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Helicobacter pylori infection combined with DENA revealed altered expression of p53 and 14-3-3 isoforms in *Gulo*$^{-/-}$ mice

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ABSTRACT

Unlike most other mammals, human bodies do not have the ability to synthesize vitamin C inside of their own bodies. Therefore, humans must obtain vitamin C through daily diet. *Gulo*$^{-/-}$ mice strain is known with deficiency, in which vitamin C intake can be controlled by diet like human, and would be valuable for investigating the molecular mechanism of various diseases. In the present study, we established *Gulo*$^{-/-}$ mice model and investigated the differentially expressed proteins in stomach tissue of *Gulo*$^{-/-}$ mice after *Helicobacter pylori*-infected, and followed by DENA, using immunohistochemistry and proteomic approach. The results of immunohistochemistry analysis of stomach tissue showed that the tumor suppressor, p53 protein, expression was significantly decreased ($p < 0.05$) but not messenger RNA (mRNA) transcriptional level, and 14-3-3ε, 14-3-3δ, Ki-67 and cleaved caspase 3 expressions were significantly increased ($p < 0.05$) by *H. Pylori* infection, and followed by DENA treatment in *Gulo*$^{-/-}$ mice. Moreover, knockdown of 14-3-3 isoforms (14-3-3ε, 14-3-3σ, 14-3-3ζ and 14-3-3η) were significantly increased sub-G1 phase (characteristics of apoptosis) in AGS cells and, phenotypic changes like cell shrinkage, density and cleaved nuclei were also observed. Proteome analyses showed that 14-3-3σ, 14-3-3η, and tropomyosin alpha-1 chain were down-regulated, and Hspd1 protein and HSC70 were up-regulated after *H. Pylori*-infection, and followed by DENA. The combined results of immunohistochemistry and proteomic analysis suggest that *H. pylori* altered the p53 and 14-3-3 isoforms expression and DENA further enhanced the *H. pylori* effect, which might be involved in carcinogenesis and metastasis of gastric cancer on *Gulo*$^{-/-}$ mice.

1. Introduction

Globally, gastric cancer (stomach cancer) is the second leading cause of cancer-related death, next to lung cancer, and approximately 989,600 new cases are diagnosed and 738,000 deaths every year [1]. Currently, the diagnosis is based on clinical, endoscopic, and histological criteria of gastric cancer patients; moreover, it is very difficult while examining many patients. The alternative possibility is to use laboratory animals, like mouse and rat for such studies. Vitamin C (ascorbic acid) is an excellent antioxidant, and an essential nutrient of most living tissues [2]. Humans must obtain vitamin C through daily diet due to a lack of L-gulono-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 vary greatly among the individuals for optimal health. The most common laboratory animals, including the mouse 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 an appropriate and prominent model for human disease studies. Recently, *Gulo*$^{-/-}$ mice model have been used for *in vivo* experiment [3] and these *Gulo*$^{-/-}$ mice strain is known with deficiency, in which vitamin C intake can be

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controlled by diet like human, would be valuable for investigating the molecular mechanism of various diseases.

Helicobacter pylori infection is a major cause of gastric cancer [4] and it is known to be an important pathogen associated with various severe gastric diseases, including peptic ulcers, chronic gastritis, and gastric cancer [5]. 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 is also higher [6,7]. However, the mechanism by which *H. pylori* promotes the gastric epithelial cells to become cancerous is not fully understood. Moreover, Nitrosamines are the most important carcinogens that are involved in development of esophageal squamous-cell cancer (SCC) and gastric adenocarcinoma [8]. Diethylnitrosamine (DENA) is a well-known N-nitrous compound that provokes esophageal cancer in laboratory animals [9,10]. It has been first reported that diethylnitrosamine (DENA) used for the induction of esophageal and gastric tumors in hamsters [11]. Presently, only limited studies are available regarding DENA effects on gastric cancer. Hence, the combined effects of *H. pylori*-infection and DENA treatment in laboratory animals like *Gulo*$^{-/-}$ mice will provide new leads to understand the mechanism between gastric cancer, *H. pylori* and DENA.

With recent advances in proteomics, protein biomarker discovery is now a major area of proteome research. Profiling methods like matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF/MS) mass spectrometry analyses of animal tissues and fluids are promising techniques for biomarker discovery. In present study, we established *in vivo Gulo*$^{-/-}$ mice model like humans (it cannot synthesis vitamin C) and to identify the proteins which are differentially expressed in *H. pylori*-infected alone and/or combined with DENA that may play important roles in the pathological mechanism of *H. pylori*-induced gastric diseases and enhancement effect of DENA. To our knowledge, this is the first study using proteomics approach to profile protein changes in *Gulo*$^{-/-}$ mice model after *H. pylori* infection alone and/or combined with DENA.

2. Materials and methods

2.1. Chemical and reagents

Materials and chemicals used for electrophoresis were obtained from BioRad (Hercules, CA, USA). Antibodies 14-3-3ε and 14-3-3δ were obtained from Bioworld Technology Inc. (St. Louis Park, MN, USA), and p53 was purchased from Millipore (Billerica, MA, USA). RPMI 1640 medium was purchased from Hyclone (Logan, UT, USA). Fetal bovine serum (FBS) and antibiotics (streptomycin/penicillin) were purchased from Gibco (BRL Life Technologies, Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), and RNase A were obtained from Sigma–Aldrich (St. Louis, MO, USA). The 14-3-3ε siRNA (h), 14-3-3σ siRNA (h), 14-3-3ζ siRNA (h) and 14-3-3η siRNA (h) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Vitamin C (Ascorbic acid) was provided by Animal Resources Research Bank (ABRB). 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.

2.2. Experimental design and sample collection

We followed Animal Science guidelines for animal experimentation. *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 (Ed note: offspring) were evaluated by PCR as recommended [12]. 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 [13]. *Gulo*$^{-/-}$ mouse was divided into three groups; the control, *H. Pylori* infected group and *H. Pylori* infected followed by DENA treatment. In all three groups, vitamin C was supplemented (20 mg/animal/day, PO) and only group 3 was treated with DENA (10 mg/L, PO). A schematic representation of whole experimental procedure showed in Fig. 1. After 48 weeks, sacrificed all animals, stomach tissues were collected. Samples were stored at −70 °C until analysis.

