

Duodenal Ulcer-related Antigens from *Helicobacter pylori*

IMMUNOPROTEOME AND PROTEIN MICROARRAY APPROACHES[□]

Yu-Fen Lin[‡], Chun-Yi Chen[‡], Mong-Hsun Tsai[§], Ming-Shiang Wu^{¶||}, Yu-Chun Wang^{**}, Eric Y. Chuang^{‡‡}, Jaw-Town Lin^{¶||}, Pan-Chyr Yang[¶], and Lu-Ping Chow^{‡§§¶¶}

Helicobacter pylori is an important risk factor of duodenal ulcer (DU). Although many virulence factors of *H. pylori* have been identified, few have been reported to show an association with the pathogenesis of DU. The aims of this study were to identify *H. pylori* antigens showing a high seropositivity in DU and to develop a platform for rapid and easy diagnosis for DU. Because DU and gastric cancer (GC) are considered clinical divergent gastroduodenal diseases, we compared two-dimensional immunoblots of an acid-glycine extract of an *H. pylori* strain from a patient with DU probed with serum samples from 10 patients with DU and 10 with GC to identify DU-related antigens. Of the 11 proteins that were strongly recognized by serum IgG from DU patients, translation elongation factor EF-G (FusA), catalase (KatA), and urease α subunit (UreA) were identified as DU-related antigens, showing a higher seropositivity in DU samples ($n = 124$) than in GC samples ($n = 95$) (FusA, 70.2 versus 45.3%; KatA, 50.8 versus 41.1%; UreA, 44.4 versus 27.4%). In addition, we found that the use of multiple antigens improved the discrimination between patients with DU and those with GC as the odds ratios increased from 1.82 (95% confidence interval (CI), 0.79–4.21; $p = 0.1607$) for seropositivity for FusA, KatA, or UreA alone to 4.95 (95% CI, 2.05–12.0; $p = 0.0004$) for two of the three antigens and to 5.71 (95% CI, 1.86–17.6; $p = 0.0024$) for all three antigens. Moreover a protein array containing the three DU-related antigens was developed to test the idea of using multiple biomarkers in diagnosis. We conclude that FusA, KatA, and UreA are DU-related antigens of *H. pylori*, and the combination of these on a protein array provided a rapid and convenient method for detecting serum antibody patterns of DU patients. *Molecular & Cellular Proteomics* 6:1018–1026, 2007.

Helicobacter pylori is the major factor involved in the pathogenesis of gastritis, gastric ulcer, duodenal ulcer (DU),¹ and gastric cancer (GC). It is known that this bacterium infects more than half the world's population (1). However, the clinical outcomes of *H. pylori* infection are highly variable and influenced by both bacterial factors and host immune responses. Most infected persons are asymptomatic with no apparent disease, but 6–20% have duodenal ulceration, and a small proportion develop GC (2). DU and GC are considered clinically divergent gastroduodenal diseases in which the pattern of gastritis is regarded as the major factor in the pathogenesis. Antral-predominant gastritis leads to increased acid production and duodenal ulceration, whereas corpus-predominant atrophic gastritis leads to acid reduction and a higher risk of developing GC (3, 4).

It has been recognized that the inflammation induced by *H. pylori* infection is a factor related to the pathogenesis of gastroduodenal diseases. Chronic active gastritis, the hallmark of *H. pylori* infection, is characterized by infiltration of the mucosa by neutrophils, lymphocytes, and monocytes/macrophages (5). A strong humoral immune response to a variety of *H. pylori* antigens is also elicited (6). *H. pylori* antigens are therefore regarded as potential candidates for biomarkers or vaccines (7). Serological tests are noninvasive methods for diagnosing *H. pylori* infection. Moreover evaluation of the humoral immune responses to *H. pylori* antigens by immunoblotting appears to be more sensitive than ELISA for detecting low abundance antibodies. Importantly ~70% of patients with DU are infected with *H. pylori* (8, 9). The aims of the present prospective study were to identify DU-related antigens by two-dimensional (2D) immunoblotting and mass spectrometry and to develop a platform for detecting antibody patterns for diagnostic use.

Protein array technology has been shown to be a useful tool for multiplexed measurements and proteomics studies. Protein arrays can be classified into two types, analytical and functional protein arrays. The most representative analytical protein arrays are antibody arrays. Functional protein arrays have been applied to many fields, including studies of protein-protein, protein-DNA, and protein-drug interactions; biomarker discovery; and clinical diagnosis (10). The binding of humoral antibodies to allergens, autoantigens, cancer-specific antigens, or infectious organisms is the basis of the design of protein arrays for biomarker discovery and clinical diagnosis

From the [‡]Graduate Institute of Biochemistry and Molecular Biology and ^{||}Department of Primary Care Medicine, College of Medicine, National Taiwan University, Taipei 10051, Taiwan, [§]Institute of Biotechnology, College of Bio-Resources and Agriculture and ^{‡‡}Department of Electrical Engineering, College of Electrical Engineering and Computer Science, National Taiwan University, Taipei 10617, Taiwan, Departments of ^{¶||}Internal Medicine and ^{§§}Medical Genetics, National Taiwan University Hospital, Taipei 10048, Taiwan, and ^{**}Institute of Environmental Health, College of Public Health, National Taiwan University, Taipei 10055, Taiwan

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¹ The abbreviations used are: DU, duodenal ulcer; 2D, two-dimensional; CI, confidence interval; GC, gastric cancer; OR, odds ratio.

(11–14). Applying this concept, we wished to develop a DU-related protein array, which can be used for immunodiagnoses in the form of miniaturized and multiplexed assays.

