



Expression of receptor interacting protein 3 and mixed lineage kinase domain-like protein-key proteins in necroptosis is upregulated in ulcerative colitis

Tielong Wu, Yuanyuan Dai, Lili Xue, Yingyue Sheng, Linlin Xu, Yuzheng Xue

Department of Gastroenterology, Affiliated Hospital of Jiangnan University, Wuxi 214062, China

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Correspondence to: Yuzheng Xue. Department of Gastroenterology, Affiliated Hospital of Jiangnan University, Wuxi 214062, China.
Email: 1480455860@qq.com.

Background: Ulcerative colitis (UC) is a chronic inflammatory colonic disease strongly associated with intestinal epithelial cell (IEC) death. Necroptosis is characterized by a newly programmed cell death through a caspase-independent pathway. Receptor interacting protein 3 (RIP3) and mixed lineage kinase domain-like protein (MLKL) are very important in the pathway of necroptosis. However, their expression in UC remains unelucidated. This study aimed to investigate the expression of RIP3 and MLKL in patients with UC, along with its correlation with disease activity.

Methods: Colonic tissue samples were taken from 22 UC patients and 19 healthy controls. RIP3 and MLKL expression levels were evaluated by immunohistochemical staining and western blotting.

Results: RIP3 and MLKL were upregulated in inflamed tissues of UC ($P < 0.01$). No variations were observed in healthy control subjects and non-inflamed colons ($P > 0.05$). RIP3 and MLKL were positively correlated with UC disease activity ($P < 0.01$).

Conclusions: Our results suggest that necroptosis is strongly associated with intestinal inflammation in patients with UC. Further studies of necroptosis may be helpful in future treatments of UC.

Keywords: Ulcerative colitis (UC); receptor interacting protein 3 (RIP3); mixed lineage kinase domain-like protein (MLKL); intestinal inflammation

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Introduction

Ulcerative colitis (UC) is a chronic intestinal inflammatory disease characterized by chronic inflammation limited to the mucosal layer of the colon. It usually affects the rectum, and inflammation may extend throughout the entire colon (1). Pathologically, it is characterized by chronic inflammation and epithelial barrier damage (2). Intestinal epithelial cells (IECs) form the first intestinal barrier. Increasing evidence has shown that abnormal IEC death plays an important role in intestinal inflammation (3,4).

Necrosis is a new caspase-independent mode of

programmed cell death. Necroptosis is characterized by cell swelling, mitochondria dysfunction, plasma membrane permeabilization, and the release of cytoplasmic content to the extracellular space (5). Necroptosis can be activated by various stimuli such as lipopolysaccharides (LPS), physical-chemical stress, ionizing radiation, calcium overload, anti-cancer drugs, DNA damage, and other causes (6). Signaling can be initiated through the activation of members of the tumor necrosis factor (TNF) family, and it has been shown that this pathway is mediated by two kinases, receptor-interacting protein 1 (RIP1) and receptor-interacting protein

Table 1 Clinicopathological characteristics of UC patients

Number	Values (n=22)
Age (years), mean \pm SD [range]	47 \pm 15 [22–56]
Gender (male/female)	13/9
Disease extent	
Proctosigmoiditis	7
Left-sided colitis	10
Pancolitis	5
Medication	
5-ASA	17
Glucocorticoids	3
Immunomodulators or biologics	2
Smoking status	
Smoking	6
Non-smoking	16
Clinical activity	
Mild	7
Moderate	9
Severe	6
Endoscopic Severity	
Grade I	6
Grade II	6
Grade III	7
Grade IV	3

ASA, aminosalicilyc acid; UC, ulcerative colitis.

3 (RIP3) (7). RIP3 has been identified as a key mediator of necroptosis (8). When the protease activity of caspase-8 is decreased, the necrosome that consists of RIP1 and RIP3 activates the pathway of necroptosis (9). Mixed lineage kinase domain-like protein (MLKL) is the main downstream component of RIP3 and RIP1 (10). Therefore, this study aimed to investigate the presence of RIP3 and MLKL in colonic mucosa in UC patients.

Methods

Patients and disease activity

Twenty-two patients with active UC and 19 healthy controls were enrolled into this study, which was conducted

from December 2012 to November 2014 (*Table 1*). The diagnosis of UC patients was based on clinical symptoms, endoscopic findings, and histological signs, according to the Lennard-Jones criteria. Disease location was categorized using the Montréal classification system (11) [proctitis (n=7), left-sided colitis (n=11), and pancolitis (n=4)]. UC disease activity was measured by three indices, in which clinical activity was measured by the Mayo score (12), and endoscopic activity was measured by the Modified Baron Score (13). These activity scores were determined on the same day RIP3 and MLKL were measured.

