Helicobacter pylori and diethylnitrosamine altered the expression of acute-phase serum proteins in vitamin C deficiency *Gulo*$^{-/-}$ mice

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Abstract

Helicobacter pylori-infects approximately half of the world's population that's known to be an important pathogen associated with various severe gastric diseases, including peptic ulcers, chronic gastritis, and gastric cancer. Even though, diethylnitrosamine (DENA) is well known hepatocarcinogen, it also provokes esophageal cancer *in vivo*. Hence, we established *Gulo*$^{-/-}$ mice model like human (it can't synthesis vitamin C) and, serum proteins were separated by using 2-dimensional gel electrophoresis (2-DE) and differentially expressed proteins in response to *H.pylori*-infection and followed by DENA treatment were identified by using matrix-assisted

laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). By comparative proteome analysis of *Gulo*$^{-/-}$ mice serum, a total of 47 statistically significant altered expression protein spots were selected (spots which shows ≥2 fold in terms of expression and $p<0.05$) and 38 proteins were successfully identified by using MALDI-TOF-MS and database searching. The results showed that the altered expressions of Alpha-1B-glycoprotein, Haptoglobin, Collagen alpha-2(IX) chain, Antithrombin-III, Hemopexin, Glutathione S-transferase, Complement C3 and Apolipoprotein A-IV in *H.pylori*-infection and DENA group when compared to control group. There proteins were involved in various physiological pathways including acute phase response signaling, inflammatory regulation and immune response. Moreover, this is the first report that combined effect of *H. pylori*-infection with DENA in *Gulo*$^{-/-}$ mice serum. These findings provide new leads for researchers to better understanding of the pathophysiologic mechanisms of *H. pylori*-induced gastric diseases and DENA, early diagnosis, and therapy of *H. pylori* and DENA associated gastric disorders.

Key words: *Gulo*$^{-/-}$ mice; Vitamin C; Gastric cancer; Helicobacter pylori; Diethylnitrosamine; Proteome analysis

{**Citation:** Arulkumar Nagappan, Hyeon Soo Park, Kwang Il Park, Jin A Kim, Gyeong Eun Hong, Silvia Yumnam, Eun Hee Kim, Won Sup Lee, Wang Jae Lee, Myung Je Cho, Woo Kon Lee, Chung Kil Won, Gon Sup Kim. Helicobacter pylori and diethylnitrosamine altered the expression of acute-phase serum proteins in vitamin C deficiency *Gulo*$^{-/-}$ mice. American Journal of Research Communication, 2013, 1(9): 215-239} www.usa-journals.com, ISSN: 2325-4076.

Introduction

Gastric cancer (stomach cancer) is second most common cause of cancer-related deaths in the world, next to lung cancer (Gonzalez and Agudo, 2012). According to the Cancer Statistics in Korea (2012) report, gastric cancer poses a major public health concern and a total of 28,078 (18,898 male and 9,180 female) new cases are estimated to occur in Korea in 2012 (Jung et al., 2012). Most stomach cancers are an adenocarcinoma type, which accounts approximately 90% (Kelley and Duggan, 2003). In advanced stage, gastric cancer is generally accompanied by metastases to the peritoneum, lymph nodes, or other organs which increase the mortality rate of patients. The average 5-year survival rate of patients with stage I was 91.2%, but patients with stage IV was only 9.4%. Preventive measures for stomach cancer are insufficient and it's difficult to cure because most patients present with advanced disease. Therefore, it is important to understand the molecular mechanism of carcinogenesis and metastasis of gastric cancer, and to identify the biomarkers for the early diagnosis and effective treatments.

A global proteomic approach is being extensively applied in cancer research (Alaiya et al, 2000). This approach uses a combination of two-dimensional gel electrophoresis (2-DE), image analysis, matrix-assisted laser desorption/ ionization-time of flight (MALDI-TOF) mass spectrometry (MS), and bioinformatics analyses to comprehensively resolve, identify, and characterize proteins in cells, tissues and animal models. The comparative proteome studies between healthy and cancer cells with the purpose of developing biomarkers and therapeutic targets. From cancer patients, serum is highly preferred sample for the early diagnosis because its need less invasive methods to collect the samples. Presently, carcinoembryonic antigen (CEA), carbohydrate antigen (CA) 19–9 and CA72–4 are the most widely serum biomarkers for gastric cancer, however the specificity is low (Marrelli et al., 1999; Takahashi et al., 2003). Moreover,

gastric cancer screening has been done using the serum ratio of pepsinogen I/II only in Asian populations (Miki, 2006; Ang et al., 2005). However, there is no genuine serum biomarker available for detection of gastric cancer at early-stage.

