INDEX COPERNICUS INTERNATIONAL

ORIGINAL ARTICLE

miRNA-146a-5p is upregulated in serum and cartilage samples of patients with osteoarthritis

Authors' Contribution:
A – Study Design
B – Data Collection
C – Statistical Analysis
D – Data Interpretation
E – Manuscript Preparation
F – Literature Search
G – Funds Collection

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Article history: Received: 24.08.2018 Accepted: 06.02.2019 Published: 08.02.2019

ABSTRACT:

Introduction: Osteoarthritis (OA) is a widely prevalent joint disease leading to motor disability and pain. However, appropriate indicators for identifying patients at risk for this progressive disease, identifying molecular events for detecting early phases of the disease, or biomarkers to screen for treatment responses are lacking. Micro RNAs (miRNAs), which play crucial roles in OA, could be potential biomarkers of OA. Because circulating miRNA levels reflect the disease state, they may be useful for OA screening and as diagnostic tools, reducing the need for invasive procedures and minimizing the cost of current diagnostic methods.

Materials and methods: The expression levels of 18 microRNAs (let-7e-5p, miR-21-5p, miR-93-5p, miR-101-3p, miR-103a-3p, miR-130a-3p, miR-146a-5p, miR-16-5p, miR-193b-3p, miR-199a-3p, miR-210-3p, miR-222-3p, miR-22-3p, miR-27a-3p, miR-27b-3p, miR-335-5p, miR-454-3p, and miR-98-5p) were analyzed by quantitative real-time polymerase chain reaction in the cartilage tissues and serum samples of 28 OA patients and were compared to those of 2 healthy controls.

Results: Expression of microRNA-146a-5p was significantly upregulated in the cartilage (P = 0.006) and serum (P = 0.002) of OA patients. The expression levels of miR-146a-5p in the serum were positively correlated with those in the cartilage (Pearson correlation coefficient R = 0.32; P = 0.002).

Conclusion: miR-146a-5p was significantly overexpressed in patients with OA, both in the articular cartilage tissue and serum, with a positive correlation between the levels in both types of samples. Therefore, the miR-146a-5p serum level could reflect the molecular processes in the cartilage, suggesting its clinical utility as a biomarker for OA management. Implementing a noninvasive biomarker using serum miRNAs involves the analysis of the misregulated miRNAs linked to the cartilage pathology.

KEYWORDS: circulating microRNA, osteoarthritis, serum, cartilage, biomarker

INTRODUCTION

Osteoarthritis (OA) is the most prevalent chronic musculoskeletal disorder, and it is increasing in frequency and severity worldwide [1]. OA is a degenerative disease in which the balance between the degradation and synthesis of cartilage structures and subchondral bone layers is disrupted [2]. Progressive cartilage degradation, joint space narrowing, and bony changes such as osteophytes and subchondral sclerosis, lead to premature motor disability and pain [3]. Degeneration of the cartilage is promoted by many factors, including mechanical injury, obesity, physical activity, and genetic factors [4]. Currently, the diagnosis of OA is based on magnetic resonance imaging and clinical features such as pain and mobility dysfunction. These methods, however, are insufficient and lack sensitivity. Moreover, these methods are not useful for detecting early stages of the developing disease based on molecular changes of the cartilage [5].

Thus, there is an urgent need for new diagnostic biomarkers for identifying patients at risk of progressive OA based on early molecular events. Ultimately, validation of a new molecular biomarker could also lead to the development of alternative therapeutic strategies and act as a clinical indicator of the treatment response to disease-modifying OA drug interventions [6]. MicroRNAs (miRNAs) are short, non-coding RNAs that are 19 to 25 nucleotides in length. They regulate gene expression post-transcriptionally by pairing with complementary nucleotide sequences in the 3'-untranslated regions of specific mRNA targets, and have an overarching regulatory role in both normal cellular function and in many diseases [7].

Aberrant miRNA expression or processing is linked to many pathologies, including OA [8]. Recent studies demonstrated the alteration of several miRNAs in OA and their potential to serve as diagnostic markers for OA [9]. Importantly, miRNAs are detectable in body fluids, including serum [10]. Their activity in a variety of body specimens, from serum to tissues, makes them appropriate targets for clinical application. miRNAs are stable against RNAse activity and external influences, and may thus serve as excellent noninvasive biomarkers for physiologic and pathologic processes [11]. Therefore, serum microRNA levels have been investigated in many disorders. The identification of a preoperative serum biomarker would allow for better risk stratification. The aim of the present study was to compare miRNAs levels between the cartilage and serum of OA patients, to assess their usefulness as disease biomarkers.

