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Helicobacter pylori Targets in AGS Human Gastric Adenocarcinoma: *In Situ* Proteomic Profiling and Systematic Analysis

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Abstract. *Background/Aim: Helicobacter pylori, a gram-negative bacterium, causes chronic stomach diseases in humans. Heat shock proteins (HSPs) are involved in cell integrity, cell growth, and gastric mucosa colonization by H. pylori. This study aimed to investigate HSP expression levels in H. pylori-infected gastric adenocarcinoma AGS cells. Materials and Methods: We determined protein expression levels using iTRAQ proteomics analysis. We analyzed the possible network interactions for H. pylori targets in AGS cells using the Ingenuity Pathway Analysis (IPA) software. Results: H. pylori-infected AGS cells potentially targeted EIF2 and BAG2 signaling pathways to regulate cell physiology. In addition, after 3, 6, and 12 h of infection, western blotting revealed significantly decreased HSP70 and HSP105 expression. Conclusion: H. pylori decreases HSPs in AGS gastric adenocarcinoma cells, and this is associated with the regulation of EIF2 and BAG2 signaling pathways.*

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Key Words: Helicobacter pylori, human gastric adenocarcinoma AGS cells, heat shock proteins family (HSP), iTRAQ proteomic, Ingenuity pathway analysis (IPA).

Helicobacter pylori (*H. pylori*) is a gram-negative bacterium. Globally, approximately 4.4 billion individuals are infected with *H. pylori* (1). *H. pylori* infection causes chronic stomach disease in humans (2-4), and infection in the stomach can lead to peptic ulcers and gastric carcinogenesis (5-8). Epidemiological studies have demonstrated that *H. pylori* infection is a risk factor for peptic ulcers and gastric carcinogenesis (1, 9-11), and has been classified as a group 1 carcinogen by the World Health Organization (WHO) (12). *H. pylori* induces the expression of specific genes in gastric epithelial cells such as cyclooxygenase-2 (COX-2) and others (13-15). It has been reported that the heat shock protein family (HSP) is involved in cell integrity, cell growth, and gastric mucosa colonization by *H. pylori* infection (15). Many studies on gene expression in *H. pylori*-infected gastric epithelial cells have used different methods (16-19). Sepulveda *et al.* and Maeda *et al.* demonstrated marked changes in more than 200 genes of *H. pylori* or CagA-positive *H. pylori* strain-infected AGS human gastric adenocarcinoma cells by microarray analysis (18, 20). However, changes in the protein profile of *H. pylori*-infected AGS cells have not been widely investigated.

iTRAQ proteomics has emerged as a promising technological platform for the identification of biomarkers, underlying mechanisms, and novel therapeutic agent targets for human diseases (21-24). In our study, we aimed to identify specific protein levels related to *H. pylori*-infected AGS cells using iTRAQ proteomics analysis. In addition, ingenuity pathway analysis (IPA) software analysis potentially targets pathways for iTRAQ proteomics results of *H. pylori* in infected gastric adenocarcinoma AGS cells.

Materials and Methods

Bacterial strains, cell lines, and culture conditions. The *H. pylori* reference strain 26695 (ATCC 700392) used in this study was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and was grown on Brucella agar (BD Biosciences, Franklin Lakes, NJ, USA), supplemented with 5% sheep blood (Taiwan Prepared Media-TPM, Taiwan) under microaerophilic conditions at 37˚C for 48-72 h. Human AGS cells (ATCC®CRL-1739™) were purchased from ATCC and were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific), 100 U/ml penicillin, and 100 μg/ml streptomycin (25-27).

Cell infection assay. AGS cells, a human gastric adenocarcinoma cell line, were seeded into 96-well plates at a density of 1×10^4 cells/well and cultured in RPMI 1640 medium at 37˚C in a 5% CO_2 incubator for 18 h. PBS-suspended *H. pylori* 26695 at an MOI ratio of 100 was co-cultured in an antibiotic-free RPMI-1640 medium supplemented with 10% FBS with AGS cells, and then cultured at 37˚C in CO_2 for 3, 6, or 12 h. Trypsin/EDTA solution (0.05%; Biological Industries, Israel) was used to detach adherent cells, which were then collected by centrifugation at 1,500×g for 5 min. These cells were subjected to protein preparation as described in the following section (25-28).

Cell viability assay. The MTT assay was used to assess the viability of *H. pylori* in infected AGS cells. The initial concentration of AGS cells was 1×10^4 cells/well in a 96-well cell culture plate and cultured in conditioned medium for 18 h. PBS-suspended *H. pylori* 26695 at a multiplicity of infection (MOI) of 100 was co-cultured in an antibiotic-free RPMI-1640 medium supplemented with 10% FBS with AGS cells, and then cultured at 37˚C in CO_2 for 3, 6, or 12 h. After the indicated time, MTT solution (0.5 mg/ml) was added, and the cells were incubated for 4 h at 37˚C. The formazan crystals were dissolved in DMSO following removal of the medium. The formazan product was analyzed spectrophotometrically at 490 nm. Data analysis was performed in triplicate (27-29).

Protein preparation. The initial concentration of AGS cells was 1×10^4 cells/well in a 96-well cell culture plate and cultured in the conditioned medium for 18 h. PBS-suspended *H. pylori* 26695 at an MOI ratio of 100 was co-cultured in an antibiotic-free RPMI-1640 medium supplemented with 10% FBS with AGS cells, and then cultured at 37˚C in CO_2 for 3, 6, or 12 h. Cells were lysed in 200 μl of dissolution buffer (AB Sciex). The lysate was centrifuged at 12,000 × *g* for 20 min at 4˚C. The supernatant containing predominantly soluble proteins was transferred to a new microcentrifuge tube, while the insoluble pellets, which consisted mostly of insoluble proteins, were discarded. The soluble protein concentration was determined using a 2D-quant kit (GE Healthcare) (30, 31).

Isobaric peptide labeling. The proteins (total 100 μg) were reduced with 5 mM tris-(2-carboxyethyl) phosphine (TCEP) (reducing reagent; Applied Biosystems) at 60˚C for 1 h; cysteine residues were blocked with 10 mM methyl methane thiosulfonate (MMTS) (blocking reagent, AB Sciex) at room temperature for 10 min, and digested with 8 mg trypsin (AB Sciex) at 37˚C for 16 h, and labeled with iTRAQ reagents according to the manufacturer's protocol (AB Sciex iTRAQ™ Reagents Chemistry Reference Guide). The iTRAQ reagent dissolved in 70 μL of ethanol was added to the protein digests, and the samples were incubated for 1 h at room temperature. Peptide digests at 0 h, 3 h, 6 h, and 12 h were labeled independently with iTRAQ tags 114, 115, 116, and 117, respectively. The four labeled peptide pools were then mixed in a 1:1:1:1 ratio and stored at –20˚C (32).

Cation-exchange fractionation. The iTRAQ-labeled four-peptide mixtures were diluted 1:10 with a Cation Exchange Buffer-Load containing 25% acetonitrile and 10 mM KH2PO4 (pH=3.0), and fractionated using a cation-exchange cartridge system (AB Sciex). Before loading the combined peptide mixture, the cartridge was conditioned with 1 ml Cation Exchange buffer-clean and 1 ml Cation Exchange buffer-load twice. A syringe was used to inject the peptide mixture under air pressure. Peptides were eluted by a gradient of 0-50 mM KCl at a flow rate of 1 drop/s and fractionated into 8 fractions. A final 8 fractions of peptide mixture were desalted using a C18 zip tip (Millipore) and dried in a speed vacuum prior to LC-MS/MS (32).

