Phosphoproteins with Stability against All Urinary Phosphatases as Potential Biomarkers

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Urine, by accumulating all kinds of changes, was proposed to be a better source for biomarker discovery. As one of the most common post-translational modifications, phosphorylation plays a vital role in many biological activities. However, the urine phosphoproteome has been largely neglected due to the low abundance of phosphoproteins and the presence of various phosphatases in urine. The low level of background phosphorylation in urine is actually advantageous, as urinary phosphopeptides/proteins that are stable to the phosphatases present in urine have the potential to serve as valuable disease biomarkers. Using a TiO2 enrichment strategy, this study aimed to create a comprehensive proteomic profile of human urinary phosphoproteins and to characterize the changes in the urine phosphoproteome after incubation of urine with cell lysates. In total, 106 urine phosphorylation sites corresponding to 64 proteins, including 80 previously unidentified human urine protein phosphorylation sites, were identified by mass spectrometry. Fifteen phosphopeptides, together averaging 47% of the total phosphopeptides, were found in samples from three individuals. Addition of cellular proteins to urine did not significantly change the phosphorylation level of urine proteins. But there were still a few phosphopeptides from cell lysates survived urinary phosphatases; such phosphopeptides represent potential biomarkers in urine.

Phosphoproteins with Stability against All Urinary Phosphatases as Potential Biomarkers in Urine

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Introduction

15 Urine, by accumulating all kinds of changes, was proposed to be a better source for biomarker

16 discovery(Gao 2013; Gao 2014a; Gao 2014b; Li et al. 2014). However, due to the masking

17 effects of highly abundant proteins in urine and to ion suppression, mass spectrometry (MS) is

18 substantially limited in its ability to detect low-abundance urine proteins and post-translational

19 modifications (PTMs) without enrichment. Thus, many urine proteomic studies have focused on

20 comparing protein abundance between healthy subjects and patients with specific medical

21 conditions but have not considered PTMs. Only urinary glycoproteins were used in some

22 biomarker studies, as high-abundance albumin is not N-glycosylated and can be subsequently

23 removed(Wang et al. 2006; Wang et al. 2008).

24   Phosphorylation, one of the most common PTMs, is a major determinant of protein-protein

25 interactions, signaling pathways, and physiological and cellular functions(Cohen 2002).

26 However, the urine phosphoproteome has largely been neglected due to the low abundance of

27 urine phosphoproteins probably due to the presence of various phosphatases in urine and the poor

28 stability of post-translational modifications(Lopez et al. 2012). However, the low level of

29 background phosphorylation in urine is actually advantageous, as urinary phosphopeptides and

30 proteins that are able to survive the phosphatases present in urine can potentially serve as

31 valuable disease biomarkers.

32   The phosphorylation of proteins present in many body fluids, including CSF, serum and

33 saliva, has been studied. Although more than 2,000 proteins have been identified in urine, only 59

34 phosphopeptides were identified in urine from healthy human subjects in a proteome study by

35 using phosphorylation as a criterion in database searching(Li et al. 2010). Urine contains more

36 than 20 different phosphatases, and previous studies have shown that the levels of several urinary

phosphoproteins increase in certain diseases and conditions. As one example, 23 unique phosphopeptides were found to be differentially expressed in pregnant and non-pregnant women(Zheng et al. 2013). The phosphorylated sodium chloride co-transporter in urinary exosomes was considered as a potential marker for aldosteronism(van der Lubbe et al. 2012), whereas profilin 1, a phosphorylated urinary protein, is a candidate biomarker for aggressive bladder cancer(Zoidakis et al. 2012).

In this study, we aimed to profile human urinary phosphoproteins. To determine which phosphoprotein species potentially present in urine can survive urinary phosphatases, we also set to illustrate changes in the urine phosphoproteome after incubation of urine with cell lysate (Figure 1).

Materials & Methods

Ethics statement

Prior to enrollment in the study, all volunteers were given a verbal explanation of the study and signed an informed consent document. The consent procedure and the research protocol in this study were approved by the Medical Ethics Committee of Peking Union Medical College (#018-2013).

