[Full Paper]

Establishment and application of a high-quality comparative analysis strategy for the discovery and small-scale validation of low-abundance biomarker peptides in serum based on an optimized novel peptide extraction method

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SUMMARY

Low-abundance native peptides are an attractive target for the discovery of disease biomarkers. However, validating candidate peptides is difficult due to challenges associated with precise peptide identification and development of high-throughput assays using specific antibodies. Therefore, a highly reproducible and sensitive strategy based on effective peptide enrichment methods is needed to identify clinically useful biomarkers. We optimized our novel differential solubilization (DS) peptide extraction method to selectively enrich peptides less than 6 kDa, using tricine-SDS-PAGE to evaluate the optimization. The modified DS method was combined with liquid chromatography-mass spectrometry (LC-MS) using conventional high-performance liquid chromatography (HPLC). The reproducibility and sensitivity of the proposed strategy were sufficient to enable discovery of low-abundance (ng/mL range) candidate biomarker peptides. A total of 40 serum samples collected pre- and post-surgery from renal cell carcinoma (RCC) patients were analyzed, resulting in discovery of 2 peptides that are upregulated and one peptide that is downregulated in pre-surgery RCC patients. These peptides were validated using 40 serum samples collected pre- and post-surgery from bladder tumor (BT) patients. Two candidate peptides that were upregulated in pre-surgery RCC patients were not upregulated in the sera of the pre-surgery BT patients. Finally, we propose 2 candidate marker peptides that could be used to detect RCC.

Key words: biomarker, peptide, serum

INTRODUCTION

Serum/plasma contains thousands of different types of proteins and peptides¹⁻⁵ and can provide valuable information about the numerous processes that take place within the body. Quantitative analysis of the proteins/peptides in serum/plasma samples is frequently employed in research aimed at discovering disease-specific biomarkers useful for the early detection of disease, for predicting drug susceptibility, and for evaluating prognosis⁶⁻⁹. The low-molecular-weight (LMW) protein/peptide component of the serum/plasma includes members of several physiologically important classes, such as cytokines, chemokines, and peptide hormones, along with proteolytic fragments of larger

proteins^{10, 11}, including those generated by cancer-specific exopeptidases¹². The LMW proteome is of great interest in proteomic studies that aim to identify disease-specific proteins.

The most widely used approaches for examining the low-molecular-weight serum/plasma proteome employ MALDI-TOF-MS following simple solid-phase extraction or functionalized bead pretreatment methods. Some of the LMW biomarkers discovered using these methods are indicative of disease onset¹⁻⁷, suggesting that native peptides may represent an unexplored archive of histological information. However, only a small proportion of peptides present in serum/plasma at concentrations above 100 ng/mL can be analyzed using conventional approaches. Obtaining a direct

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reflection of physiological or pathological states necessitates analysis of low-abundance peptides^{8,9}. Various strategies employing peptide enrichment methods followed by separation using methods such as reverse-phase high-performance liquid chromatography (RP-HPLC)^{10–13} or isoelectric focusing (IEF)¹⁴ have been applied to analyze low-abundance LMW proteins/peptides in serum/plasma in detail. However, typical peptide enrichment methods such as organic solvent precipitation or ultrafiltration do not work well due to low efficiency of peptide extraction and the difficulty of extracting peptides bound to carrier proteins such as albumin. In 2010, we developed a novel differential solubilization (DS) method for extracting LMW proteins/peptides from serum/plasma. The DS method consists of two steps. In the first step, the serum is mixed with a denaturing solution, and is then dropped into acetone, which causes all of the proteins and peptides to precipitate. In the second step, those LMW proteins/peptides that are easily dissolved in 70% ACN containing 12 mM HCl are separated from most of the other proteins. The DS method has good reproducibility and a high extraction efficiency as compared to the conventional peptide enrichment methods described above. Using the DS method combined with reverse-phase HPLC fractionation followed by MALDI-TOF-MS, we conducted high-quality comparative analyses of more than 1,500 peptides from 1- μ L serum samples, including low-abundance peptides in the subnanomolar range¹⁵. When compared to peptide ligand libraries (ProteoMiner) and ultrafiltration, DS method allowed the identification of the highest number of peptides. Moreover, the DS method enabled also the quantitative comparison of samples¹⁶. However, technical difficulties precluded application of this strategy to biomarker validation studies. Furthermore, it can be difficult to obtain specific antibodies that react only with a peptide of interest because peptides are low-molecular-weight and are often part of larger proteins. Therefore, there is a real need for high-quality strategies for comparative analysis of low-abundance peptides for use in discovery and small-scale verification studies. Ideally, such a strategy should be directly applicable to large-scale validation studies through selected reaction monitoring using LC-MS.