Mass spectrometric analysis. Dried peptide fractions were re-dissolved in 10 ml of 5% (v/v) acetonitrile/0.1% (v/v) formic acid. Peptide separation was performed on an Ultimate nanoflow HPLC system (UltiMate 3000 RSLCnano system; Dionex, the Netherlands.) via the partial loop mode and then interfaced with a hybrid Q-TOF mass spectrometer (MaXis impact, Barker, Germany). Individual cation exchange fractions were injected and captured onto a 375 μm × 2 cm trap column (5-μm C18 Dionex-LC Packings) followed by elution onto a 75 mm × 250 mm analytical column Acclaim PepMap C18, 2 μm, Thermo Scientific using an automated binary gradient (200 nl/min) from 95% buffer A (5% ACN, 0.1% FA) to 65% buffer B (80% ACN, 20% H2O, and 0.1% FA) over 125 min, followed by 90% buffer B for 5 min. Afterward, the column was re-equilibrated to 5% buffer B. Separated peptides were directly eluted into an electrospray ionization tandem mass spectrometer. Survey scans were acquired from m/z 350-1600 with up to two precursors selected for MS/MS from m/z 65-2000 using dynamic exclusion. Peptides with +2 to +4 charge states were selected, and the two most abundant charged peptides above a 20-count threshold were selected for MS/MS (32).

iTRAQ proteomics analysis. Peptide and protein identifications were performed using ProteinScape software (Braker,Germany). Proteome data were searched against the *Homo sapiens* (human) protein database. Fixed cysteine alkylation was selected for methyl methane thiosulfonate (MMTS); furthermore, the tryptic digest was considered up to one missed cleavage for the search algorithms. Quantification was determined by calculating the ratio of the areas under the reporter peaks (114 of control, 115 of 3 h treatment, 116 of 6 h treatment, and 117 of 12 h treatment). ProteinPilot software was used to pool data from all fractions (32).

Ingenuity pathway analysis. To generate a corresponding molecular network from iTRAQ protein targets, we used a core analysis tool in IPA (IPA 2021; Qiagen Sciences, Inc.). The enriched molecular networks and associated ontology groups in their respective canonical pathways, upstream regulators, diseases, and rankings of network analysis were calculated based on statistical significance. All pathways were deemed to be statistically significant according to the Fisher's exact *t*-test ($p<0.05$). Specifically, the network was started from the focus molecule, and each molecule was connected with others (32).

Western blotting. AGS cells were seeded into 10 cm dishes at a density of 1×10^7 cells/75 T flask and cultured in RPMI 1640 medium at 37˚C in a 5% CO_2 incubator for 18 h. PBS-suspended *H. pylori* 26695 at an MOI ratio of 100 was co-cultured in an antibiotic-free RPMI-1640 medium supplemented with 10% FBS with AGS cells, and then cultured at 37˚C in CO_2 for 0, 3, 6, and 12 h. The cells were lysed in 200 μl of dissolution buffer (AB Sciex). Cells were lysed in Trident RIPA Lysis Buffer and collected. Protein concentrations were determined using the Pierce BCA Protein Assay Kit. Equal amounts of 50 μg protein were prepared and loaded onto 10% sodium dodecyl sulfate-polyacrylamide gels and separated according to molecular weight. Proteins were transferred to an Immobilon-P polyvinylidene difluoride transfer membrane prior to blocking with 5% skim milk for 1 h at room temperature. The membrane was subsequently incubated at 4˚C overnight with primary antibodies against HSP70 (GTX111088), HSP105 (GTX64587), and β-actin (GTX109639) at a dilution of 1:1,000. Membranes were then incubated with appropriate anti-mouse and anti-rabbit IgG HRP-linked secondary antibodies at a dilution of 1:10,000 for 1 h at room temperature. Blot visualization was performed using the Immobilon Western Chemiluminescent HRP Substrate, and all bands of immunoblots were normalized to the densitometric value of β-actin. The bands were quantified by densitometry using ImageJ software (version 1.41; National Institutes of Health, Bethesda, MA, USA) (33, 34).

Statistical analysis. The results are presented as the mean±standard deviation for the indicated numbers of independent experiments. Statistical comparison of data was performed using one-way ANOVA followed by Tukey's test. ***$p<0.001$ was considered to indicate statistically significant differences between the control and *H. pylori* infected groups.

Results

Analysis of cell viability of H. pylori-infected AGS cells. First, the viability of *H. pylori* (MOI 100)-infected AGS cells for 3, 6, or 12 h was detected by the MTT assay. As shown in Figure 1, *H. pylori*-infected AGS cells for 3 h and 6 h did not significantly reduce cell viability by MTT assay. However, the viability of *H. pylori* in infected AGS cells was significantly reduced after 12 h of treatment.

Identification of protein targets in H. pylori-infected AGS cells by iTRAQ proteomics analysis. Second, iTRAQ proteomics was performed to identify the protein targets of *H. pylori*-infected AGS cells. After 3, 6, and 12 h of infection, cell lysates were collected and the proteins were profiled using iTRAQ coupled to LC-MS/MS-based methods. The derived peptides were reacted with iTRAQ reagents (control samples were labeled with 114; 3 h infection samples were labeled with 115; 6 h infection samples were labeled with 116 and 12 h infection samples were labeled with 117). The labeled samples were pooled and analyzed using LC-MS/MS to identify and quantify the target proteins (Figure 2). Proteins with an iTRAQ ratio >1.2 were considered overexpressed, whereas those with a ratio <0.8, were considered under-expressed. A total of 321 proteins were found, and 159 proteins were differentially expressed after *H. pylori* infection compared with uninfected AGS cells. Among them, the expression of 89 proteins increased in the course of infection, and 70 protein levels were decreased (Table I).

Biological pathway analysis of H. pylori-infected AGS cells. We performed IPA analysis of major pathways targeting *H. pylori*-infected AGS cell targets. We collected the protein targets by iTRAQ proteomics and then analyzed the biological pathways involved in *H. pylori*-infected AGS cells. Our results showed clustering analysis and biological pathways (Figure 3 and Table II). The biological pathways associated with *H. pylori*-infected AGS cells include 'Coronavirus', 'Pathogenesis Pathway', 'NAD Signaling Pathway', 'Spliceosomal Cycle', 'eNOS Signaling', 'Huntington's Disease Signaling', 'Coronavirus Replication Pathway', 'Natural Killer Cell Signaling', 'PPARα/RXRα Activation', 'PPAR Signaling', 'Xenobiotic Metabolism AHR Signaling Pathway', 'Sirtuin Signaling Pathway', 'Paxillin Signaling', 'VEGF Signaling', 'tRNA Charging', 'Inhibition of ARE-Mediated mRNA Degradation Pathway', 'Role of PKR in Interferon Induction and Antiviral Response', 'RhoA Signaling', 'Actin Cytoskeleton Signaling', 'Integrin Signaling', 'ILK Signaling', 'EIF2 Signaling', 'Telomerase Signaling', 'Xenobiotic Metabolism CAR Signaling Pathway', 'PI3K/AKT Signaling', 'HIF1α Signaling', 'NRF2-mediated Oxidative Stress Response', 'Estrogen Receptor Signaling', 'Unfolded protein response', 'mTOR Signaling', 'Regulation of eIF4 and p70S6K Signaling', 'BAG2 Signaling Pathway', 'p70S6K Signaling' and 'Gluconeogenesis I'. Furthermore, the top 10 major biological pathways associated with *H. pylori*-infected AGS cells include 'EIF2 Signaling', 'BAG2 Signaling Pathway', 'Regulation of eIF4 and p70S6K Signaling', 'Glycolysis I', 'mTOR Signaling', 'Protein Ubiquitination Pathway', 'Huntington's Disease Signaling', 'FAT10 Signaling Pathway', 'Gluconeogenesis I', and 'Spliceosomal Cycle' (Figure 4). As shown in Figure 5 and Figure 6, the EIF2 signaling and BAG2 signaling pathways are involved in the regulation of the HSP family. In addition, a network of the associations of *H. pylori*-infected AGS cells with infected cell networks was generated following IPA analysis (Figure 7).

