miR-223 is overexpressed in T-lymphocytes of patients affected by rheumatoid arthritis

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ABSTRACT

miRNAs have recently emerged as key regulators of the immune system, being involved in lymphocyte selection and proliferation, in Treg cells differentiation, and in hematopoiesis in general. Rheumatoid arthritis (RA) is an autoimmune pathology the etiology of which is still obscure. Although a multifactorial pathogenesis has been hypothesized, the precise mechanisms leading to the disease are still poorly understood at the molecular level. miRNA expression profile analysis highlighted that miR-223 is the only miRNA that is strikingly deregulated in peripheral T-lymphocytes from RA patients compared with healthy donors. Further analysis by quantitative reverse transcription–polymerase chain analysis confirmed that miR-223 is overexpressed in T-lymphocytes from RA patients (n = 28) compared with healthy donors (n = 10). Moreover, purification of different T-lymphocyte populations from RA patients highlights that miR-223 is expressed at higher levels in naive CD4+ lymphocytes, whereas its expression is barely detectable in Tc17 cells. In summary, our data provide a first characterization of the miRNA expression profiles of peripheral T-lymphocytes of RA patients, identifying miR-223 as overexpressed in CD4+ naive T-lymphocytes from these individuals. A deeper analysis of the biologic functions and effects of the expression of miR-223 in T-lymphocytes is needed to clarify the exact link between our observation and the disease.

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1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by inflammation of joint synovial tissue and progressive damage to the cartilage and bone tissue, ultimately leading to disability [1]. The mechanisms underlying pathology initiation and progression are still poorly understood. However, it is largely accepted that an important role is played by the immune system. In fact, synovia are infiltrated with macrophages and CD4+ T-lymphocytes [2,3]. Moreover, a decrease of TCR repertoire diversity has been observed in RA patients. This decrease is paralleled by an age inappropriate telomere erosion in peripheral T-lymphocytes, suggesting a defect in T-cell homeostasis [4].

Recently, several studies highlighted that microRNAs (miRNAs) are important regulators of the immune response [5]. These tiny noncoding RNAs, 19–23 nt long, act as negative regulators of gene expression at post-transcriptional level, inhibiting target mRNA translation and/or inducing specific degradation of their target mRNAs [6]. miRNAs playing a role in the immune system include miR-181a, which has been shown to control TCR sensitivity [7], thus possibly affecting thymic selection; miR-150, which plays a key role in lymphocyte differentiation [8,9]; miR-155, acting as a regulator of B-lymphocyte proliferation [10]; and the miRNA cluster 17-92, the expression of which leads to uncontrolled lymphocyte proliferation, lymphomas, and impairment of peripheral tolerance [11,12]. miRNAs have also been shown to play a role in innate immunity: for example, miR-146a is a negative regulator of TLR-4 signaling involved in innate immune response [13] and miR-223 is specifically expressed upon retinoic acid induced differentiation of the promielocytic leukemia cell line HL-60 and has been shown to play a role in granulocytic differentiation [14–16].

The role of miRNAs in inflammatory diseases has been recently explored. Stanczyk et al. [17] revealed a prominent upregulation of miR-155 and miR-146a in RA synovial fibroblasts (RASFs) compared with those in patients with osteoarthritis (OA). These authors also demonstrated that the expression of miR-155 in synovial fibroblasts could be further induced by tumor necrosis factor (TNF)−α, interleukin-1β, lipopolysaccharide, poly(I-C), and bacterial lipoprotein. Nakasa et al. [18] confirmed the increased expression of...
miRNA-146 in synovial tissue from patients with RA compared with OA and normal controls also by in situ hybridization and immunohistochemistry of tissue sections. The authors also concluded that miR-146 expressed in RA synovial tissue may be induced by stimulation with TNF-α and IL-1β. More recently, a significant increase of miR-146a, miR-155, miR-132, and miR-16 in peripheral blood mononuclear cells from RA patients compared with healthy and diseased control individuals has been highlighted, suggesting that miRNAs can be involved at different levels in the pathogenesis of RA [19].

