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Circulating MicroRNA-23b as a New Biomarker for Rheumatoid Arthritis

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Abstract

MicroRNA-23b (miR-23b) is associated with inflammation and autoimmune diseases. This study evaluated miR-23b expression and assessed its potential as a biomarker of disease activity for rheumatoid arthritis (RA). Differential expression of microRNAs was determined by miRNA microarray analysis in fibroblast-like synoviocytes (FLSs) from four trauma patients as healthy controls (HCs) and eight RA patients. The microarray results showed elevated expression of miR-23b in FLSs from RA patients and this finding was corroborated by real-time quantitative polymerase chain reaction (RT-qPCR) and *in situ* hybridization using synovial tissues (STs). Furthermore, we found miR-23b levels in plasma of RA patients were significantly higher than in HCs, and plasma miR-23b levels positively correlated with the erythrocyte sedimentation rate (ESR), hypersensitive C-reactive protein (hs-CRP), C-reactive protein (CRP), DAS28, and platelet

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Authors' contributions
YW and MW contributed to the conception and design of the study. XL and SN performed the main experiments. NX, MW, and XL are responsible for the acquisition of clinical samples. WC and CL completed the miRNA *in situ* hybridization analysis. SN and XL contributed to the analysis and interpretation of the data. XL and SN contributed to the drafting of the article. AVW and YW contributed critical revisions of the paper and provided important intellectual feedback. All the authors read and approved the final manuscript.

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Conflict of interest
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(PLT) count ($P < 0.05$). MiR-23b levels in plasma inversely correlated with the levels of hemoglobin (Hb), total bilirubin (TBIL), direct bilirubin (DBIL), indirect bilirubin (IBIL), total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) ($P < 0.05$), but not with rheumatoid factor (RF) or anti-cyclic citrullinated peptide antibodies (ACPA) ($P > 0.05$). Moreover, patients with anorexia showed higher levels of miR-23b in plasma than those without anorexia. Similar results were observed with fatigue. Appropriate treatment for RA not only ameliorated the disease condition but also reversed the elevated plasma miR-23b level remarkably. These results suggest that circulating miR-23b may be a promising biomarker for RA disease activity.

Keywords

Circulating microRNA-23b; rheumatoid arthritis; biomarker

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease (Smolen, Aletaha, & McInnes) which affects about 1% of the global population (Mcinnes & O'Dell, 2010). It chronically impairs the heart, skin, and many other organs. It is characterized by erosive alterations of the joint surfaces leading to destruction and deformity of the joints and eventual disability (Hellmich, 2014; Torpy, 2011). In addition to the joint-related symptoms, extra-articular symptoms and systemic lesions, such as anorexia, fatigue, morning stiffness, lymph nodes, and interstitial lung disease (ILD) can also be observed in patients with active RA (Benaglio et al., 2015; Kim, Kim, Moon, Lee, & Kim, 2015; Zhang, Li, Wu, Xin, & Yi, 2017). Nevertheless, the pathology of RA is not fully elucidated and diverse factors, such as genes, environment, gender, infection, and even dietary components are reported to get involved in the initiation and progression of RA (Vojdani, 2014). Recently, a growing body of evidence has pointed out that a cohort of microRNAs (miRNAs) existing in the peripheral blood gets elevated in the plasma or serum of RA patients and are involved in the pathogenesis of RA (Churov, Oleinik, & Knip, 2015).

miRNAs are endogenous small (approximately 22 nucleotides long) noncoding RNAs (Murata et al., 2010). They destabilize the target mRNAs to inhibit protein synthesis and regulate crucial pathways and cellular processes, such as cell growth, differentiation, proliferation, and cell death (Hruskova et al., 2016). An increasing number of miRNAs are being found to be involved in inflammatory and autoimmune responses, such as miR-346, which indirectly regulates the release of the pro-inflammatory cytokine interleukin (IL)-18 (Alsaleh et al., 2009). Wang, et al. have identified the circulating miRNAs that are specifically altered in patients with systemic lupus erythematosus (SLE) as compared to RA and healthy controls (HC). Eight miRNAs, namely, miR-126, miR-16, miR-451, miR-223, miR-21, miR-125a-3p, miR-146a, and miR-155 were given importance in subsequent clinical studies since these miRNAs were identified as important regulators of the immune cells responsible for the pathogenesis of SLE (Reid, Kirschner, & van Zandwijk).

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MicroRNA-23b (miR-23b), a member of the miR-23b/27b/3074/24-1 cluster, harbored in chromosome 9, has been found to play a vital role in tumorigenesis (Au Yeung, Tsang, Yau, & Kwok, 2017; Cao et al., 2017; Fukumoto et al., 2016). An increasing number of reports have suggested that miR-23b has a close relationship with inflammation and autoimmune diseases (Zheng et al., 2012). Ayyadurai, et al. demonstrated that miR-23b was secreted and transported between the cells to impose a gene-silencing effect on the recipient intestinal macrophages, and regulated Marcksl-1 in the macrophages during intestinal inflammation (Ayyadurai et al., 2014). miR-23b has also been found to regulate the expression of inflammatory factors in vascular endothelial cells during sepsis (M. Wu, Gu, Yi, Tang, & Tao, 2015). In bone marrow mesenchymal stem cells, blocking the activation of NF-κB pathway through miR-23b overexpression resulted in the repression of maturation and differentiation of dendritic cells (DCs), which is the initiating factor in immune response (J. Wu et al., 2017). Moreover, Qi, et al. have demonstrated that a decreased level of miR-23b was involved in the activation of the Notch signaling pathway which contributes to the increase in the number of Th1/Th17 cells leading to Behcet's disease (Jian et al., 2014). Most importantly, the level of miR-23b was reported to be decreased in both synovial tissues of RA patients and kidney tissues of SLE patients after suppressing IL-17-associated autoimmune inflammation through TAB2, TAB3, and IKK-α (Zhu et al., 2012).