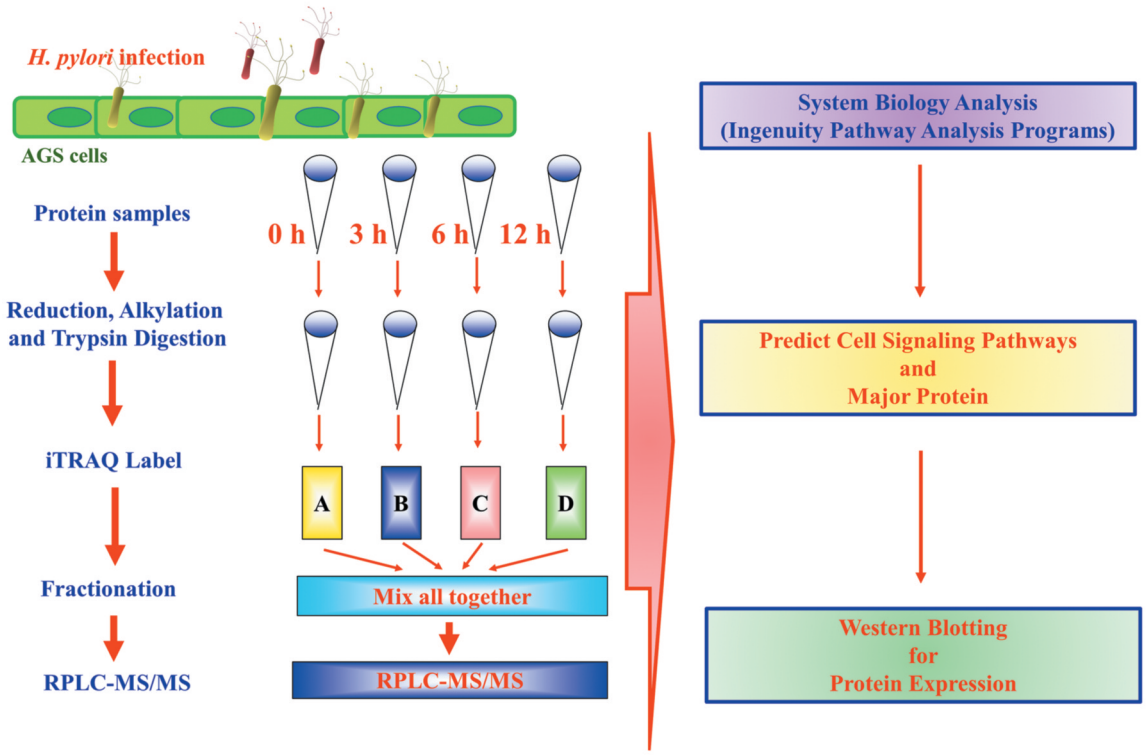

Figure 1. *Flowchart of iTRAQ proteomics study protocol on Helicobacter pylori-infected AGS cells.*

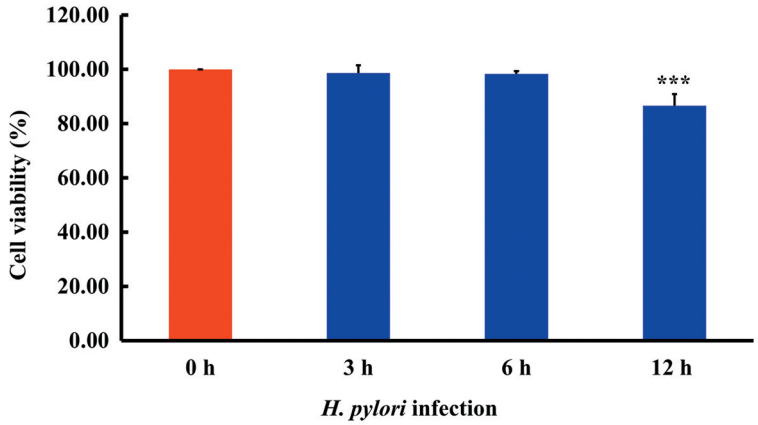

Figure 2. *In vitro cell viability results of Helicobacter pylori-infected AGS cells. AGS cells (1×10^4 cells/well) in a 96 well plate cultured in condition medium 18 h. PBS-suspended H. pylori 26695 at a MOI of 100 was co-cultured in an antibiotic-free RPMI-1640 medium supplemented with 10% FBS with AGS cells and then cultured at 37˚C in CO_2 for 3h, 6h and 12 h. The values were obtained from MTT assay and shown as mean±SEM (n=3). ***p<0.001 compared to untreated controls.*

Inhibition of HSP70 and HSP105 protein levels in H. pylori-infected AGS cells. Our pathway analysis highlighted EIF2 signaling and the BAG2 signaling pathway as the top two canonical pathways in which the targets of *H. pylori*-infected AGS cells are involved, suggesting that the HSP family may be affected by *H. pylori* infection. We examined the protein level changes of HSP70 and HSP105 by western blotting in *H. pylori*-infected AGS cells. As shown in Figure 8, after 3, 6, and 12 h of *H. pylori* infection, the protein expression of HSP70 and HSP105 was significantly decreased. These results indicate that *H. pylori* infection can potently inhibit HSP70 and HSP105 protein levels.

Table I. *The representative protein targets identified and ratio in Helicobacter pylori-infected human gastric adenocarcinoma AGS cells by iTRAQ proteomics*.

GI Accession	3 h/0 h	6 h/0 h	12 h/0 h	% Cov
62897671	0.75	0.72	0.75	49.10
53791219	1	0.96	1.03	15.60
372466572	0.96	1.01	0.97	44.00
62896661	0.93	1.21	1.77	43.50
3298597	1.39	0.79	1.43	8.10
62897129	1.16	0.72	0.72	20.70
75517570	1.67	1.43	1.36	38.20
30311	1.08	0.88	0.88	35.90
693933	0.85	0.48	0.82	44.20
332164775	1.21	1.37	1.42	34.10
62897609	1.01	0.92	1.02	33.40
968888	1.2	1.34	1.56	35.40
32488	1.22	0.64	0.48	29.90
296080693	0.61	0.58	0.52	17.90
221045918	0.61	0.34	0.35	22.40
2804273	0.92	0.97	1.04	17.00
73909156	1.04	1.01	1.05	20.50
62089306	1.91	1.98	2.4	6.20
32097	0.96	0.7	0.05	52.40
553596	1.04	1.12	1.2	8.30
31645	1.29	1.72	1.89	31.90
49456715	1.14	1.04	1.58	27.50
6807647	1.56	1.02	0.89	25.00
189053217	0.91	0.74	0.77	52.30
47682755	0.9	1.57	1.91	32.50
39644794	0.96	0.94	0.92	12.20
66365795	1.28	1.11	1.2	42.00
48734884	0.99	0.97	0.98	23.20
33869643	1.04	1.11	1.43	19.70
194373909	0.49	0.21	0.04	21.20
40788952	1.09	0.99	0.97	9.30
62896853	0.52	0.58	0.61	20.50
1049053	0.86	0.87	0.79	4.20
14042261	0.94	0.91	0.91	18.70
62898960	1.1	1.25	1.17	15.60
496902	0.84	0.77	0.7	11.20
119626900	1.01	1.07	1.13	24.30
62087582	0.35	0.33	0.09	32.00
50592994	0.94	0.31	0.26	11.40
88900491	1.16	0.96	1.02	7.40
238624110	0.83	0.31	0.24	47.00
6959304	0.9	0.61	0.74	7.30
77415419	1.43	1.53	2.33	30.60
119602580	0.87	0.82	0.94	7.60
60552474	0.87	0.76	0.79	8.20
182592	1.08	1.18	1.08	21.80
337580	0.59	0.52	0.6	15.10
1314310	1.24	0.97	0.92	16.00
45219796	0.98	0.81	0.13	35.30
62087534	2.44	2.03	1.96	12.70
40789059	0.95	0.93	1.39	6.60
62896517	0.92	0.9	0.86	12.20
36146	0.63	0.58	0.57	32.60
5762315	0.93	1.04	1	12.60
46249758	0.89	0.71	0.7	12.60
9802310	1.22	1.39	1.6	7.00
51476906	2.11	2.86	3.28	7.40
1905998	0.68	0.5	0.51	10.30
48145549	1.47	0.74	0.77	16.80
21040388	0.99	0.97	0.98	26.90
6470150	0.95	1.5	1.07	6.10
49456447	0.02	0.03	0.02	19.70
42542700	0.12	0.02	0.03	14.90
194387570	1.34	1.51	1.2	10.80
119573146	0.35	0.35	0.28	30.20
119572383	19.77	33.73	37.33	18.90
971270	0.81	0.9	1.08	13.00
719269	1.1	1.17	1.03	46.80
62898359	0.72	0.57	0.5	4.80
4099506	0.98	2.33	1.38	12.90
119601067	0.9	0.9	0.82	16.30
10800858	1.54	1.31	1.6	7.80
10439439	1.12	1.11	1.11	34.90
73983054	0.86	0.76	0.69	22.80
62088494	0.87	0.67	0.63	5.30
51094802	0.38	0.14	0.26	8.30
119585358	0.86	1.79	1.98	10.00
80478622	1.24	1.22	1.08	13.70
1263196	0.66	0.3	0.44	4.70
42542645	0.86	0.82	0.77	10.90
62089222	0.99	0.94	0.86	19.20
47496627	2.4	2.36	2.42	12.10
73909106	1.04	1.32	1.28	33.60
460789	1.37	1.02	0.9	25.20
62988906	1.11	0.7	0.59	5.20
62898357	1.08	1.57	1.04	5.60
194387272	0.9	0.7	1.04	28.60
62897681	0.24	0.07	0.04	3.20
375331879	1.04	1.21	0.97	9.40
60817455	0.83	0.98	1.29	10.10
403282323	0.9	0.8	0.79	5.90
83674986	1.13	0.8	0.93	21.90
296040438	0.77	0.76	0.93	7.80
118764023	1.33	1.02	1.66	33.60
37196760	1.66	1.11	1.01	5.30
63102455	0.75	0.67	0.64	6.80
62088648	1.14	1	0.82	12.90
693937	0.86	0.48	0.47	8.40
194378426	0.86	1.22	1.22	9.00
47115163	1.04	0.93	1.24	20.40
4503249	0.14	0.12	0.11	14.40
19423868	0.15	0.15	0.11	22.10
119608470	0.74	0.64	0.77	8.60
332217748	1.2	1.09	0.87	23.10
48145663	0.51	0.07	0.36	20.20
194382012	1.12	1.32	0.15	44.10
62087698	0.85	0.8	1.08	10.50
62897717	1.32	0.79	0.79	32.20
62087230	1.11	0.99	0.61	15.60
332817238	1.16	0.96	1.06	10.10
158255138	1.36	1.31	1.07	24.20
194378804	0.98	1.96	2.21	9.80
62897773	1.58	1.32	1.14	17.20
62896765	0.24	0.08	0.06	5.30

