

ORIGINAL ARTICLE

Plasma miR-146a and miR-365 expression and inflammatory factors in patients with osteoarthritis

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Abstract

Objective: To investigate the expression levels of micro-ribonucleic acid (miR)-146a and miR-365 in the plasma of osteoarthritis (OA) patients, to study their expression with the inflammatory factors and the severity of disease in patients and to analyse their diagnostic significance. **Materials and Methods:** A total of 42 OA patients diagnosed with OA and treated in our hospital from January 2017 to January 2018 were selected as the subjects, and 28 healthy people were enrolled as controls. The expressions of interleukin-1 beta (IL-1 β) and IL-6 in the plasma of OA patients were detected via immunohistochemical staining. Moreover, the knee joint function of OA patients was evaluated by Lysholm score, Western Ontario and McMaster Universities (WOMAC) score and Visual Analogue Scale (VAS) score. The expression levels of plasma miR-146a and miR-365 in OA patients were measured through RT-PCR. Besides, the significance of the expression levels of miR-146a and miR-365 for the diagnosis of OA was analysed by ROC curves. **Results:** As compared with healthy people, OA patients had elevated expression levels of plasma IL-1 β and IL-6, decreased Lysholm score, increased WOMAC and VAS scores as well as significantly up-regulated levels of plasma miR-146a and miR-365, which were of important significance for diagnosis. **Conclusion:** The expression levels of plasma miR-146a, miR-365 and inflammatory factors are notably higher, the disease is more severe, and the function of knee joint movement is weaker in OA patients than those in healthy controls. It can be concluded that the levels of both miR-146a and miR-365 can serve as biomarkers of OA diagnosis.

Keywords: miR-146a, miR-365, osteoarthritis, inflammatory factors, diagnostic significance

INTRODUCTION

Osteoarthritis (OA), also known as osteoarthropathy, is a type of degenerative disease that frequently occurs in middle-aged and elderly people. Studies have demonstrated that OA is associated with obesity, trauma, strain and other factors.^{1,2} Arthralgia, stiffness, joint swelling and joint deformity are the major clinical manifestations of OA.³ The treatment protocols for OA include reduction of joint bearing and excessive vigorous movement and administration with analgesics, anti-inflammatory drugs and cartilage protective agents. Besides, the patients in the advanced stage can be treated by artificial joint replacement surgery under acceptable conditions, but the postoperative effects need to be further improved.^{4,5} Due to the insidious onset

and atypical clinical symptoms of OA in the early stage as well as extremely limited regeneration ability in the advanced stage, such a degenerative disease is irreversible, seriously threatening people's health and bringing economic burdens to the society. Therefore, exploring the schemes or means for early intervention or diagnosis of OA is particularly important.

With the constant development of molecular biology in recent years, some studies on non-coding ribonucleic acids (RNAs) are gradually conducted.⁶ Notably, related research on micro-RNAs (miRNAs), a category of short-chain non-coding RNAs, becomes more and more mature. miRNAs can inhibit the translation of genes into proteins or promote their degradation by binding to these genes.⁷ Moreover, miRNAs are

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highly conservative and tissue-specific *in vivo*. It has been proven in studies that the abnormally expressed miRNAs can predict the occurrence and development of disease in organisms, and they are closely involved in regulating vital biological behaviours of cells.^{8,9}

Recently, a large amount of literature has illustrated that miRNAs probably participate in the pathogenesis of bone diseases and autoimmune diseases. Given the existence of certain specific miRNAs in bone joints, researchers have assumed that miRNAs can be used as a new kind of biomarkers to predict and diagnose bone-related diseases. For example, Niimoto *et al.* studied and found that the level of miR-146a is up-regulated in the knee joint synovial cells of patients with synovitis.¹⁰ Guan *et al.* revealed that miR-146a expression is also up-regulated in OA patients¹¹, implying that miR-146a is intimately involved in the progression of bone diseases. In addition, Yang *et al.* denoted that miR-365 is capable of facilitating chondrocyte differentiation and aggravating inflammatory responses at the site of OA¹², indicating that miR-365 may be a biomarker of OA. However, the correlations of the expressions of miR-146a and miR-365 with the expression of inflammatory factors and OA severity in patients and their diagnostic significance are rarely reported. In view of the above research levels, therefore, the levels of miR-146a and miR-365 in the plasma of OA patients in our hospital were detected via quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in this research, as well as to investigate their relations with the inflammatory factors and OA severity in patients and to explore their application value in the early diagnosis of the disease.

