Toxicomics Report

Urinary proteome profile of renal papillary necrosis in experimental model rats

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ABSTRACT — We aimed to identify novel biomarkers using toxicoproteomics to profile the urinary proteome of renal papillary necrosis (RPN) in experimental model rats. RPN was induced in rats by a single intraperitoneal injection of 2-bromoethylamine hydrobromide (BEA). Urinary proteins from four groups (control group; BEA-treated but no RPN; BEA-treated with RPN but no increase in blood urea nitrogen [BUN]; and BEA-treated with RPN and increase in BUN) were pooled. Relative quantitation of pooled urinary proteins was then performed by two-dimensional liquid chromatography-tandem mass spectrometry with the isobaric labeling technique. We found 77 proteins were changed in RPN model rat urine.

Key words: Renal injury, Nephrotoxin, Proteomics, iTRAQ

INTRODUCTION

Renal papillary necrosis (RPN) is a well-known condition arising as one of kidney injury. Several etiological factors of RPN are recognized, such as analgesic abuse or overuse, diabetes mellitus, urinary obstruction, and sickle cell disease (Sutariya and Pandya, 2016; Jung *et al.*, 2006). Although RPN has a lower incidence than other injuries such as proximal tubular or glomerular injury, it is a serious condition because the regenerative ability of the kidney papilla is low and no biomarker that can detect RPN before the injury becomes too severe for treatment has yet been identified. For a start to discover novel urinary biomarkers for RPN, we aimed to profile urinary proteome of RPN using toxicoproteomics (TPx) in the current study. Urinary proteins from rats with RPN that had been induced by BEA injection, before an increase in the levels of BUN, were analyzed by two-dimensional liquid chromatography-tandem mass spectrometry with a peptide isobaric labeling technique to quantify levels relative to those in control rats.

MATERIALS AND METHODS

Reagents

BEA was obtained from Sigma-Aldrich (St. Louis, MO, USA). Thermo 660 nm protein assay reagent used for quantification of total protein and Dithiothreitol (DTT) No-Weigh Format used for reduction were purchased from Thermo Scientific (Waltham, MA, USA). Acrylamide solution used for alkylation was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Sequencing Grade Modified Trypsin and Lysyl Endopeptidase Mass Spectrometry Grade (LysC), used for protein digestion for TPx quantitation, were purchased from Promega (Fitchburg, WI, USA) and Wako (Tokyo, Japan), respectively. The iTRAQ reagents multiplex kit used for sample labeling was purchased from AB Sciex (Framingham, MA, USA).

Animal studies

Animal experimental procedures were conducted at Kashima Facilities in Astellas Pharma, Inc. Male Sprague Dawley rats (8 weeks of age at the start of treatment) were purchased from Charles River Laboratories

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(Shiga, Japan). Rats were given a single intraperitoneal injection of BEA (0, 3, 10, 30, 100 mg/kg, 5 animals per dosing group) and sacrificed 24 hr after administration. Water was available *ad libitum* through the experiment and food was available before urine sampling. Preserved urine samples were collected under fasting conditions from after administration to before the necropsy. Blood samples were collected from the abdominal aorta at the time of necropsy. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc. Furthermore, Astellas Pharma Inc., Kashima Facilities was awarded Accreditation Status by the AAALAC International.

Histopathology and clinical pathology

At necropsy, both kidneys were removed and the right kidney was fixed in 10% phosphate-buffered formalin and subsequently embedded in paraffin blocks, sectioned, stained with hematoxylin and eosin and examined by light microscopy. Histopathological change was graded as none (−), minimal (+), mild (++), moderate (+++), and severe (++++). BUN was measured using a Hitachi 7170S (Tokyo, Japan). Statistical analysis of BUN was performed by Dunnett's test using StatFlex Ver. 6 (Artech Co., Ltd., Osaka, Japan). Values that were significantly different from control values are indicated as $^{*}p < 0.05$ or $^{**}p < 0.01$. TIBCO™ Spotfire® 6.5.3 (TIBCO Software Inc., Palo Alto, CA, USA) was used to draw graphs.

