protective effect of an oral adsorbent on renal function in chronic renal failure: Determinants of its efficacy in diabetic nephropathy. Ther Apher Dial 8:232–40.
63. Owada A, Nakao M, Koike J, Ujiie K, Tomita K, Shiigai T. 1997. Effects of oral adsorbent AST-120 on the progression of chronic renal failure: A randomized controlled study. Kidney Int Suppl 63:S188–S190.
64. Soga T, Ohashi Y, Ueno Y, Naraoka H, Tomita M, Nishioka T. 2003. Quantitative metabolome analysis using capillary electrophoresis mass spectrometry. J Proteome Res 2:488–494.
65. Ueda S, Yamagishi S, Matsumoto Y, Fukami K, Okuda S. 2007. Asymmetric dimethylarginine (ADMA) is a novel emerging risk factor for cardiovascular disease and the development of renal injury in chronic kidney disease. Clin Exp Nephrol 11:115–121.
66. Kielstein JT, Zoccali C. 2008. Asymmetric dimethylarginine: A novel marker of risk and a potential target for therapy in chronic kidney disease. Curr Opin Nephrol Hypertens 17:609–615.
67. Taes YE, Marescau B, De Vriese A, De Deyn PP, Schepers E, Vanholder R, Delanghe JR. 2008. Guanidino compounds after creatine supplementation in renal failure patients and their relation to inflammatory status. Nephrol Dial Transplant 23:1330–1335.
68. De Deyn PP, Vanholder R, Eloot S, Glorieux G. 2009. Guanidino compounds as uremic (neuro) toxins. Semin Dial 22:340–345
69. Saffran M, Prado JL. 1949. Inhibition of aconitase by transaconitate. J Biol Chem 180:1301–1309.
70. Fujii-Kuriyama Y, Miura J. 2005. Molecular mechanism of AhR functions in the regulation of cytochrome P450 genes. Biophys Res Commun 228:311–317.
71. Wu L, Whitlock JP Jr. 1993. Mechanism of dioxin action: Receptor–enhancer interactions in intact cells. Nucleic Acids Res 21:119–125.
72. Nioi P, Hayes JD. 2004. Contribution of NAD(P)H: Quinone oxidoreductase 1 to protection against carcinogenesis, and regulation of its gene by the Nrf2 basic-region leucine zipper and the arylhydrocarbon receptor basic helix-loop-helix transcription factors. Mutat Res 555:149–171.
73. Denison MS, Nagy SR. 2003. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu Rev Pharmacol Toxicol 43:309–334.

Ⅲ　メタボロミクス

CE-MSメタボローム測定法

平山　明由 *Akiyoshi Hirayama*（慶應義塾大学先端生命科学研究所）
曽我　朋義 *Tomoyoshi Soga*（慶應義塾大学先端生命科学研究所教授）

Key Words
メタボロミクス
メタボローム
キャピラリー電気泳動-質量分析法（CE-MS）
糖尿病性腎症
バイオマーカー

はじめに

ポストゲノム研究の新しい手法として近年注目を集めているメタボロミクスは，細胞内代謝産物を網羅的に探索することによって生命現象を包括的に理解しようとする方法論である。代謝物は生命システムにおける最終産物であり，その変動は生物の環境応答や適応変化などを最も鋭敏に反映していると考えられている。また，疾病などによって代謝の変動がある場合，血液や尿などに存在する代謝物質の組成や濃度にも変化が起こると考えられるため，各種の疾患バイオマーカーの探索なども精力的に行われている。

メタボローム測定における分析化学的なアプローチとしては，質量分析装置（Mass Spectrometer：MS）を用いたものが主流であるが，特に筆者らはイオン性化合物に対して高分離能を示すキャピラリー電気泳動（Capillary Electrophoresis：CE）とMSをタンデムに接続したCE-MS法を世界に先駆けて開発し，数千種類の代謝物の一斉測定を可能にした[1]。本稿では，CE-MS（図1）を用いたメタボローム解析法の概略とそれを糖尿病性腎症のバイオマーカー探索に適用した例について紹介する。糖尿病性腎症のバイオマーカー探索は名古屋大学医学部，中部ろうさい病院との共同研究の成果である。