Materials

Colonic biopsy specimens from patients with active UC included inflamed mucosa and non-inflamed mucosa. Control specimens were obtained from healthy volunteers during endoscopy. Two adjacent biopsies were taken from one spot: one for histology, which was immediately fixed in 10% neutral buffered formaldehyde; and another for RNA or protein analysis, which was snap-frozen in liquid nitrogen for protein extractions.

Immunohistochemistry

Colonic specimens were fixed in formalin and embedded in paraffin. Paraffin-embedded tissue sections (2 μ m) were dewaxed in dimethylbenzene solution and rehydrated in graded alcohols. Then, 1.5% of H₂O₂ was used to suppress endogenous tissue peroxidase activity. Antigen retrieval was conducted by boiling slides in 10 mM of sodium citrate buffer. Subsequently, 3% of BSA was used to block non-specific binding sites for 1 h at room temperature. Slides were incubated with rabbit anti-human antibody (RIP3: ab152130, MLKL: ab32125; Abcam, Cambridge, UK) at 4 °C overnight. Biotinylated anti-IgGs (Maixin, Fuzhou, China) were used as a secondary reagent. Next, slides were incubated with peroxidase-conjugated avidin-biotin complex. DAB (brown) peroxidase substrates were used to develop stains, and hematoxylin was used as a counterstain.

Immunoblot analysis

Western blotting analysis was performed as previously described (14). Fresh colon tissue samples were grounded into powder in liquid nitrogen, and a total of 20 μ g of total proteins were fractionated by SDS-PAGE. Proteins

were transferred onto PVDF membranes, and membranes were blocked by 5% fat-free milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature. Anti-RIP3 (1:1,000; Abcam, Cambridge, UK), anti-MLKL (1:500, Sigma) and anti- β -actin (1:5,000; Sigma) antibodies were diluted in TBS-T and incubated overnight at 4 °C. Then, the membranes were washed with horseradish peroxidase-conjugated rabbit anti-mouse IgG, and developed with ECL Plus. β -actin was used as a loading control. Densitometric analysis of the blots was performed using ImageJ software. Average integrated optical density was obtained by analyzing 5 fields of view for each slide evaluated by Image-Pro Plus version 5.0.

Statistical analysis

All statistical analysis was conducted using SPSS 22.0. For comparison between two groups, Student's *t*-test was used for normally distributed variables and Mann-Whitney U-test was used for non-normally distributed variables. For the comparison of more than two groups, ANOVA test was used for normally distributed variables while Kruskal-Wallis test was used for non-normally distributed variables. Correlations among protein levels and disease activity were evaluated by Spearman's rank correlation coefficient (*r*) for non-parametric correlations. $P < 0.05$ was considered statistically significant.

Ethics

This study was approved by The Scientific Ethics Committee of Wuxi Third People's Hospital (No. 0228). All patients provided informed consent before participation, and this project abided the guidelines outline by the Declaration of Helsinki.

Results

Expression levels of RIP3 and MLKL were upregulated in inflamed tissues of UC patients

Immunohistochemical staining was applied to determine the localization of RIP3 and MLKL in biopsies obtained from 22 patients with active UC and 19 healthy volunteers (controls). Immunohistochemistry revealed that RIP3- and MLKL-positive staining was mainly in inflamed tissues. Semi-quantitative analysis was performed to examine the expression of RIP3 and MLKL. This revealed that

the expression of RIP3 and MLKL in IECs increased in inflamed tissues of UC patients, compared to non-inflamed tissues and controls (*Figure 1A,B*). Average optical density (AOD) of RIP3 and MLKL was significantly higher in inflamed tissues compared to non-inflamed tissues and control groups (RIP3: AOD 0.1580 ± 0.0639 vs. 0.1016 ± 0.0442 or 0.0979 ± 0.0425 , MLKL: AOD 0.2060 ± 0.0639 vs. 0.1310 ± 0.0570 or 0.1230 ± 0.0639 ; $P < 0.01$ respectively) (*Figure 1C*).