2.3. Immunohistochemistry

We performed immunohistochemistry to evaluate the expression of p53, 14-3-3ε, 14-3-3δ, Ki-67 and cleaved caspase 3 in *Gulo*$^{-/-}$ mice mouse stomach tissue of all three groups, as mentioned in the experimental design. Collected samples were immersed in 4% prarformaldehyde for 48 h, and then processed for paraffin wax histology. In addition, 5 and 10 μm thick paraffin embedded lung slices were collected. To estimate histological changes, Hematoxylin–eosin (H–E) staining was carried out. Immunohistochemistry for p53, 14-3-3ε, 14-3-3δ, Ki-67 and cleaved caspase 3 were performed using rabbit monoclonal antibodies. Immunoreactivity was visualized with an avidin–biotin peroxidase reaction (PK-4001, Vectastain ABC Kit). The peroxidase reaction was developed using a 3, 3′-diaminobenzidine tetrahydrochloride (D-5905, Sigma). The sections were counterstained with hematoxylin before being mounted.

2.4. Real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from *Gulo*$^{-/-}$ mice stomach tissues of control, *H. pylori* infected group and *H. pylori* infected combined

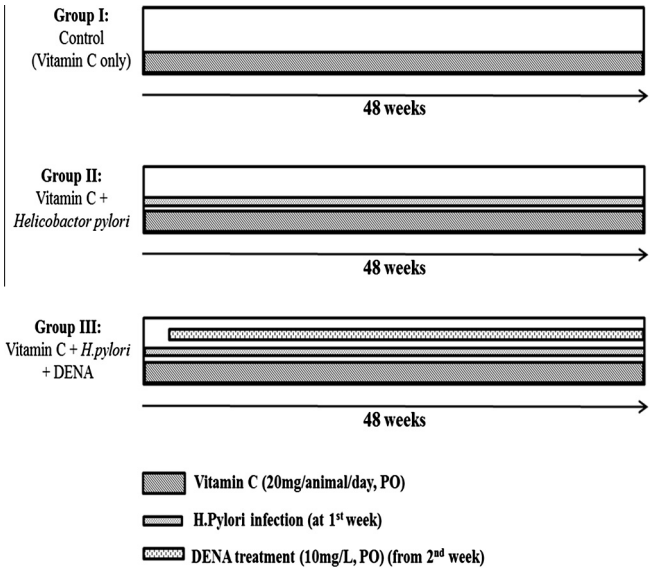


Fig. 1. A schematic representation of experimental procedure followed in *Gulo*$^{-/-}$ mice.

with DENA treated group using TRIzol reagent (GeneALL Biotechnology, Seoul, Republic of Korea) and reverse-transcribed into cDNA by commercially available cDNA synthesis kits (iScript cDNA Synthesis Kit; Bio-Rad) according to the manufacturer protocol. The real-time PCR was performed on a CFX96 real-time PCR system (Bio-Rad) with QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA, USA). The primers were designed with the following sequences: p53, 5′-ACATGACGGAGGTCGTGAGA-3′ (forward), 5′-TTTCCTTCCACCCGGATAAG-3′ (reverse); and β-actin, 5′-GGCTGTATTCCCCTCCATCG-3′ (forward) and 5′-CCAGTTGGTAAC AATGCCATGT. All reactions were performed in triplicate and the messenger RNA (mRNA) expression of p53 was normalized to β-actin mRNA in the same sample.

2.5. Protein extraction and two-dimensional gel electrophoresis (2-DE)

The *Gulo*$^{-/-}$ mice stomach tissues were lysed in a lysis buffer (7 M urea, 2 M thiourea, and 4% (w/v) CHAPS) on ice for 1 h. The lysates were centrifuged at 14,000 rpm for 15 min at 4 °C, and the collected supernatants were stored at −80 °C until analysis. Stomach tissue proteins were precipitated in lysates with equal volume (1:1) of 20% v/v trichloroacetic acid and dissolved in 7 M urea, 2 M thiourea and 4% (w/v) CHAPS, 0.5% (v/v) IPG buffer, 1% dithiothreitol (DTT). Protein concentration was determined by the Non-Interfering™ protein assay kit (G-Biosciences, St. Louis, MO, USA) according to the manufacturer's protocol. Immobilized 18 cm linear pH gradient (IPG) strips, pH 4–7, were rehydrated in rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.002% Bromophenol blue). For the first dimension, 100 μg protein was focused 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, 10,000 V for 1 h, 10,000 V for 13 h, and 50 V for 3 h. The focused strips were equilibrated twice for 15 min each time, first with 10 mg/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 12% linear gradient SDS–PAGE with a constant current of 20 mA/gel at 20 °C. Gels were run until the Bromophenol dye front reached the end of the gel.

2.6. 2-DE gel analysis, protein detection and in-gel digestion

The gels were stained with silver nitrate, similar to the method described by Swain and Ross (1995) [14] 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 automatically matching the detected protein spots. Those spots differing significantly ($p < 0.05$) in their intensities with a fold-change $\geqslant 2$ were used for further analysis. Selected protein spots were excised manually from the two-dimensional electrophoresis (2-DE) gel and protein digestion was performed [15] with slight modifications. Briefly, the excised gel pieces were washed with 100 μl of 100 mM NH_4HCO_3 for 5 min and then dehydrated in 100 μl 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 50 mM NH4HCO3, containing 20 ng/μl trypsin (Promega, Madison, WI, USA) on ice. After 45 min, the trypsin solution was removed and replaced with 10–20 μl of 50 mM NH4HCO3 without trypsin, and digestion was carried out for a minimum of 16 h at 37 °C. These peptide mixtures were collected and analyzed by mass spectrometry.

