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Original Article

Effect of Glycyrrhiza uralensis against ulcerative colitis through regulating the signaling pathway of FXR/P-gp

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Abstract: Objective: Ulcerative colitis (UC) is a moderate to severe inflammatory bowel disease, with a characteristic inflammatory response. Chinese herbal medicine can play a role in UC treatment. Herein, we aimed to investigate the function of Glycyrrhiza uralensis in UC treatment and the underlying mechanism. Methods: After establishing an animal model of UC, different agents of kuijieguanchang prescription, Glycyrrhiza uralensis, mesalazine, and GW4064 were administrated to mice. The apoptosis rate was measured by TUNEL assay, and the expression of different biomarkers was tested by western blot and qPCR. Results: Glycyrrhiza uralensis could regulate apoptosis of intestinal mucosal cells, through regulating the expression of apoptosis-related proteins and protective proteins of intestinal mucosa. The administration of Glycyrrhiza uralensis could greatly enhance the expression of muc1, muc3, and the pro-apoptotic protein, BAX. The proteins involved in malignancy from UC, such as Bcl-2 and fgf-15, were dramatically downregulated after using the Glycyrrhiza uralensis. Moreover, it was illustrated that Glycyrrhiza uralensis acted against UC by activating the signaling of P-gp through upregualting its expression. The upregulation of FGFR4, SHP, and P-gp in liver conferred protective function in UC. Conclusion: Glycyrrhiza uralensis could regulate apoptosis of intestinal mucosal cells, through regulating the expression of apoptosis-related proteins and protective proteins of intestinal mucosa. The results provide novel options for UC treatment, as well as a rationale for pharmacology of Chinese traditional medicine, that is favorable for use of herbal medicine.

Keywords: Glycyrrhiza uralensis, ulcerative colitis, FXR/P-gp

Introduction

Ulcerative colitis (UC) is a chronic inflammatory disease in the colon and rectum, presenting a characteristric T cell dysfunction, cellular inflammation, and aberrant cytokine production [1]. The incidence of ulcerative colitis has increased in past decades due to the upregulation of emotional stress, pollution, and unhealthy lifestyles [2]. The progression and development of UC can result in several complications, that include toxic megacolon, reduction of gastrointestinal hemorrhage, chronic inflammation, and even ultimately colorectal cancer [3]. Thus, the ulcerative colitis is hurtful to health and causes a heavy burden to patients.

The current therapies for UC contain corticosteroids, glucocorticosteroids, aminosalicylates, and immunosuppressive agents. However, both the steroid and immunosuppressive agents have side effects for patients [4]. Currently, research on UC is focusing on immune regulation through typical immune cell types or involved signaling transduction, such as the T cells, and JNK-STAT signaling [5, 6].

Compared with treatment by western medicine, Chinese herbal medicine had a long history in the treatment of UC. Many traditional herbals were found effective to the treatment of UC, for instance, Sanhuangshuai decoction, Gegenqinlian decoction, and Qingchang Wenzhong decoction [7-9]. Besides the evaluation of effec-

tiveness and safety of Chinese herbals on UC treatment, the underlying mechanism based on the research of cellular and molecular signaling regulation has also been preliminary illustrated. For example, the NF-κB signaling pathway was proven to be inhibited by rhein for treatment of UC [10]. However, because Chinese herbals were the majority of compound preparation, the function and mechanism of specific substrate in the therapeutic application of UC remain largely unknown, and still need further exploration.

Glycyrrhiza uralensis is an efficacious ingredient commonly used in Chinese traditional medicine for the treatment of colorectal disease, including UC. For instance, the HuangqinTang (HQT), containing Glycyrrhiza uralensis Fisch, was reported beneficial in UC treatment [11]. Besides, Glycyrrhiza uralensis had potential for the treatment of other colorectal disease, such as the anticancer and immunodulatory effect in colon carcinoma [12]. Moreover, the function and toxicity research on Glycyrrhiza uralensis found that it improved the degradation functions of mucus and aromatic amino acids on intestinal bacteria, which ultimately reduce the intestinal urotoxin and other harmful substances in the colon [13]. These findings derived from traditional medicine and current pharmacology indicated that Glycyrrhiza uralensis was protective to the colon.

