MiR-221-5p Is Involved in the Regulation of Inflammatory Responses in Acute Gouty Arthritis by Targeting IL-1 β

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Research

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Abstract

Background: Gout is caused by the accumulation of deposited sodium urate (MSU) crystals in the joints. Recent studies have shown that interleukin-1 β (IL-1 β) is a key inflammatory mediator of acute gouty arthritis (AGA), and its level is regulated by microRNAs (miRNAs). However, the molecular mechanism of its regulation is still unclear.

Methods: 100 patients with AGA and 94 healthy individuals were recruited. The expression of serum miR-221-5p was determined by qRT-PCR. The receiver operating curve (ROC) was applied for diagnostic value analysis. A luciferase reporter assay was performed to confirm the interaction of miRNA and the 3'-untranslated region (UTR) of IL-1 β . ELISA was used to detect serum and proinflammatory factors.

Results: miR-221-5p was lower expressed in the serum of AGA patients. The AUC was 0.884, the sensitivity was 82.0%, and the specificity was 80.9%. Serum miR-221-5p was negatively correlated with the expression levels of VAS and IL-1 β . Cell experiments showed that overexpression of miR-221-5p significantly inhibited the expression of inflammatory factors tumor necrosis factor- α (TNF- α), interleukin-8 (IL-8), and IL-1 β , while down-regulation of miR-221-5p was the opposite. Luciferase analysis showed that IL-1 β was the target gene of miR-221-5p.

Conclusions: This study confirmed that miR-221-5p regulates the production of inflammatory cytokines during the pathogenesis of AGA. These results suggest that miR-221-5p can be used as potential therapeutic targets for the treatment of AGA.

Background

Gout attacks can be caused by hunger, trauma, surgery, ingestion of high-purine foods, excessive alcohol consumption, and medications that affect urate concentrations[1]. It is divided into three clinical stages: acute gout arthritis (AGA), critical gout, and chronic gout. AGA is an acute inflammation caused by the precipitation of urate crystals in joints[2]. It is one of the most common types of autoimmune inflammatory arthritis, characterized by a sudden onset and significant pain that resolves spontaneously within a week[3, 4]. Interleukin-1 β (IL-1 β) is a central cytokine in the initiation of the acute inflammatory response, which plays a key role in the pathogenesis of gout[5], especially its role in the pathology of acute gouty arthritis. It has been reported in the pathological mechanism of gout treatment by gout capsule that reducing the levels of IL-1 β and tumor necrosis factor- α (TNF- α), can inhibit the expression of MDA and nitric oxide (NO), and promote the release of SOD, to achieve the purpose of treating gout[6]. Moreover, the mechanism of action of IL-1 β in AGA is unclear.

MicroRNAs (miRNAs) are evolutionary conserved non-coding small RNA molecules that act as negative post-transcriptional gene regulators [7]. Since a single miRNA molecule can target hundreds of messenger RNAs (mRNAs), the abnormal expression of miRNA is related to the occurrence of many diseases [8]. Recent research suggests that miRNAs may be involved in the development of arthritis [9, 10]. For example, The expression level of miR-155 in patients with gout arthritis is significantly higher

than that in healthy individuals, and the overexpressed miR-155 can promote the production of MSU induced inflammatory cytokines by reducing SHIP-1 level[9]. Considering the important role of miRNAs in inflammatory diseases, especially AGA, more studies on miRNAs are urgently needed[11] It is well known that diabetes is one of the main complications of AGA. More importantly, studies have reported that Mir-221-5P is involved in the research process of diabetes[12].Up to now, the molecular mechanism of miR-221-5p in AGA is unclear.

In summary, miR-221-5p is critical for human cell inflammation and AGA. However, the functional role of miR-221-5p in AGA is not yet clear. Therefore, the purpose of this study was to study the role of miR-221-5p in the pathogenesis of AGA.

