

Revealing the Therapeutic Targets and Mechanism of Baicalin for Anti-chronic Gastritis Through TMT Proteomic Approach

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Research

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Abstract

Background

Baicalin (BCL) is a natural compound with beneficial activities, including antioxidant, anti-inflammatory and immunomodulatory. To investigate the therapeutic action of baicalin treatment in ethanol-induced chronic gastritis. Here, we investigated the proteome changes in the gastric tissue to elucidate the therapeutic targets of baicalin in chronic gastritis by TMT-based quantitative proteomics.

Results

Using TMT-based quantitative proteomics, a total of the 4,452 proteins were identified and quantified in the gastric antrum tissue of Sprague–Dawley rats. Of these, 107 differentially expressed proteins, including 44 up-regulated and 63 down-regulated proteins, were uncovered in the baicalin-treated group as compared with the untreated group with ethanol-induced gastritis. Furthermore, the expression of TPM2, GIMAP4, and Mpc1 was validated using Western Blot. Baicalin could decrease the production of interleukin (IL)-2, IL-8 and tumor necrosis factor- α (TNF- α), while increase the expression of epidermal growth factor (EGF) and B-cell lymphoma-2 (Bcl-2). Notably, protein-protein interaction network analysis revealed the widespread interactions mediated by baicalin.

Conclusions

We investigated the effects and potential mechanism of baicalin in chronic gastritis. Proteomic technology was used to explore baicalin-affected proteins and some signaling pathways. The results may provide important insights into the discovery of potential target proteins for the treatment of chronic gastritis.

Introduction

Long-term alcoholism may cause chronic gastritis, and approximately 10% of the world's population has gastritis or gastric ulcer [1]. Ethanol exerts direct effects on the digestive system and interferes with the digestive functions [2]. It was demonstrated that ethanol-induced chronic gastritis is highly likely to trigger gastric mucosal injury, owing to the high sensitivity of the stomach mucosal tissue, which is easily irritated by external factors. In particular, ethanol may severely corrode and destroy the physiological environment required for the normal function of the gastric mucosa [3, 4]. Furthermore, the damage caused to the gastric mucosa is accelerated upon binding of acetaldehyde to gastric mucosal proteins [5]. High concentrations of ethanol ($\geq 7\%$ v/v) exert a wide variety of toxic effects, including alteration in both passive and H⁺-K⁺-ATPase dependent transport of protons across cell membranes [6].

At present, the treatment of gastritis is designed with the aim to reduce the secretion of gastric acid by through the administration of anti-histamines, proton pump inhibitors, and antacids [7]. The etiology of gastritis remains unclear and is thought to be associated with the imbalance between protective and

destructive factors. Cytokines, such as IL-8 and TNF- α , play important roles in the inflammatory reactions involved in the progression of gastritis [8]. Overexpression and translocation of nuclear factor kappa B (NF- κ B) subunits to the nucleus increase the expression of proinflammatory mediators such as TNF- α [9]. Inhibition of IL-8 secretion in TNF- α treated human gastric epithelial cells by suppressing NF- κ B signaling may exert anti-inflammatory effects [10]. Studies have shown that the inhibition of NF- κ B activity may reduce the severity of inflammation [11]. Among the drugs used for the treatment of gastritis, antacids (including omeprazole; OME) are the most common agents that alleviate the associated symptoms. However, these drugs may exert adverse effects such as dry mouth, nausea, bloating, constipation, insomnia and peripheral neuritis [12]. Therefore, alternative and complementary remedies that may exhibit anti-inflammatory effects and regenerate stomach tissues without adverse effects have received global attention.

Emerging evidence indicates that some flavonoid compounds, such as baicalin and baicalein, exert anti-inflammatory effects. Baicalin belongs to flavonoid compounds, respectively. Baicalein, baicalin, and wogonin could exert an anti-inflammatory effect on LPS-mediated vascular inflammatory response by inhibiting the MAPK and NF- κ B pathways [13]. Baicalin and baicalein promote health by preventing *H. pylori* infection, which interferes with *H. pylori* growth and virulence [14]. Given that, antioxidant and anti-inflammatory natural molecules may be potential candidates for gastritis therapy, most of all, they performed the characteristics of efficacy and safety. Baicalin is a flavonoid with good anti-inflammatory, antioxidant properties, and antimicrobial activities, which is a great potential in preventing and inhibiting tumors [15]. Therefore, baicalin is one kind of active components with the important synergies, they have been widely used in China due to their crucial pharmacological effects, including enhancing immune function and anti-inflammatory [16]. Here, we employed a Tandem Mass Tag (TMT) labeling system, a high throughput, efficiency, and sensitivity tool, to analyze the expression of various proteins in the rat gastric antrum following ethanol-induced gastritis. Proteomics technology may uncover the molecular machinery underlying signaling pathways [17].