Focusing on the pathology of RA, we applied microarray analysis with microRNAs obtained from RA patients and HC. Our data showed a substantial elevation of miR-23b in fibroblast-like synoviocytes (FLS) obtained from RA patients. Till date, there is no report on the level of circulating miR-23b in RA. The present study was aimed to evaluate the miR-23b levels in synovial tissues (STs) and plasma and to further explore the clinical potential of miR-23b as a biomarker for RA disease activity.

MATERIAL AND METHODS

Samples obtained from RA patients and HC

All RA patients included in this study were diagnosed with RA according to the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) criteria (Aletaha et al., 2010). The samples of synovial tissues from 34 RA patients were obtained by total knee joint replacement. The control knee tissue samples were taken from 36 trauma patients (as re. These trauma patients had healthy joints with intact synovium. All tissue samples were stored at −80 °C immediately upon collection until use for miRNA extraction.

Peripheral blood was collected from 109 RA patients and 48 HC having no history of autoiimnune disease, severe cardiovascular disease, hepatic disease, renal disease, and any other chronic disease. Before venipuncture, the skin was disinfected with alcohol, and the blood samples were collected in EDTA-coated tubes. The demographic characteristics of the RA patients and the HC are shown in Table 1.

The levels of platelet (PLT), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), high sensitivity C-reactive protein (hs-CRP), low density lipoprotein cholesterol (LDL-C), aspartate transaminase (AST), total bilirubin (TBIL), indirect bilirubin (IBIL), direct

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bilirubin (DBIL), antinuclear antibody (ANA), rheumatoid factor (RF), anti-cyclic citrullinated peptide antibody (ACPA), and other laboratory parameters were estimated in a laboratory. The patients were evaluated by Disease Activity Score 28 (DAS28) using ESR, number of tender and swollen joints, and a 100 mm visual analog scale (VAS) for calculating the pain score. The disease characteristics of the RA patients are shown in Table 2 and 3.

Collection of STs and blood samples was performed following the medical ethics regulation of Nanjing Medical University and Suzhou University. This study was approved by Nanjing Medical University and Suzhou University Review Board, and written consent was requested and obtained from all the patients and HC.

miRNA microarray with FLSs

FLSs were isolated and cultured as previously described (Ni et al., 2015). After the cells were grown to 80–90% confluence on passage 3, they were harvested by trypinizing and total RNAs were purified with NucleoZOL (MACHEREY-NAGEL GmbH & Co. KG, Germany) according to the manufacturer's protocol. For microarray analysis, the samples are labeled using miRCURY™ Hy3™/Hy5™ Power Labeling Kit and hybridized using miRCURY™ LNA Array (v.11.0, Exiqon, Demnark). Axon GenePix 4000B microarray scanner was used for scanning. GenePix pro v 6.0 was used to read the raw intensity of the image. The ratio of red signal to green signal was calculated after background subtraction and normalization using the global Lowess (Locally Weighted Scatter Plot Smoothing) regression algorithm (MIDAS, TIGR Microarray Data Analysis System). Between slides normalization was performed by scale normalization to reduce the between-slide variability. The replicated spots on the same slides were averaged by obtaining a median ratio of the replicated spots. The threshold value used to screen the differentially expressed miRNAs was 1.5-fold change or 0.67-fold change (p 0.05).

Real time-PCR

Total RNA was extracted from STs and plasma using mirVana™ PARIS™ Kit (Thermo Fisher, USA) according to the manufacturer's instructions. The total RNA was eluted from a Filter Cartridge using 60 μl nuclease-free water. On-column digestion of the contaminated DNA was performed using RNase-Free DNase Set (Qiagen, Germany). Total RNA was reverse-transcribed using TaqMan® miRNA Assays and TaqMan® miRNA Reverse Transcription Kit in an Eppendorf AG. Following that TaqMan® miRNA Assays and TaqMan® Universal PCR Master Mix II no UNG were used to quantify miRNA expression by quantitative polymerase chain reaction (qPCR) in a ViiA 7 Real-Time PCR System (all the TaqMan® kits, assays, and probes were purchased from Applied Biosystems, Thermo Fisher Scientific, USA). The primer sequences used for amplifying matured miR-23b and U6 control were designed by Applied Biosystems. The PCR cycles were performed following the manufacturer's instructions as follows: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The threshold cycles (Ct) for the target miRNAs and the internal reference gene were used to represent their relative expression.

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miRNA in situ hybridization

The ST samples were subjected to standard overnight fixation in 10% neutral-buffered formalin followed by paraffin embedding and were cut into 10 μm-thick slices. The slides were then deparaffinized in xylene and ethanol, incubated with Proteinase-K for 10 min at 37 °C, dehydrated with gradient ethanol, and hybridized with 1 nM LNA™ U6 snRNA probe and 50 nM double-DOG LNA™ miRNA probe complementary to miR-23b and LNA™ scrambled miRNA probe, respectively (all the probes were purchased from Exiqon, Demnark). After incubating with and removing the blocking solution, the slides were sequentially incubated with anti-DIG reagent and AP substrate (all were procured from Roche, Mannheim, Germany) protecting from light. Finally, KTBT buffer was added to stop the reaction. Nuclear Fast Red (Solarbio, China) was used for counterstaining of the nucleus. The slides were finally observed under light microscopy.

Statistical Analyses:

All statistical analyses were performed using Prism 6.0 (GraphPad Prism, San Diego, CA, USA) and SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Mann-Whitney U test was performed to compare the expression of miR-23b in STs and plasma between RA and HC. The relationships between the expression of miR-23b in plasma and other parameters were evaluated using the Spearman correlation. Wilcoxon matched paired test was used to compare the miRNA-23b levels of 30 patients before and after treatment. All tests were two-tailed, and a P value of < 0.05 was considered as statistically significant.