Materials And Methods

Subject and sample collection

100 AGA patients and 94 healthy individuals matched in age and gender frequency participated in the study. The 100 AGA patients recruited excluded the following conditions. (1) infection (2) tumor (3) rheumatoid. 5 ml peripheral blood samples were collected from each subject and centrifuged immediately. Subsequently, the serum samples were stored at -80°C for further analysis. All controls had no history of systemic inflammation or tumor. Clinical data including erythrocyte sedimentation rate (ESR), serum uric acid (SUA), Visual Analogue Scale (VAS), serum creatinine (SCR), age, sex, body mass index (BMI), leukocyte count, neutrophils count, and lymphocyte count were recorded in all participants.

The protocol of this study was approved by the Ethics Committee of Qingdao Municipal Hospital, and written informed consent was collected from each participant.

Cell culture and transfection

The human monocyte THP-1 cell line was cultured in RPMI 1640 medium (Life Technologies) and cultured in a 37°C, 5% CO₂ constant temperature incubator. 1.5×10^6 /ml THP-1 cells were incubated in the 96 well plates. The THP-1 cells were stimulated for 3 h with 0.5 μ M phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA) the day before stimulation. Then, the cells were stimulated with 250ug/mL MSU crystal (Invivogen, San Diego, CA, USA) for 24 h, causing inflammation, and presenting a variety of features of AGA. In order to regulate the expression level of miR-221-5p, cells were transfected with miR-221-5p mimic, miR-221-5p inhibitor, or their negative control (miR-NC), which was produced by Ribo Bio (Guangzhou), China). Liposome 2000 (Invitrogen, Carlsbad, CA, USA) was used for transfection according to the manufacturer's instructions.

Total RNA extraction and quantitative real-time PCR (qRT-PCR) assay

Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). The miRNA bulge loop was reverse transcribed using the PrimeScript RT Reagent Kit(TaKaRa, Dalian, China). qRT-PCR was

performed to detect gene expression using SYBR premix ExTaq M. II commercial kit (Takara, Dalian, China) and the Applied Biosystems 7900 Real Time PCR System (Applied Biosystems, Foster City, CA). PCR parameters were as follows: 95°C for 3 minutes, followed by 40 cycles of 95°C for 10 seconds, 60°C for 20 seconds, and 72°C for 1 second. The relative gene expression was normalized to that of the internal control U6 according to the comparative delta CT ($2^{-\Delta\Delta C_t}$) method.

Evaluation of inflammatory cytokines

The concentrations of IL-1 β , IL-8, and TNF- α proteins in THP-1 cell culture supernatant were determined using an ELISA kit (UK Abeam) in accordance with the manufacturer's instructions. Each sample was analyzed three times.

Luciferase reporter assay

The putative binding sites of miRNAs in the 3'-UTR of the human IL-1 β gene transcript were predicted by the combinatorial use of Target Scan (<http://targetscan.org/>), and then verified by luciferase reporter gene experiment. Cells were co-transfected with miR-221-5p mimic or inhibitor, and miR-221-5p wide type (WT) or mutant seed region (MUT) of IL-1 β 3'-UTR. Lipofectamine 2000 (Invitrogen, USA) was used for cell transfection. Relative luciferase activity was measured by the dual luciferase reporting system (Promega, INC., USA) according to the manufacturer's instructions. The fluorescent activity of renal cells was used as an internal reference.

Statistical analysis

In our study, all statistical analysis was performed with Prism 6 (GraphPad Software, San Diego, CA, USA) and IBMSPSS statistics 20 statistical software. The data were expressed as mean and standard deviation (SD). The differences between the two groups were compared by Student t-test or one-way ANOVA. Receiver operating characteristic (ROC) curves were used to determine the specificity and sensitivity of the diagnostic value of miR-221-5p for AGA. Correlation analysis was performed using Pearson correlation coefficients.

Results

Clinical characteristics of different patient groups

Table 1 reports the main characteristics of the study population and laboratory results. A total of 194 individuals were included, the range of age was 27–65. Among them, 94 subjects were healthy controls group (44 males / 50 females), and 100 AGA patients (48 males / 52 females). There was no difference in age, gender distribution, BMI, erythrocyte sedimentation rate (ESR), and lymphocytes count between the groups ($P > 0.05$). There were significant differences in serum uric acid (SUA), leukocyte count, neutrophils counts ($P < 0.001$). The average visual analog scale (VAS) score in patients was 6.16 ± 2.36 .