The DS method we developed permits high-yield extraction of LMW proteins/peptides from serum/plasma, including species bound to albumin. However, in addition to peptides, the DS method results in extraction of a small amount of protein, which limits the amount of sample that can be analyzed using liquid chromatography-mass spectrometry (LC-MS) due to the risk of ion suppression and column overloading. In this study, we optimized the DS method for the selective extraction of peptides of less than 6 kDa. We combined the modified DS method with a highly reproducible LC-MS method, and this strategy enabled us to discover candidate peptide biomarkers from tens of sera/

plasma samples in only a few weeks. Using this highly reproducible and sensitive strategy, we discovered 2 candidate renal cell carcinoma (RCC) biomarker peptides via a small-scale validation study of 80 serum samples.

MATERIALS AND METHODS

Human serum samples

Serum samples were allowed to clot at room temperature and were then centrifuged at 2000 $\times g$ for 15 min at room temperature. Aliquots (55 μ L) were stored at -80°C until use. Pooled sera from 5 healthy volunteers were used to assess the modified method. A total of 40 pre- and post-surgery serum samples from 20 RCC patients were used for biomarker discovery (Table 1), and 40 pre- and post-surgery serum samples from 20 bladder tumor (BT) patients were used to verify the candidate biomarkers (Table 2). All subjects provided written informed consent and the Ethics Committee of Kitasato University approved the study. Sera were kept frozen, thawed only once, used rapidly, and the remainder was discarded. Clotting time was measured for each sample as described in Tables 1 and 2.

Peptide extraction using the DS method

Differential solubilization was carried out according to Kawashima *et al.*¹⁵. Briefly, a mixture of 50 μ L of serum and 100 μ L of denaturation solution was slowly added dropwise into 2 mL of ice-cold acetone. After centrifugation, the precipitate was redissolved with 1 mL of redissolving solution and centrifuged again. The supernatant containing extracted peptides was lyophilized and stored at -80°C until use. In optimizing the DS method for LC-MS, peptides were extracted with redissolving solution composed of 60%, 70%, 80%, or 90% acetonitrile (ACN) and 12 mM HCl.

Synthetic Peptides

An isotope-labeled synthetic peptide was obtained commercially from AnyGen Co., Ltd. (Kwangju, Korea). The amino acid sequence of the peptide was VNPFRPGDSEPP-PAPGAQRAQ with the underlined amino acids synthesized with ^{13}C , ^{15}N uniformly labeled 9-fluorenylmethoxycarbonyl (FMOC) amino acids, L-PHENYLALANINE-N-FMOC ($^{13}\text{C}_9$, 98%; ^{15}N , 98%); and L-PROLINE-N-FMOC ($^{13}\text{C}_5$, 98%; ^{15}N , 98%). The peptide was a fragment of zyxin with the sequence $^{36}\text{VNPFRPGDSEPPAPGAQRAQ}^{56}$ and was one of the biomarker candidates of colon cancer discovered using DS method by Kawashima *et al.*¹⁵. The molecular weight increment of this peptide relative to the nonlabeled peptide was 22.

Tricine-SDS-PAGE

Lyophilized LMW proteins/peptides extracted from serum samples were dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample

Table 1. Clinical Features of RCC Patients.

Patient No.	Gender	Age	Stage	Grade	Clotting time (hours) ^{a)}		Post-surgery time ^{b)} (weeks)
					pre-surgery	post-surgery	
1	Female	56	T2	G2	*	3	53.1
2	Male	59	T1	G2	*	*	4.1
3	Male	59	T3	G3	*	*	4.7
4	Female	75	T2	G2	*	4	13.6
5	Male	43	T1	G2	2	4	3.9
6	Male	60	T1	G2	4	4	6.7
7	Male	75	T3	G2	*	3	8.3
8	Male	56	T3	G2	2	3.5	4.3
9	Male	52	T1	G2	2	3	3.7
10	Male	61	T3	G2	2	2	4.1
11	Male	69	T1	G1	2	3	8.7
12	Male	67	T1	G1	2	4.5	3.4
13	Male	40	T1	G2	3	4	3.9
14	Male	73	T3	G2	2	3	8.9
15	Male	61	T1	G2	2	3	13.1
16	Male	43	T1	G1	4	3.5	3.3
17	Male	63	T1	G2	2	6.5	4.3
18	Male	57	T1	G2	2	4	8.9
19	Male	77	T1	G1	2	5	2.6
20	Male	57	T1	G2	2	2	5.3

^{a)} Period between blood collection and centrifugation.