Vitamin C (ascorbic acid) is a water-soluble antioxidant and, an essential nutrient of most living tissues (Acunzo et al., 2012). Humans must obtain vitamin C through daily diet due to lack of L-*Gulo*no-g-lactone oxidase (*GULO*), gene encoding the key enzyme in ascorbic acid biosynthesis of most mammalian species. Even though humans overcome ascorbic acid deficiency by diet, its requirements are vary greatly among individuals for optimal health. The most common laboratory animals including the mice and rat possess a functional *GULO* gene that can readily synthesize ascorbic acid when destitute of this vitamin in the diet. In such case, ascorbic acid deficiency animal would be appropriate and prominent model for human disease studies. Recently, *Gulo*$^{-/-}$ mice model have been used for *in vivo* experiment (Li et al., 2008) and these *Gulo*$^{-/-}$ mice strain is known with deficiency, in which vitamin C intake can be controlled by diet like human, would be valuable for investigating the molecular mechanism of various diseases.

The primary factors for a high incidence of stomach cancer are *Helicobacter pylori* infection or/and diet (Marchetti et al., 1995; Ray, 2005). *H.pylori* -infects approximately half of the world's population that colonizes the mucosal layer overlying the gastric epithelium of the human stomach. This bacterium has been classified as a group 1 carcinogen by the World Health Organization and it's known to be an important pathogen associated with various severe gastric diseases, including peptic ulcers, chronic gastritis, and gastric cancer (Houghton et al., 2002). Epidemiological studies have reported that H. pylori infection is a risk factor for gastric carcinoma and vitamin C deficiency humans linked to more severe *H. pylori*-associated gastritis

and a gastric cancer risk also higher (Zhang et al., 1998; Correa et al., 1998). Moreover, Diethylnitrosamine (DENA) is a well-known N-nitrous compound that provokes esophageal cancer in laboratory animals (Sallet et al., 2002; Sallet et al., 2002). Presently, only limited studies reported regarding DENA effects on gastric cancer. Hence, the study of both *H. pylori* infection and DENA treatment in laboratory animals will provide further evidence supporting a link between gastric cancer, *H. pylori* and DENA.

The aim of the present study was to separate proteins from serum of *Gulo*$^{-/-}$ mice for quantitative comparison, and to identify proteins which showing altered expression by MALDI-TOF-MS. To our knowledge, this study is the first report that profiling of protein changes in *Gulo*$^{-/-}$ mice serum after *H. pylori*-infection alone and/or combined with DENA by proteome analysis. The findings of this study will also provide new leads for researchers to better understanding of the pathophysiologic mechanisms of *H. pylori*-induced gastric diseases and combined effect of DENA, early diagnosis, and therapy of *H. pylori* and DENA associated gastric disorders.

Materials and methods

Chemical and reagents

Materials and chemicals used for electrophoresis were obtained from BioRad (Hercules, CA, USA). All other chemicals used in this study were purchased from AMRESCO (Solon, OH, USA) and Sigma-Aldrich (St. Louis, MO, USA). All the chemicals used were of the highest grade available commercially.

Animals

Gulo$^{-/-}$ mice were kindly provided by Prof. Wang Jae Lee (Department of Anatomy, Seoul National University College of Medicine). *Gulo*$^{-/-}$ breeding pairs were originally obtained from the Mutant Mouse Regional Resource Centers, University of California at Davis. Genotypes of the off springs were evaluated by PCR as recommended (Maeda et al., 2000). Female *Gulo*$^{-/-}$ and C57BL/6 mice at 6-7 weeks of age were used, and they were maintained in specific pathogen free condition in the animal facility at the Gyeongsang National University School of Medicine with the animal experiments protocol reviewed and approved by Ethics Committee of the Gyeongsang National University. *Gulo*$^{-/-}$ mice were supplemented with 1.0 g/L of vitamin C in drinking water to prevent the death by vitamin C deficiency (Kim et al., 2012). We followed animal Science guidelines for animal experimentation.

Experimental design and sample collection

Gulo$^{-/-}$ mouse was divided the animal groups into three groups such control, *H. Pylori* infected group and *H. Pylori* infected followed by DENA treatment (Table 1). These all three groups, vitamin C was supplemented (20mg/animal/day, PO) and only group 3 treated DENA (10mg/L, PO). After 48 weeks, sacrificed all animals, serum were collected. Samples were stored at -70°C until analysis.

Protein extraction and two-dimensional gel electrophoresis

100µg of serum protein was focused in the first dimension using the Ettan IPG Phor II (GE Healthcare) at 50 V for 1 h, followed by 200 V for 1 h, 500 V for 30 min, 4000 V for 30 min, 4000 V for 1 h, 10000 V for 1 h, 10000 V for 13 h, and 50 V for 3 h. The focused strips were

equilibrated twice for 15 min each time, first with 10mg/ml DTT and then with 40 mg/ml iodoacetamide (IAA) prepared in equilibration buffer containing 50 mM Tris–HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 0.002% (w/v) Bromophenol blue. The focused proteins were then separated in the second dimension by 10% linear gradient SDS-PAGE with a constant current of 20mA/gel at 20°C. Gels were run until the Bromophenol dye front reached the end of the gel.