Urine sample preparation

Three healthy volunteers were enrolled in the study. All subjects were asked to maintain a comfortable supine position at a room temperature between 20°C and 24°C, and each subject was instructed to collect a 24-h urine sample. Seven hundred milliliters of urine was collected from each study volunteer. Four hundred milliliters of urine from each volunteer was pooled, and the pooled sample was divided into three equal parts. To two of these portions, 1 mg or 100 μg of protein from cell lysate was added, and each sample was then incubated at 37°C for 4 hours. The

60   remaining 300 mL urine from each volunteer was centrifuged at 3,500 g for 30 min. After

61   discarding the debris, the supernatant was centrifuged at 12,000 g for 30 min. After the removal

62   of precipitates, urinary proteins were extracted by acetone precipitation and re-dissolved in 25

63   mM ammonium bicarbonate.

Cell lysates

65   Human A498 cells (Cell Resource Center, Chinese Academy of Medical Sciences) were cultured

66   in DMEM media with 10% fetal bovine serum and 1% penicillin and streptomycin. The cell

67   pellets (1×10^8 cells) were lysed in 3 mL of lysis buffer containing 25 mM Tris (pH 8) and 8 M

68   urea. The mixture was subjected to ultrasonication at 60 W using 30 cycles at 50% duty. Cell

69   debris was removed by centrifugation at 12,000 g for 15 min at 4°C. Protein concentration was

70   measured with a Bradford assay. Four milligrams of total protein was resuspended in 25 mM

71   ammonium bicarbonate.

In-solution digestion and phosphopeptide enrichment

73   The proteins in the sample were reduced in 20 mM DTT at 56°C for 1 hour and were

74   subsequently alkylated in the dark with 50 mM iodoacetamide at room temperature for 45 min.

75   Trypsin was added (1:50, w/w), and digestion was conducted overnight at 37°C before quenching

76   by the addition of formic acid to a final concentration of 0.1%. The digestions were centrifuged

77   to remove insoluble materials, and the supernatants were desalted by passage over an Oasis HLB

78   cartridge (Waters, Milford, MA) and then dried by vacuum evaporation.

79   TiO$_2$ enrichment of the peptide mixtures was performed using the TiO$_2$ Phosphopeptide

80   Enrichment Kit (Thermo Fisher Scientific, Bremen, Germany). The peptide samples were re-

81   dissolved in 150 μL of 28% lactic acid and applied to the TiO$_2$ spin column, and phosphopeptide

82   isolation was performed according to the manufacturer's instructions. The phosphopeptides were

83   eluted with 50 μL of 5% pyrrolidine.

LC-MS/MS analysis

Samples were analyzed on an LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The peptide mixture was loaded onto a C18 trap column (5 cm, 5 µm, 200 A, Proxeon, Co.). Captured peptides were directed to a reversed-phase analytical column (25cm, 5 µm, 120 A, homemade) with an integral fritted nanospray emitter and eluted using a linear gradient of acetonitrile (3-30% in 80 min) in 0.1% formic acid at a flow rate of 300 nL/min. Spectra were recorded in data-dependent acquisition mode and the dynamic exclusion option of 30 seconds was enabled. MS scan in the Orbitrap system with high resolution was set to r=120,000 (all Orbitrap system resolution values are given at m/z 400), followed by Collision Induced Dissociation fragmentation of the top 15 most abundant precursor ions or MSA (neutral losses of m/z 98, 49, and 32.6) both with target values of 1,000 and 35% normalized collision energy Each sample was analyzed three times.

Data analysis

The data was analyzed using a pre-release version of Thermo Scientific Proteome Discoverer™ software version 1.3. SEQUEST and Mascot search engines were used for identification against a Uniprot_2013_06 database. Validation based on separate target and decoy searches and subsequent calculation of classical score-based false discovery rates or Percolator(Kall et al. 2007) determined q-values were used for assessing the statistical significance of the identifications. The Percolator algorithm uses a set of features related to the quality of the peptide-spectrum matches (PSMs) to classify between correct and incorrect matches. The mass tolerance was set at 10 ppm for precursors and 1 Da for product ions. Modifications selected were set as fixed carbamidomethylation of cysteines, variable oxidation of methionine, phosphorylation of serine, threonine, and tyrosine residues(S, T and Y) were chosen. Maximum missed cleavage sites were set to be 2. False-positive assignments were removed by the

