ORIGINAL ARTICLE

H19 is involved in the regulation of inflammatory responses in acute gouty arthritis by targeting miR-22-3p

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Abstract

A great number of studies have confirmed that long noncoding RNA (lncRNA) are involved in the regulation of inflammatory response in acute gouty arthritis (AGA). This paper aimed to survey the regulatory mechanism of H19 on AGA. The expression of serum H19 in all subjects was examined by qRT-PCR. The ROC curve was used to estimate the diagnostic value of H19 for AGA. THP-1 cells were induced by MSU to establish in vitro AGA cell model. The concentrations of cytokines such as IL-1β, IL-8, and TNF-α were tested by ELISA. Luciferase reporter gene analysis was used to verify the interaction between H19 and the 3′-UTR of miR-22-3p. Expressions of serum H19 in AGA patients were significantly higher than that in controls. The ROC curve indicated the potential of H19 as a diagnostic marker for AGA. Cell experiments revealed that the downregulation of H19 significantly inhibited the expressions of IL-1β, IL-8, and TNF-α. The luciferase reporter gene assay manifested that miR-22-3p is the target gene of H19. And knockdown of miR-22-3p overturned the downregulation of inflammatory factors caused by H19 inhibition. H19 aggravated MSU-induced THP-1 inflammation by negatively targeting miR-22-3p, suggesting a new regulatory mechanism and potential therapeutic target for AGA.

Keywords H19 · miR-22-3p · Acute gouty arthritis · Inflammatory response

Introduction

Gout, a chronic metabolic disorder syndrome caused by purine metabolic disorder, is mainly divided into two categories, primary gout, and secondary gout [1]. Clinically, patients with primary gout are more common. Acute gouty arthritis (AGA), the most common initial clinical manifestation of primary gout, was obviously a kind of progressive inflammatory response [2]. The accumulation of uric acid in the blood leads to supersaturation of monosodium urate (MSU) crystals in the periosteum or articular cartilage and other bone tissues, and then excessive MSU activated macrophages, which engulf MSU crystals and release chemical attractants such as leukotriene and interleukin, promoting neutrophil aggregation and triggering inflammatory cascade reactions [3, 4]. The pathogenesis of AGA is related to the immune dysfunction and activation of multiple

inflammatory signaling pathways [5]. In recent years, the exploration of AGA from the perspective of genetics has gradually increased. This study shows that there are significant differences in the expression profiles of long noncoding RNA (lncRNA) in serum between AGA patients and healthy people [6].

LncRNA is an important part of gene transcriptome, with a length of more than 200nt, which does not have the function of protein coding [7]. It is widely expressed in dendritic cells, macrophages, and NK cells, participates in the differentiation of immune cells, and regulates the stability of messenger RNA and microRNA by activating or inhibiting transcription factors, thus participating in various diseases [8]. H19, located on human chromosome 11, was originally found to play a role as an oncogene in cancer regulation [9]. Recently, several studies reported the relationship between H19 and inflammation. In ischemic stroke, H19 accelerates neuroinflammation by promoting polarization of M1 microglial [10]. Li et al. reported that H19 promoted the inflammatory reaction and nerve injury in rats with cerebral ischemia–reperfusion injury by negatively regulating miR-138-5p [11]. H19 has also previously been explored to act as a regulator of inflammatory response in arthritis. One

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study reported that H19 exacerbates inflammatory damage in osteoarthritis (OA) by acting as a molecular sponge for miR-130a [12]. Another study indicated that H19 activates NF-κB and JNK/p38 MAPK pathways by promoting TAK1 phosphorylation in the rheumatoid arthritis (RA) cell model, thus aggravating the level of inflammatory factors [13]. In response to this, the efficacy of H19 in inflammatory diseases such as arthritis has been established, but its expression and mechanism in gout arthritis are still unclear.

In this study, we intended to inspect the association between H19 and inflammatory responses in AGA, as well as the regulatory mechanism of H19 in AGA. Interestingly, it was found that H19 knockdown effectively alleviates the inflammatory response induced by MSU, and this effect may be achieved through negative regulation of miR-22-3p.