Table 1. Experimental procedure followed in *Gulo*$^{-/-}$ mice

	Vitamin C[a]	H.pylori	DENA[b]
Group I (Control)	√	-	-
Group II (H.pylori infected group)	√	√	-
Group II (H.pylori infected followed by DENA treatment)	√	√	√

[a] Vitamin C - 20mg/animal/day, PO
[b] DENA - 10mg/L, PO

Protein detection, analysis, and in-gel digestion

The gels were stained with silver nitrate, similar to the method described by (Swain and Ross, 1995) with slight modifications. Three independent gels were performed in triplicate. Gels were scanned and Image analysis was performed using Progenesis Samespots software (Nonlinear Dynamics, Newcastle, UK). Using this software, the differentially expressed spots were identified by automatic matching of the detected protein spots. Those spots differing significantly ($p<0.05$, one-way ANOVA test) in their intensities with a fold-change ≥2 were used

for further analysis. Selected protein spots were excised manually from the two-dimensional electrophoresis (2-DE) gel and protein digestion was performed (Shevchenko et al., 1996) with slight modifications. Briefly, the excised gel pieces were washed with 100 µl of 100mM NH4HCO3 for 5 min and then dehydrated in 100µl of acetonitrile for 10 min. After dried in a lyophilizer (SFDSM06, Samwon Freezing Engineering Co., Busan), the gel pieces were rehydrated in 5–10 µl of 50mM NH4HCO3 containing 20ng/µl trypsin (Promega, Madison, WI, USA) on ice. After 45 min, the trypsin solution was removed and replaced with 10–20 µl of 50mM NH4HCO3 without trypsin, and digestion was carried out for a minimum of 16 h at 37oC. These peptide mixtures were collected and analyzed by mass spectrometry.

MALDI-TOF/MS analysis and database searching

Tryptic peptides obtained as described above were subsequently extracted by addition of 10µl of extraction buffer followed by addition of 10–15 µl of acetonitrile. Pooled extracts were dried in a lyophilizer (SFDSM06, Samwon Freezing Engineering Co., Busan) and the extracts were re-dissolved in 1µl of extraction buffer and 1µl of matrix solution (α-acyano- 4-hydroxycinnamic acid, HCCA) and targeted onto a MALDI-TOF plate. After drying the samples completely onto the targeting plate, MALDI-TOF/MS was conducted using a Voyager- DE STR mass spectrometer (Applied Biosystems, Franklin Lakes, NJ, USA) equipped with delay ion extraction. Mass spectra were obtained over a mass range of 800–3,000 Da. For identification of proteins, the peptide mass fingerprinting data were used to search against the Swissprot database using the Mascot program (http://www.matrixscience.com). The following parameters were used for database searches: taxonomy, Mus musculus (Mouse); cleavage specificity, trypsin with one missed cleavage allowed; peptide tolerance of 100ppm for the fragment ions; allowed

modifications, Cys Carbamidomethyl (fixed), oxidation of Met (variable). Protein scores >56 were considered statistically significant ($p<0.05$).

Statistical analysis

The data represent mean±standard deviation (SD) of three independent experiments. The statistical significance between control and test groups were calculated by the Student's t-test. A p value <0.05 was considered as significant.

Results

Protein separation by 2-DE and image analysis

To construct a 2-DE map of *Gulo*$^{-/-}$ mice serum after *H.pylori* infected and followed by DENA treatment, 100µg of total protein were separated by IEF on 18 cm IPG strips in the first dimension and 10% SDS-PAGE in the second dimension. Silver staining was applied to visualize the protein spots, because of its compatibility and sensitivity with trypsin digestion and MS analysis. Also, silver staining is the most sensitive method for permanent staining of proteins in polyacrylamide gels. The experiment was repeated three times. Approximately 300 protein spots were detected in this silver-stained 2-DE image of all three groups (Fig. 1). All protein spots exhibited molecular weights (MWs) of 10–175 kDa. Use of pH 4–7 IPG strips provided increased resolution of the acidic species. Gels images were analyzed by using Progenesis Samespots software (Nonlinear Dynamics, Newcastle, UK). Totally 47 protein spots differing significantly ($p<0.05$, one-way ANOVA test) in their intensities with a fold-change ≥2 were used for MALDI-TOF/MS analysis.

