Long non-coding RNA Mirt2 interacts with long non-coding RNA IFNG-AS1 to regulate ulcerative colitis

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Abstract. Long non-coding RNAs (lncRNAs) Mirt2 and interferon-γ antisense RNA I (IFNG-AS1) play opposing roles in lipopolysaccharide (LPS)-induced inflammation, a key initiator of ulcerative colitis (UC). The present study aimed to analyze the potential interaction between Mirt2 and IFNG-AS1 in UC. Levels of IFNG-AS1 and Mirt2 in plasma samples from UC patients were measured using reverse transcription-quantitative PCR. Receiver operating characteristic curves were used to evaluate the diagnostic values of IFNG-AS1 and Mirt2 fr UC. The role of Mirt2 and IFNG-AS1 in colonic epithelial cell apoptosis was analyzed by cell apoptosis assay. In patients with UC, Mirt2 and IFNG-AS1 exhibited an inverse correlation, in which Mirt2 was downregulated while IFNG-AS1 was upregulated. Altered expression of IFNG-AS1 and Mirt2 separated patients with UC from healthy controls. In colonic epithelial cells, lipopolysaccharide treatment led to the downregulation of Mirt2 and the upregulation of IFNG-AS1. Furthermore, overexpression of Mirt2 in colonic epithelial cells resulted in downregulation of IFNG-AS1, and vice versa. Overexpression of Mirt2 led to a decreased rate of colonic epithelial cell apoptosis, while overexpression of IFNG-AS1 led to an increased rate of apoptosis. Moreover, IFNG-AS1 overexpression attenuated the effects of Mirt2 overexpression. Therefore, Mirt2 may interact with IFNG-AS1 during UC to participate in colonic epithelial cell apoptosis.

Introduction

Ulcerative colitis (UC) is a form of chronic inflammatory bowel disease that affects the colon and large intestine, causing swelling and irritation (1). UC is typically diagnosed during young adulthood and affects an individual for their entire life (2). The incidence rate of UC varies across the world, with high incidence rates observed in North America and Western Europe (3). With the development of a prevention program, the prevalence of UC has been stabilized in areas that were typically associated with high incidence (3). However, an increasing trend in UC incidence has been observed in Eastern Europe and Asia in the last 10 years (3). It is well established that UC not only reduces the quality of life of patients, but also increases the risk of colorectal cancer (4,5).

As UC is a type of inflammatory disease (6), the inhibition of inflammation is a promising approach for UC treatment (7). Inflammatory factors, including interleukin (IL)-36α, IL-17 and IL-23, are critical mediators of UC (8,9). In addition to these cytokines, long non-coding RNAs (lncRNAs; >200 nucleotides in length) also play pivotal roles in inflammation, and the regulation of the expression of certain lncRNAs can contribute to the amelioration of UC symptoms (10). In a previous study, Padua *et al* (11) reported a novel lncRNA, interferon-γ antisense RNA I (IFNG-AS1), as an enhancer of inflammation in UC. In another study, lncRNA Mirt2 was characterized as a lipopolysaccharide (LPS)-inducible inflammation inhibitor (12). It is known that LPS-induced inflammation and UC share a similar pathogenesis (13), suggesting that the opposite roles of IFNG-AS1 and Mirt2 indicate a possible interaction between the two lncRNAs during UC. Therefore, the present study aimed to investigate the interactions between IFNG-AS1 and Mirt2 in UC.

Materials and methods

Participants and plasma samples. The present study included 60 patients with UC (34 males and 26 females; age range, 19-64 years; mean ± SD, 40.1±6.2 years) and 60 healthy controls (34 males and 26 females; age range, 20-65 years; mean ± SD, 39.8±6.5 years). All the participants were recruited at the Department of Gastroenterology, Hainan General Hospital between April 2016 and April 2019. The 60 healthy volunteers were recruited from the Physical Health Center of Hainan General Hospital to match the age and gender distributions of the patients with UC. The present study was approved by the Ethics Committee of Hainan General Hospital. All participants were informed of the details of the protocols of the present study and provided written informed consent. The inclusion criteria for patients with UC were as follows: i) No

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treatment received within 100 days before admission; and ii) newly diagnosed cases. The exclusion criteria for patients with UC were as follows: i) Presence of other clinical disorders; and ii) patients transferred from other hospitals.

