Original Article

Expression of Transcription Factor FOXO3a is Decreased in Patients with Ulcerative Colitis

Min Min[1], Jing Yang[2], Yun-Sheng Yang[2], Yan Liu[1], Li-Mei Liu[3], Yang Xu[1]

[1]Department of Gastroenterology and Hepatology, Affiliated Hospital of Academy of Military Medical Sciences, Beijing 100071, China
[2]Department of Gastroenterology and Hepatology, Institute of Digestive Diseases, Chinese People's Liberation Army General Hospital, Beijing 100853, China
[3]Department of Gastroenterology and Hepatology, Tonghua Hospital, Tonghua, Jilin 134001, China

Min Min and Jing Yang contributed equally to this work.

Abstract

Background: Ulcerative colitis (UC) is associated with differential expression of genes involved in inflammation and tissue remodeling, including *FOXO3a*, which encodes a transcription factor known to promote inflammation in several tissues. However, FOXO3a expression in tissues affected by UC has not been examined. This study investigated the effects of FOXO3a on UC pathogenesis.
Methods: FOXO3a expression, in 23 patients with UC and in HT29 cells treated with tumor necrosis factor-α (TNF-α) for various durations, was detected by quantitative real-time polymerase chain reaction and Western blotting analysis. Enzyme-linked immunosorbent assay was used to quantify interleukin (IL)-8 expression in FOXO3a-silenced HT29 cells treated with TNF-α for various durations.
Results: The messenger RNA and protein expression of FOXO3a were significantly lower in UC tissues than those in normal subjects ($P < 0.01$). TNF-α treatment for 0, 0.5, 1, 6, and 24 h induced FOXO3 degradation in HT29 cells. FOXO3a silencing increased IL-8 levels in HT29 cells treated with TNF-α for 6 h ($P < 0.05$).
Conclusion: FOXO3a may play an important role in the intestinal inflammation of patients with UC.

Key words: FOXO3a; Interleukin-8; Ulcerative Colitis

Introduction

Ulcerative colitis (UC) is a type of nonspecific colon inflammation, with lesions mainly at colonic mucosa and submucosa. Its etiology and pathogenesis are yet to be elucidated. Currently, it is believed that immune dysfunction of intestinal mucosa against bacteria in the lumen, loss of intestinal homeostasis, and excessive inflammatory responses against pathogens leads to increased secretion of proinflammatory cytokines, resulting in intestinal inflammation.[1,2] Several studies have found that patients with UC have higher secretion of many cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, and IL-8. This finding indicates that cytokine imbalance plays an important role in the occurrence and the development of UC.

The transcription factor FOXO3a is an important member of the forkhead family. It is widely expressed in many tissues and organs in adults and regulates many cellular functions such as growth, metabolism, differentiation, apoptosis, and immunity through transcriptional regulation.[3-5] Some studies suggest that FOXO3a plays an important role in the occurrence and the development of inflammation through regulation of nuclear factor kappa B (NF-κB).[6] However, FOXO3a expression has not been reported in UC. In this study, we explored the role of FOXO3a in the pathogenesis of UC by examining FOXO3a levels in the intestinal mucosa of patients with UC and in the intestinal HT29 cells stimulated by TNF-α, as well as by observing changes in IL-8 expression in these cells after silencing FOXO3a using small interfering RNA (siRNA).

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.4103/0366-6999.167314

Address for correspondence: Dr. Yun-Sheng Yang, Institute of Digestive Diseases, Chinese People's Liberation Army General Hospital, 28 Fuxing Road, Beijing 100853, China
E-Mail: sunny301ddc@126.com

Received: 08-04-2015 **Edited by:** Li-Shao Guo
How to cite this article: Min M, Yang J, Yang YS, Liu Y, Liu LM, Xu Y. Expression of Transcription Factor FOXO3a is Decreased in Patients with Ulcerative Colitis. Chin Med J 2015;128:2759-63.

