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Original Research Article

Molecular Network Analysis of the Urinary Proteome of Alzheimer's Disease Patients

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Keywords
Alzheimer's disease · Urine · Label-free mass spectrometry · Case-control study · Proteomics

Abstract
Background/Aims: The identification of predictive biomarkers for Alzheimer's disease (AD) from urine would aid in screening for the disease, but information about biological and pathophysiological changes in the urine of AD patients is limited. This study aimed to explore the comprehensive profile and molecular network relations of urinary proteins in AD patients. ***Methods:*** Urine samples collected from 18 AD patients and 18 age- and sex-matched cognitively normal controls were analyzed by mass spectrometry and semiquantified with the normalized spectral index method. Bioinformatics analyses were performed on proteins which significantly increased by more than 2-fold or decreased by less than 0.5-fold compared to the control ($p < 0.05$) using DAVID bioinformatics resources and KeyMolnet software. ***Results:*** The levels of 109 proteins significantly differed between AD patients and controls. Among these, annotation clusters related to lysosomes, complement activation, and gluconeogenesis were significantly enriched. The molecular relation networks derived from these proteins were mainly associated with pathways of lipoprotein metabolism, heat shock protein 90 signaling, matrix metalloproteinase signaling, and redox regulation by thioredoxin. ***Conclusion:*** Our findings suggest that changes in the urinary proteome of AD patients reflect systemic changes related to AD pathophysiology.

Y.W. and Y.H. contributed equally to this work.

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease and the most common cause of dementia [1]. The main neuropathological changes associated with AD are extracellular accumulation of amyloid-β plaques, intracellular accumulation of neurofibrillary tangles of τ protein, inflammation, and brain atrophy [1]. Unfortunately, no cure currently exists for AD. Recent studies have shown that brain changes associated with AD start more than a decade before the onset of clinical symptoms such as progressive memory deficits [2–5]. Thus, in order to reduce the incidence and prevalence of AD, it will be necessary to focus on the stage before clinical symptoms appear [6]. Accumulating evidence suggests that systemic metabolic dysfunction such as diabetes, vascular dysfunction, and systemic inflammation underlie the development of AD [7–9]. These systemic changes also precede the onset of clinical symptoms of the disease. The discovery of a panel of biomarkers that reflect these systemic changes and could therefore predict the development of AD would be valuable for screening those at risk.

Urine is one of the most preferred biofluids for biomarker discovery because urine collection is simple and noninvasive. Moreover, repeated urine sampling from the same individual is easy, as is collection of a sufficient volume for analysis compared to other biofluids [10, 11]. Urine also contains systemic information since approximately 30% of urinary protein originates from plasma via blood filtration, with the remainder coming from the kidneys and the urinary tract [10, 12]. With technological advances in mass spectrometry (MS), MS-based proteomics has been used to identify a large number of proteins belonging to the urinary proteome [11, 13]. While the discovery of predictive biomarkers for AD from urine would be highly beneficial, information regarding biological and pathophysiological changes in the urine of AD patients is currently limited. In the present study, urinary proteomes of AD patients and cognitively normal elderly controls were compared to explore the comprehensive profile and molecular-network relations of the urinary proteome of AD patients.

Materials and Methods

Participants and Classification

AD patients were recruited from outpatients of Niigata University Hospital who were diagnosed with the disease based on criteria of the National Institute of Neurological and Communicative Disorders and Stroke AD and Related Disorders Association (NINCDS-ADRDA) and took the Mini-Mental State Examination (MMSE) [14] within a year of urine collection. The clinical characteristics of the AD group are summarized in online supplementary Table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000496100).

Age- and sex-matched cognitively normal controls (MMSE score >27) were selected from a subcohort (Sekikawa cohort) of the Murakami cohort, a population-based cohort study that targeted individuals aged between 40 and 74 years living in areas of the northern Niigata Prefecture (Murakami region) [15]. Participants provided urine samples at specific health checkups held by the national health insurance of Japan and underwent the MMSE within a year of urine collection.

Urine Sample Collection and Laboratory Test

Spot urine samples were obtained from participants. No restrictions on diet, drinking, or exercise were required prior to urine sampling. Urinary protein, urinary sugar, and occult blood were checked using urine test strips (Pretest 5bII [Wako, Japan] for AD samples and Hema-Combistix-long [Siemens Healthcare, Japan] for control samples). Urinary albumin and

Table 1. Participant characteristics and results of the general urinalysis

	AD (n = 18)	Control (n = 18)	p value
Age, years	72.9±5.6	72.8±5.2	0.951
Males, n	8	8	1.000
MMSE points	21.6±4.5	28.8±0.7	<0.001
Urinary albumin[a], μg/mL	46.18±24.8 (11)	18.6±4.7 (10)	0.298
Urinary creatinine, mg/dL	106.4±13.8	77.7±10.5	0.107
Albumin/creatinine[b], mg/gCr	22.7±6.6	22.4±6.0	0.976
Results of the urine test strip			
Urinary protein level	– (17), ± (1)	– (18)	0.486
Urinary blood level	– (16), 2+ (28)	– (14), 1+ (1), 3+ (1)	0.486
Urinary glucose level	– (17), 2+ (1)	– (16), ± (1), 1+ (1)	0.019

Results are presented as means ± SD for continuous variables. Values in parentheses are numbers of patients. p values were calculated using an unpaired t test and Fisher's exact test for continuous variables and categorical variables, respectively. [a] Undetectable (<5 μg/mL) in 7 AD and 8 control group patients. The mean ± SD of urinary albumin was calculated from values of detected samples. [b] For calculation of this ratio, undetected albumin was substituted with 5 μg/mL.

creatinine were measured by latex immunological nephelometry using a SPOTCHEM D-01 analyzer (SD-3810; Arkray Global Business, Inc. Japan). Urine samples were centrifuged at 1,000 g for 15 min and the supernatant was stored at –20 °C until use.