2.7. MALDI-TOF/ TOF MS analysis and database searching

Peptide mixtures were targeted onto a MALDI-TOF/TOF plate and analyzed by 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 800–3000 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 100 ppm for the fragment ions; allowed modifications, Cys Carbamidomethyl (fixed), oxidation of Met (variable). Protein scores >84 were considered statistically significant ($p < 0.05$).

2.8. Cell culture

AGS human gastric cancer cells obtained from the Korea Cell Line Bank (Seoul, Korea) were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin and streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

2.9. Cell viability assay and morphological examination

The AGS cells transfected with 14-3-3ε siRNA (h), 14-3-3σ siRNA (h), 14-3-3ζ siRNA (h) and 14-3-3η siRNA (h), the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based assay was performed on AGS cells after 24 h incubation. Cells were seeded in a 12-well plate and incubated with siRNA for 24 h. After incubation, 100 μl of a MTT solution (5 mg/ml in 1× phosphate buffered saline, PBS) was added to the wells and incubated for 3 h. Then, 500 μl of dimethyl sulphoxide (DMSO) was added to each well after medium was removed completely to dissolve the cellular crystalline deposits. The optical density was measured of at 540 nm using an ELISA plate reader. For phenotypic morphological examination, cells were grown on 6-well plates, treated with siRNA for 24 h for 24 h, and then examined under light microscopy (×400).

2.10. Cell cycle analysis

A total of 60×10^4 cells/well was plated in 6-well plates and incubated overnight at 37 °C in an atmosphere of 5% CO_2. The cells were transfected with 14-3-3ε siRNA (h), 14-3-3σ siRNA (h), 14-3-3ζ siRNA (h) and 14-3-3η siRNA (h) and incubated for 24 h. Cells were trypsinised, washed twice with cold PBS, and centrifuged. The pellet was fixed using cold 70% ethanol (v/v) for 30 min at 4 °C. The cells were washed with PBS and stained with propidium iodide (PI) solution (50 μg/ml) containing RNase A (0.1 mg/ml) in PBS (pH 7.4) for 30 min in the dark. Flow cytometry analyses were performed using a FACSCalibur apparatus (Becton Dickinson, San Jose, CA, USA). The data were analyzed using CellQuest software (Becton Dickinson).

2.11. Nuclear morphology

For nuclear morphological analysis, cells were seeded onto cover glasses and transfected with 14-3-3ε siRNA (h), 14-3-3σ siRNA (h), 14-3-3ζ siRNA (h) and 14-3-3η siRNA (h) for 24 h. Cells were fixed with 3.7% paraformaldehyde for 15 min, washed three times (10 min each), permeabilized with 1% Triton X-100 and stained with Hoechst 33342 fluorescent dye (0.5 μg/ml) for 15 min. Nuclear morphology was examined by fluorescence microscopy (Leica) using a 1000× objective.

2.12. Statistical analysis

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

3. Results and discussion

Ascorbic acid (vitamin C) is an essential dietary antioxidant in humans. As a result of a lack of L-gulonolactone oxidase, unlike most other mammals, humans are unable to synthesize vitamin C; thus, humans must intake by their daily diet, is critical for maintaining normal levels of vitamin C [16]. Epidemiological studies have shown that dietary ascorbic acid appears to protect against gastric cancer [17,18]. However, role of vitamin C in cancer treatment is still controversial. In the present study, we established *Gulo*$^{-/-}$ mice model like human, that vitamin C should be orally supplemented daily. *Gulo*$^{-/-}$ mice were divided into three groups, such as the control group, *H. pylori* infected group and *H. pylori* infected, followed by DENA treatment. In all three groups, vitamin C was supplemented (20 mg/animal/day, PO), since *Gulo*$^{-/-}$ mice is unable to synthesize vitamin C and it will die within 6 weeks due to severe scurvy. Previous studies have reported that vitamin C insufficiency affects severe defects on cardiac function and skeletal systems due to the deficiency on the production of hormones and collagen [19,20]. Recently, Kim et al. [13] have first demonstrated phenotypic changes of *Gulo*$^{-/-}$ mice upon vitamin C withdrawal (i.e.) *Gulo*$^{-/-}$ mice supplemented with 0.33 g/L could not reach at the concentration in wild-type and *Gulo*$^{-/-}$ mice supplemented with 3.3 g/L of vitamin C. In the present study, vitamin C supplemented *Gulo*$^{-/-}$ mice may not be physiologically identical with wild-type mice but rescued the *Gulo*$^{-/-}$ mice from vitamin C deficiency and protected from scurvy. Taken together, we investigated the expression of p53, 14-3-3ε, 14-3-3δ, Ki-67 and cleaved caspase 3, and differentially expressed protein in *Gulo*$^{-/-}$ mice by using immunohistochemistry and 2-DE combined with MALDI-TOF/TOF MS analysis, respectively.