In this study, we used a proteomics approach to identify DU-related antigens of *H. pylori* by comparing the profiles of 2D immunoblots of an acid-glycine extract of *H. pylori* probed with DU and GC sera. We identified three DU-related antigens, FusA, KatA, and UreA, all of which were more frequently recognized by DU sera than GC sera. Statistical analysis of the data showed that the discrimination power of the three-antigen combination was greater than that of a single antigen. We therefore developed a DU-related protein array that can be used to simultaneously and rapidly detect the reactions of serum antibodies to different DU-related antigens.

EXPERIMENTAL PROCEDURES

Isolation of the *H. pylori* Strain and Culture Conditions—*H. pylori* strain HD12 was isolated from endoscopic biopsy samples from the stomach of a patient with DU at the National Taiwan University Hospital. The bacteria were cultured on a BBL™ Stacker™ plate (BD Biosciences) at 37 °C under microaerobic conditions.

Patients and Serum Samples—Serum samples were prospectively collected from individuals who participated in a national project for the investigation of *H. pylori* and gastroduodenal disorders in Taiwan between December 1999 and December 2001. Our study protocol was approved by both the Institutional Research Board and the Department of Health, Executive Yuan, Taiwan. One hundred and twenty-four patients with DU who received an upper gastrointestinal endoscopic examination were enrolled. Ninety-five patients with GC who underwent curative gastrectomy at our institution were also enrolled. *H. pylori* status was determined by culture and/or histological examination of gastric biopsy specimens. In addition, 40 subjects with a normal appearance of the gastric mucosa and no evidence of *H. pylori* infection were selected as controls. Fasting serum samples were collected from all participants, catalogued, aliquoted, and stored at –80 °C. Aliquots were only thawed once prior to analysis.

Two-dimensional Electrophoresis and Immunoblotting—Cell surface proteins were extracted from *H. pylori* using an acid-glycine extraction procedure as described previously (15). Proteins in the *H. pylori* acid-glycine extract were precipitated using trichloroacetic acid (20%) and separated by 2D electrophoresis as described previously (16). Briefly the protein extract was incubated with 2D sample buffer (8 M urea, 2% Pharmalyte, pH 3–10, 60 mM DTT, 4% CHAPS, bromophenol blue), the first dimension of the 2D gel was run on IPG strips (Immobiline DryStrip, pH 3–10, 11 cm, GE Healthcare), and the second dimension was run on 12.5% SDS-polyacrylamide gels. For immunodetection, the proteins on the gel were transferred to a PVDF membrane (Millipore, Bedford, MA). Then the membrane was blocked by incubation for 1 h at room temperature in blocking buffer (26 mM Tris-HCl, 150 mM NaCl, pH 7.5, 1% skimmed milk) and incubated overnight at 4 °C with serum samples from DU patients or GC patients or pooled normal sera (1:1000 in blocking buffer containing 0.05% Tween 20) and then for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-human IgG (Chemicon, Temecula, CA). Then bound antibody was detected using 3-amino-9-ethylcarbazole (Sigma) as substrate.

Protein Identification—The individual Coomassie Blue-stained protein spots were excised and subjected separately to in-gel tryptic digestion. Briefly the spots were destained using 50 mM NH₄HCO₃ in 50% acetonitrile and dried in a SpeedVac concentrator. The protein was then digested by incubation overnight at 37 °C with sequencing grade trypsin (Promega, Madison, WI) in 50 mM NH₄HCO₃, pH 7.8. The resulting peptides were extracted sequentially with 1% TFA and

0.1% TFA, 60% acetonitrile, and the combined extracts were lyophilized and analyzed using a QSTAR™ XL Q-TOF mass spectrometer (Applied Biosystems, Framingham, MA) coupled to an UltiMate™ nano-LC system (Dionex/LC Packings, Amsterdam, Netherlands). Peak lists of MS/MS spectra were created using Mascot Search version 1.6b4 in Analyst® QS 1.1 (Applied Biosystems) and uploaded to the Mascot MS/MS Ions Search program (Mascot version 2.0) on the Matrix Science public website, and protein identification was performed against the National Center for Biotechnology Information non-redundant (NCBI nr) database (containing 3,957,439 protein entries at the time searched). Up to one missed cleavage was allowed. Cysteine carbamidomethylation, glutamine/asparagine deamidation, and methionine oxidation were set as possible modifications. The error windows for peptide and MS/MS fragment ion mass values were 0.3 and 0.5 Da, respectively. MH₂²⁺ and MH₃³⁺ were selected as the precursor peptide charge states in the search. Ion scores greater than 54 indicate a significant match; the individual score for the MS/MS spectrum of each peptide was more than 20. From the hit lists, the protein names and locus_tag for *H. pylori* strain 26695 were selected and are listed in Table I and Supplemental Table 1.

Cloning and Purification of the Recombinant Proteins—*H. pylori* was lysed, the lysate was subjected to RNase treatment, and then genomic DNA was extracted using phenol-chloroform and precipitated with 70% ethanol. To amplify the DNA fragments containing the *H. pylori* *fusA* gene, *katA* gene, *ureA* gene, and *flaA* gene (control) by PCR, the primer pairs used were: *fusA*: sense, 5'-GGT ACC ATG GCT AGA AAA ACC CCA-3'; antisense, 5'-CTG CAG TCA GCC TTT GCG TTT TTC-3'; *katA*: sense, 5'-GAA TTC GAT GGT TAA TAA AGA TGT GAA-3'; antisense, 5'-CTC GAG CTT TTT CTT TTT TGT GTG GT-3'; *ureA*: sense, 5'-GGA TCC ATG AAA CTC ACC CCA-3'; antisense, 5'-GGA TCC TTA CTC CTT AAT TGT T-3'; and *flaA*: sense, 5'-GGA TCC ATG GCT TTT CAG GTC AAT-3'; antisense, 5'-GGT ACC CTA AGT TAA AAG CCT TAA G-3'. PCR was performed using 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min followed by a final extension at 72 °C for 15 min. The *fusA*, *ureA*, and *flaA* gene fragments were cloned into the expression vector pQE30 (Qiagen, Hilden, Germany) and transformed into *Escherichia coli* strain M15, whereas the *katA* gene fragment was cloned into the pET-22b(+) vector (Novagen, Madison, WI) and transformed into *E. coli* strain BL21. For expression of recombinant proteins, cells were grown to an A₆₀₀ value of 0.6, induced with 1 mM isopropyl β-D-thiogalactoside, and harvested after 6 h at 25 °C (for UreA), 2 h at 37 °C (for FlaA), or 4 h at 37 °C (for FusA and KatA). The recombinant proteins were dissolved in binding buffer (20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, pH 7.9) containing 8 M urea and purified on a Ni²⁺-chelating Sepharose column (GE Healthcare).