Materials and methods

Study population and sample collection

This research approach employed 90 male patients who presented to this hospital with sudden onset of severe joint pain at night and were recruited from July 2016 to May 2020. The inclusion criteria of AGA follow the diagnostic criteria of AGA established by the American Rheumatology Association in 1977 [14]. In addition, 86 healthy male subjects were recruited as the control group. The exclusion criteria for volunteers in this study are as follows: (1) the non-acute phase of gouty arthritis; (2) recently or currently taking hormone medication; (3) patients with tumor, bone tuberculosis, and osteomyelitis; and (4) patients with hematopathy, nephropathy, and autoimmune diseases. This study obeys the ethical principles in the Declaration of Helsinki and has been authorized by the ethics committee of First People's Hospital of Lianyungang. All subjects recruited in this protocol have signed written informed consent. After obtaining informed consent, all subjects included in the study were collected and summarized general clinical data, including age and body mass index (BMI). The peripheral venous blood was collected in anticoagulation tubes, and the erythrocyte sedimentation rate (ESR), serum uric acid (SUA), leukocyte count, neutrophils count, and lymphocyte count were detected. After the blood was centrifuged, the upper serum was gathered and stored for later use.

Cell culture and treatment

Human peripheral blood mononuclear cell line THP-1 cells were gained from SIBCB. Culture conditions of THP-1 cells: RPMI 1640 medium contains 10% FBS and 1% penicillin/streptomycin and is incubated at 37 ℃ in an incubator containing 5% CO_2. THP-1 cells were treated with 100-ng/ mL PMA for 48 h to differentiate into THP-1–derived macrophages. Induction concentrations of MSU were selected in accordance with previously published methods [15]. After PMA treatment, the culture medium was removed, and 250-μg/L MSU crystal was added to stimulate the cells for 6 h to induce inflammation.

Cell transfection

The levels of H19 and miR-22-3p in THP-1 cells were regulated by in vitro cell transfection. First, THP-1 cells were inoculated in a 12-well plate and treated with 100-ng/mL PMA for 48 h. After PMA treatment, according to the product instructions, small interfering RNA-negative control (si-NC), si-H19, miR-22-3p mimic, miR-NC, and miR-22-3p inhibitor were, respectively, mixed with lipofectamine 3000 (Invitrogen) and then added dropwise into the above cells for transfection for 48 h. Finally, the cells were stimulated with 250-μg/mL MSU crystal for 6 h.

RNA extraction and qRT-PCR

Total RNA was isolated from serum by the TRIzol and then reversely transcribed into cDNA using the iScript™ cDNA Synthesis Kit. miScript SYBR® Green PCR kit (Qiagen GmbH, Germany) and SYBR green I Master Mix kit (Invitrogen) were used for amplification on real-time fluorescence quantitative PCR system (Applied Biosystems, Foster City, CA, USA). GAPDH and U6 were used as internal reference, and the relative expression of H19 and miR-22-3p was calculated by the $2^{-\Delta\Delta Ct}$ method.

Enzyme-linked immunosorbent assay (ELISA)

After the cells were treated according to the prescribed experimental steps, the supernatant of cells was extracted, and the concentration of inflammatory factors such as IL-1β, IL-8, and TNF-α was detected by ELISA kit. All experimental procedures were carried out in accordance with the instructions of the ELISA kit.

Luciferase reporter gene assay

Target gene prediction using StarBase V2.0 online program revealed that miR-22-3p had binding sites with the 3′-UTR region of H19 gene transcript, which was subsequently verified by a dual luciferase reporter gene assay. Firstly, the 3′-UTR fragments of H19 were cloned into the pmirGLO vector, and then the wild-type reporter vector (WT-PVT1) and mutant-type reporter vector (MUT-PVT1) were constructed. Secondly, according to the product specification, THP-1 cells were co-transfected with the above reporter vectors and miR/inhibitor-NC and miR-22-3p mimic/inhibitor,

respectively, for 48 h using lipofectamine 3000. Finally, cells were collected, and the luciferase activity of each group was measured by dual luciferase assay system (Promega, Madison, WI, USA).

Data analysis

GraphPad Prism software version 7.0 and SPSS software version 21.0 were used for statistical analysis. All data are expressed as mean ± SD. ROC curve was used to evaluate the diagnostic value of H19 in patients with AGA. Pearson correlation coefficient analyzes the correlation between continuous variables. Students t test and one-way ANOVA were used for intergroup and multigroup comparisons, respectively. Chi-square test was used for classification variables comparison. $P < 0.05$ is statistically significant. Each experiment was repeated at least three times in parallel.

Results

Comparison of clinical data of subjects

General information and biochemical indexes of subjects are shown in Table 1. There was no significant difference in age, BMI, erythrocyte sedimentation rate (ESR), and lymphocyte count between the two groups ($P > 0.05$), but the level of serum uric acid (SUA), leukocyte count, neutrophil count, and VAS pain score in the AGA group was significantly enhanced in comparison to the control group ($P < 0.001$, Table 1).