108 thresholds of two parameters, Xcorr and ΔCn (obtained using the target-decoy database search

109 method) with the false-discovery rate (FDR) of 1%. Peptides were assigned again using the

110 PhosphoRS algorithm(Shiromizu et al. 2013), which calculated the possibility of the

111 phosphorylation site from the spectra matched to the identified peptides. The score threshold for

112 peptide identification was set at a 1% false discovery rate (FDR) and a 70% PhosphoRS site

113 probability. Peptides <7 amino acids in length were discarded due to their reduced specificity for

114 a single protein. Further filtering criteria for peptides included detection in at least 2 of 3

115 technical replicates per biological sample.

Results and Discussion

Urine phosphorylation sites identified in this study

118 The three individual urine samples were analyzed by LC-MS/MS in triplicate; the average

119 technical variation of the analysis was 64%. In total, 106 phosphorylation sites (97 pSer, 6 pThr

120 and 3 pTyr) corresponding to 64 phosphoproteins were identified in the urine proteome. The ratio

121 of pSer:pThr:pTyr was close to the reported ratio in cells(Olsen et al. 2006). Approximately 50% of the

122 high-confidence phosphorylation sites showed an acidophilic SXE motif (Motif-x

123 algorithm(Amanchy et al. 2007)). Of the 64 phosphorylated proteins, 54 (84%) contained one or two

124 unique phosphorylated peptides. Ten proteins yielding more than 3 different phosphopeptides

125 were detected, and one protein (osteopontin) was detected that yielded more than 40

126 phosphorylated peptides. Some of the high-confidence phosphoproteins (> 3 phosphopeptides)

127 are of great importance for biomarker discovery. For example, expression of human fibulin-2 was

128 reported to be associated with acute kidney injury(Jin et al. 2012), whereas upregulation of

129 human aquaporin in urine has been associated with nephrogenic diabetes insipidus(Kanno et al.

130 1995).

131   A total of 15 phosphopeptides derived from 8 phosphoproteins were found to be common to

132   the three individual urine samples; these peptides contributed, on average, 47% of the

133   phosphopeptide abundance in each sample. The spectral counts of each peptide from each

134   individual are listed in Table 1. Among the 8 phosphoproteins identified as common to the three

135   individuals in this study, alpha-2-HS-glycoprotein has been considered a reliable indicator of

136   many glomerular diseases, such as diabetic nephropathy, in previous studies not considering

137   PTMs(Rao et al. 2007). A relationship between osteopontin and renal calculi has also been

138   confirmed(Nene et al. 2013).

139   Of the 64 phosphoproteins identified in urine, 19 are present in the Urinary Protein

140   Biomarker Database(Shao et al. 2011). Twelve of these are associated with renal diseases such as

141   obstructive nephropathy, IgA nephropathy and renal calculi (Table S1).

142   The data obtained in this study were compared with two previous phosphoproteome data

143   sets. In one previous urine proteome study, 59 phosphorylation sites were identified without

144   enrichment(Li et al. 2010). Although different enrichment strategies and identification methods

145   were adopted and different samples were included in our study, 40.7% of the sites reported in

146   previous studies (24 phosphorylation sites) were also found in our study. In another urinary

147   exosome study, 19 phosphorylation sites were identified after IMAC enrichment(Gonzales et al.

148   2009). Although our study analyzed urine rather than exosome phosphopeptides and used a TiO_2

149   enrichment method, 4 phosphorylation sites common to previous exosome studies were

150   identified.

