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ORIGINAL ARTICLE

# Analyzing proteins in colonic tissues from mice with ulcerative colitis using the iTRAQ technology*

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## Abstract

**Objective** The aim of the study was to investigate the expression of proteins in colonic tissues of mice with ulcerative colitis (UC) by using isobaric tags for relative and absolute quantitation (iTRAQ), probe into the pathogenesis of UC, and find potential biomarkers of UC.

**Methods** Forty C57 mice were randomly divided into the control and model groups (20 mice in each group). The mice in the model group were administered dextran sulphate sodium (DSS) for 7 consecutive days ad libitum to induce acute colitis, and the colon tissue was extracted on the 8th day after the successful establishment of the UC model. Proteins were identified by the iTRAQ and tandem mass spectrometry techniques,and the identified proteins were analyzed by bioinformatics.

**Results** A total of 4019 proteins were identified among the two groups. Among them, 317 significant differentially expressed proteins (DEPs) were detected according to the screening criteria for selecting DEPs, i.e. fold change ratios ≥ 1.5 or ≤ 0.67 and *P*-values < 0.05, of which 156 were upregulated and 161 were downregulated. In the Gene Ontology (GO) analysis, the DEPs were classified into 48 functional categories, which contained biological process, cellular component, and molecular function. Based on the 317 DEPs, the KEGG pathway analysis identified 160 vital pathways.

**Conclusion** DEPs in colonic tissues of mice with UC were screened using the iTRAQ technique, which laid a foundation for further studies regarding the pathogenesis of UC.

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**Key words:** ulcerative colitis (UC); isobaric tags for relative and absolute quantitation (iTRAQ); colonic tissue; differentially expressed proteins (DEPs)

Ulcerative colitis (UC) is a chronic non-specific inflammatory bowel disease with an unclear etiology and pathogenesis; it mainly affects the colon and is characterized by the inflammation of the colorectal mucosa and submucosa. The alternating periods of the relapse and remission of mucosal inflammation can have a serious impact on the life quality of the patients. The main clinical manifestations of UC are abdominal pain, diarrhea, and hematochezia [1–2]. In recent years, the incidence of UC is on the rise, and patients with UC are at an increased risk for colorectal cancer with the extension of the course of UC [3]. The risk of carcinogenesis 10 years after the onset of UC is higher; hence, UC is considered an intractable disease [4]. Most scholars believe that its pathogenesis is the result of the interaction of immune system disorders, genetic factors, environmental factors, microbial infection and so on [5]. Although new drugs and treatments have been emerging, their efficacy is not satisfactory. Therefore, there is an urgent need for a better understanding of the mechanism of UC and to find more effective and alternative molecular targets for treatment of this disease in clinical practice.

With the rapid development of proteomic techniques, isobaric tags for relative and absolute quantitation (iTRAQ) coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) serves asa more powerful methodology for quantitative proteomics [6]. The iTRAQ technique is a quantitative technique involving isotope labeling that can simultaneously study up to eight different samples with good accuracy and repeatability; it has been applied in proteomics research very well [7–8]. In the present study, iTRAQ coupled with LC-MS/MS was

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used to analyze the expression of proteins in the colonic tissue of mice with UC, and to explore the mechanism of UC, which laid a foundation for further studies on UC.

## Materials and methods

### Experimental animals

Forty wild-type male C57BL/6 mice that weighed 18–22 g (6–8 weeks old) and were of specific pathogen-free (SPF) grade were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). They were housed in a pathogen-free-grade animal room under standard conditions [room temperature: (21 ± 2) °C, room relative humidity: 40%–60%, 12 h day/night cycle with lights], and fed with standard chow diet and water ad libitum for one week to adapt to the environment. All procedures involving animals were approved by the Animal Care Committee of the University of Wuhan, China (certificate No. SYXK(E)2015-0027).