Serologic Study—An *H. pylori* acid-glycine extract and recombinant FusA, KatA, UreA, and FlaA were electrophoresed on 12.5% SDS-polyacrylamide gels, transferred to the PVDF membrane, and then subjected to immunoblotting using serum samples from patients with DU or GC or from normal controls diluted 1:1000 as described above.

Statistical Analysis—Statistical analysis was performed using SAS version 9.1 (SAS Institute Inc., Cary, NC). The odds ratio (OR) and 95% confidence interval (CI) were estimated by multiple logistic regression analysis. Comparisons between tests of serum reactions on protein arrays were made using Student's *t* test. A *p* value of <0.05 was considered statistically significant.

Protein Array Fabrication—Poly(L-lysine)-coated slides were placed in an OmniGrid arrayer (GeneMachines, San Carlos, CA) and four SMP3 printing pins (TeleChem, Sunnyvale, CA) with a tip diameter of 100 μm were used to generate the protein arrays. All proteins were spotted in quadruplicate at three different concentrations (100, 300, and 600 μg/ml) diluted with 0.14–2 M urea in PBS. Each protein array

contained the control spots, human IgG in three dilutions (one normal serum was diluted with PBS to 1:1500, 1:1000, and 1:500). The resulting protein chips were stored in a desiccator.

Serum Reaction on Protein Arrays—All reactions were performed at room temperature. The chips were blocked for 2 h with 2% BSA in TBST (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.6) in a humidified atmosphere and then incubated with the test sera (1:100 in 2% BSA in TBST) for 1 h. Following three TBST washes, the chips were incubated for 1 h in the dark with Cy3-conjugated anti-human IgG antibody (1:500 in 2% BSA in TBST; Abcam, Cambridge, UK) and then washed three times in TBST and twice in distilled water. All incubation steps were performed in a volume of 50 μ l underneath a cover slide. Following air drying, the protein chips were imaged using a GenePix™ 4000B scanner (Axon Instruments, Union City, CA), and

image analysis was performed using GenePix Pro 6.0 (Axon Instruments).

RESULTS

Identification of DU-related Antigens of *H. pylori* by 2D Immunoblot Analysis—To identify the DU-related antigens of *H. pylori*, we first examined the one-dimensional immunoblot profiles of the acid-glycine-extracted bacterial proteins probed with sera from *H. pylori*-infected patients with DU. The molecular masses of the immunoreactive proteins ranged from 23 to 97 kDa (Fig. 1A). The binding pattern of each serum sample was distinct and unique. Moreover several proteins were recognized by sera from normal individuals (Fig. 1B). We then performed 2D electrophoresis on the bacterial proteins and examined the patterns of the 2D immunoblots probed with sera from 10 patients with DU in the active stage. Fig. 2A shows the complex 2D profile of the *H. pylori* acid-glycine-extracted proteins after silver staining. The representative 2D immunoblot probed with one DU serum is shown in Fig. 2B. Numerous protein spots recognized by the DU sera were observed. The majority of the recognized antigens had molecular masses greater than 30 kDa under reducing conditions, and those showing the strongest reaction had molecular masses greater than 50 kDa. To determine which antigens were DU-related and non-DU-related, we compared the frequency of recognition of these by sera from patients with DU and GC ($n = 10$) and identified the proteins by nano-LC-MS/MS analysis (Table I and Supplemental Table 1). The representative 2D immunoblot probed with one GC serum is shown in Fig. 2C. Several proteins recognized at a high frequency by both DU and GC sera were flagellar hook protein, flagellar hook-associated protein (FliD), molecular chaperone

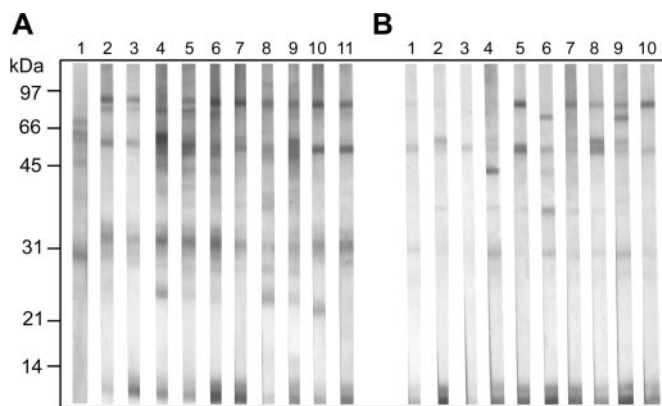


FIG. 1. **One-dimensional immunoblotting of an *H. pylori* extract using serum samples.** An acid-glycine extract of cell surface proteins from *H. pylori* was separated by 12.5% SDS-PAGE, and then the separated proteins were transferred to a PVDF membrane that was stained with Coomassie Blue (A, lane 1) or immunoblotted with sera from *H. pylori*-infected patients with DU (A, lanes 2–11) or *H. pylori*-negative normal individuals (B, lanes 1–10).