Sample preparation for 2D-LC/MS/MS

To identify biomarker candidates that are more sensitive than traditional biomarkers such as BUN, urine samples were grouped as follows: Group i, 0 mg/kg group (n = 4); Group ii, 10 mg/kg group and RPN was not observed (n = 4); Group iii, 30 mg/kg group and RPN was observed (n = 2); Group iv, 30 mg/kg group, RPN and an increase in BUN were observed (n = 2). The urine samples in the same group were pooled to obtain equal protein amounts and concentrated using an Amicon ultra 10,000 molecular weight cut-off filter (Merck Millipore, Billerica, MA, USA) and each sample was subsequently exchanged with DPBS buffer. Protein concentrations in these samples were then determined using Thermo 660 nm protein assay reagent and 30 µg of protein in each sample was used for further analysis.

Protein digestion and isobaric tag labeling

Samples were precipitated using 9 volumes of ethanol, and the resulting precipitation was resolved with 20 µL of iTRAQ dissolution buffer, 1 µL of 2% SDS, and 2 µL of 50 mM DTT. Reductive alkylation of cysteine residues was performed with heating at 110°C for 30 min followed by the addition of 0.6 µL of 500 mM acrylamide solution at 37°C for 30 min. Protein digestion was performed with 4 µg of trypsin and LysC at 37°C overnight. Isobaric tag labeling was used with iTRAQ reagent and was set as follows: 114 tags for Group i, 115 tags for Group ii, 116 tags for Group iii, and 117 tags for Group iv. Two vials of iTRAQ reagents dissolved in 140 µL of ethanol were added into sample tubes, and were then adjusted to pH 8 using trimethylamine and incubated at 37°C for 2 hr. The solutions were evaporated and each pellet was reconstituted with 100 µL of 100 mM ammonium acetate. These 4 samples were mixed into one tube and 1.6 mL of acetonitrile was added.

Two-dimensional liquid chromatography coupled with tandem mass spectrometry proteome analysis (TPx)

Offline separation of peptides via hydrophilic interaction chromatography was performed using SeQuant™ ZIC-HILIC PEEK HPLC Columns (main column, 100 x 2.1 mm, 5 µm 200 Å; pre-column, 20 x 2.1 mm, 5 µm 200 Å; Merck Millipore), Nano Space SI-2 pump system (Pump: 3101 x 2, Detector (UV): 3002-UV, Heater and Injector: 3014, Shiseido, Tokyo, Japan) and Fraction collector FC-203B (Gilson, Middleton, WI, USA). Mobile phase A contained 20 mM ammonium acetate in 80% acetonitrile, and mobile phase B contained 20 mM ammonium acetate in 40% acetonitrile. Separation was obtained by employing a gradient of 0% to 100% mobile phase B at 200 µL/min over 55 min, 100% mobile phase B for 5 min, and 100% mobile phase A for 15 min. Fractions were collected every minute, evaporated, and dissolved in 0.1% formic acid in 2% acetonitrile. Each fraction was analyzed via a NANO HPLC capillary column (75 µm x 150 mm, packed with 3 µm ODS silica particles, Nikkyo Technos Co., Ltd, Tokyo, Japan). The LC-MS/MS system was composed of an auto sampler (HTC-PAL; CTC Analytics AG, Zwingen, Switzerland), liquid chromatography (Ultimate 3000 capillary LC system; Dionex corporation, Sunnyvale, CA, USA), and mass spectrometer (LTQ-Orbitrap XL; Thermo Scientific) interfaced with a nanoelectrosplay ion source. Mobile phase C contained 0.1% formic acid in 2% acetonitrile, and mobile phase D contained 0.1% formic acid in 90% acetonitrile. Flow rate was 200 nL/min and the gradient conditions in the chromatographic run were as follows: D 2% (0 min) → 15% (40 min) → 45% (80 min) → 90% (80 min) → 90% (100 min) → 10% (105 min) → 2% (120 min). Mass spectrometry was performed in positive ion data-dependent analysis mode. Each scan cycle consisted of one full

MS scan (*m/z* 400-1500) in profile mode at a resolution of 15,000, followed by "Top-three" HCD product ion scans in centroid mode at a resolution of 7500 with a normalized collision energy of 40.