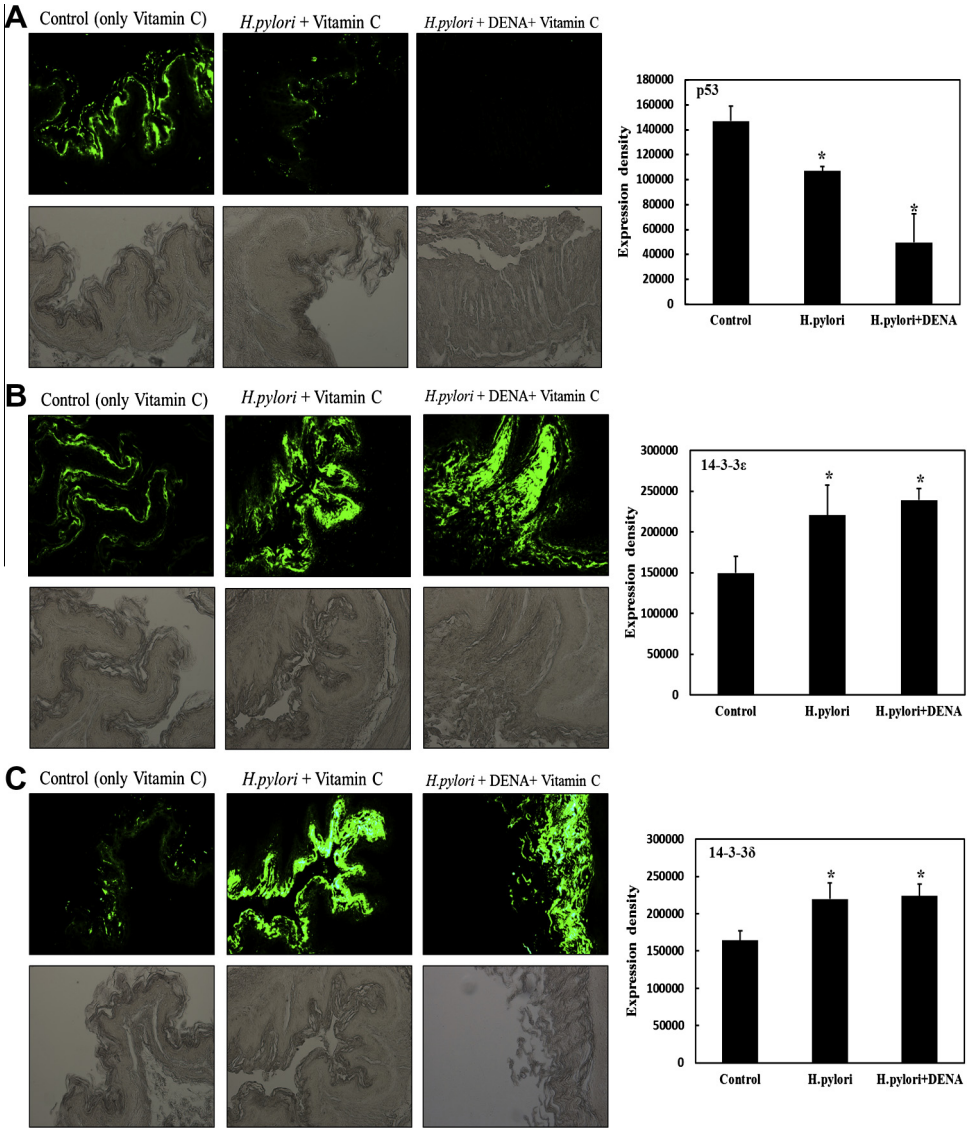
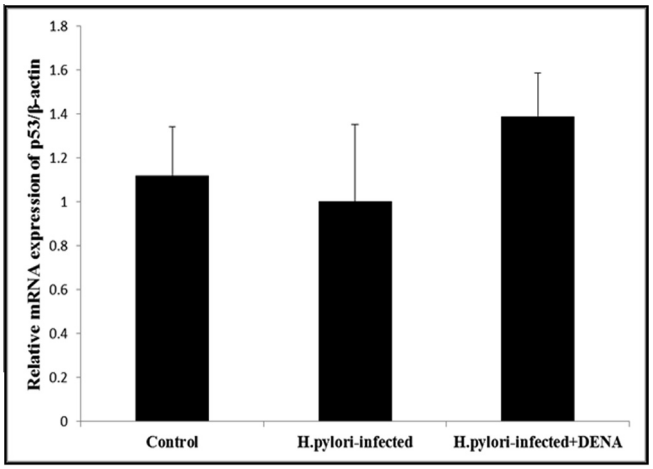


Fig. 2. Immunohistochemistry of p53 (A), 14-3-3ε (B), 14-3-3δ (C) in *Gulo*$^{-/-}$ mice stomach tissue. After 48 weeks, stomach tissue were collected and fixed with 4% paraformaldehyde. Microscopic observations were conducted by LEICA DM 6000 B microscope (×200). Data represent the mean ± SD of three replicates independent experiments. The asterisk (*) indicates a significant difference from the control group (*p < 0.05).



	mRNA expression of p53 normalization with β-actin	SEM
Control (Only vitamin C)	1.11787	0.22281
H.pylori-infected	1	0.35025
H.pylori-infected+DENA	1.38898	0.1969

Fig. 3. The mRNA expression of p53 in *Gulo*$^{-/-}$ mice stomach tissue. The real-time PCR was performed on a CFX96 real-time PCR system (Bio-Rad). Data represent the mean ± SED of three replicates independent experiments and the mRNA expression of p53 was normalized to β-actin mRNA in the same sample. The asterisk (*) indicates a significant difference from the control group (*$p < 0.05$).

3.1. The expression of p53 was decreased in H. pylori-infected and, combined with DENA treated groups

H. Pylori, a gram negative bacterium that colonizes the mucosal layer overlying the gastric epithelium of the human stomach [21]. We tested the expression of p53 protein (tumor suppressor gene), known to be involved in G1 cell cycle arrest to allow DNA repair as well as induction of apoptosis after DNA damage [22], which is reported to be frequently mutated in a wide variety of human cancers [23–25]. The previous studies have reported that the complete loss of p53 expression greatly enhanced stomach carcinogenesis [26]. In the present study, immunohistochemistry result shows that p53 was significantly decreased in *H. pylori*-infected and combined with DENA ($p < 0.05$) treated *Gulo*$^{-/-}$ mice stomach tissue when compared with control (Fig. 2A). Especially, no expression was found in the *H. pylori*-infected combined with DENA treated group. However, no significant changes were found in mRNA expressions of all three groups (Fig. 3). This expression at transcription and translational level in p53 was vary because of transcriptional activity of a gene (mRNA) is not always representative of cellular protein expression due to posttranslational modifications of p53. This result revealed that p53 decreased expression at translational level might be involved in stomach carcinogenesis due to *H. pylori*-infection and DENA enhanced the gastric cancer with *H. pylori*-infection in *Gulo*$^{-/-}$ mice.