Methods

Tissue collection

Colonic mucosa pinch biopsies were obtained from the sigmoid colon of patients with active UC ($n = 23$) and healthy subjects ($n = 20$) undergoing screening colonoscopies at the Institute of Digestive Diseases, Chinese People's Liberation Army General Hospital from May to November 2011. UC diagnosis was confirmed by colonoscopy and pathological findings. Disease activity was defined using the Mayo criteria.[7] The clinical characteristics of the patient groups are listed in Table 1. The biopsies were either embedded in paraffin blocks or snap frozen in liquid nitrogen and subsequently stored at −80°C. This study was approved by the Chinese People's Liberation Army General Hospital Institutional Review Board.

Cell culture and tumor necrosis factor-α treatment

The human intestinal epithelial HT29 cells (ATCC, Rockville, USA) used in this study were routinely cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, USA) supplemented with 10% fetal calf serum, penicillin, and streptomycin at 37°C in a 5% CO_2 incubator. For protein analysis, cells were plated in 6-well plates and used at 60–70% confluence. Semiconfluent HT29 monolayers were treated with TNF-α at a final concentration of 10 ng/ml for 0.5, 1, 6, and 24 h.

Quantitative real-time polymerase chain reaction

FOXO3a expression was validated by an SYBR green-based quantitative real-time polymerase chain reaction (qRT-PCR) assay (Qiagen, Valencia, USA). Primers for FOXO3a and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Invitrogen and are listed in Table 2. The qRT-PCR was performed in an ABI7900 cycler (Applied Biosystems, Foster City, USA), and the cycle threshold (Ct) value was recorded.

Table 1: Clinical characteristics of patients

Variables	Control	UC
Number of patients	20	23
Age, years (mean ± SD)	38.2 ± 12.2	35.6 ± 9.4
Range	32–52	30–45
Gender (male/female), *n*	12/8	17/6
Duration of symptoms, months	NA	20
Medications, *n*		
5-ASA	0	20
Antibiotics	0	0
Steroids	0	5
Immunomodulators	0	0
Biologics	0	0

NA: Not available; SD: Standard deviation; UC: Ulcerative colitis.

Western blotting analysis

Protein extracts were prepared from the human tissues and cells using a lysis buffer supplemented with ethylenediamine tetraacetic acid (EDTA)-free complete protease inhibitors (Roche, Mannheim, Germany). Proteins were extracted and subjected to sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) on a 10% gel. The bands were transferred to a polyvinylidene difluoride membrane. After blocking with 5% nonfat dry milk in Tris-buffered saline supplemented with 0.1% Tween 20 for 4 h at room temperature, the membranes were incubated overnight with either anti-FOXO3a or anti-β-actin primary antibodies (all used at 1:1000 dilution; Bioworld, Shanghai, China) at 4°C. The membranes were then incubated with a secondary antibody at 37°C for 1 h. Normalization was performed by blotting the same membranes with anti-β-actin antibody.

RNA interference

The siRNAs targeting FOXO3a were designed and synthesized by GenePharma (Shanghai, China) and are listed in Table 2. All siRNA transfections were performed with Lipofectamine 2000 (Invitrogen). HT29 cells were transfected with 100 nmol/L siRNA for 48 h.

Enzyme-linked immunosorbent assay

To quantify IL-8 expression, the culture medium from the monolayers was analyzed using an enzyme-linked immunosorbent assay kit (R and D Systems, Minneapolis, USA) according to the manufacturer's protocol.

Statistical analysis

All experiments were repeated at least 3 times. The results were expressed as mean ± standard deviation values. The differences between groups were determined by two-tailed Student's *t*-tests and one-way analysis of variance (ANOVA) performed using the software SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered as statistically significant.

