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Preliminary report

Analysis of miRNA expression in patients with rheumatoid arthritis during remission and relapse after a 5-year trial of tofacitinib treatment

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ABSTRACT

The physiopathology of rheumatoid arthritis (RA) is mediated by proinflammatory cytokines, some of which are regulated by the JAK/STAT pathway. Tofacitinib is a JAK inhibitor, but its role in the regulation of microRNAs (miRNAs) is unknown. There is also no information regarding the role of miRNAs in the clinical relapse/remission of RA. The present project aims to identify a signature profile of miRNA expression in a subgroup of RA patients who had to discontinue tofacitinib treatment (because of the ending of a 5-year open-label clinical trial) and to describe the expression of miRNAs during RA remission or flare-up. The relative expression of 61 miRNAs was determined in serum samples with the Firefly™ BioWorks assay. Statistical analysis was performed by means of Student's *t*-test and heatmap analysis was performed with Firefly™ Analysis Workbench software and in the software GraphPad® Prism v5.0. Target prediction and Gene Ontology analysis were carried out using bioinformatic tools. We found a distinctive signature of miRNA expression associated with relapse, featuring upregulated expression of hsa-miR-432-5p ($p < 0.05$). We also found upregulation of hsa-miR-194-5p ($p < 0.05$) in samples of patients with RA flare-up. Gene Ontology analysis of the target genes for hsa-miR-432-5p was performed to identify relevant pathways associated with relapse; the implications of these pathways in the physiopathology of RA are discussed. Tofacitinib treatment does not have a direct effect on the expression of measured miRNAs. The changes in hsa-miR-432-5p and hsa-miR-194-5p are associated with the regulation of proinflammatory pathways and RA flare-up.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory and autoimmune disease that affects joints and other systems [40]. The etiology of RA is multifactorial, including genetic and environmental factors (air pollution, infections, etc.). RA predominantly affects women of reproductive age and is estimated to affect about 1% of the world's population [41].

The physiopathological mechanisms of RA are mediated by proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), granulocyte-colony stimulating factor (GM-CSF), interleukin (IL)-6, IL-1β and IL-17, among others [7, 18]; the intracellular signaling of these cytokines is mediated through the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathway. When a cytokine binds to its receptor, it leads to the tyrosine phosphorylation of associated JAKs and to the recruitment, phosphorylation, and dimerization of STATs, which translocate to the nucleus and promote or regulate the transcription of target genes related to inflammation [1, 35].

The therapeutic arsenal in rheumatology has improved drastically

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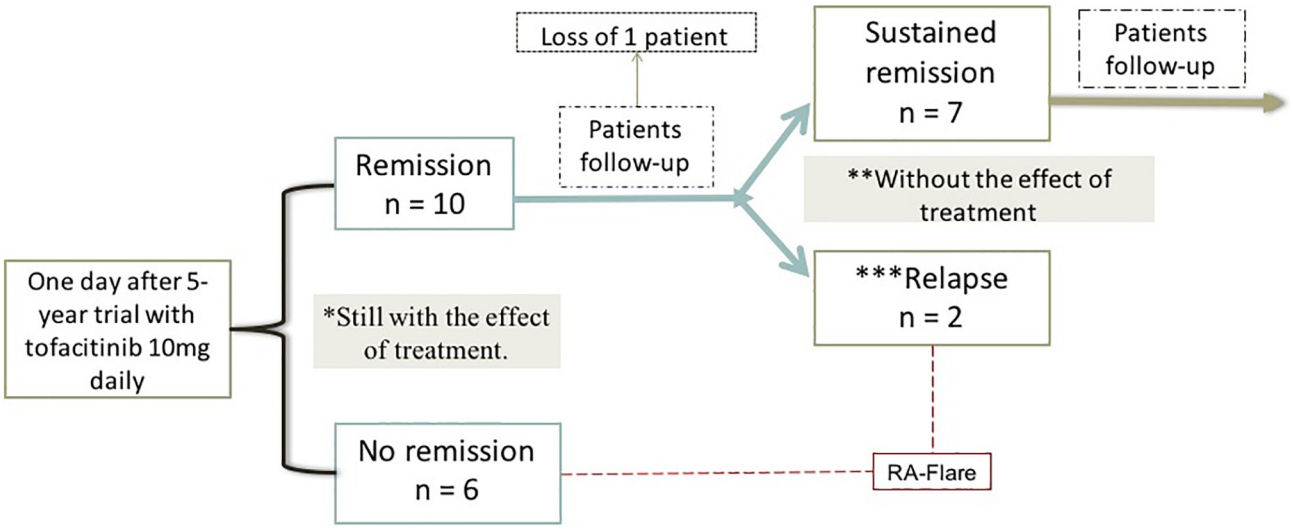