Table I. *Continued*

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GI Accession	3 h/0 h	6 h/0 h	12 h/0 h	% Cov
38327625	0.79	0.24	0.39	10.30
7022744	0.94	0.9	0.93	4.90
194377686	0.94	0.98	0.93	7.80
194097350	0.52	0.45	0.38	12.80
62088766	1.09	1.02	0.94	10.70
397489099	1.22	1.27	1.29	8.40
6633953	0.87	0.98	1.07	6.70
46249756	1.15	1.11	1.14	6.00
45861372	0.9	1	0.96	5.20
62988842	1.63	1.21	1.85	4.00
915284	0.69	1.58	1.28	5.40
62113341	0.12	2.83	2.54	11.50
3152942	1.08	1.24	0.81	19.50
194386684	1.02	0.98	0.51	5.10
187609784	1.14	0.84	0.77	9.50
62897953	1.11	1.16	1.17	9.00
62089046	1.03	0.86	1.08	8.30
393007760	1.32	1.43	1.29	7.40
33879558	0.42	0.16	0.52	10.90
158256542	1.33	0.99	1.08	10.10
77415483	0.88	1.03	1.02	12.60
7211188	1.03	1.12	1.42	10.30
51477708	1.14	1.09	1.05	21.60
40788905	0.6	0.44	0.49	4.50
291084635	1.06	1.1	1.15	9.30
194382320	0.92	1.37	1.67	11.80
119586485	1	1.49	1.25	4.50
8489021	1.03	1	0.99	23.40
79750824	1.31	1.39	1.2	5.50
62630180	1.37	1.53	1.32	7.80
62088312	1.08	0.88	1.16	11.20
5031973	1.31	1.18	1.11	8.90
48146259	1.11	1.32	1.13	5.40
221045312	0.82	1.07	1	14.60
221042312	1.24	1.42	1.43	7.80
119608226	1.26	1.26	1.63	16.00
913174	1.67	1.57	1.2	4.60
904032	1.22	1.09	1.66	9.50
7513316	0.86	1.13	1.19	15.90
71891715	0.8	0.65	0.97	1.40
7020733	1.31	1.03	1.19	13.80
68800138	1.09	1.05	1.06	15.00
687239	0.77	0.79	0.91	3.30
62896539	1.27	0.97	0.81	8.00
62088770	1.39	1.13	1.32	4.10
56972026	1.32	1.07	1.37	8.80
4689134	1.17	0.78	0.92	17.10
397526013	0.93	0.93	0.92	6.00
397511518	0.96	1.49	0.6	15.00
397487389	1.21	1.18	1.22	12.60
3450955	0.98	1.05	1.17	5.00
332840081	1.17	1.04	1.09	9.90
32492946	1.07	1.31	0.51	8.20
31873732	1.37	1.45	1.8	21.50
284164	1.02	1.17	1.14	14.10
221040244	0.84	0.79	0.77	6.40
194385288	0.59	0.37	0.5	10.70
194377348	0.96	0.97	1.05	12.60
119576944	0.86	0.41	0.85	18.30
89573979	0.84	0.72	0.9	5.40
82408395	1.2	1.14	1.57	3.90
78070376	0.25	0.13	0.28	5.00
73486658	1.14	1.75	1.53	6.30
71052048	0.76	1.14	1.2	6.70
62898319	0.2	0.13	0.08	10.40
62898301	0.61	0.93	0.68	4.70
62897547	1.12	1.04	1.07	9.80
62897053	1.05	1.01	0.98	15.30
62896799	1.41	1.42	1.51	8.80
62896525	0.99	1	1.05	7.70
62088010	1.08	0.72	0.58	5.40
553640	0.53	0.33	0.45	15.50
49456445	0.88	1.1	1.24	9.20
48734757	0.25	0.17	0.15	11.20
48146951	0.98	1	1.11	6.50
48146909	0.71	0.73	0.65	4.30
46250408	0.67	0.6	0.42	14.30
4587127	0.86	0.91	0.87	20.30
41351350	0.92	1.14	1.2	3.40
221045034	0.98	0.56	1.14	19.10
193787899	0.96	0.98	0.96	18.90
158256566	0.89	1.41	1.61	8.00
119623416	1.87	2.75	2.38	13.30
119598231	1.03	1.01	0.82	6.80
119597116	1.1	1.19	1.01	21.90
119592809	1.36	1.45	1.96	11.00
119579121	0.98	0.9	0.91	20.90
9022435	0.99	1.14	1.36	8.20
8923900	0.92	1.13	0.99	3.50
7706497	1.54	1.33	1.04	7.00
73622130	0.87	1.2	1.04	12.50
6841470	1.04	1.29	1.25	5.00
65506891	1.24	1.08	1.19	1.90
62087740	1.21	0.63	1.53	1.60
5834273	0.92	1.04	1.03	8.90
49457384	1.26	1.06	1.03	12.50
4503117	1.05	1.04	1.16	21.40
371872799	0.64	0.82	0.9	8.50
291393360	0.98	0.83	0.79	9.40
1237406	1.17	1.17	1.27	21.40
109948283	1.27	0.86	0.72	6.00
83405045	1.16	0.53	1.24	19.10
16924231	0.37	0.11	0.16	9.60
55961080	0.69	0.37	0.54	17.00
190267	1.19	1.25	1.16	8.60
332814230	1.11	1.17	0.98	23.80
68533125	1.64	1.2	0.83	2.70
16552475	0.85	1.13	1.2	15.20
7688673	0.71	0.6	0.5	10.50
30819	0.74	0.89	0.77	5.30
221044236	1.11	1.29	1.43	7.50
119599372	0.91	0.91	0.72	8.00
27370952	0.85	0.84	0.9	5.80
3668141	0.82	0.63	0.67	4.30
119618576	1.04	1.5	1.2	31.60
55959640	1.43	1.53	1.92	6.00