Consistent with the expression levels assessed by immunohistochemical analyses, immunoblot analyses revealed that RIP3 and MLKL protein levels were elevated in inflamed tissues of UC patients compared with non-inflamed tissues and controls (*Figure 1D*).

Correlation of RIP3 and MLKL with disease activity

The AODs of RIP3 and MLKL were higher in moderate and severe activity UC than in mild activity UC (RIP3: AOD 0.1742 ± 0.0486 vs. 0.1212 ± 0.0469 , 0.2166 ± 0.0519 vs. 0.1212 ± 0.0469 ; MLKL: AOD 0.2127 ± 0.0581 vs. 0.1528 ± 0.0405 , 0.2579 ± 0.0503 vs. 0.1528 ± 0.0405 , respectively; $P < 0.01$) (*Figure 2A*). The AODs of RIP3 and MLKL in grades III–IV were also higher than in grades I and II (RIP3: AOD 0.2152 ± 0.0424 vs. 0.1114 ± 0.0454 , 0.2152 ± 0.0424 vs. 0.1492 ± 0.0328 ; MLKL: AOD 0.2458 ± 0.0594 vs. 0.1589 ± 0.0448 , 0.2458 ± 0.0594 vs. 0.1867 ± 0.0505 ; respectively; $P < 0.05$) (*Figure 2B*). In addition, AODs of RIP3 and MLKL in UC-inflamed tissues were positively correlated with disease activity including clinical activity, colonoscopic activity, and histologic activity (*Table 2*), suggesting that RIP3 and MLKL levels are associated with UC disease activity. However, RIP3 and MLKL staining did not reveal any correlation with disease extent (*Table 2*).

Discussion

IECs form the first barrier between the inner environment and the lumen. In general, IEC death is restricted to the villous tip. Intestinal barrier function is disrupted when IEC death is increased and is diffusely distributed throughout the villous. Increased IEC death and intestinal inflammation represent two key features of UC. The major pathway of programmed cell death is apoptosis, which consists of the extrinsic pathway (triggered by the death receptor and ligand) and intrinsic pathway (controlled by the Bcl-2 family). Previous studies have shown that IECs were not sensitive to