In this study, we used quantitative proteomics to elucidate the mechanism of baicalin to explain the differences in outcomes of ethanol-induced chronic gastritis. Furthermore, we focused on the differentially expressed proteins (DEPs) and signaling pathways, which may reveal potential targets for the development of drugs for the effective treatment of chronic gastritis.

Methods

Reagents and materials

Baicalin were obtained from Yuanye Biological; Omeprazole (Luoxin, China); IL-2, IL-8, and TNF- α were obtained from Sinobest Biotech (Shanghai, China); TPM2, GIMAP4, Bcl-2, and Mpc1 were supplied by Biosynthesis (Beijing, China); EGF was provided by Affinity (USA) and Tandem Mass Tags (TMTs)¹⁰ were purchased from (Thermo Fisher, USA); Tris (2-carboxyethyl) phosphine (TCEP) was obtained from Thermo Fisher Scientific (USA) and ACQUITY UPLC BEH C18 column was procured from Waters (USA).

Animal experiments and sample preparation

Male Sprague-Dawley (SD) rats, weighing 180 ± 20 g, were obtained from the Animal Experiment Center of Shanghai University of Traditional Chinese Medicine, Shanghai, China. The animals were housed in a controlled environment (12 h light and 25°C), and were given free access to food and water. All experimental procedures were carried out in accordance with the Chinese Guidelines for the Care and Use of Laboratory Animals (Permit No. SZY201712009). Thirty-two rats were randomly assigned to four groups as follows (n=8): normal group, model group, BCL group (50 mg/kg of baicalin), and OME group (20 mg/kg of OME; positive group). Gastritis rat model was induced with 56% ethanol according to an established procedure [18]. After 12 h fasting, the rats received 56% ethanol (8 g/kg) through gastrogavage twice a week (every Tuesday and Friday). After 4 weeks, the chronic gastritis model was fully established. In the model therapy groups, BCL, or OME were orally administered. The normal and model groups received saline following the same protocol. All the therapeutics were administered via gastrogavage once daily for 7 days. On the eighth day, all rats were anaesthetized by 2% pentobarbital sodium (0.3 ml/100g) via intraperitoneal injection and blood was taken from the abdominal aorta, then the stomach was immediately excised.

Histopathological analysis

The stomach tissue was carefully cut along the large curvature, cleaned with saline and blotted with filter paper. Tissue samples close to the gastric antrum were obtained from each group. The gastric antrum tissues were fixed in a 4% paraformaldehyde for 24 h, and sectioned and embedded in paraffin. Sections (5 mm thickness) were deparaffinized, stained with hematoxylin and eosin (H&E), and examined under a light microscope.

Determination of IL-2, IL-8, and TNF- α levels in serum

Plasma was separated from 4 mL of blood by centrifugation at 4°C for 15 min at $3000 \times g$. Serum levels of IL-2, IL-8 and TNF- α were measured with enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions.

Protein isolation, digestion, and labeling with TMT reagent

Tissue samples (5×5 mm) close to the gastric antrum were collected in all groups, ground into a powder in liquid nitrogen, and extracted with lysis buffer in a ratio of 1:12 (8 M urea, 1% sodium dodecyl sulfate [SDS], including protease inhibitors). These samples were chilled on ice for 30 min and finally lysed with sonication on ice for 3 min. These homogenized samples were centrifuged for 20 min at $12,000 \times g$ to obtain protein containing supernatants. TCEP was added to each sample (each representing 100 μ g of protein) and left to react for 60 min before iodoacetamide was added. The solution was incubated at 20°C in the dark for 40 min. Finally, acetone was added to each tube for precipitation. The precipitate was collected and dissolved in TEAB. Trypsin was added to the samples at a ratio of 1:50 (enzyme: protein)

and the samples were incubated at 37°C overnight. Tandem mass tags TMT¹⁰ of different molecular weights (126 to 131 Da) served as isobaric tags for relative quantification.