Before the initiation of any therapies, ~5 ml blood was extracted from the median cubital vein under fasting conditions. To separate the plasma, blood was centrifuged in EDTA tubes for 20 min at 1,200 x g at room temperature.

Human colonic epithelial cells (HCnEpCs) and transfections. HCnEpCs from Cell Applications were used in the present study. The rationale behind using HCnEpCs was that HCnEpCs are usually affected by UC (6). Cell culture was performed according to the manufacturer's instructions. HCnEpCs were cultured in colonic epithelial cell medium (ScienCell Research Laboratories, Inc.) at 37˚C with 5% CO_2 containing 5 μg/ml LPS (Sigma-Aldrich; Merck KGaA) for 12, 24, 36 and 48 h at 37˚C with 5% CO_2 prior to further experimentation.

Mirt2 and IFNG-AS1 overexpression vectors were constructed using the pcDNA3.1 vector (Sangon BioTech Co., Ltd.). HCnEpCs were harvested and counted, and $4x10^6$ cells were transfected with 10 nM vector using Lipofectamine® 2000 (Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions. Empty vectors were used for the negative control group. All transfections were performed using un-transfected HCnEpCs as control cells. Subsequent experimentation was performed after cells were incubated at 37˚C for 24 h.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). The total RNA in 0.2 ml plasma and $4x10^5$ HCnEpCs (collected at 24 h post-transfection) was extracted using RiboZol (Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions. To remove genomic DNA, all RNA samples were digested with DNase I for 90 min at 37˚C. The digested RNA samples were reverse transcribed into cDNA using a Tetro Reverse Transcriptase kit (Bioline) at the following thermal conditions: 55˚C for 20 min and 80˚C for 10 min. qPCR was performed using Power SYBR® Green PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. GAPDH was used as an endogenous control to measure the expression levels of Mirt2 and IFNG-AS1. The following thermocycling conditions were used for qPCR: 95˚C for 1 min, then 95˚C for 10 sec and 55˚C for 40 sec for a total of 40 cycles. The following primer sequences were used: Mirt2 forward, 5'-TCA ACACTTTCCATAGGT-3' and reverse, 5'-ATTGTGAGG TCCAGATAG-3'; IFNG-AS1 forward, 5'-GCTGATGATGGT GGTGGCAATCT-3' and reverse, 5'-TTAGCAGTTGGTGGG CTTCT-3'; and GAPDH forward, 5'-GTCTCCTCTGACTTC AACAGCG-3' and reverse, 5'-ACCACCCTGTTGCTGTAG CCAA-3'. All qPCR reactions were performed in triplicate. mRNA levels were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to the loading control GAPDH (14).

Cell apoptosis analysis. HCnEpCs were harvested and counted at 24 h post-transfection. Single-cell suspensions were prepared by mixing $4x10^3$ HCnEpCs with 1 ml colonic epithelial cell medium. Cells were transferred to a 6-well cell culture plate (2 ml per well) and were supplemented with 5 μg/ml LPS per well to induce cell apoptosis. Cells were incubated at 37˚C for 48 h. After incubation, cells were harvested and washed with PBS. Subsequently, a fluorescein isothiocyanate-labeled Annexin V and propidium iodide kit (cat. no V13242; Thermo Fisher Scientific, Inc.) was used to stain cells in the dark at 4˚C for 20 min, according to the manufacturer's instructions). Early apoptosis was analyzed using a flow cytometer. Data were analyzed using Invitrogen Attune NxT flow cytometry software (version 3.1, Thermo Fisher Scientific, Inc.).

Statistical analysis. The mean ± SD values of data derived from three biological replicates of each experiment were calculated. All data analysis was performed using mean values. Correlation analysis was conducted using the Pearson's correlation test. Differences between two groups of participants and among different cell groups were compared by the unpaired Student's t-test and one-way ANOVA followed by Tukey's post hoc test, respectively. Receiver operating characteristic (ROC) curve analysis was used for diagnostic analysis. All statistical analyses were performed using GraphPad Prism software (version 6.0; GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Mirt2 and IFNG-AS1 are inversely correlated in plasma from patients with UC. RT-qPCR and the Student's t-test were used to measure and compare levels of Mirt2 and IFNG-AS1 in plasma derived from patients with UC and healthy controls. Compared with the control group, significantly lower levels of Mirt2 and significantly higher levels of IFNG-AS1 were observed in plasma derived from patients with UC ($P<0.05$; Fig. 1A and B). The relationship between Mirt2 and IFNG-AS1 was analyzed by the Pearson's correlation test. Plasma levels of Mirt2 were inversely and significantly correlated with plasma levels of IFNG-AS1 in patients with UC ($P<0.05$; $R^2=0.7179$; Fig. 1C). However, this correlation was not significant in the control group ($P>0.05$; $R^2=0.004$; Fig. 1D).