In recent years, studies have found that multidrug resistance (MDR) gene polymorphism is related to the pathogenesis of ulcerative colitis [14]. The P-glycoprotein encoded by the MDR gene plays an important role in loss of the normal mucosal barrier function in UC [15]. P-glycoprotein, also known as P-gp, is a glycoprotein encoded by the multi-drug resistance gene (MDRI). Its physiologic role is to protect cells from poisons or metabolic wastes to maintain the stability of the internal environment. There is a high concentration of P-gp in the colon and small intestinal epithelial cells of healthy people, which can prevent the absorption of drugs and toxins in the intestinal lumen [16]. With a prolonged course of ulcerative colitis, the expression of P-gp decreased. Therefore, exploring the mechanism of regulating P-gp expression is helpful in studying the pathogenesis of UC.

We designed this project to explore the effect of Glycyrrhiza uralensis on UC treatment. Because of the relationship between P-gp and UC, this will provide a rationale and treatment basis for the diagnosis, treatment, and prevention of ulcerative colitis.

Materials and methods

Ulcerative colitis animal model

All animal experiments fully complied with the guidelines of the Institutional Animal Care and Use Committee (IACUC) and were approved by the Ethics Committee for Experimental Animals of our hospital. The C57BL/BJ mice weighing 20-30 g were adopted to establish the acute inflammatory model. A total of 72 mice (half male and half male) were randomly divided into 6 groups with 12 per group: NC group, NS group, Kuijieguanchang Prescription group, Mesalazine group, Glycyrrhiza uralensis group, and GW4064 group. To induce intestinal inflammation, the DSS of 3.5% concentration was added to water for raising the mice. After feeding with DSS for 7 days, an animal model of UC was used for subsequent experiments. The DAI score was adopted to measure the status and grade of UC. After measurement of the indicators of UC, the mice were treated by different kinds of agents, such as physiologic saline solution, kuijieguanchang prescription (Nanjing University of Traditional Chinese Medicine), Glycyrrhiza uralensis extraction solution (Nanjing University of Traditional Chinese Medicine), mesalazine solution (Merck, Germany), and GW4064 (MCE, USA).

TUNEL assay

Apoptosis of colorectal cells was determined by terminal deoxynucleotidyl transferase-mediated fluorescein-dUTPnick-end labeling (TUNEL) assay. The mice were sacrificed, and the colon sections were prepared. The colon sections were treated for 5 min with a permeabilizing solution containing 0.1% Triton X-100 and 0.1% sodium citrate in PBS. Then, the permeabilized sections were incubated with TUNEL reaction buffer at 37°C for 1 h. The slides were stained with FITC-conjugated rabbit anti-mouse IgG (1:100, Abcam, USA) and DAPI (Sigma, USA). The luciferase value was captured by immunofluorescence microscope to evaluate the apoptosis of cells.

Table 1. Sequences of primers used for real-time PCR

Primer	Sequence 5'-3'
muc1-F	ACAATTGACTCTGGCCTTCCG
muc1-R	TGGGTTTGTGTAAGAGAGGCT
muc3-F	TGT CAG CTC CAG ACCAGATG
muc3-R	CCT GCT CAT ACT CGCTCTCC
Bcl-2-F	TCT TCCAGGAACCTCTGTGATG
Bcl-2-R	CAATGCCGCCATCGCTTACACC
Bax-F	ATCCAGAGACAAGACATGTAC
Bax-R	TTCAGATGTTCTAAGCCTACGG
fgfl15-F	AGTGGAGTGGGCGTATTG
fgfl15-R	CGTAGGGACCAGCAGAGTA
fgfr4-F	GTGGTCAGTGGGAAGTCTGG
fgfr4-R	TGCCATGTCTTCTGTCGTTC
SHP-F	ATGAGCTCCAGCCAATCGGGGTC
SHP-R	TCACCTCAACAAAAGCATGTCTTCG
GAPDH-F	GCACCGTCAAGGCTGAGAAC
GAPDH-R	ATGGTGGTGAAGACGCCAGT

Quantitative real-time PCR (qRT-PCR)

The experimental mice were sacrificed and the colon tissues were harvested. The colon tissues were firstly washed using PBS (pH 7.4), and the total RNA was extracted by Trizol (Takara, Japan) according the protocol provided by manufacturer. And then the cDNA was synthesized by PrimeScript RT-PCR kit (Takara, Japan). The expression of indicated genes was examined by real-time PCR methods conducted by the 7500 real-time PCR system (Applied Biosystems, USA). The method of the relative quantitation method $2^{-\Delta\Delta CT}$ was used to calculate the relative expression of specific genes, and GAPDH was adopted as the housekeeping gene. The primer sequences are shown in **Table 1**.

