Comparative Proteome Analysis of Cyanidin 3-O-glucoside Treated *Helicobacter pylori*

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Some virulence proteins of *Helicobacter pylori*, such as vacuolating cytotoxic protein A (VacA) and cytotoxin-associated gene protein A (CagA) have been reported to be causative agents of various gastric diseases including chronic gastritis, gastric ulcer or gastric adenocarcinoma. The expression level of these virulence proteins can be regulated when *H. pylori* is exposed to the antibacterial agent, cyanidin 3-O-glucoside (C3G) as previously reported. In this study, we analyzed the quantitative change of various virulence proteins including CagA and VacA by C3G treatment. We used 2-dimensional electrophoresis (2-DE) to analyze the quantitative change of representative ten proteome components of *H. pylori* 60190 (VacA⁺/CagA⁺; standard strain of Eastern type). After 2-DE analysis, spot intensities were analyzed using ImageMasterTM 2-DE Platinum software then each spot was identified using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) or peptide sequencing using Finnigan LCQ ion trap mass spectrometer (LC-MS/MS). Next, we selected major virulence proteins of *H. pylori* among quantitatively meaningful ten spots and confirmed the 2-DE results by Western blot analysis. These results suggest that cyanidin 3-O-glucoside can modulate a variety of *H. pylori* pathogenic determinants.

Key Words: *Helicobacter pylori*, Cyanidin 3-O-glucoside, 2-dimensional electrophoresis, VacA

INTRODUCTION

Numerous attempts have been made to identify markers for virulent *H. pylori* strains by proteomics analysis. Vacuolating cytotoxic protein A (VacA) and cytotoxin-associated protein A (CagA) have been reported as representative pathogenic determinants in *H. pylori* (Censini et al., 1996; Cover et al., 1993). Especially the genes located within the Cag pathogenicity island (Cag PAI) are thought

to be responsible for the increased virulence of *H. pylori* (Israel et al., 2001; Glocker et al., 1998; Meyer-ter-Vehn et al., 2000; Selbach et al., 2002). Although other bacterial factors such as urease (Megraud et al., 1992), flagella (Megraud et al., 1996), adhesin (Doig et al., 1992), and ferritin (Waidner et al., 2002) also have been reported as pathogenic determinants, understanding the cellular pathogenesis of *H. pylori* in the pathogenesis of gastric diseases, as well as identifying virulent strains, requires further studies. Currently, the complete genome sequences of *H. pylori* strains 26695 and J99 have been reported (Alm et al., 1999; Tomb et al., 1997). In addition, further genomic information is accumulating rapidly which combined with identification techniques to find disease or organism-specific markers have greatly improved our understanding of pathogenic factors in *H. pylori*. In this study, 2-dimensional electrophoresis (2-DE)

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and protein identification techniques were used to analyze the effect of cyanidin 3-O-glucoside on the expression of various *H. pylori* pathogenic proteins.

MATERIALS AND METHODS

Cyanidin 3-O-glucoside (C3G)

C3G (Extrasynthese; Lyon, France) was dissolved in sterile dimethyl sulfoxide (DMSO; Sigma Aldrich, St. Louis, MO, USA) as a 50 mM stock solution and stored at -20°C prior to usage.

Bacterial strain and culture

Helicobacter pylori reference strain 60190 (Eastern type: CagA⁺/VacA⁺) was purchased from American Type Cell Collection (ATCC; Manassas, VA, USA). Bacteria were maintained under microaerophilic conditions at 37°C on Brucella agar plates (Becton-Dickinson, Baintree, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Long Island, NY, USA). For experiments, *H. pylori* were cultured in Mueller-Hinton broth (Becton-Dickinson) containing 10% FBS under microaerophilic condition for 3~4 days at 37°C and 100% humidity.