MS and Semiquantification of Proteome Data

Detailed methods for protein extraction, MS analysis, and semiquantification of proteome data can be found in the online supplementary material. In brief, urine proteins were precipitated by the methanol precipitation method, dissolved, and digested in solution by trypsin. Digested samples were purified using a C18 spin column and peptides (500 ng) were analyzed by liquid chromatography coupled to tandem MS (MS/MS). All MS and MS/MS spectrums were analyzed by MASCOT (v4.2; Matrix Science) for protein and peptide identification. Data were queried against the Uniprot/Swiss-Prot database. Identification of proteins and peptides was carried out with a significance threshold of $p < 0.05$. The normalized spectral index (SI_N), a label-free quantification method [16], was used to compare protein abundance between different samples.

Bioinformatics Analysis

Gene enrichment analysis was performed using functional annotation clustering of DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/home.jsp) [17, 18]. Molecular network analysis was performed using KeyMolnet software (KM Data, Tokyo, Japan) [19]. Details of the KeyMolnet analysis are provided in the online supplementary material.

Statistical Analysis

Statistical analysis was performed using SAS® Studio 3.7 software (SAS Institute Inc., Cary, NC, USA). Means of protein abundance were compared using a t test with Welch's correction ($\alpha = 0.05$). Pearson's ρ (r) was used to assess expression-level correlations of proteins among and between AD and control groups. Multiple comparisons were accounted for by using a false discovery rate adjustment ($q = 0.05$). Graphs were prepared using GraphPad software (GraphPad Prism version 7.0a for Mac; La Jolla, CA, USA).