Table 1 Comparison of clinical indicators between healthy individuals and AGA patients

Characteristics	Subjects		P value
	Controls ($n=86$)	AGA ($n=90$)	
Age (years)	42.19 ± 7.96	43.57 ± 7.83	0.248
BMI (kg/m²)	23.85 ± 2.70	24.41 ± 2.49	0.157
ESR (mm/h)	4.69 ± 2.52	5.12 ± 2.67	0.272
SUA (umol/L)	204.16 ± 54.77	421.36 ± 42.83	< 0.001
VAS (umol/L)	-	6.35 ± 1.26	-
Leukocyte count (10⁹/L)	5.88 ± 1.10	19.06 ± 9.45	< 0.001
Neutrophils count (10⁹/L)	3.54 ± 0.95	19.42 ± 7.88	< 0.001
Lymphocytes count (10⁹/L)	2.10 ± 0.59	2.29 ± 1.11	0.154

Note: AGA, acute gouty arthritis; BMI, body mass index; SUA, serum uric acid; ESR, erythrocyte sedimentation rate; VSA, visual analog scale. Mean ± standard deviation

Serum H19 expression levels in AGA patients

To find out whether there is a difference between serum H19 expression levels of AGA and control group, serum H19 was tested by qRT-PCR. The results showed that the level of serum H19 in AGA patients was significantly higher than that in controls ($P < 0.001$, Fig. 1), which indicated that H19 might play an important role in the development of AGA.

Diagnostic value of serum H19 for AGA

As can be seen from Fig. 2, the AUC value of this curve is 0.938, and the sensitivity and specificity are 90.00% and
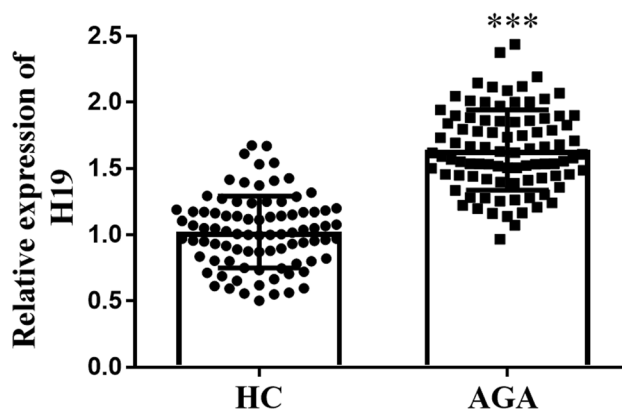