Protein sample preparation & 2-dimensional gel electrophoresis

The harvested samples were suspended in 0.5 ml of 50 mM Tris buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, and 16 µl protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IA, USA). For 2-DE analysis, pH 4~7 IPG strips (Amersham, Pittsburgh, PA, USA) were used. The 2-D separation was performed on 8~16% (w/v) linear gradient SDS-polyacrylamide gels. Protein spot detection and 2D pattern matching were carried out using ImageMasterTM 2D Platinum software (Amersham). For comparison of protein spot densities between control and treated samples, more than 20 spots throughout all gels were correspondingly marked and normalized. The quantified spots of candidate proteins were compared with the aid of histograms. To ensure reproducibility of 2-DE experiments, each sample was analyzed in triplicate.

In-gel digestion with trypsin and extraction of peptides

In-gel digestion of protein spots from Coomassie Blue stained gels was performed by ProteomeTech (Seoul, South Korea). In brief, protein spots were excised from stained gels and cut into pieces. The excised protein spots from a silver stained gel were cut into pieces and in-gel digested with trypsin.

Analysis of peptides using MALDI-TOF MS and identification of proteins

Mass measurement of tryptic peptides were carried out with a Voyager-DE STR mass spectrometer (PerSeptive Biosystems, Bedford, MA, USA). Close external calibration was performed for every sample with calibration mixtures. The proteins were identified by peptide mass fingerprinting (PMF) searching, against the Swiss-Prot and NCBI databases, using the search program ProFound (http://129.85.19.192/profound_bin/WebProFound.exe, Rockefeller University, Version 4.10.5), MASCOT (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF), or MS-Fit (<http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm>, University of California San Francisco, Version 4.0.5).

Identification of proteins by LC-MS/MS

The resulting tryptic peptides were separated and analyzed using reversed phase capillary HPLC directly coupled to a Finnigan LCQ ion trap mass spectrometer (LC-MS/MS). For tandem mass spectrometry, a full mass scan range mode was $m/z = 450\sim 2000$ Da. The individual spectra from MS/MS were processed using the TurboSEQUENT software (Thermo Quest, San Jose, CA). The generated peak list files were used to query either MSDB database or NCBI using the MASCOT program (<http://www.matrixscience.com>). Only significant hits as defined by MASCOT probability analysis were considered initially.

Western blot analysis

H. pylori were lysed in ice-cold RIPA lysis buffer (Millipore, Billerica, MA, USA) for 30 minutes on ice and sonicated for 2 minutes with 10 second intervals (Sonicator XL-2020, Heat Systems Ultrasonics, Pittsburgh, PA, USA).

KATO III cells (5×10^6) were plated in 10-cm plates. Following infection, cells were washed with PBS, lysed with 150 μ L of RIPA buffer on ice for 30 min. Lysates of culture dishes treated identically were pooled and clarified at $13,000 \times g$ for 10 min at 4°C. Protein concentration was determined by the Lowry method and quantified using NanoQuant spectrophotometer (Infinite M200, TECAN, Männedorf, Switzerland). Protein extracts were resolved on 7.5%, 10% or 4~15% gradient SDS-PAGE and then transferred to a nitrocellulose membrane (Millipore). Membranes were blocked with 5% skim milk for 30 minutes and then incubated with rabbit anti-VacA polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-*H. pylori* SecA polyclonal antibody (Kim et al., 2014) or rabbit anti-*H. pylori* polyclonal antibody (Kim et al., 2012). Appropriate

horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) were used and protein bands were visualized using enhanced chemiluminescence and X-ray film. The bacterial supernatant was centrifuged at 3,000 rpm three times and filtered with 0.22 μ m syringe filters to remove remained all bacteria. The prepared supernatant was concentrated 10-fold using Centricon centrifugal filters (Millipore; 3 kDa cut-off) at 3,000 rpm for 1~2 hours at 4°C.