### Grouping and model preparation

Forty C57 mice were randomly divided into the control and model groups ($n$ = 20 in each group) after 7 days of adaptive feeding. The mice in the control group were fed standard chow diet and water ad libitum for a week, while those in the model group were fed with drinking water containing 3% dextran sodium sulfate (DSS, molecular weight 36 000–50 000; MP Bio, Santa Ana, CA, USA) for 7 consecutive days to induce acute colitis. At day 8 of the experiment, the mice were sacrificed following isoflurane (GeneCreate Biological Engineering Co., Ltd., Wuhan, China) inhalation and cervical dislocation, and their colons were dissected. Then, the colon tissues were frozen in liquid nitrogen immediately and stored in a refrigerator at –80 ℃ for future use.

### Protein extraction and quantification

The colonic tissues of mice from the control and model groups were randomly divided into two groups each, namely control 1, control 2, model 1, and model 2 ($n$ = 10 each group). Then, the four samples were ground into a powder after being frozen in liquid nitrogen. The powder was dissolved with lysis buffer (containing 7 M urea, 2 M thiourea, 4% SDS, 40 mMtris-HCl, 1 mM PMSF, 2 mM EDTA, all which were procured from Sinopharm Chemical Reagent Co., Ltd, China). After five minutes, 10 mM DTT (Solarbio Science & Technology Co., Ltd, Beijing, China) was added to the samples. The suspension was sonicated for 15 minutes and then centrifuged at 4 ℃ and 13 000 rpm for 20 minutes. The precipitate was collected with chilled acetone (Sinopharm Chemical Reagent Co., Ltd, China) and incubated at –20 ℃ overnight. The precipitate was collected by treatment with a solution of 10 mM DTT for 1 hour at 56 ℃. Centrifugation was performed again, at 13 000 rpm for 20 min at 4 ℃; then, the precipitate was collected and dried. Protein concentrations were measured using the Bradford method [9]. All samples were stored at –80 ℃ prior to further analysis.

### Protein digestion and iTRAQ labeling

First, 100 μg of each protein sample was reduced, alkylated, and then digested with trypsin (GeneCreate Biological Engineering Co., Ltd., Wuhan, China) overnight at 37 ℃. The samples were labeled with iTRAQ Reagent-8 plex Multiplex Kit (AB Sciex, USA) according to the manufacturer's instructions as follows: control 1 (tag 115), control 2 (tag 116), model 1 (tag 117), and model 2 (tag 118). Each group included two biological replicates, and each sample was pooled from ten individuals. Finally, all the labeled samples were mixed in equal amounts.

### Protein fractionation and LC-MS/MS analysis

The labeled samples were fractionated using high-performance liquid chromatography (HPLC) system (Thermo Dinoex, USA) using a Durashell C18 column (5 be, 100 Å, 4.6 mm × 250 mm). Finally, 12 fractions were collected. LC-MS/MS analysis was performed using a Triple TOF 5600 plus system (AB Sciex, USA).

### Data analysis

The original MS/MS file data were loaded into the Protein Pilot Software version 4.5 (AB Sciex, USA) for data analysis. For protein identification, the data was searched against the Uniprot database. Only unique peptides were considered for iTRAQ labeling quantification. In addition, only data with a false discovery rate (FDR) of < 1% were used for subsequent analyses. Proteins with fold change ratios ≥ 1.5 or ≤ 0.67 and $P$-values ≤ 0.05 were considered to be significantly differentially expressed. To determine the biological and functional properties of all the identified proteins, the identified protein sequences were mapped with Gene Ontology (GO) terms (http://geneontology.org/). The GO term matching was performed using blast2go v4.5 pipeline 5. The GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were considered statistically significant at $P$ < 0.05.