^{b)} Period between surgery and blood collection.

* Clotting time unclear, but less than 7 hours.

Table 2. Clinical Features of BT Patients.

Patient No.	Gender	Age	Stage	Grade	Clotting time (hours) ^{a)}		Post-surgery time ^{b)} (weeks)
					pre-surgery	post-surgery	
1	Male	73	–	G2	*	*	3.9
2	Male	74	–	G2	*	*	4.3
3	Male	74	Ta	G3	*	*	56.7
4	Female	78	–	G2	*	*	2.9
5	Male	79	–	G1	*	*	50.9
6	Male	84	Ta	G2	*	*	2.9
7	Male	64	Ta	G1	*	*	3.0
8	Male	65	T1	G2	*	*	3.0
9	Male	73	Ta	G2	*	*	2.9
10	Male	68	T2	G2	*	*	3.1
11	Male	72	Ta	G1	*	*	4.1
12	Male	72	T2	G2	*	*	2.9
13	Female	70	Ta	G1	*	*	3.0
14	Male	57	T1	G2	*	*	3.9
15	Male	59	–	G2	2	6	4.0
16	Female	69	–	G3	2	4	2.9
17	Male	73	–	G2	2	4	1.7
18	Male	76	–	G2	2	4	15.9
19	Male	66	Ta	G1	2	3	19.1
20	Male	79	T1	G3	2	2	2.7

^{a)} Period between blood collection and centrifugation.

^{b)} Period between surgery and blood collection.

* Clotting time unclear, but less than 7 hours.

buffer (50 mM Tris–HCl, pH 6.8, containing 50 mM DTT, 0.5% SDS, and 10% glycerol). Serum LMW peptides/proteins along with molecular weight markers (Mark 12TM; Life Technologies Co., Carlsbad CA, USA) were resolved

on tricine-SDS-PAGE gels (Perfect NT Gel W, NTH-7A6T, 15–20%, 20 wells; DRC Co., Ltd., Tokyo, Japan) according to the manufacturer's protocol. Gels were stained with silver nitrate (2D-Silver Stain Reagent II; Cosmo Bio Co.,

Ltd., Tokyo, Japan) or Coomassie brilliant blue (CBB, PhastGel Blue R; GE Healthcare, Little Chalfont, UK). Gel images were converted to densitograms using Image J software (<http://rsb.info.nih.gov/ij/>).

LC-MS analysis

Lyophilized peptides extracted from 40- μ L serum samples were dissolved in 80 μ L of 0.1% trifluoroacetic acid (TFA). The sample was then loaded onto a C18 column (2.0 mm i.d. $\times$ 100 mm, Cadenza CD-C18; Imtakt Corp., Kyoto, Japan) attached to a Nanospace SI-2 HPLC system (Shiseido Fine Chemicals, Tokyo, Japan). The column was maintained at 40°C and the flow rate was 200 μ L/min. The mobile phase composition was programmed to change over 39 min by varying the mixing ratios ($r = [B]/([A] + [B]) \times 100$) of solvent A (0.05% formic acid (FA)) and solvent B (90% ACN in 0.05% FA) as follows. A constant mixing ratio ($r = 0\%$) was used from time (t) = 0–4 min, followed by a linear gradient ($r = 0$ –52%) from $t = 4$ –30 min, followed by washing at $r = 95\%$. Mass spectra were acquired with an LCQ DECA mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) over the m/z range 340–2000 from $t = 4$ –30 min with 1-s survey spectra. The heated capillary temperature and spray voltage were held at 300°C and 5.0 kV, respectively. To obtain high resolution and sensitive comparative analyses, the abundance of each peptide was compared to the peak intensity of the averaged mass spectra collected every 1 min from 4–20 min and every 1.5 min from 19.5–30 min.

Statistical analysis

The statistical significance of differences in peak intensity for candidate peptides was determined using the Student's t -test. Graphpad Prism (version 5.02) was used to construct receiver operator characteristic (ROC) curves and calculate area under curve (AUC) values.

