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ORIGINAL ARTICLE

Analysis of the urinary peptidome associated with *Helicobacter pylori* infection

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Abstract

AIM: To investigate the relationship between urinary peptide changes and *Helicobacter pylori* (*H. pylori*) infection using urinary peptidome profiling.

METHODS: The study was performed in volunteers (n = 137) who gave informed consent. Urinary peptides were enriched by magnetic beads based weak cation exchange chromatography and spectrums acquired by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). ClinProTools bioinformatics software was used for statistical analysis and the recognition of peptide patterns. The marker peptides were identified by LTQ Obitrap XL tandem MS.

RESULTS: Approximately 50 proteins or peptides which loaded onto the magnetic beads were detected by MALDI-TOF MS. By optimizing the parameters of the model, the Genetic Algorithm model had good recognition capability (97%) and positive predictive value (94%). Based on the model, 2 markers with molecular masses of 6788 and 1912 Da were found that differentiated between *H. pylori* positive and negative volunteers. The m/z 1912 sequence was parsed as SKQFTSSTSYNRGDSTF. The peptide was identified as isoform 1 of the fibrinogen α chain precursor, whose concentration in urine was markedly higher in *H. pylori* infected volunteers than in *H. pylori* non-infected ones.

CONCLUSION: The appearance of urinary fibrinogen degradation products is caused by an active *H. pylori*-induced process.

Key words: Urinary peptidome profiling; MB-matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; *Helicobacter pylori*; Fibrinogen degradation products

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative, microaerophilic bacterium adapted for survival in the human stomach, where it can cause chronic gastritis and peptic ulcer disease and is an important risk factor that may lead

to gastric cancer. Great progress has been made in understanding *H. pylori* pathogenicity since its discovery 25 years ago. *H. pylori* infection has been proposed as a risk factor not only for gastrointestinal diseases but also for cardiovascular diseases such as peripheral arterial disease[1] and atherosclerosis[2]. In addition, some studies have shown that *H. pylori* infection is associated with Henoch-Schonlein purpura[3,4] and membranous nephropathy[5,6]. Purpura nephritis is one of the serious complications of Henoch-Schonlein purpura[7]. As a result of its long delitescence, rapid growth of drug resistance and the ease of infection, *H. pylori* infection has become a prominent chronic digestive system disease.

Recent progress in proteomic analysis and strategies for the identification of clinically useful biomarkers in biological fluids has shown that urine can be an excellent non-invasive reservoir[8-10]. By virtue of its noninvasiveness and the availability of specimens, peptidome profiling of human urine is now becoming an important method for detecting novel disease-associated markers[11,12]. Bruker Daltonics provides the mass spectrometry (MS)-based ClinProt™ system solution for preparation, measurement and visualization of peptides and proteins in body fluid[13]. The Profiling Kit MB-WCX (Magnetic Beads based Weak Cation Exchange Chromatography) was developed for the enrichment of proteins and peptides from biological samples based on cation exchange chromatography prior to matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS) analysis. Successful applications and reproducibility of the MB-WCX beads using serum, plasma and urine samples was demonstrated in various studies[14-16]. Nanoliquid chromatography coupled to micro-electrospray ionization tandem MS (ESI-MS/MS) has become a powerful tool for identification and quantification in peptide analysis due to its higher sensitivity[17,18]. In this study, we analyzed the urine peptidome profiles of *H. pylori* infected and non-infected volunteers by the ClinProt™ system, followed by MALDI-TOF MS, and we identified the biomarkers using Aquity nano-ultra-performance liquid chromatography coupled to a Thermo LTQ Orbitrap high resolution/high accuracy ultra-performance liquid chromatography (UPLC)-ESI-MS/MS.

MATERIALS AND METHODS

Protein/peptide marker discovery in urine

Urine specimen collection: Urine samples were collected from healthy volunteers (n = 137, 70 male, 67 female) who did not have cardiovascular diseases and had received a health checkup 3 mo prior to the study, and gave written informed consent before participation. The volunteers received ^{13}C-urea breath tests to determine whether they were infected with *H. pylori,* and their midstream urine was collected the following morning[19]. Urine samples were kept at a low temperature with ice and were transferred to the laboratory within 2 h, centrifuged at 3000 g for 20 min, aliquotted and stored at -80℃ until use.