## Results

### Identification of differentially expressed proteins (DEPs) in the model and control groups

In this study, the mice in the model group showed obvious diarrhea, hematochezia, weight loss and so on, which indicated that the DSS administration had successfully induced acute UC. Using iTRAQ combined with LC-MS/MS proteomics technology, a total of 4019

proteins were identified, and 317 DEPs were identified in the model group, compared to the control group; of these, 156 proteins were upregulated and 161 were downregulated according to the criteria for defining DEPs (fold change ratios of ≥ 1.5 or ≤ 0.67, and $P \leq 0.05$). The top 10 upregulated proteins and top 10 downregulated proteins have been listed in Table 1.

## GO analysis of the DEPs

GO analysis has been widely used to describe the molecular function of proteins [10]. In the GO analysis, all the DEPs were mapped to the terms in the GO database. The DEPs were classified into 48 functional categories, which contained biological process, cellular component, and molecular function. Biological processes accounted for 26 GO terms (such as cellular process, metabolic process, biological regulation, and response to stimulus), cellular components accounted for 10 GO terms (such as cell, cell part, organelle, and extracellular region), and molecular functions accounted for 12 GO terms (the most frequent were binding and catalytic activity), as shown in Fig. 1.

## KEGG pathway analysis of the DEPs

KEGG pathways were constructed to better understand the biological pathways and elucidatethe molecular mechanisms involved in the progress of UC. Based on 317 differentially expressed proteins, the KEGG pathway analysis identified 160 vital pathways. We discovered that most of the DEPs were enriched in the metabolic pathways, microbial metabolism in diverse environments, and oxidative phosphorylation (Table 2 and Fig. 2).

**Table 1** Top 10 upregulated and downregulated proteins (model vs. control, $P < 0.05$)

| Accession | Protein name (Gene name) | Ratio (Model/control) | *P*-value |
|---|---|---|---|
| Upregulation | | | |
| Q53X15 | S100 (S100a8) | 26.2 | 0.001660956 |
| Q3UBS3 | Haptoglobin (Hp) | 24.4 | 1.17489E-06 |
| Q3TGR2 | Fibrinogen (Fgb) | 22.8 | 3.48954E-09 |
| Q3UEM7 | Fgg | 18.6 | 9.71003E-06 |
| Q3V2G1 | Apoa1 | 18.4 | 4.85304E-06 |
| O35744 | Chil3 | 16.9 | 0.001728216 |
| Q91VE7 | Cramp | 16.6 | 0.02424784 |
| Q542I8 | Itgb2 | 15.9 | 0.01233843 |
| E9PV24 | Fga | 12.9 | 6.94809E-07 |
| Q9DCE9 | Igtp | 11.8 | 0.001547599 |
| Downregulation | | | |
| A0A0R4J077 | Pentaxin (Mptx1) | 0.05 | 0.000801551 |
| Q9D7Z6 | Clca1 | 0.19 | 2.86455E-10 |
| Q8C7U7 | Galnt6 | 0.21 | 0.007778632 |
| Q53YP5 | Fabp2 | 0.21 | 0.028193399 |
| O88312 | Agr2 | 0.22 | 6.66103E-05 |
| E9QNL5 | Sult1a1 | 0.22 | 0.000238775 |
| Q3UER1 | Aldob | 0.26 | 0.000422554 |
| A0A0R4J034 | Pdxdc1 | 0.26 | 4.42615E-07 |
| P05784 | Keratin (Krt18) | 0.26 | 1.57795E-06 |
| Q545S0 | Sulfurtransferase (Tst) | 0.26 | 0.024794869 |