Peptide Identification by LC-MS/MS

Candidate biomarker peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an LTQ-Orbitrap (ThermoFisher). The resolution of MS/MS spectra was 7,500, and peptides were identified by database searching with SwissProt 57.15 using MASCOT, version 2.3.02 for Windows (Matrix Science, London, UK), with the following search parameters: a monoisotopic precursor mass tolerance of 2 ppm and a fragment mass tolerance of 0.5 Da, with no specified protease cleavage site.

RESULTS AND DISCUSSION

To optimize the DS method, we first examined varying the concentration of ACN in the redissolving solution. A representative tricine-SDS PAGE gel stained with CBB is shown in Fig. 1Aa, and Fig. 1Ba shows the results of densitometric analysis of this gel. The sample resolved in lane 3

was redissolved using 80% ACN as the redissolving solution, which resulted in a dramatic decrease in the number of proteins larger than 6 kDa, but had a minimal impact on the peak representing peptides smaller than 6 kDa compared to samples extracted with 60% ACN (lane 1) and 70% ACN (lane 2) (Fig. 1Ba), as per the original DS method. The results of more sensitive analyses using silver staining are shown in Fig. 1Bb, which clearly show that redissolution using 80% ACN did not significantly diminish the major components of peptides less than 6 kDa compared to redissolution using 60% or 70% ACN. These results indicate that the use of 80% ACN as the redissolving solution enriches peptides less than 6 kDa with very high efficiency.

We then developed a strategy combining a modified DS method and LC-MS using conventional HPLC for the high-quality comparative analysis of low-abundance peptides in serum. To assess the reproducibility of the combined strategy, peptides in 20 portions of a pooled serum sample were extracted using the modified DS method and analyzed by LC-MS. Total ion current (TIC) chromatograms and MS spectra for 6 of the 20 sequential analyses are shown in Fig. 2A and 2B, respectively. The results were highly consistent across the entire retention time range. The coefficients of variation (CVs) for the relative intensities of MS

Fig. 1. Evaluation of the modified DS method using tricine-SDS-PAGE.

(A) Tricine-SDS-PAGE analysis of human serum (10 μ L) extracted using redissolving solution containing 60% (lane 1), 70% (lane 2), 80% (lane 3), or 90% (lane 4) acetonitrile. Lane M: molecular weight markers. (a) CBB-stained and (b) silver-stained gels. (B) Densitometric analyses of the gels shown in (A). Lanes are as shown in (A).

Fig. 2. Reproducibility and sensitivity of the modified DS/LC-MS combined method.

(A) TIC chromatograms of the 1st, 4th, 8th, 12th, 16th, and 20th LC-MS analyses of peptides extracted using the modified DS method. Boxes marked a, b, and c denote retention times of 12–13, 18–19, and 24–25 min. (B) Average MS spectra collected over the retention times denoted by boxes a, b, and c in (A). (C) TIC chromatogram of LC-MS (a) and average MS spectra collected over the retention time 17–18 min (b) for 6 serum samples spiked with a stable isotope-labeled peptide and extracted using the modified DS method. The concentration of the peptide in serum was 40 ng/mL (1), 20 ng/mL (2), 10 ng/mL (3), 5 ng/mL (4), 2 ng/mL (5), and 0.8 ng/mL (6).

peaks selected randomly from each of the 20 sequential analyses were in the range of 6–15 CV%. These results indicated that the modified DS/LC-MS combined strategy is highly reproducible.

The detection limit of the combined strategy was evaluated using a stable isotope-labeled peptide (Fig. 2C). The limit of detection for comparative analyses in serum was quite low (a few ng/mL), even though conventional HPLC is used for the LC-MS analysis.

Our new strategy is highly sensitive and reproducible due to the combined use of the modified DS method and conventional HPLC. With respect to sensitivity, the peptide enrichment rate was dramatically increased by the modified DS method, and the amount of sample that could

be analyzed by LC-MS was increased by employing conventional HPLC. In addition, the robustness of the LC-MS system we employed enables direct analysis of crude samples prepared using the modified DS method, without prior desalting, even though such samples cannot be analyzed directly using nano-flow HPLC. Direct analysis of crude samples without prior desalting provides another advantage because some peptides could not be detected after desalting when analyzed on an LC-MS instrument equipped with a nano-flow HPLC system. Thus, the overall sensitivity of this new strategy is high even though we use conventional HPLC for LC-MS. Furthermore, the number of peptides detected using this LC-MS system in analyses of samples prepared using the modified DS method increased

Fig. 3. Discovery of candidate RCC biomarker peptides.

