Online Appendix

Discovery and validation of urine markers of acute pediatric appendicitis using high accuracy mass spectrometry

Discovery of diagnostic markers by using urine proteomic profiling

In order to identify candidate urinary markers of acute appendicitis, we assembled a discovery urine proteome dataset, derived from the analysis of 12 specimens, without any clinical urinalysis abnormalities, collected at the onset of the study, and distributed equally between patients with and without appendicitis. Six of these specimens were collected from patients who were found to have histologic evidence of appendicitis (2 mild, 3 moderate, 1 severe). Three specimens were collected from patients without appendicitis (1 with non-specific abdominal pain, 1 with constipation, 1 with mesenteric adenitis). From 3 patients with appendicitis, we collected additional control specimens at their routine post-surgical evaluation 6-8 weeks after undergoing appendectomies, at which time they were asymptomatic and in their usual state of health. These specimens were included in the analysis in order to minimize the potential effect of individual variability in urinary composition that may arise due to age, gender, physiologic state or possible genetic variation.

The urine proteome compositions of these 12 (9 original urines from index encounter and 3 from follow-up) were discovered by using protein capture and fractionation coupled with high accuracy mass spectrometry, as described in detail below, and schematized in Figure 1. As urine is a complex mixture with abundant proteins such as albumin obscuring the detection of less concentrated, potentially diagnostic proteins such as secreted cytokines and mediators of the inflammatory response, we devised a fractionation method that reduced mixture complexity while minimizing loss of material (Figure 1).

Aliquots were thawed and centrifuged at 17,000 *g* for 15 minutes at 10 °C to sediment cellular fragments. Absence of intact cells in the sediment was confirmed by light microscopy (data not shown). Subsequently, supernatant was centrifuged at 210,000 *g* for 60 minutes at 4 °C to sediment vesicles and high molecular weight complexes. Resultant pellets were resuspended in 0.5 ml of 0.1× Laemmli buffer, concentrated 10-fold to 0.05 ml by vacuum centrifugation and stored at -80 °C.

Supernatant remaining after ultracentrifugation was diluted 5-fold with 0.1 M acetic acid, 10 % (v/v) methanol, pH 2.7 (Buffer A) and incubated with 1 ml 50 % (v/v) slurry of SP Sephadex (40-120 μm beads, Amersham) for 30 minutes at 4 °C to adsorb peptides that are < 30 kDa molecular weight. Upon washing the beads twice with Buffer A, peptides were eluted by incubating the beads in 5 ml of 0.5 M ammonium acetate, 10% (v/v) methanol, pH 7 for 30 minutes at 4 °C. Eluted peptides were purified by reverse phase chromatography by using PepClean C-18 spin columns, according to manufacturer's instructions (Pierce). Residual purification solvents were removed by vacuum centrifugation and small proteins and peptides were resuspended in aqueous 50 mM ammonium bicarbonate buffer (pH 8.5).

Proteins remaining in solution after cation exchange were precipitated by adding trichloroacetic acid to 20 % (w/v), with deoxycholate to 0.02 % (w/v) and Triton X-100

to 2.5 % (v/v) as carriers, and incubating the samples for 16 hours at 4 °C. Precipitates were sedimented at 10,000 *g* for 15 minutes at 4 °C and pellets were washed twice with neat acetone at 4 °C with residual acetone removed by air drying. Dried pellets were resuspended in 0.1 ml of 1× Laemmli buffer.

Laemmli buffer suspended fractions (from 17,000 *g* and 210,000 *g* centrifugation, and from protein precipitation) were incubated at 70 °C for 15 min and separated by using NuPage 10% polyacrylamide Bis-Tris gels according to manufacturer's instructions (Invitrogen). Gels were washed three times with distilled water, fixed with 5% (v/v) acetic acid in 50% (v/v) aqueous methanol for 15 minutes at room temperature, and stained with Coomassie. Each gel lane was cut into 6 fragments and each fragment was cut into roughly 1 mm^3 particles, which were subsequently washed 3 times with water and once with acetonitrile.

Protein containing gel particles and cation exchange purified proteins were reduced with 10 mM dithiotreitol in 50 mM ammonium bicarbonate (pH 8.5) at 56 °C for 45 minutes. They were subsequently alkylated with 55 mM iodoacetamide in 50 mM ammonium bicarbonate (pH 8.5) at room temperature in darkness for 30 minutes. Gel particles were washed 3 times with 50 mM ammonium bicarbonate (pH 8.5) prior to digestion. Alkylated peptides were purified by using PepClean C-18 spin columns as described above to remove residual iodoacetamide from the cation exchange fraction. They were then digested with 12.5 ng/μl sequencing grade bovine trypsin in 50 mM ammonium bicarbonate (pH 8.5) at 37 °C for 16 hours. Tryptic products were purified by using PepClean C-18 spin columns as described above, vacuum centrifuged and stored at -80 °C.