Fig. 1 The relative expression level of H19 in AGA group was increased compared with control group ($^{***}P < 0.001$)
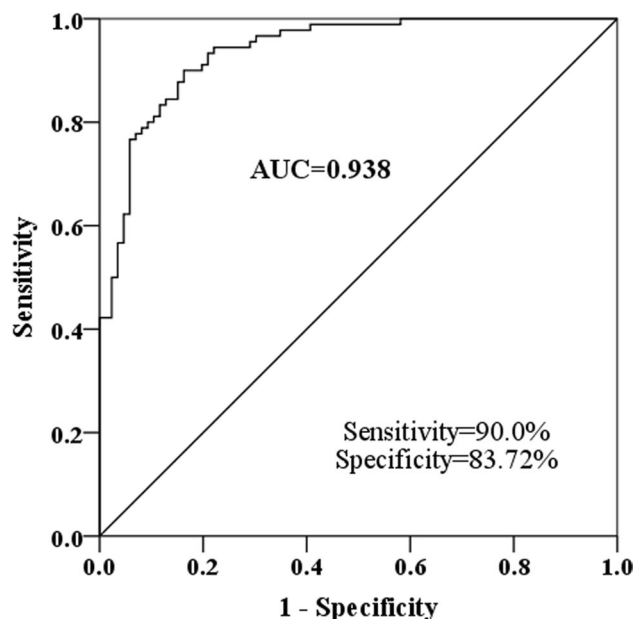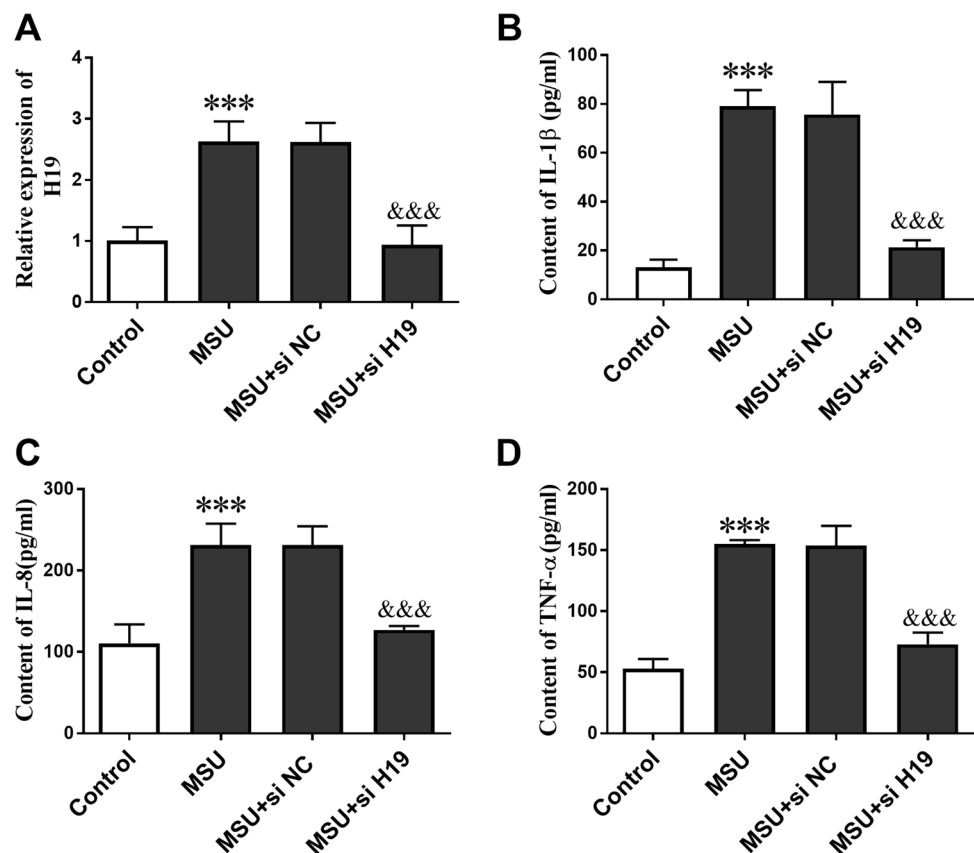

Fig. 2 The ROC curve analysis of H19, AUC was 0.938, the sensitivity was 90.00%, and the specificity was 83.72%

83.72%, respectively, indicating that H19 has the potential as a diagnostic marker for AGA in clinical practice, that is, H19 showed clinical diagnostic value for AGA.

Effects of H19 on inflammatory response in MSU-treated THP-1 cells

In this study, MSU stimuli were used for establishing the in vitro gouty inflammatory cell model. The results showed that the level of H19 after MSU stimulation was significantly upregulated compared with the control group, and this phenomenon can be neutralized by the knockout of H19 (Fig. 3A, $P < 0.001$). Additionally, the relationship between H19 and inflammation was confirmed by cell transfection technique and ELISA. It was observed that the concentration of inflammatory factors increased significantly after MUS stimulation, indicating that the inflammation model was successfully established. However, the expression of IL-1β, IL-8, and TNF-α was significantly reduced after transfection with si-H19. These results suggested that H19 overexpression promoted inflammatory response, while inhibition of H19 suppressed the production of inflammatory factors (Fig. 3B–D, $P < 0.001$).

Validation of downstream target genes of H19

StarBase V2.0 predicted that the miR-22-3p was the target of H19, and the putative binding sites of them are shown in Fig. 4A. Based on luciferase reporter gene analysis, mir-22-3p mimic or miR-22-3p inhibitor could significantly weaken or enhance the luciferase activity of WT-H19. However, neither miR-22-3p mimic nor inhibitor affected MUT-H19 luciferase activity (Fig. 4B, $P < 0.001$). In addition, the results from clinical serum samples showed that miR-22-3p was decreased in the AGA group (Fig. 4C, $P < 0.001$). In view of the above results, we analyzed the relationship between the level of H19 and miR-22-3p in serum of AGA patients. The results showed that there was a negative correlation between H19 and miR-22-3p ($r = -0.7319$, $P < 0.0001$, Fig. 4D). And finally, miR-22-3p was effectively reduced in MSU-induced THP-1 cells, whereas transfection of si-H19 strikingly enriched the miR-22-3p expression (Fig. 4E, $P < 0.001$).