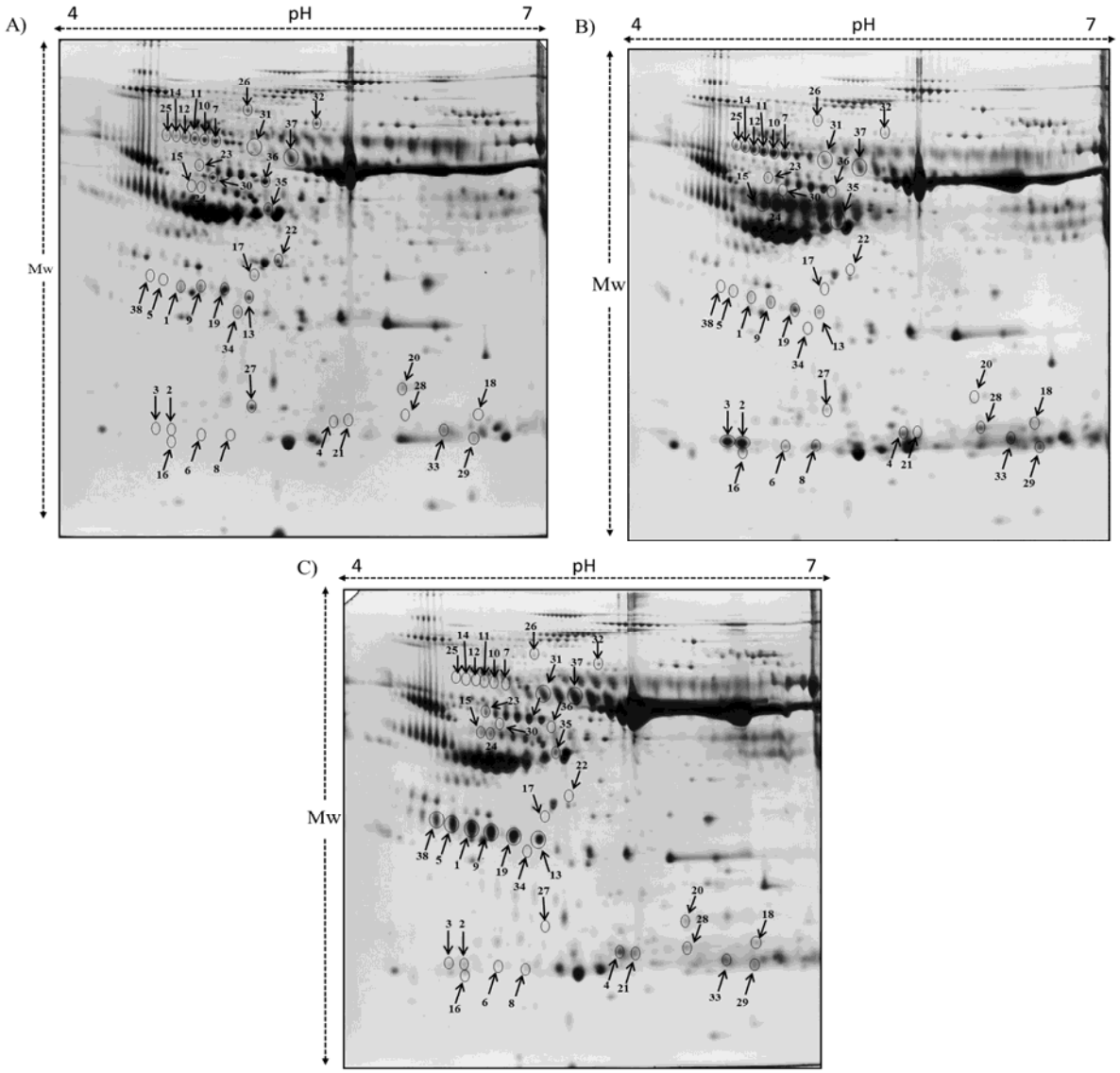

Figure 1. Two-dimension electrophoresis pattern of *Gulo*$^{-/-}$ mouse serum. (A) Control (only vitamin C), **(B)** *H.Pylori*-infected, **(C)** *H.Pylori*-infected and DENA treated. 100µg of serum total proteins were separated on IPG-strips with pH 4–7(18 cm) in the first dimension and then on 12% polyacrylamide gels in the second dimension, which were stained silver nitrate. Protein spots which showed significantly altered expression levels (Max fold change ≥2 and $p<0.05$) were marked on the gels. Three independent experiments were performed.

Identification of differentially expressed proteins in H.pylori infected and combined with DENA treated *Gulo*$^{-/-}$ mice serum by MALDI-TOF- MS

The differentially expressed proteins in *H.pylori* infected and/or combined with DENA treated *Gulo*$^{-/-}$ mice serum were investigated by 2-DE analysis in comparison with control. The 2-DE maps of three groups of *Gulo*$^{-/-}$ mice serum are shown in Fig. 1. A total of 47 statistically significant altered protein spots were selected (≥2 fold, $p<0.05$) and successfully 38 proteins were identified by a MALDI-TOF/MS using the MASCOT search engine and the SwissProt database (Table 2). The name, accession number, number of matching peptides, theoretical isoelectric point (pI), molecular weights, sequence coverage of the differentially expressed protein identified are listed in Table 2. We noticed some of the important proteins such Alpha-1B-glycoprotein, Haptoglobin, Collagen alpha-2 (IX) chain, Antithrombin-III, Hemopexin and Glutathione S-transferase, Complement C3 and Apolipoprotein A-IV were differentially expressed in *H.pylori* infection and followed by DENA groups. The expression patterns of identified proteins were shown in Table 3. These differentially expressed proteins were involved in various biological processes such as immune system process, response to stimulus, metabolic process, cellular process, system process and cell communication due to this *H.pylori*-infection and DENA treatment.