(A) Change in the serum concentration of the m/z 733.3 (a), 1263.6 (b), and 548.7 (c) peptides in sera of pre- and post-surgery RCC patients. Dashed lines in (a) and (b) denote patient serum samples in which the peptide level increased following surgery. (B) ROC curves of candidate peptides for the diagnosis of RCC. Graphs (a)–(c) correspond to graphs (a)–(c) in (A). The AUC values of the comparisons between groups were 0.756 (a), 0.725 (b), and 0.775(c).

due to suppression of the overlap of intense peaks induced by multi-charged proteins.

We used our strategy to analyze serum samples collected pre- and post-surgery from 20 RCC patients (Table 1), and discovered 8 potential biomarker candidates ($P < 0.05$). In these 8 peptides, 3 peptides at m/z 549.2, 733.8 and 1263.6 showed the same change in more than 16 of 20 patients following surgery. Fig. 3A illustrates the pre- and post-surgery change in the levels of these 3 peptides in sera of the 20 RCC patients participating in the study. The serum level of the peptide at m/z 549.2 increased in most of the patients following surgery, but decreased slightly in 4 patients. In most cases, the serum levels of the m/z 733.8 and 1263.6 peptides decreased after surgery. However, the serum level of both of these peptides increased following surgery in 3 patients (Nos. 7, 10, and 14). Patients 7, 10, and 14 were among the 5 patients in stage T3. The AUCs for the ROC curves for the 3 candidate biomarker peptides as calculated from the 40 RCC patient serum samples were 0.775, 0.756, and 0.725 (Fig. 3B).

Identification of the 3 candidate markers revealed that the m/z 549.2 peptide was a 5+ charged fragment of alpha-2-HS-glycoprotein (fetuin-A), with the amino acid sequence TVVQPSVGAAAGPVVPPCPGRIRHFKV (MW=2738.52 Da, MASCOT Score=57). The m/z 733.8 and 1263.6 peptides were doubly and singly charged fragments, respectively, of fibrinogen α -chain, with the respective amino acid

sequences DSGEGDFLAEGGGVR (MW=1464.64 Da, Mascot Score=101) and GEGDFLAEGGGVR (MW=1262.59 Da, Mascot Score=100).

As the first step in the validation of the 3 candidate biomarkers, we analyzed serum samples from 20 pre-surgery BT patients and serum samples from 20 post-surgery BT patients using the same strategy described above. Fig. 4 shows a comparison with the results obtained from RCC patient sera. The 2 peptides derived from fibrinogen α -chain were specifically upregulated in the sera of the pre-surgery RCC patients, while the peptide derived from fetuin-A was upregulated in the sera of post-surgery RCC patients. However, the levels of the peptide from fetuin-A of pre-surgery RCC patients and both pre- and post-surgery BT patients were the same. Table 3 summarizes data regarding the 3 identified peptides, together with reports describing them as candidate biomarkers of several diseases.

Fetuin-A is a protease inhibitor reportedly upregulated in the serum of patients with pregnancy-induced hypertension¹⁷, but has not been reported as a candidate cancer biomarker. Araki *et al.*¹⁷ reported that the fetuin-A peptide is significantly depleted by albumin/IgG, and they were able to detect the peptide bound to albumin using a newly developed BLOTCHIP MALDI-MS target plate. As the DS method is a powerful strategy for extracting peptides bound to albumin, we were able to detect this peptide with good

Fig. 4. Results of a small-scale validation of the m/z 733.3 (a), 1263.6 (b), and 548.7 (c) candidate biomarker peptides using 20 sets of pre- and post-surgery serum samples collected from RCC patients and BT patients.