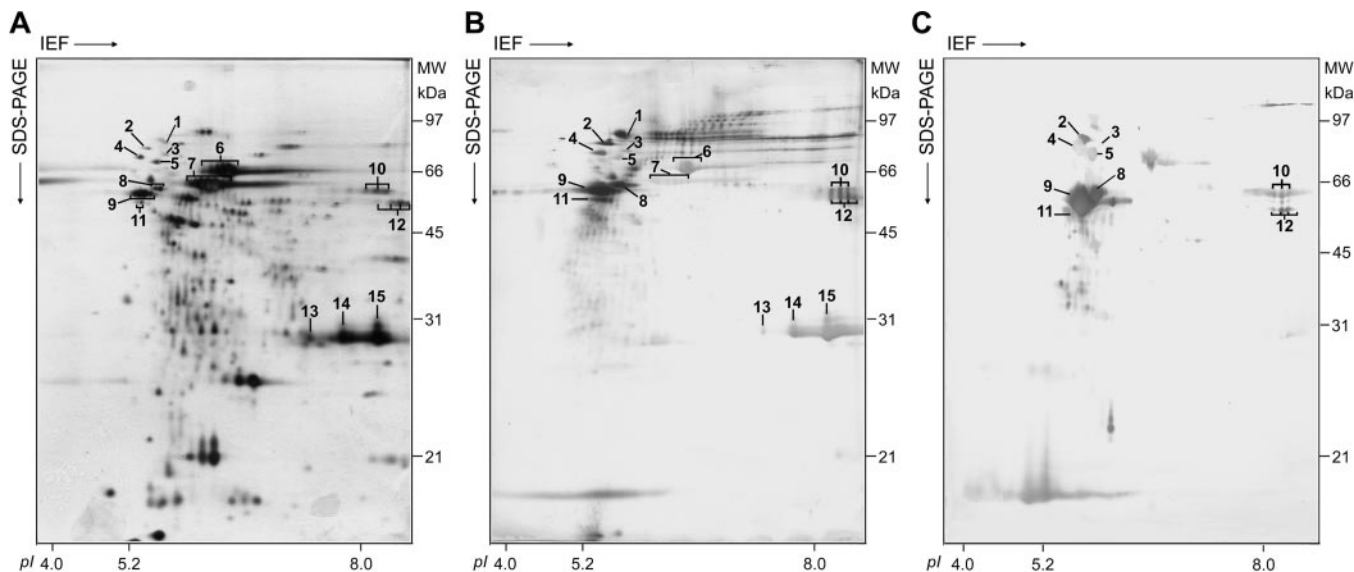


FIG. 2. **2D profiles of immunoreactive proteins in the *H. pylori* sample.** The acid-glycine extract of cell surface proteins from *H. pylori* was separated by 2D electrophoresis using a linear pH 3–10 gradient in the first dimension and 12.5% SDS-PAGE in the second dimension. The separated proteins were detected by silver staining (A) or were transferred to a PVDF membrane and probed with serum from an *H. pylori*-infected patient with DU (B) or GC (C).

TABLE I
Frequency of seropositivity for the indicated *H. pylori* antigens in the DU and GC groups identified by nano-LC-MS/MS analysis

Spot no.	Protein	Locus_tag	Theoretical pl/molecular mass (Da)	Sequence coverage	Score	Seropositivity	
						DU (n = 10)	GC (n = 10)
				%		%	
1	Translation elongation factor EF-G (FusA)	HP1195	5.25/76,972	5	68	70	40
2	Flagellar hook protein	HP0870	5.04/76,160	36	292	100	100
3	Flagellar hook-associated protein (FlhD)	HP0752	5.15/74,043	34	183	100	80
4	Chaperone protein DnaK (<i>Actinobacillus actinomycetemcomitans</i>)		4.88/68,352	28	436	90	80
5	Molecular chaperone DnaK	HP0109	5.04/67,011	36	330	100	70
6	Urease β subunit (UreB)	HP0072	5.71/61,676	52	788	90	80
7	Chaperonin GroEL	HP0010	5.44/58,236	52	500	70	80
8	Flagellin B (FlaB)	HP0115	5.95/53,923	29	153	100	100
9	Flagellin A (FlaA)	HP0601	5.58/53,235	43	575	100	100
10	Catalase (KatA)	HP0875	8.57/58,599	40	259	100	50
11	Flagellin A (FlaA)	HP0601	5.58/53,235	5	72	100	100
12	Serine protease (HtrA)	HP1019	9.18/51,593	56	443	100	80
13	Urease α subunit (UreA)	HP0073	8.47/26,537	46	144	40	20
14	Urease α subunit (UreA)	HP0073	8.47/26,537	66	288	80	20
15	Urease α subunit (UreA)	HP0073	8.47/26,537	61	313	80	20

DnaK, urease β subunit, flagellin A, flagellin B, and serine protease (HtrA). Importantly comparison of DU and GC prevalence data showed that translation elongation factor EF-G (FusA; DU versus GC, 70 versus 40%), catalase (KatA; 100 versus 50%) and urease α subunit (UreA; 40 and 80% versus 20%) were potential candidates for DU-related antigens. Fig. 3 shows the reactivity of FusA, KatA, and UreA in 2D immunoblots probed with 10 DU sera. The location of FusA was close to a spot with strong immunoreactivity. The different isoforms of KatA were recognized equally well by DU sera; in contrast, 40% of DU sera recognized two of three UreA isoforms, and 40% of DU sera recognized all UreA isoforms.