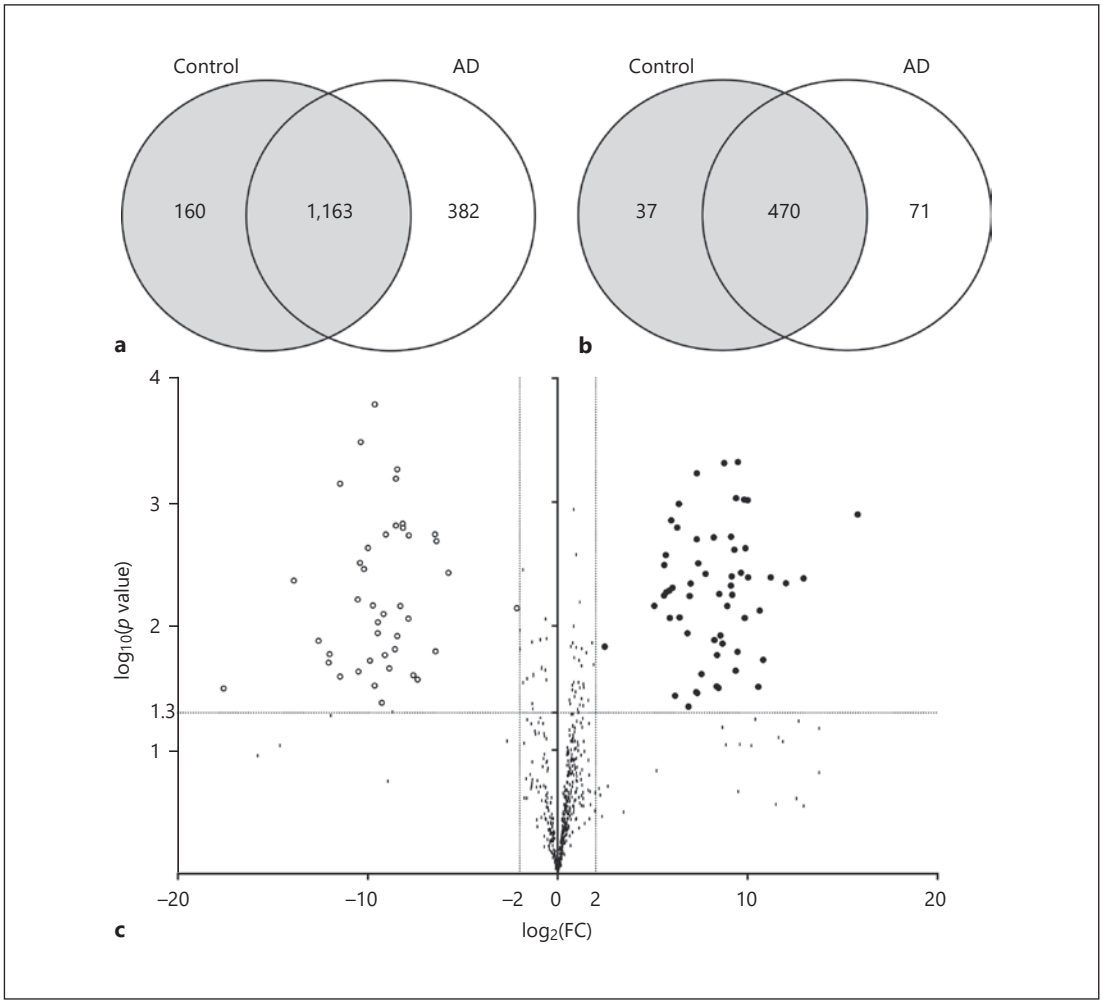

Fig. 1. Venn diagrams of all of the identified proteins (**a**) and proteins identified in at least 9 samples of either group (**b**). **c** Volcano plot displaying differentially expressed proteins between the AD and control groups. The *x*-axis displays the log 2-fold change (FC) of the mean SI_N value between the groups, while the *y*-axis corresponds to the absolute value of log 10 (*p* value) of the *t* test with Welch's correction. Closed black circles represent the 73 increased proteins in the AD group (FC >2, $p < 0.05$) and open circles represent the 36 decreased proteins in the AD group (FC <0.5, $p < 0.05$).

Results

Protein Identification by Liquid Chromatography-MS/MS and Semiquantification by SI_N

Urine samples were collected from 18 AD patients (8 males and 10 females) and 18 cognitively normal (MMSE >27 points) controls selected from participants of the Murakami cohort in an age- and sex-matched manner. General characteristics of the participants and the results of general urinalysis are shown in Table 1.

For AD and control urine samples, 613.2 ± 117.7 and 589.7 ± 87.3 (mean ± SD) proteins were identified, respectively. Of the total of 1,705 unique proteins identified, 382 and 160 proteins were uniquely identified in AD and control groups, respectively, and 1,163 proteins were shared between the 2 groups (Fig. 1a). For further analysis, 578 proteins identified in at least 9 samples of either group, excluding 28 keratin isoforms, were selected. Of these, 71 and 37 proteins were uniquely identified in AD and control groups, respectively, and 470

