The polymorphism rs2227513 can affect expression of IL-22 and proliferation of fibroblast-like synoviocytes, which leads to progression of rheumatoid arthritis

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Abstract

Introduction: MiR-101 rs7536540 may influence the expression of miR-101 and another polymorphism, rs2227513, which in turn affects the expression of interleukin (IL)-22, a gene with an essential role in the pathogenesis of rheumatoid arthritis (RA). However, studies on the combined effect of these polymorphisms are still scarce.

Material and methods: DAS28 (ESR) and Clinical Disease Activity Index (CDAI) scores were measured to evaluate the severity of RA. Quantitative real-time PCR was performed to analyze the expression of miR-101 and IL-22 mRNA. ELISA and Western blot were carried out to examine the expression of IL-22 protein. MTT assay and flow cytometry were used to assess the cellular proliferation and apoptosis of fibroblast-like synoviocytes (FLS) incubated in the synovial fluid (SF) collected from RA patients carrying differential genotypes. Immunofluorescence was performed to measure the expression of p-STAT3. Luciferase assay was carried out to explore the inhibitory role of miR-101 in the expression of IL-22.

Results: The severity of RA was progressively increased in patients with rs7536540 GG + rs2227513 AA, rs7536540 GG + rs2227513 AG, rs7536540 CC/CG + rs2227513 AA and rs7536540 CC/CG + rs2227513 AG genotypes. The CC/CG alleles at rs7536540 were correlated with up-regulated miR-101 expression in the serum and SF of RA patients, whereas both CC/CG alleles at rs7536540 and AG alleles at rs2227513 were correlated with elevated expression of IL-22. Incubation of FLS with SF isolated from RA patients carrying the CC/CG alleles at rs7536540 and AG alleles at rs2227513 remarkably increased the cell proliferation and inhibited the apoptosis of FLS. Luciferase assay demonstrated that the expression of IL-22 was notably suppressed by miR-101 in THP-1 cells.

Conclusions: In this study, we investigated the combined effect of polymorphisms rs7536540 and rs2227513 on the expression of IL-22 and the proliferation of FLS as well as their association with the severity of RA, so as to gain a deep insight into the molecular mechanisms underlying the severity of RA.

Key words: rheumatoid arthritis, fibroblast-like synoviocyte, miR-101, IL-22, rs2227513.

Introduction

Rheumatoid arthritis (RA) is a systemic, inflammatory autoimmune disease which is commonly considered as a joint disease despite the fact that multiple additional organ systems such as pulmonary, cardiovascular,

ocular, and cutaneous systems are known to be involved [1]. Rheumatoid arthritis often triggers evident joint damage, inflammatory arthritis (IA), and bone loss [2, 3]. However, despite all these symptoms, the presence of IA is the hallmark of clinical diagnosis of RA. The diagnostic criteria comprise a series of joint examination and imaging, testing of RA-related autoantibodies, systemic inflammation, and the duration of joint symptoms [1, 4].

Non-coding RNAs (ncRNAs) are primarily separated into 2 sub-types based on their lengths: long non-coding RNAs (lncRNAs) and microRNAs (miRNAs). LncRNAs are specified as a type of transcripts with no protein coding ability that are greater than 200 nucleotides in length. LncRNAs were at first regarded as genome noise [5]. However, current proof shows that lncRNAs are key factors in many biological processes, including transcription as well as epigenetic regulation [6]. MiRNAs are short, single-stranded RNAs participating in the control of different biological activities, including cell growth, apoptosis and inflammation, as well as tumorigenesis [7].

Previous research revealed that rs7536540 on miR-101-1 can bind to various gene regulators. More lately, the ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (ATP5B) was revealed to be inhibited by miR-101 [8]. Given that ATP5B expression is selectively suppressed in human tumors, it is suspected that rs7536540 induces miR-101 overexpression to reduce ATP5B expression while indirectly influencing the progression of HBV-related illness [9].

Interleukin (IL)-22 is an IL-10 family member of cytokines, and it has also been demonstrated that IL-22 expression is dysregulated in RA patients and the aberrant IL-22 is significantly correlated with the onset of RA [10]. Accordingly, in a previous study, IL-22 was suggested to exhibit an therapeutic effect in the treatment of RA from the aspect of immune mechanisms rather than the aspect of genetic susceptibility [10]. A vast array of immune cells, including T cells, natural killer (NK) cells, dendritic cells, monocytes, as well as lymphoid cells, can generate IL-22 [11–13]. However, the key resource of IL-22 is activated T cells, especially Th17, Th1, and Th22 cells [12]. Ikeuchi *et al*. demonstrated that IL-22 can boost phospho-ERK1/2 expression in synovial fibroblasts [14]. Taking into consideration that STAT3-mediated signaling is an often seen route shared by the members of the IL-10 family, the STAT3 pathway is indeed activated by rhIL-22 through boosting phospho-STAT3 expression, which is due to the fact that AG490 inhibits the expansion of fibroblast-like synoviocytes (FLS) [15]. Similarly, STAT3 phosphorylation is a vital pathway moderating the effect of IL-22 on FLS expansion. Previous research making use of stratified evaluation (through gender) revealed a raised danger of HIV infection amongst females carrying the A/G genotype of rs2227513. An additional examination of IL-22 in plasma revealed that the A/G genotype of rs2227513 is linked with evidently greater IL-22 expression.

