Role of miRNA Let-7 in Plasma of Rheumatoid Arthritis

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ABSTRACT

Objectives: Micro ribonucleic acids (miRNAs) are noncoding RNAs, recently implicated as potential biomarkers or therapeutic targets for autoimmune diseases such as rheumatoid arthritis (RA). The aim of this study is to assess the role of miRNA Let-7 in the plasma of RA.

Materials and methods: Trained medical staff already enrolled for the study collected blood samples from healthy controls (N = 42) and RA patients (N = 44). In the laboratory, these samples were centrifuged at 2000 rpm for 8 minutes to separate serum from the sample, which was then transferred to a plain vial. Until transport to the genetic lab, samples were stored at −20°C. Deoxyribonucleic acid (DNA) was isolated using a standard protocol cohort of controls and patient blood samples. Quantification of DNA was conducted using ultraviolet (UV) spectroscopy, and DNA quality was assessed on 0.8% agarose gel. A comparison of genotype frequencies in the different study groups was performed using the Chi-squared test, while a comparison of allelic frequencies was conducted using Fisher’s exact test.

Results: Variations of alleles, such as 16539423 (G>T), 16539433 (T>G), and 16539629 (A>T) were found only in RA patients. On the other hand, 16539617 (T>A), 16539622 (G>T), and 16539624 (T>C) were found only in control cases. Five sequences (three RA variants and two control variants) with minimal alignment were compared to the wild-type sequence. We found that the sequence modification of pre-miRNA 16539623 del G was significantly higher and had a risk allele in the study group [odds ratio (OR) = 3.29].

Conclusion: Rheumatoid arthritis (RA) is an autoimmune disorder that presents with a variety of clinical manifestations. Genetic factors possibly account for about 60% of disease susceptibility and expression, thus playing a very important role in disease pathogenesis.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, multisystem inflammatory disorder of autoimmune origin that involves multiple body parts. However, it primarily affects the articular system, resulting in inflammation and synovitis, often progressing to damage of articular cartilage and eventually stiffness and fixation of the joints.1 The incidence of RA is three cases per 10,000 populations per year. The incidence of RA increases with age until the age of 80, while its onset is uncommon before the age of 15 years. The prevalence of RA is 1%. Females are three to five times more commonly involved compared to men2 and four times more common among smokers compared to nonsmokers.

Insights gained from multiple clinical research over the past two decades have miraculously improved the paradigms for the diagnostic approach and management of RA. Serum antibodies to cyclic citrullinated peptides (anti-CCPs) are now identified as a significant serum biomarker for diagnostic and prognostic purposes. Advances in imaging techniques like ultrasound and MRI have facilitated early detection of joint inflammation and subsequent destruction in RA. The science behind the pathogenesis of RA has taken a great step forward with an understanding of new disease-related genes and their molecular pathways of disease pathogenesis. The relative importance of these different mechanisms is evidenced by the adequate benefits of the new class of highly targeted biologic therapies. However, partial desory of the initiating disease-related pathways of RA still remains a significant hurdle.

Rheumatoid arthritis (RA) is strongly associated with human leukocyte antigens (HLA)—DR4 (most specifically DR0401 and 0404), making family history one of the important risk factors.2 HLA-DRB1 and protein tyrosine phosphatase non-receptor type 22 1858T gene variants are important risk factors for the progression of joint destruction in RA. Presently, many immunopathogenetic study models of other genes are undergoing discussion. The outcome of a few of the gene polymorphisms related to RA and pharmacogenetic concepts are being applied to different classes of available medical therapeutics, such as classical disease-modifying antirheumatic drugs and newer biological agents.3

The lethal-7 (Let-7) gene was first found among nematodes, where it acts as the main developmental regulator and now has become one of the first two known micro ribonucleic acids (miRNAs) (the other one is lin-4).4 It plays an important role in posttranscriptional regulation of innate immune responses to pathogenic agents. Numerous reports have depicted a lower frequency of expression levels of Let-7, whereas chromosomal clusters of Let-7 are often found to be deleted in many cancers.5 Let-7 is also a potential therapeutic agent to prevent tumor growth and angiogenesis, specifically the cancers with under-expression of Let-7.4 Intranasal use of Let-7 was found to be highly useful in controlling tumorigenesis in a transgenic mouse model of lung cancer.6 Similar restoration of Let-7 has been observed to be beneficial in controlling tumor growth in other cancers (like breast, colon cancers and uterine leiomyoma).7 The aim of this study is to define the role of miRNA Let-7 in RA patients.

MATERIAL AND METHODS

This is a case-control study that involved all patients visiting our hospital and volunteers for blood investigations. Blood samples of patients were drawn at Medicine and Rheumatology, Outpatient Department of Sardar Patel Medical College, Bikaner, Rajasthan, India after properly performed informed consent from the patients or from patient guardians.

