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MiRNA expression profile in serum of osteoarthritis patients

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Abstract

Introduction: Osteoarthritis (OA) is the most common form of arthritis that affects millions of people worldwide. Despite advances in medicine, OA still remains incurable. Scientists are still seeking candidates for biomarker-based risk stratification and the early detection of disease. Measurement of miRNAs in the serum may become a powerful tool in the development of diagnostic biomarker.

Purpose: The aim of this study was to examine the expression profiles of 20 miRNAs in the serum of patients with OA.

Methods: We used RT-qPCR method to measure the expression profile of selected miRNAs.

Results: Six miRNAs showed differential expression between OA and normal serum samples. miR-146a-5p and miR-98-5p were significantly upregulated ($P < 0.05$), while miR-222-3p, miR-22-3p, miR-27a-3p and miR-93-5p were downregulated ($P < 0.05$) compared to the control group. To the best of our knowledge, this is the first work that shows that expression of these six miRNAs is different between serum OA samples and

healthy controls. However, we did not find any statistically significant relationship between the miRNAs expression and clinical characteristics.

Conclusions: Our findings suggest that miRNAs can be used as potential biomarkers for OA, but further research is needed to assess the usefulness of serum miRNAs in miRNA-based prognostic and therapeutic approaches.

Key words: expression; miRNA; osteoarthritis; serum

1. Introduction

Degenerative joint disease, also known as osteoarthritis (OA), is the most common chronic disease of the musculoskeletal system. It develops as a result of impaired quality and quantity of articular cartilage, whose task is to cushion the movements of the joint and other articular surfaces [1]. OA leads to a deterioration of the disability and quality of life. Many risk factors affect the pathogenesis, presentation and prognosis of OA. These include systematic risk factors such as age, gender and hormones, race/ethnicity, congenital/developmental conditions, genetics, diet, and also local risk factors including obesity, injury/surgery, physical activity/sport, occupation or mechanical factor [2]. Therefore, a multifactorial etiology of OA can be considered the product of an interplay of local and systemic factors. Until recently, OA was considered a sign of aging and simple "wear and tear" of the articular cartilage. Results from several studies have shown that age is one of the strongest risk factors for OA [3, 4], but joint injury and obesity have been found to be strongly associated with the disease [5]. However, for past several years, OA is the subject of increasing interest for scientists and doctors, as also applies to young adults [6, 7]. OA is still in the realm of researches and orthopaedic surgeons who are trying to find effective treatment for inhibiting its development. Because current knowledge about OA is incomplete, there is a pressing

need to find molecular biomarkers for early detection and in consequence prevention of this disease.

There are promising potential biomarkers for OA, e.g. biochemical, genetic and epigenetic, which are still under investigation and in the near future could be used in clinical decision making [8]. The special attention is deserved by miRNAs, which are one of the forms of RNA and are involved in a number of physiological and pathological processes [9]. MicroRNAs (miRNAs) are a family of non-coding, endogenous regulatory molecules, produced from double-stranded precursors known as pre-miRNAs. They consist normally of 21-23 single-nucleotides. The function of miRNAs is associated with posttranscriptional regulation of the expression of numerous genes by binding to specific sequence within target messenger RNA (mRNA). A single molecule miRNA can simultaneously control the expression of hundreds of target genes [10]. It is estimated that miRNAs account for 1-5% of the human genome and more than 30% of protein-coding genes in human cells are regulated by these molecules [11]. Numerous scientific reports have shown that miRNAs play a very important role in a number of physiological processes in humans such as cell growth, proliferation, cell differentiation, apoptosis but also in oncogenesis [9]. In fact, their expression levels are dysregulated in response to pathological abnormalities, thereby resulting in the pathogenesis of many human diseases [12] including degenerative diseases, such as OA [13]. Therefore, we aimed to identify the expression pattern of selected miRNAs in the serum of patients with OA in comparison with healthy controls that can predict the risk of early development of disease and in the future can be used as potential clinical applications.