Clinical Significance of FusA, KatA, and UreA Seropositivity—To further examine the clinical significance of FusA, KatA, and UreA as potential candidates for DU-related antigens, we expressed the recombinant His-tagged fusion proteins in *E. coli*. Recombinant FusA, KatA, and UreA with apparent molecular masses of 80, 63, and 28 kDa, respectively, were successfully expressed and purified (Fig. 4, lanes 1–3). The identity of the purified proteins was confirmed by nano-LC-MS/MS, and their antigenicity was examined by immunoblot analysis using pooled sera from DU patients (Fig. 4, lanes 4–6). They were then used in an immunoblot assay using a series of clinical samples including sera from *H. pylori*-infected patients with DU ($n = 124$) or GC ($n = 95$) or non-*H. pylori*-infected normal individuals (control, $n = 40$). As shown in Table II, the prevalence of FusA seropositivity was significantly higher in DU patients than in GC patients (70.2 versus 45.3%; OR = 2.68; 95% CI, 1.52–4.72; $p = 0.0007$) or controls (70.2 versus 20%; OR = 8.42; 95% CI, 3.23–22.0; $p < 0.0001$). The corresponding values for KatA seropositivity in patients with DU, GC, and control were 50.8, 41.1, and 5%. KatA seropositivity in DU patients was significantly higher than in controls (OR = 17.7; 95% CI, 3.83–81.8; $p = 0.0002$) but not significantly different from that in GC patients (OR =

1.45; 95% CI, 0.82–2.56; $p = 0.1976$). UreA seropositivity in DU patients was significantly higher than in GC patients (44.4 versus 27.4%; OR = 2.05; 95% CI, 1.13–3.71; $p = 0.0183$) or controls (44.4 versus 15%; OR = 4.43; 95% CI, 1.51–13.1; $p = 0.0069$). Moreover the seropositivity of each protein in DU patients was significantly different from that in the GC patients and normal individuals combined (FusA: OR = 3.37; 95% CI, 1.97–5.76; $p < 0.0001$; KatA: OR = 2.16; 95% CI, 1.25–3.73; $p = 0.0055$; UreA: OR = 2.38; 95% CI, 1.35–4.20; $p = 0.0029$). As a result, *H. pylori* FusA, KatA, and UreA were antigens related to DU disease.

Combination of Multiple Antigens as Biomarkers of DU Disease—To evaluate whether a combination of multiple antigens increased the ability to distinguish patients with DU from those with GC and normal individuals, we compared the frequency of seroreactivity with a single antigen (FusA, KatA, or UreA) and with combinations of these antigens in gastroduodenal diseases (Table III). Comparing the results for the DU samples with those for the GC samples, the odds ratios increased with the number of recognized antigens, going from 1.82 (95% CI, 0.79–4.21; $p = 0.1607$) for a single recognized antigen to 4.95 (95% CI, 2.05–12.0; $p = 0.0004$) for two recognized antigens and to 5.71 (95% CI, 1.86–17.6; $p = 0.0024$) for three recognized antigens. Similarly when the results for the DU samples were compared with those for the GC samples and normal samples combined, the odds ratios again increased with the number of recognized antigens: 3.14 (95% CI, 1.46–6.77; $p = 0.0035$) for a single antigen, 9.87 (95% CI, 4.35–22.4; $p < 0.0001$) for two antigens, and 12.5 (95% CI, 4.23–36.8; $p < 0.0001$) for three antigens. Using combinations of two of the three antigens, each pair had a significantly higher odds ratio than the single antigen when DU samples were compared with GC samples alone or the population of GC and normal individuals combined. Importantly the combination of KatA and UreA led to the best

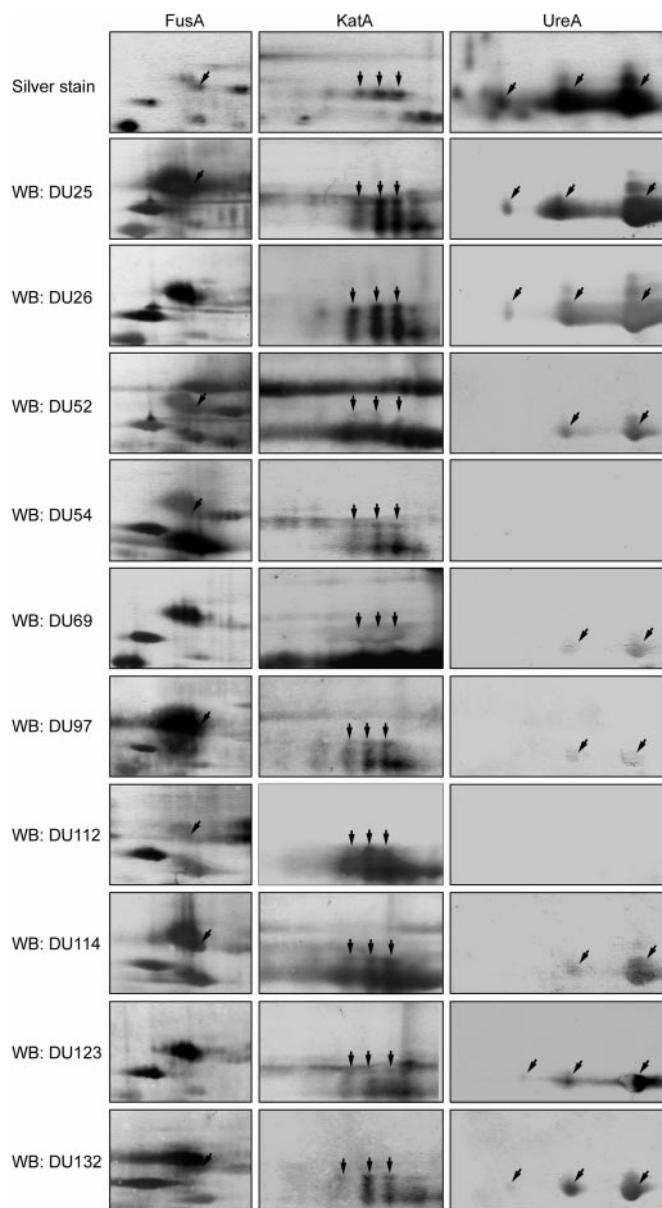


FIG. 3. The patterns of DU-related antigens on 2D immunoblots. The acid-glycine extract of cell surface proteins from *H. pylori* was separated by 2D electrophoresis and transferred to the PVDF membrane; the portions of the silver-stained gel and the immunoblots containing FusA, KatA and UreA protein spots are shown (indicated by arrows). The 2D immunoblots were analyzed by blotting with sera from 10 DU patients. WB, Western blot.

discrimination of DU disease from GC (OR = 16.0; 95% CI, 1.77–144; $p = 0.0136$) or a population of GC and normal individuals (OR = 34.9; 95% CI, 3.95–308; $p = 0.0014$). According to the statistical data, we can use two-antigen and three-antigen combinations of FusA, KatA, and UreA as biomarkers of DU.