RESULTS

miR-23b levels were elevated in FLSs, STs, and plasma in RA patients

The expression profiles of miRNA in FLSs in 8 RA patients were determined by miRNA microarray analysis. Compared to FLSs obtained from 4 HC, the expression of 5 miRNAs (miR-28-3p, miR-378, miR-BART10, miR-432, and miR-23b) was significantly increased in FLS obtained from RA (Figure 1a).

To confirm our microarray data, miR-23b levels were estimated by RT-QPCR in the STs obtained from 34 RA patients. Compared to the STs obtained from 36 control trauma patients, the miR-23b levels were significantly up-regulated in that obtained from RA patients (Figure 1b). To obtain a deeper insight into the expression of miR-23b in STs, miRNA *in situ* hybridization was performed to compare the tissue levels of miR-23b in STs obtained from RA and HC. Consistent with the results of PCR, the miR-23b staining was at a very low level in the normal STs from HC, but was highly intense in the STs from RA patients. The miR-23b staining was clearly seen in the synoviocytes of the lining and sublining from the STs of RA patients (Figure 1c).

Increased miR-23b expression in plasma of RA patients

Escaping from RNase in the circulation, miRNA can exist in a stable form as an effective marker of a variety of diseases (Chakraborty & Das, 2016). Since miR-23b is a promising novel autoimmunity regulator molecule (Remakova, Svitalkova, Skoda, Vencovsky, & Novota, 2013), we evaluated the miR-23b expression in plasma. The relative expression of miR-23b was

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compared between 109 RA patients and 48 HC. As shown in Figure 2, the plasma miR-23b expression in RA was significantly higher than that in HC. We further analyzed the difference between the male and female patients, but there was no significance as P=0.254 (data not shown).

Plasma miR-23b level correlated with the clinical patterns of RA

Considering that miR-23b is an important inflammatory mediator [Zheng et al., 2012] and the activity of RA is closely related to the severity of inflammation [Smolen et al.], the plasma miR-23b level and the pattern of RA disease activity was thoroughly analyzed. The plasma miR-23b level showed a positive correlation with CRP, ESR, hs-CRP, and DAS28 (Table 2 and Figure 3a).

RA is a systemic inflammatory disease, and hence, we collected other clinical indices in addition to the patterns of disease activity, such as white blood cells (WBC), platelet (PLT), alanine aminotransferase (ALT), aspartate transaminase (AST), etc. The correlation between the levels of plasma miR-23b and the levels of these parameters was analyzed (Table 2). The levels of plasma miR-23b positively correlated with PLT count but negatively correlated with Hb, TBIL, DBIL, IBIL, TC and LDL-C levels (Figure 3a). The plasma miR-23b levels, however, showed no correlation with the other parameters (Table 2).

Many types of antibodies are present in the blood of RA patients because of the autoimmune nature of the disease. We further estimated the levels of 3 crucial antibodies, ACPA, RF, and ANA, and evaluated their correlation with miR-23b expression. The results showed that plasma miR-23b expression in the ANA-positive RA patients was higher as compared to that in the ANA-negative RA patients (Figure 3b). However, the plasma levels of miR-23b did not correlate with RF or ACPA (Table 3).

We further obtained data regarding the clinical features of RA patients involved in this study and compared the plasma miR-23b levels in patients having positive clinical features with those having negative clinical features. The patients having anorexia showed higher levels of miR-23b in plasma than those without anorexia. A similar result was found in patients with fatigue (left and middle panel in Figure 3c). There was no significant difference between patients having extra-articular symptoms, systemic lesions, and articular symptoms including swollen joints, tender joints, and joint deformity and the patients not having these symptoms (Table 4). However, the patients having ILD showed a remarkably lower level of miR-23b compare to those without ILD (right panel in Figure 3c).

Effects of treatment on plasma miR-23b levels in RA patients

We performed follow-up studies with plasma miR-23b levels in 30 RA patients after treatment with infliximab and/or disease-modifying antirheumatic drags (DMARDs) for an average of 37 days. Among these 30 RA patients, 8 achieved ACR20, 9 achieved ACR50, 4 achieved ACR70, and 9 were non-responders. The treatment plan included infliximab combined with DMARDs and non-steroidal anti-inflammatory drags (NSAIDs) for 1 patient, DMARDs only or combined with traditional Chinese medicinal preparation for 5 patients, NSAIDS combined with DMARDs and/or traditional Chinese medicinal preparation for 19 patients, glucocorticoid combined with DMARDs and/or traditional

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Chinese medicinal preparation for 4 patients, and irregular treatment during two follow-up visits for 1 patient. As shown in Figure 4, the plasma miR-23b levels in 21 patients with active RA were markedly decreased following clinical improvement with treatment (Wilcoxon matched paired test, $P = 0.029$). Importantly, for the 9 patients who did not achieve an ACR20 response, the plasma miR-23b levels did not change significantly before and after treatment (Wilcoxon matched paired test, $P = 0.25$).