Table I. *Continued*

Table I. *Continued*

GI Accession	3 h/0 h	6 h/0 h	12 h/0 h	% Cov
55960772	0.34	0.31	0.39	23.20
62896895	0.94	0.94	1.02	6.40
397138385	1.47	1.69	1.46	14.20
145942462	6.67	11.17	13.68	26.90
347446676	0.77	0.55	0.64	7.90
426383484	1.16	1.04	1.64	6.70
119570025	2.31	0.85	2.27	5.00
410058693	1	1.05	1.28	14.30
119601958	0.69	0.76	0.79	11.10
1814274	0.88	1.63	1.04	12.10
13654237	0.7	0.28	0.48	3.90
194387264	1.05	0.3	0.78	13.60
4530441	0.76	1.27	1.24	6.30
71051299	0.95	0.73	0.88	2.80
55959888	1.25	1.92	1.8	55.70
773575	1.22	1.34	1.37	5.90
62088522	0.96	0.65	1.21	16.80
337518	1.34	1.17	1.21	16.70
47938338	1.14	1.19	1.18	13.10
58530840	0.8	0.56	0.61	7.00
4033735	1.37	1.38	0.89	3.60
10440418	1.38	2.4	1.94	4.00
62898948	0.74	0.74	0.9	3.00
62896731	0.83	0.61	0.82	2.70
63003907	1.89	1.67	1.58	2.20
6648547	1.09	1.06	1.11	9.20
47496673	1.28	1.45	1.43	9.20
194377152	1.31	1.17	1.13	17.90
20381196	0.11	0.07	0.06	20.50
119614889	0.66	0.31	0.53	15.00
15012043	0.9	0.84	0.77	22.80
119610254	0.74	0.42	0.77	29.60
119571409	0.63	0.71	0.59	4.90
214010226	0.98	1.06	0.92	22.20
395822376	0.82	0.9	0.79	5.40
6469374	1.36	0.9	1.21	3.60
48145985	1.13	1.27	1.29	36.80
10438983	0.53	0.23	0.48	15.50
62897941	0.94	1.61	2.19	8.10
71052098	1	1.2	1.19	5.10
46020022	0.99	1.07	0.98	2.00
1526426	0.9	0.76	0.64	9.50
9295176	0.84	1.38	0.91	26.10
20521838	1.17	1.1	1.06	7.70
55859528	0.81	0.88	1.37	4.80
9802306	1.53	1.49	1.15	12.80
193786052	0.84	1.02	0.86	2.90
7959903	1.34	1.18	1.33	9.80
31419730	25.12	37.33	39.45	4.90
119599647	1.04	0.46	0.99	11.80
221046276	0.74	0.69	0.69	2.30
62241042	1.36	1.19	1.32	2.90
33341656	0.97	0.98	1	4.70
158258244	1.61	2.73	2.42	4.30
189069230	0.21	0.05	0.04	3.10
62898045	0.99	1	0.8	6.10
120444924	0.75	0.67	0.9	3.00
116283290	5.97	7.18	13.18	8.50
47076940	1.98	1.57	0.86	7.80
284178790	1.06	0.33	0.49	8.80
119580675	1.11	1.57	1.8	2.20
4586876	0.81	0.59	0.7	2.10
21750118	0.76	0.86	0.58	19.60
33469917	0.84	0.72	0.77	4.30
62896515	9.38	11.8	18.88	11.80
402888626	32.51	43.65	51.05	40.50
119569526	1.26	1.08	1.09	3.90
194388628	1.02	0.8	0.9	4.30
31873738	0.37	0.51	0.37	1.80
57162594	1.21	1.89	1.54	3.50
6010211	1.94	2.61	2.54	2.10
48145665	2.07	1.32	1.33	3.10
180758	0.92	0.9	1.51	7.00
71891782	0.71	0.53	0.5	2.20
14133233	1.32	3.6	2.56	4.00
11321634	1.57	1.6	1.09	4.70
10433642	0.49	0.36	0.66	8.90
73921242	0.39	0.15	0.25	1.30
62087566	1.18	1.07	0.92	2.00
33338040	67.3	83.95	86.3	18.90
33317134	10.57	17.06	29.38	19.20
2588783	13.06	24.21	23.55	3.40
51599156	1.02	0.74	0.95	1.90
119616342	0.79	0.42	0.89	50.00
90265805	0.44	0.2	0.27	1.10
440308	0.71	0.67	0.86	12.60
62087784	3.19	4.79	5.2	9.10
14270502	0.77	0.89	0.92	1.30
119567980	4.25	7.59	6.85	6.00
62897427	0.74	0.69	0.69	1.00
204306657	2.21	3.31	3.19	0.80

Discussion

Increasing evidence demonstrates that *H. pylori* infection is an important factor for gastric diseases such as peptic ulcers and gastric carcinogenesis (35-39). It is necessary to study human gastric cell responses to *H. pylori* infection. To understand the human responses to *H. pylori* and its interaction with the host, high-throughput technologies, such as microarrays and proteomic focus studies, gene level profiles are very important (40). In our present studies, we used iTRAQ proteomics to characterize the differentially expressed proteins in *H. pylori*-infected AGS cells for the discuses pathologic mechanism. Simultaneously, we performed IPA analysis of the major pathway in *H. pylori*-

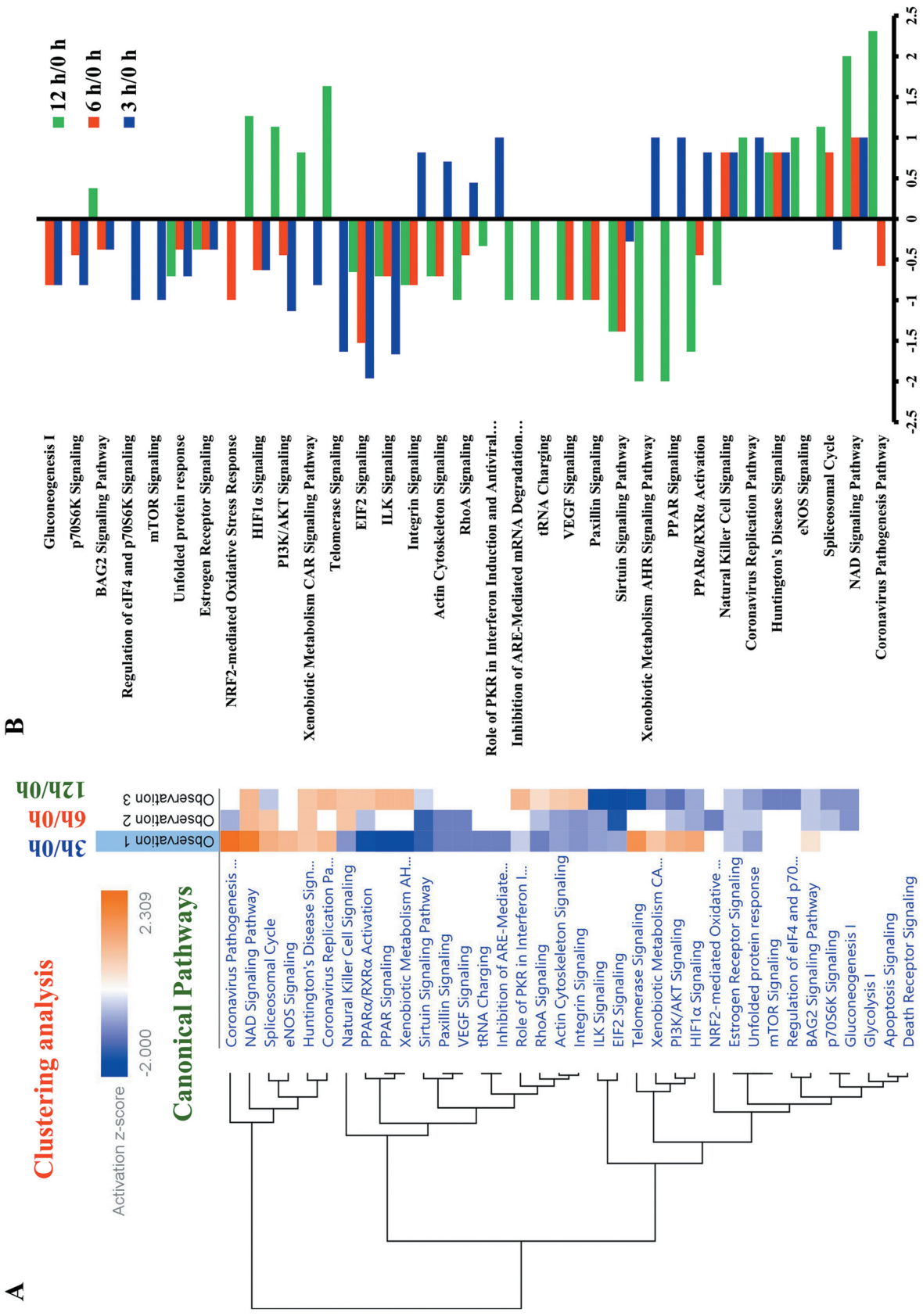

Figure 3. *Clustering analysis (A) and biological pathways (B) of iTRAQ proteomics study results on Helicobacter pylori-infected AGS cells using ingenuity pathway analysis.*