MiR-101 rs7536540 may influence the expression of miR-101 and another polymorphism, rs2227513, to affect the expression of IL-22 [16–18]. In this study, we investigated the combined effect of these two polymorphisms on the expression of IL-22 and the proliferation of FLS as well as their association with the severity of RA.

Material and methods

Human subjects and sample collection

We recruited a total of 84 RA patients in this study. The serum and synovial fluid (SF) of the RA subjects were collected and subjected to genotyping of rs7536540 and rs2227513 as well as measurement of miR-101 and IL-22 expression. Subsequently, the RA patients were partitioned into 4 groups according to their genotypes of rs7536540 and rs2227513: 1) miR-101 rs7536540 GG + IL-22 rs2227513 AA (n = 18); 2) miR-101 rs7536540 GG + IL-22 rs2227513 AG (n = 22); 3) miR-101 rs7536540 CC/CG + IL-22 rs2227513 AA (n = 24) and 4) miR-101 rs7536540 CC/CG + IL-22 rs2227513 AG (n = 20). The demographic and clinicopathological features of the subjects, such as their age, gender, disease duration, C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR), were summarized and compared using Student's t test. The institutional ethical committee has approved the protocol of this study.

RNA isolation and real-time PCR

The expression of miR-101 and IL-22 mRNA in the serum samples collected from the subjects was measured using realtime PCR. In brief, the samples were treated with the assay kits of mirVana (Ambion, Thermo Fisher Scientific, Waltham, MA), Trizol Reagent (Invitrogen, Carlsbad, CA), and miRNeasy (Qiagen, Redwood City, CA) following the instructions of the manufacturers to collect their total RNA contents. In the next step, the RNA extract was rehydrated on a column and then centrifuged at 4000 g and 4°C to accumulate the RNA eluate. Finally, all extracted RNA was precipitated first and then resuspended in nuclease-free water (Invitrogen, Carlsbad, CA) for subsequent analysis.

During real-time PCR, the extracted RNA was first reversely transcribed into cDNA using a TaqMan miRNA Reverse Transcription Assay kit following the instructions of the manufacturer, followed by real-time RT-PCR carried out using a SYBR Green real-time PCR kit (Thermo Fisher Sci-

entific, Waltham, MA) following the instructions of the manufacturer.

Cell culture and transfection

THP-1 cells were purchased from European Collection of Cell Cultures (Salisbury, United Kingdom) and incubated at 37°C in a humidified carbon dioxide incubator. The culture medium was Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (Gibco, Thermo Fisher Scientific, Waltham, MA) as well as 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA). Prior to the transfection experiments, the cells reached logarithmic growth and were divided into 3 groups, i.e., 1) NC; 2) miR-101 mimics; and 3) IL-22 siRNA. The cells were transfected with respective oligonucleotides (Saigon, Shanghai, China) for 48 h using Lipofectamine 3000 (Invitrogen, Carlsbad, CA) following the instructions of the manufacturer. Then, the cells were harvested to measure the expression of target genes.

Vector construction, mutagenesis and luciferase assay

We carried out binding site screening to search potential target genes of miR-101 and found that the 3' UTR of IL-22 contained a potential miR-101 binding site. Then, the 3' UTR of IL-22 containing the miR-101 binding site was inserted into a pcDNA3.1 vector downstream of the firefly luciferase reporter gene to generate the wild type vector of IL-22 3' UTR. At the same time, a Quick Change mutagenesis kit (Stratagene, San Diego, CA) was used to generate a site directed mutation in the 3' UTR of IL-22, which was also inserted into a pcDNA3.1 vector to create the mutant type vector of IL-22 3' UTR. In the next step, the wild type or mutant type vector of IL-22 3' UTR was co-transfected with miR-101 mimics into THP-1 cells, which were pre-seeded in 96-well tissue culture plates at 50000 cells/well at 24 h prior to transfection. After 48 h of transfection, the luciferase activity of transfected cells was assayed using a Bright Glo luciferase assay kit (Promega, Madison, WI) following the instructions of the manufacturer. The reading of luciferase activity was carried out on a TD 20/20 luminometer (Turner Styles, Sunnyvale, CA).

Cell proliferation assay

THP-1 cells were treated and plated into 96-well cell culture plates at 50000 cells/well in 100 µl of medium. The proliferation status of the cells was assayed using an MTT assay kit (Sigma Aldrich, St. Louis, MO) following the instructions of the manufacturer. The optical density values of various wells were measured at 450 nm on a plate reader.