Urinary peptide enrichment

The urine samples were thawed at room temperature for 30 min, adjusted to pH 7, and centrifuged again. Urinary peptides were separated using MB-WCX kit (Bruker Daltonics, Bremen, Germany; particle size < 1 μm; mean pore size, 40 nm; specific surface area, 100 cm^2/g). The magnetic beads were mixed thoroughly on a vortex device for 1 min, then a 30 μL urine sample was diluted in 60 μL MB-WCX binding solution, and 10 μL WCX beads were added. After thorough stirring, sample mixtures were incubated for 1 min at room temperature. The tube was placed into the magnetic separator and the beads at the wall of the tube were collected for 1 min. The supernatant was removed by using a pipette. Wash buffer (100 μL) was added to the tube, which was moved back and forth in the magnetic separator 10 times. The beads were collected at the tube wall for 1 min and the supernatant was removed carefully using a pipette. Elution buffer (5 μL) was added and the beads dissolved at the tube wall by pipetting up and down intensively 10 times. The beads were collected at the tube wall for 2 min and the clear supernatant was transferred into a fresh tube. Stabilization buffer (5 μL) was added to the eluate.

MALDI-TOF data acquisition

Sample solution (1 μL) was dropped onto an AnchorChip™ 600-μm target (Bruker Daltonics) and dried. Next, 1 μL of freshly prepared α-cyano-4-hydroxycinnamic acid [0.4 mg/mL matrix solution in ethanol/acetone (2:1, v/v)] was added onto the sample and crystallized. MALDI-TOF MS analysis of the peptidome profile was performed using an autoflex™ instrument (Bruker Daltonics), equipped with a N_2 laser (λ = 377 nm), with the ion source voltage as follows: source 1, 120 kV; ion source 2, 18.6 kV; lens 7.6 kV. The pulsed ion extraction delay was 320 ns and operated in positive ion linear mode (LP-ClinProt) with a total of 450 shots (30 shots at each of 15 different spot positions) per sample. All signals with a signal-to-noise ratio > 3 in a m/z range of 1000-10 000 Da were collected with the AutoXecute tool of the flexControl™ acquisition software (version 3.0; Bruker Daltonics). Mass calibration was performed with the standard calibration mixture of peptides and proteins (CPS, preparation method in the MB-WCX operation manual, MW range 1000-10 000 Da).

Statistical data analysis

The spectra were analyzed statistically using Clin-Prot™ (version 2.2 β; Bruker Daltonics) bioinformatics software. Parameters were as follows: peak definition: signal to noise ratio > 3; statistical analysis: Wilcoxon/Kruskal-Wallis; area normalization: against total ion count; integration: end point level; mass recalibration: maximal peak shift of 500 ppm; sort mode: t-test P-value/analysis of variance (ANOVA). The spectra from 90 samples (40 in the *H. pylori* infected group and 50 in *H. pylori* non-infected group) were used to build models and 47 samples (23 in the *H. pylori* infection group and 24 in the *H. pylori*

non-infected group) were used in model verification by the Genetic Algorithm (GA), Quick classifier, and Supervised Neural mathematical algorithms. The parameters k-nearest neighbor classification (KNN), maximal number of generations (MNG) were optimized and the best model was determined. The performance of the models was evaluated by recognition capability (RC) and positive predictive value (PPV): RC = TP/*n* where TP is the number of true positives (correctly classified) in a data set and *n* is the number of samples in a data set and PPV = TP/(TP + FP) where FP is the number of false positives (misclassified). The best model (RC and PPV values are a maximum one of 1) was implemented to determine the marker peptides. The *P*-value of the Anderson-Darling test (PAD) which can give information about the normal distribution: < 1 not normally distributed, > 1 normally distributed, the *P*-value of the *t*-test (2 classes) or ANOVA test (> 2 classes) (PTTA, preferable for normal distributed data) or the *P*-value of the Wilcoxon test (2 classes) or Kruskal-Wallis test (> 2 classes) (PWKW, preferable for abnormally distributed data) was used to confirm significant differences. If the PWKW or PTTA value was < 0.05, the protein/peptide was confirmed to be significantly different.