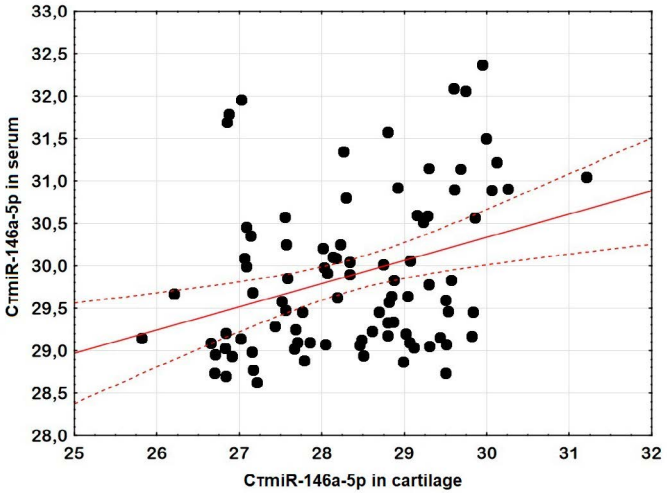

Fig. 1. Scatterplot of CT cartilage miR-146a-5p vs. CT serum miR-146a-5p; y = 22.1367 + 0.2733*x; R = 0.3273; P = 0.002.

MATERIALS AND METHODS

All patients provided written informed consent and the study was approved by the local ethics committee (number: 5/01/2014). Samples were collected from 2014 to 2015, and the patients were recruited from the Orthopedics and Traumatology Department at Hospital no. 2 in Rzeszow, Poland. We obtained 28 serum samples and 28 articular cartilage samples from patients with OA who were diagnosed according to the criteria of the American College of Rheumatology.

The control group comprised 2 patients with femoral neck fractures who had no previous hip joint pain and no radiologic symptoms of OA (Kallgren Lawrence scale I, II). Femoral cartilage samples were collected during surgery for total hip replacement. All surgeries were performed at the same hospital by the same team of orthopedists. During hospitalization after obtaining written informed consent from the patients, 5 mL of blood was drawn into Serum S-Monovette Gel tubes with clotting activator (Sarstedt, Nümbrecht, Germany). The samples were immediately transported to the Laboratory of Molecular Biology, Center for Innovative Research in Medical and Natural Sciences. The serum samples were centrifuged twice at 2000 g for 10 min and stored at -80°C until analyzed.

RNA EXTRACTION

Total RNA, including microRNA, was isolated from 200 µL of serum using the miRCURY™ RNA Isolation Kit – Biofluids (Exiqon, Denmark), according to the manufacturer's protocol. Frozen cartilage tissue was homogenized using a Tissue Ruptor (Qiagen, Germany). Total RNA, including microRNA, was extracted from the cartilage using the miRVana miRNA isolation kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) with modification. Prior to RNA extraction, the RNA quality and concentration were assessed using a NanoDrop ND2000c Spectrophotometer (ThermoFisher Scientific, Waltham, Mass). RNA was evaluated by determining the ratio of absorbance at 260 nm and 280 nm (260/268) and at 260 and 230 nm (260/230). RNA integrity was confirmed on a 1% standard agarose gel. All RNA samples were stored in a -80°C freezer until further analysis.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (QRT-PCR)

We selected the following 18 miRNAs based on the published data and the miRNA database: let-7e-5p, miR-21-5p, miR-93-5p, miR-101-3p, miR-103a-3p, miR-130a-3p, miR-146a-5p, miR-16-5p, miR-193b-3p, miR-199a-3p, miR-210-3p, miR-222-3p, miR-22-3p, miR-27a-3p, miR-27b-3p, miR-335-5p, miR-454-3p, and miR-98-5p. Expression levels of 18 miRNAs were examined in all cartilage and serum samples. Each RNA sample was reverse-transcribed into cDNA using the miRCURY LNA™ Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon, Denmark). Each miRNA was assayed twice by qRT-PCR on the microRNA Ready-to-Use PCR, Custom Pick- &-Mix panel. The relative amount of miRNA was normalized to the endogenous miRNAs hsa-miR-103a-3p, hsa-miR-423-5p, and hsa-miR-191-5p as a reference. The target cDNA sequences were amplified by qRT-PCR in a LightCycler® 480 Real-Time PCR System (Roche, Switzerland). The amplification curves were analyzed using Roche LC software, both for determination of Cp (by the 2nd derivative method) and melting curve analysis.