Table 2. List of 109 significantly increased or decreased proteins

Accession No.	MW, kDa	Protein description (gene name)	log_2FC (AD/control)	Mean SI_N value[a]		Detected cases, *n*		Welch's *p* value	FDR
				AD	control	AD (*n* = 18)	control (*n* = 18)		
P02042	16.0	Hemoglobin subunit δ (HBD)	–17.59	0.0001	19.7571	0	10	0.032	0.166
Q8N2U0	11.7	Transmembrane protein 256 (TMEM256)	–10.53	0.0001	0.1478	0	9	0.006	0.068
A0AVF1	64.1	Intraflagellar transport protein 56 (TTC26)	–10.49	0.0001	0.1440	0	12	0.023	0.138
P81605-2	12.4	Isoform 2 of dermcidin (DCD)	–10.41	0.0001	0.1360	0	13	0.003	0.056
P06703	10.2	**Protein S100-A6 (S100A6)**	**–10.37**	**0.0001**	**0.1320**	**0**	**15**	**0.000**	**0.048**
P54710	7.3	Sodium/potassium-transporting ATPase subunit γ (FXYD2)	–10.20	0.0001	0.1178	0	9	0.004	0.058
Q15485	34.0	Ficolin-2 (FCN2)	–9.99	0.0001	0.1017	0	12	0.002	0.050
P20827	23.8	Ephrin-A1 (EFNA1)	–9.88	0.0001	0.0941	0	10	0.019	0.123
P21926	25.4	CD9 antigen(CD9)	–9.73	0.0001	0.0852	0	9	0.007	0.070
P01127	27.3	Platelet-derived growth factor subunit B (PDGFB)	–9.64	0.0001	0.0796	0	9	0.030	0.164
P02656	10.8	**Apolipoprotein C-III (APOC3)**	**–9.63**	**0.0001**	**0.0793**	**0**	**12**	**0.000**	**0.048**
O00241	43.2	Signal-regulatory protein $β_1$ (SIRPB1)	–9.48	0.0001	0.0714	0	12	0.011	0.095
P55259-3	59.1	Isoform α of pancreatic secretory granule membrane major glycoprotein GP	–9.47	0.0001	0.0709	0	9	0.009	0.081
Q7LBR1	22.1	Charged multivesicular body protein 1b (CHMP1B)	–9.26	0.0001	0.0612	0	13	0.042	0.196
Q6UXB4	32.5	C-type lectin domain family 4 member G (CLEC4G)	–9.17	0.0001	0.0576	0	12	0.008	0.077
Q96PP9	73.1	Guanylate-binding protein 4 (GBP4)	–9.10	0.0001	0.0550	0	9	0.017	0.114
P36915	68.7	**Guanine nucleotide-binding protein-like 1 (GNL1)**	**–9.05**	**0.0001**	**0.0532**	**0**	**10**	**0.002**	**0.048**
P61981	28.3	14-3-3 protein γ (YWHAG)	–8.87	0.0001	0.0467	0	9	0.022	0.135
P09972	39.4	Fructose-bisphosphate aldolase C (ALDOC)	–8.57	0.0001	0.0381	0	11	0.015	0.110
Q6UY14-3	118.7	**Isoform 3 of ADAMTS-like protein 4 (ADAMTSL4)**	**–8.52**	**0.0001**	**0.0366**	**0**	**14**	**0.001**	**0.048**
Q00796	38.3	**Sorbitol dehydrogenase (SORD)**	**–8.52**	**0.0001**	**0.0368**	**0**	**12**	**0.002**	**0.048**
O00592	58.6	**Podocalyxin (PODXL)**	**–8.45**	**0.0001**	**0.0350**	**0**	**12**	**0.001**	**0.048**
Q6FHJ7	39.8	Secreted frizzled-related protein 4 (SFRP4)	–8.45	0.0001	0.0349	0	10	0.012	0.099
P02748	63.1	Complement component C9 (C9)	–8.29	0.0001	0.0313	0	10	0.007	0.070
P08238	83.2	**HSP 90-β (HSP90AB1)**	**–8.16**	**0.0001**	**0.0286**	**0**	**10**	**0.002**	**0.048**
Q9Y3B3	25.2	**Transmembrane emp24 domain-containing protein 7 (TMED7)**	**–8.15**	**0.0001**	**0.0284**	**0**	**9**	**0.002**	**0.048**
O14578	231.4	Citron ρ-interacting kinase (CIT)	–7.86	0.0001	0.0233	0	10	0.009	0.079
P07711	37.5	**Cathepsin L1 (CTSL)**	**–7.84**	**0.0001**	**0.0229**	**0**	**10**	**0.002**	**0.048**
P19823	106.4	Inter-α-trypsin inhibitor heavy chain H2 (ITIH2)	–7.60	0.0001	0.0194	0	12	0.025	0.145
Q8IUL8	126.2	Cartilage intermediate layer protein 2 (CILP2)	–7.38	0.0001	0.0166	0	10	0.027	0.155
P16284	82.5	**Platelet endothelial cell adhesion molecule (PECAM1)**	**–6.46**	**0.0001**	**0.0088**	**0**	**10**	**0.002**	**0.048**
P20774	33.9	Mimecan (OGN)	–6.43	0.0001	0.0086	0	9	0.016	0.111
Q9NPY3	68.5	**Complement component C1q receptor (CD93)**	**–6.38**	**0.0001**	**0.0083**	**0**	**9**	**0.002**	**0.049**
P16234	122.6	Platelet-derived growth factor receptor α (PDGFRA)	–5.75	0.0001	0.0054	0	10	0.004	0.058
P25311	34.2	Zinc-α-2-glycoprotein (AZGP1)	–2.15	5.6354	25.0614	17	18	0.007	0.072
P00746	27.0	Complement factor D (CFD)	–1.30	0.0796	0.1959	9	13	0.048	0.217
Q9NQ84-2	49.4	Isoform 2 of G-protein coupled receptor family C group 5 member C (GPRC5C)	1.06	0.2948	0.1411	18	18	0.049	0.217
Q6GTX8	31.4	Leukocyte-associated immunoglobulin-like receptor 1 (LAIR1)	1.10	3.4443	1.6068	18	18	0.029	0.163
P21810	41.6	Biglycan (BGN)	1.13	0.1803	0.0822	15	17	0.020	0.124
P11047	177.5	Laminin subunit γ-1 (LAMC1)	1.17	0.0121	0.0054	16	12	0.007	0.070
P07602	58.1	Prosaposin (PSAP)	1.17	0.3748	0.1664	18	18	0.033	0.166
P10643	93.5	Complement component C7 (C7)	1.17	4.3890	1.9449	18	18	0.045	0.206
P35241-5	71.0	Isoform 5 of radixin (RDX)	1.26	0.2582	0.1081	16	18	0.022	0.134
P33908	72.9	Mannosyl-oligosaccharide 1,2-α-mannosidase IA (MAN1A1)	1.26	0.1469	0.0611	13	10	0.040	0.191
P10253	105.3	Lysosomal α-glucosidase (GAA)	1.27	1.3006	0.5395	18	18	0.016	0.111
Q9H3G5	54.1	Probable serine carboxypeptidase CPVL (CPVL)	1.34	0.2208	0.0870	15	11	0.045	0.206
P07686	63.1	Hexosaminidase subunit β (HEXB)	1.35	0.1201	0.0472	15	12	0.030	0.164
Q12860	113.2	Contactin-1 (CNTN1)	1.37	0.0319	0.0124	16	11	0.032	0.166
P0DJD8	42.0	Pepsin A-3 (PGA3)	1.51	4.3450	1.5285	18	17	0.017	0.114
P06744-2	64.3	Isoform 2 of glucose-6-phosphate isomerase (GPI)	1.61	0.0687	0.0224	14	11	0.041	0.194
Q16651	36.4	Prostasin (PRSS8)	1.81	0.8232	0.2355	18	18	0.014	0.106
P34059	58.0	N-acetylgalactosamine-6-sulfatase (GALNS)	1.90	0.0533	0.0143	14	12	0.021	0.132
Q5JS37	38.3	NHL repeat-containing protein 3 (NHLRC3)	2.48	0.0701	0.0126	12	10	0.015	0.109
Q5JRA6	213.6	Melanoma inhibitory activity protein 3 (MIA3)	5.09	0.0034	0.0001	12	0	0.007	0.070
P13591	94.5	Neural cell adhesion molecule 1 (NCAM1)	5.60	0.0048	0.0001	9	0	0.006	0.065
Q7Z7M0	302.9	Multiple epidermal growth factor-like domain protein 8 (MEGF8)	5.61	0.0049	0.0001	13	0	0.003	0.056
Q92859	159.9	Neogenin (NEO1)	5.69	0.0052	0.0001	12	0	0.003	0.052
Q6UX71	59.5	Plexin domain-containing protein 2 (PLXDC2)	5.71	0.0052	0.0001	11	0	0.005	0.065
Q9NZV1	113.7	Cysteine-rich motor neuron 1 protein (CRIM1)	5.88	0.0059	0.0001	9	0	0.005	0.065
Q92563	46.7	Testican-2 (SPOCK2)	5.91	0.0060	0.0001	12	0	0.009	0.079
P08253	73.8	**72-kDa type IV collagenase (MMP2)**	**5.98**	**0.0063**	**0.0001**	**10**	**0**	**0.001**	**0.048**