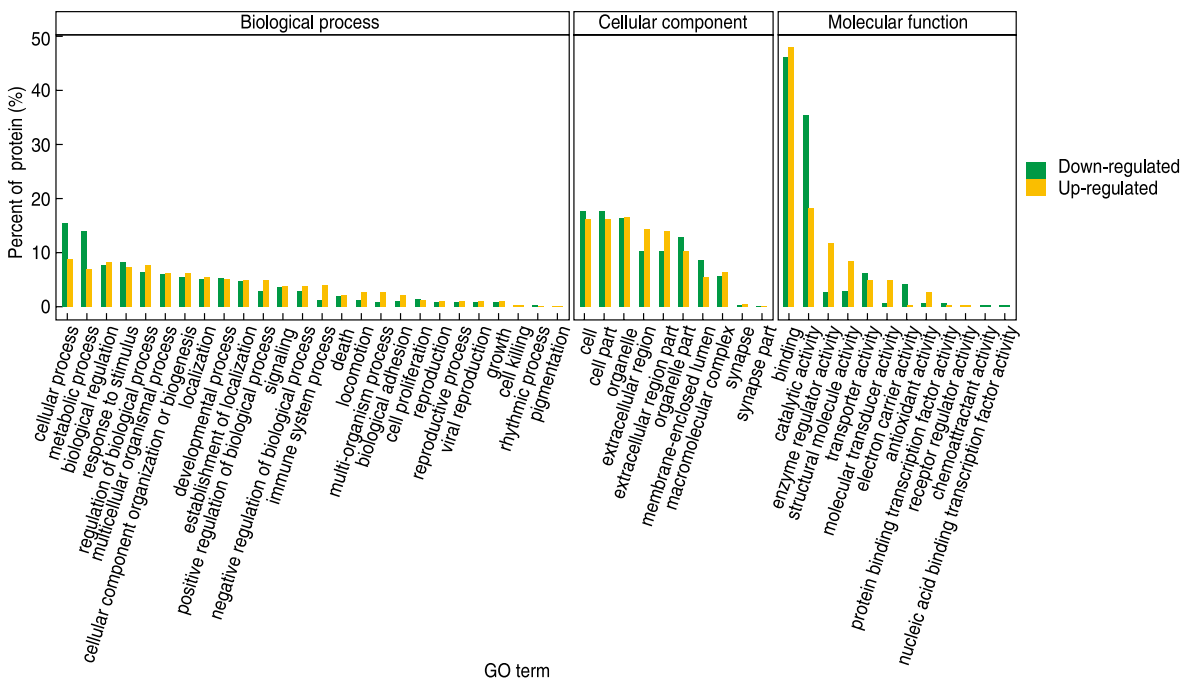


**Fig. 1** GO classification. GO (Gene Ontology) analysis of all DEPs (model vs. control) according to the biological processes (left), cellular component (middle), and molecular function (right). Percent of proteins in each category are shown. The yellow color represents the upregulated proteins and the green color represents the downregulated proteinsk

## Discussion

UC is a chronic condition in which the overreacting immune system may play an important role. Some studies have found that the incidence of UC has increased steadily in developed countries, while in developing countries, its incidence has rapidly been on the rise over the past few decades. This high incidence is manifested mainly in European and American countries; this could possibly be associated with the living environment and lifestyle of the West (such as drug use, environmental pollution, life stress) [11–12]. Therefore, there is an urgent need to understand the pathogenesis of UC and look for

**Table 2** The top 10 pathways in the pathway enrichment analysis of DEPs in the model and control groups

| Pathway ID | Pathway name | Proteins quantity |
|---|---|---|
| Ko00020 | Citrate cycle (TCA cycle) | 14 |
| Ko01100 | Metabolic pathways | 89 |
| Ko01120 | Microbial metabolism in diverse environments | 32 |
| Ko04610 | Complement and coagulation cascades | 14 |
| Ko00190 | Oxidative phosphorylation | 19 |
| Ko05150 | Staphylococcus aureus infection | 12 |
| Ko05012 | Parkinson' disease | 19 |
| Ko00620 | Pyruvate metabolism | 11 |
| Ko04972 | Pancreatic secretion | 13 |
| Ko04512 | ECM-receptor interaction | 12 |