Western blot

The colorectal tissues and liver tissues were harvested after sacrificing the mice and were washed by pre-cooled PBS for three times and protein was extracted by IP lysis buffer (Thermo Fisher, USA) with proteinase and phosphatase inhibitor cocktail (Roche, Switzerland). The protein concentration was measured by BCA protein assay kit (Thermo Fisher, USA) and added with loading buffer for western blot. An 8% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) was utilized to separate proteins by molecular weight and then the proteins were transferred to NC membrane (Merck, Germany). The membrane was blocked by 5% fat-free milk at room temperature for 1 h and incubated with the primary antibody at 4°C overnight through gentle shaking. Antibodies against the proteins were: Bcl-2 (1:1000, Bioworld Technology, Cat#: BS70205), FGFR4 (1:1000, Bioworld Technology, Cat#: BS60719), FGF-15 (1:1000, Abcam, Cat#: ab229630), MUC1 (1:500, Bioworld Technology, Cat#: BS60935), MUC3 (1:1000, Abcam, Cat#: ab199260), Bax (1:1000, Bioworld Technology, Cat#: BS1725), p-gp (1:500, Bioworld Technology, Cat#: BS3523), SHP-1 (1:500, Bioworld Technology, Cat#: BS9843M), and GAPDH (1:10000, Bioworld Technology, Cat#: AP0063). The next day, the membrane was washed by TBST (pH 7.4) for three times and incubated with the secondary antibody (Thermo Fisher, USA) at room temperature for 1 hour. Finally, the bands of protein were visualized by Odyssey imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

The SPSS 13.0 and GraphPad 7.0 were used for statistical analysis. Comparation between groups was calculated by two-tailed Student's t-test, and the significance among three or more groups was validated by ANOVA. Pearson analysis was performed to calculate the correlation. In this study all error bars were presented the mean ± standard deviation ($\bar{x}$ ± sd). Statistical significance was set at $P<0.05$.

Results

Glycyrrhiza uralensis relieved apoptosis in vivo

To measure the function of Glycyrrhiza uralensis in the treatment of UC, we tested the apoptosis rate after treatment of Glycyrrhiza uralensis as well as the positive Chinese herbal, Kuijieguanchang prescription, which was shown to ease the symptoms of UC in prior research; the standard treatment of mesalazine, and the agonist of FXR receptor. Compared with the negative control group, the use of Glycyrrhiza uralensis significantly promoted cell apoptosis, which was identical to the standard treatment and positive control groups, indicating the potential of Glycyrrhiza uralensis in the treatment of UC (**Figure 1**).