Table 3. Characteristics of candidate biomarker peptides

Observed Peak (m/z)	MW (Da)	Uni-Prot accession number	Origin of the peptide	Amino acid number (N-/C-terminus)	Peptide sequence	MASCOT search		Result in this study	Reports	
						Score	Expect		Upregulated	Downregulated
733.3	1464.64	P02671	fibrinogen α -chain	21–42	DSGEGDFLAE-GGGVR	101	$6.8e^{-9}$	Upregulated in pre-surgery RCC patients	Crohn's disease ⁽¹⁰⁾ , Gastric cancer ^(19, 20)	Prostate cancer ⁽¹⁾ , Bladder cancer ⁽¹⁾ , Abstinence in an alcoholic subject ⁽²⁾ , Hepatocellular carcinoma ⁽¹¹⁾ , Thyroid carcinoma ⁽¹⁸⁾ , Colon carcinoma ⁽²¹⁾
1263.6	1262.59	P02671	fibrinogen α -chain	23–42	GEGDFLAE-GGGVR	100	$1.2e^{-8}$	Upregulated in pre-surgery RCC patients	Crohn's disease ⁽¹⁰⁾	Prostate cancer ⁽¹⁾ , Bladder cancer ⁽¹⁾ , Breast cancer ⁽¹⁾ , Colon carcinoma ⁽²¹⁾
548.7	2738.52	P02765	α -2-HS-glycoprotein (fetuon-A)	341–367	TVVQPSV-GAAAGPVVP-PCPGRIHFKV	57	$7.3e^{-5}$	Upregulated in post-surgery RCC patients	Pregnancy-induced hypertension ⁽¹⁷⁾	–

* The peptide was cysteinylated on Cys 358

sensitivity. However, unfortunately, this peptide may not be suitable as a biomarker because it is upregulated specifically in the serum of post-surgery RCC patients but not in that of pre-surgery RCC patients, nor is it upregulated in the serum of either pre- or post-surgery BT patients.

As illustrated in Table 3, the m/z 733.3 peptide derived from fibrinogen α -chain was downregulated in sera obtained from patients with 4 types of cancer^(1, 11, 18, 21) and upregulated in sera of patients with gastric cancer^(19, 20). The m/z 1263.6 peptide was also downregulated in sera obtained from patients with 4 types of cancer^(1, 21). Most of these data

were obtained by comparative analyses of patients and healthy controls. In contrast, our results showed that both peptides are upregulated in the serum of pre-surgery RCC patients, and in BT patients are found at the same level as post-surgery RCC patients.

Native peptides in serum samples can be produced from the cleavage of a variety of proteins, which may be influenced by such as clotting time, the kinds of blood collection tubes used, and storage conditions. However, there was no clear correlation between clotting time and the serum levels of the peptides we identified (Tables 1 and 2), as all

serum samples were collected and stored under the same conditions. Furthermore, there were no correlations between serum levels of these peptides and post-surgery time. Therefore, it is unlikely that the observed changes in the serum levels of these peptides were the result of artifacts or influence of inflammation after surgery. One potential explanation for the differences between our results and those of other researchers could be differences in pretreatment methods, which can skew results for peptides such as fetuin-A, which can interact with albumin or other serum proteins. To date, there is no biomarkers capable of detecting RCC and predicting its recurrence. Therefore, we believe that the 2 peptides we identified in this study should be validated in comparative studies of sera from healthy subjects and patients with a wide variety of diseases, including RCC.

CONCLUSION

We optimized a novel differential solubilization peptide extraction method for use in LC-MS analyses. Combining the modified DS method with highly reproducible LC-MS using conventional HPLC allowed us to achieve high overall sensitivity through a decrease in ion suppression effects and also allowed us to increase the amount of peptide that could be analyzed in one experiment. This strategy is well-suited to comparative analyses for the discovery of candidate biomarker peptides in serum and for use in small-scale validation studies using tens of samples, even though it employs classical LC-MS. With this strategy, we discovered 2 candidate biomarker peptides for detecting RCC. However, it will likely be necessary to discover a more sensitive biomarker that provides direct indication of the presence of small tumors. Recent and anticipated future advances in mass spectrometry²²⁾ suggest that the strategy described here will increase the chance of discovering more sensitive biomarker peptides.

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ABBREVIATIONS

DS, differential solubilization; LC-MS, liquid chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; RCC, renal cell carcinoma; BT, bladder tumor; SRM, selected-reaction monitoring; LMW, low-molecular-weight; RP, reverse-phase; IEF, isoelectric focusing; MALDI-TOF-MS, matrix-assisted laser desorption/ionization-time of flight-mass spectrometry; ACN, acetonitrile; TFA, trifluoroacetic acid; FA, formic acid; ROC, receiver operating characteristic; AUC, area under curve; LC-MS/MS, tandem mass spectrometry; TIC, total ion current; CV, coefficient of variation; FMOC; 9-

fluorenylmethoxycarbonyl; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CBB, Coomassie brilliant blue

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