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Plasma miR-200c-3p, miR-100-5p, and miR-1826 serve as potential diagnostic biomarkers for knee osteoarthritis

Randomized controlled trials

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Abstract

Objective: To study the potential diagnostic value of plasma miR-200c-3p, miR-100-5p, and miR-1826 levels in knee osteoarthritis (KOA).

Methods: Real-time quantitative PCR (RT-PCR) was used to measure the expression levels of serum miR-200c-3p, miR-100-5p, and miR-1826 in 150 KOA patients and 150 control controls. In addition, the levels of *DNMT3A*, *ZEB1*, *MMP13*, and *CTNNB1* mRNAs in the synovial fluid were also measured by RT-PCR.

Results: The expression levels of miR-100-5p, miR-200c-3p, and miR-1826 in the synovial fluid of 150 KOA patients were significantly lower than those in 54 controls ($P < .001$). In the synovial fluid, the miR-100-5p and *DNMT3A* mRNA levels, miR-100-5p and *ZEB1* mRNA levels, miR-200c-3p and MMP13 mRNA levels, and miR-1826 and *CTNNB1* mRNA levels were all negatively correlated ($r = -0.83, -0.81, -0.83, -0.58$, respectively). The AUCs of the diagnosis for KOA using the plasma levels of miR-200c-3p, miR-100-5p, and miR-1826 were 0.755, 0.845, and 0.749, respectively.

Conclusion: The plasma levels of miR-200c-3p, miR-100-5p, and miR-1826 are of potentially high value in the diagnosis of KOA.

Abbreviations: AUC = the area under the curve, BMI = body mass index, CTNNB1 = catenin beta 1, DNMT3A = DNA methyltransferase 3 alpha, EDTA = ethylenediaminetetraacetic acid, KOA = knee osteoarthritis, MMP13 = matrix metallopeptidase 13, OA = osteoarthritis, ROC = receiver operating curve, RT-PCR = Real-time quantitative PCR, SD = standard deviation, ZEB1 = zinc finger E-box binding homeobox 1.

Keywords: biomarker, knee osteoarthritis, miR-100-5p, miR-1826, miR-200c-3p

1. Introduction

Osteoarthritis (OA) is a common chronic and progressive joint disease that occurs in middle-aged and elderly people. The clinical manifestations are different degrees of joint swelling and pain, limited mobility, and other symptoms. The pathological features are a progressive destruction of the articular cartilage and subchondral bone, and synovial inflammation of the joints.[1,2] In recent years, the incidence of knee osteoarthritis (KOA) has been on the rise, and it has become the primary factor that causes pain and disability in the elderly.[3] Owing to the poor ability of articular cartilage to repair itself, and the limited efficacy of the currently used conservative treatment, most patients need artificial knee replacements as the disease progresses.[4] According to several studies, biochemical changes in the damaged articular cartilage precede morphological changes, so an ability to detect relevant enzymes and cytokines, as well as other biological markers, in body fluids (e.g., synovial fluid, serum, or urine) from patients could provide a new means for the early diagnosis of KOA.[5–7]

MicroRNAs (miRNAs) are known to be involved in the development of numerous diseases such as cancer,[8] liver fibrosis and cirrhosis,[9] and OA.[10] To maintain cartilage, chondrocytes upregulate anabolic and catabolic processes by responding to growth factors and cytokines. Studies have shown that miRNAs are involved in regulating the expression of genes in the signaling pathway that controls cartilage transformation.[11,12] Studies have shown that the interaction between miR-200c-3p and ZEB1 may contribute to chondrocyte hypertrophy, so that chondrocytes change from a quiescent state to a proliferative state, and then differentiate.[13] The important role of miR-100–5p in the pathogenesis of various cancers has been confirmed by many

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studies.[14,15] miR-1826 is involved in the occurrence and development of malignant tumors. Studies have found that the upregulation of miR-1826 may be a marker of the prognosis of CRC, and could be a potential therapeutic target for the treatment of CRC.[16]

The action of miRNAs is also crucial in the occurrence and development of KOA. At present, a variety of miRNAs with potential diagnostic KOA values have been predicted by computational means, and include miR-200c-3p, miR-100–5p, and miR-1826.[17] This study aimed to investigate the potential value of plasma miR-200c-3p, miR-100–5p, and miR-1826 levels in the diagnosis of KOA.