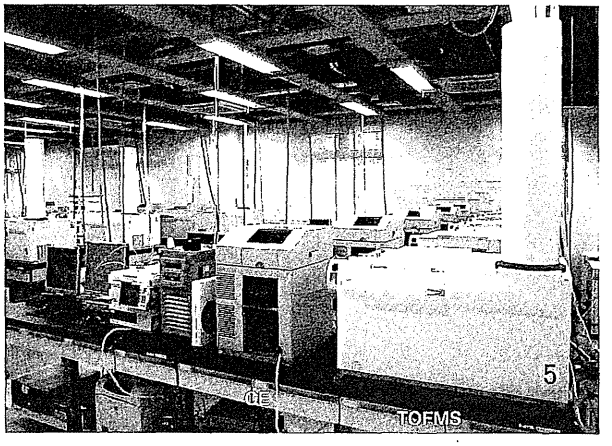

図1．キャピラリー電気泳動-飛行時間型質量分析装置（CE-TOFMS）

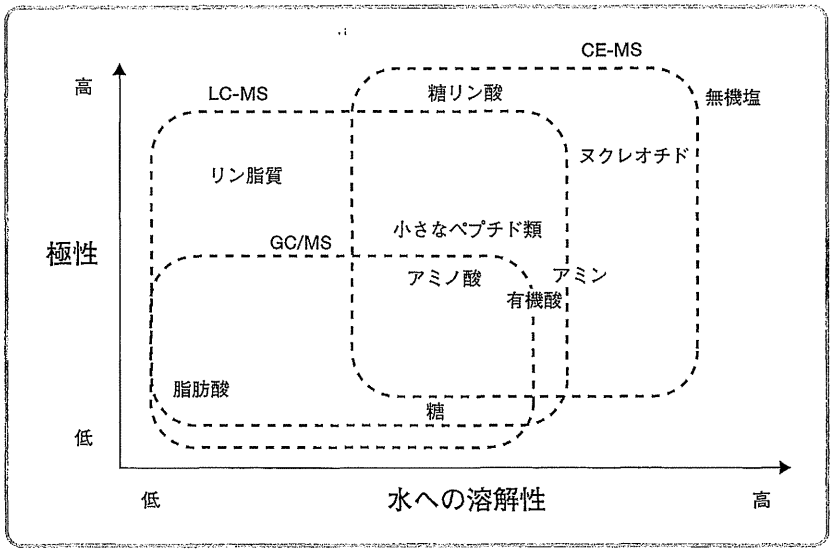

図2．代謝物の物理化学的性質による分類と各分析法の適用可能範囲

Ⅰ　メタボロミクスとその測定手法

生命活動は代謝と呼ばれる種々の酵素反応の連鎖によって営まれており，代謝によって産生された代謝中間体や代謝産物の総体をメタボロームと呼ぶ。メタボロームの総数は種によって異なるが，大腸菌などの微生物では約800～1,600種類，ヒトなどの哺乳類では2,500～8,000種類，植物では2万～10万種類程度存在すると見積もられている。

メタボロミクス（メタボローム解析）は対象とするサンプル中に存在するこれらの代謝産物を包括的に扱う学問分野の1つであるが，現在のところこれらの代謝物を一斉に測定する決定的な分析法は存在しない。対象とする代謝物の物理的・化学的性質が多岐に渡っているため，単独の測定法ですべての代謝物を網羅するのは不可能である。したがって，比較的性質の似ている化合物群に対し，ガスクロマトグラフィー-質量分析法（GC/MS），液体クロマトグラフィー-質量分析法（LC-MS），キャピラリー電気泳動-質量分析法（CE-MS）などのメタボローム測定法を使い分けているのが現状である（図2）。

Ⅱ　CE-MSによるメタボローム測定法

メタボローム測定を始めるにあたり，どの測定手法を用いるかは重要な問題である。前述のように，単独ですべての代謝物を網羅する分析法はないので複数の分析手法を組み合わせるのが最も良い方法ではあるが，装置の導入に多額の資金が必要になってくる。筆者らはほとんどの生物が共通に有している，解糖系，クエン酸回路，ペントースリン酸回路に代表されるエネルギー代謝やその周辺のアミノ酸，核酸などの生合成経路に存在する代謝中間体の多くが，水酸基，カルボキシル基，アミノ基，リン酸基などを有する低分子のイオン性の物質であることを見い出した。実際に，生物学の分野でよく用いられており，代謝物の多くがわかっている大腸菌の代謝物データベースを詳細に調べてみたところ，主要な代謝物のうち，実に88％がイオン性の代謝物であることがわかった[2]。そこで筆者らは，イオン性代謝物の一斉分析に威力を発揮するCE-MSを用いたメタボローム解析法を微生物[3]や植物[4]，動物[5][6]などのサンプルに適用してきた。