Effects of miR-22-3p on inflammatory response in MSU-induced THP-1 cells

The cell transfection method was conducted to regulate the level of miR-22-3p in MSU-induced cells and to verify the effect of miR-22-3p on cell inflammation. Figure 5A
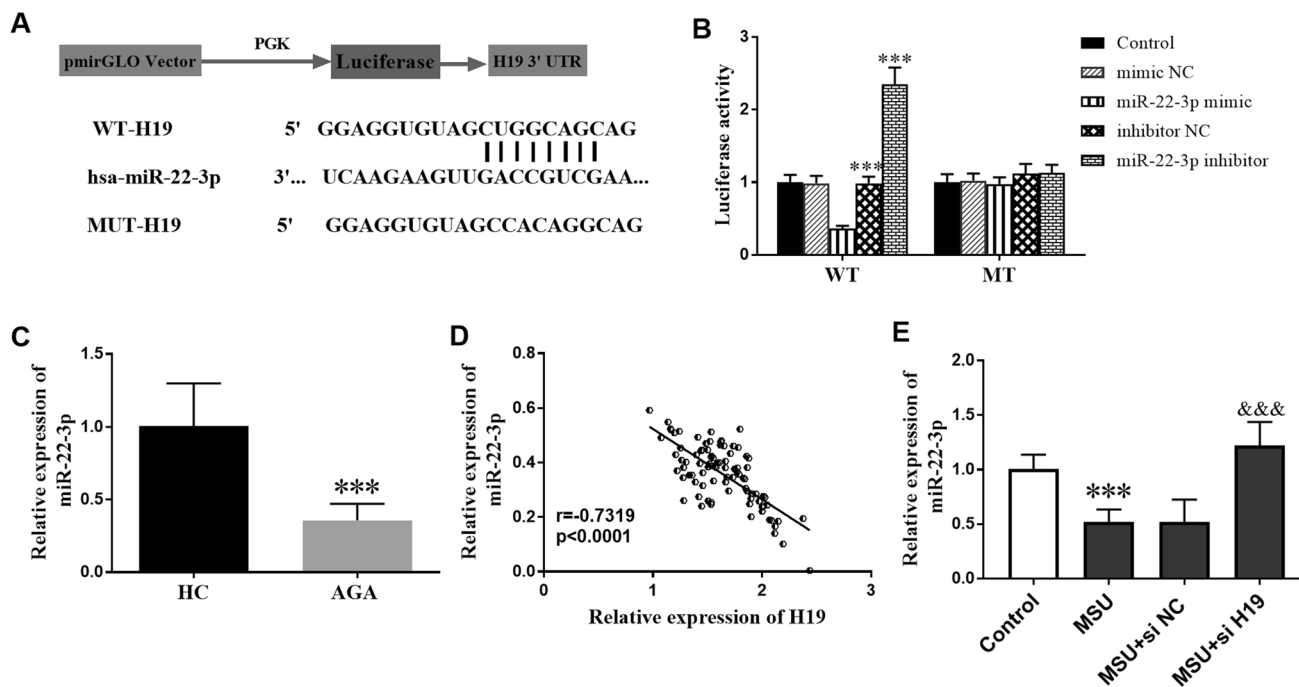

Fig. 3 The effects of MSU on H19 expression and inflammatory response in THP-1 cells. MSU induced the upregulation of H19 level in THP-1 cells, but this effect could be offset by transfection of si-H19 (A). MSU induction promoted the inflammatory response of THP-1 cells, which showed that the levels of IL-1β (B), IL-8 (C) and TNF-α (D) increased, while transfection of si-H19 could reduce the expression of inflammatory factors (*** $P < 0.001$, $^{\&\&\&}$ $P < 0.001$)

Fig. 4 Verification of the interaction between H19 and miR-22-3p. Complementary sequence of H19 and miR-22-3p (A). Transfection of miR-22-3p mimic significantly reduced luciferase activity in WT-H19 group (B). The relative expression level of miR-22-3p in AGA group was decreased compared with control group (C). The expression of miR-22-3p was negatively correlated with the expression of H19 (D). MSU induced the downregulation of miR-22-3p level in THP-1 cells, but this effect could be reversed by transfection of si-H19 (E) ($^{***}P < 0.001$, $^{\&\&\&}P < 0.001$)

demonstrated that the expression of mir-22-3p in MSU-induced cells was decreased. The level of miR-22-3p could be upregulated or downregulated by transfection with miR-22-3p mimic or inhibitor ($P < 0.01$). Besides, inflammatory factors such as IL-1β, IL-8, and TNF-α increased in cell models. However, it can be seen from the results that after upregulating the level of miR-22-3p, the contents of inflammatory factors in cells are declined, while the opposite results are presented after inhibiting the expression of miR-22-3p (Fig. 5B–D, P < 0.001).