Development of a DU-related Antigen Protein Array—To improve the efficiency and convenience of diagnostic usage, we designed a multiple protein array including DU-related

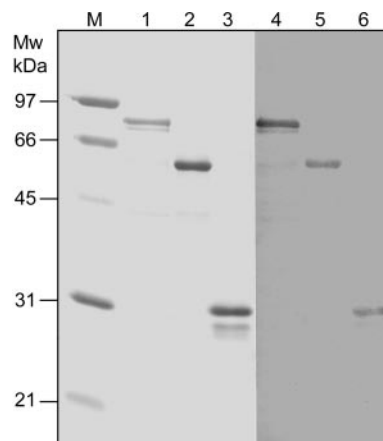


FIG. 4. Immunoreactivity of the recombinant proteins. Purified recombinant FusA (lanes 1 and 4), KatA (lanes 2 and 5), and UreA (lanes 3 and 6) were electrophoresed on a 10% SDS gel and detected by Coomassie Blue (lanes 1–3) or transferred to a PVDF membrane and immunoblotted with pooled DU sera (lanes 4–6). Lane M, molecular mass markers.

antigens and a non-disease-related antigen in a poly(L-lysine)-coated slide. The DU-related antigens were FusA, KatA, and UreA, whereas FlaA was selected as a positive control antigen to monitor the experimental performance. We found that FlaA was recognized by sera from *H. pylori*-infected persons and non-infected persons. All antigens were spotted at three different concentrations (100, 300, and 600 $\mu\text{g/ml}$) to show the trend of antigen-antibody recognition. Human IgG was also included at three serial dilutions for interchip comparison. The chips were incubated with sera from six DU patients and seven normal individuals. Based on the immunoblot analysis, six DU patients whose serum IgG recognized all the DU-related antigens were selected. In contrast, none of the DU-related antigens were recognized by serum IgG from the seven normal individuals. The results of chip reactions with six DU sera and four normal sera are shown in Fig. 5A. The analysis of human IgG showed a low coefficient of variation (26.4, 28.9, and 3.6% for 1:1500, 1:1000, and 1:500, respectively), indicating good reproducibility between chip-serum incubations. When comparing DU sera with normal sera using the average intensity of fluorescence, obviously distinct two groups were observed at each concentration of each protein (Fig. 5B). This experiment demonstrated that the diagnosis testing of a combination of antigens could be performed using a protein array.

DISCUSSION

H. pylori is an important factor in the pathogenesis of DU (17). Clinically about 70% of endoscopically diagnosed DU patients are infected with *H. pylori* (8, 9). To identify potential candidate *H. pylori* antigens related to DU, we used a proteomics approach to analyze *H. pylori* antigens from a strain clinically isolated from a patient with DU in the active stage and found 11 proteins, which reacted strongly with sera from DU patients. Most of these were recognized by all of the DU

TABLE II

Frequency of seroreactivity with a single recombinant antigen in patients with gastroduodenal diseases or controls and the odds ratio

Antigens	Positive no. (%)			OR (95% CI), <i>p</i> value ^a		
	DU (<i>n</i> = 124)	GC (<i>n</i> = 95)	Control (<i>n</i> = 40)	DU vs. GC	DU vs. control	DU vs. (GC + control)
FusA	87 (70.2)	43 (45.3)	8 (20)	2.68 (1.52–4.72), 0.0007	8.42 (3.23–22.0), <0.0001	3.37 (1.97–5.76), <0.0001
KatA	63 (50.8)	39 (41.1)	2 (5)	1.45 (0.82–2.56), 0.1976	17.7 (3.83–81.8), 0.0002	2.16 (1.25–3.73), 0.0055
UreA	55 (44.4)	26 (27.4)	6 (15)	2.05 (1.13–3.71), 0.0183	4.43 (1.51–13.1), 0.0069	2.38 (1.35–4.20), 0.0029

^a The OR, 95% CI, and *p* value were obtained by multiple logistic regression analysis.

TABLE III

Effect of an increased number of recognized antigens on the odds ratio

No.	Recognized antigens	Positive no. (%)			OR (95% CI), <i>p</i> value ^a	
		DU (<i>n</i> = 124)	GC (<i>n</i> = 95)	GC + control (<i>n</i> = 135)	DU vs. GC	DU vs. (GC + control)
0	None	11 (8.9)	22 (23.2)	48 (35.6)	1.0	1.0
1	Any one antigen	41 (33.1)	45 (47.4)	57 (42.2)	1.82 (0.79–4.21), 0.1607	3.14 (1.46–6.77), 0.0035
2	Two of the three antigens	52 (41.9)	21 (22.1)	23 (17.0)	4.95 (2.05–12.0), 0.0004	9.87 (4.35–22.4), <0.0001
	FusA and KatA	26 (21.0)	12 (12.6)	12 (8.9)	4.33 (1.60–11.7), 0.0039	9.45 (3.67–24.4), <0.0001
	KatA and UreA	8 (6.5)	1 (1.1)	1 (0.7)	16.0 (1.77–144), 0.0136	34.9 (3.95–308), 0.0014
	FusA and UreA	18 (14.5)	8 (8.4)	10 (7.4)	4.50 (1.49–13.6), 0.0075	7.85 (2.85–21.6), <0.0001
3	All three antigens	20 (16.1)	7 (7.4)	7 (5.2)	5.71 (1.86–17.6), 0.0024	12.5 (4.23–36.8), <0.0001