Results

FOXO3a expression decreased in patients with active ulcerative colitis

As shown in Figure 1, the messenger RNA (mRNA)

Table 2: Primer sequences used for real-time PCR and siRNA-FOXO3a

Items	Forward primers	Reverse primers
FOXO3	5'-AGCGGATGCCCAAATAAAAG-3'	5'-CCTCCTTCACATTTGTTTAC-3'
GAPDH	5'-GACCACAGTCCATGCCATCAC-3'	5'-GTCCACCACCCTGTTGCTGTA-3'
siRNA-1620	5'-GGACCUUCAUCUCUGAACUTT-3'	5'-AGUUCAGAGAUGAAGGUCCTT-3'
siRNA-1886	5'-CCAGGGAAGUUUGGUCAAUTT-3'	5'-AUUGACCAAACUUCCCUGGTT-3'
siRNA-2370	5'-GACCCUCAAACUGACACAATT-3'	5'-UUGUGUCAGUUUGAGGGUCTT-3'
Negative control	5'-UUCUCCGAACGUGUCACGUTT-3'	5'-ACGUGACACGUUCGGAGAATT-3'

PCR: Polymerase chain reaction; siRNA: Small interfering RNA.

expression of FOXO3a was significantly lower in the colon tissues of active UC patients than in those of the controls ($P < 0.01$). FOXO3a protein level was significantly lower in active UC tissues than in the tissues of healthy subjects ($P < 0.01$) [Figure 2].

Tumor necrosis factor-α regulates FOXO3a in HT29 cells

Treatment of HT29 cells with TNF-α for 0, 0.5, 1, 6, and 24 h resulted in decreased FOXO3a mRNA expression [Figure 3]. TNF-α treatment also induced FOXO3 protein degradation [Figure 4].

Silencing FOXO3a increases interleukin-8 level in tumor necrosis factor-α–treated HT29 cells

FOXO3a silencing by siRNA was confirmed by Western blotting analysis [Figure 5]. We chose siRNA-1886 for the use in further investigation as it was found to be the most effective. Transfection of HT29 cells with siRNA against FOXO3a inhibited FOXO3a mRNA expression by 90% (data not shown). As shown in Figure 6, FOXO3a silencing significantly increased the level of IL-8 in HT29 cells after 1-h treatment with TNF-α.

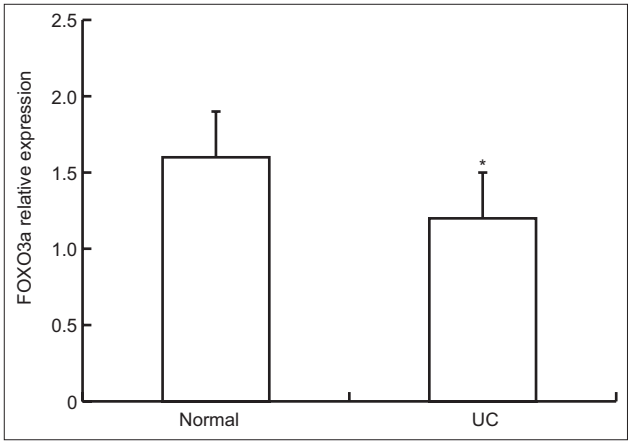

Figure 1: Messenger RNA expression of FOXO3a as determined by quantitative real-time polymerase chain reaction. The results were normalized to GAPDH expression. *$P < 0.05$.

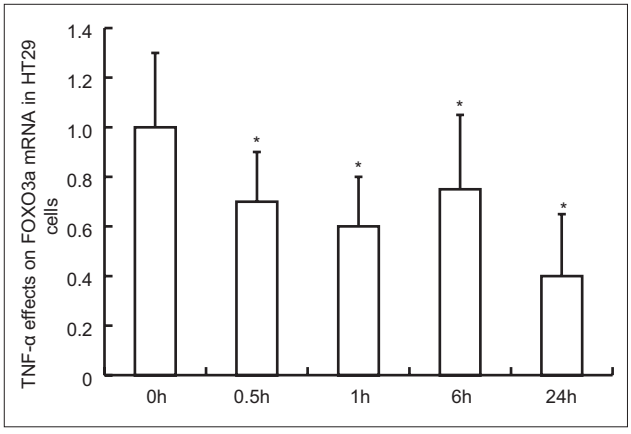

Figure 3: Effects of tumor necrosis factor-α on FOXO3a messenger RNA expression in HT29 cells. FOXO3a messenger RNA expression was assessed at 0.5, 1, 6, and 24 h after tumor necrosis factor-α treatment by quantitative real-time polymerase chain reaction. *$P < 0.05$.