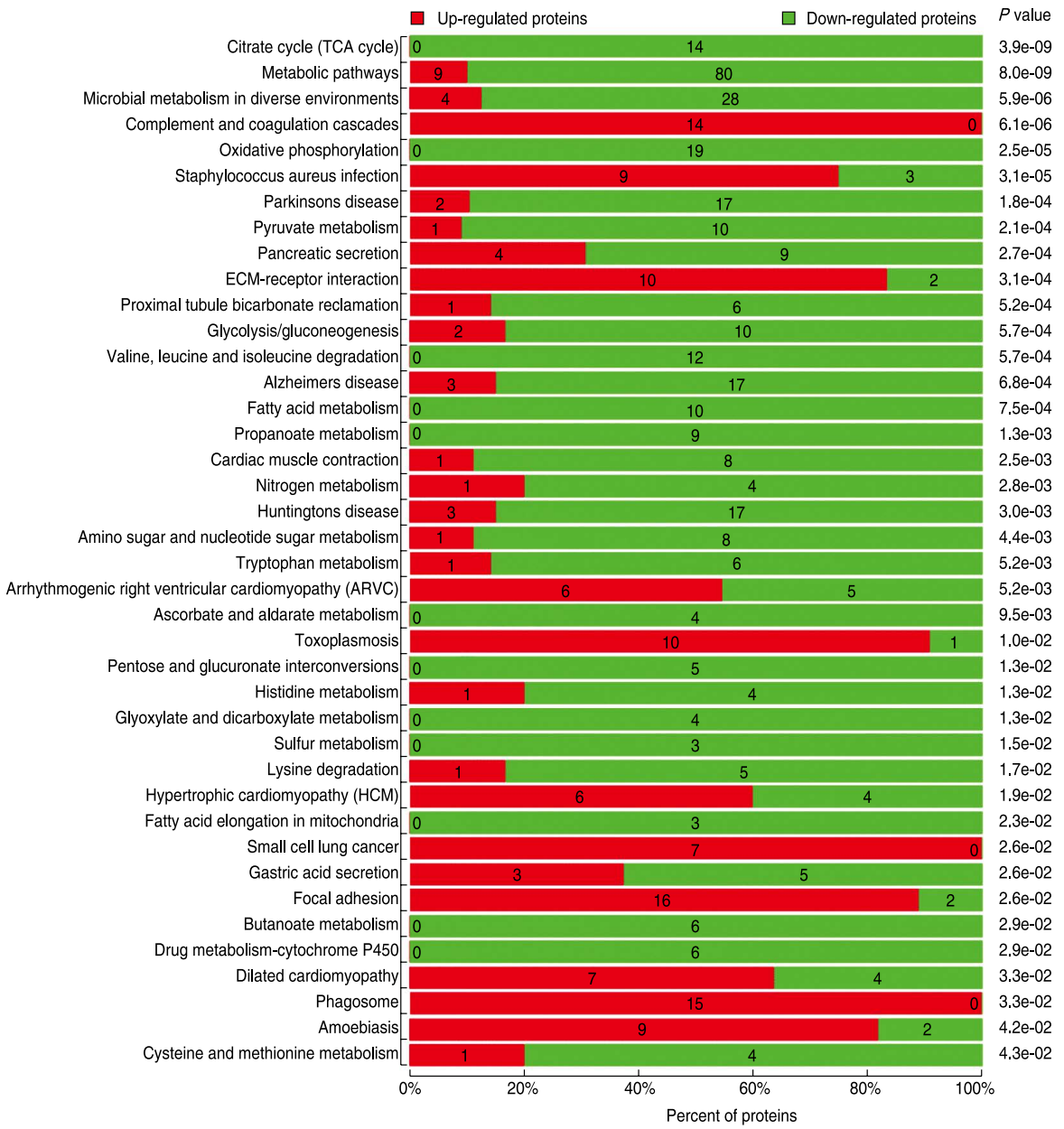


**Fig. 2** Statistics of KEGG pathways (The red color represents the upregulated proteins and the green color represents the downregulated proteins)

effective biomarkers.