Table 1
Baseline characteristics of the subjects.

Characteristics	Controls (n = 94)	AGA (n = 100)	P value
Age (years)	46.54 (11.83)	43.21 (11.66)	0.796
Gender (male/female), n	44/50	48/52	0.868
BMI (kg/m ²)	20.72 (1.73)	21.05 (1.64)	0.553
ESR (mm/h)	4.68 (2.81)	4.99 (3.01)	0.517
SUA (umol/L)	185.15 (8.20)	227.44 (12.36)	< 0.001
VSA (umol/L)	-	6.16 (2.36)	-
SCR (umol/L)	89.34 (26.48)	87.32 (26.27)	0.600
Leukocyte count (10 ⁹ /L)	7.06 (1.83)	25.92 (8.73)	< 0.001
Neutrophils count (10 ³ /mL)	4.49 (1.44)	31.19 (9.05)	< 0.001
Lymphocytes count (10 ⁹ /L)	2.55 (0.94)	2.29 (0.86)	0.125
Note: AGA, acute gouty arthritis; BMI, body mass index; SUA, Serum uric acid; ESR, erythrocyte sedimentation rate; VSA, visual analog scale; SCR, serum creatinine; SD,. Data are expressed as n or mean and standard deviation			

The expression level of miR-221-5p and its correlation with VAS in AGA

We first studied the serum levels of miR-221-5p in healthy controls and AGA groups. The results of the study were shown in Fig. 1A: The expression level of miR-221-5p in the serum of patients with AGA was significantly lower than that of the healthy control group ($P < 0.05$). The results indicated that miR-221-5p may be a key biomolecule for AGA and play an important biological role in its disease progression. In addition, in order to further explore the relationship between miR-221-5p and AGA, we also made a correlation between VAS and miR-221-5p. As shown in Fig. 1B, serum miR-221-5p was negatively correlated to the VAS ($r = -0.7671$, $P < 0.0001$) in AGA patients. We concluded that miR-221-5p might be associated with the occurrence and severity of AGA.

Diagnostic value of miR-221-5p in patients with AGA

ROC curves were drawn based on the expression level of miR-221-5p in AGA patients and the control group to evaluate the diagnostic value of miR-221-5p in AGA patients. As shown in Fig. 2, the miR-221-5p expression may be used to distinguish AGA patients from healthy individuals. AUC was 0.884, cut off

value was 0.800, sensitivity was 82.0%, specificity was 80.9%. The results of this study confirmed the diagnostic value of miR-221-5p in differentiating AGA patients from healthy individuals.

Effect of miR-221-5p on inflammatory responses in THP-1 cells

As shown in Fig. 3A, the transfection of miR-221-5p mimic/inhibitor had a significant effect on the expression of miR-221-5p ($P < 0.001$), transfection with miR-221-5p mimic significantly increased the expression of miR-221-5p, while transfection with miR-221-5p inhibitor had the opposite effect. As shown in Fig. 3B-D, the expression levels of TNF- α , IL-8, and IL-1 in the M5 group were significantly increased compared with the control group. Compared with the M5 group, the expression levels of inflammatory factors were significantly decreased in the miR-221-5p mimic transfection group ($P < 0.001$), and significantly increased in the miR-221-5p inhibitor group ($P < 0.001$).

MiR-221-5p directly targets IL-1 β in THP-1 cell

MiRNAs are known to function by inhibiting the expression of their target genes. According to the Target scan analysis results, the binding sites of miR-221-5p in IL-1 β was shown in Fig. 4A(a). Luciferase reporter assay results showed that miR-221-5p mimic significantly inhibited luciferase activity of IL-1 β WT 3'-UTR (Fig. 4A(b), $P < 0.001$), while miR-221-5p inhibitor significantly increased luciferase activity. In addition, the luciferin activity of the mutant group was not affected by transfection with miR-221-5p mimic or miR-221-5p inhibitor. As shown in Fig. 4B, the correlation between miR-221-5p expression and target gene IL-1 β in AGA patients was also analyzed. The results showed that the expression of miR-221-5p in AGA patients was negatively correlated with IL-1 β level ($r = -0.6762$, $P < 0.0001$)