Western blot analysis

Western blot was carried out using a conventional method. In brief, the collected clinical and cell culture samples were homogenized in a TNEC lysis buffer (pH 8) containing 1.5 mM Tris-HCl, 2 mM NaCl, 3% IGEPAL CA-630, 4 mM EDTA and a cocktail of protease inhibitors (Roche, Switzerland). The protein content in the homogenate was assayed by making use of a BCA assay kit (Pierce, Waltham, MA) following the instructions of the kit manufacturer. About 40 µg of protein sample from each specimen were denatured using an LDS buffer containing a NuPAGE denaturing agent (both from Invitrogen, Carlsbad, CA) that was warmed up to 72°C and incubated for 10 min. In the next step, the proteins were resolved on a 8% Tris-Acetate gel (Invitrogen, Carlsbad, CA), moved to a nitrocellulose membrane, blocked in a TBST buffer containing 5% non-fat milk, and incubated consecutively with primary anti-IL-22 antibodies and appropriate secondary antibodies (Santa Cruz Biotechnology, San Diego, CA) before the relative protein expression of IL-22 in each sample was evaluated using an AlphaEaseFC system (Alpha Innotech, San Leandro, CA).

Apoptosis

The apoptosis of cells was evaluated using flow cytometry. In brief, THP-1 cells were collected at 48 h post transfection when the cell confluency reached about 80%. Then, 0.4×10^6 cells were obtained for each sample and treated with an Annexin V Apoptosis Assay Kit (Thermo Fisher Scientific, Waltham, MA) following the instructions of the kit manufacturer. The assay values were read on a FACSCalibur flow cytometer using Summit 3.1 software (Beckman Coulter, San Jose, CA).

Immunofluorescence

The expression of p-STAT3 in samples was determined using an immunofluorescence assay. In brief, the cells were seeded on glass cover slides and then fixed with PBS containing 4% (v/v) paraformaldehyde. After being permeabilized using PBS with 0.5% (v/v) Triton X-100 for 15 min, the samples were blocked using PBS with 5% (v/v) FBS for 1 h. In the next step, the cells were incubated with primary anti-p-STAT3 antibody and corresponding AF-488-tagged Ig G secondary antibody (Molecular Probes, Carlsbad, CA) following the instructions of the manufacturer. The positive expression of p-STAT3 in samples was analyzed underneath a fluorescence microscope (Axio Imager A1, ZEISS, Germany).

ELISA

The expression of IL-22 in SF samples was measured using an ELISA assay kit (BD, Franklin

Table I. Demographic and clinicopathological parameters of recruited subjects

Characteristics	miR-101 rs7536540 GG + IL-22 rs2227513 AA (n = 18)	miR-101 rs7536540 GG + IL-22 rs2227513 AG (n = 22)	miR-101 rs7536540 CC/CG + IL-22 rs2227513 AA (n = 24)	miR-101 rs7536540 CC/CG + IL-22 rs2227513 AG (n = 20)	P-value
Age at enrolment [years]	42.8 ±5.4	43.3 ±5.7	40.3 ±3.6	41.7 ±5.7	0.284
Female sex, n (%)	12 (66.7)	15 (68.2)	18 (75.0)	15 (75.0)	0.594
Disease duration [years]	6.0 ±1.1	6.4 ±0.9	6.0 ±1.3	6.4 ±0.8	0.698
CRP [mg/l]	25.4 ±5.7	25.0 ±6.7	25.3 ±5.7	25.4 ±5.7	0.753
ESR [mm/h]	51.2 ±6.0	51.4 ±6.2	49.2 ±7.9	50.6 ±5.3	0.374

Lakes, NJ) following the instructions of the kit manufacturer, and the results were read at an excitation wavelength of 450 nm and an emission wavelength of 620 nm on a plate reader (Bio-Rad Laboratories, Richmond, CA).

Statistical analysis

All statistical evaluations were carried out using GraphPad Prism 6.0 software (GraphPad, San Diego, CA). The experimental values were expressed as mean ± SEM. Inter-group comparisons were made using Student's *t*-test (twotrailed, non-parametric) and the statistical significance was determined by $p \leq 0.05$.

Results

Demographic and clinicopathological characteristics of participants

We recruited a total of 84 RA patients in this study and partitioned them into 4 groups according to their genotypes of rs7536540 and rs2227513: 1) miR-101 rs7536540 GG + IL-22 rs2227513 AA (n = 18); 2) miR-101 rs7536540 GG + IL-22 rs2227513 AG (n = 22); 3) miR-101 rs7536540 CC/CG + IL-22 rs2227513 AA (n = 24) and 4) miR-101 rs7536540 CC/CG + IL-22 rs2227513 AG (n = 20). The demographic and clinicopathological features of the subjects are summarized in Table I. The differences among the four groups were compared using Student's *t* test and no significant differences were found.

Severity of rheumatoid arthritis was elevated in patients with CC/CG alleles at rs7536540 and AG alleles at rs2227513

DAS28 (ESR) was measured in each group of patients to evaluate the severity of RA. The DAS28 (ESR) was the smallest in patients with GG alleles at rs7536540 and AA alleles at rs2227513, but the largest in patients with CC/CG alleles at rs7536540 and AG alleles at rs2227513. The DAS28 (ESR) was at a moderate level in patients with GG alleles at rs7536540 + AA alleles at rs2227513 and CC/CG genotypes at rs7536540 + AA alleles at rs2227513 (Figure 1 A). Furthermore, the Clinical Disease Activity Index (CDAI) scores in the patients showed the same trends as those of DAS28 (ESR) in different groups (Figure 1 B). These results indicated that CC/CG at rs7536540 and AG at rs2227513 significantly increased the severity of RA.