MATERIALS AND METHODS

Reagents

First-strand complementary deoxyribonucleic acid (cDNA) synthesis kit, miRcute miRNA extraction kit and fluorescence qRT-PCR kit were purchased from TIANGEN Biotech (Beijing) Co., Ltd., and primers of miR-146a and miR-365 and immunohistochemistry kit from Invitrogen, USA.

Instruments

Fluorescence qPCR instrument (Bio-Rad, USA), high-speed low-temperature centrifuge (Eppendorf, Germany), -80°C refrigerator and

pipette (Thermo Fisher Scientific, USA), and microscope (Olympus Corporation, Japan).

General data

A total of 42 OA patients (average age: 55.39±7.19 years old) treated and diagnosed in our hospital from January 2017 to January 2018 were selected as the subjects, and 28 healthy people who were aged 49 years old on average and received physical examination in our hospital were enrolled as controls. The KL grade of those OA patients was I-III grade. All patients had pain, swelling, limited activity and other main clinical manifestations of OA, while none of the controls had any clinical manifestation. This research was approved by the Ethics Committee of the hospital Ethical approval code: AH-1036).

Detection of protein expression levels of interleukin-6 (IL-6) and IL-1 β in OA patients via immunohistochemical staining

Human serum was obtained from each group, and the plasma was collected, fixed in 4% paraformaldehyde, permeabilised and incubated with 3% BSA for 1 h for surface antigen retrieval. After washing in phosphate-buffered saline (PBS), the plasma was incubated with IL-6 and IL-1 β primary antibodies (1:500) at 37°C for 1 h and washed with PBS again for 5 min \times 3 times. Later, HRP-labeled secondary antibodies were added for 20 min of incubation at 37°C, and the plasma was washed with PBS for 3 times, followed by colour development with DAB and observation of staining results under the microscope.

Detection via Lysholm score for OA patients

Lysholm knee scoring criteria, proposed by Lysholm in 1982, refer to a kind of method for assessing the knee joint function. The scoring system consists of 8 questions, with a total score of 0-100 points, including pain (25 points), instability (25 points), locking (15 points), swelling (10 points), limp (5 points), stair climbing (10 points), squatting (5 points) and support (5 points). The score indicated the excellent (95 points and above), good (85-94 points), fair (65-84 points) and poor function (below 65 points).

Detection via Western Ontario and McMaster Universities (WOMAC) score for OA patients

Every patient enrolled filled the WOMAC questionnaire by reference to the WOMAC Osteoarthritis Index scale. The changes in the

structure and function of the knee joint are evaluated mainly from the aspects of pain (5 items), stiffness (2 items) and physical function (17 items), and each item is scored 0-4 points, which correspond to none (0 point), mild (1 point), moderate (2 points), severe (3 points) and extremely severe (4 points). In summary, the WOMAC score ranges from 0-96 points, which is classified as extremely severe (≥ 70 points), severe (48-70 points), moderate (21-48 points) or mild (≤ 20 points).

Detection via Visual Analogue Scale (VAS) for OA patients

The pain in the case of OA was evaluated using VAS. Basically, a cursor (about 10 cm long) with 10 scale bars on one side, ranging from 0 point to 10 points. In clinical evaluation, the VAS score indicates no pain (0 point), excellent (1-2 points), good (3-5 points), poor (6-8 points), fairly poor (9 points) and unbearable severe pain (10 points).

Detection of expression levels of plasma miR-146a and miR-365 in OA patients via qRT-PCR
The venous blood (3 mL) was drawn, placed at room temperature for 0.5 hour and centrifuged at 4°C, 8000rpm for 15 minutes. Then 100 μ L of plasma was aspirated, added with miRcute miRNA extracting solution, chloroform and absolute alcohol to extract the miRNAs in the plasma. Next, the miRNAs were synthesised into corresponding cDNAs according to the instructions of the first-strand cDNA synthesis kit. Subsequently, the primers of miR-146a and miR-365 (Table 1) were added into the system for 35 cycles of fluorescence qPCR amplification. As for the experimental results, the single peak in melting curve was set as the evaluation criterion of specificity and validity of experiments, and the expressions of miRNAs in the plasma were calculated by $2^{-\Delta\Delta Ct}$.