DISCUSSION

We investigated the relationship between miR-23b and RA in the present study. Our results revealed that RA patients show higher levels of miR-23b in STs and plasma than HCs. The levels of plasma miR-23b correlated with the serological and clinical features of RA disease activity. Further, we have observed that plasma miR-23b levels in RA patients diminished following successful treatment and clinical improvement.

miR-23b lias been demonstrated to play complicated roles in several pathological and physiological process, such as diabetes [Grieco et al., 2017] tumor [Kou, Zhou, Han, Zhuang, & Qian, 2016], and inflammation and immune disorders [Zheng et al., 2012]. Previous reports have demonstrated that miR-23b is dysregulated in inflammatory and autoimmune diseases. For instance, miR-23b was found to be up-regulated in the colonic mucosa of patients with Crohn's disease [Lin et al., 2013] and in the kidneys of mice with SLE [He et al., 2016]. The present study demonstrated that higher miR-23b levels were detected in plasma in RA patients as compared to that in HC. We found a significant increase in the miR-23b levels in STs in RA patients as compared to that in HC. This is contrary to the results of Zhu, et al. who showed that miR-23b levels deceased in STs in RA patients as compared to that in HC [Zhu et al., 2012]. The inconsistency between these results might be attributed to the difference in sample size. The number of ST samples obtained from RA patients and HC were 17 and 3, respectively, in the study of Shu Zhu, et al. [Zhu et al., 2012], whereas, the number of ST samples obtained from RA patients and HC were 34 and 36 in our study. Besides, our result of RT-QPCR with STs is consistent with the result of *in situ* hybridization. The plasma miR-23b levels correlated well with the clinical features of RA and the level was down-regulated after treatment. However, an increasing miR-23b level has been reported to promote Tregs differentiation, while number of Tregs has been demonstrated to be decreased in RA patients [Zheng et al., 2012]. We hypothesized that the up-regulation of plasma miR-23b might be due to feedback from the significantly decreased number of Tregs in RA[Al-Zifzaf et al., 2015].

We further assessed the relationship between miR-23b and clinical patterns of RA. Our results revealed that plasma miR-23b levels correlated well with several important biological and clinical indices which could assess the RA disease activity, including CRP, ESR, hs-CRP[Benhadjmohamed et al., 2017], and DAS28 score. The RA patients with anorexia showed higher levels of miR-23b in plasma than those with preserved appetite, and similar results were obtained with fatigue. Importantly, the level of plasma miR-23b was significantly decreased along with improvement of clinical symptoms. These results indicated that plasma miR-23b might be a potential biomarker for RA disease activity. The existing laboratory markers of RA often lack sensitivity and specificity without being 100% truly positive in RA

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or 100% truly negative in normal subjects (Pincus & Sokka, 2009). The plasma miR-23b level could be a useful marker in RA patients who are negative to the existing test results.

In addition, our results showed that plasma miR-23b level positively correlated with PLT count and negatively correlated with Hb, TBIL, DBIL, IBIL, LDL-C and TC levels. Previous reports have shown that the PLT count in the peripheral blood and synovium of RA patients is higher than that in HC and this count decreases in the peripheral blood following treatment with TNF-α inhibitor (Łukasik, Makowski, & Makowska, 2018). Low Hb in patients with RA upon TNF-α inhibitor treatment indicates an ongoing radiographic progression of the disease(Möller et al., 2017). Pan, et al. indicated that RA patients have a lower serum bilirubin level than HCs and that serum bilirubin level negatively correlates with the DAS28 score (Peng, Wang, & Pan, 2017). Serum bilirubin level is an indicator of hepatic function, and hence, miR-23b may take part in the mechanism of hepatic injury in RA. Meanwhile, reports have shown that the TC and LDL-C level in RA patients is lower than that in HCs(Plutzky & Liao 2018) and LDL-C negatively correlates with CRP, ESR, and DAS28(Charles-Schoeman et al., 2016). The increase in TC and LDL-C level in RA patients after treatment with DMARDs correlates with the alleviation of inflammation(Plutzky & Liao 2018). Taking together, we can conclude that plasma miR-23b could serve as a new biomarker for RA disease activity.

Reports before have found that miR-23b can alleviate fibrosis in diabetic nephropathy(Zhao et al., 2016), and the miR-23b/27b cluster suppresses activation of hepatic stellate cells which plays a pivotal role in the fibrogenesis process during chronic liver injury(Zeng et al., 2016). And we found that miR-23b levels in RA patients without ILD were higher than those with ILD. But The role of miR-23b playing in the pathological process of RA complicating with ILD needs further research to explore.

Interestingly, the plasma miR-23b level in ANA-positive RA was higher than in ANA-negative RA, which may be because of the fact that miR-23b can silence B cell Blimp-1 expression, thereby dampening the class-switch and hypermutated autoantibody response including ANA and immunopathology(White et al., 2014).

Elucidation of the specific mechanism regulating miR-23b expression can provide additional support for the involvement of miRNAs in the pathogenesis of RA. This needs to be investigated in further studies. Further research is also required to explain the correlation between plasma miR-23b level and ANA and ILD in RA patients and to examine the specificity of our proposed biomarkers by analyzing plasma from patients with infection, injury, and other inflammatory diseases.

CONCLUSION

The miR-23b level was found to be increased in the synovial tissues and plasma of RA patients as compared to that in HCs. Furthermore, the plasma miR-23b level in RA patients positively correlated with CRP, ESR, hs-CRP, DSA28, and PLT, and negatively correlated with TBIL, DBIL, IBIL, Hb, TC and LDL-C. In addition, the plasma miR-23b level declined significantly following treatment. Since miRNA has high stability in body

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fluids(Mitchell et al., 2008) and plasma miR-23b can be easily accessed through various methods, miR-23b possesses every property for being considered as a promising biomarker reflecting the degree of inflammatory disease activities and therapeutic effects in patients with RA.