Liquid chromatography with tandem mass spectrometry (MS/MS) analysis

The peptides were subjected to elution on Acquity UPLC BEH C18 column in an ultra high-pressure liquid chromatography (UPLC) system (Waters, USA) with a mobile phase of 2-80% acetonitrile, using ammonia to pH10. The flow rate was 200 µL/min. The peptides were subjected to nanoelectrospray ionization and followed by MS/MS analysis on Q-Exactive instrument (Thermo, USA) coupled to an HPLC system (EASY-nLC1200) with a reverse-phase C18 column. For MS scans, the *m/z* scan range was 350-1,300 Da.

Bioinformatics and statistical analysis

MS/MS spectra were collected from raw data and converted into an MGF format in Proteome DiscovererTM Software 2.1 (containing 29,969 reference protein sequences). The parameters of the protein database were as follows: Max.Missed Cleavage Sites were 2, Precursor Mass Tolerance was 20 ppm, Fragment Mass Tolerance was 0.02 Da, validation was based on p-values and the peptide filtration parameter was false discovery rate (FDR) ≤ 0.01. By means of the GO database, genes and proteins were classified in accordance with their participation in biological processes, cellular components and molecular function (<http://www.geneontology.org/>). In the KEGG database (<http://www.genome.ad.jp/kegg/pathway.html>), the DEPs involved in various signaling pathways and biological metabolic processes were identified. The DEPs and the predicted PPI network were analyzed with STRING database (<https://string-db.org/>) [19]. The proteins we identified had to satisfy the criteria fold change (FC) < 0.83 or > 1.20 and *P* < 0.05 to be considered DEPs. All results were reported as mean ± S.D. The data were evaluated using the SPSS 16.0 software. The significance level was set to *P* < 0.05.

Western Blot analysis of DEPs

The total protein extract of the gastric antrum samples corresponding to 20 µg of proteins from each group was resolved by SDS-polyacrylamide gel electrophoresis using 10% gel and then transferred to PVDF membranes, blocked with 5% non-fat dried-milk in TBST buffer for 1 h, and incubated with antibodies (1:1000) against TPM2, GIMAP4, Mpc1, EGF, Bcl-2 and β-actin at 4°C overnight. The membranes were subsequently incubated with a secondary antibody (anti-IgG) at 1:2000 dilution for 1 h at room temperature and the membrane was washed thrice. Finally, the antibody-antigen complexes and protein bands were quantified by densitometry using a Quantitative Gel and Western Imaging System (FluorChem Q).

Results

Protective effects of baicalin against ethanol-induced chronic gastritis in rat

After administration of ethanol to the tissues in the model group, extensive lymphocyte infiltration was observed in the intrinsic, submucosal, and serosal layer. Edema and congestion were apparent in the submucosal and intrinsic layers. Lymphocyte infiltration and edema reduced in BCL group as compared with model group, the symptoms of gastritis were significantly alleviated in BCL group (Fig.1A). The levels of IL-2, IL-8, and TNF- α significantly increased ($P < 0.01$) in model group. BCL treatment may down-regulate the expression of IL-2, IL-8 and TNF- α (Fig. 1B). Meanwhile, EGF and Bcl-2 were significantly up-regulated following BCL treatment (Fig. 1C). These findings demonstrated that baicalin exerted therapeutic effects on ethanol-induced chronic gastritis in rats.

TMT-based quantification of the gastric antrum proteomes

The proteome was performed on the gastric antrum divided into four groups based on different treatments. Our proteomic workflow was shown in Fig.2A. In total, 4,452 proteins were identified from 20,842 peptides with high confidence. A cutoff value of 1.2-fold change and P -values < 0.05 were used in our study [20, 21]. According to the matching degree of the peptides, it was found that the DeltaM [ppm] of most peptides is concentrated in -10 to 10 (Fig.2B). Compared to the control group, 318 DEPs were identified, of which 216 and 102 were up- and down-regulated respectively in ethanol-treated rats. Furthermore, compared with the model group, there were 107 DEPs in baicalin, of which 44 and 63 were up- and down-regulated, respectively (Fig.2C).

Biological function analysis

To examine the biological functions of DEPs in the therapeutic effect of BCL on chronic gastritis, DEPs were categorized according to their associated biological processes, molecular function and cell localization with the Gene Ontology (GO). In the biological processes category, DEPs were significantly enriched in cell killing, immune system process, and multicellular organismal process. In the cellular component category, these DEPs were involved in cell part, extracellular region, and extracellular region part. In the molecular function category, the DEPs were significantly enriched in binding, catalytic activity and molecular transducer activity. Thus, BCL-affected DEPs may play important regulatory roles in fundamental biological processes, immune and binding during BCL treating chronic gastritis.