Discussion

Unlike most other mammals and animals, humans do not have the ability to make vitamin C inside of their own bodies. Therefore, humans must obtain vitamin C through daily diet and its requirements are vary greatly among individuals. In present study, we established *Gulo*$^{-/-}$ mice like human (it does not have ability to synthesis vitamin C) must orally supplemented daily because *Gulo*$^{-/-}$ mice can't survive without vitamin C. Vitamin C deficiency mice will lost body

Table 2. List of statistically significant differentially expressed proteins identified in serum of *Gulo*$^{-/-}$ mice after infected with *H.pylori* alone and/or combined treatement with DENA by MALDI-TOF- MS

Spot No.	Protein name[1]	Accession number[1]	Theoretical Mr (kDa)[2]	Theoretical pI Value[2]	Fold change	p Value (t-test)	Peptides matched	Sequence Coverage (%)	Mowse Score[3]
1	Haptoglobin	Q61646	39.24	5.88	9.1	7.208e-005	6/20	22	61
2	Collagen alpha-2(IX) chain	Q07643	65.51	9.40	15.6	1.254e-004	7/19	14	63
3	Collagen alpha-2(IX) chain	Q07643	65.51	9.40	19.7	1.967e-004	8/20	13	62
4	Histidine protein methyltransferase 1 homolog	Q4KM84	40.55	5.54	4.7	2.454e-004	8/43	20	60
5	Haptoglobin	Q61646	39.24	5.88	6.0	2.460e-004	8/48	30	58
6	Vasculin-like protein 1	Q3KR53	52.86	6.82	8.4	6.580e-004	5/10	15	60
7	Alpha-1B-glycoprotein	Q19LI2	57.20	6.33	4.5	8.789e-004	15/45	22	126
8	Alpha-1B-glycoprotein	Q19LI2	57.20	6.33	15.6	9.135e-004	56/16	25	123
9	Haptoglobin	Q61646	39.24	5.88	5.9	0.001	8/26	24	69
10	Alpha-1B-glycoprotein	Q19LI2	57.20	6.33	4.6	0.003	15/47	24	122
11	Alpha-1B-glycoprotein	Q19LI2	57.20	6.33	3.7	0.003	17/47	29	149
12	Alpha-1B-glycoprotein	Q19LI2	57.20	6.33	4.0	0.004	11/48	18	74
13	Growth arrest-specific protein 6	Q63772	76.53	5.63	6.4	0.005	13/83	21	58
14	Alpha-1B-glycoprotein	Q19LI2	57.20	6.33	4.8	0.005	10/37	17	76
15	Collagen alpha-2(IX) chain	Q07643	65.51	9.40	6.1	0.005	7/22	14	58

16	Antithrombin-III	P32261	52.48	6.10	23.2	0.006	12/75	29	64
17	Apolipoprotein A-IV	P06728	45.00	5.34	6.2	0.007	8/23	22	74
18	UPF0361 protein C3orf37 homolog	Q8R1M0	40.77	8.86	3.2	0.007	15/109	33	72
19	Haptoglobin	Q61646	39.24	5.88	3.0	0.008	7/28	24	64
20	Collagen alpha-2(IX) chain	Q07643	65.51	9.40	2.7	0.008	7/21	10	58
21	Serum albumin	P02770	70.68	6.09	4.0	0.010	10/37	22	72
22	Apolipoprotein A-IV	P06728	45.00	5.34	4.3	0.013	13/67	30	81
23	Glutathione S-transferase	P10649	26.07	7.71	2.1	0.013	7/38	32	58
24	Antithrombin-III	P32261	52.48	6.10	3.9	0.013	9/36	27	73
25	Alpha-1B-glycoprotein	Q19LI2	57.20	6.33	4.3	0.015	9/31	14	72
26	MACRO domain-containing protein 1	Q922B1	35.84	9.07	2.4	0.016	10/53	23	75
27	NF-kappa-B-repressing factor	Q8BY02	78.38	9.08	4.6	0.020	7/16	9	60
28	Serum albumin	P02770	70.68	6.09	3.8	0.024	10/51	21	57
29	Serum albumin	P07724	70.70	5.75	3.1	0.027	14/97	26	62
30	Stabilin-2	Q8CFM6	161.86	7.48	4.0	0.033	9/16	8	69

31	Hemopexin	Q91X72	52.03	7.92	2.4	0.033	20/118	43	119
32	Creatine kinase M-type	P07310	43.25	6.58	2.7	0.040	7/27	18	57
33	Microtubule-associated tumor suppressor candidate 2 homolog	Q3UHD3	147.95	8.42	2.8	0.044	12/39	12	58
34	G protein-regulated inducer of neurite outgrowth 1	Q3UNH4	96.07	8.14	6.3	0.046	16/93	21	59
35	1,25-dihydroxyvitamin D(3) 24-hydroxylase, mitochondrial	Q64441	59.93	9.16	2.4	0.042	9/27	15	64
36	Complement C3	P01027	187.91	6.29	2.2	0.020	28/71	17	118
37	Hemopexin	Q91X72	52.03	7.92	2.2	0.034	19/79	38	142
38	Haptoglobin	Q61646	39.24	5.88	2.7	0.027	8/40	27	64