Table 2 (continued)

Accession No.	MW, kDa	Protein description (gene name)	log_2FC (AD/control)	Mean SI_N value[a]		Detected cases, *n*		Welch's *p* value	FDR
				AD	control	AD (*n* = 18)	control (*n* = 18)		
Q15375	112.0	Ephrin type-A receptor 7 (EPHA7)	6.05	0.0066	0.0001	10	0	0.005	0.063
P16112	250.0	Aggrecan core protein (ACAN)	6.18	0.0072	0.0001	9	0	0.037	0.179
P07357	65.1	**Complement component C8 α chain (C8A)**	**6.29**	**0.0078**	**0.0001**	**9**	**0**	**0.002**	**0.048**
Q92954	151.0	**Proteoglycan 4 (PRG4)**	**6.38**	**0.0084**	**0.0001**	**11**	**0**	**0.001**	**0.048**
Q66K79-2	72.5	Isoform 2 of carboxypeptidase Z (CPZ)	6.42	0.0086	0.0001	9	0	0.009	0.079
O75787	39.0	Renin receptor (ATP6AP2)	6.83	0.0114	0.0001	9	0	0.011	0.095
O43405	59.4	Cochlin (COCH)	6.89	0.0118	0.0001	9	0	0.045	0.206
O00622	42.0	Protein CYR61 (CYR61)	6.95	0.0124	0.0001	9	0	0.006	0.065
Q9UJ96	51.2	Potassium voltage-gated channel subfamily G member 2 (KCNG2)	7.00	0.0128	0.0001	9	0	0.005	0.060
P50897	34.2	Palmitoyl-protein thioesterase 1 (PPT1)	7.29	0.0157	0.0001	9	0	0.034	0.172
P55083-2	31.1	**Isoform 2 of microfibril-associated glycoprotein 4 (MFAP4)**	**7.32**	**0.0159**	**0.0001**	**10**	**0**	**0.002**	**0.048**
O75309	89.9	**Cadherin-16 (CDH16)**	**7.33**	**0.0161**	**0.0001**	**12**	**0**	**0.001**	**0.048**
Q08345-5	101.7	Isoform 4 of epithelial discoidin domain-containing receptor 1 (DDR1)	7.35	0.0164	0.0001	10	0	0.035	0.173
P35858-2	70.2	Isoform 2 of insulin-like growth factor-binding protein complex acid labile subun	7.40	0.0169	0.0001	10	0	0.003	0.056
Q8NI32-2	23.3	Isoform 2 of Ly6/PLAUR domain-containing protein 6B (LYPD6B)	7.57	0.0189	0.0001	11	0	0.024	0.142
Q8N307	71.9	Mucin-20 (MUC20)	7.79	0.0221	0.0001	11	0	0.004	0.058
P55957-2	26.8	**Isoform 2 of BH3-interacting domain death agonist (BID)**	**8.22**	**0.0299**	**0.0001**	**9**	**0**	**0.002**	**0.048**
P34896	53.0	Serine hydroxymethyltransferase, cytosolic (SHMT1)	8.25	0.0304	0.0001	12	0	0.013	0.103
Q6UX73	45.4	UPF0764 protein C16orf89 (C16orf89)	8.37	0.0332	0.0001	11	0	0.031	0.164
Q13145	29.1	BMP and activin membrane-bound inhibitor homolog (BAMBI)	8.39	0.0335	0.0001	9	0	0.017	0.114
Q8IV08	54.7	Phospholipase D3 (PLD3)	8.47	0.0355	0.0001	12	0	0.032	0.166
P17174	46.2	Aspartate aminotransferase, cytoplasmic (GOT1)	8.51	0.0365	0.0001	9	0	0.006	0.065
P23526	47.7	Adenosylhomocysteinase (AHCY)	8.57	0.0381	0.0001	9	0	0.012	0.098
P00491	32.1	Purine nucleoside phosphorylase (PNP)	8.68	0.0410	0.0001	9	0	0.014	0.106
Q9BRK5	41.8	**45-kDa calcium-binding protein (SDF4)**	**8.77**	**0.0437**	**0.0001**	**14**	**0**	**0.001**	**0.048**
P10092	13.7	Calcitonin gene-related peptide 2 (CALCB)	8.93	0.0488	0.0001	10	0	0.007	0.070
Q9H444	24.9	Charged multivesicular body protein 4b (CHMP4B)	9.12	0.0556	0.0001	12	0	0.005	0.062
P15121	35.8	**Aldose reductase (AKR1B1)**	**9.13**	**0.0559**	**0.0001**	**13**	**0**	**0.002**	**0.048**
O95445	21.2	Apolipoprotein M (APOM)	9.16	0.0572	0.0001	10	0	0.004	0.058
P41181	28.8	Aquaporin-2 (AQP2)	9.19	0.0585	0.0001	10	0	0.006	0.065
P01889	40.4	HLA class I histocompatibility antigen, B-7 α chain (HLA-B)	9.30	0.0631	0.0001	11	0	0.002	0.050
P60953	21.2	Cell division control protein 42 homolog (CDC42)	9.37	0.0660	0.0001	9	0	0.023	0.138
P78380	30.9	**Oxidized low-density lipoprotein receptor 1 (OLR1)**	**9.39**	**0.0670**	**0.0001**	**12**	**0**	**0.001**	**0.048**
P17050	46.5	α-N-acetylgalactosaminidase (NAGA)	9.47	0.0709	0.0001	12	0	0.016	0.111
P17936-2	32.2	**Isoform 2 of insulin-like growth factor-binding protein 3 (IGFBP3)**	**9.49**	**0.0720**	**0.0001**	**11**	**0**	**0.001**	**0.048**
P62873	37.4	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit β-1 (GNB1)	9.65	0.0801	0.0001	11	0	0.004	0.058
P16152	30.4	**Carbonyl reductase (NADPH) 1 (CBR1)**	**9.82**	**0.0903**	**0.0001**	**11**	**0**	**0.001**	**0.048**
P49441	44.0	Inositol polyphosphate 1-phosphatase (INPP1)	9.85	0.0921	0.0001	9	0	0.009	0.079
P15153	21.4	Ras-related C3 botulinum toxin substrate 2 (RAC2)	9.88	0.0942	0.0001	12	0	0.002	0.050
P29622	48.5	**Kallistatin (SERPINA4)**	**10.00**	**0.1021**	**0.0001**	**13**	**0**	**0.001**	**0.048**
P18669	28.8	Phosphoglycerate mutase 1 (PGAM1)	10.02	0.1038	0.0001	10	0	0.004	0.058
P31944	27.7	Caspase-14 (CASP14)	10.56	0.1510	0.0001	9	0	0.031	0.164
P10599	11.7	Thioredoxin (TXN)	10.64	0.1599	0.0001	10	0	0.008	0.073
P01225	14.7	Follitropin subunit β (FSHB)	10.82	0.1804	0.0001	9	0	0.019	0.121
P62979	18.0	Ubiquitin-40S ribosomal protein S27a (RPS27A)	11.22	0.2393	0.0001	9	0	0.004	0.058
Q07654	8.6	Trefoil factor 3 (TFF3)	12.02	0.4156	0.0001	12	0	0.005	0.060
P04155	9.1	Trefoil factor 1 (TFF1)	12.95	0.7906	0.0001	13	0	0.004	0.058
P06312	13.4	**Ig κ chain V-IV region (fragment) (IGKV4-1)**	**15.80**	**5.6997**	**0.0001**	**12**	**0**	**0.001**	**0.048**

