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Expression and function of peripheral blood miRNA16a in patients with ankylosing spondylitis

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Abstract. – OBJECTIVE: Ankylosing spondylitis (AS) is a progressive spinal disease presented as rheumatoid factor negative. As an autoimmune disorder, AS is featured with an inflammatory change of tendon and ligament, accompanied by elevates serum levels of inflammatory factors. MicroRNA (miR) participates in the regulation of various diseases including tumor, inflammation, cardiovascular disease and immune response. MiR16a exerts critical roles in inflammatory disease. Its function in AS, however, has not been fully illustrated.

PATIENTS AND METHODS: AS patients (at stable and active phase) and healthy controlled individuals were recruited to test peripheral expression of miR16a by Real-time PCR (RT-PCR). Enzyme-linked immunosorbent assay (ELISA) was used to test serum helper T cell 1 (Th1) cytokine levels including interferon (IF-N)-γ, tumor necrosis factor-α (TNF-α) and Th2 cytokines including interleukin-4 (IL-4) and IL-10. The correlations between miR16a and cytokine levels, C reactive protein (CRP), erythrocyte sedimentation rate (ESR) and AS activity, were analyzed.

RESULTS: MiR16a expression in peripheral blood of AS patients was significantly higher compared to control people ($p<0.05$ compared to control group). AS patients at active phase had significantly higher miR16a levels, compared to stable phase ($p<0.05$). Serum IL-4 and IL-10 levels in AS patients were significantly increased, while IFN-γ and TNF-α expressions were depressed ($p<0.05$ compared to healthy controls). MiR16a expression was positively correlated with IL-4/IL-10 or disease active index, and was negatively correlated with IFN-γ and TNF-α levels ($p<0.05$), but not with CRP or ESR.

CONCLUSIONS: Peripheral miR16a was up-regulated in AS patients, and reflected disease activity, probably via regulating Th1/Th2 balance.

Key Words:
Ankylosing spondylitis, MicroRNA16a, Th1, Th2, Inflammatory factor.

Introduction

Ankylosing spondylitis (AS) is a multi-gene congenital disorder. It is commonly occurred in 20-30 years males[1,2] and has different incidences across ethnics groups, with higher frequency in the Asian population. AS has an insidious onset and a slow progression. No effective treatment has been discovered so far, causing its high morbidity[3,4] and severe pain and economic burdens on patients, making it a major health concern worldwide. AS is presented as a progressive spinal disease that affects middle axial joints, and is one autoimmune disorder in rheumatoid disorders, although showing negative results for rheumatoid factors[5]. AS pathology is featured with inflammatory changes of tendon, ligament, and sacroiliac joint, accompanied by increased serum inflammatory factor and chronic inflammation[6,7]. AS is presented as peripheral arthritis, inflammation of tendon and ligament in clinics, and progresses into hip/buttock pain, wrist pain and limited spine activity, affecting major joints and causing ossification/fibrosis of intervertebral disc and peripheral tissues, or affecting multiple tissues including lung and muscle[8]. AS has been confirmed to be strongly correlated with HLA-B27, but has unknown pathogenesis mechanism, which includes genetic, immune and environmental factors[9].

MicroRNA (miR) is a non-coding single-stranded RNA with highly conserved structure. It can specifically bind with target genes to induce their mRNA degradation for inhibiting protein translation[10]. MiR is widely distributed in various tissues of eukaryotes and plays an essential role in cell growth, development, embryonic development, apoptosis and proliferation/differentiation[11,12]. MiR participates in the modulation of various pathological or physiological processes including tumor, immune response, inflammation, cardiovascular diseases[13]. MiR down-regulation or

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up-regulation may affect body development an disease progression, with unique expressional profiles in specific disease[14]. MiR16a has been demonstrated to exert critical roles in various diseases including inflammation[15]. However, the expression and role of miR16a in AS has not been fully understood. Thus, we investigated the expression of miR16a in peripheral blood samples of AS patients with different disease stages, in an attempt to analyze its value as the molecular target of disease diagnosis/treatment.