KEGG pathway enrichment analysis demonstrated that BCL-affected signaling pathways were significantly enriched in complement and coagulation cascades, PI3K-Akt and NF- κ B. Interestingly, several common pathways including MAPK signaling pathway, such as Col4a2 and HSP27, which were strongly associated with BCL treatment (Fig.3A).

Protein interaction networks of BCL-affected DEPs

STRING was carried out to analyze the interactions between DEPs that showed functional interactions [22]. A protein-protein interaction (PPI) network may integrate and analyze the known PPIs via proteomic and gene data. 44 Up- and 63 down-regulated proteins and constructed PPI networks were used to study the relations between chronic gastritis and BCL treatment. In the network, Acta, Prothrmbin,

Fibrinogen beta chain, and TPM2 were associated with other DEPs in PPI. Interestingly, BCL-affected DEPs were classified into immune process, regulation of actin cytoskeleton and inflammation process, which formed the large network associated with gastritis (Fig.3B).

Western Blot confirmation

From the data of proteomics, the expression level of some proteins performed significant change between BCL and model groups. From the results of Western Blot (Fig. 4), compared to model group, it was confirmed that the expression of ICAM-1 and GIMAP4 were reduced in baicalin group. On the contrary, TPM2 and Mpc1 expression increased in baicalin group. These results demonstrated that the expression trend of these proteins in tissue samples was consistent with our proteomics findings and confirmed the reliability of the proteomics data.

Discussion

A previous study has illustrated the therapeutic effects of baicalin on ethanol-induced gastritis through a reduction in the generation of inflammatory cytokines. It have demonstrated that the intake of ethanol causes acute or chronic gastritis [23]. The molecular structure of ethanol includes both hydrophobic alkyls and hydrophilic hydroxyls that may attenuate the gastric mucosal barrier defense system and reduce the ability of the gastric mucosa to defend against gastric acid invasion, leading to mucosal edema, erosion, hemorrhage, and necrosis [24]. Simultaneously, EGF receptor ligands have been implicated in the induction of gastric cell proliferation and function [25]. Baicalin significantly up-regulated the expression of EGF and protected the damaged gastric mucosa. Among various the regulatory factors involved in apoptosis, Bcl-2 regulation is particularly important, because reduction in Bcl-2 level causes apoptosis-mediated cell death [26, 27].

Quantitative proteomics technology is an effective tool to study the mechanisms underlying natural compounds-mediated gastritis alleviation. Proteomic technology may identify novel potential diagnostic biomarkers in the gastrointestinal stroma [28]. Previous study demonstrated that TPM2 may be involved in cancer formation. Beta-tropomyosin (β -tropomyosin, TPM2) gene is known to contribute to series of rare myopathies including many kinds of diseases, and is crucial for the regulation of muscle contraction associated with actin and the troponin complex [29]. It was also essential for the stability of actin thin filaments, as well as many functions of cell processes such as migration. Report showed that TPM2 was down-regulated in colorectal adenomas and cancers as compared to normal colon tissues [30]. Several early studies also demonstrated that TPM2 was down-regulated in human esophageal squamous cell carcinoma, while unregulated in ovarian and breast cancer [31-33]. Form the validation analysis of Western Blot, BCL could up-regulate the expression level of TPM2, which was consistent with results of proteomics. From the results of TMT proteomics, TPM2 was down-regulated in model group, while up-regulated in BCL group, TPM2 may take part in the gastritis formation, so we speculated it may be a biomarker candidate for ethanol-gastritis. Additionally, GO analysis demonstrated that TPM2 was involved in molecular function.

GIMAP4 is reported to exhibit GTPase activity and function in cellular transport processes. GIMAP4 may affect the levels of IFN- γ and is involved in the IFN- γ signaling pathway in Th1 cells [34]. Mpc1 takes part in the control of rate-limiting pyruvate transport through the inner mitochondrial membrane [35]. Mpc1 overexpression inhibits the proliferation, migration, and invasiveness of gastric cancer cells [36].

Therefore, we can speculate that TPM2, GIMAP4, and Mpc1 are potential targets of baicalin in treating chronic gastritis. Baicalin affected several signaling pathways, including PI3K-Akt, NF- κ B, and MAPK signaling pathways. The underlying mechanism involves anti-inflammatory, anti-apoptotic effects, and induction of endogenous factors for cytoprotection.