## H19 regulates inflammation by negatively regulating miR-22-3p expression

To verify whether H19 regulates the inflammatory response of MSU-treated THP-1 cells through targeting miR-22-3p, the THP-1 cells were co-transfected with si-H19 and miR-22-3p inhibitor. The results showed that H19 knockdown dramatically increased miR-22-3p expression, while transfection of miR-22-3p inhibitor also observably restrained miR-22-3p (Fig. 6A, P < 0.001). Furthermore, the suppression of H19 restricted the inflammatory response of THP-1 cells; meanwhile, after downregulating the expression of miR-22-3p, the inflammatory response was strengthened, which showed that the release of inflammatory factors

increased, such as IL-1β, IL-8, and TNF-α (Fig. 6B–D, P < 0.001).

Discussion

Arthritis is a general term for joint diseases. The most common forms of arthritis are osteoarthritis, gouty arthritis (GA), and rheumatoid arthritis (RA) [16]. GA is responsible for the most severe episodes of acute pain. Uric acid is the final metabolite of purine, and hyperuricemia is a necessary condition for the formation of gout [17]. Therefore, any decrease in uric acid excretion or increase in uric acid production can indicate metabolic problems in the organism. Gout often affects the joints and soft tissues, and AGA is the most common initial symptom of primary gout [18]. In this study, we observed that the expression of H19 was enhanced in the serum of AGA patients, and the high expression of H19 showed good clinical diagnostic value for AGA. Additionally, in in vitro studies, we observed elevated expression of H19 in MSU-treated THP-1 cells, and H19 overexpression may induce an inflammatory response by targeting negative regulation of miR-22-3p. In addition, a high level of miR-22-3p was found to have the effect of inhibiting cellular inflammatory reaction. AGA has an acute and rapid onset, which is mainly characterized by severe pain in unilateral

Fig. 5 Effects of miR-22-3p on inflammatory response of MSU-induced THP-1 cells. Transfection of miR-22-3p mimic or inhibitor can significantly improve or reduce the miR-22-3p level of MSU-induced cells (A). MiR-22-3p knockdown induced the inflammatory response of THP-1 cells, including the upregulation of the expression levels of IL-1β (B), IL-8 (C), and TNF-α (D) ($^{***}P<0.001$, $^{###}P<0.001$, $^{##}P<0.01$)
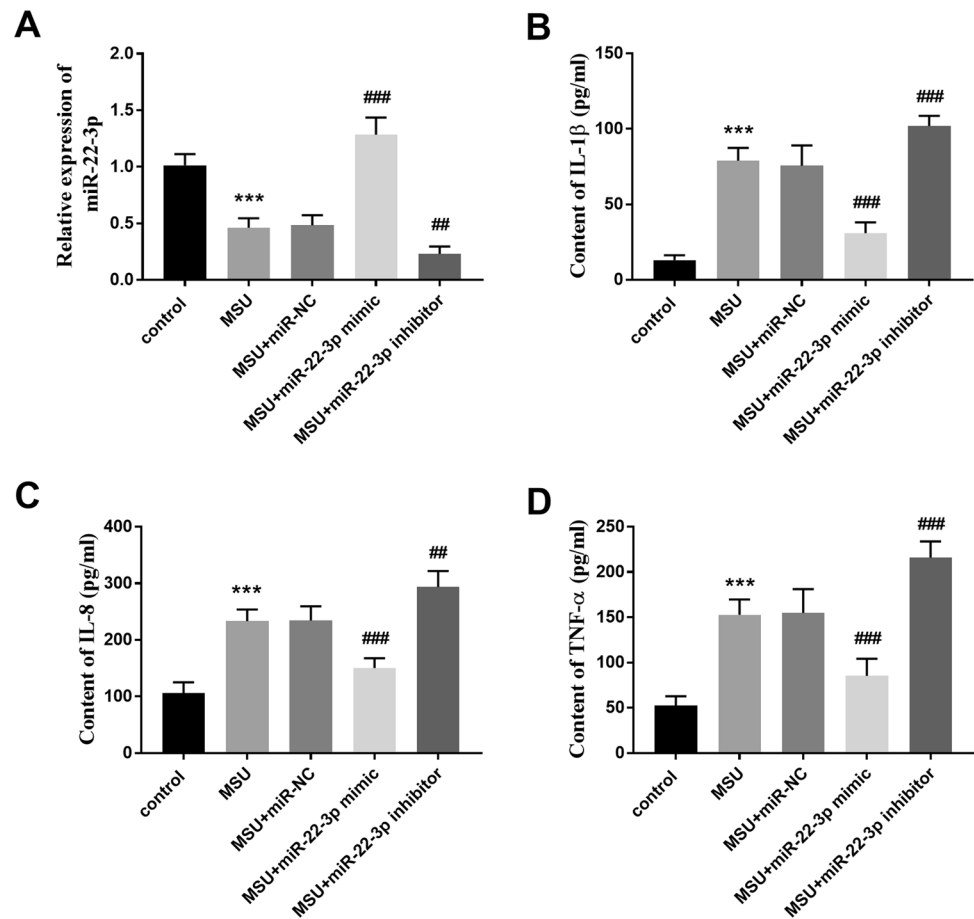