2. Material and methods

2.1. Patients

Ethical approval for this study was granted by the Bioethical Committee of the Medical Faculty of Rzeszow University (number: 5/01/2014). Study subjects were recruited in The Clinical Department of Orthopedics and Traumatology Adults and Children at the Clinical Regional Hospital No. 2 for them. St. Jadwiga in Rzeszow. Patients were diagnosed according to the American College of Rheumatology (ACR)

criteria for this disease. Informed consent was obtained from all patients who took the part in the study. We included into the study serum samples of 36 OA patients (sex: 15 Females and 21 Males, mean age: 64±16) and 3 healthy controls without OA (sex: 1 Females and 2 Males, mean age: 55±26). Among the patients 12 were classified as early and 24 as late stage of the disease. Every subject was interviewed to obtain the individuals' complete clinical history including general information, previous injuries, BMI, occupational or sports activities, and clinical manifestations of OA.

2.2. RNA extraction from serum

Serum samples were centrifuged twice at 3000 x rpm for 5 minutes and stored in a -80°C freezer until analysis. For miRNA qPCR analyses, total RNA (including miRNAs) was isolated from serum using the miRCURY™ RNA isolation kit – biofluids (Exiqon, Vedbaek, Denmark) following the manufacturer's suggested protocol.

2.3. Reverse transcription and quantification of miRNAs by real-time PCR

Based on previous reports in the literature and the miRNA databases we selected 20 miRNAs (14-30). In all 39 serum samples (3 from healthy control group and 36 from patients with OA) we analyzed 20 miRNAs: let-7e-5p, miR-101-3p, miR-127-5p, miR-130a-3p, miR-138-5p, miR-146a-5p, miR-16-5p, miR-193b-3p, miR-199a-3p, miR-210-3p, miR-21-5p, miR-222-3p, miR-22-3p, miR-27a-3p, miR-27b-3p, miR-335-5p, miR-454-3p, miR-9-5p, miR-98-5p and miR-93-5p. For normalization of the data, we have applied three miRNAs: miR-423-5p, miR-103a-3p and miR-191-5p as those were found by Exiqon (Copenhagen, Denmark) to be good reference genes in serum and plasma samples and are proved to be the most stable normalizers. As technical controls, an RNA spike-in (UniSp6) was used for the quality control of the RNA isolation and cDNA synthesis [31]. For the reverse transcription and quantitation of miRNAs by real-time PCR, reagents from Exiqon (Copenhagen, Denmark) were used. Reverse transcription and real-time PCR were performed like as described previously [31]. Briefly, the miRCURY LNA™ Universal RT microRNA PCR, Polyadenylation and

cDNA synthesis kit was used for reverse transcription 2 µl of RNA samples into cDNA in 10 µl reactions (a single reaction step). cDNA was diluted 50x and assayed in 10 µl PCR reactions in a protocol designed for miRCURY LNA™ Universal RT microRNA PCR; each microRNA was assayed once by qPCR on the microRNA Ready-to-Use PCR, Custom Pick and Mix using ExiLENT SYBR® Green master mix. The target cDNA sequences were amplified by qRT-PCR in 384-well plates in a LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland). We analyzed the amplification curves using the Roche LC software both for determination of Cp (by the 2nd derivative method) and melting curve analysis.

2.4. *Data analysis*

For data analysis we used the methods used in Valassi et al. study. To calculate amplification efficiency algorithms similar to the LinReg software were used. Assays were inspected for distinct melting curves and Tm to be within assays known specifications. Analysed assays were detected with 3 Cqs less than the negative control, and with Cq<37 (data that did not pass these criteria were omitted from further analysis). In the study Cq was calculated as the 2nd derivative. According to NormFinder, the average of the tests found in all samples turned out to be the best normalizer (miR-103a-3p, miR-423-5p and miR-191-5p) [33]. We calculated the normalizes Cq using the following formula: normalized Cq = average Cq (n=93) – assay Cq (sample).

Statistical analysis

All statistical analyses were performed using Statistica 12.5 PL software (Statsoft, Poland). The distribution of variables was tested with Shapiro-Wilk test and Kolmogorov-Smirnov test with Lilkefors' correction. For all non-normal distributed data, we used non-parametric test. P values less than 0.05 were considered to be statistically significant.