Conclusions

In this study, a total of 107 DEPs were identified from the BCL group, when compared to the ethanol-induced gastritis group, the results showed for the first time that quantitatively evaluating the gastric antrum proteins by TMT in ethanol-induced gastritis and BCL-treated gastritis rats performs a feasible and effective strategy for identify gastritis associated with proteins, pathways, networks, and potential therapeutic targets. This study may also demonstrate that baicalin played a crucial role in treating ethanol-induced gastritis. Consequently, the present study widens the range of treatment strategies for chronic gastritis and highlights potential drug targets for modulation of inflammation and apoptosis.

Abbreviations

TMT: Tandem mass spectrometry tag; BCL: Baicalin; IL-2: Interleukin-2; TNF- α : Tumor necrosis factor- α ; EGF: Epidermal growth factor; Bcl-2: B-cell lymphoma-2; TPM2: Tropomyosin beta chain 2; GIMAP4: GTPase IMAP family member 4; Mpc1: Mitochondrial pyruvate carrier 1.

Declarations

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Author Contributions

Wan-li Ji: Data curation, Formal analysis, Methodology, Visualization, Writing- Original draft preparation. Yan Huo: Data curation, Methodology, Validation; Yu Zhang: Methodology, Validation; Xin-hong Wang: Conceptualization, Writing—review and editing. Yi-fan Zhang: Conceptualization, Methodology, Writing—review and editing, Supervision, Funding acquisition.

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Availability of data and materials

The MS proteomics raw data have been deposited in the iProX system (<http://www.iprox.org/index>) under the identifier IPX0001246000.

Ethics approval and consent to participate

All experimental procedures were carried out in accordance with the Chinese Guidelines for the Care and Use of Laboratory Animals (Permit No. SZY201712009).

Consent for publication

Not applicable.

Competing interests

There are no competing interests.