2. Materials and methods

2.1. General information

From February 2015 to March 2018, this study enrolled 150 patients with KOA (KOA group), aged 35 to 87 years, with an average age of 58.33 ± 11.13 years. The American College of Rheumatology diagnostic KOA criteria were used for patient diagnosis.[18] The severity of KOA was classified according to the Kellgren and Lawrence scoring systems, and included 75 patients with grade 2, 54 patients with grade 3, and 21 patients with grade 4. We recruited 150 non-KOA patients as a control group. These patients included 54 cases of traumatic amputation or meniscus injury surgery, and 96 healthy subjects. All the control patients had Kellgren and Lawrence scores of 0. The study protocol was approved by our ethics committee, and all participants in the study signed an informed consent form.

2.2. RNA extraction

Approximately 10 mL of venous blood was collected from all patients in an ethylenediaminetetraacetic acid (EDTA) collection tube, allowed to stand for 2 hours, and then centrifuged at 2000 r/m for 25 min. In addition, 1 mL of knee joint synovial fluid was collected from all of the KOA patients, as well as the 54 subjects from the control group, and then centrifuged at 13,000 r/m for 20 minutes within 1 hour after sampling, and the supernatant was stored in an Eppendorf tube at 80°C before testing. Total RNA was purified using the AllPrep DNA/RNA Micro kit (Qiagen, Valencia, CA).

2.3. cDNA synthesis

cDNA synthesis was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, CA). From the kit protocol, 10 × RT buffer, dNTP mix w/dTTp (100 M total), RNase inhibitor (20 U/μL), MultiScribeTM RT enzyme (50 U/μL), water, and primers were then used to extract total RNA as a template to synthesize cDNA.

2.4. Real-time quantitative PCR (RT-PCR)

The synthesized cDNA was used as a template, using primers with the sequences shown in Table 1 for the RT-PCR reaction, and an ABI 7900 HT Fast Real-Time PCR system (Applied Biosystems). The RT-qPCR conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 60 seconds. The expression levels of miRNAs relative to U6 were calculated using the 2-ΔΔCt method.

Table 1

RT-PCR primer information.

	Primer sequence
miR-200c-3p	Forward: 5′-GGG GTA GGG GAA GGT GGT TTA-3′
	Reverse: 5′-CAC CAC CCC AAT CCC TAA AAA CAC T-3′
miR-100-5p	Forward: 5′-CGG GTA CCG GTT CAG ACA TGT CAC AGC CCC-3′
	Reverse: 5′-CGG AAT TCA AAA AGG AAA CTA AGG GGA AGA AG-3′
miR-1826	Forward: 5′- GCA TTG ATC ATC GAC ACT TCG A-3′
	Reverse: 5′- AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT-3′
MMP13 mRNA	Forward: 5′-TTG CAG AGC GCT ACC TGA GAT CAT-3′
	Reverse: 5′-TTT GCC AGT CAC CTC TAA GCC GAA-3′
U6	Forward: 5′-CTT CGG CAG CAC ATA TAC-3′
	Reverse: 5′-GAA CGC TTC ACG AAT TTG C-3′
DNMT3A mRNA	Forward: 5′-GGA AGG CTA CCT GGC TAA AGT CAA G-3′
	Reverse: 5′-ACT GAA AGG GTG TCA CTG TCC GAC-3′
ZEB1 mRNA	Forward: 5′-GCT TCT CAC ACT CTG GGT CTT A-3′
	Reverse: 5′-CCT CAT TCT CTG CCT CTT CTA CC-3′
CTNNB1 mRNA	Forward: 5′-CCA AGT GGG TGG TAT AGA G-3′
	Reverse: 5′-GGG ATG GTG GGT GTA AGA-3′

CTNNB1 = catenin beta 1, DNMT3A = DNA methyltransferase 3 alpha, MMP13 = matrix metallopeptidase 13, RT-PCR = real-time quantitative PCR, ZEB1 = zinc finger E-box binding homeobox 1.