Altered plasma levels of Mirt2 and IFNG-AS1 exhibit diagnostic values for UC. ROC curve analysis was performed to evaluate the diagnostic potential of measuring plasma Mirt2 and IFNG-AS1 levels in UC. Patients with UC were always the true positive cases and healthy volunteers were always the true negative cases. An area under the curve (AUC) >0.65 indicated diagnostic value. For plasma Mirt2, the AUC was 0.86 (95% confidence interval, 0.80-0.93; standard error, 0.032; Fig. 2A). For plasma IFNG-AS1, the AUC was 0.84 (95% confidence interval, 0.78-0.91; standard error, 0.035; Fig. 2B).

LPS treatment leads to altered expression of Mirt2 and IFNG-AS1 in HCnEpCs. HCnEpCs were cultured in cell culture medium containing 5 μg/ml LPS for 12, 24, 36 and 48 h, prior to the measurement of Mirt2 and IFNG-AS1 expression levels. LPS treatment led to the downregulation of Mirt2 in a time-dependent manner ($P<0.05$; Fig. 3A). Moreover, LPS treatment led to the upregulation of Mirt2 in a time-dependent manner ($P<0.05$; Fig. 3B).

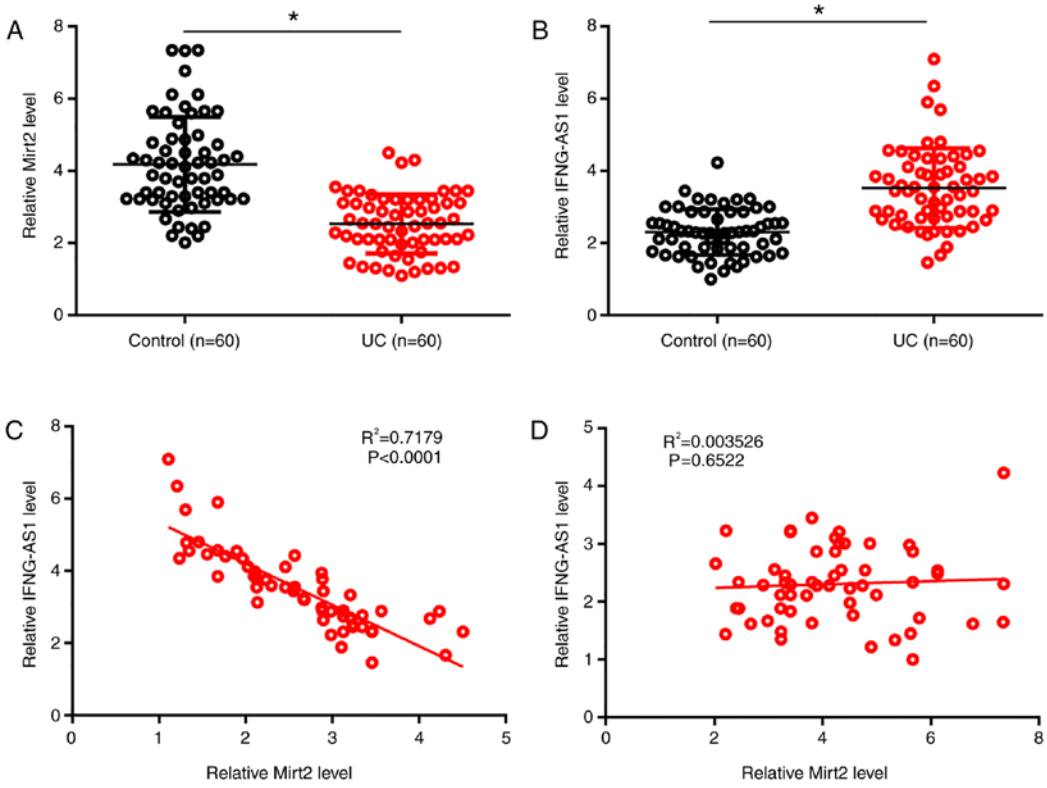

Figure 1. Mirt2 and IFNG-AS1 were inversely correlated in plasma from patients with UC. Reverse transcription-quantitative PCR and the Student's t-test were used to measure and compare expression levels of (A) Mirt2 and (B) IFNG-AS1 in plasma derived from patients with UC and healthy controls. Correlations between Mirt2 and IFNG-AS1 in (C) patients with UC and (D) healthy controls were analyzed by the Pearson's correlation test. Experiments were performed in triplicate and mean values are presented. *P<0.05, as indicated. IFNG-AS1, interferon-γ antisense RNA I; UC, ulcerative colitis.