3. Results

From previously reported miRNA studies we selected 20 miRNAs which have been described as being involved in the pathogenesis of OA [14-30]. miRNAs for the

study were generated by extracting total RNA from serum of healthy donors (n=3) and OA patients (n=36). qRT-PCR technique was used to identify the expression profiles of selected miRNAs. Five miRNAs (miR-127-5p, miR-138-5p, miR-193b-3p, miR-21-5p and miR-9-5p) have been excluded from the analysis because of the frequent failure to detect the samples. The remaining fifteen (let-7e-5p, miR-101-3p, miR-130a-3p, miR-146a-5p, miR-16-5p, miR-199a-3p, miR-210-3p, miR-222-3p, miR-22-3p, miR-27a-3p, miR-27b-3p, miR-335-5p, miR-454-3p, miR-98-5p and miR-93-5p) were tested using U Mann-Whitney test in order to demonstrate the differences between OA groups and the control. We found that expression of miR-146a-5p and miR-98-5p was increased significantly, while the expression of miR-222-3p, miR-22-3p, miR-27a-3p and miR-93-5p was decreased significantly in the serum of patients with OA (Fig. 1).

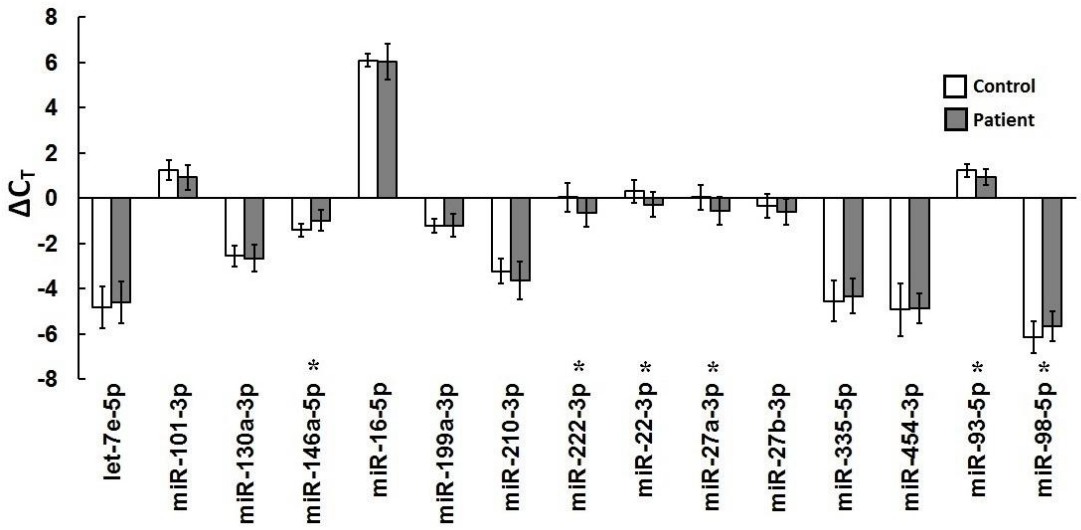

Fig. 1. Differential expression of serum miRNAs in osteoarthritis. *Statistically significant differences between the two groups ($p<0.05$)

The heat map diagram shows the result of differentially expressed miRNAs in patients with OA and healthy controls. The relative miRNA expression levels were calculated based on the two-way hierarchical clustering of miRNAs and samples. The distance between two clusters was defined as the average distance between all pairs of the two clusters' members (average linkage), and Pearson correlation. Hierarchical

clustering was done in R (version 3.2.3) using scripts from Bioconductor (3.3). The colour scale illustrates the relative level of miRNA expression: green color represents downregulation, red color represents upregulation (Fig. 2).