Bold text corresponds to proteins that remained significant after FDR correction. MW, molecular weight; FDR, false discovery rate. [a] Proteins not identified in certain samples were assumed to be at levels under the detection limit and thus were assigned a value that was half of the minimum SI_N value (i.e., 0.0001).

Table 3. Significantly enriched annotation clusters of significantly increased or decreased proteins (FC >2 or <0.5, $p < 0.05$) calculated by DAVID Bioinformatics Resources 6.8

Term	*p* value	Enrichment score	Gene names
Cluster 1			
GO:0043202~lysosomal lumen	<0.001	4.25	PSAP, PPT1, OGN, GAA, GALNS, BGN, HEXB, ACAN, NAGA, CTSL, GOT1
hsa04142: lysosome	<0.001		
GO:0005764~lysosome	0.011		
Cluster 2			
GO:0006957~complement activation, alternative pathway	<0.001	2.38	LAMC1, C7, C8A, IGKV4-1, PNP, FCN2, C9, HLA-B, ITIH2, PRG4, HBD, NCAM1, CFD
hsa05020: Prion diseases	<0.001		
GO:0005579~membrane attack complex	0.001		
GO:0006956~complement activation	0.002		
GO:0072562~blood microparticle	0.002		
GO:0030449~regulation of complement activation	0.014		
hsa04610: complement and coagulation cascades	0.018		
GO:0006958~complement activation, classical pathway	0.022		
GO:0006955~immune response	0.042		
hsa05322: systemic lupus erythematosus	0.296		
Cluster 3			
GO:0006094~gluconeogenesis	0.002	1.7	PGAM1, ALDOC, SHMT1, GPI, GOT1
GO:0061621~canonical glycolysis	0.011		
hsa01200: carbon metabolism	0.013		
GO:0006096~glycolytic process	0.018		
hsa01230: biosynthesis of amino acids	0.022		
hsa01130: biosynthesis of antibiotics	0.092		
hsa00010: glycolysis/gluconeogenesis	0.102		

proteins were shared between the 2 groups (Fig. 1b). To estimate protein abundance, SI_N, a label-free quantification method, was used. Proteins that were not identified in certain samples were assumed to be at levels under the detection limit and thus they were assigned a value that was half of the minimum SI_N value (i.e., 0.0001).