Discussion

The forkhead family of transcription factors includes at least 17 sub-families from FOXa to FOXq. They play very important roles in regulating the expression of genes related to cellular functions such as apoptosis, cell cycle, oxidative stress, and differentiation.[8-10] In mammals, the FOXO protein sub-family includes FOXO1, O3, O4, and O6, which are important transcription factors that function downstream of the PI3K/Akt signaling pathway. Various external stimuli and cytokines can stimulate PI3K and activate Akt, leading to phosphorylation of the transcription factor FOXO3a and its transport from the nucleus into the cytoplasm. This translocation of FOXO3a decreases the transcriptional activation of the target gene, which finally affects the corresponding cellular signaling pathway.[11,12] Some studies have shown that FOXO3a knockout mice develop spontaneous, multi-organ systemic immune responses, which increase with increasing cytokines production and NF-κB activity.[6]

The pathological basis of UC is nonspecific intestinal inflammation, characterized by infiltration of lymphocytes, plasma cells, and neutrophils into the colon. These activated inflammatory cells can release inflammatory mediators and cytokines, which contribute to the occurrence and the

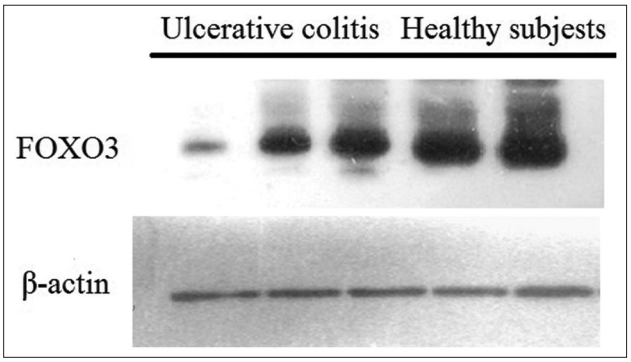

Figure 2: Protein expression of FOXO3a in tissues from both patients with UC and healthy subjects as determined by Western blotting analysis.

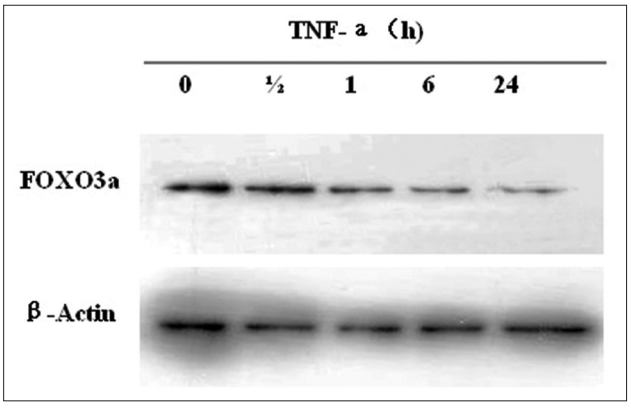

Figure 4: Effects of tumor necrosis factor-α on FOXO3a protein expression in HT29 cells. FOXO3a protein expression was assessed at 0.5, 1, 6, and 24 h after tumor necrosis factor-α treatment by Western blotting analysis.