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Abbreviations:

ACPA: Anti-Cyclic Citrullinated Peptide Antibody

ANA: Anti-Nuclear Antibody

AST: Aspartate Transaminase

CRP: C-Reactive Protein

DBIL: Direct Bilirubin

ESR: Erythrocyte Sedimentation Rate

FLS: Fibroblast-Like Synoviocytes

HC: Healthy Controls

Hs-CRP: High Sensitivity C-Reactive Protein

IBIL: Indirect Bilirubin

ILD: Interstitial Lung Disease

LDL-C: Low Density Lipoprotein Cholesterol

miRNAs: microRNAs

PLT: Platelet Count

RA: Rheumatoid Arthritis

RF: Rheumatoid Factor

SLE: Systemic Lupus Erythematosus

STs: Synovial Tissues

TBIL: Total Bilirubin

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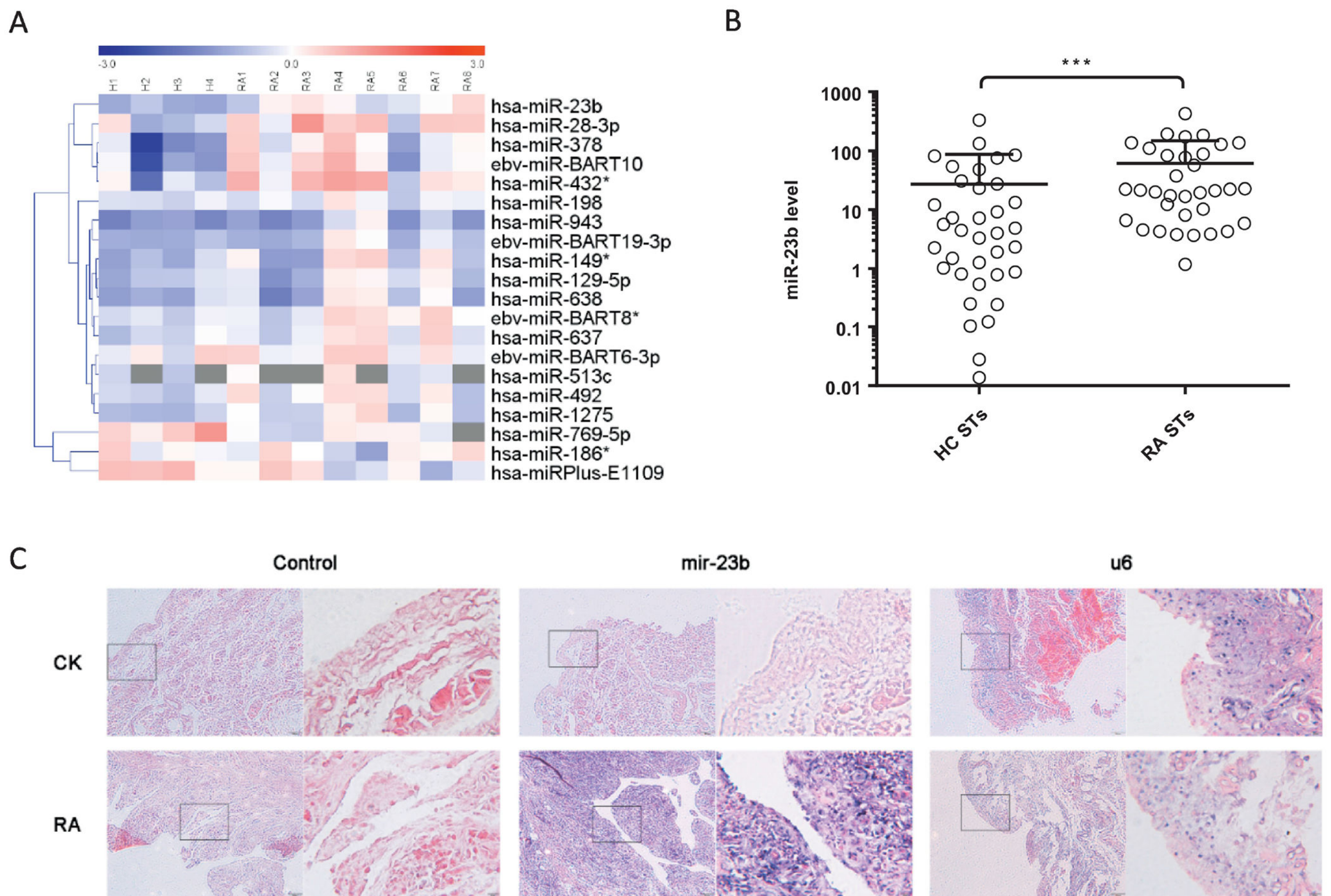

Figure 1. Increased miR-23b expression in FLS and STs obtained from RA patients.

(A) Compared to the trauma controls (H1-4), significantly altered expression of 20 miRNAs was found in FLS obtained from RA by miRNA microarray analysis (red: up regulation, blue: down regulation, grey: signals not detected). The threshold value of differentially expressed miRNAs was 1.5-fold change or 0.67-fold change (p 0.05). **(B)** miR-23b levels determined using RT-PCR in the STs obtained from RA patients (n = 34) were higher than that in HC (trauma control, n = 36). The *P* value derived by using Mann-Whitney U test is indicated. **(C)** *In situ* hybridization analysis showed that miR-23b expression in the STs obtained from RA patients was higher than that obtained from HC (scramble means negative control; u6 means positive control; FLS, fibroblast like synoviocytes; STs, synovial tissues; RA, rheumatoid arthritis).