MS data analysis

All raw MS/MS data were merged into an MGF file using Mascot Daemon (Matrix Science, London, UK) with Extract-MSN (Thermo Scientific). Database search was done using the Mascot search engine (ver. 2.2.04.1, Matrix Science) against the in-house customized database based on the Rat_REFP (January 10, 2010, 15,863 sequences, downloaded from NCBI) with the addition of proteases (trypsin and LysC) and commonly contaminating proteins (e.g. human keratin). Database search settings were as follows: fixed modifications of the cysteine residue (propionamide), lysine residue and N-terminal (iTRAQ tag), and variable modifications of the methionine residue (oxidation). Peptide mass tolerance was 10 ppm and fragment mass tolerance was 25 mDa. Up to two missed trypsin cleavages were allowed. Proteins with a significance threshold $p < 0.05$ were selected and the relative expression levels in each group (Groups i to iv) were calculated on MASCOT.

RESULTS

Histopathology and BUN levels of RPN model

A single injection of BEA induced RPN as described in our previous report (Sasaki *et al.*, 2011). In histopathology, RPN and associated findings were observed at 30 mg/kg or more (Table 1). Basophilic tubules in the cortex were also observed in one rat at a dose of 3 mg/kg, but the findings were judged to be toxicologically insignificant because they were found only one animal and not observed higher dosing groups. On examination of clinical chemistry, BUN levels were increased at a dose of 30 mg/kg or more (Fig. 1).

Urinary proteome profiling of RPN by TPx

Subsets of urine samples were grouped into four groups (Group i, ii, iii, and iv) based on the results of BUN and histopathology (See the "Sample preparation for 2D-LC/MS/MS" in a section of "materials and methods"). These samples were pooled, iTRAQ-labeled, and analyzed with off-line 2D-LC/MS/MS. Criteria for selection of proteins for biomarker candidates were as follows: proteins increased or decreased in a sequential order from Group i to Group iv; proteins in Group iii (the group in which kidney injury was not detected by measuring BUN) increased over two-fold or decreased less than ½-fold compared with proteins in Group i. Identified proteins that fulfilled both criteria were determined to be biomarker candidates. A total of 77 biomarker candidates passed

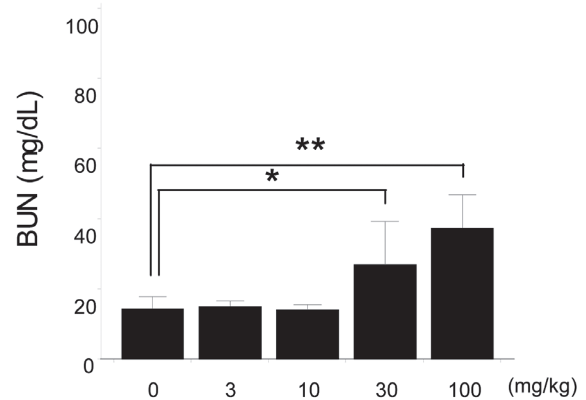

Fig. 1. BUN concentration in the serum of rats treated with BEA. Data are presented as mean and S.D.

Table 1. Histopathological changes in rat kidney.

Histopathological findings	Dose (mg/kg)				
	0	3	10	30	100
Papillary necrosis (RPN)	-	-	-	+ to ++ (5/5)	+ to ++ (4/5)
Infiltration of inflammatory cells in the papilla	-	-	-	+ (3/5)	+ (4/5)
Eosinophilic droplets in collecting ducts in the papilla	-	-	-	+ (3/5)	+ to ++ (4/5)
Basophilic tubules in the cortex	-	+ (1/5)	-	-	-
Dilatation of proximal tubules in the cortex	-	-	-	+ to ++ (3/5)	+ (4/5)

Histopathological grade: - lesion not observed; +, minimal, ++ mild severity of lesion
Numbers in parentheses describe "number of animals with findings" / "number of animals examined"

Table 2. Urinary proteins changed in RPN model rat.