[1]Entry name, protein name and accession number from SWISS-PROT database identified by MALDI-TOF/MS

[2]Theoretical molecular weight (kDa) and pI from SWISS-PROT database

[3]Score is –10*log (p), where p is the probability that the observed match is a random event, Protein scores greater than 56 are significant ($p<0.05$).

Table 3. Expression patterns of differentially expressed proteins identified in serum of *Gulo*$^{-/-}$ mice after infected with H.pylori alone and/or combined treatement with DENA by MALDI-TOF- MS

Spot No.	Protein name[1]	Accession number[1]	Control Vs H. Pylori infected[2]	Control Vs H.Pylori + DENA[2]
1	Haptoglobin	Q61646	↓	↑
2	Collagen alpha-2(IX) chain	Q07643	↑	↑
3	Collagen alpha-2(IX) chain	Q07643	↑	↑
4	Histidine protein methyltransferase 1 homolog	Q4KM84	↑	↑
5	Haptoglobin	Q61646	↓	↑
6	Vasculin-like protein 1	Q3KR53	↑	↑
7	Alpha-1B-glycoprotein	Q19LI2	↑	↓
8	Alpha-1B-glycoprotein	Q19LI2	↑	↓
9	Haptoglobin	Q61646	↓	↑
10	Alpha-1B-glycoprotein	Q19LI2	↑	↑
11	Alpha-1B-glycoprotein	Q19LI2	↑	↓
12	Alpha-1B-glycoprotein	Q19LI2	↑	↓
13	Growth arrest-specific protein 6	Q63772	↓	↑
14	Alpha-1B-glycoprotein	Q19LI2	↑	↓
15	Collagen alpha-2(IX) chain	Q07643	↑	↑
16	Antithrombin-III	P32261	↑	↑
17	Apolipoprotein A-IV	P06728	↓	↓
18	UPF0361 protein C3orf37 homolog	Q8R1M0	↑	↑

19	Haptoglobin	Q61646	↓	↑
20	Collagen alpha-2(IX) chain	Q07643	↑	↑
21	Serum albumin	P02770	↑	↑
22	Apolipoprotein A-IV	P06728	↑	↓
23	Glutathione S-transferase	P10649	↑	↑
24	Antithrombin-III	P32261	↑	↑
25	Alpha-1B-glycoprotein	Q19LI2	↑	↓
26	MACRO domain-containing protein 1	Q922B1	↓	↓
27	NF-kappa-B-repressing factor	Q8BY02	↓	↓
28	Serum albumin	P02770	↑	↑
29	Serum albumin	P07724	↑	↑
30	Stabilin-2	Q8CFM6	↓	↓
31	Hemopexin	Q91X72	↑	↑
32	Creatine kinase M-type	P07310	↓	↓
33	Microtubule-associated tumor suppressor candidate 2 homolog	Q3UHD3	↑	↑
34	G protein-regulated inducer of neurite outgrowth 1	Q3UNH4	↓	↑
35	1,25-dihydroxyvitamin D(3) 24-hydroxylase, mitochondrial	Q64441	↑	↑
36	Complement C3	P01027	↓	↓
37	Hemopexin	Q91X72	↑	↑
38	Haptoglobin	Q61646	↓	↑

[1]Entry name, protein name and accession number from SWISS-PROT database identified by MALDI-TOF//MS

[2]Image analysis was performed using Progenesis Samespots software (Nonlinear Dynamics, Newcastle, UK). Those spots differing significantly ($p<0.05$, one-way ANOVA test) in their intensities with a fold-change ≥2.

weight and die within 6 weeks due to severe scurvy. With recent advances in proteomics protein biomarker discovery is now a major area of proteome research. Using *Gulo*$^{-/-}$ mice model, we investigated the protein expression pattern of serum in response to *H.pylori* infection and followed by DENA treatment. Comparison of serum protein profiles of *H.pylori* infection group and followed by DENA treated groups with control group revealed that the differentially expressed proteins were involved in various physiological pathways including acute phase response signaling, inflammatory regulation and immune response.

Complement components plays a pivotal role as mediators of inflammation and regulation of the immune response (Jurianz et al., 1999). Complement C3 is protein of immune system and plays a central role in the activation of the complement system (Sahu and Lambris, 2001; Markiewski and Lambris. 2007). Complement C3 is a multipotent protein that's involved in the immune response, as including complement activation, antigen presentation, cell-cell interactions, and cell proliferation (Frade, 1999). The people with complement C3 deficiency are susceptible to bacterial infection (Litzman et al, 2003). In this study, complement C3 (Spot no. 36) expression was significantly decreased in in *H. Pylori* infected group and *H. Pylori* infected followed by DENA treated group when compare with control group (Fig.2A). The down-regulation of complement C3 suggest that *Gulo*$^{-/-}$ mice infected with *H.pylori* and further DENA enhanced the *H.pylori* effect and complement C3 might be involved in carcinogenesis of gastric cancer.