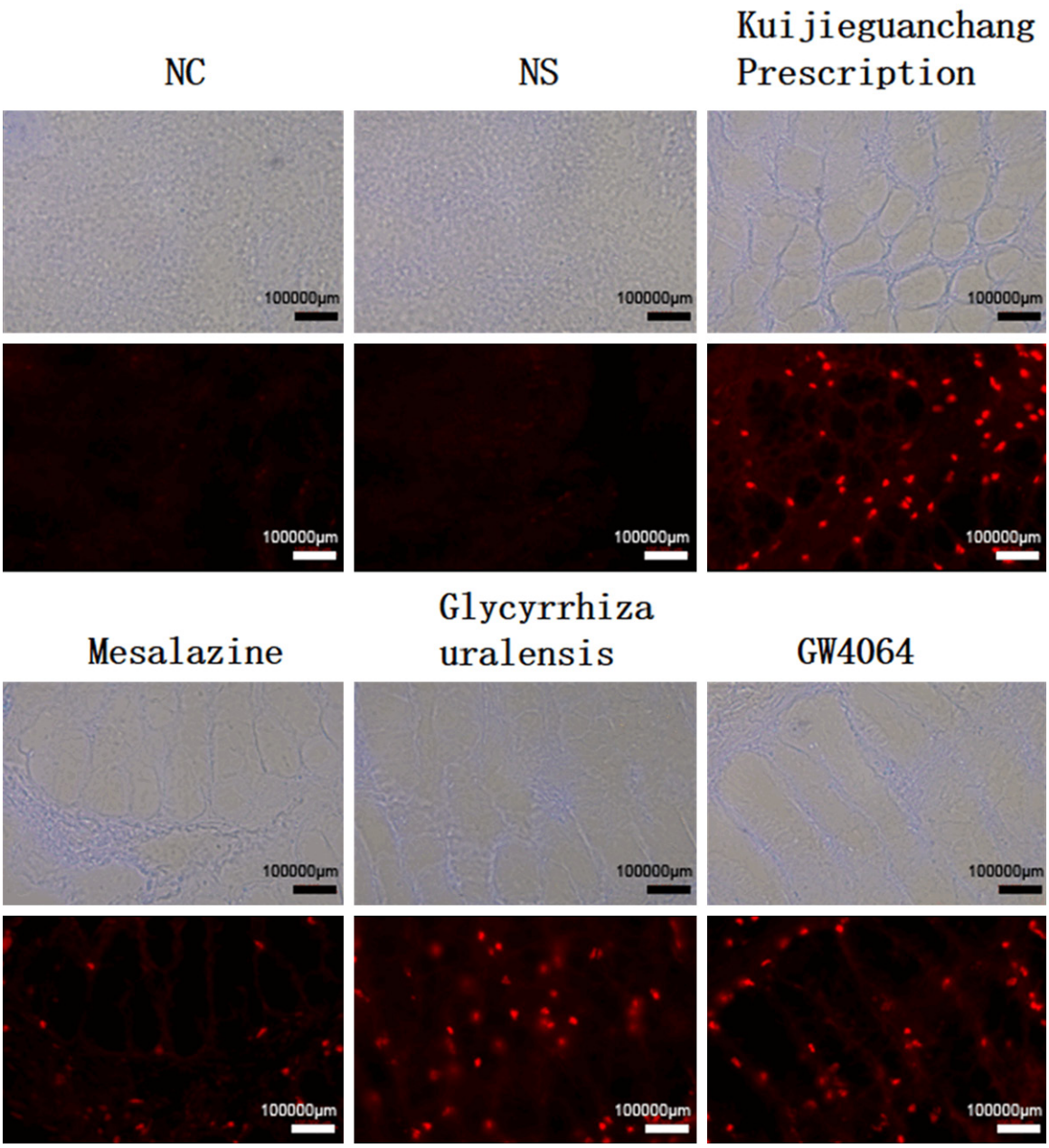
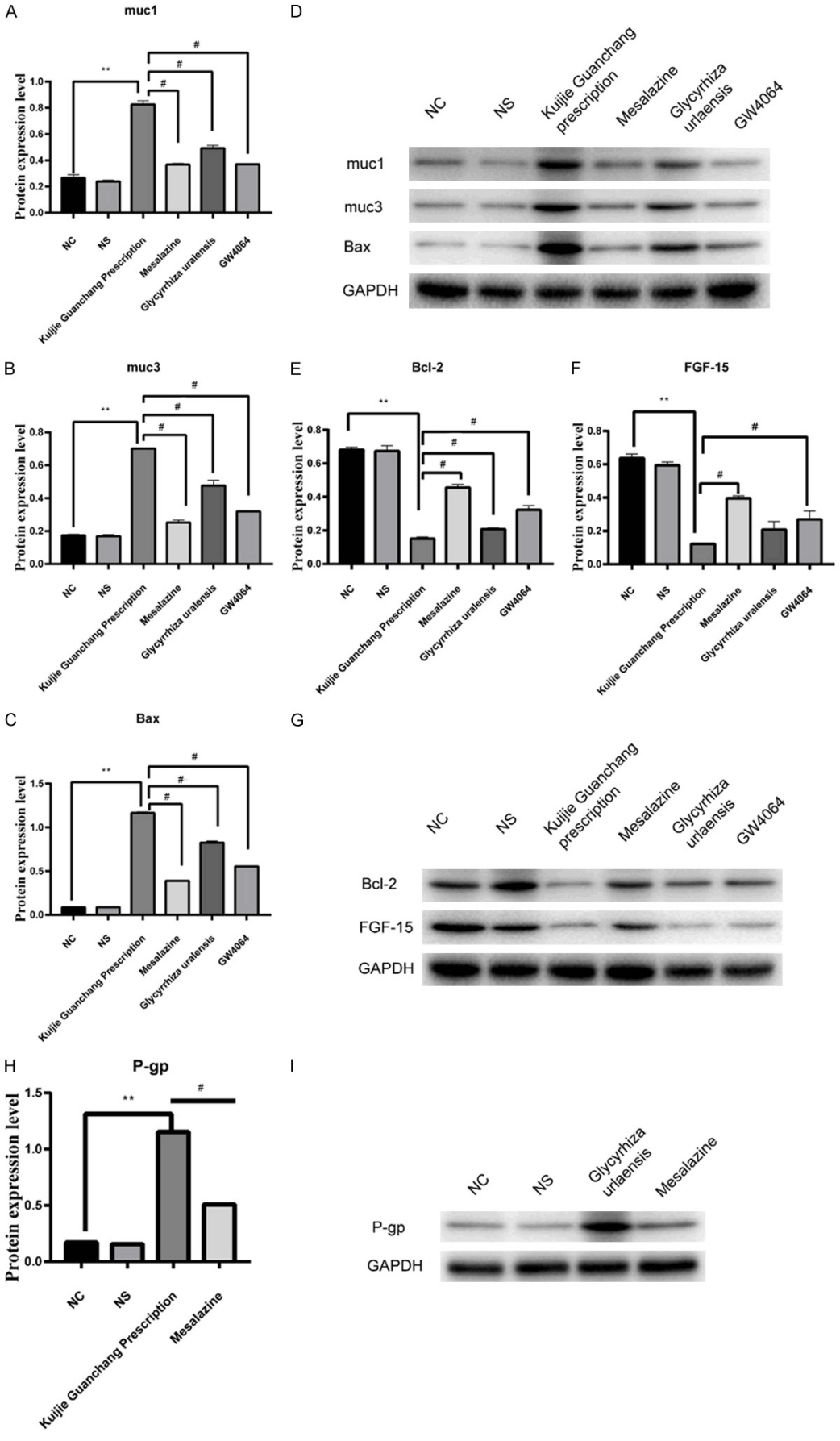