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Figures

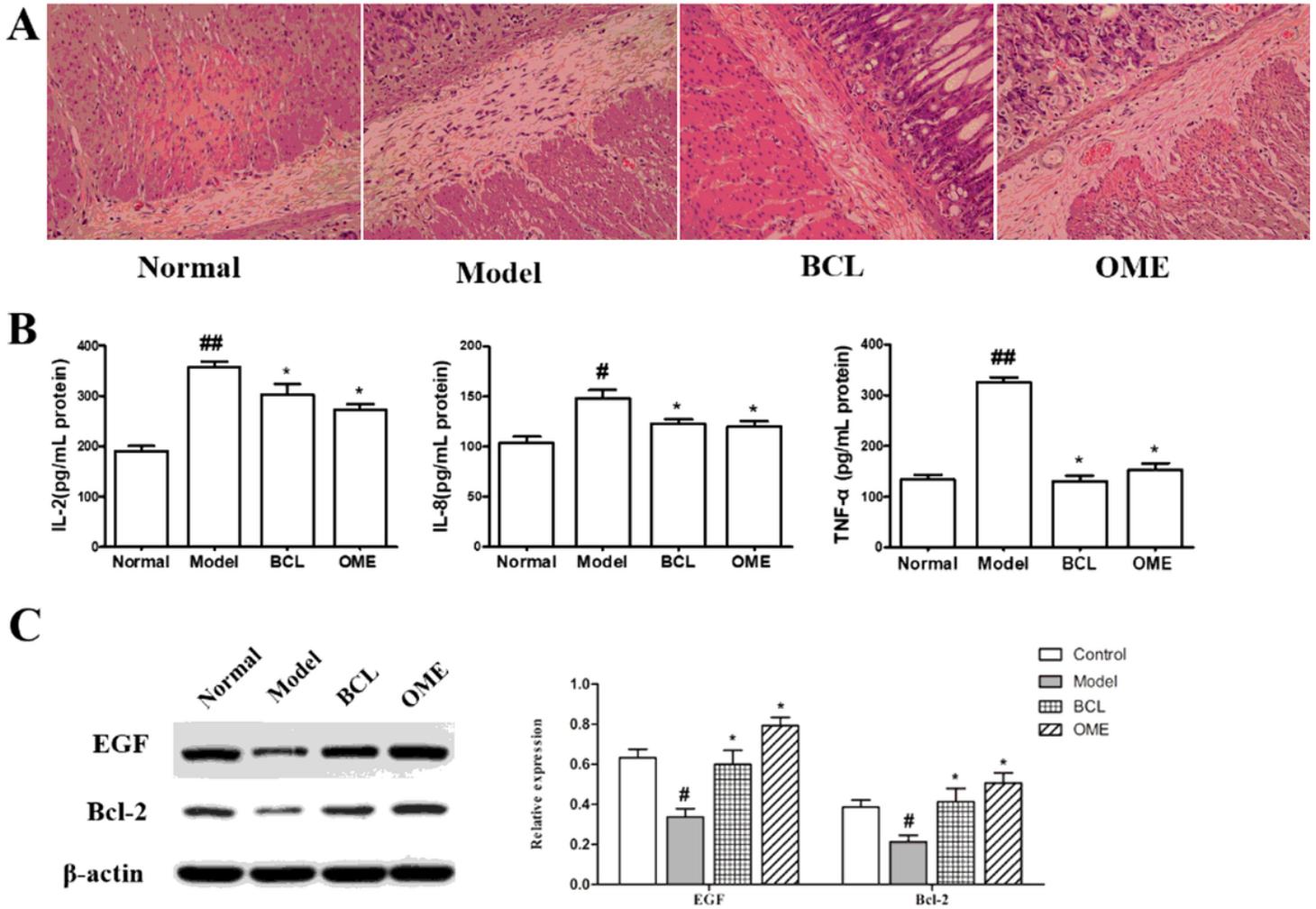


Figure 1

Effects of baicalin on chronic gastritis in rats. A: H&E staining (magnification 200×); B: Determination of IL-2, IL-8 and TNF-α levels in serum and cytoprotective factors; C: Western Blot analysis of EGF and Bcl-2, #P < 0.05 vs. Normal group, *P < 0.05 vs. Model group.