Expression of miR-101 and IL-22 was elevated in patients with CC/CG alleles at rs7536540 and AG alleles at rs2227513

Next, the serum and SF of RA patients were collected and subjected to measurement of miR-

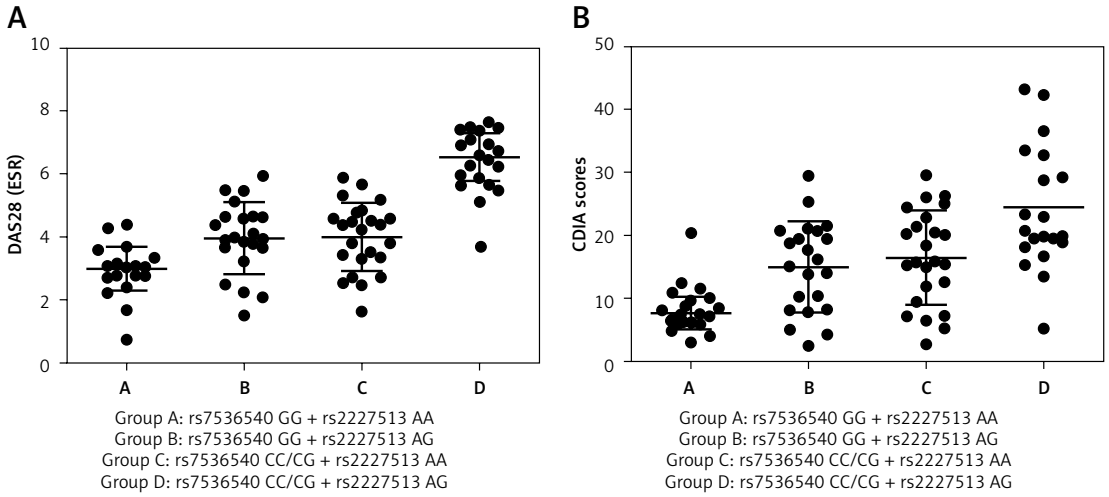

Figure 1. CC/CG alleles at rs7536540 and AG alleles at rs2227513 were correlated with elevated severity of rheumatoid arthritis (RA). **A** – DAS28 (ESR) was progressively increased in RA patients carrying the genotypes rs7536540 GG + rs2227513 AA, rs7536540 GG + rs2227513 AG, rs7536540 CC/CG + rs2227513 AA, and rs7536540 CC/CG + rs2227513 AG. **B** – Clinical Disease Activity Index (CDAI) score was progressively increased in RA patients carrying the genotypes rs7536540 GG + rs2227513 AA, rs7536540 GG + rs2227513 AG, rs7536540 CC/CG + rs2227513 AA, and rs7536540 CC/CG + rs2227513 AG

101 and IL-22 expression. No significant difference was observed for the expression of miR-101 between patients carrying AA or AG alleles at rs2227513, whereas the serum and SF expression of miR-101 in patients with CC/CG alleles at rs7536540 was significantly higher than that in patients with GG alleles at rs7536540 (Figures 2 A, 3 A). However, the expression of IL-22 in the SF progressively increased in patients with rs7536540 GG + rs2227513 AA, rs7536540 GG + rs2227513 AG, rs7536540 CC/CG + rs2227513 AA and rs7536540 CC/CG + rs2227513 AG (Figures 2 B, 3 B). These results demonstrated that miR-101 rs7536540 effectively altered the expression of miR-101, and both miR-101 rs7536540 and IL-22 rs2227513 altered the expression of IL-22 in the serum and SF of RA patients.

CC/CG alleles at rs7536540 and AG alleles at rs2227513 were correlated with elevated proliferation and decreased apoptosis of fibroblast-like synoviocytes incubated with synovial fluid isolated from rheumatoid arthritis patients

Fibroblast-like synoviocytes were incubated for 48 hours with SF collected from RA patients carrying differential genotypes, followed by MTT and flow cytometry analysis to evaluate their proliferation and apoptosis. The proliferation showed an ob-