Figure 1. Glycyrrhiza uralensis relieved cell apoptosis in vivo. The apoptosis rate of intestinal mucosa treated by Kuijieguanchang prescription, Glycyrrhiza uralensis, mesalazine, or GW4064 was measured by TUNEL assay (Scale bar: 100,000 μm).

Glycyrrhiza uralensis upregulated the expression of pro-apoptotic proteins and downregulated the expression of anti-apoptotic proteins in intestinal mucosa

To further elucidate the underlying mechanism of Glycyrrhiza uralensis in the treatment of UC, we measured the expression level of proteins involved in apoptosis and other biomarkers involved in UC progression. Bcl-2 was reported to anti-apoptotic function, and the fgf-15 was reported as downregulated in intestinal inflammation [17, 18]. The O-glycosylated proteins, muc1 and muc3, were reported to protect colon cells from several kinds of stress, and is secreted by goblet cells [19]. Furthermore, we also examined the expression of Bax, which accelerates the process of apoptosis. The results showed that the administration Glycyrrhiza uralensis significantly promote the expres-


A
muc1
Protein expression level
NC
NS
Kuijie Guanchang Prescription
Mesalazine
Glycyrrhiza uralensis
GW4064
D
NC
NS
Kuijie Guanchang prescription
Mesalazine
Glycyrrhiza urlaensis
GW4064
muc1
muc3
Bax
GAPDH
B
muc3
E
Bcl-2
F
FGF-15
C
Bax
G
Bcl-2
FGF-15
GAPDH
H
P-gp
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P-gp
GAPDH

Figure 2. Glycyrrhiza uralensis upregulated the expression of pro-apoptotic proteins and downregulated the expression of anti-apoptotic proteins in intestinal mucosa. A: Quantitative analysis of western blot result of muc1 expression; B: Result of muc3 expression; C: Result of BAX expression; D: The bands of the western blot examined the expression of muc1, muc3, and BAX among the NC, NS, kuijieguanchang prescription, Glycyrrhiza uralensis, mesalazine and GW4064 group; E: Quantitative analysis of Bcl-2 expression; F: The quantitative analysis of FGF-15 expression; G: The bands of the western blot examined the expression of Bcl-2 and FGF-15 among the NC, NS, kuijieguanchang prescription, Glycyrrhiza uralensis, mesalazine, and GW4064 groups; H: Quantitative analysis of western blot result of P-gpexpression; I: The bands examined the expression of P-gp among the NC, NS, Glycyrrhiza uralensis, and mesalazine group. (NC representing negative control; $^{**}P<0.01$; #not statistically significant).

sion of muc1, muc3, and Bax at the protein level, compared with the NC and NS groups, which is consistent with the effects of control groups, such as kuijieguanchang prescription, FXR agonist, and mesalazine (**Figure 2A-D**). According to the results of **Figure 2E-G**, the treatment of Glycyrrhiza uralensis significantly reduced the expression of Bcl-2 and FGF-15. Ultimately, we also tested the effect of Glycyrrhiza uralensis in the regulation of P-gp expression, and we found that the administration of this Chinese traditional agent could upregulate the expression of P-gp (**Figure 2H**, **2I**).