The present study is the first to report the proteomic profiling of mice with UC using iTRAQ combined with the LC-MS/MS technique; 4019 proteins were identified using the iTRAQ technique. Among the relative quantitative results, 317 significant DEPs were identified (proteins with fold change ratios ≥ 1.5 or ≤ 0.67 and $P \leq 0.05$), among which 156 proteins were found to be upregulated and 161 proteins were found to be downregulated.

Of these proteins, a significant increase of S100A8 expression was observed in the model group samples, when compared to the control group samples. As we all know, S100A8 is a low-molecular-weight (10.8-kDa) calcium-binding protein. In addition, it is also an important member of the S100 protein family. S100A8 and S100A9 form noncovalent homodimers and a heterodimer (S100A8/A9) in a calcium-dependent manner [13]. The S100 proteins participate in the onset of many inflammatory diseases such as rheumatoid arthritis (RA) [14], inflammatory bowel disease (IBD) [15], and so on. The bioinformatics analysis showed that S100A8 was mainly located in the cytoplasm, cell membrane, and cytoskeleton, mediating immune and biological functions. Some scholars believe that S100A8 can stimulate the migration and adhesion of human neutrophils to inflammatory sites and other pro-inflammatory activities [13]. At present, S100A8 has been used as a biomarker of inflammatory response to monitor the disease status and evaluate the therapeutic effects [15]. Its proinflammatory activity may be explained by the fact that the immune complex can stimulate the secretion of S100A8/A9 by neutrophils. By binding with RAGE and TLR4, it can induce the secretion of IL-6, IL-1β, TNF-α, and other cytokines via the intracellular MAPK and NF-kappa B signaling pathways, which can produce a series of inflammatory reactions [16]. In addition, S100A8/A9 also promotes the activation of the MAPK and NF-kB signaling pathways and the proliferation of cancer cells in colon-associated cancer [17]. The above studies suggest that S100A8 is an important biochemical marker that can be used in the clinical diagnosis and treatment of UC.

From Table 1, we found that the protein of Agr2 was downregulated markedly in the model group samples, compared to the case for the control group samples. The bioinformatics analysis showed that Agr2 was mainly located in the extracellular region, organelle, and cell part that participated in the regulation of biological processes. Previous researches have reported Agr2 mutationsin mice associated with diarrhea and goblet cell dysfunction; similar changes have been observed in patients with UC. Agr2 was expressed in tissues or organs that possess mucous-secreting cells or functions. Thus, Agr2 may be involved in the epithelial barrier function [18]. It is well known that UC is related to the destruction of colonic epithelial barriers that sustain the inflammation of the colon mucosa caused by the recruitment of lymphocytes and neutrophils into the lamina propria [19]. Some researchers have found that the expression level of Agr2 in UC patients was significantly lower than that in healthy people, and that Agr2 may be involved in the maintenance of epithelial integrity inmouse models [18]. In addition, Park has found that Agr2 is notably required for MUC2 mucin production, which is essential for immune regulation. Moreover, the expression of pro-inflammatory cytokines would increase in the absence of Agr2 [20]. Importantly, previous studies have shown that $Agr2^{-/-}$ mice spontaneously develop severe colitis [21]. The observations of all the above studies are consistent with our findings. Thus, we can select Agr2 as an effective and potential candidate biomarker for in-depth future investigations. However, the mechanism underlying this observation has not yet been fully explored.

In summary, this study used iTRAQ-based quantitative proteomics to analyze the DEPs in UC to unravel the unknown molecular mechanisms of UC. In addition, the present study demonstrates that S100A8 and Agr2 maybe potential biomarkers for UC. However, this study is only a preliminary study, and the specific functions and mechanisms of a series of DEPs need to be further verified.

### Conflicts of interest

The authors indicate no potential conflicts of interest.

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