Accession No.	Gene ID	Protein name	MS score	MS peptides	emPAI*	Fold change to Group i		
						Group ii	Group iii	Group iv
NP_758823	Afm	afamin precursor	1043	90	2.48	1.108	2.742	6.463
NP_599153	Alb	albumin precursor	3610	2669	152.62	1.087	14.481	36.442
NP_036630	Akr1b1	aldo-keto reductase family 1, member B1	46	1	0.09	1.420	10.751	20.797
NP_001033064	LOC297568	alpha-1-inhibitor III precursor	1616	130	1.26	1.384	2.417	8.885
NP_036958	Azgp1	alpha-2-glycoprotein 1, zinc-binding precursor	136	6	0.47	1.098	5.627	10.207
NP_037030	Ahsg	alpha-2-HS-glycoprotein precursor	621	174	2.70	1.730	1.552	3.260
NP_446409	Apbb3	amyloid beta (A4) precursor protein-binding, family B, member 3	24	1	0.06	1.046	3.405	6.732
NP_570106	Asz1	ankyrin repeat, SAM and basic leucine zipper domain containing 1	22	1	0.00	1.131	12.567	20.912
NP_036870	Apoa1	apolipoprotein A-I precursor	388	24	2.21	1.107	9.740	41.136
NP_036869	Apoa4	apolipoprotein A-IV precursor	508	35	1.09	1.212	10.623	37.957
NP_036911	Aqp5	aquaporin 5	34	8	0.00	1.065	8.219	26.326
NP_001030174	Atad1	ATPase family, AAA domain containing 1	48	5	0.00	1.104	2.022	6.498
NP_058812	Capn2	calpain 2 precursor	38	7	0.04	1.226	7.793	26.354
NP_036664	Cp	ceruloplasmin precursor	1215	56	0.89	1.050	5.036	12.562
NP_444180	Clu	clusterin	297	14	0.67	2.095	1.408	3.614
NP_001014028	F12	coagulation factor XII	314	17	0.49	1.117	3.665	8.455
NP_058690	C3	complement component 3	2914	195	2.67	0.999	8.548	32.967
NP_001100025	C8g	complement component 8, gamma polypeptide	125	2	0.44	1.315	8.948	25.606
NP_476487	C9	complement component 9	726	37	1.15	1.084	5.606	13.789
NP_997631	Cfb	complement factor B	891	66	1.31	1.158	5.903	16.653
NP_569093	Cfh	complement factor H	215	22	0.15	1.512	3.472	15.552
NP_077071	Cfi	complement factor I precursor	274	12	0.46	1.296	4.572	10.746
NP_001003964	Dnmt3l	DNA (cytosine-5-)-methyltransferase 3-like	28	1	0.07	1.418	13.115	22.414
NP_062243	Enpp3	ectonucleotide pyrophosphatase/phosphodiesterase 3	50	4	0.07	1.210	2.086	9.758
NP_001101978	Eps8l2	EPS8-like 2	31	3	0.00	1.064	3.078	5.735
NP_058700	Es2	esterase 1	769	65	1.73	1.275	5.680	15.400
NP_001028241	Eif4ebp2	eukaryotic translation initiation factor 4E binding protein 2	22	1	0.00	1.131	12.567	20.912
NP_446334	Ecm1	extracellular matrix protein 1 precursor	31	2	0.05	1.057	6.869	19.728
NP_598221	Expi	extracellular proteinase inhibitor	47	1	0.51	1.553	4.779	8.769

Table 2. (Continued).