The aim of the present study was to investigate the role of miRNAs in RA; in particular, as T-lymphocytes have been reported to play a role in the pathogenesis of RA, we focused on the role of miRNAs in this cell lineage. Our analyses highlight miR-223 as the only miRNA dramatically upregulated in peripheral blood CD3+ lymphocytes from RA patients compared with healthy controls. Moreover, our data suggest that miR-223 overexpression occurs mainly in CD4+ naive T-lymphocytes. We suggest that this aberrant overexpression of miR-223 in RA patients lymphocytes could contribute to pathogenesis of the disease. Identification of miR-223 targets in T-lymphocytes could therefore contribute to elucidate at least in part some of the molecular mechanisms that lead to RA.

2. Subjects and methods

2.1. Patients and controls

We studied 37 RA patients, 27 female and 10 male, 22–82 years of age (median 58 years), with a disease duration between 2 and 336 months (median 132 months). All patients fulfilled the 1987 ACR criteria for the classification of RA [20], all had active disease as defined according to DAS28 score. In all, 75% were IgM rheumatoid factor (RF) positive, and 67.5% had anticyclic citrullinated peptide antibodies (anti-CCP) in serum. Nine of them were taking no drug at all, whereas 28 were under treatment with less than 10 mg of prednisone and paracetamol at the time of blood collection. No patient was taking disease modifying antirheumatic drugs (DMARDs) or was under biologic (anticytokine) therapy. Control samples were taken from local blood donor bank.

Demographic and clinical characteristics of patients are summarized in Table 1. In all patients DAS28 was calculated at the time of recruitment, and IgM RF, and anti-CCP antibodies were tested by commercial kits. This study was approved by the University of Siena local Ethic Committee, and written permission was obtained from all who participated in the study.

2.2. miRNA expression profile

Microarray assay was performed using a service provider (LC Sciences, Houston, TX). A 10-μg quantity of total RNA were size fractionated using a YM-100 Microcon centrifugal filter (Millipore, Billerica, MA) and the small RNAs (<300 nt) isolated were 3’-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining. Hybridization was performed overnight on a μParafflo microfluidic chip using a micro-circulation pump (Atactic Technologies) [21]. On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to target microRNA (from miRBase, http://

Table 1

<table>
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<tr>
<th>ID</th>
<th>Gender</th>
<th>Age (y)</th>
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<th>Therapy</th>
<th>Experimental procedure</th>
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</table>
microrna.sanger.ac.uk/sequences/) or other RNA (control) and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. The detection probes were made by in situ synthesis using photogenerated reagent chemistry. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization buffer consisted of 100 μl 6 × SSPE (Sodium chloride, Sodium Phosphate, EDTA) buffer (0.90 mol/l NaCl, 60 mmol/l Na₂HPO₄, 6 mmol/l ethylenediaminetetraacetic acid (EDTA), pH 6.8) containing 25% formamide at 34°C. After hybridization, detection was performed using tagspecific Cy3 dye. Hybridization images were collected using a laser scanner (GenePix 4000B, Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics).

2.3. Quantitative reverse transcription–polymerase chain reaction analysis

Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) of miR-223 was performed using the Applied Biosystems (Foster City, CA) kit for measuring miR-223; small RNA U6B was used for normalization. Polymerase chain reactions were performed in triplicate.

2.4. Statistical analysis

We used the miRHuman 9.0 chip from LC Sciences to profile miRNA expression. This chip contains probes for 470 different mature miRNAs; each miRNA probe is repeated seven times on the array. Signal values from each array were background subtracted (array background value was set as quantified by the service provider), quantile-normalized between the seven intra-array replicates and the signal value for each miRNA summarized as the average of repeating spots. Processed miRNA intensity values were normalized between arrays by quantile normalization. Quantile normalization was performed using function “normalize.quantiles” from R package “affy” using default settings [22]. p Values were calculated using Wilcoxon rank-sum test.