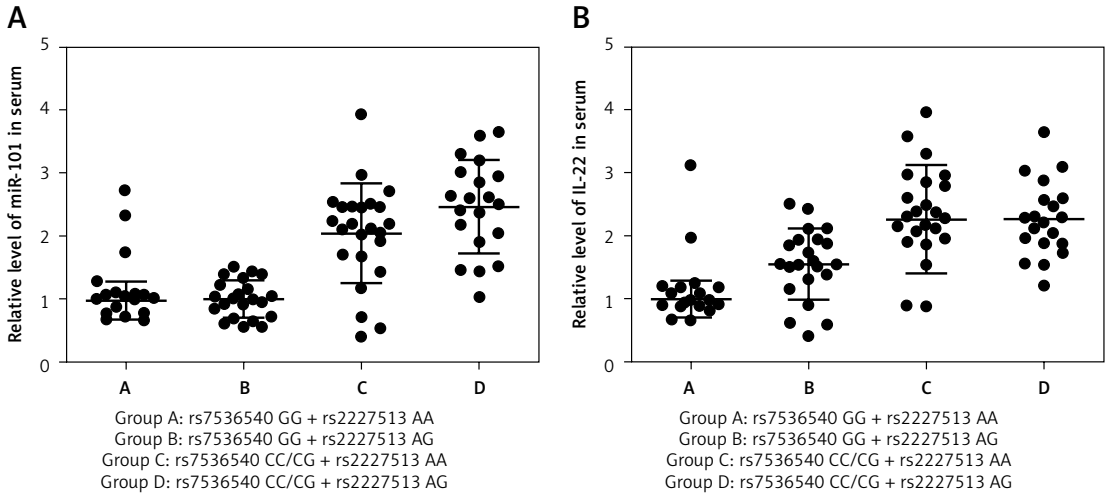

Figure 2. CC/CG alleles at rs7536540 and AG alleles at rs2227513 were correlated with enhanced expression of miR-101 and IL-11 in the serum of rheumatoid arthritis (RA) patients. **A** – Expression of miR-101 was significantly elevated in serum of RA patients with CC/CG alleles at rs7536540. **B** – Expression of IL-22 was gradually enhanced in serum of RA patients carrying the genotypes rs7536540 GG + rs2227513 AA, rs7536540 GG + rs2227513 AG, rs7536540 CC/CG + rs2227513 AA, and rs7536540 CC/CG + rs2227513 AG

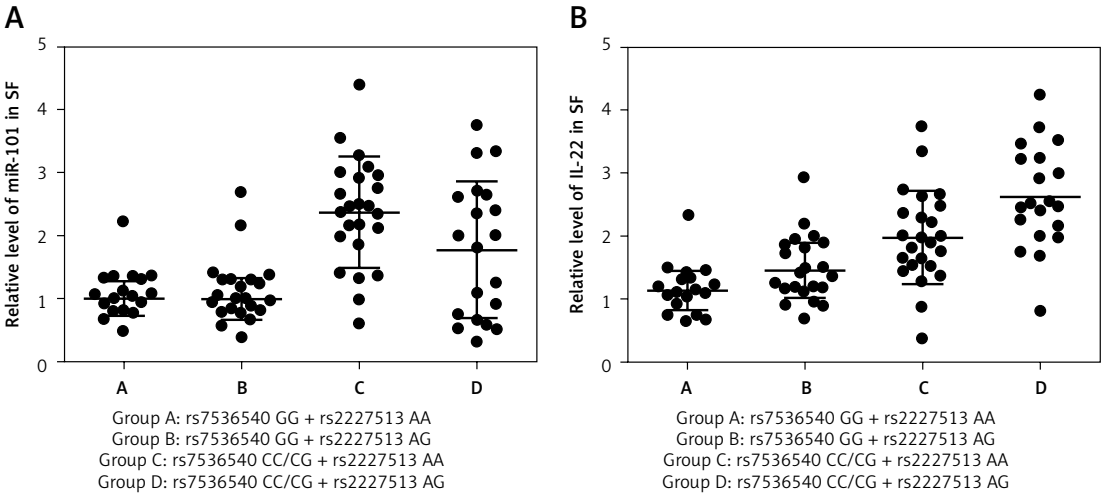

Figure 3. CC/CG alleles at rs7536540 and AG alleles at rs2227513 were correlated with enhanced expression of miR-101 and IL-11 in synovial fluid (SF) of rheumatoid arthritis (RA) patients. **A** – Expression of miR-101 was significantly elevated in SF of RA patients with CC/CG alleles at rs7536540. **B** – Expression of IL-22 was gradually enhanced in SF of RA patients carrying the genotypes rs7536540 GG + rs2227513 AA, rs7536540 GG + rs2227513 AG, rs7536540 CC/CG + rs2227513 AA, and rs7536540 CC/CG + rs2227513 AG

viously elevating trend when fibroblast-like synoviocytes were incubated with SF from RA patients with genotypes rs7536540 GG + rs2227513 AA, rs7536540 GG + rs2227513 AG, rs7536540 CC/CG + rs2227513 AA, and rs7536540 CC/CG + rs2227513 AG (Figure 4). Also, the apoptosis of fibroblast-like synoviocytes was gradually suppressed when they were incubated with SF from RA patients carrying genotypes rs7536540 GG + rs2227513 AA, rs7536540 GG + rs2227513 AG, rs7536540 CC/CG + rs2227513 AA, and rs7536540 CC/CG + rs2227513 AG (Figure 5). Moreover, immunofluorescence analysis showed that the expression of p-STAT3, which was crucial for cell proliferation, in the fibroblast-like synoviocytes was progressively enhanced when the cells were incubated with SF collected from RA patients carrying genotypes rs7536540 GG + rs2227513 AA, rs7536540 GG + rs2227513 AG, rs7536540 CC/CG + rs2227513 AA, and rs7536540 CC/CG + rs2227513 AG (Figure 6).