joints. At the early stage of the disease, the patients' joint intima proliferated, and the white blood cells, neutrophils, and lymphocytes increased in different degrees, showing typical characteristics of inflammatory reaction [19, 20]. In this study, the serum level of H19 in AGA patients was enhanced, and the serum uric acid level, leukocyte count, and neutrophil content of patients with AGA were significantly higher than those of healthy controls, suggesting that there was an infection or inflammatory reaction in AGA patients. In a previous study on OA, Yang et al. found that H19 was upregulated in OA cartilage tissues [21]. The previous study by Stuhlmuller et al. reported that H19 expression was significantly higher in synovial tissues of RA and OA patients than in normal or joint trauma controls [22]. All the evidence confirmed the effect of H19 on arthritis. Clinically, the diagnosis of AGA generally includes blood biochemical examination, joint X-ray examination, and electrocardiogram monitoring, with various means and complicated steps [23]. At present, more and more studies are devoted to exploring the value and significance of serum lncRNA as a biomarker of disease diagnosis and prognosis due to its easy acquisition, convenient detection, and stable properties [24, 25]. In consideration of the abnormal expression of H19 in AGA, the ROC curve was performed to analyze the

diagnostic significance of H19 for AGA. The results showed that the curve has a high AUC value, high sensitivity, and specificity, which indicated that H19 had the ability to distinguish AGA patients from healthy people.

Increasing evidence has proved that H19 promotes disease progression by modulating inflammatory responses. For instance, Wang et al. found that H19 deficiency alleviated bleomycin-induced idiopathic pulmonary fibrosis and pulmonary inflammation [26]. Xu et al. illustrated that metformin reduced the inflammatory response and promoted the proliferation of rat glomerular membrane epithelial cells treated with high glucose by inhibiting H19 [27]. In the in vitro experiments of our study, our data exhibited that the level of H19 in MSU-induced THP-1 cells was upregulated. Functionally, H19 knockdown can relieve MSU-induced inflammatory response, as evidenced by suppressed inflammatory cytokines production, including IL-1β, IL-8, and TNF-α. Therefore, combined with the above literature evidence, we can infer that there is indeed an inseparable relationship between high expression of H19 and inflammation in AGA.

LncRNA, as a competitive endogenous RNA (ceRNA), is often acted as a molecular sponge for specific miRNAs to play a role in gene regulation [28]. As reported by Bi et al., PICSAR participates in RA by targeting miR-4701-5p to