^a The OR, 95% CI, and *p* value were obtained by multiple logistic regression analysis.

sera used in the 2D immunoblot analysis in our study and most have been reported as immunoreactive proteins in previous studies (18–20). Kimmel *et al.* (18) identified 29 antigens that reacted with sera from patients with different gastroduodenal diseases but failed to show a correlation between antibody patterns in patients and particular diseases. Haas *et al.* (19) probed *H. pylori* proteins from the strain 26695 with sera from patients with active *H. pylori* infection suffering from gastritis, ulcer, or gastric cancer. They identified 32 *H. pylori* antigens reactive with *H. pylori*-positive sera and characterized several antigens differentially recognized by sera from gastritis and ulcer patients. Mini *et al.* (20) analyzed the *H. pylori* proteins of three strains isolated from patients with chronic gastritis, duodenal ulcer, and GC and probed with sera from patients with GC and DU. They identified 30 antigens and divided these into three groups: pathogen-unspecific, ulcer-associated, and gastric cancer-associated groups. Many immunoreactive proteins of *H. pylori* have been reported by 2D immunoblot studies, such as those mentioned above, but, as far as we are aware, only GroES has been confirmed to be associated with a particular disease (21). In this study, translation elongation factor EF-G (FusA) and flagellar hook protein were identified as *H. pylori* antigens for the first time. Furthermore we also identified three of the 11 proteins as candidate DU-related antigens by comparing the antigenic profile of *H. pylori* proteins from a DU strain probed with sera from patients with DU and GC. In addition to the preliminary data derived from 2D immunoblots, recombinant FusA, catalase (KatA), and urease α subunit (UreA) were used to screen a larger sample size of clinical sera from patients with DU and GC, and the results confirmed the association with DU.

The physiological function of FusA is important for the translocation of peptidyl-tRNA from the A site to the P site within the ribosome (22). In addition, it is also an essential protein in the dissociation of the post-termination complex (23). In *Staphylococcus aureus* and most Gram-negative bacteria, such as *Salmonella typhimurium*, mutation of FusA causes fusidic acid resistance (24, 25). *H. pylori* UreA, the α subunit of urease, is mainly found in the cytoplasm but is also presents at the cell surface and is secreted out of the bacterium (26, 27). *H. pylori* urease, an important *H. pylori* virulence factor, hydrolyzes urea into NH₃ and CO₂ and allows this bacterium to survive in the acidic gastric environment (28). Haas *et al.* (19) reported that UreA showed a significant difference in reactivity with sera from patients with gastroduodenal ulcer compared with gastritis. From our 2D immunoblot data, the frequency of reactivity with FusA and UreA in DU patients was 70 and 40 and 80% (*n* = 10), respectively. It was similar when a larger sample size was screened using the recombinant FusA (70.2%, *n* = 124), whereas it was lower using the recombinant UreA (44.4%, *n* = 124).

H. pylori KatA was purified and characterized by Hazell *et al.* (29) and was found to be located in both the cytoplasm and periplasm (30). In addition, it is reported to be secreted out of *H. pylori* (31), and its catalase activity is essential for the survival of the bacterium at the phagocyte surface (32). Atanassov *et al.* (33) showed that the frequency of KatA seropositivity in patients with gastroduodenal ulcer was greater than 90% (*n* = 55), whereas that in patients with non-ulcer dyspepsia was 67% (*n* = 30) using an extract of a gastritis strain of *H. pylori* and 93% using the ATCC 43579 strain. In our study, as with the UreA data, the frequency of KatA seroreactivity in DU patients was 100% (*n* = 10) by 2D immunoblot