Table II. *Top canonical pathways generated and ratio by ingenuity pathway analysis from protein profiles in Helicobacter pylori-infected human gastric adenocarcinoma AGS cells.*

Canonical pathways	3 h/0 h	6 h/0 h	12 h/0 h
Coronavirus pathogenesis pathway	0	–0.577	2.309
Nicotinamide adenine dinucleotide signaling pathway	1	1	2
Spliceosomal cycle	–0.378	0.816	1.134
Endothelial nitric oxide synthase signaling	0	0	1
Huntington's disease signaling	0.816	0.816	0.816
Coronavirus replication pathway	1	0	1
Natural killer cell signaling	0.816	0.816	–0.816
PPARα/RXRα activation	0.816	–0.447	–1.633
Peroxisome proliferator-activated receptor (PPAR) signaling	1	0	–2
Xenobiotic metabolism aryl hydrocarbon receptor signaling pathway	1	0	–2
Sirtuin signaling pathway	–0.277	–1.387	–1.387
Paxillin signaling	0	–1	–1
Vascular endothelial growth factor signaling	0	–1	–1
Transfer RNA charging	0	0	–1
Inhibition of AU-rich element (ARE)-mediated mRNA degradation pathway	0	0	–1
Role of protein kinase R in interferon induction and antiviral response	1	0	–0.333
RhoA signaling	0.447	–0.447	–1
Actin cytoskeleton signaling	0.707	–0.707	–0.707
Integrin signaling	0.816	–0.816	–0.816
Integrin-linked kinase signaling	–1.667	–0.707	–0.707
Eukaryotic initiation factor 2 signaling	–1.964	–1.528	–0.655
Telomerase signaling	–1.633	0	1.633
Xenobiotic metabolism chimeric antigen receptor signaling pathway	–0.816	0	0.816
Phosphatidylinositol 3-kinase/protein kinase B signaling	–1.134	–0.447	1.134
Hypoxia-inducible factor 1α signaling	–0.632	–0.632	1.265
Nrf2-mediated oxidative stress response	0	–1	0
Estrogen receptor signaling	–0.378	–0.378	–0.378
Unfolded protein response	–0.707	–0.378	–0.707
Mammalian target of rapamycin signaling	–1	N/A	0
Regulation of eIF4 and p70S6 kinase signaling	–1	N/A	0
Bcl2-associated athanogene 2 signaling pathway	–0.378	–0.378	0.378
p70S6 kinase signaling	–0.816	–0.447	0
Gluconeogenesis I	–0.816	–0.816	0

infected AGS cells (Figure 2). The findings of the present study provide further understanding of the effects of *H. pylori*, which may be useful for further exploration of the pathological mechanism.

Our results showed that the two major biological pathways associated with *H. pylori*-infected AGS cells at 3, 6, and 12 h of infection were the EIF2 signaling and BAG2 signaling pathways (Figure 5 and Figure 6). The biological function of EIF2 signaling is involved in protein synthesis, cellular growth, cell proliferation, cell development, cellular stress, cellular injury, and second messenger signaling (41-45). eIF2 (eukaryotic initiation factor-2) is a GTP-binding protein that escorts the initiation-specific form of met-tRNA onto the ribosome. Importantly, the functions of eIF2 include delivery of the charged initiator methionyl-tRNA to the ribosome as well as a role in identifying the translational start site (41, 44, 46, 47). Sokolova *et al*. demonstrated that *H. pylori* also regulates protein synthesis. In early protein translation, the Met-tRNA-eIF2-GTP pre-initiation complex mediates methionyl-tRNA via eIF2 to the 40S ribosome (48). The biological function of the BAG2 signaling pathway is involved in cell death, cell survival, embryonic development, organismal injury, and abnormalities (49). The BAG2/HSP70 complex plays an essential housekeeping role in the maintenance of the neuronal cytoskeleton, inducing proteasome-independent degradation of phosphorylated tau protein (49, 50). BAG2 is a target of miR-128a, which decreases BAG2-mediated Tau degradation and promotes Alzheimer's disease (50, 51). The BAG2 signaling pathway includes family proteins and HSPs (52). BAG family proteins regulate HSP70/HSC70-mediated chaperone activity and apoptosis (52). BAG2 contains a carboxyl-terminal domain that promotes substrate release from HSP70/HSC70 chaperones (52).

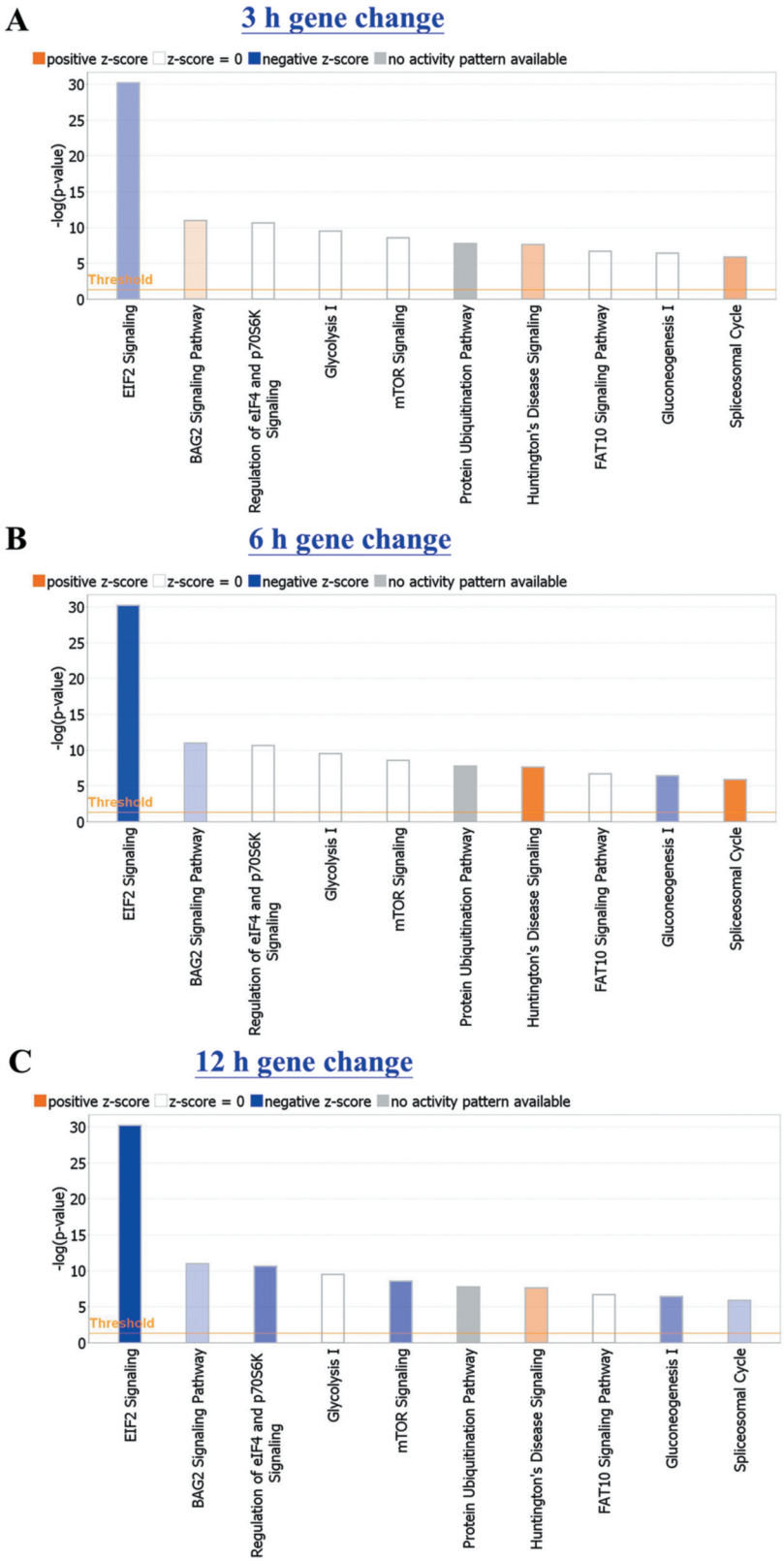

Figure 4. *Statistical results of the top 10 related biological pathways associated with the potential targets on Helicobacter pylori 3 h (A), 6 h (B) and 12 h (C) infected AGS cells using ingenuity pathway analysis.*