Statistical analysis

SPSS 19.0 software was adopted for statistical analysis, data were presented as mean \pm standard deviation, and *t*-test was used for comparison

of statistical data between groups. The receiver operating characteristic (ROC) curves of OA patients were plotted to determine the optimal diagnostic critical value, and the area under ROC curve (AUC) was utilised to assess the sensitivity of miR-146a and miR-365 in the diagnosis of OA patients. $p < 0.05$ suggested that the difference was statistically significant.

RESULTS

Expression levels of plasma IL-6 and IL-1 β were elevated in OA patients

The results of immunohistochemical staining (Figure 1A-B) indicated that the expression levels of plasma IL-6 and IL-1 β were elevated in OA group compared with those in control group, suggesting that the expression level of inflammatory factors in the plasma is raised markedly, and apparent inflammatory responses occur in OA patients.

Declined Lysholm score of OA patients

The knee joint function of OA patients was judged by means of Lysholm score. As shown in Table 2, OA group had poorer Lysholm scores than control group [(45.81 \pm 5.27) points (poor) vs. (89.68 \pm 6.94) points (good), $*p < 0.05$], implying that the knee joint function declines in OA patients (Table 2).

Increased WOMAC score of OA patients

The WOMAC score was applied to assess the osteoarthritis index. The results (Table 3) manifested that the pain index ($*p < 0.05$), stiffness index ($*p < 0.05$) and physical function index ($*p < 0.05$) were increased in the OA group compared with those in the control group. The WOMAC score indicated the severe condition in the OA group, demonstrating that pain and stiffness of knee joints are the clinical manifestations of OA patients, causing inconvenience to daily life (Table 2).

Raised VAS score of OA patients

The degree of knee pain was determined in accordance with the VAS scoring standards. It

TABLE 1: Primer information

List	5'-3' primer
MiR-146a	TGCAGGGTCCGAGGTATTCGCAC
MiR-365	TAATGCCCTAATAAATCCTTAT
β -actin	GGCTGTATTCCCCTCCATCG

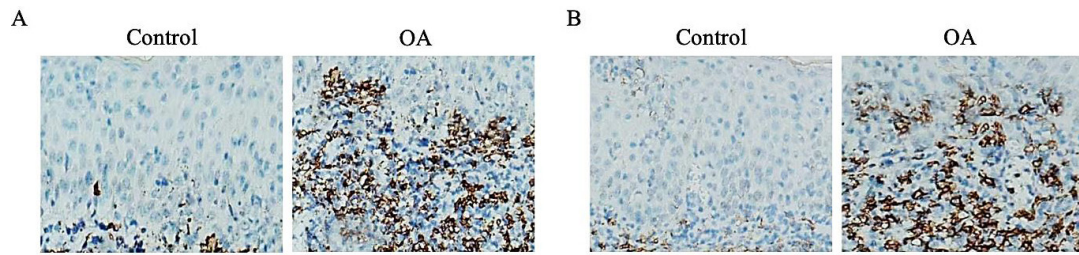


FIG. 1: Comparisons of protein levels of IL-6 and IL-1β via immunohistochemical staining. A: IL-6 expression, B: IL-1β expression (20×)

was revealed that the pain index was raised in OA group in comparison with that in control group ($p < 0.05$) (Table 2). Moreover, the OA group exhibited a higher VAS score [(7.26±1.33) points] and a poorer pain index, suggesting that the VAS score is high in OA patients (Table 2), besides, we learned from the medical history the unbearable pain has affected patients' sleep.

Expressions of plasma miR-146a and miR-365 were decreased in OA patients

To investigate the changes in miRNAs between the OA group and the control group, the levels of plasma miR-146a and miR-365 at 1 d and 7 d were measured via qRT-PCR. According to the results, the levels of plasma miR-146a and miR-365 rose distinctly on the 1st and 7th days in OA group in comparison with those in control group ($p < 0.05$, $p < 0.05$), and they were increased more obviously on the 7th day (Figure 2A-B).