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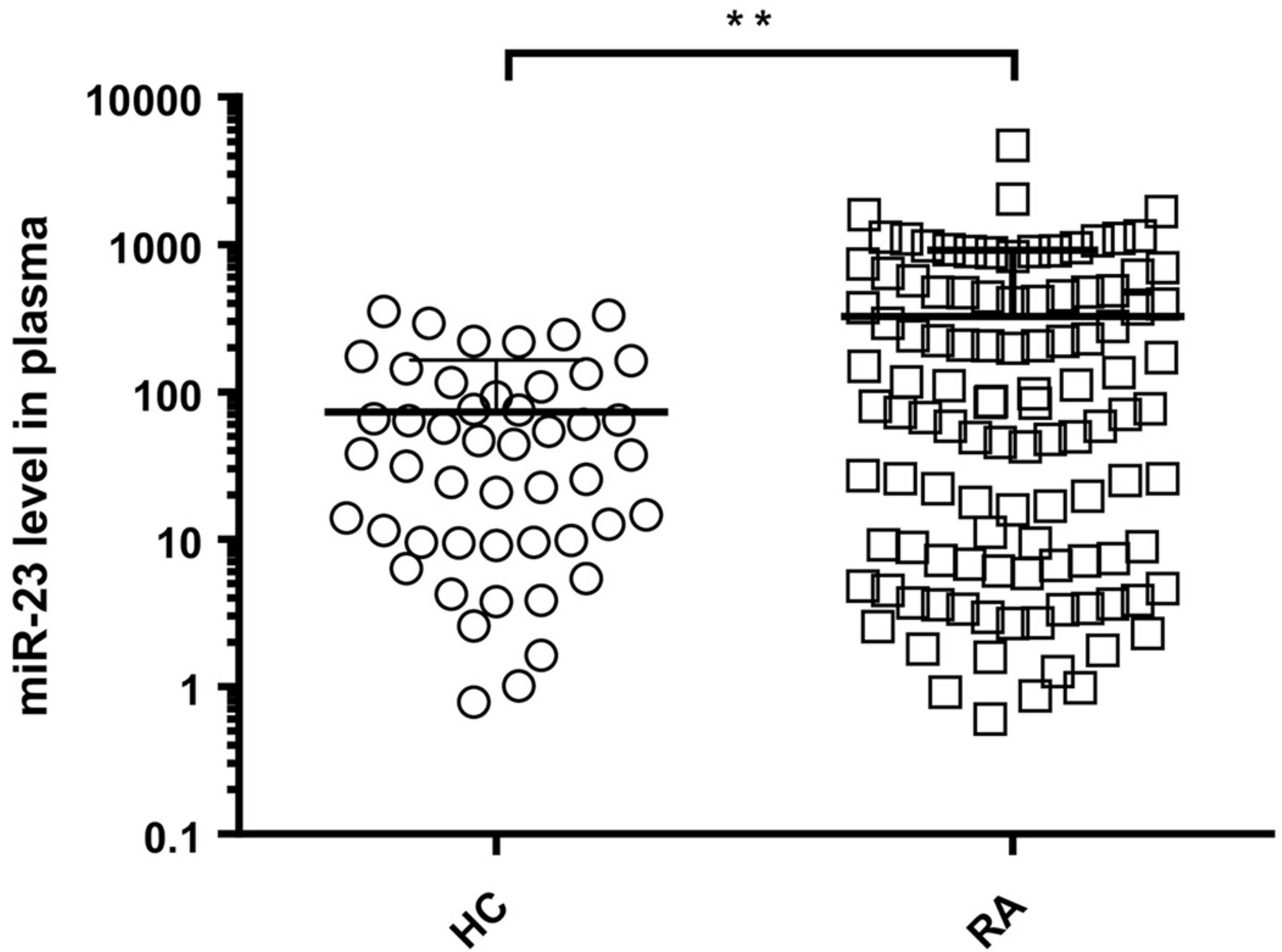

Figure 2. Increased miR-23b expression in the plasma of RA patients.

The levels of miR-23b in plasma of 109 RA patients were higher than that in 48 healthy controls (HC). The *P* value derived using Mann-Whitney U test are indicated (RA, rheumatoid arthritis).

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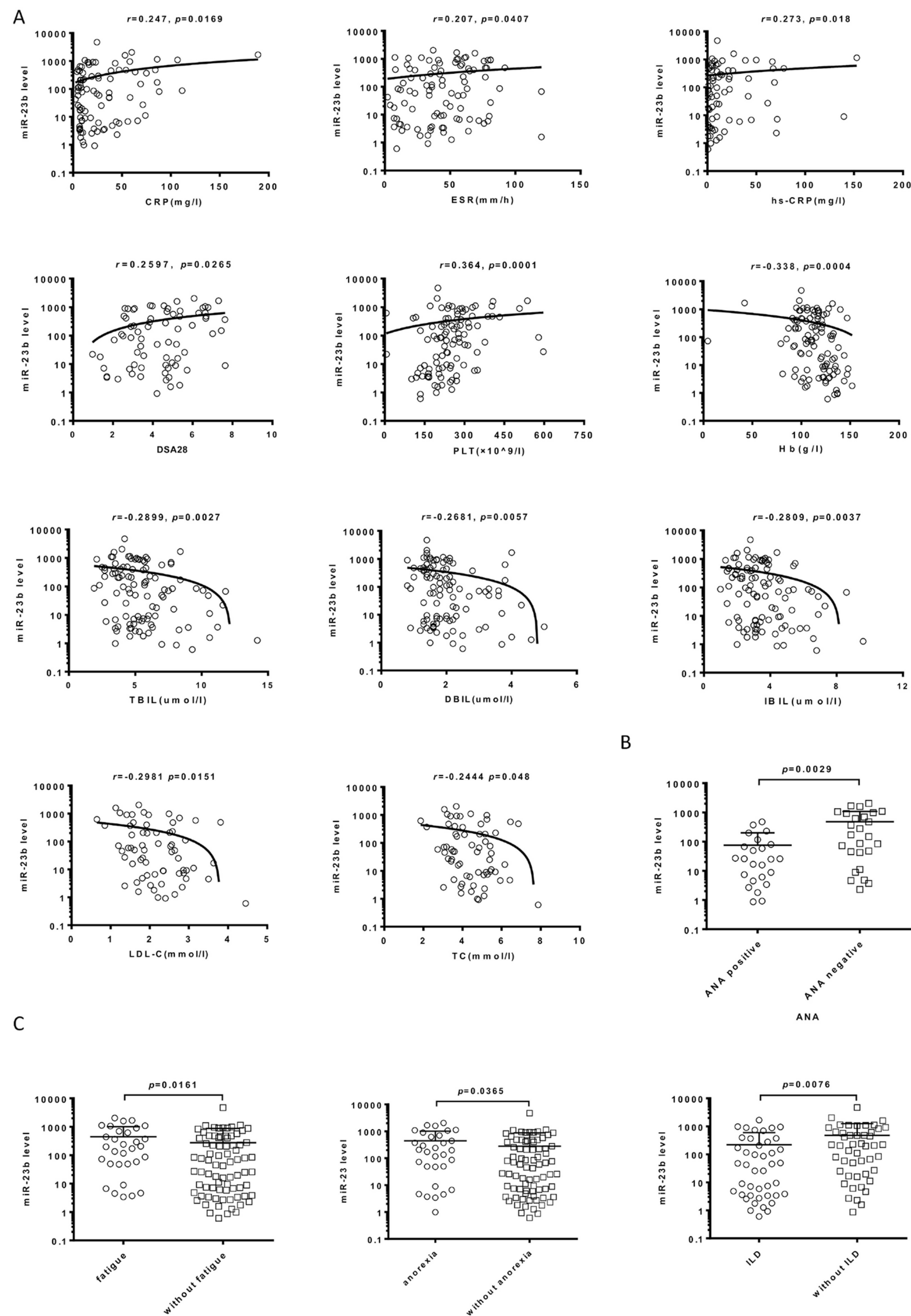