2.5. Cell purification

Blood samples were drawn into EDTA-supplemented tubes. Resuspended cells from peripheral blood samples were separated by lymphoprep (Nyegaard, Oslo, Norway) density gradient centrifugation.

Fig. 1. Microarray screening for miRNAs differentially expressed in rheumatoid arthritis. (Upper panel) miRNAs expression profile for peripheral blood T-lymphocytes (CD3⁺) of three RA patients (patients 1–3) and a healthy donor (control) is depicted as a heatmap. (Lower panel) Expression values for miR-223 are represented.
2.6. Pan T-cell isolation

T cells were purified from RA patients’ and healthy donors’ PBLs by using the Pan T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, non-T cells (i.e., B cells, NK cells, dendritic cells, monocytes, granulocytes, and erythroid cells) were depleted by separation over a MACS Column after labeling with a cocktail of biotin-conjugated mAbs (anti-CD14, -CD16, -CD19, -CD36, -CD56, -CD123, -Glycophorin A), as primary labeling reagent, and anti-Biotin MicroBeads, as secondary labeling reagent (Miltenyi Biotec).

2.7. CD4+CD45RA+ and CD4+CD45RO+CCR4CCR6 isolation

CD4⁺ T cells were purified from RA patients’ and healthy donors’ PBLs by using the Pan T cell isolation kit (Miltenyi Biotec). Briefly, non-CD4⁺ cells were depleted by separation over a MACS Column after labeling with a cocktail of biotin-conjugated mAbs (anti-CD8, -CD14, -CD16, -CD19, -CD36, -CD56, -CD123, -Glycophorin A) and anti-Biotin MicroBeads (Miltenyi Biotec). The enriched CD4⁺ T-cell fraction was then recovered, conjugated with CD45RO MicroBeads (Miltenyi Biotec), and separated using a MACS Column. The unlabeled CD4⁺CD45RO⁺RA⁺ T-cell fraction was retained collecting the effluent, instead the magnetically labeled CD4⁺CD45RO⁺CD45RA⁺ T cells were retained on the column and eluted after removal of the column from the magnetic field.

2.7. CD4⁺CD45RA⁺ and CD4⁺CD45RO⁺CCR4CCR6 isolation

CD4⁺CD45RA⁺ T cells were purified from PBLs by using CD4⁺ T-cell isolation kit II (Miltenyi Biotec). Briefly, non-CD4⁺ cells were depleted by separation over a MACS Column after labeling with a cocktail of biotin-conjugated mAbs (anti-CD8, -CD14, -CD16, -CD19, -CD36, -CD56, -CD123, -Glycophorin A) and anti-Biotin MicroBeads (Miltenyi Biotec), and separated using a MACS Column. The unlabeled CD4⁺CD45RO⁺RA⁺ T-cell fraction was then recovered, conjugated with CD45RO MicroBeads (Miltenyi Biotec), and separated using a MACS Column. The unlabeled CD4⁺CD45RO⁺RA⁺ T cells were retained on the column and eluted after removal of the column from the magnetic field.

Fig. 2. qRT-PCR analysis of a larger cohort of patients confirms overexpression of miR-223 in peripheral blood T-lymphocytes from RA patients compared with healthy controls. miR-223 expression is depicted. Dark gray box: healthy controls (n = 10); light gray box: treated RA patients (n = 21); white box: untreated RA patients (n = 8). Boxes represent two and three quartile range. Whiskers extend to the first data point 1.5 times the IQR below box lower end and to the first point 1.5 IQR higher than the upper end of box. Representative fluorescence-activated cell sorter (FACS) analysis to document cell purity are showed.

Fig. 3. miR-223 is expressed predominantly in CD4⁺ cells in RA patients. CD4⁺ and CD8⁺ T-lymphocytes were purified from peripheral blood of three different RA patients and three healthy controls. miR-223 expression levels are depicted.