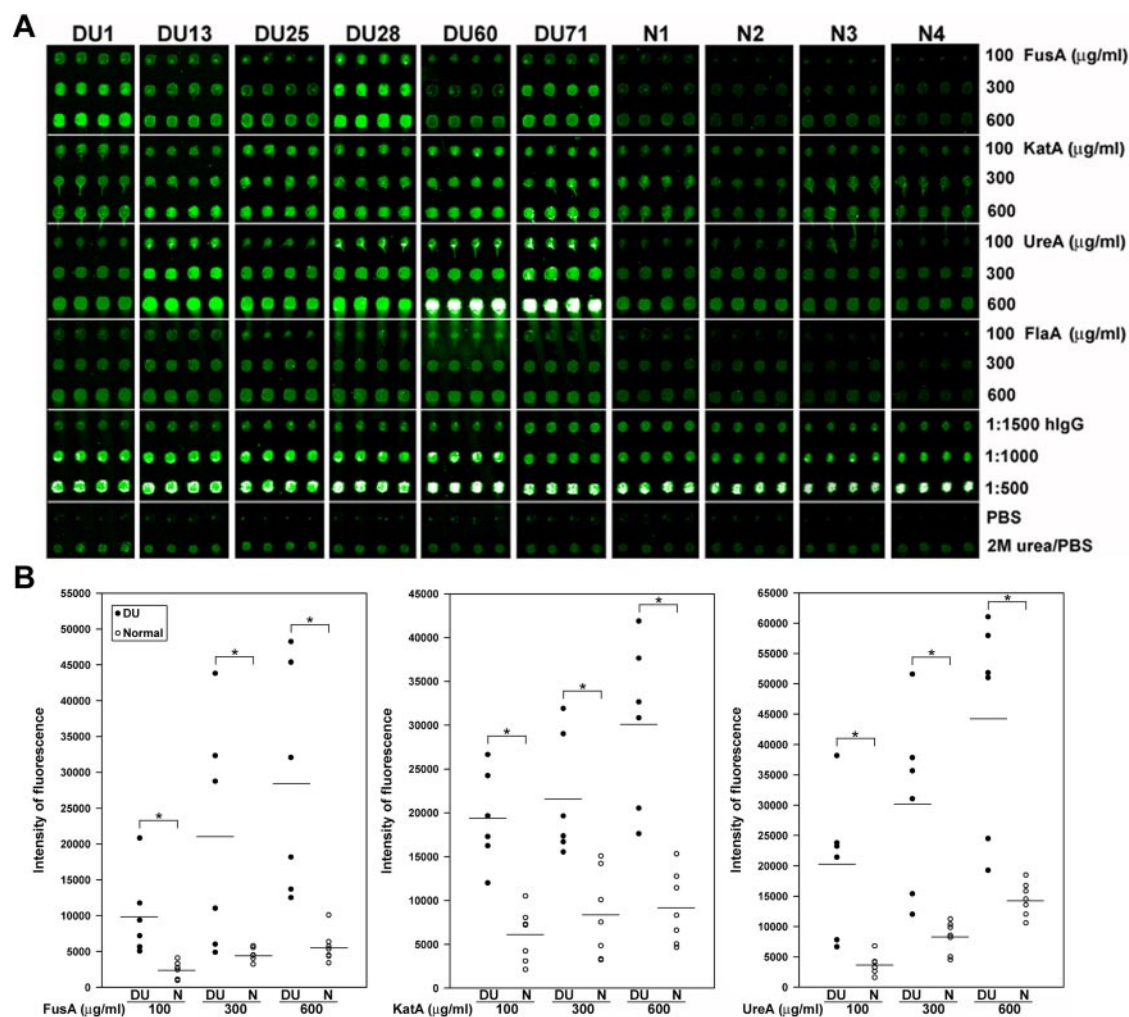


FIG. 5. Development of a DU-related protein array for diagnosis. *A*, all proteins were spotted in quadruplicate at three different concentrations (100, 300, and 600 $\mu\text{g/ml}$) diluted with 0.14–2 M urea in PBS. Each protein array contained human IgG at three dilutions (one normal serum was diluted with PBS to 1:1500, 1:1000, and 1:500) for interchip comparison. FlaA was used as a positive control antigen to monitor the experimental performance. The chips were incubated with sera from DU patients and normal individuals, and bound antibody was detected by the fluorescence of the Cy3-conjugated anti-human IgG (*hIgG*) antibodies. *B*, comparison of the serum reactions of six DU patients and seven normal individuals with the three DU-related antigens, FusA, KatA, and UreA. Student's *t* test was used for statistical evaluation (*, $p < 0.05$). *N*, normal.

analysis but was 50.8% ($n = 124$) by larger sample size screening using recombinant KatA. Although KatA seropositivity was not associated more with DU than with GC, it improved the prediction of DU when combined with FusA and/or UreA (Table III). KatA is therefore also an important DU-related antigen.

Our statistical analysis revealed that combinations of antigens improved the ability to distinguish between patients with DU and GC and normal individuals. The three-antigen combination gave excellent discrimination, and this phenomenon also implies the value of each antigen related to DU disease. However, heterogeneity of the host immune response to different *H. pylori* antigens may explain the relatively low occurrence of multiseropositivity in particular individuals in this study. For the preliminary examination of the risk of DU in the

clinic, we suggest a rule considering positivity of two or three antigens as high risk to DU to compensate for this phenomenon.

H. pylori infection can be diagnosed by noninvasive methods or by endoscopy. Serologic testing, a noninvasive method, is inexpensive and widely used for the diagnosis of *H. pylori* infection before treatment (34). Generally patients only agree to invasive endoscopy when they have alarming symptoms, such as stomachache, anemia, or gastrointestinal bleeding. Despite advances in therapies, gastrointestinal bleeding is still a cause of morbidity and mortality (35). Serologic tests are more acceptable and can be carried out in a routine physical examination. Following a finding of seropositivity for two or three antigens (*i.e.* FusA, KatA, and UreA), patients would be recommended to undergo endoscopic ex-

amination. We believe this concept will promote the early detection and treatment of DU disease.

To apply the idea of diagnosis using multiple biomarkers, we constructed a protein array containing the three DU-related antigens and compared the antibody binding patterns of sera from DU patients and normal individuals. At each concentration of each of the three antigens, the signal intensity was higher with DU sera than with normal sera. Although the serum reaction using an antigen concentration of 100 $\mu\text{g/ml}$ was able to distinguish the DU group from the controls, the use of a single cutoff value for a single protein concentration might lead to false positive or false negative results. We therefore used three concentrations of each antigen to monitor the trend of signal intensities. As long as two of the three signal intensities were higher than each cutoff value of protein concentrations, the sample was considered seropositive for a particular antigen. This results in fewer false positives.

In conclusion, here we report a comparison of the reactivity of serum antibodies from DU and GC patients to the *H. pylori* proteome, leading to the identification of FusA, KatA, and UreA as DU-related antigens of *H. pylori*. We further demonstrate that the combination of these three antigens can be used as a biomarker as a preliminarily means of distinguishing patients with DU from those with GC. Moreover we have developed a DU-related protein array using the combination of these three antigens for the purpose of clinical diagnosis.

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✉ To whom correspondence should be addressed: Graduate Inst. of Biochemistry and Molecular Biology, College of Medicine, National Taiwan University, No. 1, Sec. 1, Jen-Ai Rd., Taipei 100, Taiwan. Tel.: 886-2-23123456 (ext. 8212); Fax: 886-2-23958814; E-mail: lupin@ha.mc.ntu.edu.tw.

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