Figure 3. Plasma miR-23b levels correlate with the clinical patterns of RA.

(A) Correlations of plasma miR-23b levels with CRP (n = 93), ESR (n = 98), hs-CRP (n = 75), DAS28 (n = 73), PLT (n = 104), Hb (n = 107), TBIL (n = 105), DBIL (n = 105), IBIL (n = 105), LDL-C (n=66) and TC (n = 66). Correlation coefficients (*r*) obtained from Spearman rank-order test are shown. **(B)** Comparison between the levels of plasma miR-23b in ANA-positive RA (n = 24) and ANA-negative RA (n = 26) are shown. **(C)** Comparison between the levels of plasma miR-23b in RA patients with (n = 31) or without anorexia (n=78), RA patients with (n = 31) or without fatigue (n=78), and RA patients with (n=43) or without

ILD (n=48) are shown. Mann-Whitney U test was used (CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; hs-CRP, high sensitivity C reactive protein; DAS28, disease activity score28; PLT, platelet; Hb, hemoglobin; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; ANA, antinuclear antibody; ILD, interstitial lung disease; TC, total cholesterol; RA, rheumatoid arthritis).

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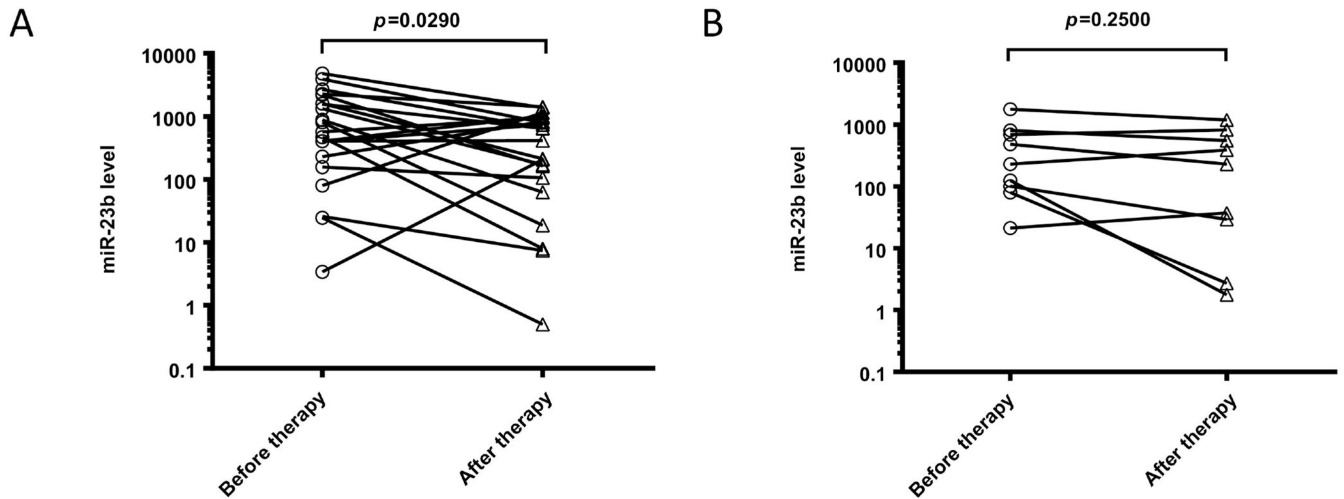

Figure 4. Plasma levels of miR-23b in RA patients before and after therapy.

Paired plasma samples were obtained from 30 patients before treatment and after an average of 37 days of treatment with DMARDs and/or NSAIDS. (a) 21 patients achieved at least an ACR20 response, (b) 9 non-responders did not achieve an ACR20 response [RA, rheumatoid arthritis; DMARDs, disease-modifying anti-rheumatic drugs; NSAIDS, nonsteroidal anti-inflammatory drugs; ACR20, improvement of 20% according to the American College of Rheumatology (ACR) response criteria].