3.2. The expression of 14-3-3ε and 14-3-3δ were increased in H. pylori-infected and combined with DENA treated groups

The 14-3-3 proteins have seven isoforms in mammals [27]. We examined the expression of 14-3-3ε and 14-3-3δ in *Gulo*$^{-/-}$ mice

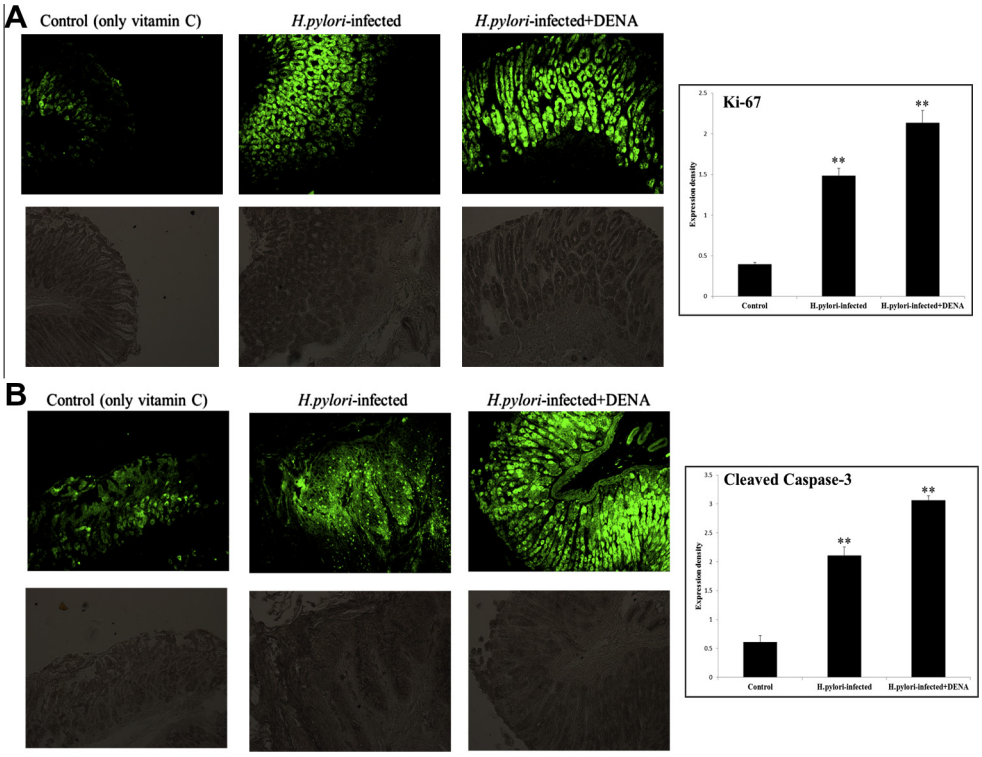


Fig. 4. Immunohistochemistry of Ki-67 and (A), cleaved caspase 3 (B) in *Gulo*$^{-/-}$ mice stomach tissue. After 48 weeks, stomach tissue were collected and fixed with 4% paraformaldehyde. Microscopic observations were conducted by LEICA DM 6000 B microscope (×200). Data represent the mean ± SD of three replicates independent experiments. The asterisk (*) indicates a significant difference from the control group (*$p < 0.05$ and **$p < 0.01$).

stomach by immunohistochemistry. The expression of 14-3-3ε and 14-3-3δ were significantly increased in *H. pylori*-infected and combined with DENA ($p < 0.05$) treated *Gulo*$^{-/-}$ mice stomach tissue when compared with the control (Fig. 2B and C). Particularly, the expression was found in mucosa layer of the stomach tissue. *H. pylori* is gram negative bacterium found in the human gastric mucosa layer. Increased expression of 14-3-3ε was found in the lung, breast, stomach and oral squamous cell carcinomas [28–31]. The expression of 14-3-3ε and 14-3-3δ in mucosa layer might be a new clue to study the protein target of *H. pylori*-infection and stomach cancer carcinogenesis. This increased expression of 14-3-3ε and 14-3-3δ indicates that *H. pylori* directly involved in gastritis and DENA enhanced the gastric cancer in *Gulo*$^{-/-}$ mice.

3.3. The expression of Ki-67 and cleaved caspase 3 were increased in H. pylori-infected and, combined with DENA treated groups

Ki-67 is a nuclear proliferation-associated antigen expressed the G1, S, G2, and M phases, but absent in quiescent and resting cells [32]. We examined Ki-67 and cleaved caspase 3 by using Immunohistochemistry to evaluate the proliferative activities and apoptosis index in stomach tissue of *Gulo*$^{-/-}$ mice after *H. pylori*-infected, and followed by DENA. The higher expression of Ki-67 in intestinal type-compared with diffuse type adenocarcinomas has been reported [33]. Moreover, the expression of Ki-67 was higher in gastric carcinomas. And also, the caspase-3 was more strongly expressed in intestinal type cancers than in the diffuse type [34]. In addition, there was marked difference in the expression of caspase-3 in tumor tissue compared to normal mucosa [35]. In the present study, the expressions of Ki-67 and cleaved caspase 3 were significantly increased in *H. pylori*-infected and combined with DENA ($p < 0.05$ and $p < 0.01$) treated *Gulo*$^{-/-}$ mice stomach tissue when compared with control (Fig. 4A and B). These results revealed that, *H. pylori* and DENA increased Ki-67 (cell proliferation index) and cleaved caspase 3 (apoptosis index) that's implicated in gastric carcinogenesis of stomach tissue of *Gulo*$^{-/-}$ mice.