An acute-phase serum proteins (APPs) such as Haptoglobin, hemopexin, Alpha-1B-glycoprotein were significantly increased in in *H. Pylori* infected group and *H. Pylori* infected followed by DENA treated group when compare with control group (Fig. 2B). APPs are circulating plasma proteins which are mainly synthesized by liver parenchymal cells following simulation by

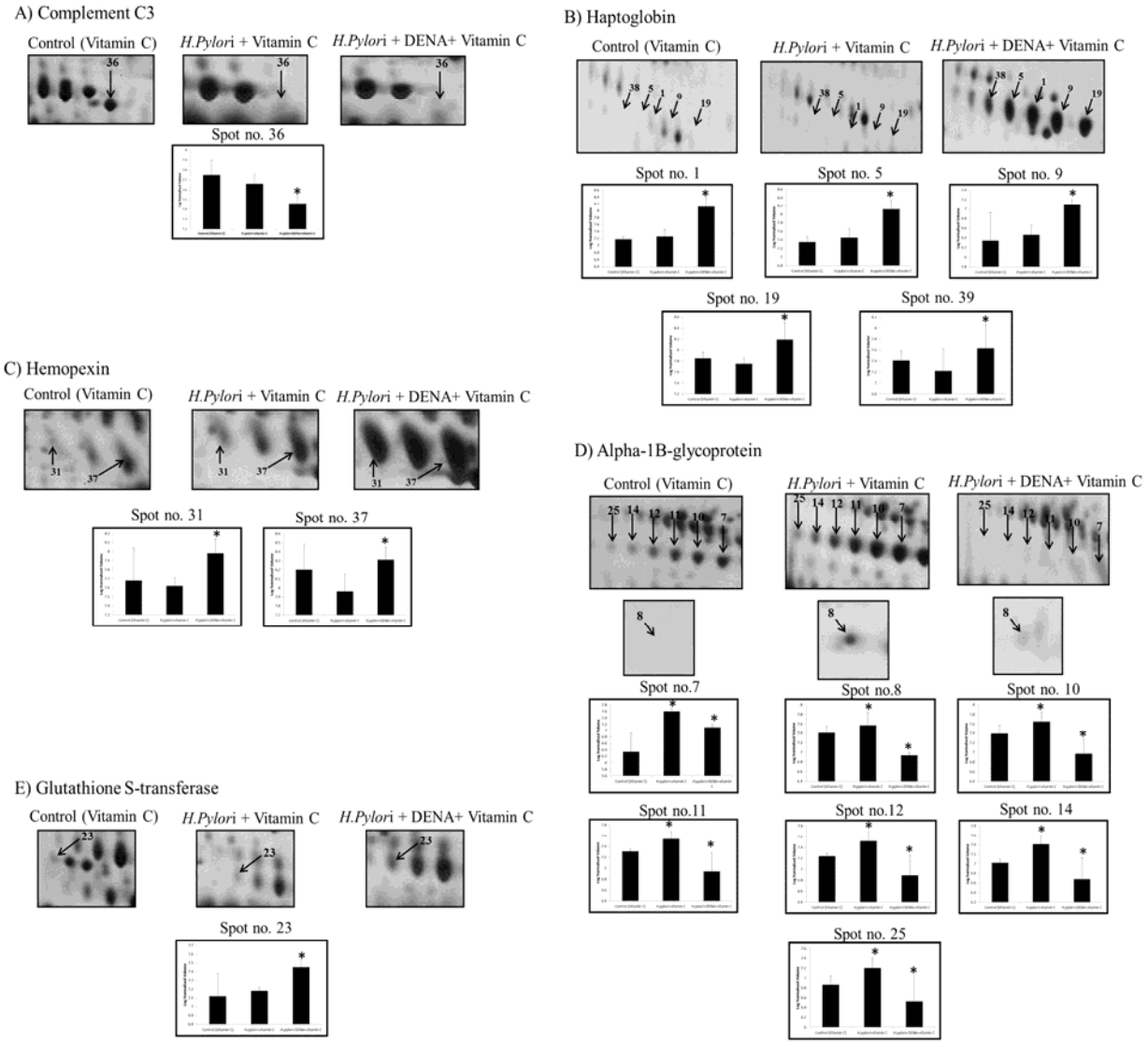

Figure 2. Enlargements of differentially expressed proteins. (A) Complement C3, **(B)** Haptoglobin, **(C)** Hemopexin, **(D)** Alpha-1B-glycoprotein and **(E)** Glutathione S-transferase from 2-DE maps of *Gulo*$^{-/-}$ mouse serum corresponding sections of gels with protein spots derived from control (only vitamin C), *H.Pylori*-infected, and *H.Pylori*-infected and DENA treated. Three independent experiments were performed and the mean ± SD was plotted (*P < 0.05 compared with control).