Patients and Methods

Patients

A total of 50 AS patients from the Department of Rheumatoid and Immune in Shuyang Hospital Affiliated to Xuzhou Medical University were recruited from January 2017 to December 2017. There were 37 males and 13 females in the patient group (aging between 19 and 31 years, average age = 20.0 ± 4.6 years). The diagnostic criteria was based on AS guideline stipulated by American College of Rheumatology (ACR) and was examined by at least 2 clinicians. Inclusive criteria referred to at least one item of clinical or imaging standards of AS. Clinical symptoms included limited lumbar spinal activity, wrist pain or morning stiffness for at least 3 months, accompanied with improved activity but not pain release after resting, lower activity of chest. Imaging diagnostic criteria included the unilateral grade III-IV or bilateral grade II or above based on sacroiliac arthritis. Exclusive criteria: recent inflammatory history, accompanied with tumor or major organ failure, with chronic inflammatory disease; pregnant or breast-feeding women.

Normal healthy control group recruited 25 volunteers from routine body examination, and excluded those with autoimmune disease or family history. There were 20 males and 5 females included, aging between 21 and 30 years (average age = 20±5.2 years). This study has been pre-approved by the Ethical Committee of Shuyang Hospital Affiliated to Xuzhou Medical University, Shuyang, China. All subjects have signed the consent forms before recruitment in this study.

Staging of AS Patients

AS patients were sub-divided into active group and stable group based on bath ankylosing spondylitis disease activity index (BASDAI) scoring system [16]. Those patients having higher than 3 points were classified in active group, while those having BASDAI score less or equal to 3 points were assigned in stable group. There were 27 patients in active stage (19 males and 8 females, aging between 21 and 30 years, average age = 20.0 ± 4.1 years), and 23 patients in stable group (18 males and 5 females, aging between 19 and 31 years, average age = 20.0 ± 5.1 years).

Disease Active Index Analysis

BASDAI disease activity index was used to analyze active index of AS disease. Visual analogue scale (VAS) approach was employed to evaluate the pain condition of disease joints of AS patients, including neck, back and hip pains or tissue swelling of other joints, in addition to the degree of tired and sleepiness. The score of morning stiffness was evaluated based on the time and degree of morning stiffness. The final score was given in a range of 1-10, in which higher score represents higher activity of disease.

Sample Collection

Heparin-sodium containing tube was used to collect 5 ml peripheral fasted venous blood from both AS patients and healthy controls. After centrifugation at 2000 r/min for 10 min, the supernatant was stored at -20°C for further use. Blood cells were placed at 4°C to extract total RNA.

Real-Time PCR for miR16a Expression

Real-time PCR was used to quantify miR16a expression from peripheral blood samples. A total of 1 ml TRIzol (Sigma-Aldrich, St. Louis, MO, USA) was mixed with peripheral blood samples until no precipitation existed. The mixture was then centrifuged at 12000 r/min for 10 min. The supernatant was saved and mixed with chloroform for 2 min vortex. Under 5 min incubation at room temperature, the mixture was centrifuged for 12000 r/min for 15 min at 4°C. The supernatant was mixed with equal volume of isopropanol for incubation at 4°C for 1.5 h. The mixture was centrifuged again at 12000 r/min for 10 min at 4°C. The supernatant was removed, followed by the addition of 75% ethanol. The precipitation was rinsed for 2-3 times using 7500 r/min centrifugation at 4°C for 10 min. RNA was re-suspended in DEPC-treated water. NanoDrop 2000 analyzer was used to quantify RNA concentration and purity. Reverse transcription kit (Axygen, Tewksbury, MA, USA) was employed to synthesize complementary DNA (cDNA). Primers were designed by Primer 6.0 (Premier, Canada) based on

Table I. Primer sequence.