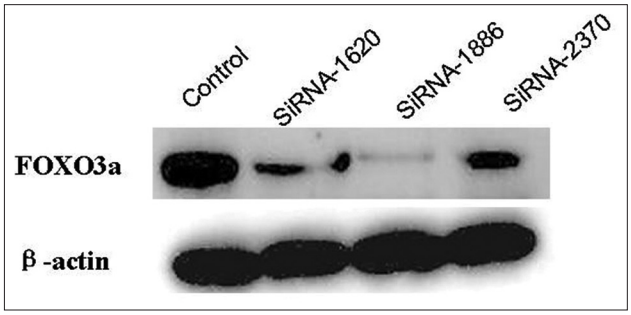

Figure 5: Effect of three small interfering RNAs on FOXO3a expression as determined by Western blotting analysis. All small interfering RNAs decreased FOXO3a level, but small interfering RNA-1886 was the most effective.

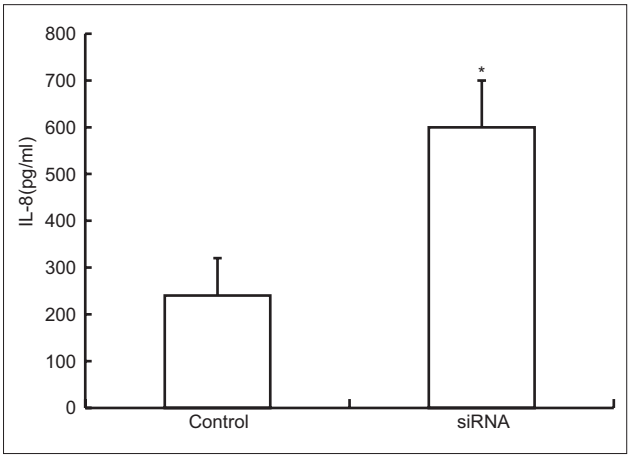

Figure 6: FOXO3a silencing in HT29 cells treated with tumor necrosis factor-α for 1 h. The medium was collected for interleukin-8 quantification. *$P < 0.05$.

development of intestinal inflammation. Some studies have found that in the dextran sulfate sodium (DSS)-induced mouse enteritis model, FOXO3a was expressed in the cytoplasm of intestinal epithelial cells, indicating FOXO3a was in an inactivated state. Also, the intestinal inflammation in DSS-induced FOXO3a knockout mice was more severe than that in wild-type mice.[13] This is the first study to examine the FOXO3a expression in the intestinal mucosa of patients with active UC. We found that FOXO3a expression was significantly lower than that in the control group, indicating that FOXO3a may play an important role in UC pathogenesis and it could be a protective factor of the intestinal mucosa.

In order to further clarify the regulatory mechanism of FOXO3a in intestinal inflammation, we observed the changes in FOXO3a expression in TNF-α-stimulated HT29 cells, as well as determined the changes in the expression of the inflammatory cytokine IL-8 in FOXO3a-silenced HT29 cells. Our results show that FOXO3a expression decreased gradually while IL-8 level increased significantly with increasing TNF-α stimulation time. IL-8 is linked to many inflammatory diseases and is known to promote inflammation, as well as induces immune responses and tissue damage. IL-8 level in colon mucosa and peripheral blood in patients with UC significantly increases with the severity of inflammation, and it plays an important role in the persistence and amplification of UC inflammation. Some current studies suggest that IL-8 is an essential inflammatory mediator in the development of UC and inflammatory reactions induced by TNF-α, IL-1, and IL-6 are to a large extent mediated by the IL-8-induced production of other chemokines.[14]

NF-κB regulates genes involved in many physiological and pathological processes, including the proliferation of inflammatory immune cells and apoptosis.[15] It also plays an important role in the pathogenesis of a range of diseases that involve cytokine inflammatory mediators and is closely linked to UC pathogenesis.[16,17] Some studies have shown that FOXO3a can inhibit NF-κB activation, reducing downstream production of inflammatory cytokines.[6] This type of regulation can allow for the control of the inflammatory reaction and help maintain the homeostasis of the intestinal mucosa. Therefore, we believe that the reduction in FOXO3a expression in the intestinal mucosa of patients with UC likely leads to increased production of inflammatory cytokines (such as IL-8) through inducing NF-κB activation. This would disrupt the cytokine balance in the intestinal mucosa, thereby inducing inflammation and ultimately damaging the mucosa.