Welch's *t* test identified 73 proteins that were significantly increased by more than 2-fold and 36 proteins that were significantly decreased by less than 0.5-fold in the AD group compared to the control group (Fig. 1c). The accession numbers, names, logarithm of the fold change ratio (AD/control) of the average SI_N values, and molecular weights of proteins that were significantly increased or decreased are listed in Table 2. Twenty-four proteins remained significant after false discovery rate correction (bold in Table 2).

The expression-level correlations of proteins listed in Table 2 were analyzed in the AD group and the control group (online suppl. Tables 2A and B). Correlations were compared between AD and control groups (online suppl. Table 2C). Eleven correlations involving proteins (PSAP, RDX, C7, CPVL, LAIR1, GAA, GALNS, BGN, CNTN1, GPRC5C, and MAN1A1) were significant in both the AD group and the control group (online suppl. Table 2D), and 4 proteins (PSAP, GAA, GALNS, and BGN) were annotated to the lysosome.

Bioinformatics Analysis

To provide a broad overview of the identified proteins in each group, gene ontology (GO) analysis was performed using DAVID. The profiles of both groups had very similar distributions of GO annotations (online suppl. Fig. 1). To assess the functional significance of signifi-

Table 4. List of the top 5 pathways contributing to the interrelation network and the AD-related network calculated by KeyMolnet

Rank	Pathway	Score
Top 5 pathways from the interrelation network		
1	HSP90 signaling pathway	103.42
2	Lipoprotein metabolism	86.74
3	Redox regulation by thioredoxin	53.19
4	MMP signaling pathway	45.63
5	Tetraspanin signaling pathway	45.32
Top 5 pathways from the AD-related network		
1	Lipoprotein metabolism	90.35
2	Transcriptional regulation by CREB	88.81
3	HSP90 signaling pathway	57.46
4	MMP signaling pathway	47.19
5	Redox regulation by thioredoxin	44.02

cantly increased or decreased proteins in the AD group, functional annotation clustering analysis with DAVID was performed using the GO database and KEGG pathway. Three annotation clusters related to lysosomes, complement activation, and gluconeogenesis were significantly enriched (Table 3; online suppl. Fig. 2).

To identify relationships between the molecular network of the urinary proteome and canonical pathway, an "interrelation" network search was performed using KeyMolnet. In the extracted molecular network, 18 pathways scored >20 and significantly contributed to the extracted network (online suppl. Table 3). The 5 pathways with the highest scores were the heat shock protein (HSP) 90 signaling pathway (score 104.424), lipoprotein metabolism (score 86.737), redox regulation by thioredoxin (score 53.188), the matrix metalloproteinase (MMP) signaling pathway (score 45.325), and the tetraspanin signaling pathway (score 45.319) (Table 4, upper panel). To extract molecular relations between AD-related molecules and the urinary proteome, a "start points and end points" network search was performed using KeyMolnet. We found that 71 proteins which were significantly increased or decreased in the urine proteome were associated with AD-related molecules directly or via an intermediate molecule (online suppl. Fig. 4), and 12 pathways significantly contributed to the extracted AD-related network (online suppl. Table 4). Of the 12 pathways, 4 of the top 5 were also listed among the top 5 pathways determined in the "interrelation" network search, i.e., lipoprotein metabolism (score 90.350), the HSP90 signaling pathway (score 57.456), the MMP signaling pathway (score, 47.188), and redox regulation by thioredoxin (score 44.021) (Table 4, lower panel).

Discussion

With recent advances in MS-based proteomics, urine has been used in biomarker studies of various diseases, not limited to renal and urogenital diseases, but also for nonurogenital diseases such as diabetes, osteoarthritis, cardiovascular disease, lung cancer, and other types of cancer [11, 20–30]. Many AD biomarker studies have used a proteomics approach, with most using CSF and blood but only a few using urine [31, 32]. A comprehensive profile of the urinary proteome is important for urine biomarker discovery [11, 13]. Therefore, in this study, we performed MS-based urine proteomics with label-free quantification, which offers a greater dynamic range and a wider proteome coverage compared to label-based methods [11], in order to gain a comprehensive view of the urinary proteome of AD patients.

In this study, we compared the urinary proteome of 18 AD patients and 18 age- and sex-matched cognitively normal elderly individuals. The average number of identified proteins in individual urine samples was 613 and 589 in the AD and control groups, respectively. This is comparable to previous reports [13, 33, 34]. Recent studies have found that systemic changes, such as insulin resistance, atherosclerosis, and increased inflammation underlie the development of AD [7–9]. Among the proteins significantly increased or decreased in the AD group compared to the control group, proteins related to lysosomes, the complement pathway, and gluconeogenesis were enriched. In the molecular network analysis, canonical pathways of lipoprotein metabolism, HSP90 signaling, MMP signaling, and redox regulation by thioredoxin significantly contributed to the molecular network of the urinary proteome and AD-related molecules.

Lysosomes are major cellular organelles that digest and recycle all types of intracellular macromolecules and thus play a major role in protein homeostasis [35]. Previous studies have suggested the involvement of impaired lysosomal activity, including lysosomal enzyme malfunction, in the AD brain starting from an early stage of the disease [36–39]. In the present study, 7 of 11 lysosome-related proteins corresponded to lysosomal hydrolase. Previous studies have also reported increased activity of lysosomal glycohydrolases at the peripheral level in AD patients [40, 41].