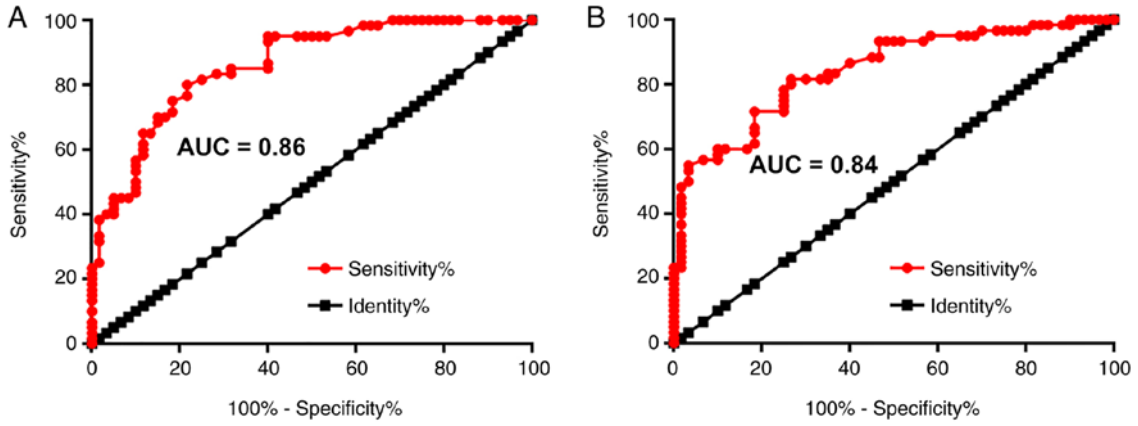

Figure 2. Altered plasma levels of Mirt2 and IFNG-AS1 exhibited diagnostic value in UC. Receiver operating characteristic curve analysis was performed to evaluate the potential of plasma (A) Mirt2 and (B) IFNG-AS1 in the diagnosis of UC. Patients with UC were true positive cases and healthy controls were true negative cases. IFNG-AS1, interferon-γ antisense RNA I; UC, ulcerative colitis.

Mirt2 and IFNG-AS1 downregulate each other in HCnEpCs. Mirt2 and IFNG-AS1 expression vectors were transfected into HCnEpCs. At 24 h post-transfection, the expression levels of Mirt2 and IFNG-AS1 were measured by RT-qPCR and compared by one-way ANOVA followed by Tukey's post hoc test. Compared with the control and negative control groups, the expression levels of Mirt2 and IFNG-AS1 were significantly upregulated following transfection with the overexpression vectors (P<0.05; Fig. 4A). Moreover, overexpression of Mirt2 and IFNG-AS1 resulted in downregulated expression levels of IFNG-AS1 and Mirt2, respectively (P<0.05; Fig. 4B). However, co-transfection of the expression vector of IFNG-AS1 and Mirt2 failed to significantly alter the expression levels of IFNG-AS1 and Mirt2 (P>0.05; Fig. 4C).