Accession No.	Gene ID	Protein name	MS score	MS peptides	emPAI*	Fold change to Group i		
						Group ii	Group iii	Group iv
NP_445800	Fetub	fetuin B precursor	571	53	1.75	1.403	17.581	33.581
NP_036691	Fgg	fibrinogen gamma chain	87	2	0.14	1.161	2.125	25.178
NP_058701	Fh1	fumarate hydratase precursor	34	3	0.06	1.124	3.723	7.073
NP_071970	Gpxp	glutathione peroxidase 3 precursor	101	3	0.29	1.173	2.398	3.096
NP_445770	Hpx	hemopexin precursor	1015	168	4.03	1.138	3.826	10.583
NP_596919	Hrg	histidine-rich glycoprotein	517	31	0.86	1.274	7.515	20.447
NP_077368	Hsd17b4	hydroxysteroid (17-beta) dehydrogenase 4	23	1	0.04	1.142	8.930	16.269
NP_001041356	RGD1310507	hypothetical protein LOC315963	595	51	1.45	1.358	11.035	30.324
NP_112308	Itpr2	inositol 1,4,5-triphosphate receptor 2	32	3	0.00	1.089	2.605	7.284
NP_037270	Itpr3	inositol 1,4,5-triphosphate receptor, type 3	32	3	0.00	1.089	2.605	7.284
NP_059047	Itih3	inter-alpha trypsin inhibitor, heavy chain 3 precursor	408	23	0.35	1.030	4.450	14.178
NP_062242	Itih4	inter-alpha-inhibitor H4 heavy chain	833	54	0.85	1.125	6.422	22.406
NP_001007742	Isg20l2	interferon stimulated exonuclease gene 20-like 2	28	1	0.00	1.001	3.288	10.570
NP_001100260	Kif21a	kinesin family member 21A	48	3	0.05	1.323	20.025	55.874
NP_036828	Kng1	kininogen 1	602	36	1.55	1.363	2.808	11.481
NP_001095888	Kng2	kininogen 2 isoform 2	291	30	0.59	1.626	3.669	14.997
NP_036903	Lyz2	lysozyme 2 precursor	66	3	0.46	1.573	4.095	5.582
NP_001006974	mrpl11	mitochondrial ribosomal protein L11 precursor	22	1	0.15	1.099	4.739	14.830
NP_001029159	Map2k5	mitogen-activated protein kinase kinase 5 isoform a	45	3	0.07	1.438	4.064	9.248
NP_075591	Mug1	murinoglobulin 1 precursor	1434	107	1.16	1.319	2.722	10.173
NP_001102065	Nat5	N-acetyltransferase 5	21	1	0.17	1.067	4.876	8.703
NP_579854	Ndel1	nuclear distribution gene E-like homolog 1	30	4	0.00	1.138	6.834	19.947
NP_001100807	Nsd1	nuclear receptor binding SET domain protein 1	24	2	0.01	1.449	4.850	6.867
NP_114466	Pon1	paraoxonase 1	17	1	0.09	1.408	5.349	12.640
NP_001102690	Pex1	peroxisome biogenesis factor 1	28	1	0.02	1.212	2.727	4.325
NP_620253	Phlpb	phospholipase B1 precursor	22	3	0.02	1.145	10.551	17.005
NP_445943	Plg	plasminogen	1097	73	1.29	1.331	3.720	9.088
NP_064476	LOC56825	prochymosin	29	1	0.00	1.264	3.496	10.856
NP_001032212	Pcdhga10	protocadherin gamma subfamily A, 10	27	5	0.00	1.284	2.657	7.811

Table 2. (Continued).

Accession No.	Gene ID	Protein name	MS score	MS peptides	emPAI*	Fold change to Group i		
						Group ii	Group iii	Group iv
NP_062097	Rgs9	regulator of G-protein signaling 9	29	4	0.04	1.102	13.297	35.527
NP_037294	Rbp4	retinol-binding protein 4, plasma precursor	117	6	0.53	1.533	11.525	21.516
NP_445890	Rnf103	ring finger protein 103	22	2	0.04	1.301	8.152	15.355
NP_445937	S100a6	S100 calcium binding protein A6	38	1	0.34	1.909	2.710	3.418
NP_001077380	Sepp1	selenoprotein P precursor	73	3	0.16	1.599	9.017	19.085
NP_077358	Serpind1	serine (or cysteine) proteinase inhibitor, clade D, member 1 precursor	72	4	0.13	1.281	2.509	8.291
NP_808788	Serpinf1	serine (or cysteine) proteinase inhibitor, clade F, member 1	224	5	0.44	1.228	3.868	7.597
NP_954524	Serping1	serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1, (angioedema, hereditary) precursor	190	6	0.27	1.007	6.004	12.789
NP_113719	Serpina3n	serine protease inhibitor 2c	979	52	3.48	1.158	3.152	11.322
NP_001102229	Siae	sialate O-acetylesterase	127	6	0.18	1.274	16.008	16.635
NP_599190	Slc12a5	solute carrier family 12 (potassium-chloride transporter), member 5	28	1	0.00	1.001	3.288	10.570
NP_001100976	Tshz3	teashirt zinc finger homeobox 3	28	1	0.00	1.001	3.288	10.570
NP_067735	Akap14	Testis-specific A-kinase-anchoring-protein	24	17	0.06	1.209	7.948	12.125
NP_001009628	Kng1l1	T-kininogen II precursor	597	53	1.54	1.627	3.606	14.124
NP_001013128	Tf	transferrin precursor	2142	570	11.70	1.026	17.504	48.955
NP_036813	Ttr	transthyretin precursor	472	136	10.43	1.083	16.142	34.992
NP_036810	Tpm4	tropomyosin 4	25	1	0.12	1.041	5.452	22.122
NP_062029	Vtn	vitronectin	296	19	0.73	1.081	4.256	10.377
NP_001128556	Zfp322a	zinc finger protein 322a	25	15	0.07	1.020	20.903	44.322