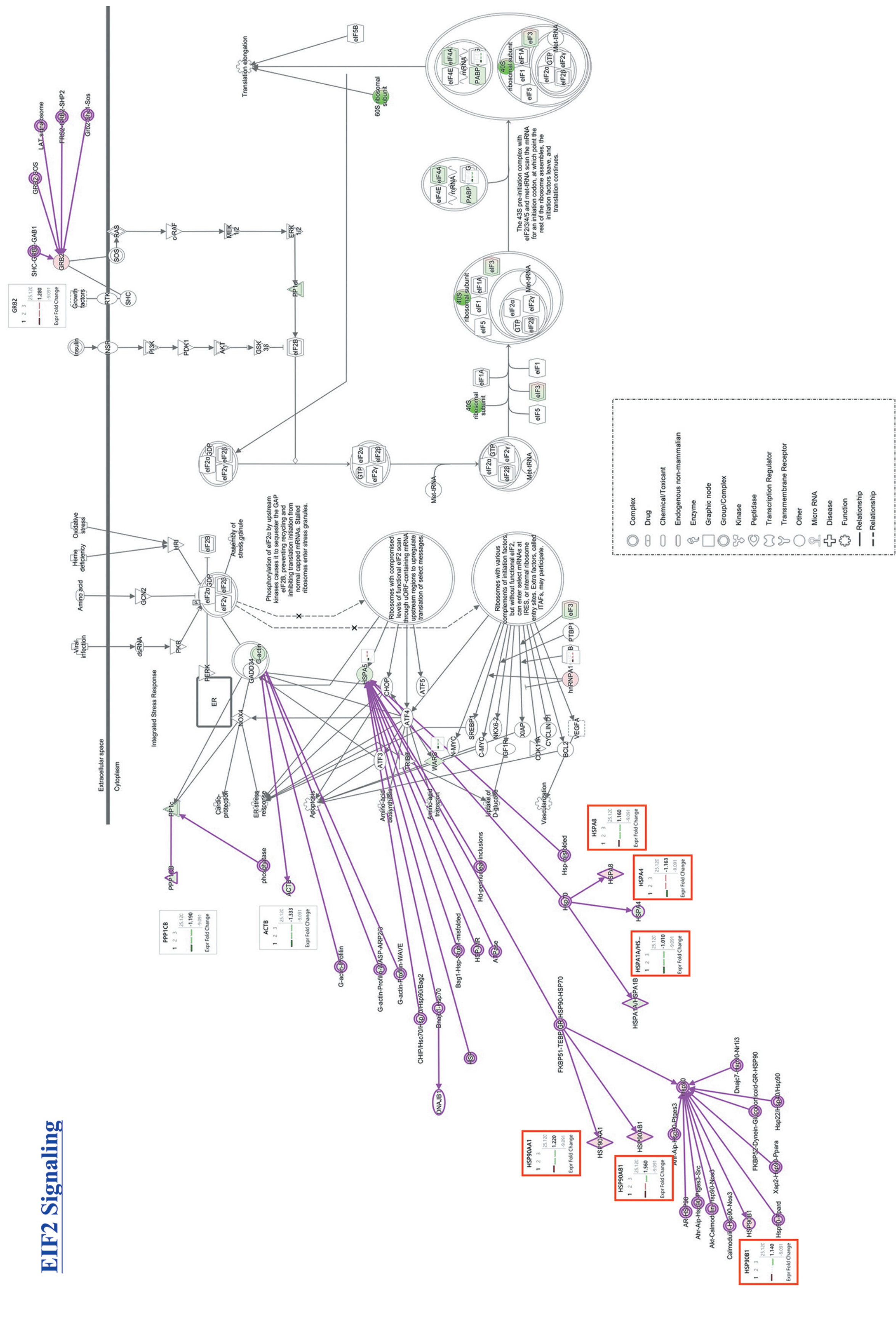

Figure 5. *Canonical EIF2 signaling pathway on Helicobacter pylori-infected AGS cells using ingenuity pathway analysis.*

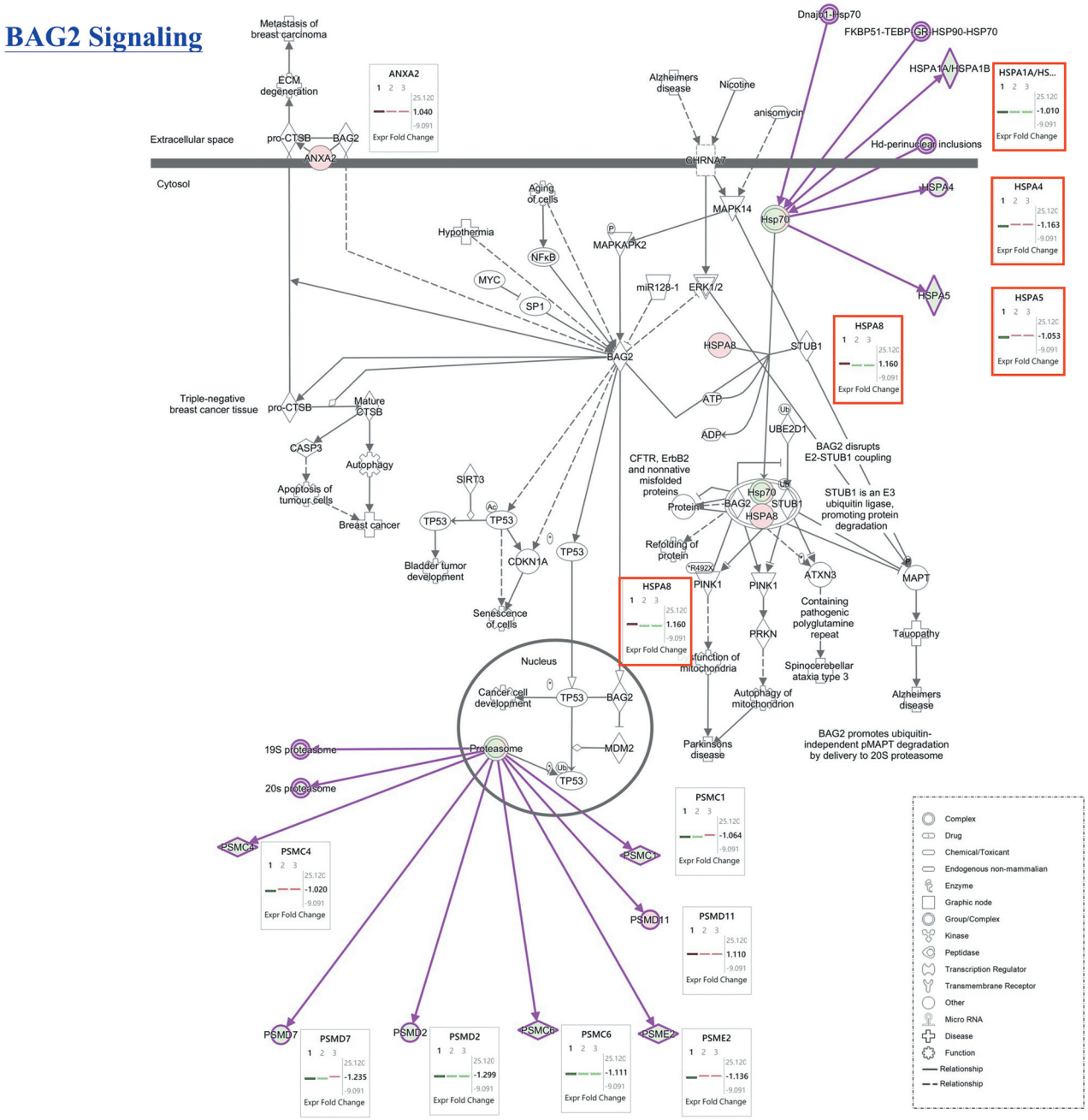

Figure 6. *Canonical BAG2 signaling pathway on Helicobacter pylori-infected AGS cells using ingenuity pathway analysis.*