151   Because urine is considered a filtrate of blood, we compared the phosphorylation sites we

152   identified in this study with phosphorylation sites reported in previous studies of serum

153   phosphoproteins(Carrascal et al. 2010; Jaros et al. 2012; Zhou et al. 2009). Some phosphorylation

154   sites, including those in kininogen-1 (ETTCSKEsNEELTESCETK), AMBP protein

155   (FSVVYAKCDSSPDsAEDVR), SPARC-like protein 1 (ISHELDSAsSEVN), protein Z-

156 dependent protease inhibitor (VVQAPKEEEEDEQE-AsEEKASEEEK) and albumin

157 (TCVADEsAENCDK), were identical to sites identified in those studies. However, the overlap

158 between serum and urine phosphorylation sites is low; this may be due to the low abundance of

159 many phosphoproteins in serum and urine, a condition that makes phosphoproteins in blood and

160 urine difficult to identify. The low overlap between the sites found in urine in this study and

161 previously reported serum phosphoproteins may also be because not all phosphoproteins in the

162 blood are passed into the urine. The phosphorylation levels of urinary proteins may reflect

163 pathological renal states and/or abnormal physiological states. Because urine reflects many of the

164 changes that occur in the body, the phosphoproteins identified in this study may have been

165 phosphorylated in their original tissues prior to release into blood and urine and thus may be

166 related to both blood condition and renal function.

167 Changes in urine phosphopeptides after the addition of cell lysates

168 Phosphatase inhibitors were not used in the urine or in cell lysates. A total of 792

169 phosphopeptides were identified in crude cell samples, whereas 74 phosphopeptides were

170 identified in the pooled urine. After incubating urine with 100 µg or 1 mg of cell lysate at 37°C

171 for 4 h, 74 or 76 phosphopeptides were identified, respectively. In addition, one peptide

172 (KGAGDGsDEEVDGKADGAEAKPAE) corresponding to myosin-9 was present in the cell

173 lysate and in the cell lysate-supplemented urine samples but not in the phosphorylation-enriched

174 urine samples (Figure 2). This finding indicates that some phosphopeptides from the cell lysate

175 are likely stable upon exposure to phosphatases in urine and that they can be identified when

176 added to urine. When 1 mg of cell lysate was added to the urine samples, 10 phosphopeptides

177 corresponding to 8 proteins could be identified both in the cell lysate and in the urine-cell lysate

178 mixture but not in the untreated urine sample. The eight proteins (AHNAK, CTTN, FLNC,

179 G3BP1, MAP1B, NDRG1, PEA15, and PGRMC1) identified after adding the larger amount (1

180 mg) of cell lysate to the urine samples are closely related to cell growth, cellular movement and

181 cancer. Figure 3 shows the peptides and proteins identified in urine alone, urine with added cell

182 lysate and cell lysate alone.

183   Although many phosphoproteins were present in the cell lysate, the addition of cellular

184 proteins did not significantly change the level of protein phosphorylation in urine, most likely

185 because urine contains more than 20 different phosphatases(Marimuthu et al. 2011). It is worth

186 noting that several phosphorylated peptides were stable to incubation with urine phosphatases.

187 Phosphorylation varies among different cells. In different cells from which proteins are released

188 into urine, some phosphorylation sites will survive the urine phosphatases; these may represent

189 potential biomarkers. The overall phosphatase activity of urine changes due to alterations in the

190 combination of phosphatases present or to the activity of individual phosphatases. Such changes

191 are also reflected in the phosphorylation status of proteins in urine. Increasing the detection

192 sensitivity of phosphorylation may aid in the discovery of additional urine phospho-biomarkers.

193 Phosphoproteins that can survive all urine phosphatases, some of which are identified here,

194 potentially represent valuable urinary biomarkers.

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Figure Legends

Figure 1. Overview of the workflow. Urine samples from three individuals, urine samples incubated with cell lysates, and cell lysates were separately digested, enriched and analyzed by LC-MS/MS.