Identification of significant peptides by nano UPLC-ESI-MS/MS

UPLC: The peptides from urine samples (the differential peptides are relatively abundant) were eluted from the magnetic beads and were analyzed by nano-UPLC-ESI-MS/MS using a nano Aquity UPLC (Waters Corporation, Milford, USA) coupled to a LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Samples of 5 μL (the sample was diluted by 2 times) were loaded on a C18 precolumn (Symmetry®C18, 5 μm, 180 μm × 20 mm, nanoAcquity™Column) at 15 μL/min in 5% acetonitrile (Sigma-Aldrich, St Louis, MO, USA), 0.05% trifluoroacetic acid (Sigma-Aldrich) for 3 min. The precolumn was switched online with the analytical column (Symmetry®C18, 3.5 μm, 75 μm × 150 mm, nanoAcquity™ Column) equilibrated in 95% solvent A (5% acetonitrile, 0.1% formic acid; Sigma-Aldrich) and 5% solvent B (95% acetonitrile, 1.2% formic acid). Peptides were eluted using a 5% to 80% gradient of solvent B over 60 min at a flow rate of 400 nL/min.

UPLC-MS/MS and data analysis

The LTQ Orbitrap XL mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. Full-scan survey MS spectra with 2 microscans (m/z 400-2000) were acquired with the Orbitrap with a mass resolution of 100 000 at m/z 400, followed by 10 sequential LTQ-MS/MS scans. Dynamic exclusion was used with 2 repeat counts, 10 s repeat duration and 60 s exclusion duration. For MS/MS, charge state 1 was rejected and precursor ions were activated using 25% normalized collision energy at the default activation q of 0.25. The mass spectra were searched against the human International Protein Index (IPI) database (IPI human v3.45 fasta with 71 983 entries) using Bioworks software (Version 3.3.1; Thermo Electron Co.) based on the SEQUEST algorithm. To reduce false positive identification results, a decoy database containing the reverse sequences was appended to the database. The parameters for the SEQUEST search were as follows: no enzyme, the variable modification was oxidation of methionine, peptide tolerance, 10 ppm, MS/MS tolerance, 1.0 Da. Positive protein identification was accepted for a peptide with Xcorr of greater than or equal to 3.20 for triply and 2.86 for doubly charged ions, and all with ΔCn ⩾ 0.1, peptide probability ⩽ 2e-3.

Table 1 Comparison of results for the classification models

Model	Algorithms	KNN	MNG	Max. peaks	RC (%)	PPV (%)
1	GA	5	60	7	90.5	83.0
2	GA	3	60	10	91.3	85.1
3	GA	7	60	15	96.5	93.6
4	GA	3	60	20	93.3	91.5
5	GA	5	60	25	93.3	87.2
6	SNN			25	78.5	63.8
7	QC			25	78.8	66.0

Model 3 was the best. GA: Genetic Algorithm; QC: Quickclassifier; SNN: Supervised Neural Network; KNN: k-nearest neighbor classification; MNG: Maximal number of generations; RC: Recognition capability; PPV: Positive predictive value.

RESULTS

Urinary peptidome profiling

^{13}C-urea breath tests showed that 74 volunteers were *H. pylori* negative and 63 volunteers were *H. pylori* positive (delta over baseline > 4). About 50 peaks with signal-to-noise ratios greater than 5 were detected between m/z 1000 and 10 000 in urine from the volunteers (Figure 1). The average intensities of peaks for the negative group and positive group are shown in Figure 2A, and the complete spectra from both the healthy group and the *H. pylori*-infected group are shown in Figure 2C.

Statistical data analysis and classification

When parameter KNN = 7, MNG = 60 and Max.peaks = 15, the GA model was the best fit: RC = 96.5%, PPV = 93.6% (Table 1). All the data PAD were < 1, so the data were abnormally distributed and PWKW was used to confirm marker peptides. Two markers that differentiated between the *H. pylori* non-infected group and the *H. pylori* infected group (PWKW < 0.05) with molecular masses of 6788 and 1912 Da were found in urine (Table 2). The content of these peptides in urine was markedly higher in *H. pylori* infected volunteers than in non-infected subjects (Figure 2B and D).