The results of this study indicate that FOXO3a could involve in the inflammation of intestinal mucosa and might play an important role in UC pathogenesis. It could also be a protective factor of the intestine, maintaining the immune hemostasis of the mucosa against lumen bacteria and the balance among inflammatory cytokines. Therefore, FOXO3a could be a potential therapeutic target for UC.

Financial support and sponsorship

This work was supported by a grant from the National Natural Science Foundation of China (No. 81300277).

Conflicts of interest

There are no conflicts of interest.

References

1. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci U S A 2007;104:13780-5.
2. Ardizzone S, Bianchi Porro G. Inflammatory bowel disease: New insights into pathogenesis and treatment. J Intern Med 2002;252:475-96.
3. Abid MR, Yano K, Guo S, Patel VI, Shrikhande G, Spokes KC, *et al.* Forkhead transcription factors inhibit vascular smooth muscle cell proliferation and neointimal hyperplasia. J Biol Chem 2005;280:29864-73.
4. Hu MC, Lee DF, Xia W, Golfman LS, Ou-Yang F, Yang JY, *et al.* IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. Cell 2004;117:225-37.
5. Yang W, Dolloff NG, El-Deiry WS. ERK and MDM2 prey on FOXO3a. Nat Cell Biol 2008;10:125-6.
6. Lin L, Hron JD, Peng SL. Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. Immunity 2004;21:203-13.
7. Kornbluth A, Sachar DB; Practice Parameters Committee of the

American College of Gastroenterology. Ulcerative colitis practice guidelines in adults (update): American College of Gastroenterology, Practice Parameters Committee. Am J Gastroenterol 2004;99:1371-85.
8. Accili D, Arden KC. FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. Cell 2004;117:421-6.
9. Nakashima K, Yakabe Y. AMPK activation stimulates myofibrillar protein degradation and expression of atrophy-related ubiquitin ligases by increasing FOXO transcription factors in C2C12 myotubes. Biosci Biotechnol Biochem 2007;71:1650-6.
10. Rathbone CR, Booth FW, Lees SJ. FoxO3a preferentially induces p27Kip1 expression while impairing muscle precursor cell-cycle progression. Muscle Nerve 2008;37:84-9.
11. Wu W, Zou M, Brickley DR, Pew T, Conzen SD. Glucocorticoid receptor activation signals through forkhead transcription factor 3a in breast cancer cells. Mol Endocrinol 2006;20:2304-14.
12. Lam EW, Francis RE, Petkovic M. FOXO transcription factors: Key regulators of cell fate. Biochem Soc Trans 2006;34(Pt 5):722-6.
13. Snoeks L, Weber CR, Wasland K, Turner JR, Vainder C, Qi W, *et al.* Tumor suppressor FOXO3 participates in the regulation of intestinal inflammation. Lab Invest 2009;89:1053-62.
14. Reddy KP, Markowitz JE, Ruchelli ED, Baldassano RN, Brown KA. Lamina propria and circulating interleukin-8 in newly and previously diagnosed pediatric inflammatory bowel disease patients. Dig Dis Sci 2007;52:365-72.
15. Rogler G, Brand K, Vogl D, Page S, Hofmeister R, Andus T, *et al.* Nuclear factor kappaB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. Gastroenterology 1998;115:357-69.
16. Hegazy SK, El-Bedewy MM. Effect of probiotics on pro-inflammatory cytokines and NF-kappaB activation in ulcerative colitis. World J Gastroenterol 2010;16:4145-51.
17. Andresen L, Jørgensen VL, Perner A, Hansen A, Eugen-Olsen J, Rask-Madsen J. Activation of nuclear factor kappaB in colonic mucosa from patients with collagenous and ulcerative colitis. Gut 2005;54:503-9.