3.4. Differentially expressed proteins in Gulo$^{-/-}$ mice stomach H. pylori-infected and combined treatment with DENA

The differentially expressed proteins in *H. pylori*-infected and combined with DENA treated *Gulo*$^{-/-}$ mice stomach tissue were

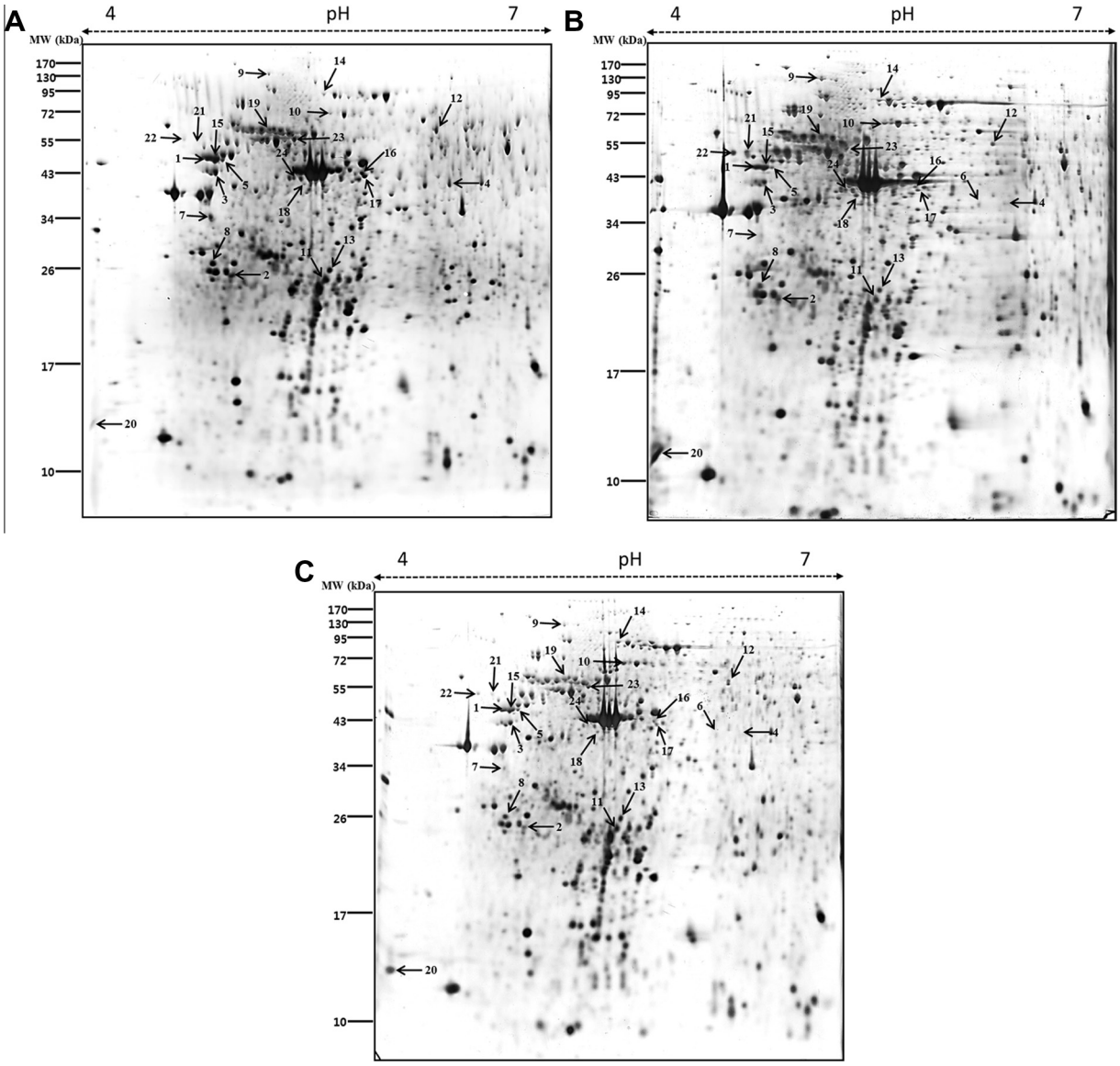


Fig. 5. Two-dimension electrophoresis pattern of *Gulo*$^{-/-}$ mice stomach (A) Control, (B) *H. pylori*-infected, (C) *H. pylori* and DENA treated. Proteins were isolated and separated on IPG-strips with pH 4–7 in the first dimension and then on 12% polyacrylamide gel on second dimension. The gels were silver stained.