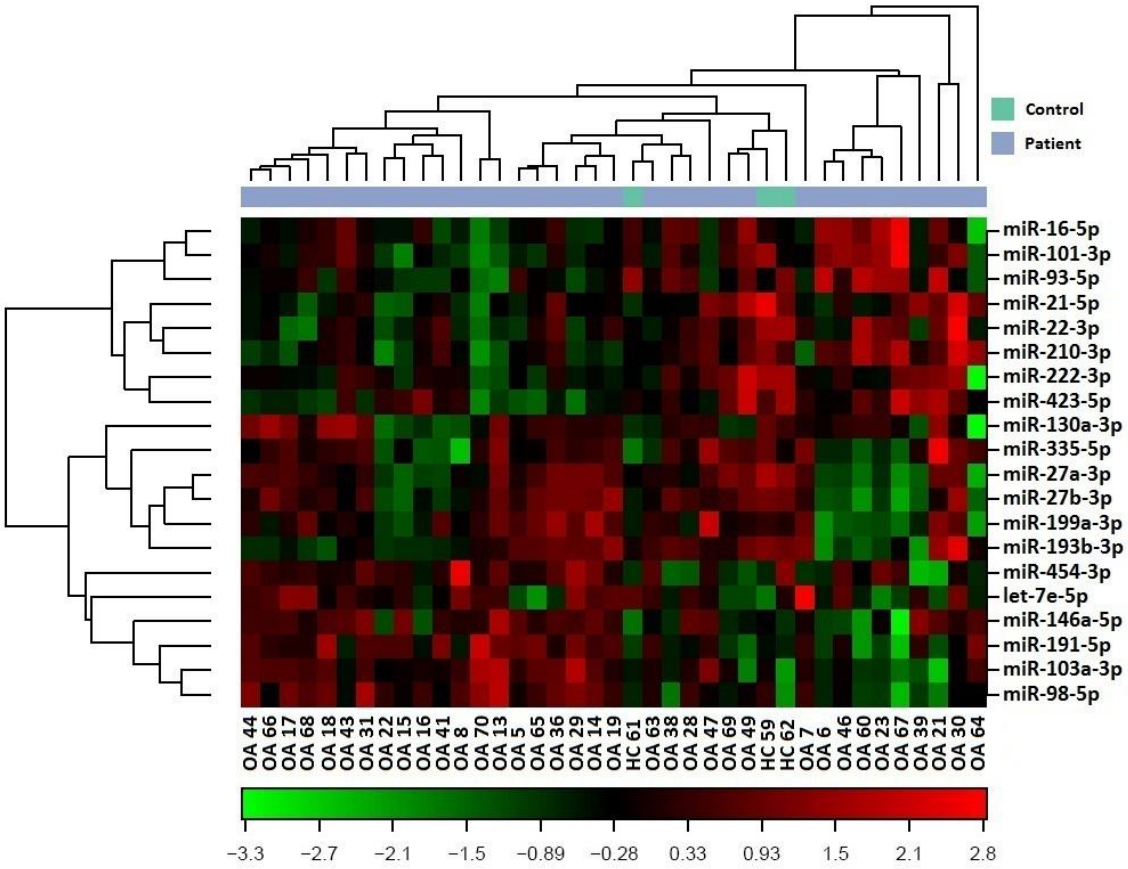

Fig. 2. Unsupervised hierarchical clustering of the serum differentially expressed miRNAs in healthy controls and patients with osteoarthritis. The clustering is performed on all samples. Each row represents one miRNA, and each column represents one sample. The miRNA clustering tree is shown on the left. The normalized (dCq) values have been used for the analysis. The colour scale illustrates the relative level of miRNA expression: red color represents an expression level above mean, green color represents expression lower than the mean

We did not find any statistically significant relationship between the expression of miRNAs and clinical characteristics. We have also observed no significant correlation between miRNA expression level between early and late stages of OA.

4. Discussion

OA arises as a result of mechanical and biological degenerative changes. These lead to instability and changes in the balance between the processes of degradation and synthesis of structures of cartilage and subchondral bone [34]. In recent years, reports of research findings have revealed various roles for specific miRNAs in physiological processes but also in OA development [19, 35, 36]. OA is an irreversible process that often results in a physical disability, therefore there is a need to find effective treatment for inhibiting its development.