2.5. Western blotting to detect protein expression levels

Articular cartilage was obtained from 3 KOA patients and 3 control subjects. These samples were used for western blotting to detect the protein levels of DNMT3A (anti-human DNMT3A antibody, #681615, R&D systems, Minneapolis, MN, USA), ZEB1 (anti-human ZEB1 antibody, #639914, R&D Systems), MMP13 (anti-human MMP13 antibody, #87512, R&D Systems), and CTNNB1 (anti-human CTNNB1 antibody, #196621, R&D Systems). The protein concentration in the cartilage samples was quantified using a BCA Kit (Sigma-Aldrich, St Louis, MO). Protein extracts were transferred to PVDF membranes, blocked with 5% skimmed milk in TBST (Tris-buffered saline and Polysorbate 20 (Tween 20) containing 50 mM Tris, 150 mM NaCl, 0.1% Tween 20), and then incubated with the appropriate primary antibody overnight at 4°C. The membranes were then incubated for 1 hour at room temperature with the secondary antibody, after which the proteins were detected using an enhanced chemiluminescence (ECL) (GE Healthcare, Chalfont St Giles, Bucks, UK) reagent.

2.6. Statistical analysis

The categorical variables are expressed as a percentage [n (%)] and a statistical analysis was performed using a Chi-square test (χ^2). Continuous variables are expressed as mean ± SD and the statistical analysis was performed using an independent sample *t* test. The receiver Operating Curve (ROC) and the area under the curve (AUC) were used to evaluate the value of miRNAs in the diagnosis of KOA. $P < .05$ indicated that the difference was statistically significant.

3. Results

3.1. Demographic characteristics

Of the 150 patients with KOA, 84 were male and 66 were female, with ages in the range of 35 to 87 years, and a BMI range of 18.9 to 29.8 kg/m^2. Of the 150 healthy controls, 88 were male and 62

Table 2

General information.

	KOA group (n = 150)	Control group (n = 150)	*P* value
Age (years, mean ± SD)	58.33 ± 11.13	58.61 ± 8.19	.80
Gender [n (%)]			
Male	84 (56.00%)	88 (58.67%)	.64
Female	66 (44.00%)	62 (41.33%)	
Body mass index (kg/m^2, mean ± SD)	24.91 ± 2.42	25.07 ± 2.65	.59
Family history [n (%)]			
Yes	15 (10.00%)	10 (6.67%)	.30
No	135 (90.00%)	140 (93.33%)	
Smoking [n (%)]			
Yes	61 (40.67%)	58 (38.67%)	.72
No	89 (59.33%)	92 (61.33%)	
Alcohol consumption [n (%)]			
Yes	65 (43.33%)	61 (40.67%)	.64
No	85 (56.67%)	89 (59.33%)	
Kellgren-Lawrence grading			
2	75 (50.00%)		
3	54 (36.00%)		
4	21 (14.00%)		

BMI = body mass index, KOA = knee osteoarthritis.

were female, with ages in the range of 35 to 85 years and a BMI range of 18.9 to 31.5 kg/m^2. Using the Kellgren–Lawrence scale, 75 cases were found to be grade 2, 54 cases were grade 3, and 21 cases were grade 4. There were no significant differences in age, gender, BMI, KOA family history, smoking history, drinking history, and other general data, between the KOA group and the healthy control group ($P > .05$) (Table 2).

3.2. Expression of miRNAs

The expression levels of miR-100-5p, miR-200c-3p, and miR-1826 in the synovial fluid from the 52 KOA patients, and the 150 healthy controls were analyzed. The results showed that the expression levels of miR-100-5p, miR-200c-3p, and miR-1826 were significantly lower in the synovial fluid from the KOA patients compared with those in the healthy control group, and this difference was statistically significant ($P < .001$) (Figs. 1 and 2).

3.3. Expression levels for the miRNA target proteins

The expression levels of DNMT3A, ZEB1, MMP13, and CTNNB1 in articular cartilage were assessed by western blotting. The data showed that in the articular cartilage from KOA patients the levels of DNMT3A, ZEB1, MMP13, and CTNNB1 were significantly higher than those in the cartilage from control healthy patients, as shown in Figure 3.

3.4. Correlation between miRNAs expression levels in synovial fluid and the corresponding target mRNA levels

RT-PCR was used to assess both the expression levels of miRNAs in synovial fluid and the levels of their target mRNAs. The results showed that the miR-100-5p levels were negatively correlated with *DNMT3A* mRNA levels (r = −0.83, Fig. 4A), and negatively correlated with *ZEB1* mRNA levels (r = −0.81, Fig. 4B). In addition, the miR-200c-3p levels were negatively correlated with the *MMP13* mRNA levels (r = −0.83, Fig. 4C), and the miR-1826 levels were negatively correlated with *CTNNB1* mRNA levels (r = −0.58, Fig. 4D).