Fig. 4. Expression of miR-223 in CD3/CD28 stimulated naive cells. Primary CD4⁺ naive T-lymphocytes from healthy donors were plated at a density of 500,000/ml and stimulated with anti-CD3/CD28 antibodies. Cells were harvested at indicated time points and miR-223 was measured by qRT-PCR.
In addition, purified CD4^+CD45RO^+ cells were stained with PE-Cy7–conjugated anti-CCR4 (BD Pharmingen, Franklin Lakes, NJ) and antigen presenting cell (APC)–conjugated anti-CCR6 (R & D Systems, Minneapolis, MN), then processed with FACSARia (Becton Dickinson, Franklin Lakes, NJ) to sort CD4^+CD45RO^+CCR4^+CCR6^+ and CD4^+CD45RO^+CCR4^CCR6^- cells.

3. Results

3.1. miR-223 is upregulated in CD3^+ T-lymphocytes from peripheral blood of RA patients

In the first step, three patients with active RA, who were taking no drugs, and a healthy control subject were tested for 470 miRNAs by microarray technique (Fig. 1A). The test was performed on CD3^+ sorted blood cells. The miRNA expression profile in the four samples was strikingly similar for all miRNAs but miR-223, which appeared to be dramatically upregulated in the three RA patients only (Fig. 1B). A few other miRNAs showed a less marked differential expression between RA patients and control (miR-142-3p, miR-30e-5p, miR-565). miR-565, originally annotated as a miRNA, has been shown to be rather a tRNA [23] and was therefore not studied further.

3.2. qRT-PCR validation in an extended cohort of RA patients and healthy donors confirms microarray data

To validate the observation that we made by microarray analysis of miRNA expression profile, we set out to measure miR-223 expression levels in peripheral blood CD3^+ lymphocytes from an extended cohort of 28 RA patients and 10 different healthy donors. Median level of miR-223 was 58.3 in RA patients, compared with 7.1 in the controls (p < 0.001 according to Wilcoxon rank sum test). Measurement of miR-142-3p and miR-30e-5p did not confirm microarray data (data not shown).

Thus, our data confirm that RA patients display an upregulation of miR-223 compared with healthy donors. There was no significant difference in the level of miR-223 expression between untreated and treated (with low-dose corticosteroids) RA patients (Fig. 2). We did not find any correlation between miR-223 levels and disease activity as assessed by DAS28 index. No correlation was found between miR-223 levels and RA severity evaluated on the base of ACR standards [24]. Furthermore, miR-223 levels do not seem to correlate with age and disease duration.

3.3. CD4^+ T-lymphocyte from RA patients express miR-223 at higher levels compared with CD8^+ cells

To understand whether a specific subpopulation of T-lymphocytes is responsible for miR-223 overexpression, we sorted CD4^+ and CD8^+ T-lymphocytes from three different RA patients and compared miR-223 expression levels, measured by qRT-PCR, in these two subpopulations. We found that miR-223 is predominantly expressed in CD4^+ cells (Fig. 3). CD4^+ cells have been highlighted previously as key players in RA pathology. It is therefore of interest that this subpopulation seems to be responsible for most of the miR-223 measured within the CD3^+ cells of RA patients.

3.4. CD3/CD28 stimulation of Jurkat cells does not result in miR-223 induction

As T-lymphocyte activation has been reported to affect miRNA expression profile, we asked whether miR-223 overexpression in RA patients is caused by T-lymphocyte activation. To this end, we have studied miR-223 expression in primary T-lymphocytes from healthy donors. Naive T-lymphocytes were stimulated with anti CD3/CD28 beads, and miR-223 levels were assessed by qRT-PCR. We did not observe any upregulation of miR-223 upon TCR stimulation (Fig. 4). We also exploited a well-established model of T-lymphocyte activation, the Jurkat cell line (an acute T-lymphocytic leukemia cell line) stimulated with anti-CD3/CD28 antibodies. Although we observed upregulation of other miRNAs known to be overexpressed in response to TCR stimulation, even in this system we failed to detect any upregulation of miR-223 upon CD3/CD28 stimulation (data not shown).

