Deregulated Blood Cellular miR-2909 RNomics Observed in Rheumatoid Arthritis Subjects

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Abstract

Background: Both clinical and experimental evidences suggest that over expression of IL-17 and CCL5 induces synovial inflammation and joint destruction leading to rheumatoid arthritis (RA). The present study investigated the inter-relationship between miR-2909 and the genes recognized widely to play crucial role in the pathogenesis of ‘RA’.

Methods: Expression levels of genes coding for IL-6, IFN-γ, Rig-1, IL-17, CCL5, and Sp1 within peripheral blood mononuclear cells (PBMCs) derived from ‘RA’ and control subjects were analyzed using quantitative real time PCR and western blotting. Sequence analysis of coding region and 3´UTR region of KLF4 within PBMCs from ‘RA’ and control subjects was analyzed using Clustal W software.

Results: Our study revealed that PBMCs from ‘RA’ subjects possessed mutant miR-2909 which was unable to repress KLF4 gene expression. The uncontrolled expression of KLF4 led to the increased expression of genes coding for IL-6, IL-17, CCL5, IFN-γ and Rig-1. Moreover, the ability of KLF4 to regulate the expression of above-mentioned genes was verified by down-regulation of KLF4 gene in PBMCs derived from ‘RA’ subjects.

Conclusion: Based upon the study, we propose that mutant miR-2909 may contribute to the initiation of ‘RA’ through its inability to repress KLF4 gene expression thereby resulting in the uncontrolled expression of KLF4 which in turn ensures predominant Th17 immune response having CCL5-induced high chemotactic activity.

Keywords: miR-2909, miR-146a, Kruppel-like factor 4, Immune response, Peripheral blood mononuclear cells, Rheumatoid arthritis

Introduction

Although rheumatoid arthritis (RA) is recognized as a chronic autoimmune disease that affects multiple joint structures and synovial membranes [1], the pathogenesis of ‘RA’ is still far from clear. Recently, epigenomic control involving microRNA (miRNA) regulation in ‘RA’ has received considerable attention and importance [1]. Most of studies carried so far revealed up- or down-regulation of different miRNAs according to the cell type analyzed from tissue sample of ‘RA’ subjects [1]. Little heed has been paid to unfold the possible correlation between miRNA expression, within blood mononuclear cells, and disease activity. Although miR-146a expression in peripheral blood mononuclear cells (PBMCs) has been shown to mimic that of ‘RA’ synovial tissue and fibroblasts [1], the elevated expression of miR-146a has found no specificity to ‘RA’ [1]. A new dimension was added to the emerging field of miRNA-based epigenomic control, by the discovery of a novel immunomodulatory microRNA (designated...
as miR-2909) encoded by human cellular AATF genome [2-4]. In this context, the present study was addressed to explore the nature of miR-2909 RNomics and its impact on immune response exhibited by PBMCs derived from human ‘RA’ subjects.

**Material and Methods**

**Human subjects: Selection criteria**

Freshly diagnosed and untreated rheumatoid arthritis (RA) patients (n=50) were selected from the outpatient “Rheumatology Clinic” of Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India with prior informed consent and approval by our Institutional Ethics (IEC) as well as control human volunteers (n=25; with their prior informed consent), who had abstained from any medication for 2 weeks before blood donation, were employed in the present study.

**Cellular model employed**

Peripheral blood mononuclear cells (PBMCs) were employed as a cellular model for the present study. Blood was drawn through venipuncture into heparinized tubes and PBMCs were isolated using density gradient centrifugation method. Briefly, 5 ml of heparinized blood (from either ‘RA’ or control subjects) was gently layered onto 4 ml of Histopaque (Sigma solution containing polysucrose and sodium diatrizoate, adjusted to a density of 1.077 ± 0.001 gm/ml) and centrifuged at 400xg in the swinging bucket rotor for 30 minutes at room temperature. After centrifugation steps, the layer of PBMCs were recovered and subsequently used for gene expression analysis and sequencing study.