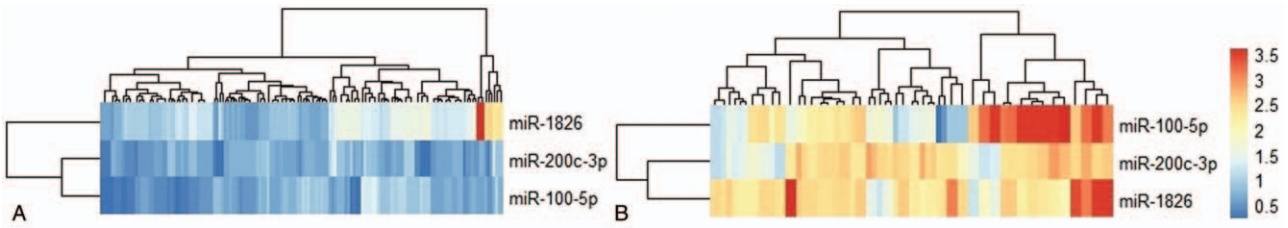

Figure 1. Heat map of miRNAs expression levels. A indicates the expression levels of miR-100-5p, miR-200c-3p, and miR-1826 in synovial fluid of KOA group; b indicates the expression levels of miR-100-5p, miR-200c-3p, and miR-1826 in synovial fluid of control group.

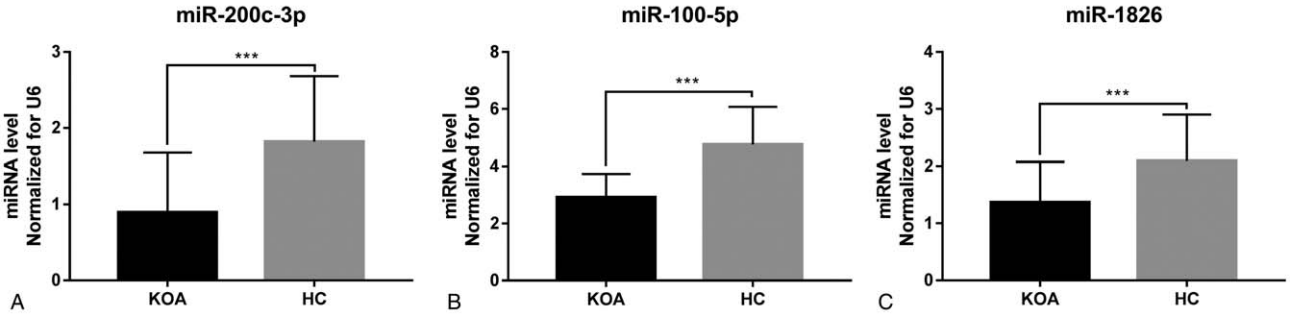

Figure 2. Comparisons of miR-100-5p, miR-200c-3p, and miR-1826 expression levels in synovial fluid of KOA patients and control groups. $^{***}P < .001$.

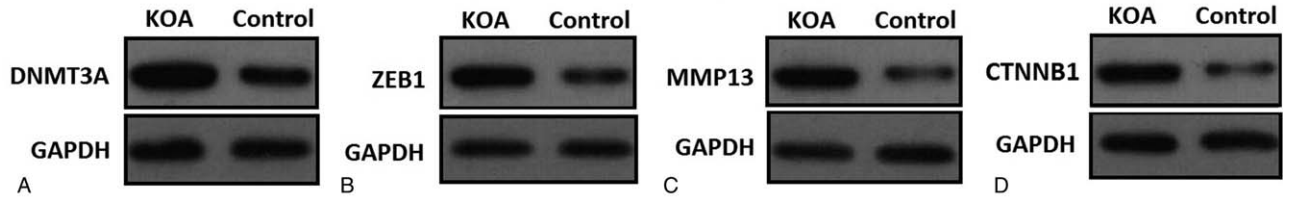

Figure 3. Detections of DNMT3A, ZEB1, MMP13, and CTNNB1 protein expression in articular cartilage tissue. Target gene/internal reference control gray value comparison. A: KOA (1.36), Control (0.64); B: KOA (1.29), Control (0.63); C: KOA (1.34), Control (0.50); D: KOA(1.06), Control (0.42).