Interaction between Mirt2 and IFNG-AS1 affects the apoptosis of HCnEpCs. The effect of Mirt2 and IFNG-AS1 overexpression on the late apoptosis of HCnEpCs was analyzed by a cell apoptosis assay. Compared with the control and negative control groups, overexpression of Mirt2 led to

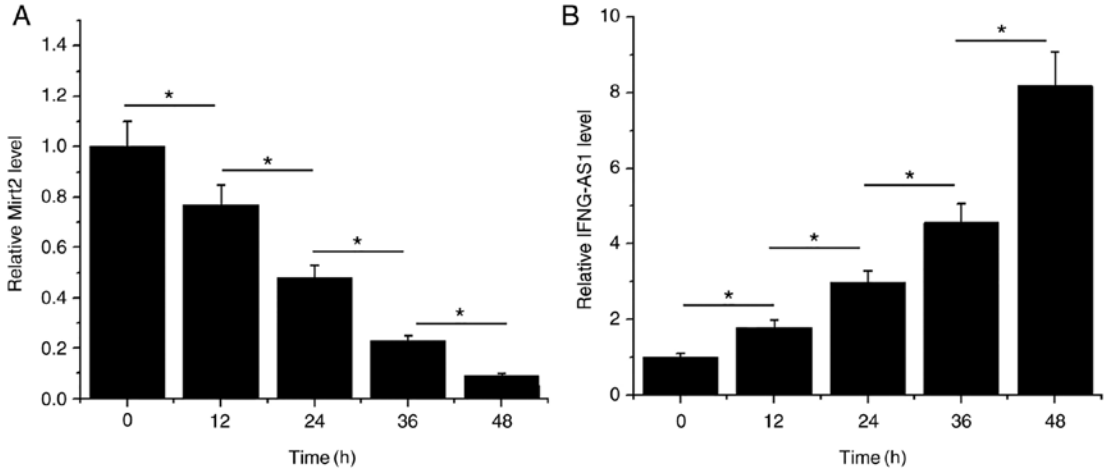

Figure 3. LPS treatment alters the expression levels of Mirt2 and IFNG-AS1 in HCnEpCs. HCnEpCs were incubated in colonic epithelial cell medium containing 5 μg/ml LPS for 12, 24, 36 and 48 h, followed by the measurement of (A) Mirt2 and (B) IFNG-AS1 expression levels. Experiments were performed in triplicate and mean values are presented. *P<0.05, as indicated. LPS, lipopolysaccharide; IFNG-AS1, interferon-γ antisense RNA I; HCnEPC, human colonic epithelial cells.