1　陽イオン性代謝物測定法[7]

陽イオン性の代謝物質の測定は，内径50 μm，全長1 m

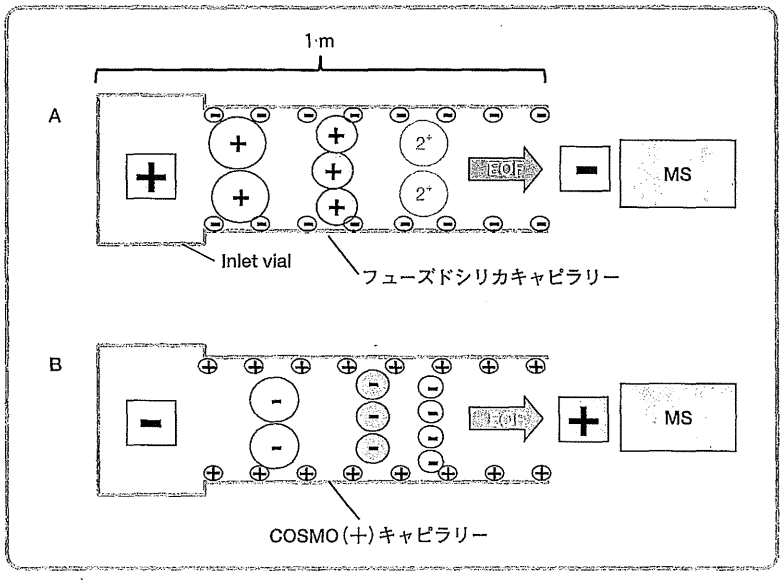

図3．CE-MSによるメタボローム測定法

A：陽イオン性代謝物質測定法，B：陰イオン性代謝物質測定法

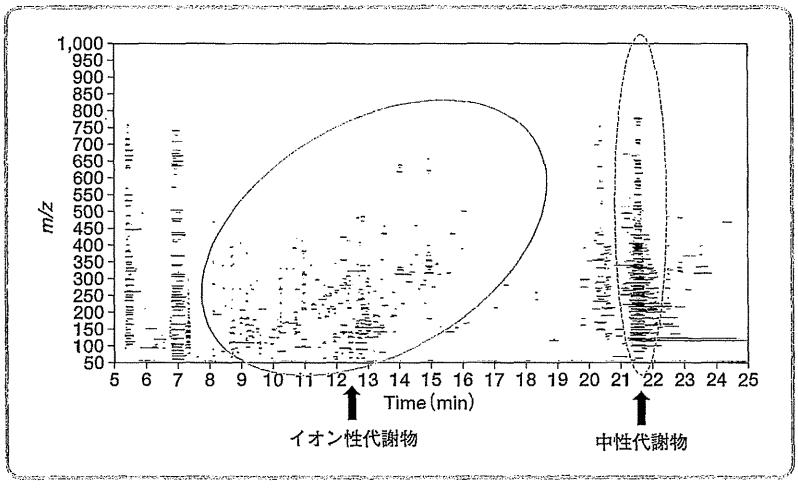

図4．CE-TOFMSによる大腸癌組織の陽イオン測定の例

イオン性の代謝物はその水和イオン半径と電荷の比に基づいてキャピラリー内で分離され，検出される。中性物質においても，キャピラリー内に発生する電気浸透流（EOF）と呼ばれる液流によってMSまで運ばれ検出されるが，キャピラリー内で分離されないので同じ時間に検出される。

のフューズドシリカキャピラリーを用いて行っている（図3A）。キャピラリー内を泳動液である1 Mのギ酸溶液で満たした後に，キャピラリーの出口（MS側）が陰極となるように両端に30kVの電圧を印加する。

キャピラリー内に導入されたサンプル中の各代謝物質は，その電荷と水和イオン半径の比に基づいた速度によってキャピラリー内で電気泳動し分離された後，キャピラリーの出口に接続されたMSによって，高感度かつ選択的に検出される。図4にCE-TOFMS（キャピラリー電気泳動-飛行時間型質量分析装置）を用いて得られた，大腸癌組織の陽イオ