3.5. Utility of plasma miRNAs to diagnose KOA using an ROC curve

The AUC for miR-200c-3p levels in the plasma was 0.755, the sensitivity was 82.00%, and the specificity was 82.00% (Fig. 5A). The AUC for miR-100-5p levels in the plasma was 0.845, the sensitivity was 82.00%, and the specificity was 86.00% (Fig. 5B). The AUC for plasma miR-1826 levels in the plasma was 0.749 with a sensitivity of 82.00% and a specificity of 86.00% (Fig. 5C).

4. Discussion

KOA is a degenerative joint disease that can cause damage to articular cartilage tissue.[19] As the population ages, more and more elderly people in China are threatened by KOA.[20]

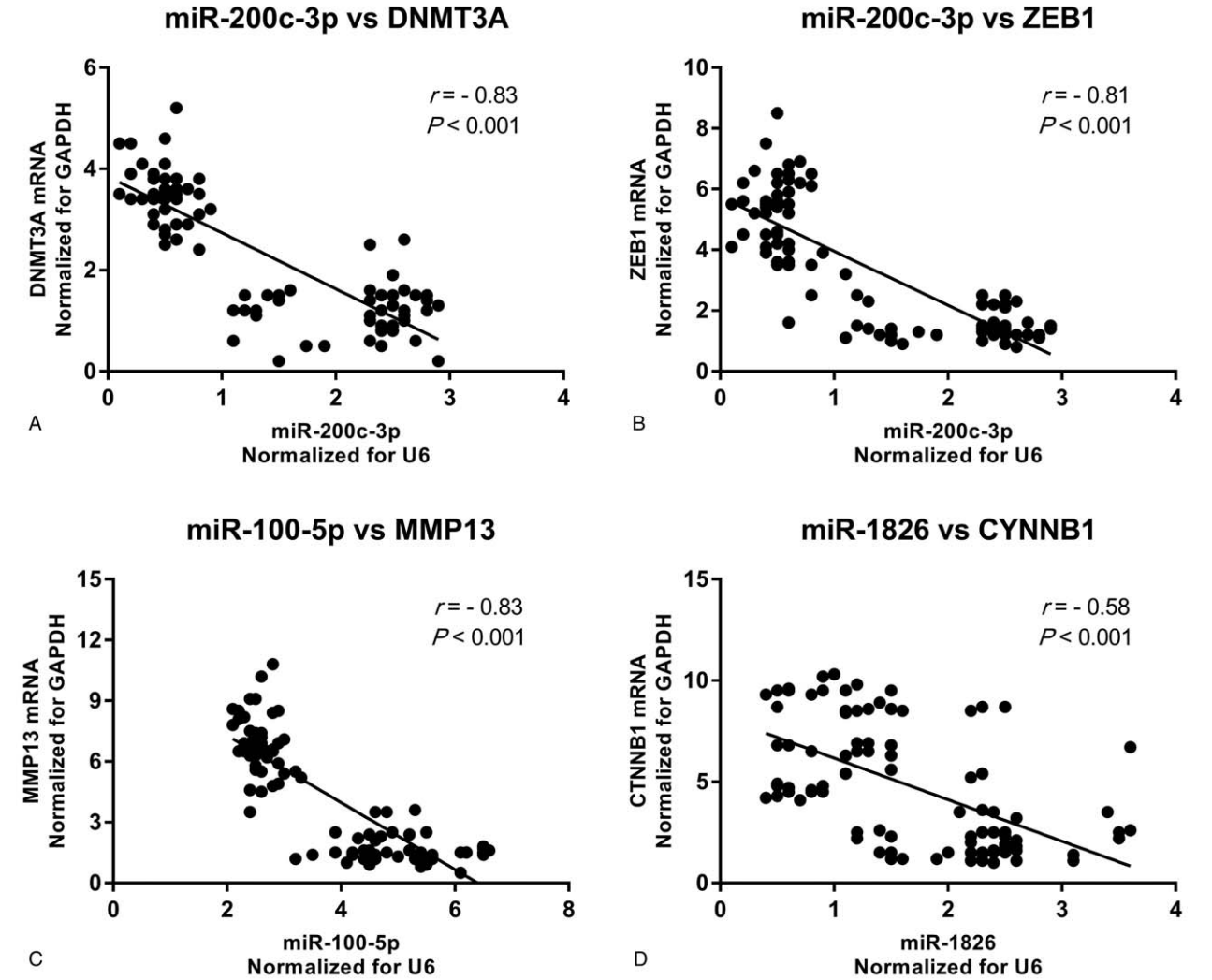

Figure 4. Correlation of miRNAs expression levels with their target mRNA levels. A is the correlation between miR-200c-3p expression level and DNMT3A mRNA level in synovial fluid; B is the correlation between miR-200c-3p expression level and ZEB1 mRNA level in synovial fluid; C is the correlation between miR-100-5p expression level and MMP13 mRNA level in synovial fluid; D is the correlation between miR-1826 expression level and CTNNB1 mRNA level in synovial fluid.

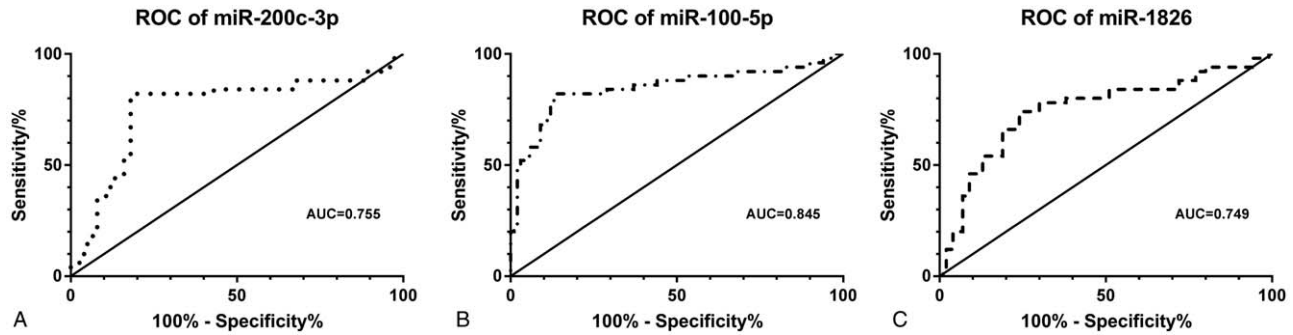

Figure 5. ROC curves for the diagnosis of KOA at the level of miRNAs. A shows the ROC curve of miR-200c-3p, AUC is 0.755; B shows the ROC curve of miR-100-5p, AUC is 0.845; C is the ROC curve of miR-1826, AUC is 0.749.

At present, the treatment of patients with KOA is a mainly conservative treatment plan that reduces pain and inflammation, but is not curative,[21] and drug treatment often causes side effects.[22,23] Therefore, it is important to explore the pathogenesis of KOA and study diagnostic and preventive measures for the treatment of KOA.

miRNAs play an important role in the regulation of many signaling pathways, especially those involved in gene regulation.[24] Previous studies have compared the expression of 365 miRNAs in the joint tissues of 10 normal controls and 33 OA patients, and explored the role of miRNAs in the pathogenesis of OA.[25,26] In the knee joint, cartilage is mainly comprised of the extracellular matrix (ECM) and chondrocytes. Chondrocytes maintain cartilage by up-regulating anabolic and catabolic processes in response to growth factors and cytokines. miRNAs play an important role in the regulation of the signaling pathways used by these growth factors and cytokines. For example, miR-140 plays an important role in the development of cartilage during aging and OA.[27,28] Some researchers have confirmed the importance of integrating information about miRNA targets and OA-related genes.[17] Using a computer simulation model 12 miRNAs have been predicted to have a potential diagnostic value for KOA. Among these, miR-200c-3p, miR-100-5p, and miR-1826 were not found to be related to the occurrence and development of KOA.