Glycyrrhiza uralensis enhanced the expression of FGFR4, SHP and P-gp in liver

The previous studies had proved the association between liver and colon, as well as the influence of UC to the liver function [20]. To comprehensively examine the efficacy, the expressions of FGFR4 and SHP were tested in liver tissues. It was reported that Src homology domain-containing tyrosine phosphatase (SHP) been associated with susceptibility to develop ulcerative colitis [21]. With the treatment of Glycyrrhiza uralensis, the expression level of FGFR4 and SHP was greatly increased compared with the negative control groups, which indicated that it could relieve the inflammatory response in UC progression (**Figure 3A-C**). The results also exhibited that utilization of the traditional agent Glycyrrhiza uralensis could enhance the signaling of P-gp by upregulating its expression (**Figure 3D**, **3E**).

Glycyrrhiza uralensis regulated the expression level of the UC-associated molecules by changing the transcription

To further clarify the manner of Glycyrrhiza uralensis in regulating the expression of the above biomarkers involved in the progression of UC, we also measured the mRNA expression of these by qPCR. The treatment of Glycyrrhiza uralensis significantly upregulated the expression of muc1 (**Figure 4A**), muc3 (**Figure 4B**), FGFR4 (**Figure 4C**), SHP (**Figure 4F**), and BAX (**Figure 4G**), but downregulated the expression of FGF-15 (**Figure 4D**) and Bcl-2 (**Figure 4E**) at the mRNA level. This was in line with the protein levels examined before. These results demonstrated that the Glycyrrhiza uralensis could regulate the signaling transduction involved in UC by altering the transcriptional level.

Discussion

In recent years, due to changes in lifestyle and emotional stress, the incidence and prevalence of ulcerative colitis has been fast-growing. The initiation and progression of UC is a complicated process involving multiple steps, which is characterized by an inflammatory response and aberrant immune cell infiltration. Due to the complexity of UC, the pathogenesis has not been fully clarified. Currently, immune suppressive agents, such as sterol hormone and TNF monoclonal antibodies are used for the treatment of UC [22]. However, unsatisfactory effectiveness and relatively severe side effects are obstacles for prognosis in UC patients. Chinese traditional medicine was used to treat UC for thousands of years. However, the underlying mechanism of the agents lacks sufficient exploration.

In this study, we found that Glycyrrhiza uralensis could significantly improve the phenotype of UC by regulating cell apoptosis of the intestinal mucosa. For exploration of a specific mechanism, we analyzed the expression of intestinal mucosa protective proteins, such as muc1 and muc3. The administration of Glycyrrhiza uralensis could greatly enhance the expression of muc1, muc3, and the pro-apoptotic protein, Bax. The proteins invloved in the malignancy from UC, such as Bcl-2 and fgf-15, were dramatically downragulated after using