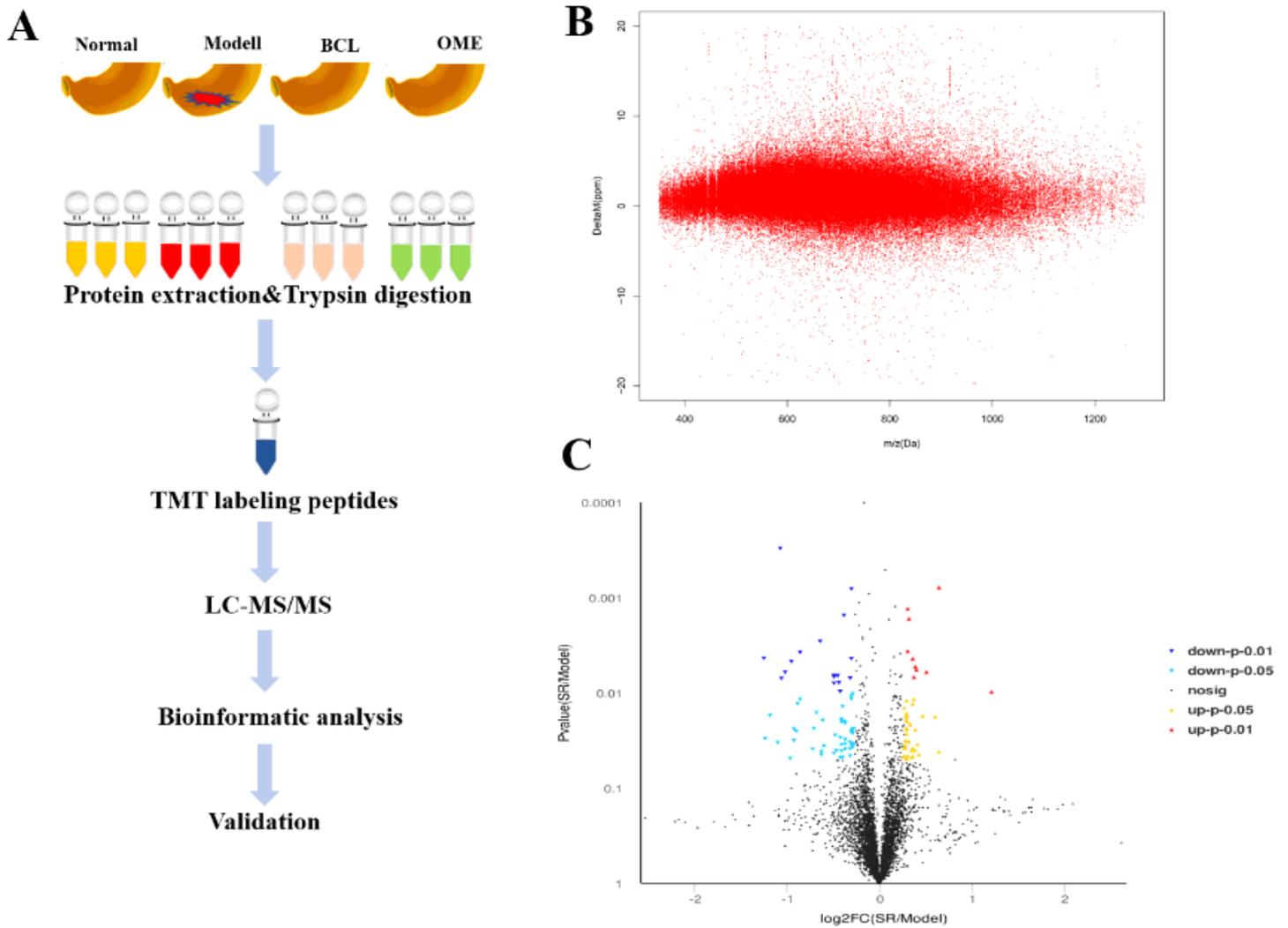


Figure 2

TMT quantitative proteomic workflow and visual analysis. A: TMT proteomic workflow; B: Gastric tissue protein peptides error; C: Volcano plot of DEPs in BCL group compared with model group. The X axis was log₂-fold change of high and low expression proteins in BCL vs model. The Y axis represents the false discovery (FDR) of the change fold. Different colors represent different FDR values.