Target gene	Forward primer 5′-3′	Reverse primer 5′-3′
GAPDH	ACCAGGTATCTGCTGGTTG	TAACCATGATGTCAGCGTGGT
MiRNA16a	CTTAGTCTCTGTATGACGT	TCACCCAGCTTGAGTCGA

target gene sequences (Table I), and were synthesized by Invitrogen/Life Technologies (Carlsbad, CA, USA). Real-time PCR was performed under the following conditions: 95°C pre-denature for 10 min, followed by 35 cycles each containing 95°C 10 s, 58°C 45 s and 72°C 35 s. The solution conditions were 60°C for 60 s and 95°C for 15 s. Each sample was tested in triplicates. Data were collected to determine amplification curve and melting curve. Cycle threshold (CT) represented initial cycle number. CT values of all samples and standards were recorded to plot the standard curve. ·Ct was calculated as sample CT value minus standard CT value. Using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference, $2^{-\cdot Ct}$ was used for semi-quantitative analysis.

Enzyme-Linked Immunosorbent Assay (ELISA) for Measuring Cytokine Levels

ELISA was employed to test serum levels of Th1 cytokines interferon (IFN)-γ, tumor necrosis factor (TNF)-α and Th2 cytokines including interleukin (IL)-4 and IL-10. Supernatants were collected from peripheral blood samples of all patients. Following the manual instruction of ELISA kit (RD Systems, Minneapolis, MN, USA), a microplate reader was used to measure optical density (OD) values of all wells. Standard curve was plotted based on OD values and respective concentrations, for calculating sample concentrations.

Clinical Record of Patients

Relevant clinical information of all patients, including family history and lab results such as C reactive protein (CRP) and erythrocyte sedimentation rate (ESR), were collected analyzed.

Statistical Analysis

All data were introduced into SPSS22.0 (IBM, Armonk, NY, USA) software for statistical analysis. Measurement data were presented as mean ± standard deviation (SD). Student's *t*-test was used to compare the differences between two groups. Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data between groups. Pearson analysis was used to reveal correlation. $p<0.05$ was treated as statistical significance.

Results

Peripheral miR16a Expression in AS Patients and Control

Real-time PCR was used to test miR16a expression in both AS patients and healthy controls. Results showed significantly higher miR16a level in peripheral blood of AS patients compared to that in healthy control people ($p<0.05$, Figure 1). In addition, active AS patients had significantly higher miR16a level than that of stable patients ($p<0.05$). These results suggested an increased expression of miR16a in peripheral blood of AS, which was correlated with the disease activity.

Disease Active Index, ESR, and CRP Levels

We analyzed both clinical and laboratory indexes of active AS, stable AS patients and healthy control individuals, including AS disease active index by BASDAI score[17], ESR and CRP changes. Results showed significantly elevated BASDAI disease active index in active AS patients ($p<0.05$ compared to stable AS patients, Table II). Both ESR and CRP levels were significantly elevated in AS patients, but showed no significant difference between active and stable patients (Table II).

Serum Levels of Th1 Cytokines IFN-γ and TNF-α

ELISA was employed to test serum levels of Th1 cytokines including IFN-γ and TNF-α. Results showed lowered IFN-γ and TNF-α levels in AS patients ($p<0.05$ compared to healthy control), with more significant change during active stage ($p<0.05$ compared to stable AS patients, Figures 2 and 3).

Serum IL-4 and IL-10 Levels in All Groups

We further employed ELISA to test serum levels of Th1 cytokines IL-4 and IL-10 in all patients. Results showed significantly elevated serum IL-4 and IL-10 levels in AS patient serum ($p<0.05$ compared to healthy controls), with more

Table II. Disease active index, ESR and CRP changes of AS patients.