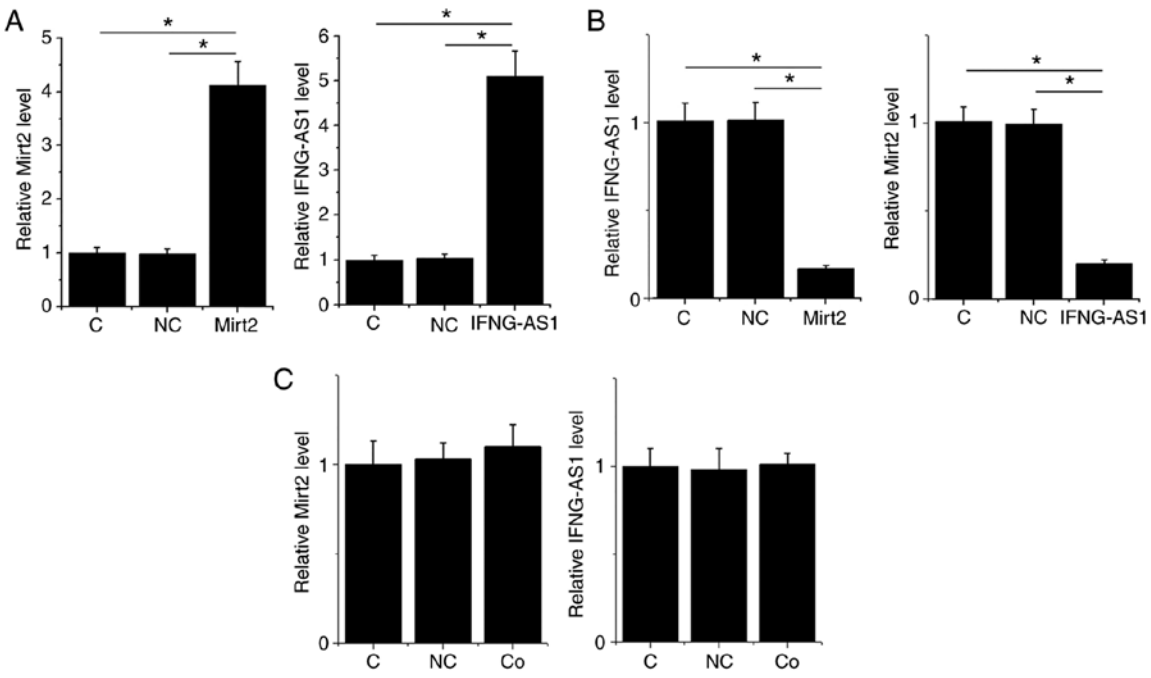

Figure 4. Mirt2 and IFNG-AS1 downregulate one another in HCnEpCs. Mirt2 and IFNG-AS1 expression vectors were transfected into HCnEpCs. At 24 h post-transfection, the expression levels of Mirt2 and IFNG-AS1 were measured by reverse transcription-quantitative PCR. Expression levels were compared by one-way ANOVA followed by Tukey's post hoc test. (A) Expression levels of Mirt2 and IFNG-AS1. (B) The effect of Mirt2 and IFNG-AS1 interaction on the expression levels of Mirt2 and IFNG-AS1. (C) The effects of co-transfection with the IFNG-AS1 and Mirt2 overexpression vectors on the expression levels of IFNG-AS1 and Mirt2. Experiments were performed in triplicate and mean values are presented. *P<0.05, as indicated. IFNG-AS1, interferon-γ antisense RNA I; HCnEpC, human colonic epithelial cells; C, control; NC, negative control; Co, co-transfection.

a decreased rate of apoptosis in the colonic epithelial cells, while IFNG-AS1 overexpression led to an increased rate of apoptosis (P<0.05; Fig. 5). Moreover, IFNG-AS1 overexpression attenuated the effects of Mirt2 overexpression (P<0.05; Fig. 5).

Discussion

The present study investigated the interactions between two inflammatory-related lncRNAs in UC (11,12). Mirt2 and IFNG-AS1 were dysregulated in UC and were inversely correlated. Furthermore, Mirt2 and IFNG-AS1 may form a negative regulation feedback loop to regulate the apoptosis of HCnEpCs.

A previous study reported that the expression level of IFNG-AS1 was increased by 5.27 times in UC (11). The study reported that in CD4 T cells, IFNG-AS1 regulated the key inflammatory cytokine, interferon-γ, to promote inflammation in patients with UC (11). Consistently, the present study also observed upregulation of IFNG-AS1 in the plasma from