*Exponentially Modified Protein Abundance Index.

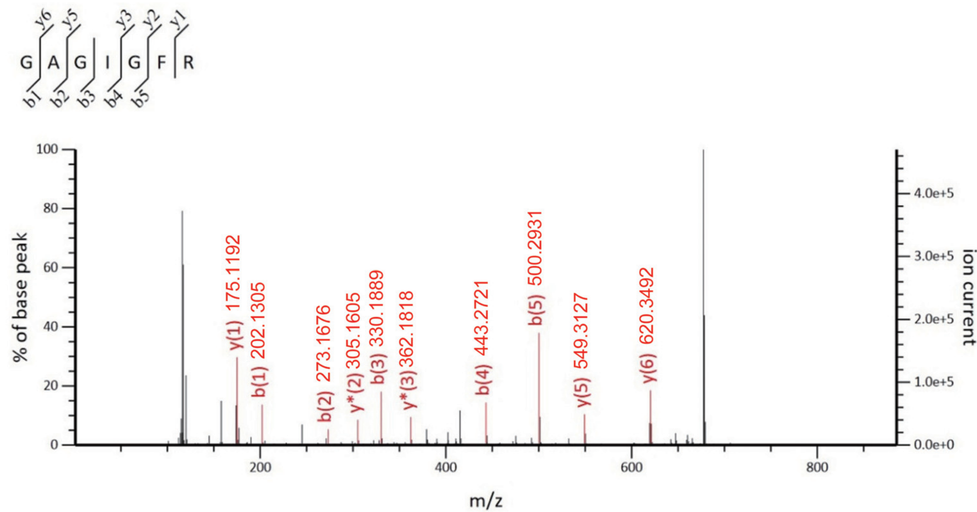

Fig. 2. MS/MS spectra of Siae peptide (Sequence: GAGIGFR). The red peaks indicate matched b- or y-ion series. The black peaks indicate unmatched peaks. * indicates desorption of NH_3 from the peptide.

the criteria (Table 2). Figure 2 shows the representative MS2 spectra. Alpha-2-HS-glycoprotein and cluterin did not fulfill the criteria but remained as candidates because they were previously reported to be or were already used as kidney injury biomarkers.

DISCUSSION

We aimed to identify novel biomarkers by profiling the urinary proteome of RPN using TPx. We found that 77 proteins were changed by RPN, some of which have previously been reported as kidney injury biomarkers, indicating that the use of TPx for profiling the urinary proteome of RPN is suitable for identifying novel biomarkers. Of note, albumin and clusterin are known biomarkers that are approved by the FDA, EMA, and PMDA for voluntary use, and formed part of the submission by the Predictive Safety Testing Consortium (Hewitt and Herget, 2009). Alpha-2-HS-glycoprotein, also known as fetuin-A, was identified in the urinary exosome of a cisplatin-induced acute kidney injury rat model (Zhou *et al.*, 2006). Fibrinogen gamma chain, hemopexin, and kininogen 2 are also associated with kidney injury (Krishnamoorthy *et al.*, 2011; Kaushal and Shah, 2012; Zager *et al.*, 2012; Bompart *et al.*, 1993). However, most of the other identified proteins have not been linked to kidney injury to date, and could therefore represent novel biomarkers. We will verify the potential for these 77 proteins as biomarkers of RPN in a future study.

Conflict of interest---- The authors declare that there is no conflict of interest.

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