Although most lysosome-related proteins were increased in AD urine, cathepsin L1 (CTSL) was not. Cathepsins are the most abundant lysosomal proteases and they have been implicated in neuronal death in AD patients [42]. Interestingly, CTSL activity is inhibited in the brains of aged animals [43]. Moreover, recent studies have found that CTSL functions as a key protease for the proteolytic processing of proneuropeptides into active neurotransmitters, and thus it is required for normal neurotransmission [44].

The complement system represents a key inflammatory pathway for the activation and execution of immune responses. Inflammatory responses in the brain are characteristic of AD pathology [45, 46]. Recent studies have also revealed the occurrence of peripheral or systemic inflammation early in the development of AD [47, 48].

Insulin is a key hormone that inhibits gluconeogenesis, and insulin resistance is a hallmark of type 2 diabetes [49]. Type 2 diabetes can cause mitochondrial dysfunction and promote an inflammatory response similar to that which triggers AD [50]. Epidemiological studies have found that the risk of AD is about 1.5-fold higher among people with diabetes than in the general population [51, 52]. In the current study, we did not have sufficient information regarding the diabetic status of our participants, particularly the control group. However, the enrichment of proteins related to gluconeogenesis in the urine of AD patients is consistent with the known relationship between AD and diabetes.

Another protein listed in Table 2 that might participate in glucose metabolism is insulin-like growth factor-binding protein-3 (IGFBP3). IGFBP3 is a major binding protein of IGF-1 and several studies have reported its association with incident diabetes [53, 54]. Although results are inconsistent, alterations of circulating IGFBP3 levels in AD patients have been reported [55, 56].

Several recent studies have concluded that intrabrain vascular dysregulation is the earliest and strongest pathologic factor associated with late-onset AD [8, 57]. Atherosclerosis is a leading cause of vascular dysfunction, and it is the result of hyperlipidemia and lipid oxidation [8, 58]. Thioredoxin is a major regulator of the cellular redox system that protects various cells from oxidative stress and is involved in atherogenesis [59]. According to one study, patients with atherosclerosis had an increased level of plasma thioredoxin-1 [60]. MMP are a large family of proteolytic enzymes and they have been implicated in the development and progression of atherosclerosis [61]. HSP90 is a molecular chaperone that prevents protein misfolding and aggregation [62]. HSP90 and HSP70 have been shown to

exert their effects on atherosclerosis by influencing LDL metabolism, and the expression of HSP90 in atherosclerotic plaques has been associated with plaque instability [63].

Another vascular related protein that was significantly increased in AD urine is oxidized low-density lipoprotein receptor 1 (OLR1), which is also known as lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1). OLR1/LOX-1 is a marker for atherosclerosis and it is induced by oxidative stress, inflammatory cytokines, and oxidized low-density lipoprotein [64]. Several studies have suggested an association of several SNP within OLR1 with AD [65].

Expression levels of several proteins in AD urine observed in the present study were inconsistent with previous reports. For example, S100A6 was significantly decreased in AD urine. In the brain, however, S100A6 has been reported to be upregulated in astrocytes of amyotrophic lateral sclerosis patients and in AD patients [66].

Although some studies have reported that hyperlipidemia is associated with AD pathogenesis, APOC3 was significantly decreased in AD urine. APOC3 is a major component of triglyceride-rich lipoproteins (chylomicrons and very low-density lipoprotein) and a minor component of high-density lipoprotein. Lin et al. [67] reported decreased levels of serum APOC3 with the progression of AD. Recent studies have reported that weight loss is a predictor of AD and may be related to the hypothalamic defects observed in AD patients [68, 69].

The present study has some limitations worth noting. First, the abundance of urinary proteins was estimated via a semiquantitative method and requires further validation by a quantitative method. Second, information regarding the comorbidities and renal function of participants was limited. Third, this study employed a cross-sectional case-control design. Further validation of our findings with a larger sample size and different populations is warranted. The above information notwithstanding, we were able to demonstrate differences in the urinary proteome of AD patients compared to cognitively normal controls and that the urinary proteome of AD patients reflects systemic changes that underlie AD pathophysiology. Further studies targeting earlier-stage AD patients or population-based prospective studies will help to clarify the potential of urine as a source of biomarkers for early screening of AD.

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Statement of Ethics

This study was approved by the human research ethics committee of Niigata University (approval No. 1836, 2015-2081). All of the patients or their guardians signed informed consent forms and all of the participants of the Murakami cohort were informed through an oral consent process.

Disclosure Statement

The authors declare that they have no conflicts of interests.

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Author Contributions

Y. Watanabe, Y. Hirao, K. Kitamura., T. Yamamoto, and K. Nakamura contributed to the study concept and design. Y. Watanabe, K. Kitamura, Y. Semizu, T. Ikeuchi, K. Kasuga, T. Tokutake, and K. Nakamura contributed to acquisition of the data. Y. Watanabe, Y. Semizu, Y. Hirao, and T. Yamamoto contributed to analysis and interpretation of the data. Y. Watanabe and Y. Hirao drafted this paper. Y. Watanabe, Y. Hirao, K. Kitamura, T. Yamamoto, Y. Semizu, T. Ikeuchi, K. Kasuga, T. Tokutake, and K. Nakamura critically edited this paper for important intellectual content. Y. Watanabe and Y. Hirao contributed equally to this work. All of the authors approved the final version of this paper.

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