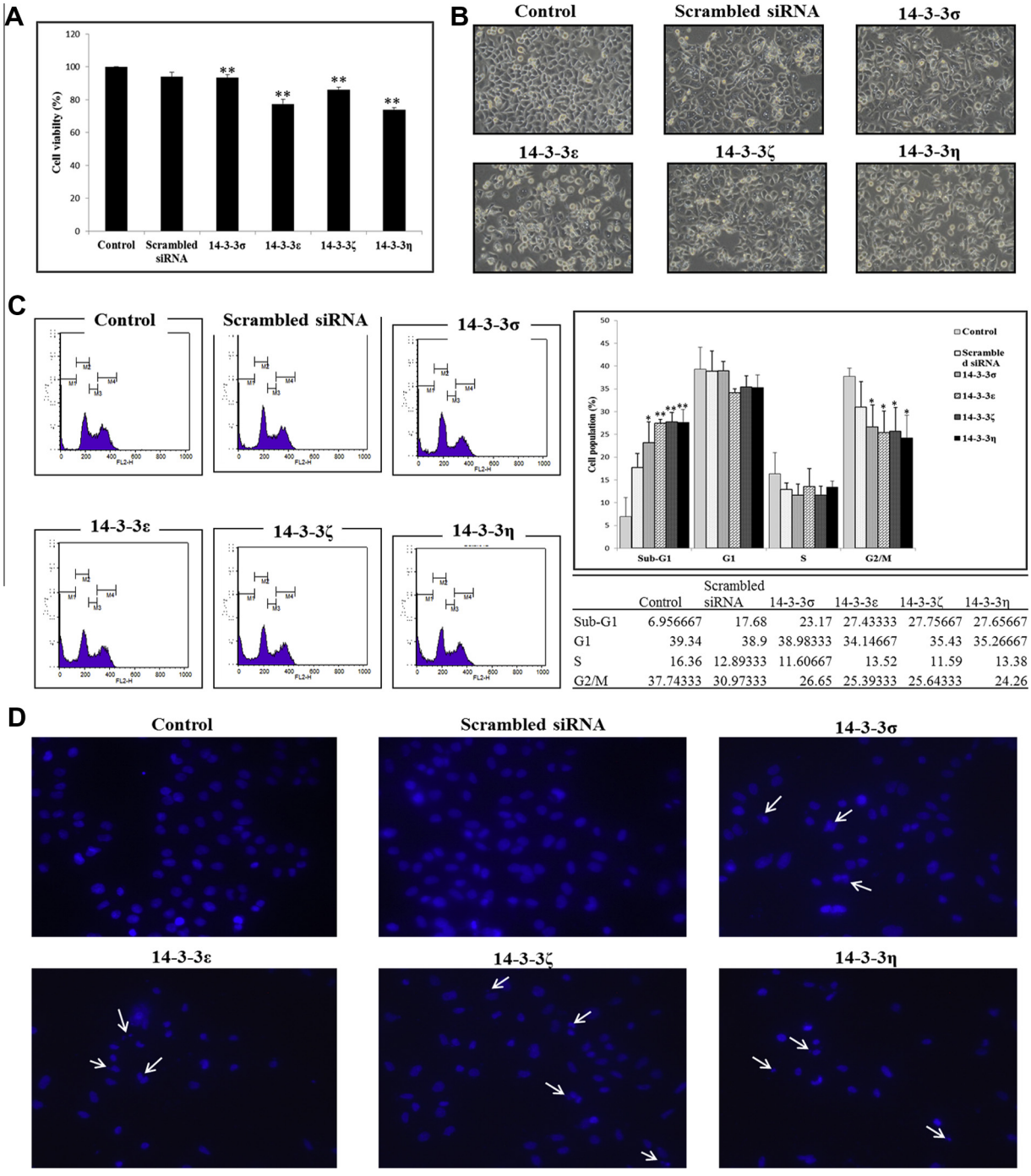
investigated by 2-DE analysis in comparison with the control group. The 100 μg of total proteins from all three groups were separated by IEF on 18 cm IPG strips in the first dimension and 12% SDS–PAGE in the second dimension. We observed a total of approximately 600 protein spots in silver stained gels. The 2-DE maps of the three groups of *Gulo*$^{-/-}$ mice stomach are shown in Fig. 5. A total of 28 statistically significant altered protein spots were identified (⩾2-fold expression, $p < 0.05$). Successfully, 24 proteins were identified by using a MALDI-TOF/TOF/MS. Table 1 lists the differentially expressed protein identified and their expression patterns. Of the 24 proteins, 17 were down-regulated, including 14-3-3σ, 14-3-3η, tropomyosin alpha-1 chain, and 7 were up-regulated, including Hspd1 protein and HSC70.

In our proteome results showed that 14-3-3σ and 14-3-3η were decreased in the *H. Pylori*-infected group, and the *H. Pylori* infected followed by DENA treated group when compare to the control group. The 14-3-3 proteins constitute a family of conserved proteins found in all eukaryotic organisms that are key regulators of a large number of processes including apoptosis, mitogenic signaling and cell-cycle checkpoints [36]. The 14-3-3 proteins are new targets of cancer research; there are in total 7 isoforms of 14-3-3 proteins that have been reported in mammals and each one has a different expression [37]. In the current study, we knock down 14-3-3 isoforms (14-3-3ε, 14-3-3σ, 14-3-3ζ and 14-3-3η) in human gastric cancer cell line AGS, to elucidate the possible roles of 14-3-3 isoforms. Firstly, AGS cells were transfected with 14-3-3 isoform siRNA and measured cell viability by MTT assay after 24 h incubation. The cell viability was decreased in 14-3-3ε, 14-3-3σ, 14-3-3ζ and 14-3-3η transfected AGS cells to 93%, 77%, 86% and 79% respectively, after 24 h incubation (Fig. 6A). These results indicated that 14-3-3 isoforms might be having possible role AGS cells proliferation. Microscopic observation showed that the AGS cells undergone many morphological change such as cell shrinkage and density, especially 14-3-3ε and 14-3-3η knockdown AGS cells compared with the control cells (Fig. 6B). When 14-3-3 is down-regulated, cells fail to maintain a G2/M arrest and induced apoptosis as a result of DNA damage [38]. Hence, flow cytometry was performed to investigate the distribution of AGS cell cycle phases after knock down 14-3-3 isoforms. There was statistically significant accumulation of cells with sub-G1 DNA content (Characteristics of apoptosis) 14-3-3ε (23.17%), 14-3-3σ (27.433%), 14-3-3ζ (27.76%) and 14-3-3η (27.66%), meanwhile G2/M phase cells were significantly decreased (Fig. 6C). To determine whether knock down 14-3-3 isoforms affect the cell viability due to apoptosis, morphological change was observed using Hoechst staining, with particular attention given to the apoptosis hallmarks of condensed chromatin near the nuclear membrane and DNA fragmentation. Condensed and cleaved nuclei were observed in AGS cells after knockdown of 14-3-3ε, 14-3-3σ, 14-3-3ζ and 14-3-3η, whereas the non-treated cells displayed a normal size and round shape nucleus (Fig. 6D). These results suggest that the knockdown of 14-3-3 isoforms could induce apoptosis in AGS cells. The one of the isoform 14-3-3σ was shown to be regulated by p53 and to play a role in the G2-M-phase checkpoint [39] and it's negatively regulates cell cycle progression through cyclin-dependent kinases association [40,41]. Moreover, the 14-3-3σ is also tumor suppressor gene as positive regulators of p53 protein, and its decreased expression reported in many cancer types [42]. In addition, overexpression of 14-3-3σ can suppress tumor growth [43,42]. Further, another isoform 14-3-3θ has been down-regulated in most of the adenocarcinomas [44]. These results revealed that the decreased expression of 14-3-3σ and 14-3-3η might be involved in *H. pylori* mediated

Table 1
List of differentially expressed proteins identified in *Gulo*$^{-/-}$ mice stomach tissue by MALDI-TOF/ TOF MS.