Discussion

Gout is a common metabolic disease and AGA is one of the important complications[13]. AGA is a group of clinical syndromes caused by monosodium urate (MSU) crystal deposition on bone, joints, and subcutaneous tissues, which is the most common initial symptom of gout[14]. It is worth noting that while hyperuricemia has been classically associated with gouty arthritis, asymptomatic hyperuricemia is frequently found in metabolic syndrome, diabetes mellitus, chronic kidney disease, or hypertension[15]. miR-221-5p has been identified to be aberrantly expressed in metabolic diseases, such as type 2 diabetes[16], therefore its role in AGA attracts our interest. In our study, it was proposed that the expression of miR-221-5p in AGA patients was significantly lower than in healthy subjects, which was consistent with the previously reported results, indicating the association of miR-221-5p with AGA.

Serum miRNAs are stable in stored samples[17], and it is more practical as a biomarker and easier to isolate than specific cell types of miRNAs, especially in AGA. Recent studies have suggested that miRNAs including miR-155 and miR-146a may be involved in the development of AGA in humans. For instance, a study has confirmed that the miR-155 was upregulated in SFMCs from patients with AGA[18]. Another

study confirmed that miR146a plays a negative regulatory role in AGA in humans[19]. However, the application of miR-221-5p in AGA has not been reported. In addition, a large number of studies have confirmed that VAS is an important indicator for the clinical assessment of AGA. Considering the dysregulation of miR-221-5p in AGA patients, we further studied the correlation between miR-221-5p and VAS, and the results showed that miR-221-5p was negatively positively correlated with VAS. Meanwhile, accurate and timely diagnosis and monitoring of treatment outcomes are critical to AGA patient prognosis. To solve this problem, reliable AGA biomarkers are urgently needed. Many studies have shown that miRNAs can be a biomarker useful for diagnosis and prognosis[20]. AGA is known to cause several complications, for example urate deposits in the kidney or urinary tract can lead to kidney disease. A report on circulating biomarkers of gastric cancer mentioned that miR-221 as a potential marker of renal cancer[21]. Therefore, we assessed the ability of serum miR-221-5p to differentiate AGA from healthy individuals by establishing ROC curves. Meanwhile, these results are consistent with our conclusion that miR-221-5p has a high diagnostic capacity in AGA patients.

AGA is one of the most painful inflammatory conditions[22]. Therefore, the onset of AGA is accompanied by all the characteristics of an acute inflammatory response. These include intimal hyperplasia, infiltration of neutrophils, mononuclear phagocytes, and lymphocytes. In the present study, THP-1 cells were stimulated with MSU crystal to mimic inflammation features of AGA, and the cell experiments demonstrated that miR-221-5p overexpression inhibited the release of inflammatory cytokines, including IL-1 β , IL-8 and TNF- α . Besides, IL-1 β is a key proinflammatory cytokine in gouty inflammation[23]. Clinical studies have demonstrated that selective blocking of IL-1 β is effective in suppressing pain and inflammation in patients with gout that are difficult to suppress with other treatments[24]. In addition, in THP-1 cells, the upregulation of miR-488 and miR-920 inhibited the expression of IL-1 β induced by MSU. Moreover, they also found that miR-488 and miR-920 can directly target the 3-UTR of IL-1 β [23]. It is demonstrated that IL-1 β can be involved in the treatment of AGA patients as a target gene. The abnormal expression of miRNAs can affect specific targets and pathways, leading to the phenotype of autoimmune diseases, which is also supported by some in vivo studies. Targeting miRNAs may be an effective option for treating autoimmune diseases in the future[25]. Specific miRNAs inhibit the release of inflammatory factors[26], including IL-1 β , IL-8 and TNF- α . In our results, we first found that miR-221-5p has a relatively high correlation with the inflammatory factor IL-1 β . Then, it is also found that IL-1 β maybe participate in the AGA process as a target gene of miR-221-5p.