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Table 1

Characteristics and plasma miR-23b levels of the subjects investigated

Characteristics	RA	HC	RA(STs)	HC (STs)
Number	109	48	34	36
Age (years)[a]	61(52-65)	56(39-66.5)	57(53-65)	54(44-59)
SEX(M/F)	20/89	13/35	16/18	17/19
MiR-23b levels[a]	55.56(16.27,229.6)	37.78(9.57,96.59)	25.93(9.40,89.85)	6.39(1.67,27.44)

[a]Expressed as the median (25th to 75th percentile). RA, rheumatoid arthritis; HC, healthy controls; MiR-23b, microRNA-23b;HC, control trauma tissues; STs, synovial tissues.

In the groups providing plasma, there was no significant difference in age (P=0.3157, Mann whitney test) and sex (P=0.1295, Chi-square) between RA patients and HC. And in the groups providing STs, there was no significant difference in age (P=0.0901, Mann whitney test) and sex (P=0.9891, Chi-square) between RA patients and HC.

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Table 2

Laboratory indicator and DAS28 of RA patients investigated[a]

Characteristics		n	*r*	*P*
WBC(×10⁹/l)[b]	6.39(5.35, 7.37)	107	0.026	0.792
Hb(g/l)[c]	113.90±20.62	107	−0.338	0.0004**
PLT(×10⁹/l)[b]	237.00(195.75, 288.00)	104	0.364	0.0001**
CRP(mg/l)[b]	19.00(7.70, 43.00)	93	0.247	0.0169*
ESR(mm/h)[c]	44.48:25.44	98	0.207	0.0407*
Hs-CRP(mg/l)[b]	9.16(3.00 22.45)	75	0.273	0.018*
ALT(u/l)[b]	15.00(11.00, 22.00)	105	−0.173	0.078
AST(u/l)[b]	18.00(14.00, 23.00)	105	−0.170	0.083
γ-GT(u/l)[b]	23.00(15.00, 31.00)	105	−0.047	0.635
ALP(u/l)[b]	78.00(65.00, 93.00)	105	0.045	0.649
LDH(u/l)[c]	200.65± 51.93	105	0.049	0.622
ALB(g/l)[c]	35.90±5.00	105	−0.026	0.790
TBIL(umol/l)[b]	5.20(4.00, 6.50)	105	−0.2899	0.0027**
DBIL(umol/l)[b]	1.80(1.50, 2.20)	105	−0.2681	0.0057**
IBIL(umol/l)[b]	3.20(2.60, 4.40)	105	−0.2809	0.0037**
Cr(umol/l)[b]	58.00(52.00, 67.00)	104	−0.1364	0.167
BUN(umol/l)[b]	4.36(3.60, 5.73)	104	−0.182	0.064
TC(mmol/l)[c]	4.41±1.13	66	−0.2444	0.048*
TG(mmol/l)[b]	1.28(0.89, 1.68)	66	−0.173	0.164
HDL-C(mmol/l)[b]	1.14(0.96, 1.35)	66	−0.154	0.217
LDL-C(mmol/l)[c]	2.17±0.72	66	−0.2981	0.0151*
TSH(uIU/ml)[b]	1.90(1.31, 3.02)	54	0.112	0.421
FT3(pmol/l)[c]	5.49±1.58	54	0.268	0.05
FT4(pmol/l)[b]	16.30(15.21, 18.34)	54	−0.134	0.334
IgG(g/l)[c]	14.22±4.38	70	0.038	0.754
IgA(g/l)[b]	3.05(2.44, 3.64)	70	−0.045	0.709
IgM(g/l)[b]	1.45(1.18, 1.69)	70	−0.195	0.105
C3(g/l)[c]	1.09±0.20	70	0.060	0.622
C4(g/l)[c]	0.21±0.06	70	−0.112	0.356
ASO(lu/ml)[b]	26.80(25.00, 62.80)	65	−0.063	0.619

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Characteristics		n	*r*	*P*
DAS28 [c]	4.35±1.63	73	0.2597	0.0265 [*]

[a]DAS28, disease activity score28; RA, rheumatoid arthritis. WBC, wliite blood cells; Fib, hemoglobin; PLT, platelet; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; hs-CRP, high sensitivity C reactive protein; ALT, Alanine aminotransferase; AST, Aspartate transaminase; γ-GT, glutamyl transpeptidase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; ALB, albumin; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; TB, total cholesterol; TG, triacylglycerol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TSH, thyroid stimulating hormone; FT3, free thyroid3; FT4, free thyroid 4; IgG, immunoglobulin G; IgA immunoglobulin A; IgM immunoglobulin M; C3, complement 3; C4, complement 4; ASO, anti-Streptolysin “O”.

[b]Expressed as the median (25th to 75th percentile)

[c]expressed as the average±standard deviation

[*]expressed as $P < 0.05$

[**]expressed as $P < 0.01$.

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Table 3

Antibodies patterns of RA patients investigated[a]

Antibodies	Positive rate(%)	n	r	*P*
RF	84.06%	68	–0.128	0.299
ACCP	87.8%	40	–0.118	0.469
ANA	48%	50	-	0.0029 **

[a]RA, rheumatoid arthritis; RF, rheumatoid factor; ACPA, anti-cyclic cirullinated peptide antibodies; ANA, antinuclear antibody.

**expressed as $P < 0.01$.

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Table 4

Clinical manifestation of RA patients[a]

Manifestation	Positive number (total number)	Percentage (%)	*p*
Morning stiffness	20 (109)	18.35	0.133
Swollen joints	79 (109)	72.48	0.519
Tender joints	86 (109)	78.90	0.782
Joint deformity	22 (109)	20.18	0.319
Fatigue	31 (109)	28.44	0.0161 *
Inappetence	31 (109)	28.44	0.0365 *
ILD	43 (91)	46.15	0.0076 **
Lymph nodes	27 (76)	35.52	0.737

[a]RA, rheumatoid arthritis; ILD, interstitial lung disease.

*expressed as $P < 0.05$

**expressed as $P < 0.01$.