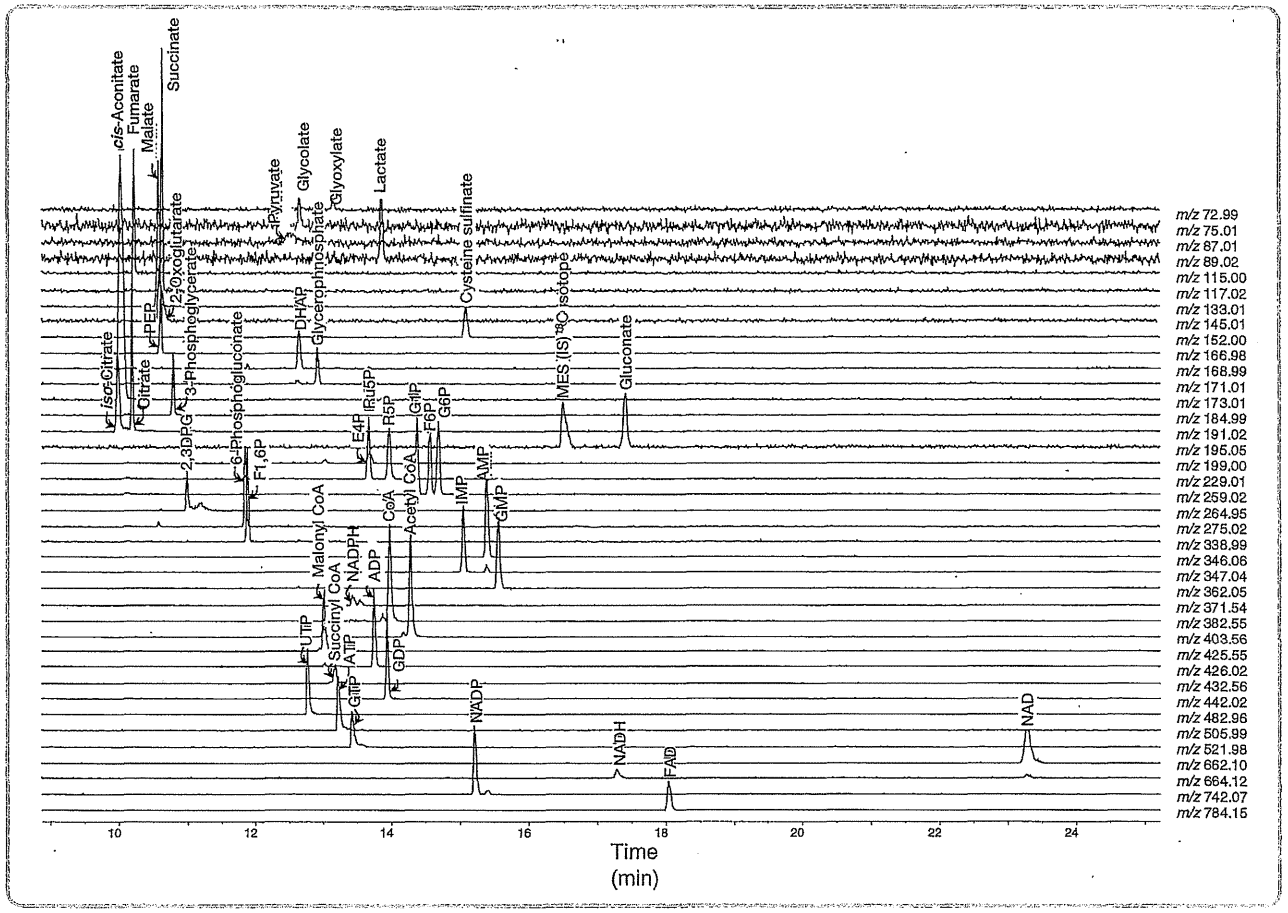

図5．CE-TOFMSによる陰イオン性代謝物の測定例

ン測定結果の1例を示す。

2　陰イオン性代謝物測定法[8)9)]

陰イオン性の代謝物質の測定は，塩基性化合物をキャピラリー内壁表面に化学修飾したCOSMO(+)キャピラリー(ナカライテスク株式会社より市販されている)を用いて行っている。泳動液にはpH8.5の50mM酢酸アンモニウム溶液を用い，陽イオン測定とは極性を反転させて電圧を印加する。COSMO(+)キャピラリーを用いることにより，電気浸透流と呼ばれる液流を反転することが可能になり，結果として陰イオン性の代謝物質を安定に測定することができるようになった(図3 B)。図5に解糖系，ペントースリン酸回路，TCA回路の代謝中間体と，ヌクレオチド類を一斉分析した例を示す。本法では，異性体であるRu5PとR5P，*iso*-CitrateとCitrate，G1P，F6PおよびG6Pなども分離することが可能である。