Fig. 6 The effects of miR-22-3p and H19 on inflammatory response of MSU-induced THP-1 cells. Transfection of miR-22-3p inhibitor can significantly reduce the increase of miR-22-3p level caused by H19 inhibition (A). Suppression of miR-22-3p induced the inflammatory response of THP-1 cells, including the upregulation of the expression levels of IL-1β (B), IL-8 (C), and TNF-α (D) ($^{***}P < 0.001$, $^{\&\&\&}P < 0.001$)
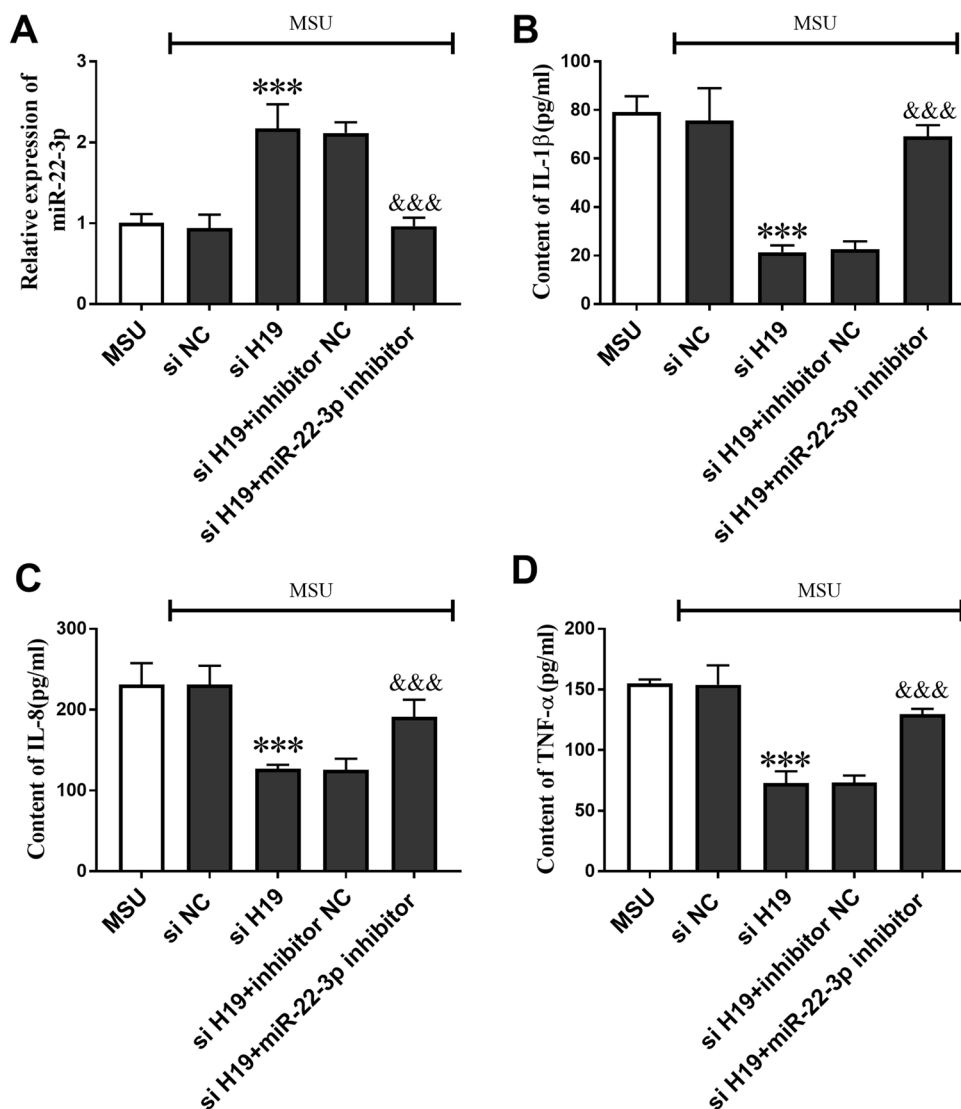
affect proliferation, migration, and inflammation of fibroblast-like synovial cells (FLSs) [29]. In the present study, bioinformatics analysis showed that H19 directly targeted miR-22-3p in MSU-treated THP-1 cells, and the expression of miR-22-3p was negatively regulated by H19. According to our preceding study, we also found that knockdown of miR-22-3p overturned the inhibitory effect of H19 suppression on inflammation in MSU-induced THP-1 cells, which suggested that H19 might facilitate the inflammatory response of MSU-induced THP-1 cells by downregulating miR-22-3p. miR-22-3p, also called miR-22, is a class of highly conserved noncoding RNAs [30]. miR-22-3p has been reported to be downregulated in MSU-stimulated THP-1 cells, which reduced the inflammatory response of gout by inhibiting NLRP3 expression [31]. Wang et al.' s study on acute kidney injury (AKI) showed that overexpression of miR-22-3p restrained inflammatory reaction and apoptosis by downregulating the level of pro-inflammatory

factors [32]. Although the molecular mechanism of H19 and miR-22-3p has been elucidated in in vitro AGA cell models, the regulation of H19/miR-22-3p–mediated signaling pathway still needs to be further explored. In addition, the results of our clinical study were consistent with those of in vitro study, which indicated that the cell model we selected for this study was reasonable. Depending on the current experimental data, we cannot determine whether the serum levels of H19 and miR-22-3p are consistent in other cell types. Therefore, it is extremely appropriate to attempt to include other disease-related cell models to verify the findings of clinical studies. In conclusion, our results showed that highly expressed H19 upregulated the release of inflammatory factors by targeting the expression of miR-22-3p in in vitro model cells, thereby promoting the inflammatory response of AGA. These results preliminarily revealed a novel pathological mechanism of AGA, which may provide a new thought for the research of AGA.

Declarations

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