Fractions containing tryptic peptides dissolved in aqueous 5% (v/v) acetonitrile and 0.1% (v/v) formic acid were resolved and ionized by using nanoflow high performance liquid chromatography (nanoLC, Eksigent) coupled to the LTQ-Orbitrap hybrid mass spectrometer (Thermo Scientific). Nanoflow chromatography and electrospray ionization were accomplished by using a 15 cm fused silica capillary with 100 μm inner diameter, in-house packed with Magic C18 resin (200 Å, 5 μm, Michrom Bioresources). Peptide mixtures were injected onto the column at a flow rate of 1000 nl/min and resolved at 400 nl/min using 45 min linear acetonitrile gradients from 5 to 40 % (v/v) aqueous acetonitrile in 0.1 % (v/v) formic acid. Mass spectrometer was operated in data dependent acquisition mode, recording high accuracy and high resolution survey Orbitrap spectra using the lock mass for internal mass calibration, with the resolution of 60,000 and *m*/*z* range of 350-2000. Six most intense multiply charged ions were sequentially fragmented by using collision induced dissociation, and spectra of their fragments were recorded in the linear ion trap, with the dynamic exclusion of precursor ions already selected for MS/MS of 60 sec.

Custom written software was used to extract the 200 most intense peaks from each MS/MS spectrum and to generate mascot generic format files. Peak lists were searched against the human International Protein Index database (version 3.36, http://www.ebi.ac.uk/IPI) by using Mascot (version 2.1.04; Matrix Science), allowing for variable formation of *N*-pyroglutamate, Asn and Gln deamidation, *N*-acetylation, and methionine oxidation, requiring full trypsin cleavage of identified peptides with 2 possible miscleavages, and mass tolerances of 5 ppm and 0.8 Da for the precursor and fragment ions, respectively. Searches allowing semi-tryptic peptides did not affect overall

search yields (data not shown). Spectral counts were calculated by summing the number of fragment ion spectra assigned to each unique precursor peptide.

Assessment of identification accuracy was carried out by searching a decoy database composed of reversed protein sequences of the target IPI database. Frequency of apparent false positive identifications was calculated by merging individual target and decoy searches for each sample. An initial estimate of the apparent false positive rate was obtained by dividing the number of peptide identifications with a Mascot score greater than the identity score obtained from the target search by the number of peptide identifications with a score higher than the identity score threshold extracted from the decoy search.[1] Only proteins identified on the basis of more than 2 peptides were included in the comparison.

As a result, we were able to identify 2,362 proteins in routinely collected urine specimens with the apparent rate of false identifications of less than 1 %, as ascertained from decoy database searching.[1] More than 1,200 identified proteins have not been detected in previous proteomic studies of urine, and more than 300 proteins appear to be filtered from serum and expressed in distal tissues, including the intestine. For the discovery of candidate appendicitis markers, we further increased the stringency of peptide identifications to less than 0.1 % false identifications, yielding essentially no false protein identifications for proteins identified on the basis of multiple peptides. For example, proteins identified on the basis of 10 unique peptides (median for the entire dataset), have an approximate identification error frequency of 10^{-19}.

In order to identify candidate markers of appendicitis, we took advantage of the quantitative information provided by tandem mass spectrometry by recording the number of fragment ion spectra assigned to each unique precursor peptide, which are proportional to peptide abundance,[2] and have been used for relative quantification of components of complex protein mixtures.[3] Though the composition and concentration of urine varies with physiologic state, there was less than 10 ± 10 % (mean $\pm$ standard deviation) difference in total protein abundance among individual specimens, similar to earlier studies of urine of children.[4-6] Individual protein spectral counts, calculated by summing spectral counts of unique peptides assigned to distinct proteins, were normalized relative to the spectral counts of albumin to account for these small differences in total protein abundance.[3]

In order to maximize the depth of candidate marker discovery, we subjected the discovery urine proteome to support vector machine (SVM) learning in order to identify candidate urine markers that may be enriched as a group but not necessarily individually, as required by the RER analysis above. This approach is implemented in a biomarker discovery program BDVAL that uses cross-validation to identify predictive biomarkers (Fabien Campagne, unpublished results, http://icb.med.cornell.edu/wiki/index.php/BDVAL), similar to established methods for microarray class discovery.[7] Because of the low number of samples, we performed cross-validation with four folds, repeated 5 times with random fold assignments (12 samples total, 6 cases, 6 controls). In this setting, 20 individual evaluation models (5 x 4) were trained. Each model was trained with a set of 50 features (normalized protein abundance levels). In each split, consisting of 9 training samples and 3 test samples, a Student *t*-test pre-filtering step prioritized up to 400 features whose average value differed the most between cases and controls in the training set. The 400 intermediate features were ranked

by decreasing support vector machine weights and the top 50 features were used to train the evaluation model (models were implemented as a support vector machine, implemented in libSVM with linear kernel, and margin parameter C=1). At the end of the evaluation, the lists of features were inspected to determine how many times a given feature has been used in any one of the 20 evaluation models. We considered features for validation only if they were found in at least 50 % of the evaluation models generated (10 models in this case).