Spot no.	Protein name[a]	Accession number[a]	Theoretical Mr. (kDa)[b]	Theoretical pI value[b]	Sequence coverage (%)	Mouse score[c]	Control vs *H. pylori*-infected[d]	Control vs *H. pylori* + DENA[d]
1	Keratin, type I cytoskeletal 13	P08730	48066	4.79	43	360	↓	↓
2	14-3-3 protein eta	P68510	28352	4.81	18	101	↓	↓
3	Keratin 13	B1AQ76	48066	4.79	43	376	↓	↓
4	Leukocyte elastase inhibitor A	Q9D154	42719	5.85	21	91	↓	↓
5	Keratin 13	B1AQ76	48066	4.79	38	320	↓	↓
6	Chitinase-3-like protein 4	Q91Z98	45289	5.80	28	180	↓	↓
7	Tropomyosin alpha-1 chain	P04692	23589	4.61	37	169	↓	↓
8	14-3-3 protein sigma	O70456	27810	4.75	48	179	↓	↓
9	Alpha-S1-casein	P19228	24477	4.85	28	93	↑	↑
10	60 kDa heat shock protein, mitochondrial	P63038	59559	8.09	17	118	↑	↑
11	Smooth muscle gamma-actin	Q61852	43251	5.36	34	302	↓	↓
12	Selenium-binding protein 1	P17563	53051	5.87	40	359	↓	↓
13	Actin, muscle 1A	P53460	42303	5.23	17	86	↓	↓
14	Heat shock cognate 71 kDa protein	P63017	71068	5.37	25	167	↑	↑
15	Keratin 13	B1AQ76	48066	4.79	42	412	↓	↓
16	Alpha-fetoprotein	P02772	48792	5.47	26	164	↓	↓
17	Adenosine deaminase	P03958	39935	5.49	40	382	↓	↓
18	Keratin 19	P19001	44515	5.28	39	248	↑	↑
19	Keratin, type I cytoskeletal 10	P02535	52824	4.91	37	357	↓	↓
20	Calmodulin	P62158	19700	4.24	26	182	↑	↑
21	Chia protein	Q91XA9	40456	4.67	13	138	↑	↑
22	Chia protein	Q91XA9	40456	4.67	35	290	↑	↑
23	Tubulin alpha-8 chain	Q9JJZ2	48221	4.97	29	258	↓	↓
24	Beta-actin	A1E0X3	40937	5.66	29	162	↓	↓

[a] Entry name, protein name and accession number from SWISS-PROT database identified by MALDI-TOF/TOF/MS.
[b] Theoretical molecular weight (kDa) and pI from SWISS-PROT database.
[c] Score is–10*log (p), where p is the probability that the observed match is a random event, Protein scores greater than 84 are significant ($p < 0.05$).
[d] 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.



	Control	Scrambled siRNA	14-3-3σ	14-3-3ε	14-3-3ζ	14-3-3η
Sub-G1	6.956667	17.68	23.17	27.43333	27.75667	27.65667
G1	39.34	38.9	38.98333	34.14667	35.43	35.26667
S	16.36	12.89333	11.60667	13.52	11.59	13.38
G2/M	37.74333	30.97333	26.65	25.39333	25.64333	24.26

Fig. 6. Phenotypic changes in 14-3-3 isoforms (14-3-3ε, 14-3-3σ, 14-3-3ζ and 14-3-3η) knockdown AGS cells. (A) The cell viability determined by MTT assay. (B) Morphology of AGS cells were examined by light microscopy (400×). (C) Cell cycle distribution and apoptotic cells were assessed by PI staining. (D) Morphological change was observed using Hoechst staining (White arrow indicates cleaved nuclei). The asterisk (*) indicates a significant difference from the control group (*$p < 0.05$ and **$p < 0.01$).

gastritis and DENA enhanced the gastritis in *Gulo*$^{-/-}$ mice. In addition, heat shock proteins (HSP) proteins are mainly involved in a wide variety of physiological and pathological conditions [45]. Over production of HSP protects malignancy transformed cells from apoptotic cell death and foasters resistance to chemotherapeutic agents [46]. In this study, Hspd1 protein and HSC70 protein were increased in the *H. Pylori* infected group and the *H. Pylori* infected followed by DENA treated group when compared to the control group. The combined results of immunohistochemistry and proteome results revealed that *H. pylori* altered the p53 and 14-3-3 isoforms expression and DENA enhanced the *H. pylori* effect in *Gulo*$^{-/-}$ mice.

In summary, immunohistochemistry analysis showed that the expression of p53 protein was significantly decreased at translational level but not mRNA level, and 14-3-3ε, 14-3-3δ, Ki-67 and cleaved caspase 3 expressions were significantly increased after *H. Pylori*-infection and followed by DENA in stomach tissue of *Gulo*$^{-/-}$ mice. In addition, proteome results showed that 14-3-3σ,

14-3-3η, and tropomyosin alpha-1 chain were down-regulated, and Hspd1 protein and HSC70 were up-regulated after *H. Pylori* infection, and followed by DENA.in *Gulo*$^{-/-}$ mice. Moreover, knock-down of 14-3-3 isoforms (14-3-3ε, 14-3-3σ, 14-3-3ζ and 14-3-3η) were significantly increased sub-G1 phase (characteristics of apoptosis) in AGS cells and, phenotypic changes like cell shrinkage, density and cleaved nuclei were also observed. These results suggest that *H. pylori* altered the p53 and 14-3-3 isoforms expression and DENA enhanced the *H. pylori* effect in stomach tissue of *Gulo*$^{-/-}$ mice, which might be involved in carcinogenesis and metastasis of gastric cancer. Some may have potential as biomarkers in the early diagnosis and therapy of gastric cancer. Moreover, these identified proteins needed to be verified by immune-blotting to make this data more valuable. In further study, we will demonstrate the protective effect of vitamin C at high dosage against gastric cancer after *H. pylori* and DENA treatment using vitamin C deficiency *Gulo*$^{-/-}$ mice model.

Conflict of interest

We declare that there are no conflicts of interest.

Acknowledgments

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