MiR-101 mimics and IL-22 siRNA significantly suppressed expression of IL-22 in THP-1 cells

Transfection of miR-101 mimics and IL-22 siRNA was performed to evaluate their effect on IL-22 expression in THP-1 cells. The expression of IL-22 in THP-1 cells transfected with miR-101 mimics and IL-22 siRNA was remarkably suppressed (Figure 7).

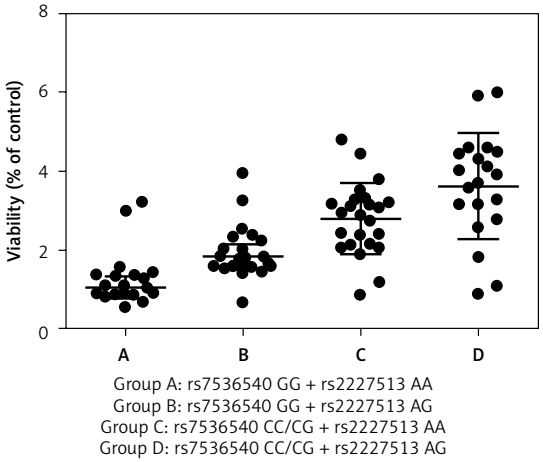

Figure 4. Viability of fibroblast-like synoviocytes was gradually elevated when incubated with synovial fluid isolated from rheumatoid arthritis patients carrying the genotypes rs7536540 GG + rs2227513 AA, rs7536540 GG + rs2227513 AG, rs7536540 CC/CG + rs2227513 AA, and rs7536540 CC/CG + rs2227513 AG

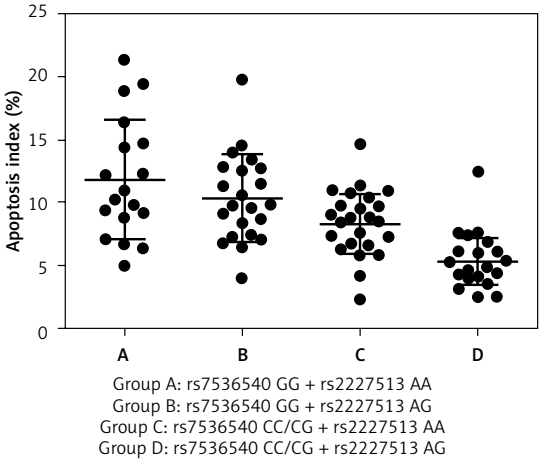

Figure 5. Apoptosis of fibroblast-like synoviocytes was progressively decreased when incubated with synovial fluid isolated from rheumatoid arthritis patients carrying the genotypes rs7536540 GG + rs2227513 AA, rs7536540 GG + rs2227513 AG, rs7536540 CC/CG + rs2227513 AA, and rs7536540 CC/CG + rs2227513 AG

MiR-101 effectively inhibited expression of IL-22 through binding to its 3' UTR

Screening of binding sites of miR-101 revealed that the 3' UTR of IL-22 was a potential target of miR-101 (Figure 8 A). Next, luciferase vectors containing wild type and mutant 3' UTRs of IL-22 were established and co-transfected into THP-1 cells with miR-101. The luciferase activity of wild type IL-22 vector was effectively suppressed by miR-101, whereas the luciferase activity of mutant IL-22 remained unchanged (Figure 8 B).

Discussion

It has been previously reported that rs7536540 may influence the expression of miR-101 while rs2227513 may affect the expression of IL-22. In this study, we hypothesized that the genotypes of these two polymorphisms may exhibit a combined effect upon the severity of RA by influencing the expression of IL-22 and the proliferation of FLS. Accordingly, our study revealed that patients with the rs7536540 genotype CC/CG and the rs2227513 genotype AG were suffering from higher severity of RA with elevated expression of miR-101 and IL-22. And for fibroblast-like synoviocytes isolated from these patients, their proliferation was promoted while the apoptosis was suppressed. Accordingly, we studied the underlying molecular mechanism and found that miR-101 negative regulated the expression of IL-22 through binding to the 3' UTR of IL-22 mRNA. Therefore, our study not only validated the correlation between polymorphism genotypes and RA severity, but also established the underlying molecular mechanisms.