Expressions of plasma miR-146a and miR-365 had early diagnostic value for OA

The ROC curves were plotted according to the expression levels of miR-146a and miR-365 in the plasma. It was demonstrated through analysis that the diagnostic efficiency of plasma miR-146a and miR-365 in OA patients was high on the 1st day. Furthermore, the AUC of miR-146a was

0.8843 ($p = 0.002$, $p < 0.05$), the AUC of miR-365 was 0.8182 ($p = 0.011$, $p < 0.05$), suggesting that the sensitivity and diagnostic accuracy of miR-146a were also higher (Figure 3), and the expressions of plasma miR-146a and miR-365 possess favourable values for early diagnosis of OA.

DISCUSSION

OA is the most common disease in rheumatology and orthopedics, whose aetiology and pathogenesis have not been clarified so far. However, it has been revealed that OA is correlated with sex, age, obesity and strain.¹³ The morbidity rate of OA is rising year by year along with population aging, and the disease has become one of the leading causes of lower limb disorders in middle-aged and elderly people, seriously affecting the daily life of patients and bringing heavy burdens to the society at the same time.¹⁴ Hence, the diagnosis and treatment of OA patients are always the hotspots of orthopedic research. Despite the continuous development of medicine and certain progress in OA treatment, OA still needs to be explored by more in-depth studies.

In this research, the conditions of 42 OA patients diagnosed and treated in our hospital were observed. Research have shown the

TABLE 2: Comparison of knee joint function, OA indexes and joint pain

List	n	Lysholm Score (mean±SD)	Pain Score (mean±SD)	Stiffness Score (mean±SD)	Physical function Score (mean±SD)	VAS Score (mean±SD)
Control	28	89.68±6.94	2.45±1.28	1.27±0.45	9.42±3.41	1.02±0.42
OA	42	45.81±5.27*	10.42±3.26*	6.71±1.73*	42.69±4.88*	7.26±1.33*
t value		23.493				4.624±0.94
p value		0.0000				0.0000

* $p < 0.05$: the difference in Lysholm knee score, WOMAC score and VAS score were statistically significant between the two groups.

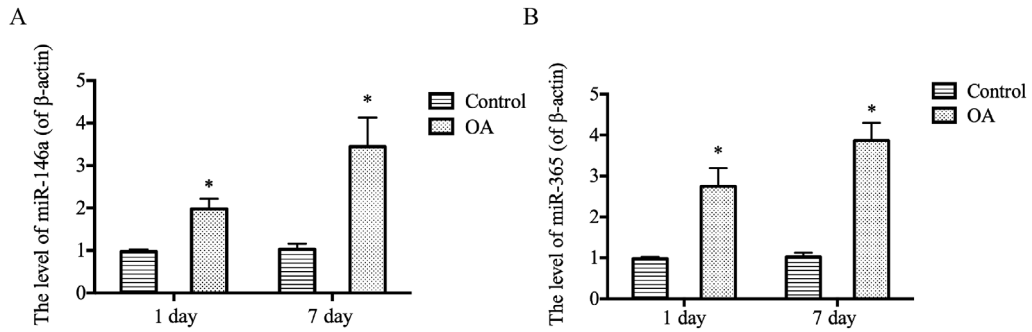


FIG. 2: Comparisons of miR-146a and miR-365 levels through qRT-PCR. A: Expression level of miR-146a, B: Expression level of miR-365 (* $p < 0.05$: OA group vs. control group).

increased inflammatory factors can influence the knee joint function in OA patients to some extent and reflect the severity of disease.¹⁵ First, we detected two cytokines in the plasma, IL-6 and IL-1 β , whose expression levels can reflect the severity of inflammation via immunohistochemical staining. As shown in Figure 1, the positive expression levels of IL-6 and IL-1 β (sepia) were remarkably higher in the OA group than those in the control group, implying distinctly up-regulated expressions of inflammatory factors and inflammatory responses in OA patients.