RESULTS AND DISCUSSION

Approximately 200 spots from silver-stained gels were recorded by the PDQUEST program. Following proteomic analysis, we selected ten quantitatively meaningful protein

Table 1. Representative virulence protein changes of C3G treated *H. pylori* 60190 strain

Spot No.	Protein ^{a)}		2-DE data ^{b)}			
	TIGR locus name	Protein name	pI / M _r	Protein-change ratio (%) ^{c)} after C3G treatment	Standard ^{d)} deviation	Reference
1	HP0547	Cytotoxin-associated gene protein A (CagA) fragment	5.75 / 81,500	+ 252.87	4.31	González L et al., 2013
2	HP0887	Vacuolating cytotoxic protein A (VacA) fragment	6.20 / 95,740	+ 644.96	2.81	Backert S et al., 2005
	HP0887	Vacuolating cytotoxic protein A (VacA) fragment	6.65 / 87,970	+ 2243.31	3.73	
3	HP0786	Preprotein translocase subunit SecA (SecA)	6.12 / 99,207	- 416.24	1.68	this study
4	HP0072	Urease subunit beta (UreB)	6.09 / 61,645	- 311.47	2.27	Backert S et al., 2005
5	HP0210	Heat shock protein 90 (Hsp90)	5.73 / 71,230	- 3513.95	4.90	this study
6	HP1205	Elongation factor thermo unstable (EF-Tu)	5.36 / 43,594	- 1144.74	12.71	Israel DA et al., 2001
7	HP0809	Flagellar basal body protein FlilL (FlilL)	5.41 / 55,134	- 714.91	1.69	this study
8	HP0779	Aconitate hydratase AcnB (AcnB)	6.44 / 118,230	- 2547.76	10.23	Backert S et al., 2005
9	HP0322	Poly E-rich protein	4.30 / 91,100	+ 200.71	3.79	Backert S et al., 2005
10	HP0480	GTP-binding protein	6.80 / 68,000	+ 188.24	21.96	Backert S et al., 2005

a) Protein designation according to *H. pylori* strain NCBI (<http://www.ncbi.nlm.nih.gov/>), KEGG database (<http://www.genome.jp/kegg/>).

b) According to peptide mass fingerprinting (PMF) data were obtained from Fig. 1.

c) The ratio of protein-change amount was calculated as follows:

$$\text{change ratio (\%)} = \frac{\Delta \text{ value (protein amount w/ C3G - protein amount w/o C3G)}}{\Delta \text{ value (protein amount w/o C3G)}} \times 100$$

d) Standard deviation of protein-change ratio in each spot was obtained from repeated five independent 2-ED analysis.

Fig. 1. A representative two-dimensional gel-electrophoresis map of *H. pylori* 60190 strain untreated (left panel) or treated with C3G (right panel). The spots were identified by MALDI-TOF-MS or TOF-MS. Molecular size markers (kDa) are shown on the left.

spots including CagA, VacA, protein translocase subunit A (SecA), urease subunit beta (UreB), heat-shock protein 90 (Hsp90), elongation factor thermo unstable (EF-Tu), flagella basal body-associated protein FliL (FliL), aconitate hydratase (AcnB), Poly E-rich protein, and GTP-binding protein (Fig. 1). The selected ten protein spots were changed in the 2-DE images and were identified by peptide mass fingerprinting using MALDI-TOF-MS or by peptide sequencing using LC-MS/MS (Table 1).

Among the ten quantitatively meaningful proteins we focused on six proteins - CagA, VacA, SecA, Hsp90, EF-Tu, and FliL - because these had been previously investigated as disease-specific markers and are used as landmarks to analyze clinical isolates (Park et al., 2006; Backert et al., 2005). These proteins were readily identified and their intensities measured in 2-DE gels of pH 4~10 (Fig. 1). Representative 2-DE spot profiles of *H. pylori* 60190 strain among total spot profiles of protein changes were compared to reference data (Choi et al., 2002; Jungblut et al., 2000). The intensities of six target protein spots in 2-DE profiles of *H. pylori* 60190 standard strain are associated with clinical diseases, such as chronic gastritis, gastric ulcers, or gastric adenocarcinoma (Park et al., 2006; Backert et al.,