Inclusion Criteria

All patients aged 16 years or older, who were diagnosed with RA using the latest RA classification criteria from 2010, were included as cases.

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Exclusion Criteria
All patients of RA who meet the following criteria are included:

• Those who are critically ill and have associated illnesses such as malignancy, renal failure, or liver failure.
• Those who have overlap syndrome (other connective tissue disorders like scleroderma, SLE, polio, etc.).

Diagnostic Criteria
The 2010 American College of Rheumatology and the European League Against Rheumatism classification criteria for RA.9

Blood Sampling Procedure
Blood samples of patients or volunteers were taken by already enrolled skilled medical staff, prior to discharge, under aseptic precautions and after immediately transferring to already labelled blood vials containing ethylenediaminetetraacetic acid as anticoagulant were carried in a cold chain from the sample collection site to the lab.

In the laboratory, these blood samples were centrifuged at 2000 rpm for 8 minutes to separate serum, which was then collected to a plain vial and kept at −20°C till shifted to the genomic lab.

Analytical Method
Clinical and Biochemistry Evaluations
Clinical data and lab investigations, including a complete blood count, random blood sugar, serum electrolytes such as sodium, calcium, potassium, blood urea, creatinine, AST/ALT ratio, serum ALP, and total proteins were conducted and were recorded from the patient’s IPD sheets.

Isolation of Deoxyribonucleic Acid (DNA)
Deoxyribonucleic acid (DNA) from blood samples of both the case and control groups was isolated as per standard protocol. DNA quantification was done using ultraviolet (UV) spectroscopy, and the quality was assessed on a 0.8% agarose gel.

Polymerase Chain Reaction (PCR)
Polymerase chain reaction (PCR) reactions were carried out with genomic DNA, using forward and reverse primers (as mentioned below), 1X PCR buffer (sigma), 200 µM of deoxynucleotide triphosphates (dNTPs) and 1 unit of Taq polymerase (sigma). The annealing temperature was standardized for the above primer pair to obtain a 245 bp amplicon. Primers used for Let-7c PCR were: 5’-GGTTTGGACGAGTCTGAA-3’ (forward) and 5’-TTGGTTTCCAGCATAGTGC-3’ (reverse) as described by Kim et al.8

Polymerase reaction (PCR) settings used were as per below:

- 94°C for 5 minutes for initial denaturation.
- 94°C for 15 seconds.
- 53°C for 25 seconds.
- 72°C for 15 seconds; 39 cycles.
- 72°C for 10 minutes for final elongation.

Agarose Gel Electrophoresis
Polymerase chain reaction (PCR) products were evaluated by electrophoresis on 1.5% agarose gels to find out their quality and amplification. Agarose gel (1.5% w/v agarose) was made by dissolving agarose in the required quantity of 0.5X TBE electrophoresis buffer by heating in a microwave oven, followed by addition of 0.5 µg/mL of ethidium bromide. This agarose solution was then spread over the gel tray with a comb, allowed to cool and solidify and then placed in an electrophoresis tank and submerged in 0.5X TBE buffer.

The PCR reaction mix was mixed with DNA loading buffer (6X; 0.2% bromophenol blue, 0.25% xylene cyanol, 40% w/v sucrose). DNA samples were loaded into the wells as per size standard. Horizontal electrophoresis was performed at approximately 75V for 1.5 hours. DNA fragments are identified after staining them with ethidium bromide visualized on a UV transillumination, and the gel picture was captured.

Purification of PCR Products
Polymerase chain reaction (PCR) products were purified to remove primer dimers and excess dNTPs and nonspecific products prior to sequencing by using a GenElute Gel Extraction Kit (sigma).

Precipitation of PCR Products
The amplified products were precipitated using the polyethylene glycol (PEG) method.

- Transferred the PCR product into 1.5 mL microfuge tubes.
- Added 0.5 V of 22% PEG to each tube (final concentration is 11%).
- Added 0.1 M MgCl2 in each tube (0.01 M final concentration).
- Mixed the sample thoroughly and kept it on ice for 20 minutes.
- Centrifuged the samples at 4°C, 14000 rpm for 15 minutes.
- Removed the supernatant carefully and again added 70% ethanol in each tube and centrifuged at 14000 rpm for 20 minutes twice.
- Decanted the ethanol carefully and air-dried the pellets.
- Pelleted PCR products were sequenced.

Statistical Analysis
Genotype distributions were determined by using the Chi-square-test for significant deviation from Hardy–Weinberg equilibrium, and a trend test was used to find out any rise in risk with the rise in the number of risk alleles. The risk associated with C17T and A118G polymorphism was calculated using odds ratios (ORs) and 95% confidence intervals, for genotypes in both control and case groups. In study groups, comparisons of genotype frequencies were done using the Chi-squared test, while comparisons of allelic frequencies were done using Fisher’s exact test.