HSP family are molecular chaperones and have been associated to be an important for the defense mechanisms at the gastric epithelial cells (53, 54). In mammalian cells, the major HSPs are HSP60, HSP70, HSP90, and HSP110 (55). HSP70 is a major molecular chaperone that accelerates cellular recovery from external stimuli by coping with unfolded or denatured proteins (55). The mucosal defense effect for ulcer and inflammation healing may occur through HSP70 biological function (55, 56). HSP105 is a member of the HSP70 superfamily of molecular chaperones and serves as a nucleotide exchange factor for HSP70 and independently prevents the aggregation of misfolded proteins. Our results demonstrated that *H. pylori* infection decreased cell viability after 12 h of treatment (Figure 1) and decreased HSP70 and HSP105 protein levels

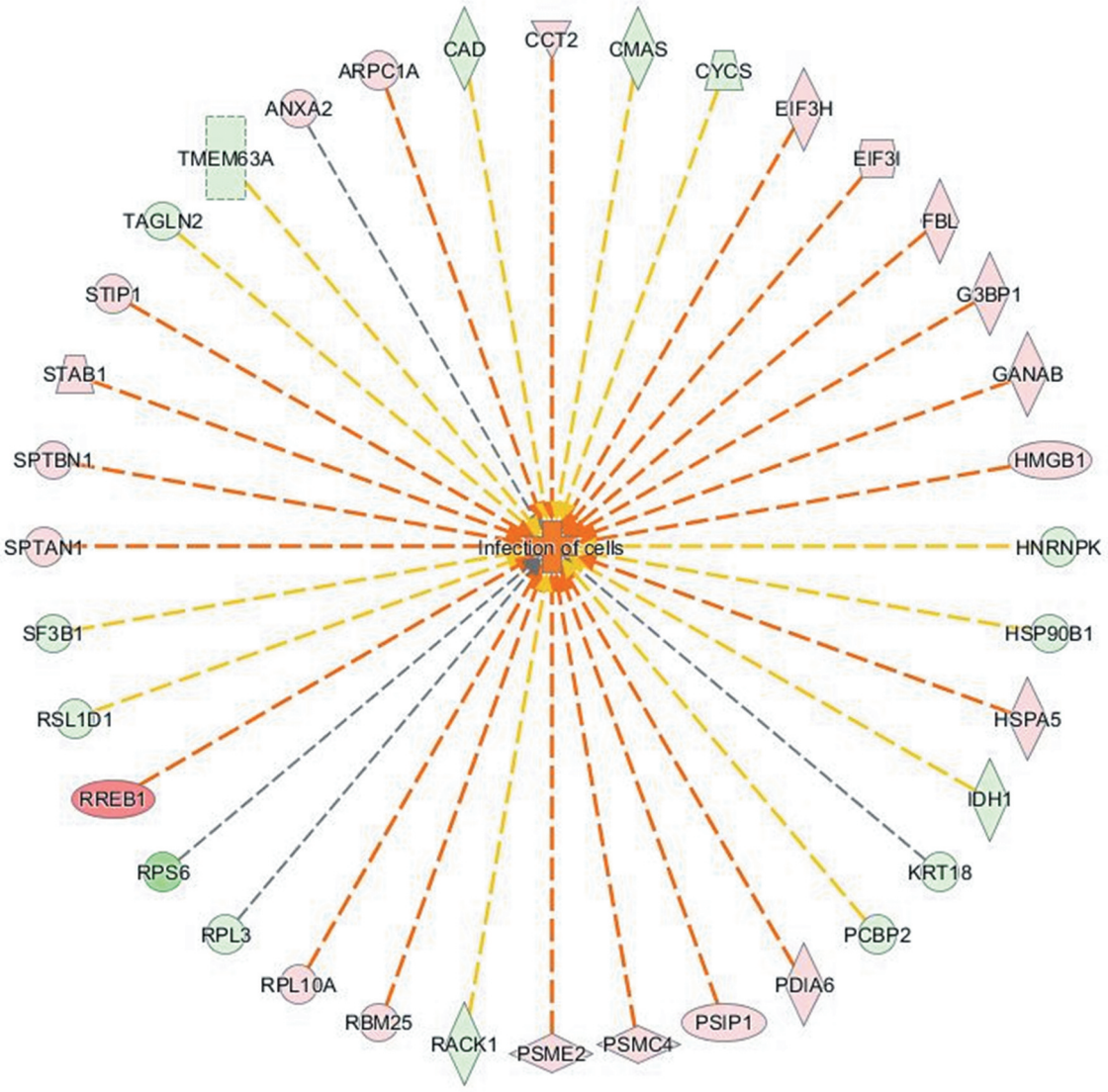

Figure 7. *Infection of the cells network constructed using ingenuity pathway analysis.*

(Figure 8). Weili Li *et al*. showed that inhibition of gastric epithelial cell growth induced cell-cycle S phase arrest and apoptosis by *H. pylori* infection through inhibition of HSP70 protein levels (53). A decrease in intracellular HSP70 protein leads to an increase in mitochondrial apoptosis-inducing factor (AIF) and cytochrome c release to the cytosol, which then triggers apoptotic cell death. Weili Li *et al*. showed that *H. pylori* downregulated HSP105, HSP72, and HSP60 expression through CagA in AGS cells. They suggested CagA as an essential bacterial protein for *H. pylori*-mediated suppression of HSP expression (57). Our previous studies demonstrated the induction of VacA and CagA gene expression in *H. pylori*-infected AGS cells (27). Aneta Targosz *et al*. reported that *H. pylori* inhibited HSP70 protein expression, which was significantly potentiated by exogenous CagA. Furthermore, *H. pylori*-infected MKN7 human gastric carcinoma cells induced cell apoptosis, activated cyclooxygenase (COX)-2, and inhibited heat shock protein HSP70 (15). We suggest that *H. pylori* infection attenuates HSP70 and HSP105 protein expression and inhibits cell viability in *H. pylori*-infected gastric adenocarcinoma cells, possibly through CagA.

Numerous cohort studies have shown that *H. pylori* infection is clearly an important risk factor for gastric cancer (58). Masahide Watada *et al*. reported that *H. pylori* cagA-related genes (jhp0045 and jhp0046) are biomarkers for predicting gastric cancer in Colombia patients (59). Furthermore, in a previous clinical study reported by Rachel Howard *et al*., CD133 significantly expresses in mucosa,

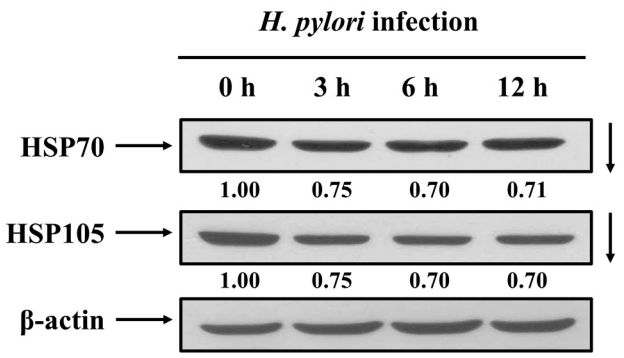

Figure 8. *The Helicobacter pylori decreased protein expression of HSP70 and HSP105 on H. pylori-infected AGS cells. Western blotting was performed to measure the expression levels of HSP70 and HSP105 proteins. The density of the bands compared with the control sample (set to 1.0) is presented above each band.*

inflammation/metaplasia, low-grade dysplasia and gastric adenocarcinoma in patients' samples for each respective patient cohort. However, there is no statistically difference of CD133 between stages of disease. These data suggest that CD133 expression may be a *H. pylori*-independent biomarker in gastric cancer cells (60). Our future studies will investigate the effects of these altered gene patterns on the pathogenesis of gastric cancer in vivo and in clinical studies.

In conclusion, our results suggest that *H. pylori* infection, through HSP family regulation, may decrease HSP70 and HSP105 to promote apoptotic cell death in *H. pylori*-infected AGS cells. We used iTRAQ proteomics study protocol to reveal the molecular targets of pathogenesis in AGS cells infected with *H. pylori*.

Conflicts of Interest

The Author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' Contributions

JSY, CYK, CHS, YJC, YMH, CJC conceived and designed the experiments. JSY, CYK, CHS, YJC, YMH, CJC performed the experiments. JSY, CHS, CJC analyzed the data. JSY, YJC, YMH wrote and modified the paper. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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