Figure 2. Phosphopeptides identified in urine, urine with added cell lysate and cell lysates. A. Urine. B. Urine incubated with 100 µg of cell lysate. C. Urine incubated with 1 mg of cell lysate. D. Cell lysates.
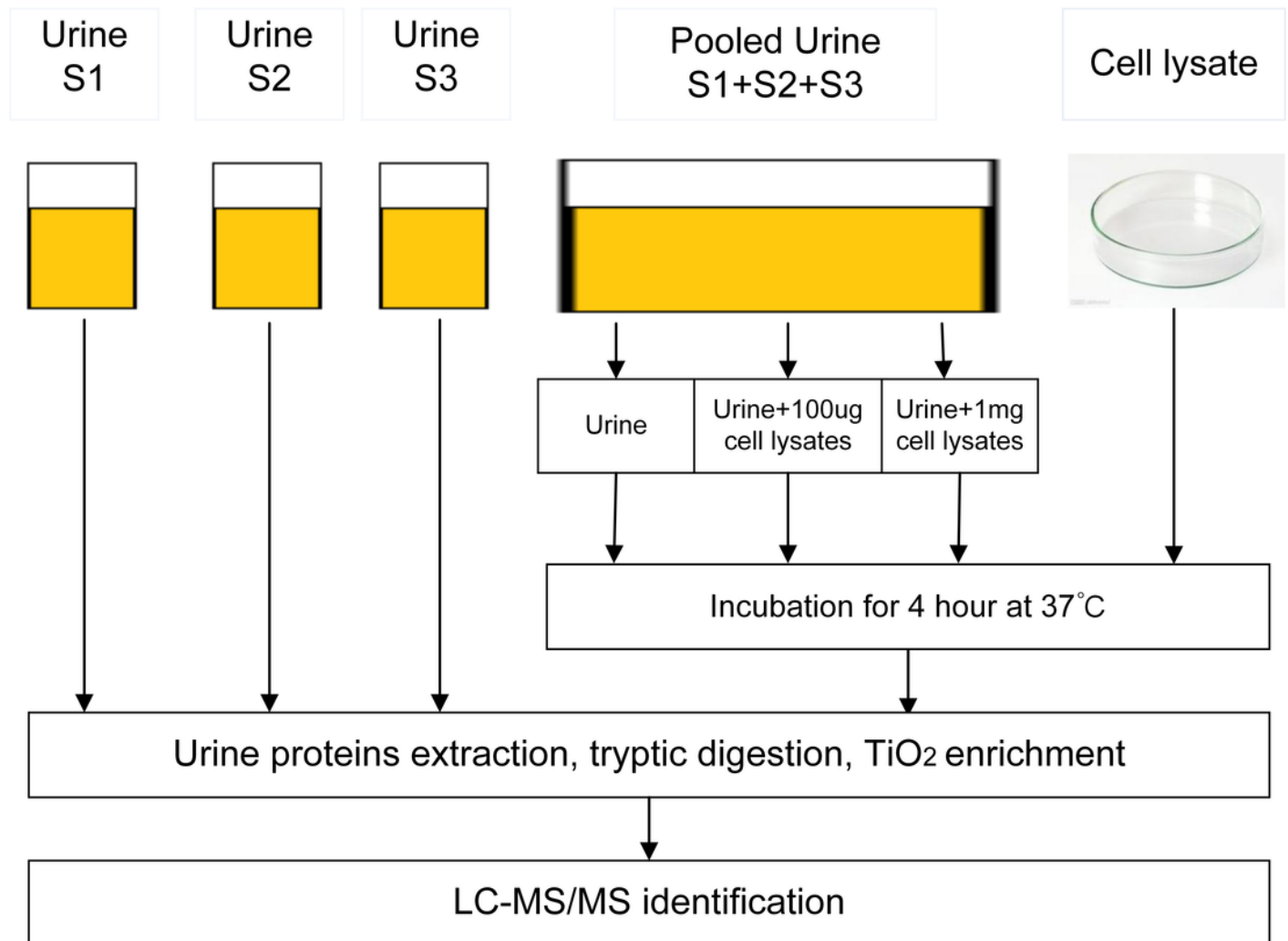
Figure 3. Representative MS/MS spectra of phosphopeptides from selected proteins that were identified only in cell lysate and in urine with added cell lysate. A. Myosin-9. B. Ras GTPase-activating protein-binding protein 1. C. Membrane-associated progesterone receptor component 1. D. Neuroblast differentiation-associated protein AHNAK.

Supporting Information Legends

**Table S1. Phosphorylation sites identified in this study and their clinical uses.**

Figure S1. Representative MS/MS spectra of peptides from selected proteins that were identified based on single-peptide evidence.

1

Overview of the workflow.

2

Phosphopeptides identified in urine, urine with added cell lysate and cell lysates.

A) Urine. B) Urine incubated with 100 µg of cell lysate. C) Urine incubated with 1 mg of cell lysate. D) Cell lysates.