Identification of peptides

The peptides from urine were separated using nano-UPLC. Product-ion-spectra of the doubly charged mol-

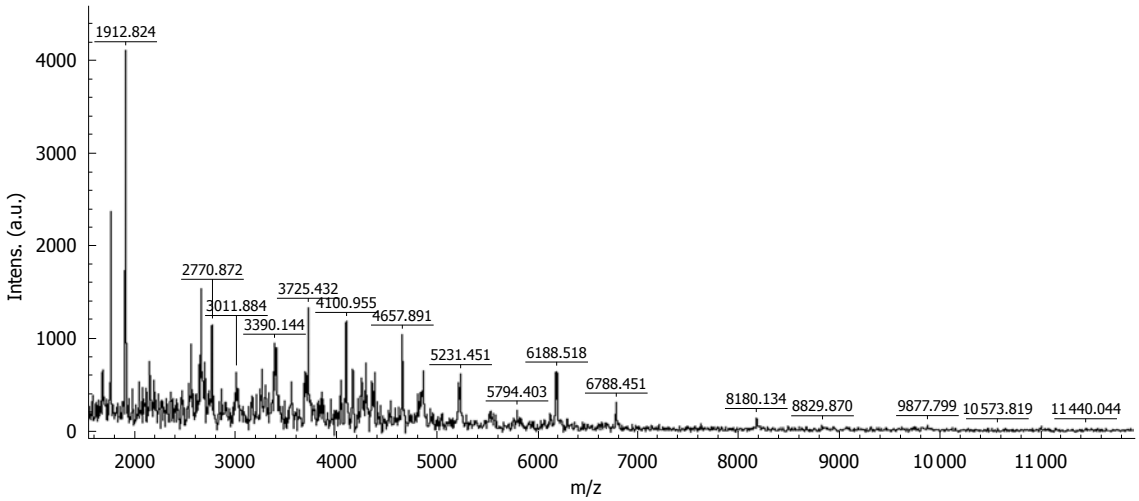


Figure 1 The mass spectrum of peptides in urine ranging between 1000 and 10 000 Da.