Index	Control	Stable AS	Active AS′
Disease active index	NA	2.07±0.71	4.12±1.91#
ESR (mm/h)	NA	30.51±2.37	31.53±2.48
CRP (mg/l)	NA	22.34±3.21	21.97±3.61

#$p<0.05$ compared to stable AS patients.

potent change during active stage ($p<0.05$ compared to stable AS patients, Figures 4 and 5).

Correlation Between miR16a and AS Disease Active Index, ESR or CRP

We performed correlation analysis between miR16a of AS patients and their disease active index, ESR or CRP levels. Results showed a positive correlation between miR16a and disease active index ($p<0.05$) but not with CRP or ESR ($p>0.05$, Table III).

Correlation Between miR16a and Th1/Th2 Cytokine Levels in AS Patients

We further tested the correlation between miR16a level and Th1/Th2 cytokine levels in AS patients. Results showed a positive correlation between miR16a and Th1 cytokines (including IFN-γ and TNF-α) and a negative correlation between miR16a and Th2 cytokines (including IL-4 and IL-10) of AS patients ($p<0.05$ in all cases, Table IV).

Table III. Correlation between miR16a and disease active index, ESR or CRP.

	Disease active index	ESR	CRP
r-value	0.517	0.018	0.327
p-value	<0.05	>0.05	>0.05

Discussion

Among various autoimmune diseases, the imbalance of Th1 and Th2 secretory cytokines is one of the major pathogenic factors. Therefore, the disruption of Th1/Th2 secretory cytokine homeostasis could be a critical factor causing the occurrence and progression of autoimmune diseases[18]. Th1 secretory cytokines mainly include IFN-γ and TNF-α, both of which exert facilitating roles in macrophage-induced anti-infection immune response, and may lead to autoimmune disease of certain organs under pathological conditions[19]. Th2 secretory cytokines mainly consist

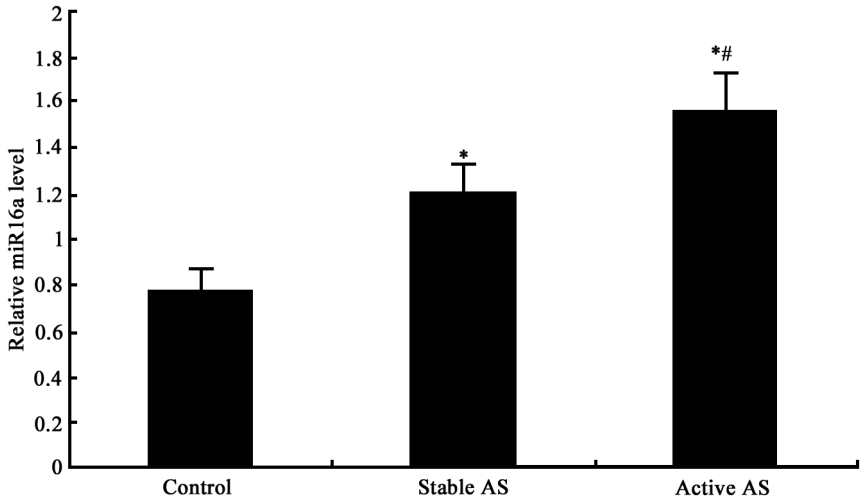

Figure 1. Peripheral blood miR16a level in AS patients and healthy controls. *$p<0.05$ compared to control group, #$p<0.05$ compared to stable AS patients.