Then the severity of OA was evaluated in this research. Firstly, the knee joint function in OA patients was assessed using the Lysholm score, which is a set of evaluation criteria for knee joint function. The research results of Briggs *et al.* also showed that the knee joint function is impaired, and the Lysholm score is fairly low.¹⁶ Based on the results in this research (Table 2), the OA patients exhibited a lowered Lysholm knee score, reduced knee joint function and limited

activities compared with the control group. Subsequently, the WOMAC scoring system was employed to evaluate the indexes of knee OA. Culvenor *et al.* conducted 15 studies involving more than 8000 participants and discovered that the more apparent the inflammation in OA patients is, the higher the WOMAC score will be.¹⁷ In this research, it was manifested that the indexes of pain, stiffness and physical function of knee joint were raised in the OA group in contrast with those in the control group (Figure 3), illustrating that there are inflammatory responses in the OA patients, and the activity of daily living are influenced severely. Moreover, the intensity of knee joint pain was assessed through VAS. Huang *et al.* discovered through study that the knee joint function of OA patients is restored, the knee joint pain is alleviated, and the VAS score is decreased after a month of isometric contraction of quadriceps femoris.¹⁸ And the results in this research revealed that the OA group had a notably higher VAS score than the control group (Table 2). The aforementioned

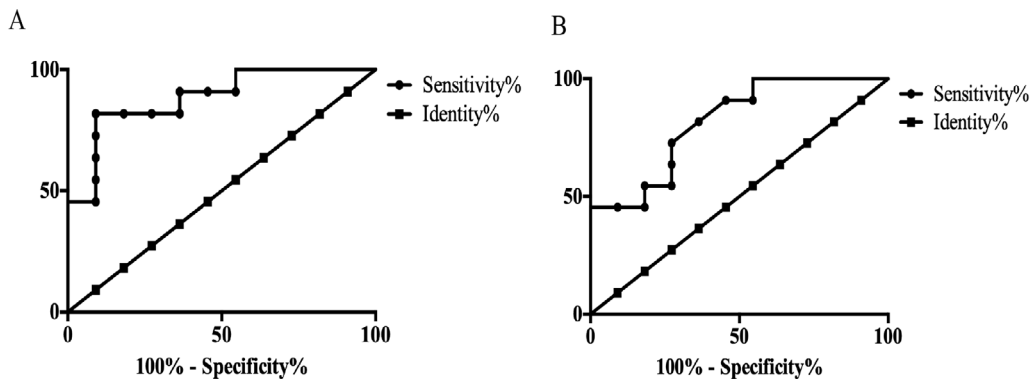


FIG. 3: ROC curves of plasma miR-146a and miR-365 in diagnosing OA patients. A: ROC curves of miR-146a, B: ROC curves of miR-365.

findings demonstrate that OA patients have stiff knee joints, weakened function, distinct pain sensation and impaired activity of daily living.

The expression levels of plasma miR-146a and miR-365 were determined in the two groups, as well as to find the biomarkers for early diagnosis of OA. As a kind of small RNAs, miRNAs can regulate the transcription of RNAs or repress the translation of RNAs into proteins by conjugating with targets.¹⁹ Currently, a growing number of studies have confirmed that miRNAs play crucial regulatory roles in bone diseases. Kopańska *et al.* conducted a study for 29 OA patients and discovered that the expression levels of 4 types of miRNAs are elevated, including miR-146a and miR-138-3p, denoting that these up-regulated miRNAs may be conducive to preventing and diagnosing OA.²⁰ In this research, the expression levels of plasma miR-146a and miR-365 in the OA patients and healthy people were measured after diagnosis and at 1 and 7 d before treatment. The results demonstrated that on the 1st and 7th days, the levels of plasma miR-146a and miR-365 were higher in OA group than those in control group, and they were remarkably higher in OA group on the 7th day than those on the 1st day (Figure 2), elucidating that the levels of miR-146a and miR-365 rise more prominently with the extension of time. Finally, the ROC curves in this research displayed that the expressions of miR-146a and miR-365 in the plasma have important significance for early diagnosis of OA, with good sensitivity and specificity.

In conclusion, multiple miRNAs in the peripheral blood are abnormally expressed in the OA patients, and their levels change along with the development of disease. According to the results in this research, the increases in plasma miR-146a and miR-365 levels signify aberrant inflammatory responses, decreased knee joint function as well as knee joint pain and stiffness in the OA patients, seriously influencing daily life and activity. As the research is deepened constantly, the detection of miRNAs in the peripheral blood has wider prospects for the early diagnosis, differential diagnosis and treatment of OA.

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MS and FR provided help and advice. HC analyzed the data. WW, XY, HC and FR wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

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