Conclusion

It is commonly known that autoimmunity plays a pivotal role in the pathology of AGA. However, the exact etiology and pathogenesis are poorly understood. In general, we found that miR-221-5p is downregulated in patients with AGA. ROC curves were drawn based on the expression level of miR-221-5p and the diagnostic value of miR-221-5p in distinguishing AGA patients from healthy people was confirmed. Therefore, it has potential as a therapeutic or biomarker for AGA. MiR-221-5p may participate in the development of AGA patients by acting on target gene IL-1 β to inhibit the release of inflammatory factors in THP-1 cells. However, our study also has some limitations. The study population should be expanded

to better verify the current study effect. Besides, only part of the role of miR-221-5p in THP-1 cells is studied in this research. In the future, we can further verify its cell function and explore the specific mechanism of the role of miR-221-5p in the development of AGA patients.

Abbreviations

IL-1 β
interleukin-1 β
miRNAs
microRNAs
ROC
receiver operating curve
UTR
untranslated region
TNF- α
tumor necrosis factor- α
IL-8
interleukin-8
AGA
acute gout arthritis
NO
nitric oxide
mRNAs
messenger RNAs
ESR
erythrocyte sedimentation rate
SUA
serum uric acid
VAS
Visual Analogue Scale
SCR
serum creatinine
BMI
body mass index
miR-NC
miR-221-5p negative control
qRT-PCR
quantitative real-time PCR
WT
wide type

MUT
mutant seed region
ROC
Receiver operating characteristic
MSU
monosodium urate

Declarations

Ethics approval and consent to participate:

The protocol of this study was approved by the Ethics Committee of Qingdao Municipal Hospital, and written informed consent was collected from each participant.

Consent for publication:

Written informed consent for publication was collected from each participant.

Availability of data and materials:

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

Competing interests:

The authors declare that they have no competing interests.

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Authors' contributions:

GL, HZ, and HM made substantial contributions to conception and design, performed all the experiment, and was a major contributor in writing the manuscript. SQ and QX contributed to acquisition of patients and tissues specimens, analysis and interpretation of data. GW has been involved in drafting the manuscript and revising it critically for important intellectual content. All authors read and approved the final manuscript.

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Figures

Figure 1

The expression level of miR-221-5p and its correlation with VAS in AGA patients. A, the expression level of miR-221-5p in the serum of AGA and control was detected by qRT-PCR. The results showed that the expression level of miR-221-5p in the serum of patients with AGA was decreased compared with the control group (** $P < 0.001$). B, the correlation between miR-221-5p and VAS in AGA patients, and a negative correlation between serum miR-221-5p and VAS were found ($r = -0.7671$, $P < 0.0001$).

Figure 2

The ROC curve was used to analyze the diagnostic value of miR-221-5p in AGA. The AUC is 0.884 , sensitivity is 82.0%, specificity is 80.9%.

Figure 3

A, the transfection of miR-221-5p mimics significantly increased the expression of miR-221-5p whereas the expression was downregulated significantly after miR-221-5p inhibitors were transfected. B-C, effects of miR-221-5p on inflammatory cytokines in THP-1 cell models treated with MSU. \$\$\$ P < 0.001, compared with the control group. *** P < 0.001, compared with the MSU group.

A (a)

Position 172-178 of IL1 β 3' UTR 5' ...AUCUCCUGUCCAUCAGCCAGGAC...
 hsa-miR-221-5p 3' UUUAGAUGUAACAUAACGGUCCA

(b)

B

Figure 4

A, IL-1 β was the target gene of miR-221-5p. (a). The binding site of miR-221-5p in IL-1 β . (b) miR-221-5p mimic significantly inhibited luciferase activity of WT 3'-UTR of IL-1 β and miR-221-5p inhibitor significantly increased its luciferase activity. Besides, the luciferin activity of the mutant type group was not affected by the transfection of miR-221-5p mimic or miR-221-5p inhibitor. *** $P < 0.001$. B, correlations between relative miR-221-5p expression with the IL-1 β expression ($r = -0.6762$, $P < 0.0001$).