STATISTICAL ANALYSIS

The statistical analysis was performed using Statistica 12.5PL software (Statsoft, Poland). Correlations between miRNA expression levels were measured by Pearson's correlation coefficient (r). Correlations with P values <0.05 were considered significant. The normality of the distribution of the variables was tested using the Shapiro-Wilk test and Kolmogorov-Smirnov test with Lilliefors correction. Because of the non-normal distribution of all analyzed variables, we used the non-parametric Mann-Whitney U-test [12].

RESULTS

We compared the expression of the 18 microRNAs in cartilage and serum of OA patients. Only the expression level of miR-146a-5p was significantly correlated between the cartilage and serum (Pearson correlation coefficient R = 0.32; P = 0.002; Fig. 1.). The Mann-Whitney U test detected a significant difference in the expression of microRNA-146a-5p in both the serum (P < 0.002) and the cartilage (P = 0.006) (Fig. 2.). In both cases, expression in the OA patients was higher than that in the controls, suggesting that increased levels of miR-146a-5p could be considered as a tool for OA management.

DISCUSSION

Several studies have demonstrated a crucial role of tissue miRNAs in intracellular processes such as apoptosis, cell proliferation, differentiation, metabolism, and cell-cycle control [13]. These molecules are thought to control many miRNAs and their influence on gene expression regulation have attracted wide research interest in miRNAs as useful biomarkers of various pathologic conditions, including cancer, neurodegenerative disease, and autoimmune disorders [14]. The regulatory role of miRNAs in OA, however, remains unclear. Here, we provide evidence for the involvement of miRNAs in OA pathology, emphasizing their possible application as a diagnostic tool. Aberrant miRNAs derived from circulat-

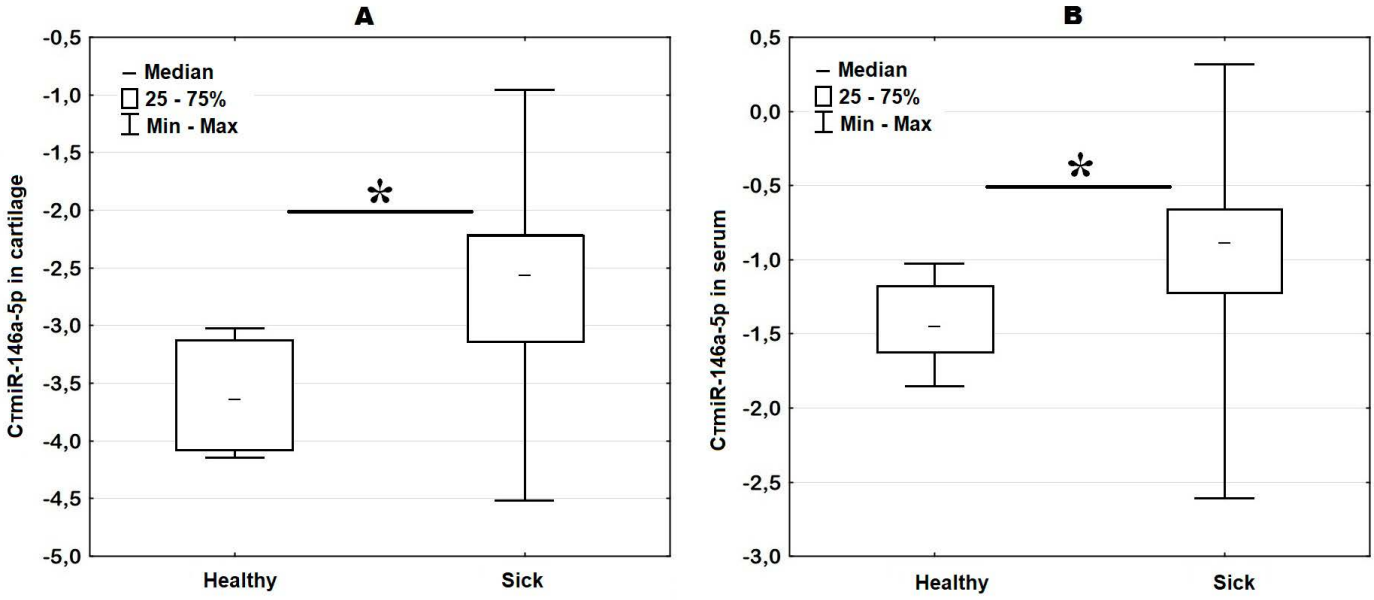

Fig. 2. Differential expression of miR-146a-5p in osteoarthritis. A – cartilage; B – serum * Statistically different from control group – Mann-Whitney U-test, $P < 0.05$.

ing blood cells as well as other pathologically altered tissues have been detected in the serum [15]. Circulating miRNAs may reflect physiologic or pathologic changes in cancer cells, and thus may be used as crucial biomarkers of disease [16]. These reports attracted our interest in the potential of miRNAs as a significant factor associated with OA disorder.