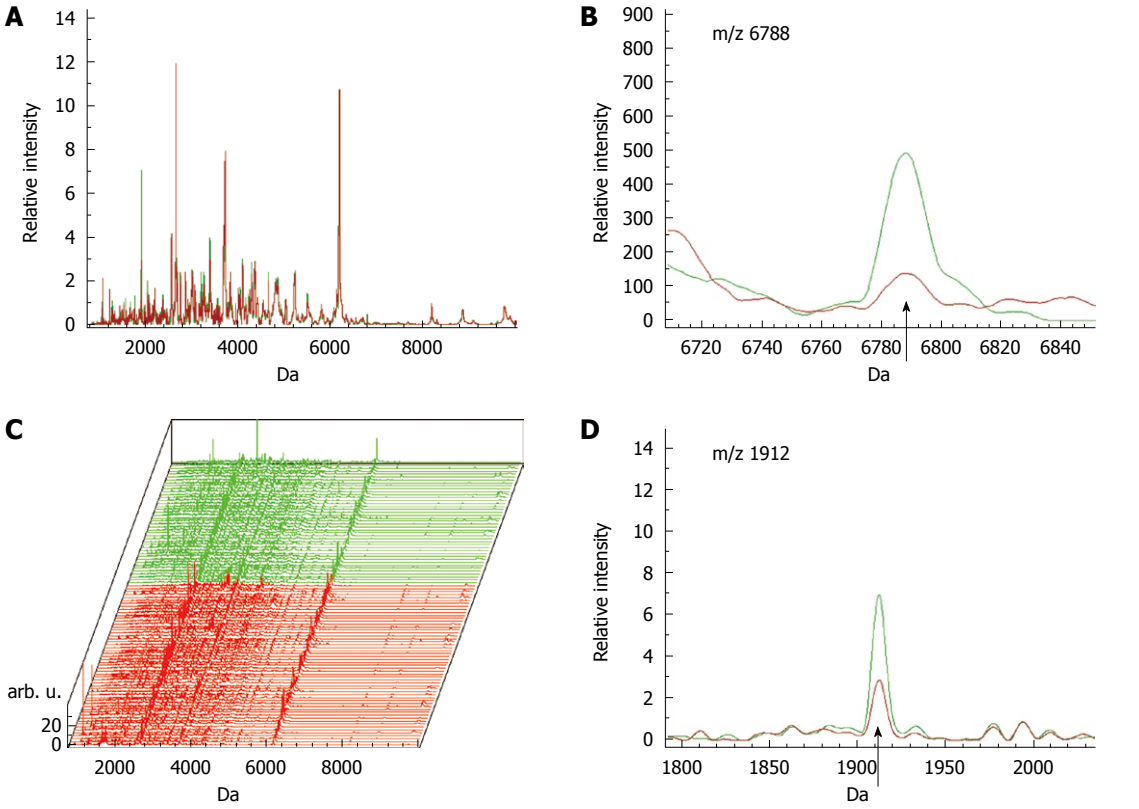


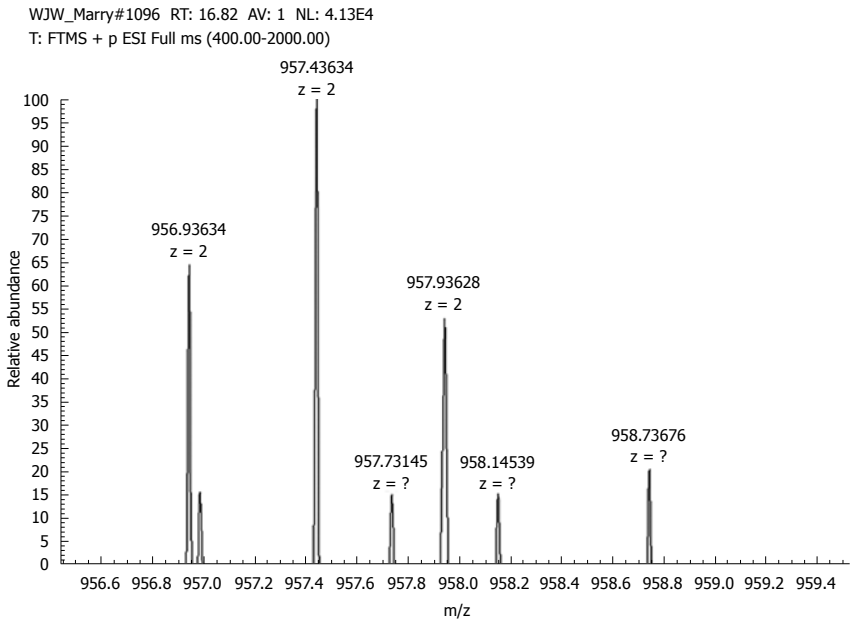
Figure 2 Differentially expressed low-mass peptides in human urine. A: The average intensities of matrix-assisted laser desorption/ionization time-of-flight peaks for the healthy group (red line), and the *H. pylori*-infected group (green line); B, D: The enlarged picture m/z 6788 and m/z 1912, respectively, the healthy group (red line) and the *H. pylori*-infected group (green line); C: The complete spectra from both the healthy group (red line) and the *H. pylori*-infected group (green line).

ecule m/z 957.436 for the 1912 Da peak was recorded with the linear ion trap (Figure 3A) and the sequence was parsed as SKQFTSSTSYNRGDSTF following MS/MS (Figure 3B). The sequence was identified as isoform 1 of fibrinogen α chain precursor (AC: IPI00021885) using the IPI database with Xcorr 3.201 (doubly charged ion), ΔCn = 0.267, P = 1.10E-04 and MS/MS tolerance 0.26 Da. Unfortunately, the m/z 6788 peak sequence was not identified. Because it was possible that the peptide m/z 1912 was from *H. pylori*, the sequence was searched against all the species in the NCBInr. The fibrinogen was identified again as a fragment of human fibrinogen [gi|4503689|ref|NP_000499.1| fibrinogen, α polypeptide isoform α-E preproprotein (Homo sapiens)].

DISCUSSION

Urine is an especially attractive medium for biomarker

A



B

	AA	B+	B2+	Y+	Y2+	
1	S	88.0	44.5	-	-	17
2	K	216.1	108.6	1825.8	913.4	16
3	Q	344.2	172.6	1697.7	849.4	15
4	F	491.3	246.1	1569.7	785.3	14
5	T	592.3	296.7	1422.6	711.8	13
6	S	679.3	340.2	1321.6	661.3	12
7	S	766.4	383.7	1234.5	617.8	11
8	T	867.4	434.2	1147.5	574.3	10
9	S	954.5	477.7	1046.5	523.7	9
10	Y	1117.5	559.3	959.4	480.2	8
11	N	1231.6	616.3	796.4	398.7	7
12	R	1387.7	694.3	682.3	341.7	6
13	G	1444.7	722.8	526.2	263.6	5
14	D	1559.7	780.4	469.2	235.1	4
15	S	1646.7	823.9	354.2	177.6	3
16	T	1747.8	874.4	267.1	134.1	2
17	F	-	-	166.1	83.5	1