**Gene expression analysis, DNA sequencing**

Human PBMCs (from ‘RA’ and control subjects) were processed for total as well as small non-coding RNA extraction using miReasy mini kit (Qiagen) in accordance to the manufacturer’s protocol as reported by us earlier [2]. The extracted RNA was reverse-transcribed using miScript reverse transcription kit (Qiagen) and subjected to quantitative real-time PCR analysis [2] using gene-specific primers. Genes coding β-Actin and U6 were used as invariant controls for studying the differential expression of cellular mRNAs (IL-6, IFN-γ, IL-17, RIG-1, CCL5, Sp1) and miR-2909 respectively as reported earlier [2,3]. For studying differential gene expression at the translational level, total cellular protein was extracted and subsequently subjected to SDS-PAGE followed by western blotting and immuno-detection using specific antibodies for genes coding for KLF4, IL-6, CCL-5, IL-17, Sp1 and β-Actin (used as invariant control). Keeping in view the fact miR-
2909 suppresses KLF4 gene expression by targeting its 3'UTR region [4], the amplicons of the coding- and 3' UTR- regions of KLF4 gene were also subjected to DNA sequencing using standard method [4] in order to explore whether or not KLF4 gene in 'RA' has any intrinsic genetic aberration. The sequence data was further analyzed using cluster x 2.0.12 software.

Transfection assays
PBMC's derived from RA subjects were transfected with siRNA specific for KLF4 (siKLF4; Sigma) to knockdown KLF4 gene expression or scramble sequence (siControl) using Lipofectamine transfection reagent (Invitrogen) according to manufacturer’s instructions. The cells were incubated for 48hr in RPMI 1640 medium supplemented with 10% (v/v) FBS (Sigma, St. Louis, USA), 100 U/ml penicillin and 100 µg/ml streptomycin under an atmosphere of 5% CO\textsubscript{2} at 37°C. After the completion of incubation period, cells were harvested and processed for analyzing the expression of genes.

Statistical analysis
Data are expressed as mean ± SD of all the experiments done independently. Unpaired Student’s T test was used for comparison between two groups. P values p<0.01 and p<0.05 were considered significant.

Results
The PBMCs derived from ‘RA’ subjects exhibited significantly higher expression of miR-2909 compared to their corresponding control cells (Figure 1A). This phenomenon was accompanied by significantly increased expression of KLF4 gene within PBMCs derived from ‘RA’ subjects as compared to their corresponding control cells (Figure 1B). Since miR-2909 has been shown to suppress KLF4 gene expression by targeting its 3'UTR region [4], the observed paradoxical high expression of KLF4 (Figure 1B) in the PBMCs of ‘RA’ subjects could be possible only if either the target sequence of miR-2909 within 3'UTR region of KLF4 mRNA is mutated or miR-2909 matured sequence is mutated. Sequence analysis of 3' UTR-region of KLF4 mRNA did not reveal any genetic abnormality consequently ruling out the existence of any mutation in the 3'UTR sequence of KLF4 (Figure 1C). However, melting-curve analysis of the amplicons pertaining to miR-2909 did show a significant change between ‘RA’ and control (Figure 1D). Since miR-146a plays crucial role in ‘RA’ coupled with the fact that it targets KLF4 3’UTR region [5,6], we performed melting-curve analysis of miR-146a to determine if any change in melting pattern exists between miRNAs (miR-2909 and miR-146a). Interestingly, on comparison melting curve pattern of miR-2909 revealed conspicuous difference between ‘RA’ and controls.

Figure 2:
Expression levels of KLF4 target genes (A). Expression level of genes for IL-6, IFN-γ and Rig-1 within PBMCs derived from ‘RA’ and control subjects. Bars represent mean ± S.D. ** indicates level of significance (p< 0.01) relative to control (B-E). Protein expression levels of genes for IL-6, IL-17, CCL5, Sp1 in RA with respect to corresponding control cells. β-actin was used as invariant control. ** indicates level of significance (p<0.01). Scion Image Analysis Software was used to determine densitometric analysis of western blot results.
resolving thereby as to why miR-2909 was unable to suppress the KLF4 gene at the translational level within PBMCs from ‘RA’ subjects (Figure 1D). We further investigated if any genetic aberration(s) exists in the coding region of KLF4 from PBMCs of ‘RA’ subjects. Sequence analysis showed no genetic aberrations in this region of KLF4 gene in PBMCs from ‘RA’ subjects compared with their corresponding control counterparts, suggesting that KLF4 protein structure is unaltered in these patient samples (Figure 1E).