Table IV. Correlation analysis between miR16a and Th1/Th2 cytokines of AS patients

	IFN-γ	TNF-α	IL-4	IL-10
r-value	-0.702	-0.815	0.562	0.639
p-value	<0.05	<0.05	<0.05	<0.05

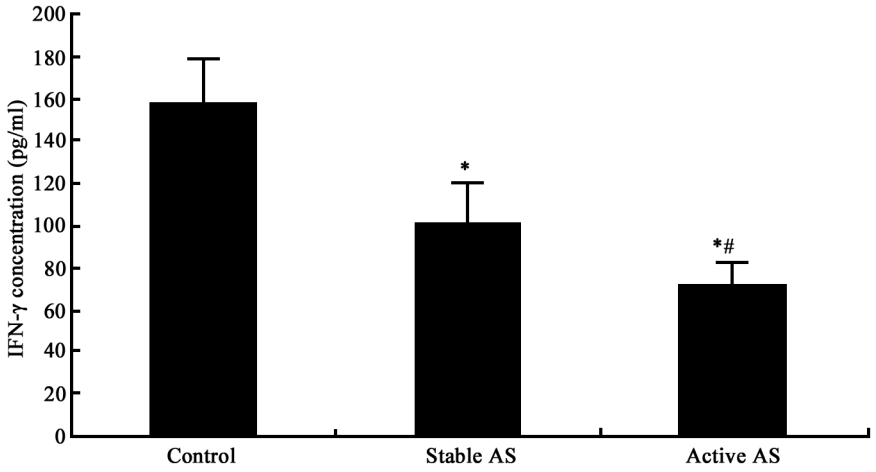

Figure 2. Serum IFN-γ level. $^{*}p<0.05$ compared to control group, $^{\#}p<0.05$ compared to stable AS patients.

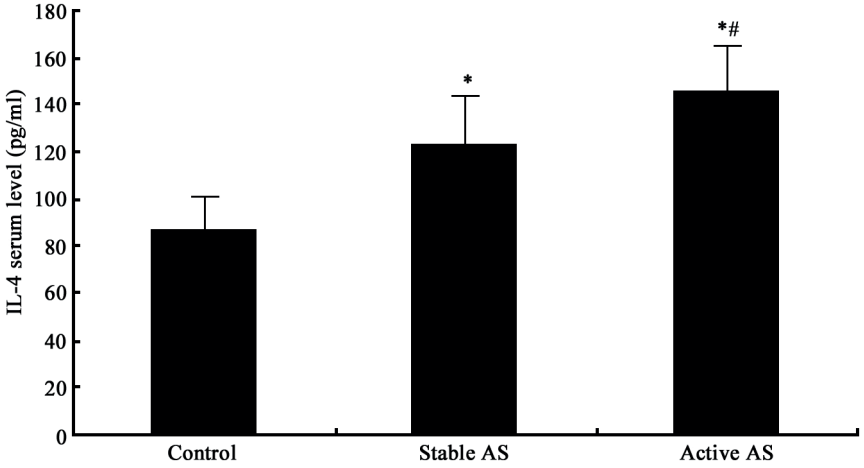

Figure 3. Serum TNF-α level. $^{*}p<0.05$ compared to control group, $^{\#}p<0.05$ compared to stable AS patients.

of IL-4 and IL-10, which assist B-lymphocytes for their proliferation, differentiation and antibody production, in addition to the participation in humoral immune response, and may be related with allergic disease such as asthma[20,21]. Therefore, the stability of Th1/Th2 determines the immune homeostasis. This study demonstrated significantly elevated IL-4 and IL-10 expression in serum of AS patients, which also had decreased IFN-γ and TNF-α expression, especially during active phase, suggesting the existence of Th1/Th2 cell imbalance in AS patients.

Bautista-Caro et al[22] showed the involvement of miR in immune modulation. Their abnormal expression in autoimmune diseases, such as SLE, may participate in the autoimmune disease. The specific expression of miR in peripheral blood can reflect the disease occurrence, progression