There are some evidences indicating that miRNAs can be useful as OA biomarkers. miRNA in serum or plasma hold great promise as minimally invasive diagnostic biomarkers for a wide range of diseases [37]. miRNAs have wide-ranging biological potential, are limited in numbers and are relatively stable in serum/plasma. It has been shown that miRNAs have potential as predictor for severe knee or hip OA [14, 38]. However, there is still a limited number of serum miRNA expression profiling studies in OA. Therefore, in the present study we determined the expression profile of selected miRNAs in serum of OA patients. We have found that expression of miR-146a-5p and miR-98-5p was increased significantly, while miR-222-3p, miR-22-3p, miR-27a-3p and miR-93-5p was decreased significantly in serum of patients with OA in comparison with healthy subjects. Moreover, literature data indicates that all of these miRNAs are associated with promotion or progression of OA [19, 25, 36, 39-41]. In this study we did not found any statistically significant relationship between the expression of selected miRNA and clinical characteristics.

miR-146a is one of the first identified miRNAs, which was differentially expressed in OA cartilage. Li *et al.* showed that in OA in both the homeostasis of the joint cells and the severity of pain symptoms, miR-146a plays an important role by maintaining the balance between the inflammatory response and expression of pain-associated factors in different joint tissue [42]. In OA, inflammation of the synovium results in the

production of tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) that are produced by mononuclear cells activated synoviocytes and articular cartilage and are involved in the pathogenesis of OA. This may cause the cascade of inflammatory factors that lead to chronic inflammation of the synovium [43]. Nakasa *et al*. demonstrated that in cartilage with low Mankin grade miR-146a is strongly expressed, and its expression is decreasing proportionally with the level of matrix metalloproteinase 13 (MMP-13) expression. [44]. Moreover, the study by Taganov *et al.* reported that in early-stage OA, miR-146a expression in cartilage tissue can be activated by IL-1β, concurrently with the onset of degenerative changes, which can be significant in repression of catabolic factors like interleukin-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) [44,45]. In late stages of OA, decreased level of miR-146a expression in OA cartilage has been observed in comparition of early stage of OA. It suggests that cartilage degradation might progress due to loss of miR-146a functioning as a repressor of catabolic signals [19]. Okuhara *et al.* observed that expression levels of miR-146a, -155, -181a, and -223 in PBMCs (Peripheral Blood Mononuclear Cells) of OA patients were significantly higher than those found in healthy controls, suggesting these miRNAs are related to the pathogenesis of OA. Moreover, in the early stages of OA, miR-146a and 223 expressions were significantly higher than they were at later stages [41]. miR-146a may also be associated with OA pathogenesis by increasing vascular endothelial growth factor (VEGF) levels and by impairing the transforming growth factor-beta (TGF-β) signalling pathway through targeted inhibition of mothers against decapentaplegic homolog 4 (SMAD4) in cartilage [46].

Jones *et al*. identified that miR-9, miR-98 were upregulated in OA cartilage and miR-146 was downregulated in both OA bone and cartilage tissue. Moreover, over-expression of these miRNAs in isolated human chondrocytes reduced IL-1β induced TNF-α production, what suggests that they mediate pathways regulated inflammatory response [40]. It is known that osteoblasts also participate in the inflammation process. It has been reported that proinflammatory cytokine, leptin, induces production of oncostatin M in osteoblast by downregulating miR-93 through the Act signalling pathway [39].

Literature data indicates that also miR-22 can be involved in inflammatory networks in OA. In chondrocytes, miR-22 directly regulates two target genes, peroxisome proliferator-activated receptor alpha (PPARα) and bone morphogenetic protein 7 (BMP-7), and its upregulation induced inflammatory and catabolic changes [25]. PPARα is a receptor involved in metabolism sub-network, which is connected with inflammatory process trough IL-1β, and its decreased expression was found in OA cartilage [47]. Expression of BMP-7 is also frequently decreased in osteoarthritic cartilage, whilst MBP7 overexpression induces formation of cartilage [48]. Moreover, inflammatory process could be blocked by inhibition of miR-22 in osteoarthritic chondrocytes and subsequently upregulate the expression of cartilage repair protein, aggrecan. Furthermore, upregulation of miR-22 expression was positively correlated with BMI in OA patients [25]. However, in our study there is no correlation between miR-22 expression and BMI in OA patients.