2005). *H. pylori* CagA is strongly associated with a significant increase of risk for the development of gastric disorders like ulcers, adenocarcinoma or MALT lymphoma (Censini et al., 1996). Especially, the Eastern Asian strains of *H. pylori* are predominantly CagA-positive, responsible for gastric diseases (Ito et al., 1997). Here, our data showed that the CagA intensity in C3G-treated group was significantly lower than the untreated group. This result suggests that CagA expression might regulated by C3G (Fig. 1 and 2). *H. pylori* VacA is associated with the apoptosis and necrosis of *H. pylori*-infected gastric epithelial cells. It has been reported that VacA forms cytotoxic vacuoles in host cells and is a cause of gastric cell death and gastric and/or duodenal inflammation and ulcer (Rassow, 2011). The level of VacA was increased in C3G treated cells (Fig. 1 and 2). This result suggests that C3G down-regulated the secretion of VacA and thus resulting in accumulation of VacA in *H. pylori* cells. We investigated this phenomenon with SecA-correlation by Western blot analysis (Fig. 3).

H. pylori Sec-related proteins are associated with bacterial type V secretion system (T5SS) (Cover, 2005). Although the mechanism of this secretory system is still under intense investigation, VacA secretion is regulated by the type V_a

Fig. 2. Quantitative changes of major pathogenic proteins identified in this study.

secretion system (T5_aSS) which is SecA-dependent (Cover, 2005; Shaffer et al., 2011). The SecA protein expression was decreased in C3G treated group compared to the untreated group. This suggests that the decreased SecA levels might affect the accumulation of VacA in cell. *H. pylori* Hsp90 showed decreased levels in the C3G treated group although the functional relevance is unclear at this time. *H. pylori* EF-Tu, the translation factor of *H. pylori* has been reported

to be highly immunoreactive to both IgG and IgA, and its expression level is significantly high in ulcer or chronic-gastritis patients (Lock et al., 2002). In our study, the expression level of EF-Tu was significantly decreased in the C3G treated group (Fig. 1 and 2). We also found that *H. pylori* FliL levels decreased after C3G treatment (Fig. 2). FliL is a transmembrane protein located adjacent to the basal body of its flagellar structure. It is thought that a bacterial-

Fig. 3. Effects of cyanidin 3-O-glucoside on secretion of *H. pylori* VacA and expression of SecA. *H. pylori* was cultured with various concentrations (0~200 μM) of C3G in Mueller-Hinton broth/10% FBS for 3 days and protein levels assessed by Western blot. (A) intracellular VacA and SecA, (B) intracellular *H. pylori* proteins reacting with rabbit anti-*H. pylori* polyclonal antibody, (C) secreted VacA in culture media, (D) secreted *H. pylori* proteins reacting with rabbit anti-*H. pylori* polyclonal antibody. Representative image from five independent experiments.

type flagellum is a motor complex composed of an extra-cellular helical protein filament coupled to a rotary motor embedded in the cell envelope. FliL has a single trans-membrane domain near its N-terminus (Lee and Belas, 2015). The function of FliL in *H. pylori* is still unclear, although it has been reported that FliL is essential for swarming in *Proteus spp* (Lee et al., 2013).

It has been reported that SecA is involved in trans-location of bacterial secretory proteins out of the bacterial plasma membrane through T5_αSS (Kim et al., 2014). Thus the decreased SecA expression by C3G offers a mechanistic explanation as to why C3G induces intracellular accumu-

lation of VacA. We already developed SecA-specific anti-bodies in our previous study (Kim et al., 2014), and it was used to determine SecA protein levels in this study. SecA expression was decreased in the C3G treated group in a dose-dependent manner (Fig. 3A and 3B). We found that the level of secreted VacA in the culture supernatant was decreased (Fig. 3C and 3D).

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Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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