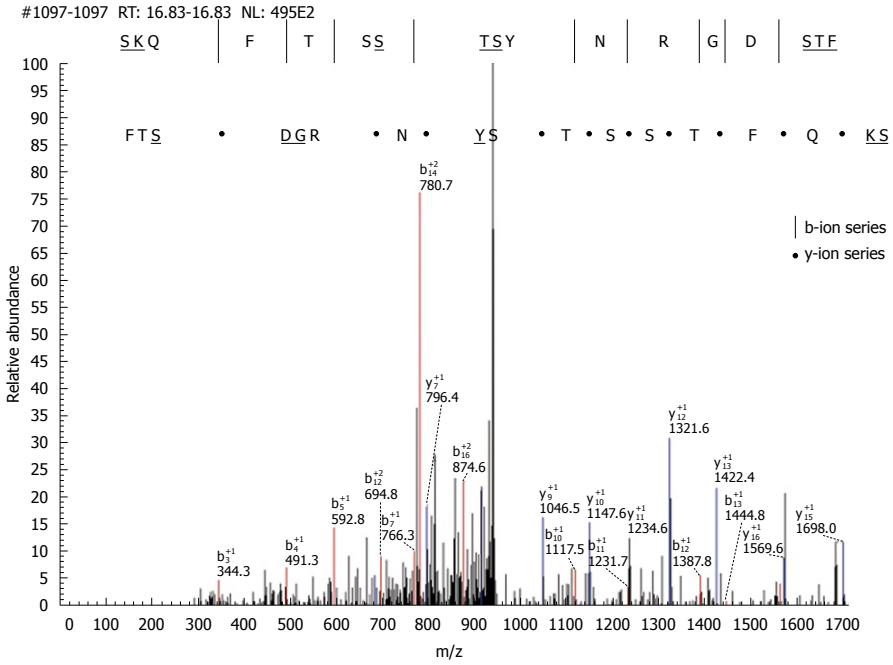


Figure 3 Protein identified by mass spectrometry/mass spectrometry. A: The enlarged picture of m/z 1912 (two charges 957.4); B: The b and y ions spectra used to identify the m/z 1912 as the fragment SKQFTSSTSYNRGDSTF. The underlined amino acids represent b or y ions of amino acids that cannot be found in the spectra. The underlined values represent the peaks where amino acids match with the b, y-ion peak.

Table 2 Statistical information for marker peptides of *Helicobacter Pylori* negative and positive groups

Index	Mass	DAve	PTTA	PWKW	PAD
56	6787.91	8.11	0.0000242	< 0.000001	0.00000418
9	1911.86	39.52	0.00847	0.00545	< 0.000001
23	3210.11	16.1	0.538	0.195	< 0.000001
27	3688.78	14.99	0.538	0.195	< 0.000001

DAve: Difference between the maximal and the minimal average peak area/intensity of all classes; PTTA: *P*-value of *t*-test (2 classes) or ANOVA test (> 2 classes); PWKW: *P*-value of Wilcoxon test (2 classes) or Kruskal-Wallis test (> 2 classes); PAD: *P*-value of Anderson-Darling test.

analysis, because urine can be obtained in large quantities using noninvasive procedures, and ample material is available for analysis and assessment of reproducibility. In addition, repeated sampling from the same individual is simple, facilitating longitudinal studies. Urine generally contains proteins and peptides of lower molecular mass (< 30 kDa) that are highly soluble. These features facilitate analysis of such polypeptides in their natural state, without any need for additional manipulation. Urinary polypeptides are stable and generally do not undergo significant proteolysis for several hours after collection[20,21]. Urine has been known, or at least has been suspected, to

reflect pathological changes for centuries. Even early pathological changes are thought to be associated with disease-specific changes in the urinary proteome[22]. In this study, we found 2 specific factors in human urine that were associated with *H. pylori* infection by urinary peptidome profiling. Urinary fibrinogen degradation products (FDP) increased with *H. pylori* infection.

Fibrinogen is a major plasma protein (340 kDa) that consists of pairs of 3 different polypeptide chains, α, β, and γ, joined by disulfide bonds to form a symmetric dimeric structure. The NH_2-terminal regions of all 6 chains form the central E-domain[23]. Fibrinogen is directly involved in the clotting process as a clotting factor and is synthesized in hepatocytes[24]. In addition, fibrinogen has a variety of other functions, such as a mediated platelet aggregation response[25]. Many studies have found that an elevated level of plasma fibrinogen is an important risk factor for cardiovascular and cerebrovascular thrombotic diseases[26,27] and renal failure[28].