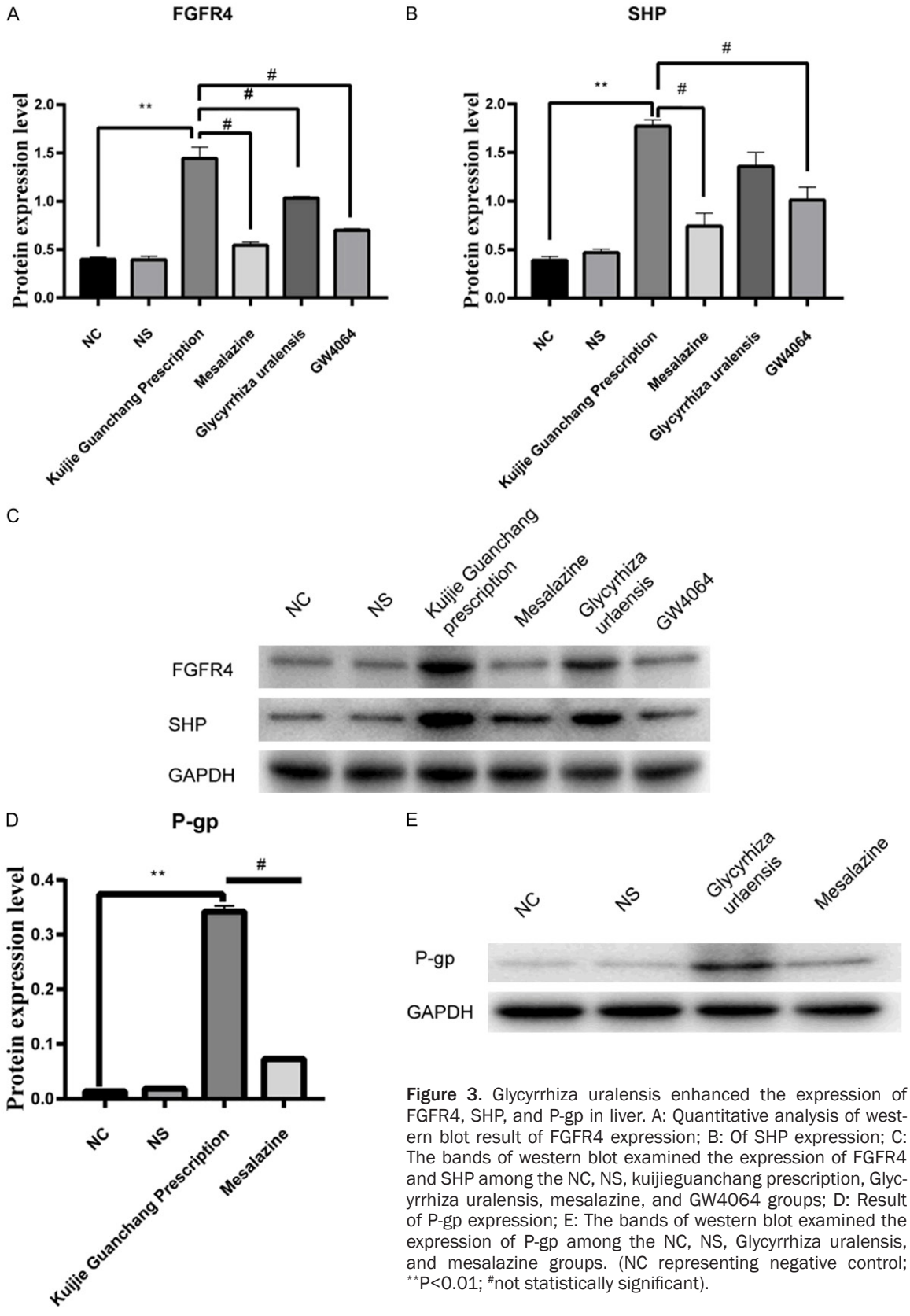

Figure 3. Glycyrrhiza uralensis enhanced the expression of FGFR4, SHP, and P-gp in liver. A: Quantitative analysis of western blot result of FGFR4 expression; B: Of SHP expression; C: The bands of western blot examined the expression of FGFR4 and SHP among the NC, NS, kuijieguanchang prescription, Glycyrrhiza uralensis, mesalazine, and GW4064 groups; D: Result of P-gp expression; E: The bands of western blot examined the expression of P-gp among the NC, NS, Glycyrrhiza uralensis, and mesalazine groups. (NC representing negative control; $^{**}P<0.01$; #not statistically significant).