cytokines such as interleukin-1, interleukin-6 and tumor necrosis factor-α mainly from macrophages at the inflammatory site (Moshage, 1997). The main biological functions of haptoglobin are to play a role in modulating host defense responses to infection and inflammation (Dobryszycka, 1997; Wassell, 2000). Haptoglobin has also been reported to be involved in immune suppression in cancer (Oh et al., 1987' Oh et al., 1990). There are five differentially expressed isoforms of Haptoglobin (Spot no.1, 5, 9, 19, 38) found in *H. Pylori* infected group and *H. Pylori* infected followed by DENA treated group. Only little expressions of Haptoglobin found in *H. Pylori* infected group but highly expressed in *H. Pylori* infected followed by DENA treated group. These results revealed that that a possible association between *H.pylori* and DENA with the disease pathogenesis and cancinogenesis.

Another APPs protein, Hemopexin two isoforms (spot no.31, 37) up-regulated protein *H. Pylori* infected group and *H. Pylori* infected followed by DENA treated group when compare with control group (Fig.2C). It provides the second line of defense against hemoglobin-mediated oxidative damage during the intravascular hemolysis (Delanghe and Langlois, 2001). Moreover, Serum hemopexin helps in scavenging free heme while the free iron is taken up and transported by transferrin (Ferreira et al., 2008). However, the relationship between tumor development and hemopexin is not very clear but matrix metallopeptidases containing a hemopexin domain have been detected in the sera of gastric cancer patients with high expression of hemopexin. These results suggest that, hemopexin might be involved *H.pylori* pathogenesis mechanism and gastric cancer carcinogenesis.

Alpha-1B-glycoprotein, 7 isoforms (spot no.7, 8, 10, 11, 12, 14, 25) were up-regulated in only *H. Pylori* infected group and completely not expressed in *H. Pylori* infected followed by DENA treated group (Fig.2D). Alpha-1B-glycoprotein is a plasma secreted protein, member of

the immunoglobulin superfamily, but its function is unknown (Ishioka et al., 1986). Recently have reported that alpha-1B-glycoprotein was detected in urinary samples from bladder cancer patients by using a glycoprotein profiling method (Kreunin et al., 2007). Also, overexpression of alpha-1B-glycoprotein has reported in saliva from patients with head-and-neck squamous cell carcinoma (Ohshiro et al., 2007). These findings suggest that alpha-1B-glycoprotein might be an *H.pylori* associated cancer-related protein and its possible functions in carcinogenesis further to be investigated.

The glutathione S-transferases (GSTs), a multigene family of dimeric enzymes that have a significant role in the detoxification of electrophilic species by catalytic conjugation with reduced glutathione (GSH) (Osada et al., 2002; Hayes and Pulford, 1995). Previous studies have reported that pi-class GST is substantially increased in the early stages of rat liver carcinogenesis (Farber, 1984; Sato et al., 1984; Satoh et al., 1985). GST pi is overexpressed in hepatic foci arising spontaneously or in animals treated with carcinogens (Sato, 1989; Sawaki et al., 1990) but almost undetectable in normal rat hepatocytes. In the present study, glutathione S-transferases (GSTs) (Spot no. 23) was highly expressed in highly expressed in *H. Pylori* infected followed by DENA treated group but no expression found in only *H. Pylori* infected group (Fig.2E). This result clearly indicates that GSTs overexpressed by carcinogen DENA treatment but *H. Pylori* not changed or involved in GSTs pathways.

In summary, we have performed a proteomic analysis to investigate the protein expression pattern in *Gulo*$^{-/-}$ mice serum in response to *H.pylori* infection and followed by DENA treatment. Comparison of protein profiles of *Gulo*$^{-/-}$ mice serum revealed that *H.pylori* infection and followed by DENA treatment altered the expressions of Complement C3, Alpha-1B-glycoprotein, Haptoglobin, Hemopexin and Glutathione S-transferase when compared to

control group. The differentially expressed proteins were involved in various biological processes such as acute phase response signaling, inflammatory regulation and immune response due to this *H.pylori* infection and DENA. These protein expressions further needed to be validated by immune-blotting. Moreover, identified proteins by MALDI-TOF/ MS in *Gulo*$^{-/-}$ serum might be involved in mechanism of H. *pylori* disease pathogenesis and/or combined effect of DENA, early diagnosis and therapy of *H. pylori* and DENA associated gastric disorders.

ACKNOWLEDGEMENTS

This work was supported by the National Research Foundation (NRF) of Korea Grant funded by the Korean government (MEST) (No. 2012045015) and the National R&D Program for Cancer Control, Ministry for Health, Welfare and Family affairs, Republic of Korea (No. 0820050).

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