Tardif *et al*. suspect that MMP-13, as well as insulin-like growth factor-binding protein 5 (IGFBP-5), are likely indirect targets of miR-27a in human chondrocytes [36]. MMP-13 is well known to be upregulated in OA and to play a major role in the pathophysiological process of disease [49]. Findings from several studies have shown that the IGFBP-5 expression level was dysregulated in human OA cartilage, chondrocytes or subchondral bone osteoblast [50-52]. MMP13 is also regulated by miR-222. In Song *et al*. study, the over-expression of miR-222 significantly reduced the cartilage destruction in DMM-induced mice, whereas it was intensified by suppression of miR-222. RNA and protein levels of MMP-13 were decreased by the over-expression of miR-222 and increased by suppression of miR-222. The histone deacetylase 4 (HDAC-4) was also decreased by the over-expression miR-222. These data indicate that miR-222 is involved in cartilage destruction by regulating MMP-13 level and targeting HDAC-4 [24]. In addition, miR-222 may be involved in an articular cartilage mechanotransduction pathway. It has been shown that expression pattern of this miRNA in articular cartilage was higher in the weight-bearing anterior medial condyle as compared with the posterior nonweight-bearing medial condyle [53].

5. Conslusions

Taken together, scientists have so far identified and described a variety of risk factors, molecular mechanisms of pathological processes that are responsible for the creation and development of OA. Despite this, OA is still an incurable disease. Due to the complexity of pathological processes occurring in the joints it is difficult to develop an effective drug therapy. miRNAs found in body fluid such as serum in a stable form have the potential to be a diagnostic and prognostic biomarker of various diseases including OA. It is important to diagnose OA earlier and to start treatment to prevent joint destruction so the distinctive profile of changes in the expression levels of miRNAs can be helpful in early diagnosis. miRNAs are also a promising target for assessment of prognosis and selection of potential therapeutic intervention in OA. We have found an alteration in expression of six miRNAs including miR-146a-5p, miR-98-5p, miR-222-3p, miR-22-3p, miR-27a-3p and miR-93-5p, but we did not find any statistically significant relationship between clinical data and the expression of selected miRNA. Therefore, further research is needed to assess the usefulness of serum miRNAs in miRNA-based prognostic and therapeutic approaches.

Abbreviations

BMP-7 – bone morphogenetic protein 7
Cq – quantification cycle
HDAC-4 – histone deacetylase 4
IGFBP-5 – insulin-like growth factor-binding protein 5
IL-1β – interleukin 1 beta
IRAK1 – interleukin-1 receptor-associated kinase 1
MMP-13 – matrix metalloproteinase 13
OA – osteoarthritis
PPARα – peroxisome proliferator-activated receptor alpha
SMAD4 – mothers against decapentaplegic homolog 4
TGF - β – transforming growth factor-beta
TNFα – tumor necrosis factor alpha
TRAF6 – TNF receptor-associated factor 6
VEGF – vascular endothelial growth factor

Conflicts of interest

The authors declare no conflict of interest with regard to this study.

Declarations

Ethics approval and consent to participate

The investigation process was approved by the Bioethical Committee of the Medical Faculty 270 of Rzeszow University (number: 5/01/2014). Written informed consent was obtained.

Consent for publication

Not applicable.

Availability of data and materials

All data and materials were presented in the main paper.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

D. Sz.: material and data collection, data interpretation, manuscript preparation, literature search

J. C.: study design, material and data collection, data interpretation, manuscript preparation, literature search

M. S.: material and data collection, data interpretation, manuscript preparation, literature search

M. K.: material and data collection

D. H.-O.: material and data collection

K. G.: statistical analysis

G. G.: manuscript preparation, literature search

S. S.: material and data collection, data interpretation

I. Z.: study design, data interpretation, manuscript preparation, literature search**. I. Z.** supervised the study.

All authors contributed to data interpretation and manuscript preparation and approved the final version submitted

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