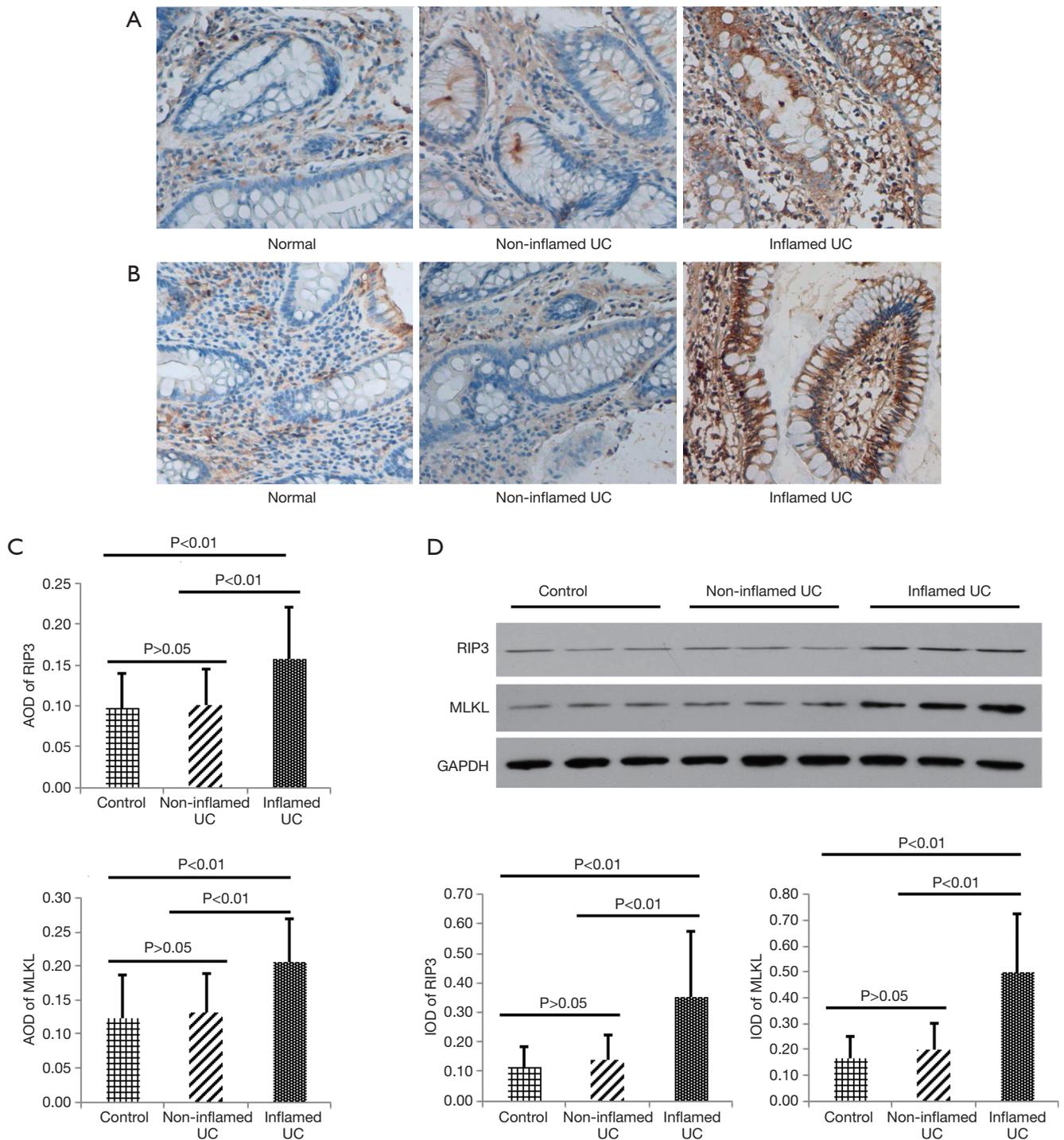


Figure 1 The expression of RIP3 and MLKL in intestinal epithelial cells increased in inflamed tissues of UC patients. (A) The expression, distribution and AOD of RIP3. (B) The expression, distribution and AOD of MLKL. Immunostaining of RIP3 and MLKL expression was robust in UC-inflamed colonic mucosa and low in UC-non-inflamed or normal colonic mucosa. The original magnifications were $\times 100$. (C) The AODs of RIP3 and MLKL were significantly higher in UC-inflamed colonic mucosa than in UC-non-inflamed or normal mucosa and the control group, $P < 0.01$. (D) Western blot analyses for RIP3 and MLKL. AOD, average optical density.

Table 2 Correlation of RIP3 and MLKL expression with disease activity indicators in UC

Clinical parameters	RIP3		MLKL	
	r	P	r	P
Mayo score	0.77	<0.01	0.75	<0.01
Re-corrected Baron score	0.81	<0.01	0.62	<0.01
Disease extent	0.04	>0.05	0.19	>0.05

RIP3, receptor interacting protein 3; MLKL, mixed lineage kinase domain-like protein; UC, ulcerative colitis.