Ⅲ　CE-MSにおける試料の前処理

サンプル中の代謝物量を正確に定量するためには代謝を瞬時に停止させることが不可欠である。また，CE-MSを用いたメタボローム測定においては，蛋白質や脂質などの夾雑物質の影響を受けやすいため，特に注意が必要である。

ここでは，血液試料の場合を例にとって述べる。50 μLの血清，または血漿をあらかじめ内部標準の入った10倍量のメタノール溶液に混合する。次いでクロロホルム，Milli-

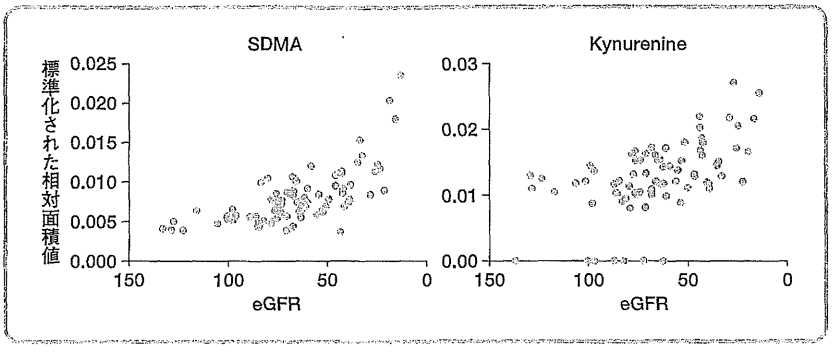

図6．SDMA，キヌレニン（Kynurenine）とeGFR（推算糸球体濾過量）との相関
両代謝物の濃度はeGFRの減少（＝腎機能の低下）と正の相関がみられた。

Q水を追加して分液操作を行うことにより，疎水性の蛋白質や脂肪酸，リン脂質などを除去する。さらに，分液後の水層を分画分子量5,000の限外ろ過フィルターに通すことによって，水溶性の蛋白質を完全に除去する。ろ液は減圧乾固した後，50 μLのMilli-Q水に再溶解して測定に用いている。

Ⅳ　データ解析

CE-MSに限らず，対象を絞らないメタボローム解析によって得られたデータには，1サンプルあたり数千から数万のピークが含まれている。これらの膨大なピークを手作業で解析するのは不可能であるので，市販のソフトウェアなどを用いて，自動的に処理を行うのが普通である。

しかしながら，CE-MS測定で得られたデータを解析する際に問題となるのが，試料間の各物質の泳動時間のずれが生じることである。筆者らの研究グループではこの問題を解決するため，CE-MS解析に特化したソフトウェア（Keio MasterHands）の開発も行っている[10)-12)]。このソフトウェアは，ピークの積分，検出時間のずれの補正，同定や定量までを自動で行うことも可能になっており，データ解析にかかる時間を飛躍的に短縮することが可能になった。

Ⅴ　糖尿病性腎症のバイオマーカー探索への応用

糖尿病性腎症は糖尿病における主要な合併症の1つであり，末期腎不全に移行する最大の原因である。最近の研究で，糖尿病性腎症のできるだけ早期に治療介入した場合，腎症の進行を有意に遅らせることができることがわかってきており，早期診断の重要性が高まっている。一般的に糖尿病性腎症の確定診断は腎生検による組織診断によって行われているが，患者が高齢である場合が多く，すべての症例に組織診断を行うことは不可能である。したがって，採血などの比較的簡便な方法によって糖尿病性腎症を早期に診断するバイオマーカーの開発は非常に重要である。

今回筆者らはCE-TOFMSを用いて，糖尿病性腎症病期を診断する血清バイオマーカーの探索を試みた。78名の糖尿病性腎症患者はその腎症の進行により，ステージⅠ（腎症前期），ステージⅡ（早期腎症期），ステージⅢ（顕性腎症期）およびステージⅣ（腎不全期）の4つのグループに分類した。CE-TOFMSを用いたメタボローム測定の結果，陽イオン測定と陰イオン測定の2回の測定によって1サンプルあたり平均4,400のピークを検出した。そこから，フラグメントイオン，アダクトイオンといった1つの代謝物から派生する関連ピークやノイズを除去した結果，最終的に糖尿病性腎症患者血清中から1サンプルあたり平均約300種類の代謝物ピークを得ることができた。

これらすべてのピークに対して統計解析を行った結果，