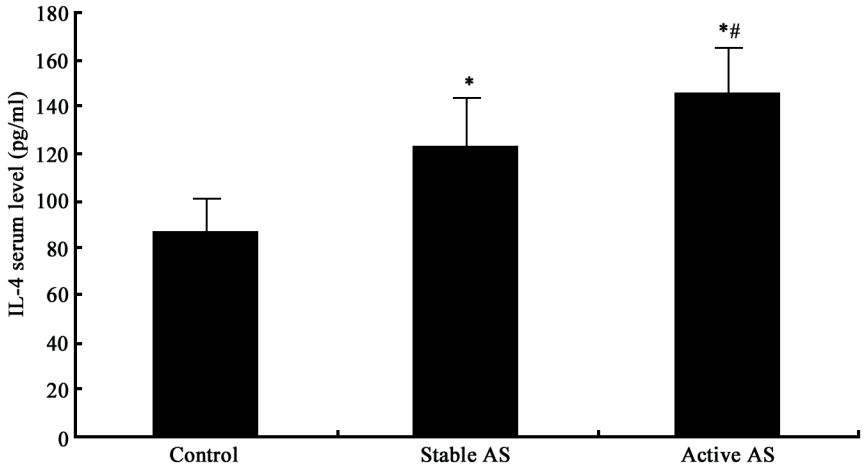

Figure 4. Serum IL-4 level. $^{*}p<0.05$ compared to control group, $^{\#}p<0.05$ compared to stable AS patients.

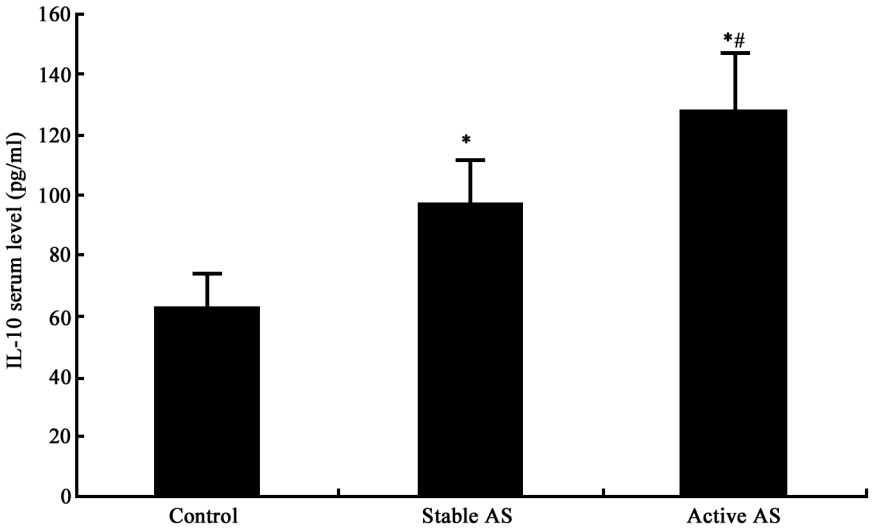

Figure 5. Serum IL-10 level. $^{*}p<0.05$ compared to control group, $^{\#}p<0.05$ compared to stable AS patients.

and severity. MiR has higher stability as it cannot be easily degraded by RNAse, making it being capable for repeated freeze/thaw and longer-term storage. MiR level is also stable regarding either quantity or quality in serum and plasma. Therefore, miR can work as a serum molecular marker for several diseases[23]. CRP and ESR can reflect AS disease activity to certain extents but lacked specificity, affecting their diagnostic value for early AS[24]. However, the molecular diagnostic marker for AS has not been determined yet. We found a significantly elevated miR16a expression in peripheral blood samples of AS patients compared to that in healthy controls, plus higher miR16a level in active AS patients compared to stable ones. In addition, miR16a level was positively correlated with IFN-γ and TNF-α or disease active index, and was negatively correlated with IL-4 and IL-10 but not with CRP or ESR. This result suggested that miR16a could reflect AS disease condition, probably relating with Th1/Th2 secretory cytokine imbalance.

Conclusions

We found that miR16a was significantly up-regulated in peripheral blood of AS patients and can

reflect the active status of the disease. Its mechanism is probably related to modulating Th1/Th2 balance. This study provided further evidence for selecting molecular targets of clinical diagnosis/treatment of AS.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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