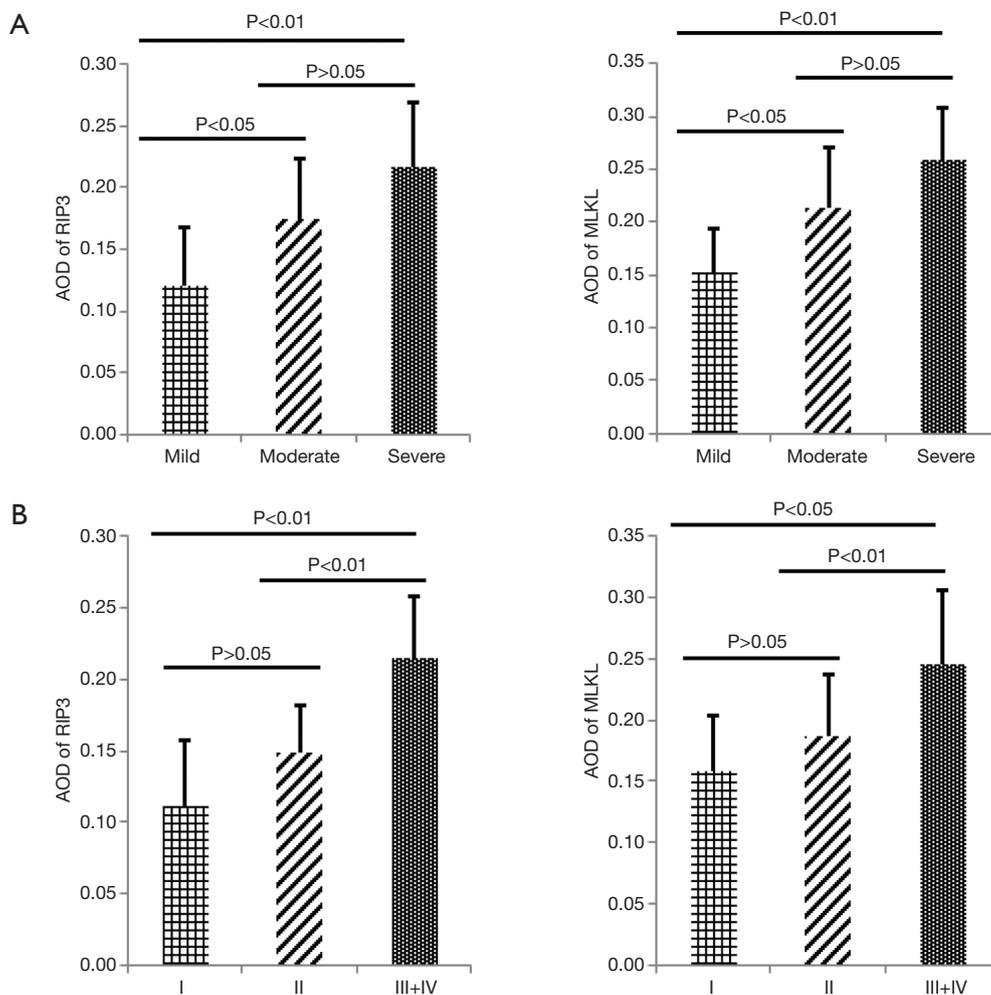


Figure 2 Expression levels of RIP3 and MLKL for clinical (12) and colonoscopic scores (13). (A) Clinical scores; (B) colonoscopic scores.

the extrinsic and intrinsic pathways (14-18). Therefore, it has been suggested that there may be another programmed death pathway involved in the dysfunction of IEC death.

Necroptosis, also called programmed necrosis, is similar to necrosis based on morphological features, but it is highly

regulated by a series of intracellular signaling pathways. Recent studies have demonstrated that necroptosis plays an important role in various physiological and pathological disorders such as COPD (19), ischemia-reperfusion injury (20), neurodegenerative diseases (6), etc. Recent

studies have suggested that necroptosis also participates in intestinal inflammation. Pierdomenico *et al.* (21) revealed that necroptosis was strongly associated with intestinal inflammation in children with inflammatory bowel disease (IBD) and contributed in heightening the inflammatory process. Günther *et al.* (22) found that necroptosis increased in the terminal ileum of patients with Crohn's disease. However, we wanted to investigate the expression of RIP3 and MLKL in UC patients and understand the relationship between necroptosis and disease activity.

Numerous studies reported that RIP3 and MLKL were key mediators in necroptosis through RIP3 interaction and MLKL phosphorylation—a process which promotes necroptosis (10,23). Our study revealed that RIP3 and MLKL protein levels in inflamed tissues of UC patients were higher than in non-inflamed tissues and healthy control groups. However, we failed to find a significant difference between non-inflamed tissues of UC patients and tissue samples of healthy controls, which is in accordance with the results of the study conducted by Pierdomenico *et al.* (21). Moreover, RIP3 and MLKL levels in inflamed colonic tissue specimens of UC were significantly and positively correlated with UC disease activity including clinical activity (Mayo scores) and endoscopic activity (Modified Baron scores), indicating that RIP3 and MLKL may have important effects on intestinal inflammation.

However, this study has some limitations. First, this study was conducted with a relatively small number of subjects, which makes our results less conclusive. Second, the levels of caspase-8 were not evaluated; thus, the relationship between caspase-8 and necroptosis proteins in the inflamed colon could not be confirmed. Finally, the contribution of RIP3 and MLKL to the intestinal inflammatory process was not determined.

Conclusions

In conclusion, we demonstrated that RIP3 and MLKL were upregulated in UC, and that it correlated positively with disease activity. Our results indicate that necroptosis may be involved in intestinal inflammation. However, the role of necroptosis in disease treatment and prognosis remains to be determined. Compared to other cell death pathways such as apoptosis and necrosis, the signal mechanism of the necroptosis pathway is not completely understood. Further studies are needed to clarify the role of necroptosis in the pathogenesis of UC, as well as its possible implications in treatment.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by The Scientific Ethics Committee of Wuxi Third People's Hospital (No. 0228) and written informed consent was obtained from all patients.

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