Table S1 lists 17 proteins identified by SVM analysis, which include several proteins that were identified by RER analysis, as well as many that were not, including additional components of the acute phase response, such as serum amyloid A, α-1-antichymotrypsin, and bikunin (AMBP). Notably, exclusion of control specimens collected from asymptomatic patients after they underwent appendectomies increased the number of candidate markers to 273 by additionally including a variety of proteins unlikely to be related to the appendicitis response, such as the universal tyrosine kinase Src for example, suggesting that individually variant factors such as those that influence protein filtration and urine production may significantly affect biomarker discovery studies.

Candidate validation targeted mass spectrometry

Thawed 1 ml urine aliquots were precipitated by adding trichloroacetic acid to 20% (w/v), and incubating the samples for 1 hour at 4 °C. Precipitates were sedimented at 10,000 *g* for 15 minutes at 4 °C and pellets were washed twice with neat acetone at 4 °C, with residual acetone removed by air drying. Dried pellets were resuspended in Laemmli buffer, resolved by SDS-PAGE, alkylated and digested with trypsin as described above. To each sample, 0.4 μg of single stranded binding (SSB) protein purified from *Escherichia coli* (USB) was added to serve as a reference standard. Target nanoLC-MS/MS was accomplished by using the LTQ-Orbitrap mass spectrometer, using the parameters described above, but operated in an inclusion list dependent acquisition mode, searching detected precursor ions against *m*/*z* values of candidate marker peptides with a tolerance of 0.05 Da, using an inclusion list of masses and charges of candidate marker peptides, derived from the analysis of the discovery proteomes. Six most intense matched ions were sequentially fragmented by using collision induced dissociation, and spectra of their fragments were recorded in the linear ion trap, with the dynamic exclusion of precursor ions already selected for MS/MS of 60 sec. Such an approach is superior to conventional data dependent acquisition methods by minimizing the detection of non-target peptides.[8] Differences in apparent protein abundance were normalized relative to exogenously added SSB reference standard to account for instrumental variability. Absence of SSB from urine specimens without its addition was confirmed by searching the data against database of *Escherichia coli* proteins (data not shown).

Recorded mass spectra were processed and identified, as described.[9] The accuracy of peptide identification was assessed by decoy database searching, enforcing a false peptide discovery rate of less than 1 %, which corresponds to essentially zero false protein discovery rate, given that all of the candidate diagnostic marker proteins were identified on the basis of at least than 9 peptides, which corresponds to an apparent false

identification frequency of less than 10^{-18}. For example, leucine-rich α-2-glycoprotein (LRG) was identified on the basis of 55 unique peptides.

Urine markers of appendiceal inflammatory response

Because acute appendicitis is characterized by the increased expression of distinct chemoattractants in the gut mucosa,[10] and specific infiltration of neutrophils,[11] we wondered if markers of acute appendicitis identified from studies of appendiceal tissue may be detected in the urine of patients with appendicitis. To this end, we compared candidate urine protein markers as identified by using urine proteome profiling (Table 3) with tissue markers identified in a different study by using microarray gene expression of diseased appendices.[12] Supplementary Figure S1 plots *RER* values of the 40 most uniformly detected ($U > 0.7$) candidate urine markers as a function of the tissue overexpression of their respective microarray profiled genes. Of these, more than 50 % exhibit a positive correlation between tissue overexpression and urine enrichment (Figure S1), consistent with the notion that tissue gene expression profiles may be used to suggest candidate disease markers. However, only 3 of the genes that are overexpressed in diseased as opposed to normal appendices were also identified as candidate markers by urine proteome profiling: SPRX2, lymphatic vessel endothelial hyaluronan acid receptor 1 (LYVE1), and α-1-acid glycoprotein 1 (orosomucoid 1), suggesting that detection of markers of local disease in the urine is not solely dependent on tissue overexpression, but likely also requires other factors, such as shedding, circulation in blood, and accumulation in urine. Table 5 lists urine protein markers that were enriched in the urines of patients with appendicitis with corresponding genes that were overexpressed in diseased appendices.