Further experiments were directed to explore the differential expression of KLF4-regulated genes within PBMCs derived from ‘RA’ and control subjects, revealed increased expression of genes coding for IL-6, IFN-γ, IL-17, RIG-1 and CCL5 as well as decreased expression of SP1 gene within the PBMCs derived from ‘RA’ subjects as compared to that in the control subjects (Figures 2A-E). These results indicated that KLF4 gene is functional in terms of downstream regulation of effector genes. Additionally we wanted to investigate if the above mentioned genes were upregulated as a consequence of KLF4 overexpression in RA subjects. PBMC’s derived from patient samples were transfected with siRNA specific for KLF4 (siKLF4) to knockdown KLF4 gene expression or scramble sequence (siControl). KLF4 protein levels were significantly decreased in patient PBMC’s transfected with siKLF4 compared with siControl transfected patient PBMC’s (Figure 3A). siKLF4 transfected cells resulted in significant down-regulation of IL-6, IL-17, Rig-1, CCL5 and IFNγ at both RNA and protein levels compared with cells transfected with siControl. However Sp1 protein levels were found to be increased in these cells (Figure 3B and C). These results thus clearly confirmed that KLF4 plays a critical role in the regulation of genes coding for IL-6, IL-17, Rig-1, CCL5, IFNγ and Sp1, recognized widely to play crucial role in innate and adaptive immune response.

**Discussion**

The human NFkB family consists of five-members; RelA (p65), RelB, C-Rel, NFkB1 (p50 and its precursor p105) and NFkB2 (p52 and its precursor p100). The members of this family form a variety of hetero-dimers and homo-dimers, each of which activates its own characteristic set of genes involved in the pathogenesis of several diseases including rheumatoid arthritis ‘RA’ [7]. miR-146a has been widely recognized to play a key role in ‘RA’ and its expression has been shown to be regulated by NFkB [5]. Recently, a novel immunomodulatory microRNA encoded by AATF genome (designated as miR-2909) was also reported to be induced...
Deregulated miR-2909 RNomics pathway in ‘RA’ subjects: Proposed pathway that defines the nature of deregulated miR-2909 RNomics and its impact upon immune response observed within the PBMCs of ‘RA’ subjects.

It is pertinent at this stage to note various reported findings: a) IL-6 plays an important role in regulating the Treg/Th17 balance [8]; b) KLF4 can not only up-regulate the expression of IL-6 [9] but also regulates the differentiation of Th17 cells independently of ROryt [10] as well as down-regulates Ikke gene expression [3]; c) RIG-1 has been shown to induce CCL5 expression [11]; d) CCL5 has been shown to mediate chemotactic activity in T cells, monocytes, dendritic cells, natural killer cells, eosinophils, and basophils [12]; e) IKKε targets IRF-1 [13] which regulates RIG1 [14] and NFkB activity is induced by RIG1 [15]. Further, high levels of IL-17 and CCL5 have been widely recognized to play crucial role in the pathogenesis of ‘RA’ [16,17]. Hence based upon the results reported here coupled with the above-mentioned findings, an attempt has been made to propose a molecular link between deregulated KLF4 expression and plasticity of T-cells repertoire within human PBMCs (Figure 4) that may be responsible for the pathogenesis of “RA”. The proposed pathway clearly indicates that mutant miR-2909 is unable to repress KLF4 and this overexpressed KLF4 induces Th17 differentiation directly as well as indirectly through the induction of IL6 expression. Further KLF4 also induces the chemokines CCL5 which in turn induces the IFNγ secretion by Th17 cells and this IFNγ further stimulates KLF4 expression. Thus KLF4 overexpression in RA subjects may be responsible for sustainance of autoimmune response in such subjects. Further, observed mutant miR-2909 may have a crucial role in the deregulation of KLF4 in the PBMCs derived from ‘RA’ subjects.

Conclusions

Human PBMCs from ‘RA’ subjects possess a mutant miR-2909 that is unable to repress KLF4 gene expression thereby resulting
in uncontrolled expression of KLF4 which in turn ensures predominant Th17 immune response having CCL5-induced high chemo-tactic activity. Based upon these findings, we propose that mutant miR-2909 may contribute to the initiation of ‘RA’. However more data is required to understand whether or not mutant miR-2909 is typical trait of ‘RA’ or generalized feature of autoimmune diseases.

References