It has been shown that miR-200c-3p has a variety of downstream regulatory targets that are associated with a variety of processes, including apoptosis (BCL2, XIAP), the up-regulation of MMPs (VEGFA), chondrocyte hypertrophy (FLT1, JAG1, VEGFA), ECM maintenance (ERRFI1, FN1), inflammation (IKBKB, NTRK2, VEGFA), angiogenesis (TIMP2, VEGFA), and cytoskeleton maintenance (TUBB3).[17] In addition, 5 target proteins have been found to be involved in the maintenance of cartilage, chondrocyte hypertrophy, and OA pathogenesis, namely DNMT3A, DNMT3B, NOTCH1, SP1, and ZEB1. In this study, 2 miR-200c-3p target proteins were selected for analysis, namely DNMT3A and ZEB1. The analysis revealed that the expression levels of miR-200c-3p and DNMT3A and ZEB1 were negatively regulated. Low levels of miR-200c-3p were detected in the blood and synovial fluid from KOA patients, whereas the expression levels of DNMT3A and ZEB1 in synovial fluid and chondrocytes were higher than those in the control group. Consistent with the results of Bellon et al,[29] the interaction between miR-200c-3p and DNMT3A and ZEB1 may contribute to chondrocyte hypertrophy, so that chondrocytes change from a quiescent state to a proliferative state and then differentiate.

miR-100-5p has six related target proteins, namely FGFR3, FLT1, ID1, IGF1R, MMP13, and PLK1.[17] The target most relevant to KOA is matrix metalloproteinase 13 (MMP-13), which is a central node in the cartilage degradation network. MMP-13 levels and activities in OA chondrocytes are associated with upstream regulatory factors, DNA methylation, and various non-coding RNAs (ncRNAs).[30] The results of this study showed that miR-100-5p levels in the plasma and synovial fluid of patients with KOA were lower than those in healthy controls, whereas MMP13 expression in the articular cartilage was higher than that in the control group. Analysis of the miR-100-5p levels and *MMP13* mRNA levels in the synovial fluid of the joint showed that the 2 were negatively correlated, indicating that miR-100-5p is involved in the negative feedback regulation of MMP13 expression.

From the miRTarBase it can be predicted that miR-1826 targets only 3 genes that are associated with the development and progression of OA, namely *CTNNB1*, *MAP2K1*, and *VEGFC*. *CTNNB1* encodes the protein β-catenin, which is a key component of the Wnt signaling pathway,[31]*MAP2K1* encodes a kinase involved in MAPK/ERK signaling,[32] and *VEGFC* encodes a VEGF receptor.[33] In this study, the CTNNB1 protein was selected for analysis to investigate the correlation between the expression of miR-1826 and the expression of CTNNB1 in the plasma and synovial fluid. The results showed that the levels of miR-1826 in synovial fluid are negatively correlated with *CTNNB1* mRNA levels. The level of miR-1826 in the plasma and synovial fluid of KOA patients was significantly lower than that in healthy controls whereas the CTNNB1 protein levels were higher than those in the healthy controls. Currently, no other studies have shown that miR-1826 levels and CTNNB1 expression are associated with the occurrence of KOA. Hirata et al[34] found that miR-1826 plays an important role as a tumor suppressor by down-regulating CTNNB1/MEK1/VEGFC in breast cancer. They showed that CTNNB1 MEK1 and VEGFC are direct targets of miR-1826, and that there is a negative correlation between miR-1826 levels and CTNNB1, MEK1, or VEGFC protein expression, which is consistent with the results of this study.

Through an ROC curve analysis, we also found that plasma levels of miR-200c-3p, miR-100-5p, and miR-1826 have high diagnostic value for the diagnosis of KOA since the AUCs were higher than 0.5. These findings provide an alternative approach

to the diagnosis of clinical KOA. However, this study also has some limitations. First, our sample size was small, and the results have not yet been confirmed with a larger sample size. In addition, we have not studied the regulatory mechanisms of these miRNAs and their related target proteins in an in vitro model. Moreover, because the acquisition of tissue samples is difficult, only a small number of tissue samples were obtained. Finally, limited by the sample size, we did not strictly distinguish between patients with different degrees of KOA severity.

In summary, the expression levels of miR-200c-3p, miR-100-5p, and miR-1826 in the plasma of KOA patients are abnormal and may be of high value for the diagnosis of KOA.

Author contributions

Conceptualization: Yanguang Cao.
Data curation: Zhen Lai.
Formal analysis: Zhen Lai.
Funding acquisition: Zhen Lai.
Methodology: Zhen Lai.
Supervision: Yanguang Cao.
Writing – original draft: Zhen Lai.
Writing – review & editing: Yanguang Cao.

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