3.5. CD4^+ CD45RA^+ T-lymphocytes (naive) from RA patients express miR-223 at higher levels compared with CD4^+ CD45RO^+ cells (memory)

Prompted by the observation that miR-223 is expressed in CD4^+ T-lymphocytes and taking into account that Th17 lymphocytes have recently been suggested as main actors in RA pathogenesis [25], we asked whether Th17 cells could be responsible for miR-223 expression. To verify this hypothesis, we set out to purify Th17 cells and, as a control, naive and memory T-lymphocytes from peripheral blood of RA patients. Because of the extremely low number of
circulating Th17 cells, T-lymphocytes from eight different patients were pooled before purification of the different T-cell subpopulations. Surprisingly, analysis of miR-223 in these samples pointed to naive CD4+ T-lymphocytes as overexpressing miR-223 (Fig. 5A), whereas miR-223 expression levels in Th17 cells appeared to be lower. To validate this preliminary and unexpected observation, we sorted naive CD4+ lymphocyte and memory CD4+ lymphocytes from peripheral blood of six different RA patients. As shown in Fig. 5B, qRT–PCR measurement of miR-223 expression levels confirms that in most RA patients miR-223 is predominantly expressed in CD4+ naive cells.

4. Discussion

MicroRNAs (miRNA) are a new class of modulators of gene expression, and a large body of evidence can be found in the literature demonstrating a role for miRNAs in adaptive immunity [26]. Because T-lymphocytes have been suggested to play a role in RA [3], we have studied miRNAs expression in T-lymphocytes from peripheral blood of RA patients and healthy donors. Notably, to avoid measuring artifactual effects on miRNAs expression caused by therapy, we focused our analysis on RA patients without any treatment or treated with low-dose corticosteroids. We demonstrated an extremely high expression, independent of treatment, of miR-223 compared with that in normal controls (−10-fold) (Fig. 2); in particular, we observed that miR-223 was predominantly expressed in naive CD4+ T-lymphocytes and not in Th17 cells, which were previously reported to play a role in RA. Moreover, miR-223 expression was not observed in Jurkat cell line after TCR stimulation. In addition, naive CD4+ T-lymphocytes from healthy donors consistently do not express miR-223 even after TCR stimulation, suggesting that miR-223 overexpression is an aberration associated with the pathology and not a consequence of T-lymphocyte activation in RA patients.

Recently, upregulation of miR-146a and miR-155 was observed in synovial fibroblasts of patients with RA compared with synovial fibroblasts from controls or patients with osteoarthritis [17,18]. Moreover, Pauley et al. [19] demonstrated that RA peripheral blood mononuclear cells exhibited between 1.8-fold and 2.6-fold increases in miR-146a, miR-155, and miR-132, and miR-16 expression, suggesting that the increased miRNAs expression in rheumatoid arthritis patients may be potentially useful as a marker for disease diagnosis, progression, or treatment efficacy. Indeed, these increases are rather mild compared with the approximately 10-fold upregulation of the miR-223 observed here. Furthermore, upregulation of miR-146a, miR-155, and miR-132, but not miR-223, is also observed upon TCR stimulation of T-lymphocytes from healthy donors (Macino G, 2008, unpublished data) and therefore their upregulation in RA patients could rather be a consequence of the activation of T-lymphocytes. In our case, it was not possible to verify correlations between miR-223 levels and clinical or serologic features, as we included only patients with active disease who were also positive for both RF and anti-CCP antibodies.

In summary, our data show a sustained expression of miR-223 in T-lymphocytes of RA patients and highlight that miR-223 in RA patients is predominantly expressed in naive CD4+ T-lymphocytes, suggesting that its expression in this cell type could contribute to the etiology of the disease. Although the role of miR-223 in the differentiation of the myeloid lineage has been characterized [14,15], little is known about its function in T-lymphocytes. Further studies are required to characterize the function of this miRNA in T-lymphocytes and to assess its role in RA.

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