3
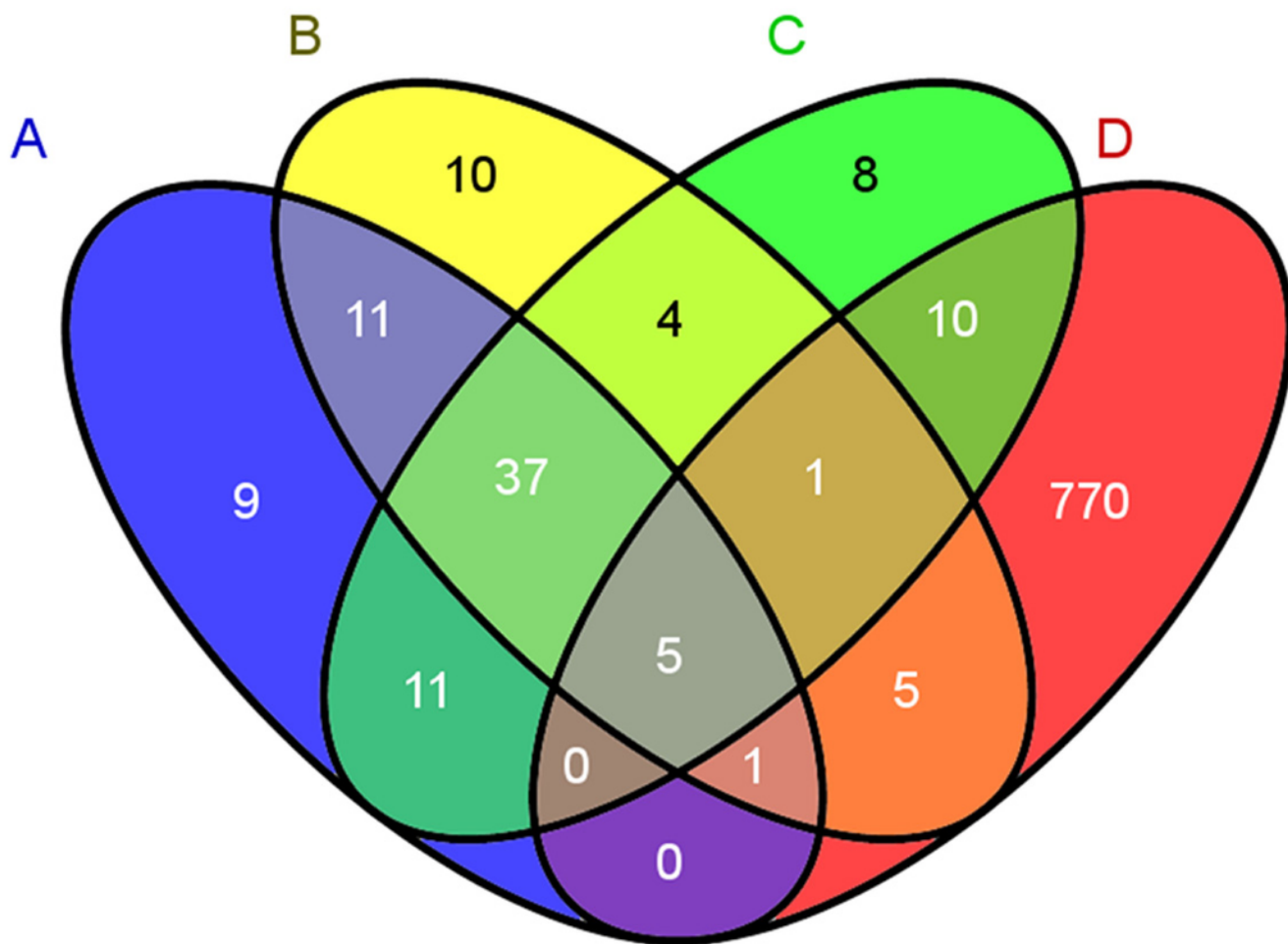
Representative MS/MS spectra of phosphopeptides from selected proteins that were identified only in cell lysate and in urine with added cell lysate.