In contrast to LRG which is expressed exclusively by the neutrophils, liver and the mesentery, S100-A8 is a cytokine expressed by diverse tissues, including a variety of endothelial and epithelial cells.[13,14] It is upregulated specifically in inflammatory states, including the processes of neutrophil activation and migration. Findings of its overexpression in appendiceal tissue during acute appendicitis,[12] and enrichment in the urine of appendicitis patients suggest that like LRG, it is also a marker of local inflammation, though its expression in a wide variety of tissues may affect its diagnostic specificity, consistent with its slightly reduced dynamic range and performance as compared to those of LRG (Table 4, Figure 3). Accordingly, it has been found to be upregulated in a wide variety of conditions, including inflammatory bowel disease,[15] arthritis,[16] Kawasaki vasculitis,[17] cancer,[18] and sepsis.[19]

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Supplementary Tables

Table S1: Candidate urine marker proteins identified using SVM analysis

Protein	Accession Number
Serum amyloid A protein	IPI00552578
α-1-antichymotrypsin	IPI00550991
Supervillin	IPI00412650
Mannan-binding lectin serine protease 2	IPI00306378
Inter-α-trypsin inhibitor	IPI00218192
VIP36	IPI00009950
Prostaglandin-H2 D-isomerase	IPI00013179
α-1-acid glycoprotein 2	IPI00020091
AMBP	IPI00022426
α-1-acid glycoprotein 1	IPI00022429
CD14	IPI00029260
Hemoglobin α	IPI00410714
Apolipoprotein D	IPI00006662
Hemoglobin β	IPI00654755
Leucine-rich α-2-glycoprotein	IPI00022417
Zinc-α-2-glycoprotein	IPI00166729

Table S2: Candidate urine marker proteins identified by comparisons with corresponding tissue gene overexpression

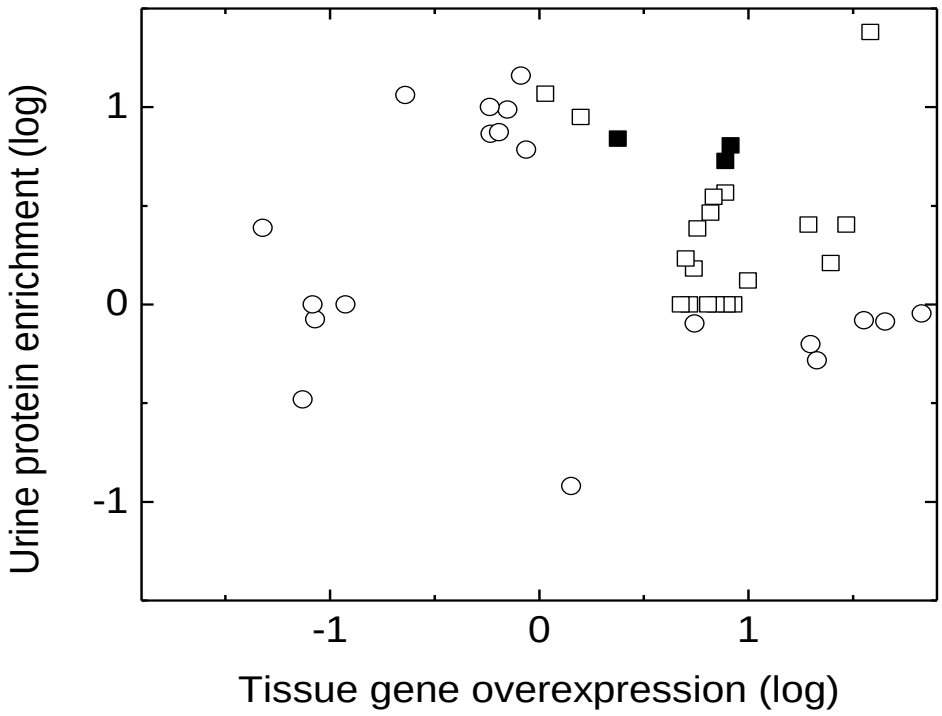
Protein	Accession Number	Affymetrix gene ID*	Fold gene overexpression*
S100-A8	IPI00007047	214370_at	67
S100-A9	IPI00027462	203535_at	45
Amyloid-like protein 2	IPI00031030	214456_x_at	38
Versican	IPI00009802	211571_s_at	11
SPRX2	IPI00004446	205499_at	8.1
α-1-acid glycoprotein 1	IPI00022429	205041_s_at	7.8
Interleukin-1 receptor antagonist protein	IPI00000045	212657_s_at	4.3
Lymphatic vessel endothelial hyaluronan acid receptor 1	IPI00290856	220037_s_at	2.0

* From [12].

Supplementary Figures

Figure S1. Relative enrichment of candidate urine protein markers as a function of appendicitis tissue overexpression of the corresponding genes, demonstrating that more than 50 % of candidate markers with tissue overexpression exhibit urine enrichment (□), but that only 3 of these (■) were identified as candidate markers by urine proteome profiling.

Fig. S1


Urine protein enrichment (log)
1
0
-1
-1
0
1
Tissue gene overexpression (log)