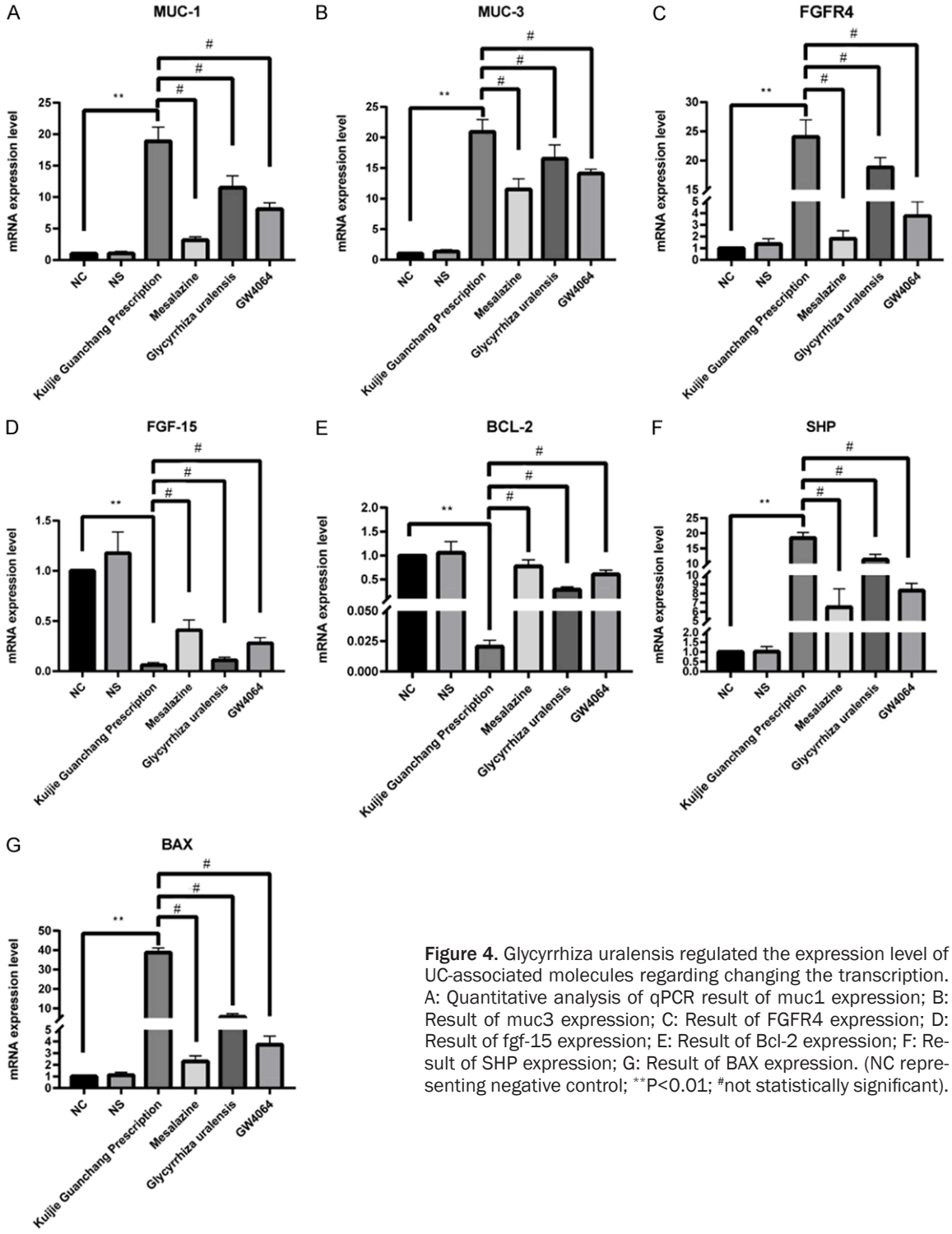

Figure 4. Glycyrrhiza uralensis regulated the expression level of UC-associated molecules regarding changing the transcription. A: Quantitative analysis of qPCR result of muc1 expression; B: Result of muc3 expression; C: Result of FGFR4 expression; D: Result of fgf-15 expression; E: Result of Bcl-2 expression; F: Result of SHP expression; G: Result of BAX expression. (NC representing negative control; $^{**}P<0.01$; #not statistically significant).

Glycyrrhiza uralensis. Moverover, it was illustrated that Glycyrrhiza uralensis performed its function in UC treatment by activating the signaling of P-gp through upregualting its expression. The upregulation of FGFR4, SHP, and p-gp in liver, which have a protective function in UC, was also shown. Furthermore, we also proved that the regulatory effect performed by Glycyrrhiza uralensis was through transcriptional adjustment.

There are some limitations in this work. As an MDR molecule, P-gp mediates substance transportation between cells, which indicates its potential for regulating crosstalk between cells, especially immune cells [23, 24]. In this study, we found that Glycyrrhiza uralensis could regulate the P-gp expression, and was benefical for treatment of UC through regulating P-gp signaling which ultimately affects apoptosis of intestinal mucosal cells. However, the downstream signaling pathway as well as the complicated network of signalling transduction were unknown. The mechanism of P-gp in immune regulation during the pathologic process in UC needs further investigation.

In summary, the results provide novel options for UC treatment, as well as a rationale for pharmacology of Chinese traditional medicine, that is favorable for widespread use of herbal medicine.

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Disclosure of conflict of interest

None.

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