Fibrinogen can be digested either by plasmin or thrombin. When fibrinogen is cleaved by plasmin, it releases 2 D fragments (the COOH termini of the α, β, and γ chains), one E fragment (the NH_2 termini of the α, β, and γ chains), and several smaller fragments including a small peptide, β 1-42 (the NH_2 terminus of the β-chain). Cleavage by thrombin releases the two fibrinopeptides A and B (FpA and FpB) from the NH_2 termini of the α and β chains, respectively, while exposed polymerization sites form electrostatic bonds between the E-domain of one molecule and the D-domain of an adjacent one. Factor XIIIa, a transglutaminase, then introduces γ-glutamyl-ε-amino-lysine isopeptide cross-links between D domains of adjacent fibrin monomers, generating a stable polymer known as fibrin. Then, fibrin can be broken down by plasmin cleavage into the 3-stranded coils found between the D and E domains, yielding a D dimer, D fragment, and fibrin E fragment (which lacks the fibrinopeptides A and B) and smaller fragments[29]. FDP, such as D-dimer, E-fragment and α, β-chain, have been widely studied in cardiovascular disease and cancer-related research fields[30,31]. The m/z 1912 peptide is a fragment of an FDP (site 580-596). Our study shows that the peptide m/z 1912 in urine was significantly increased in patients with *H. pylori* infection.

The normal glomerular basement membrane has a filtration function, and the average pore size is 5.5 nm. Therefore, under normal circumstances, some small molecular weight proteins can filter through tiny pores in the glomerular membrane. Because of endocytosis, the major proteins are normally reabsorbed when they pass through the proximal tubule, so there is low protein content in urine, a random urinary protein of 0-80 mg/L. Although there are many kinds of fibrinogen degradation fragments, large fragments are retained by the glomerulus or are taken up by the renal tubule, therefore only small peptides are normally seen in the urine. In this study, the peptides or proteins below 10 kDa in the urine were captured by weak cation beads, so only the marker peptides 1912 and 6788 were detected, while the fragments of FDP that exceeded 10 kDa were not captured.

The reasons why *H. pylori* infection results in an FDP increase in urine are not clear. Our preliminary studies have shown that *H. pylori* will lead to human gastric adenocarcinoma epithelial cell calreticulin phosphorylation, and dephosphorylation of its calcium-binding protein (nucleobindin-2), which affects cell calcium ion channels[32]. Fibrinogen achieves its biological functions by being degraded by plasmin or thrombin. The activities of plasmin and thrombin are regulated or progressively activated by calcium ions; therefore, the changes in the calcium ion channels will affect the fibrinolytic system. In short, the changes in FDP in urine are important for gaining a comprehensive understanding of the pathogenesis of *H. pylori*.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection has been proposed as a risk factor not only for gastrointestinal diseases but also for cardiovascular diseases and nephropathy. The pathogenic mechanisms of *H. pylori* are not yet clear since its discovery 25 years ago.

Research frontiers

The peptidome has been widely used in finding biomarkers with the development of mass spectrometry (MS). As it can be obtained in large quantities using noninvasive procedures, urine is an especially attractive medium for biomarker analysis. In this study, the authors analyzed the urine peptidome profiles of *H. pylori* infected and non-infected volunteers using the ClinProt™ system, followed by matrix-assisted laser desorption/ionization time-of-flight MS, and identified the marker peptides using liquid chromatography coupled to MS.

Innovations and breakthroughs

Cardiovascular diseases and nephropathy have been reported which associated with *H. pylori* infection. To date, the pathogenic mechanism is not clear. This study suggests that the appearance of urinary fibrinogen degradation products is caused by an active *H. pylori*-induced process. The results of this study are important to further the comprehensive understanding of the pathogenesis of *H. pylori*.

Applications

This study suggests that fibrinogen degradation products are associated with *H. pylori* infection. This result can help researchers in this field further understand the potential mechanism associated with *H. pylori* infection and cardiovascular diseases and nephropathy, and provide important information for prevention and control of *H. pylori*-related diseases.

Peer review

It would be of great interest in future experiments to investigate whether the urine fibrinogen peptide correlates with serum fibrinogen levels and *H. pylori* infection, as serum fibrinogen levels have been investigated in *H. pylori* infection in many studies.

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