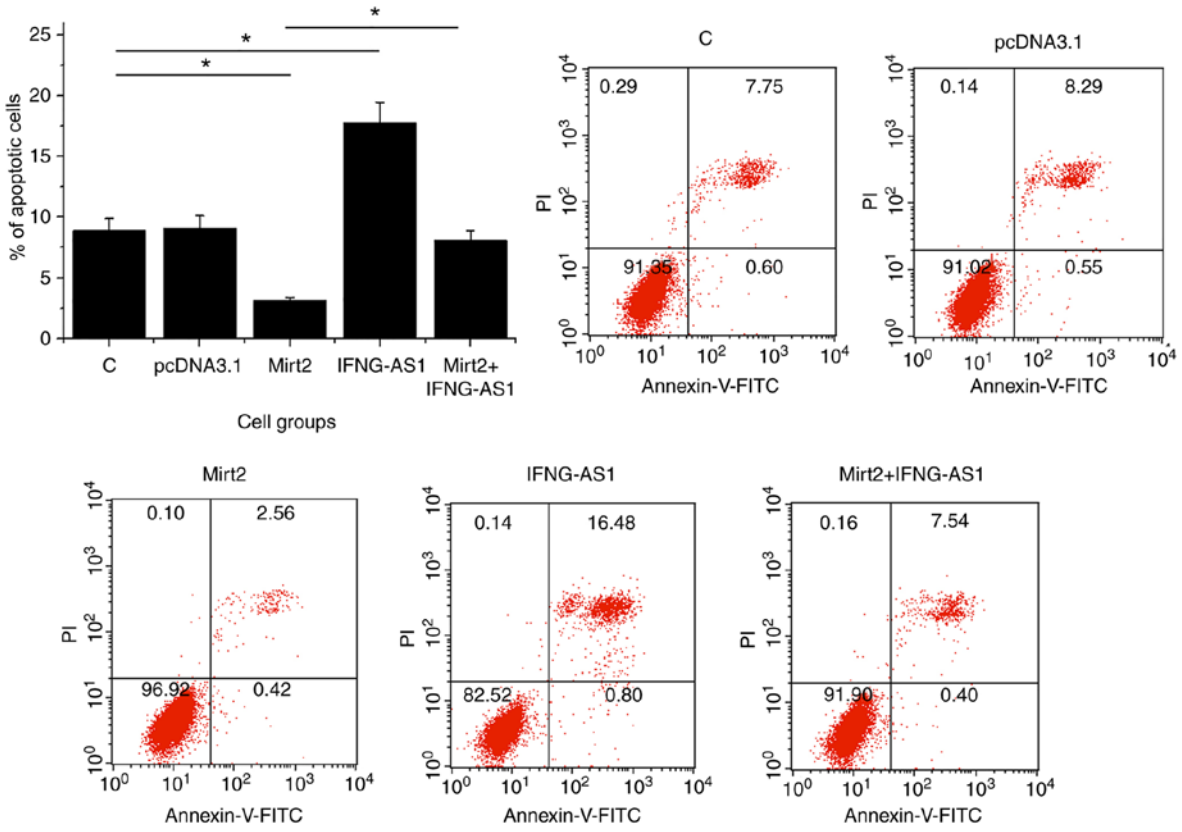

Figure 5. Interaction between Mirt2 and IFNG-AS1 alters the rate of apoptosis of HCnEpCs. The effect of Mirt2 and IFNG-AS1 overexpression on the apoptosis of HCnEpCs was analyzed by a cell apoptosis assay. Experiments were performed in triplicate and the mean values are presented. *P<0.05, as indicated. HCnEpC, human colonic epithelial cells. IFNG-AS1, interferon-γ antisense RNA I; C, control; PI, propidium iodide; FITC, fluorescein isothiocyanate.

patients with UC and in HCnEpCs treated with LPS. In another study, Mirt2 was reported to interact with toll-like receptor 4 to negatively regulate inflammation (12). In the present study, Mirt2 was downregulated in plasma from patients with UC and in HCnEpCs treated with LPS. Collectively, these results suggested that IFNG-AS1 and Mirt2 may be involved in UC.

Cell apoptosis, including the apoptosis of HCnEpCs, contributes to the progression of UC (15,16). Therefore, suppression of HCnEpC apoptosis is a potential therapeutic approach for UC (15,16). lncRNAs have been characterized as key players in the regulation of cell behavior (17). In cellular processes, lncRNAs regulate the expression of downstream genes or sponge miRNAs (18,19). To the best of our knowledge, the interactions between different lncRNAs have not been well characterized in general. In the present study, the results suggested that IFNG-AS1 and Mirt2 may negatively regulate the expression of one another to regulate the apoptosis of HCnEpCs. However, the mechanism that mediates the interaction between the two lncRNAs requires further investigation. Based on the findings obtained in the present study, it could be speculated that the interaction between IFNG-AS1 and Mirt2 is mediated by certain pathological mediators. This speculation is supported by the observation that IFNG-AS1 and Mirt2 were only inversely correlated in patients with UC, but not in healthy controls. Further investigation is required to identify the possible mediators between IFNG-AS1 and Mirt2.

To the best of our knowledge, the present study is the first to report the involvement of Mirt2 in UC and to analyze the interaction of two lncRNAs in UC. However, the present study did not include a detailed analysis of the mechanism of the roles of Mirt2 and IFNG-AS1 in UC, therefore further investigation is required. Additionally, the present study did not analyze the effects of Mirt2 on inflammatory and apoptotic factors in HCnEpCs. However, it is known that LPS induces multiple inflammatory and apoptotic pathways in HCnEpCs, such as the mitogen-activated protein kinase signaling pathway (20). Mirt2 is an LPS-inducible inhibitor of inflammation (12), therefore, it is reasonable to speculate that Mirt2 may also interact with inflammatory pathways, such as the mitogen-activated protein kinase, NF-κB and JAK-STAT pathways, in HCnEpCs.

In conclusion, IFNG-AS1 was upregulated in UC, while Mirt2 was downregulated. IFNG-AS1 and Mirt2 may negatively effect the expression of one another and participate in the pathogenesis of UC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CL performed the clinical studies, experimental work, data analysis and wrote the manuscript. LC, SL and ML performed the experiments and carried out literature research. XM conceived and designed the study and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Hainan General Hospital. Written informed consent was provided by all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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