Here, we aimed to determine the usefulness of circulating miRNAs for monitoring of degeneration processes in cartilage affected by OA disease. We determined microRNAs expression level in serum and cartilage samples obtained from patients with OA. Then, correlation analysis between altered miRNAs circulating in the serum and those in diseased tissue was conducted. Our findings indicated that miR-146a-5p levels showed the same trend in the serum as in the cartilage tissues. This finding suggests the potential use of serum miRNA as a noninvasive indicator of OA pathogenesis. Although a role for miRNAs in OA pathogenesis has recently been proposed, that role has not yet been elucidated [17]. In this report, we demonstrated differences between the levels of miRNAs in the serum and in the cartilage within the same patient group.

MiR-146a is one of the first miRNAs reported to be upregulated in human OA cartilage, and it has been linked to OA pathogenesis [18]. The functions of miR-146a are related to cartilage degradation, synovial inflammation, neoangiogenesis, and osteoclastogenesis [19]. Several reports indicate that miR-146a is strongly expressed in the articular cartilage of patients with OA [20-22]. Our results confirmed these findings. Nakasa et al.20 reported that miR-146a levels in synovial fibroblasts may be involved in the immune response in rheumatoid arthritis. miR-146a expression is induced in response to an increase in the levels of inflammatory cytokines such as tumor necrosis factor α and interleukin (IL)-1β, and might have a role in regulating the negative feedback loop that attenuates the release of the inflammatory factors, mainly TRAF6 and IRAK1 genes [20, 21]. Yamaski et al. [21]. confirmed the presence of higher levels of miR-146a in early-stage OA cartilage induced by stimulation of IL-1β. These studies support the notion that miR-146a plays a protective anti-inflammatory role in OA through inhibiting IL-1–induced expression of cartilage-degrading enzymes such as metalloproteinase-13 (MMP-13) and ADAMTS-5. Another study suggested that miR-146a has catabolic effects on human chondrocytes. Li et al. [18] found that miR-146a directly decreases Smad4 expression in chondrocytes, thereby disrupting signal transduction of transforming growth factor β, and ultimately overexpression of miR-146a leads to reduced cellularity in cartilage tissue.

Moreover, in the same study, the authors demonstrated that miR-146a inhibition of Smad4 leads to increased expression of vascular endothelial growth factor [18]. Vascular endothelial growth factor adversely affects cartilage homeostasis, and induces matrix degradation, chondrocyte apoptosis, blood vessel invasion into the cartilage, MMP secretion, and extracellular matrix remodeling [22]. Together, the findings of these studies suggest the immense potential for future application of this particular miRNA as a diagnostic and therapeutic tool for OA. The role of miR-146a in OA pathogenesis appears to be the regulation of genes involved in catabolic and anabolic signals of cartilage homeostasis and inflammatory functions.

In conclusion, in the present study we demonstrated that miR-146a-5p is deregulated in OA either in cartilage and plasma samples. Tissue- and pathology-specific patterns of circulating miRNAs does strongly suggest that the analysis of miRNAs signatures in serum or plasma will evolve into an important biomarker for prognosis, diagnosis, patient stratification, down to personalized medicine approaches. To our knowledge, the present study is the first comparative analysis of miRNA levels in articular cartilage tissue as well in the serum from OA patients. These results confirmed that circulating miRNAs provide a new genetic material easily collectable from blood samples of OA patients.

However, discoveries of miR-146a-5p for noninvasive biomarkers should be improved by deeper analyses in a larger cohort of patients and additional studies should be performed to investigate the possible gene targets of miR-146a-5p.

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Word count: 2210 Page count: 5 Tables: – Figures: 2 References: 22

DOI: 10.5604/01.3001.0012.0135 Table of content: https://ppch.pl/issue/11974

Competing interests: The authors declare that they have no competing interests.

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Cite this article as: Skrzypa M., Szala D., Gabło N., Czech J., Pająk J., Kopańska M., Trzeciak M., Gargasz K.3, Snela S., Zawlik I.: miRNA-146a-5p is upregulated in serum and cartilage samples of patients with osteoarthritis; Pol Przegl Chir 2019: 91 (3): 1–5