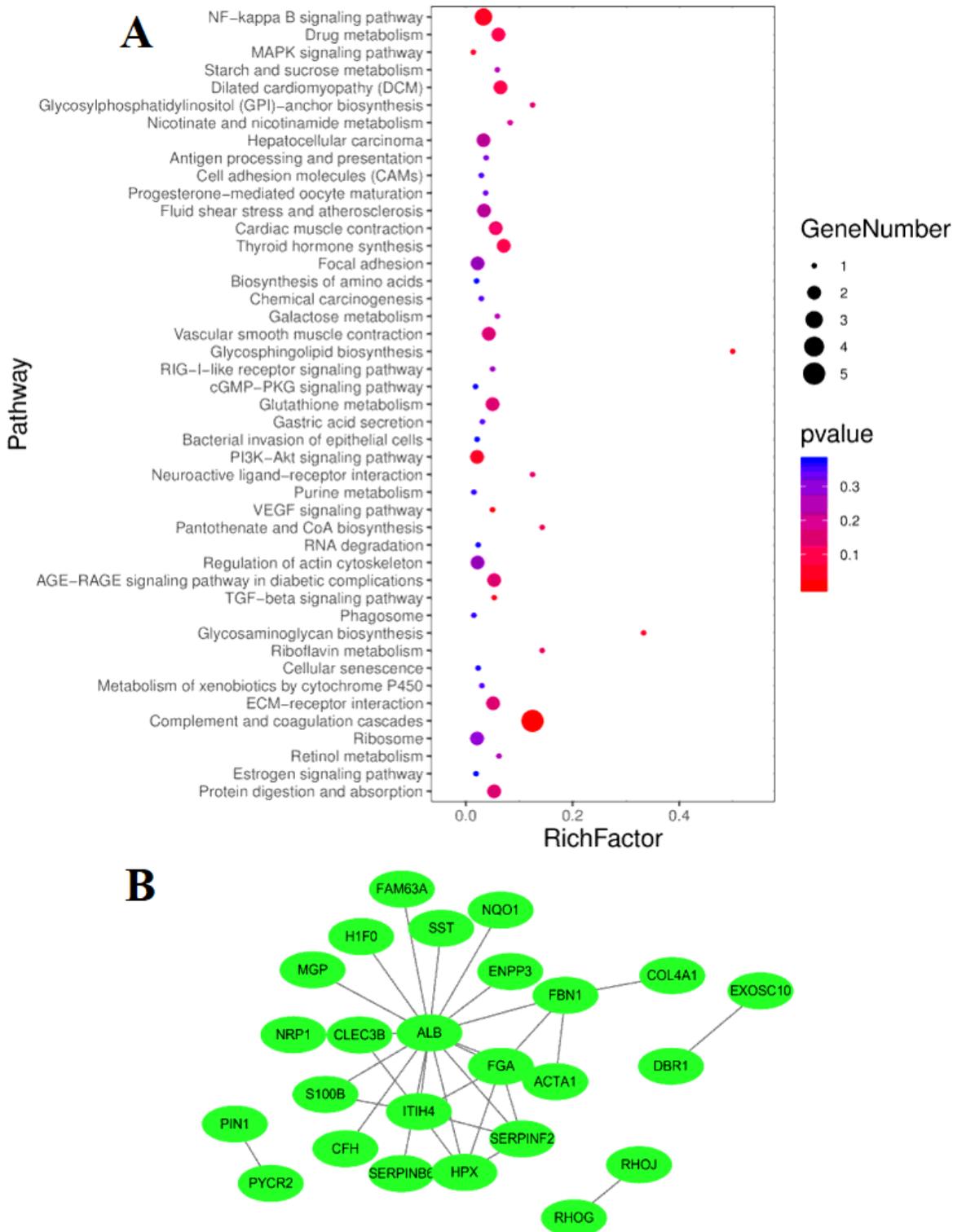


Figure 3

The KEGG and PPI analysis of BCL-affected DEPs. A: The KEGG analysis, the size of the circle represents the amount of protein enrichment, colour represents the significance of enrichment, and the right-hand colour gradient represents P value. B: The PPI analysis of BCL-affected DEPs, 25 DEPs out of 107 DEPs were involved in the PPI networks, which contained 25 nodes and 30 edges.

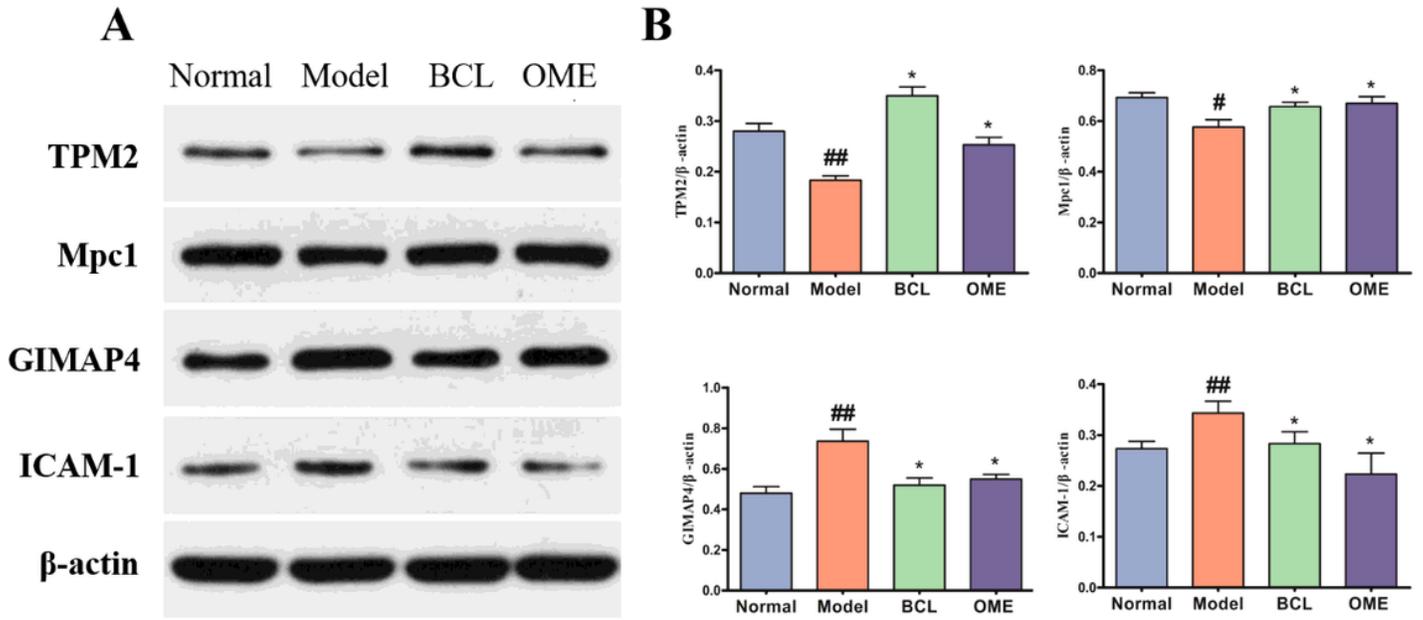


Figure 4

DEPs-affected by BXD and Western Blot validation of DEPs. A: In rats subjected to BCL treatment, the expression levels of GIMAP4 and ICAM-1 decreased as compared with the rat from the model group, while the expression levels of TPM2 and Mpc1 were increased by BCL. B: Expression levels of the DEPs by western blot was normalized to β -actin expression. The relative intensity of bar chart demonstrated quantification of proteins, the bars showed means \pm SEM of three independent experiments. ##P < 0.01 vs. Control group, #P < 0.05 vs. Control group, *P < 0.05 vs. Model group.