Result and Discussion
The PCR product obtained was purified and sent for DNA sequencing. The DNA sequences were subjected to multiple sequence alignment using NC_000021.9 as a reference sequence for the region under study. The Basic Local Alignment Search Tool was used to analyze the differences in the sequences. Various insertions, deletions, and variations were observed in the 245 bp amplicon (Table1).10

Several variations were found both in RA patients and controls, namely, 16539425 (A>C), 16539427 (A>G), and 16539594 (T>C) were found in both RA and control cases. 16539423 (G>T), 16539433 (T>G), and 16539629 (A>T) were found only in RA patients. 16539617

Table 1: Deletions present in RA and control cases

<table>
<thead>
<tr>
<th>Sequence modifications</th>
<th>Cases</th>
<th>Controls</th>
<th>Odds ratio</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>16539609 del T</td>
<td>7</td>
<td>12</td>
<td>0.17</td>
<td>Protective</td>
</tr>
<tr>
<td>16539611 del G</td>
<td>10</td>
<td>10</td>
<td>0.37</td>
<td>Protective</td>
</tr>
<tr>
<td>16539615 del G</td>
<td>1</td>
<td>3</td>
<td>0.15</td>
<td>Protective</td>
</tr>
<tr>
<td>16539617 del T</td>
<td>13</td>
<td>9</td>
<td>0.62</td>
<td>Protective</td>
</tr>
<tr>
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<td>4</td>
<td>0.58</td>
<td>Protective</td>
</tr>
<tr>
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<td>2</td>
<td>3</td>
<td>0.30</td>
<td>Protective</td>
</tr>
<tr>
<td>16539623 del G</td>
<td>6</td>
<td>1</td>
<td>3.29</td>
<td>Risk allele</td>
</tr>
</tbody>
</table>
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Longer primary transcripts are referred to as pre-miRNAs. These act as the initial substrates to generate the miRNA precursor forms by the nuclear Drosha RNase. Albeit sequences outside of the pre-miRNA hairpin are important for RNA processing or stability but actual composition of pre-miRNAs is still a mystery. Sequences were aligned using Clustal W and based on the minimal alignment, the variants were chosen for MFold analysis. Five sequences (three RA variants and two control variants) with minimal alignment were compared to the wild-type sequence. It is obvious from the folding of the messenger ribonucleic acid (mRNA) structures that the mutations (deletions and insertions) cause a variation in the secondary structure of the mRNA as a consequence of which the processing of pre-miRNA to miRNA could be affected leading to long-ranging effects on gene expression in the prechondrocytes and chondrocytes. Specific changes occurring due to these mutations in the upstream region of the Let-7 gene cluster need to be further characterized in a larger cohort and the resultant changes in expression/regulation of expression of genes involved in the etiology and/or progression of the immunological events underlying the development of RA.11

In multiple studies, aberrant expression of miRNAs has been documented in RA patients. The majority of them focused on T cell differentiation (Th17), which results in the formation of inflammatory cytokines, and B cell activation. These inflammatory markers and miRNAs that control their expression might be used as genetic biomarkers in RA.12

Murata et al. in a study concluded that the involvement of miR-16, miR-146a, miR-155, and miR-223 in the pathogenesis of RA.13

The expression of miR-155 and miR-146a is enhanced in synovial fibroblasts of RA patients after stimulation by pro-inflammatory mediators. This enhanced expression of miR-155 in RA patients' synovial fluid suppresses the production of MMP-3 and counters MMP-3 and -3 induction by pro-inflammatory cytokines and toll-like receptor ligands.14

Eight miRNA (miR-126-3p, Let-7d-5p, miR-431-3p, miR-221-3p, miR-24-3p, miR-130a-3p, miR-339-5p, Let-7i-5p) were significantly elevated in RA serum compared to HC (all p < 0.01) and one miRNA (miR-17-5p) was significantly lower in RA (p < 0.01).

CONCLUSION

Rheumatoid arthritis (RA) is a systemic inflammatory disease with autoimmunity with multiple clinico physical presentations. It affects approximately 70% of women. Genetic factors, accounting for about 60% of disease susceptibility, play a crucial role in pathogenesis. Several studies performed in past to evaluate the specific genes and chromosomal markers attributing to RA in European and American populations, but no such study has been conducted on Indian populations by any rheumatologist or molecular biologist so far. We found that sequence modification of pre-miRNA 16539623 del G was significantly higher and had a risk allele in the study group (OR = 3.29).

REFERENCES