Fig. 1. Diagram of patient follow-up and sample collection (*first sample collection, **second sample collection, ***at least one inflamed joint).

during the last 20 years with the availability of biological and target synthetic disease-modifying anti-rheumatic drugs (bDMARDs and tsDMARDs). Recently, other approaches have been explored, particularly the inhibitors of JAK kinases such as tofacitinib (TOFA), a new kind of tsDMARD. TOFA was recently approved for the treatment of RA in several countries [11, 34, 39]. Smolen et al. found that TOFA was effective at inducing remission after 3 months of treatment using various established and new remission criteria; the remission rates were generally greater with TOFA at 10 mg twice a day [39]. It is known that patients with RA who are in remission can suffer relapse after the suspension of any of the DMARDs; however, the causes of this are unknown, although several risk factors for relapse have been identified [6, 15, 32]. Moreover, little information on the molecular mechanisms involved in RA relapse has been obtained.

There are natural mechanisms that regulate important molecules of signaling pathways, as well as the expression of pro- and anti-inflammatory cytokines. One of the components in these mechanisms is the microRNAs (miRNAs). miRNAs are small noncoding RNA molecules that bind target messenger RNA (mRNA) to negatively regulate the translation of genes involved in the immune system, such as those encoding cytokines, chemokines, and signaling molecules [3, 23]. The changes in the expression of miRNAs and the regulation of their targets during periods of remission and relapse of RA are unknown; how these miRNAs change upon TOFA treatment is also unclear.

Current treatment guidelines clearly define the criteria for using bDMARDs or tsDMARDs for RA treatment; however, there are no current guidelines available as to when to stop therapy. Some studies have described certain clinical variables that can predict relapse, such as methotrexate (MTX) discontinuation, basal anti-citrullinated protein antibody (ACPA) levels, and high rheumatoid factor (RF) [6, 15, 32]. The present project aims to find a signature profile of the miRNAs in a subgroup of RA patients who had to discontinue TOFA because of the ending of a 5-year open-label clinical trial and to describe the expression of miRNAs during RA remission or flare-up.

2. Materials and methods

2.1. Study design

This was a prospective cohort study of patients with RA assembled at the last TOFA intake because of the ending of an open-label, industry-sponsored, long-term extension trial (study number A3921024) conduced in a single research center. All patients included in the study had a failure to MTX treatment. None of them had any previous use of bDMARDs.

2.2. Patients

All patients were enrolled in an open-label extension study using TOFA (10 mg per day) for 5 years at one participating center in Mexico. Patients received treatment with MTX (10 mg per day) during the open-label study. The remission criterion was that, at the last TOFA dose, all patients had a 28-Joint Disease Activity Score (DAS28) < 2.6 and no swollen joints. The inclusion criteria were: patients who failed MTX therapy, patients naive to treatment with bDMARDs and patients with 18 years and older. The exclusion criterion was: subjects with serious medical conditions that would make treatment with TOFA potentially unsafe such as infections.

2.3. Procedures and follow-up

After the end of the extension study, patients no longer received TOFA but continued with MTX at the same dose as before. Given the elimination kinetics of TOFA, serum levels of the drug are detectable 30 days after stopping therapy [17]. We obtained a blood sample at the first month after the last dose of TOFA in order to describe the levels of miRNAs associated with treatment and also to monitor the changes in miRNAs associated with relapse or RA flare-up. Each patient was instructed to communicate with our center if they believed that they had a swollen joint; RA flare-up was confirmed by in-office assessment by the attending rheumatologist. In cases with confirmed RA relapse, a blood sample was taken and the occasion was considered as the last visit for the follow-up (Fig. 1).

2.4. Analysis of miRNA expression by flow cytometry using firefly™ BioWorks technology

We designed a panel of 61 miRNAs to be analyzed in the patients' samples, in which miRNAs related to immune response, inflammatory response, JAK/STAT pathway, and RA were included (Table 1). Measurements were performed using the Firefly™ Circulating miRNA Assay (Abcam, Cambridge, MA) [8]. In general, the protocol consists of adding 40 µl of serum to each well and miRNAs hybridizing to complementary oligonucleotide sequences covalently attached to encoded hydrogel microparticles for target capture. The bound target was ligated to oligonucleotide adapter sequences that served as universal PCR priming sites. The miRNA–adapter hybrid molecules were then dehybridized from the particles and reverse-transcription polymerase chain