A) Myosin-9. B) Ras GTPase-activating protein-binding protein 1. C) Membrane-associated progesterone receptor component 1. D) Neuroblast differentiation-associated protein AHNAK.
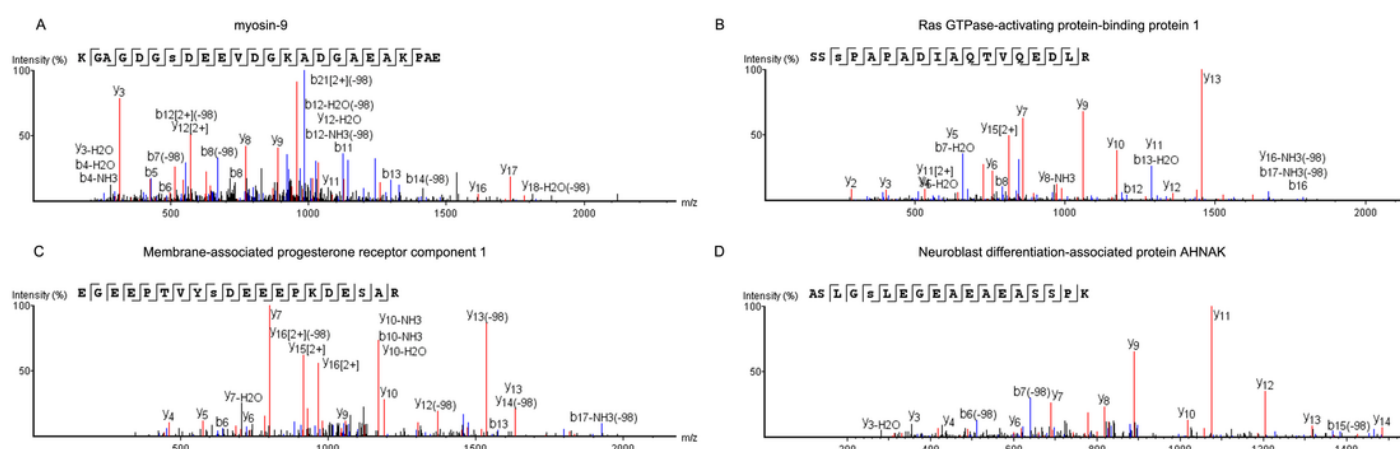
Table 1 (on next page)

Fifteen phosphopeptides corresponding to 8 phosphoproteins were common to urine samples from three individuals.

Accession	Protein name	Peptides	Modifications	S1 spectra	S2 spectra	S3 spectra
P04792	Heat shock protein beta-1	QLsSGVSEIR	S3(Phospho)	2	2	2
P01042	Kininogen-1	ETTCSKEsNEELTESCETK	S8(Phospho)	12	22	17
P02765	Alpha-2-HS-glycoprotein	CDSSPDsAEDVR	S7(Phospho)	31	4	7
P10451	Osteopontin	ANDEsNEHSDVIDsQELSK	S5(Phospho); S14(Phospho)	6	6	8
		ISHELDsAsSEVN	S7(Phospho); S9(Phospho)	3	29	20
		KANDESNEHSDVIDsQELSKVSR	S15(Phospho)	37	15	13
		AIPVAQDLNAPSDWDsR	S16(Phospho)	23	24	28
		QNLLAPQNAVsSEETNDFKQETLPSK	S11(Phospho)	18	5	31
		QNLLAPQNAVSsEETNDFK	S12(Phospho)	48	17	38
		GKDSYETSQLDDQsAETHSHK	S14(Phospho)	10	18	13
		KANDEsNEHSDVIDsQELSK	S6(Phospho); S15(Phospho)	15	30	13
Q8NBJ4	Golgi membrane protein 1	GFGGAGELGQTPQVQAALSVsQENPEMEGPER	S21(Phospho)	2	1	1
Q969E1	Liver-expressed antimicrobial peptide 2	RCsLSVAQE	S3(Phospho)	5	14	1
Q9NZH0	G-protein coupled receptor family C group 5	TAGFPNGsLGK	S8(Phospho)	4	2	3
Q9ULI3	Protein HEG homolog 1	SYSESSSTSsSESLNSSAPR	S10(Phospho)	5	5	5