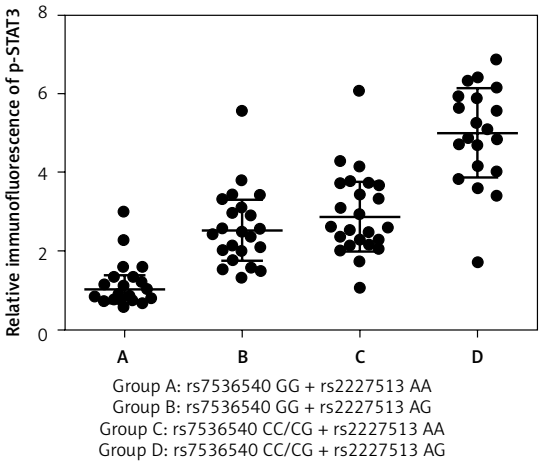

Figure 6. Expression of p-STAT3 was gradually elevated when incubated with synovial fluid isolated from rheumatoid arthritis patients carrying the genotypes rs7536540 GG + rs2227513 AA, rs7536540 GG + rs2227513 AG, rs7536540 CC/CG + rs2227513 AA, and rs7536540 CC/CG + rs2227513 AG

Rheumatoid arthritis is a persistent and inflammatory condition that affects the synovial joint [19]. FLS are the cells first impacted by the development of arthritis [20]. It was shown that IL-22 as well as its receptors in synovial cells and FLS are overexpressed along with increased levels of Th17 cells in the joint of individuals with RA [14, 21, 22]. Likewise, high IL-22 expression in the synovial fluid of RA subjects is associated with disease development [23, 24]. FLS show a main function in RA subjects with synovial hyperplasia [20]. The level of FLS is evidently raised in the synovial cells of RA subjects, along with damaged cartilage materials as well as activation of pro-inflammatory

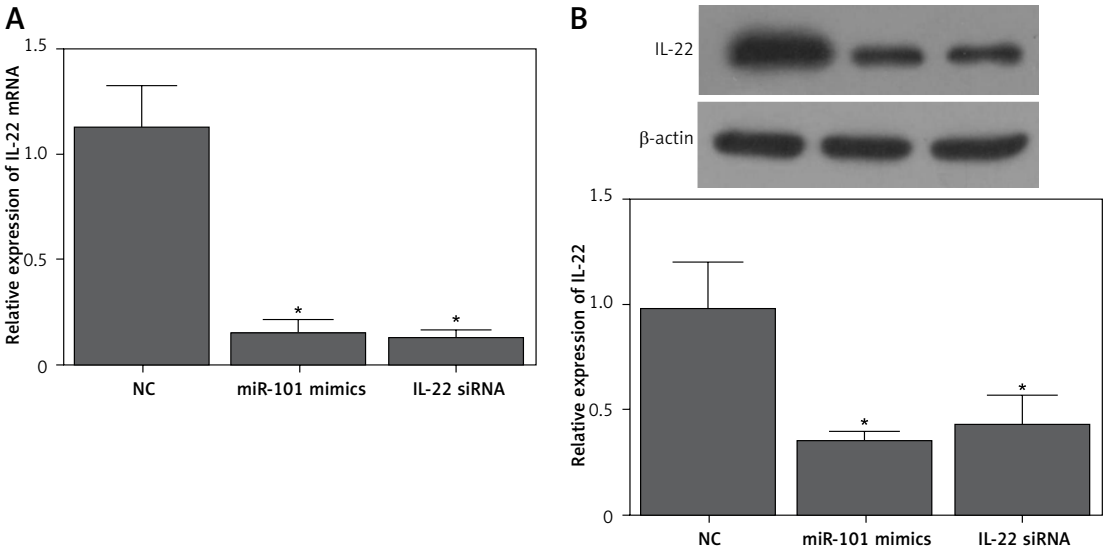

Figure 7. Expression of IL-22 in THP-1 cells was inhibited by miR-101 mimics and IL-22 siRNA. **A** – Expression of IL-22 mRNA was significantly suppressed by miR-101 mimics and IL-22 siRNA (*$p < 0.05$, vs. NC group). **B** – Expression of IL-22 protein was significantly suppressed by miR-101 mimics and IL-22 siRNA (*$p < 0.05$ vs. NC group)

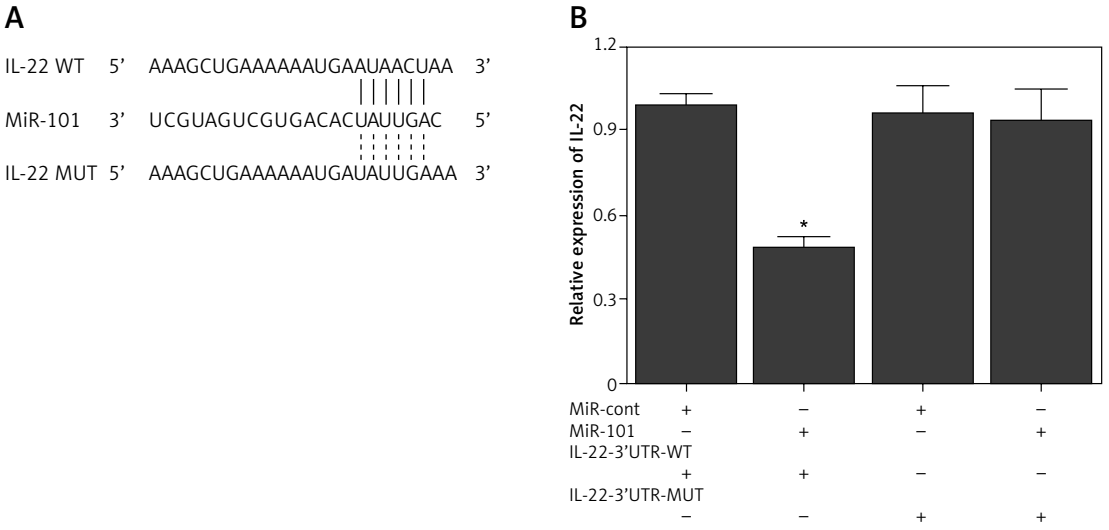

Figure 8. MiR-101 inhibited expression of IL-22 through binding to its 3' UTR. **A** – Sequence analysis indicated a potential binding site of miR-101 on IL-22. **B** – Luciferase activity of wild type IL-22 was effectively repressed by miR-101 (*$p < 0.05$ vs. control group)

cytokines, matrix metalloproteinases (MMPs), and chemokines [20, 25].

The function of IL-22 in antiviral protection remains debatable. Located on the IL-22 gene, the A/G genotype of rs2227513 is shown to raise the danger of HIV infection, no matter whether the subject is HIV seronegative or seropositive, indicating a pathogenic effect of IL-22 on the infection of HIV [26, 27].

Rs2227513 is situated on an intron in IL-22. It is known that introns can work as crucial gene regulatory elements exerting different impacts, including RNA modification [26, 27]. A statistical correlation was seen between genotype distribution and rs2227513 alleles in kidney diseases, indicating a close association between the genotypes of rs2227513 and the risk of HIV infection amongst females [28].

Current research shows that miR-101-3p level is evidently lowered in synovium as well as FLS collected from AIA animals. Transfection with miR-101-3p mimic evidently minimized the expression of CUL4B, while miR-101-3p inhibitor evidently upregulated the level of CUL4B amongst AIA FLS. Previous studies have revealed that miR-101 is closely associated with tumor malignancy. The irregular expression of miR-101 has been verified in several studies [2, 3]. Research has also revealed that miR-101 is linked to tumor metastasis. As an example, subduing miR-101 expression enhanced the metastasis of cancer cells [4]. It was also revealed that miR-101 silencing could prevent cartilage destruction [29]. Dual specificity phosphatase 1 (DUSP1), or MKP-1, could undermine the activation of MAPK signaling in arthritis [30]. Wei *et al*. (2015) confirmed that DUSP1 acts as a target of miR-101. In summary, it was hypothesized that miR-101 overexpression may target DUSP1 to enhance cartilage deterioration in RA [31]. In this study, we recruited RA patients and sub-grouped them into 4 groups according to their genotypes of rs7536540 and rs2227513. Accordingly, the severity of RA was remarkably elevated in patients carrying the genotypes of rs7536540 CC/CG + rs2227513 AG compared with other patient groups. CC/CG at rs7536540 was correlated with up-regulated miR-101 expression, while CC/CG at rs7536540 and AG at rs2227513 were correlated with up-regulated IL-22 expression in the serum and SF of RA patients.

IL-22 is generated by NK cells, activated T cells, and lymphoid cells [32]. The secretion of IL-22 as well as CCL2 by eESCs promoted the recruitment of macrophages to advance endometriosis. Similarly, the co-culture of NK cells with eESCs promoted the secretion of IL-22 and CCL2 to promote the recruitment of macrophages. The IL-10 family includes cytokines such as IL-26, IL-10, IL-22, IL-19, IL-24, as well as IL-20, which are generated by various immune cells to play roles as effectors in the immune system. IL-22 can trigger numerous immune responses. For example, IL-22 promotes the immune responses in the liver by activating different anti-microbial pathways, including HPX, C3, and hepcidin. In this study, we incubated FLS for 48 h in SF isolated from RA patients and performed MTT assay and flow cytometry to evaluate the proliferation and apoptosis of FLS. SF isolated from patients carrying the genotypes rs7536540 GG + rs2227513 AA, rs7536540 GG + rs2227513 AG, rs7536540 CC/CG + rs2227513 AA, and rs7536540 CC/CG + rs2227513 AG gradually increased the proliferation and decreased the apoptosis of FLS.

Formerly, Ikeuchi *et al*. revealed that IL-22 is likewise synthesized as a type of proinflammatory cytokine in macrophages as well as synovial fibroblasts to enhance inflammatory reactions via the IL-22 receptor 1 located on the surface of FLS in RA subjects [14]. The results of MTT assays showed that the supernatants collected from the culture of NKp44þNK cells enhanced the expansion of FLS, while IL-22 antagonist could suppress the effects of the above supernatants.

In conclusion, the findings of this study demonstrated that miR-101 rs7536540 may influence the expression of miR-101 and another polymorphism, rs2227513, to affect the expression of IL-22. In this study, we investigated the combined effect of these two polymorphisms on the expression of IL-22 and the proliferation of FLS as well as their association with the severity of RA.

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Conflict of interest

The authors declare no conflict of interest.

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