The Effects of microRNA-515-5p on the Toll-Like Receptor 4 (TLR4)/JNK Signaling Pathway and WNT1-Inducible-Signaling Pathway Protein 1 (WISP-1) Expression in Rheumatoid Arthritis Fibroblast-Like Synovial (RAFLS) Cells Following Treatment with Receptor Activator of Nuclear Factor-kappa-B Ligand (RANKL)

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Background: This study aimed to investigate the effects of microRNA-515-5p (miR-515-5p) on the expression of the WNT1-inducible-signaling pathway protein 1 (WISP-1) gene in rheumatoid arthritis fibroblast-like synovial (RAFLS) cells following treatment with the receptor activator of nuclear factor-kappa-B ligand (RANKL).

Material/Methods: RAFLS cells were cultured in vitro and were divided into six study groups: a normal control group; a miR-515-5p mimic group; a miR-515-5p inhibitor group; a RANKL (50 ng/ml) treatment group; a miR-515-5p mimic+RANKL treatment group; and a miR-515-5p inhibitor+RANKL treatment group. The luciferase assay was used to determine the effects of miR-515-5p on the WISP1 expression. Cell proliferation, cell apoptosis, the cell cycle, and protein expression were determined using the Cell Counting Kit-8 (CCK-8) assay, flow cytometry, Western blot, and real-time polymerase chain reaction (RT-PCR).

Results: The luciferase assay showed that the effects of miR-515-5p on the 3'UTR of WISP1 inhibited the gene expression. The miR-515-5p mimics promoted cell proliferation, reduced apoptosis, and promoted the cell cycle. The miR-515-5p mimics reduced the expression of TLR4, WISP1, and JNK at the mRNA level, while the miR-515-5p inhibitor promoted the expression of TLR4, WISP1, and JNK. Both the miR-515-5p inhibitor and mimic promoted the phosphorylation of AKT in RAFLS cells treated with or without RANKL compared with the control, and the miR-515-5p inhibitor promoted the phosphorylation of JNK in the RAFLS cells.

Conclusions: In RAFLS cells, miR-515-5p inhibited the expression of the WISP1 gene, and treatment with RANKL inhibited the TLR4/JNK signaling pathway.

MeSH Keywords: Arthritis, Juvenile • MicroRNAs • Toll-Like Receptor 4

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Background

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by chronic inflammation of joint synovium [1,2]. Worldwide, in 2015, there were about 24.5 million patients with RA [3]. The pathogenesis of RA involves environmental factors, genetic susceptibility factors, and altered immune function [4]. Delays in the treatment of RA may destroy the affected joints [5,6].

Currently, drug treatments for RA include compounds and biologics that target inflammation and cytokine-associated signaling pathways, including inhibitors of tumor necrosis factor (TNF) combined with methotrexate [7]. Recently developed targeted therapies for RA, including baricitinib and tofacitinib, inhibit Janus kinase (JAK), and the target of rapamycin (TOR) signaling pathway, respectively [8,9]. The junction between the synovium and the cartilage and bone of the joint represents an environment for the aggregation of osteoclasts, resulting in joint erosion in RA [10]. Also, fibroblast-like synoviocytes of RA can promote the differentiation of osteoblasts to osteoclasts [11].

High-throughput sequencing studies involving clinical studies and animal models of osteoarthritis (OA) have shown that receptor activator of nuclear factor-kappa B ligand (RANKL) and Wnt-1-induced secreted protein (WISP1) are OA-related genes [12,13]. The RANKL protein belongs to TNF superfamily, which can promote the differentiation of osteoclasts by activating TNFR-associated factor 6 (TRAF6). Drugs that target RANKL have been used to treat osteoporosis and other bone diseases [14,15]. The secreted protein is enriched in the extracellular matrix (ECM) and is induced by the Wnt signaling pathway. WISP1 also upregulates the activity of the Wnt signaling pathway, and promotes the inflammatory response, and promotes osteoblast proliferation and differentiation through the Toll-like receptor 4 (TLR4) signaling pathway [16–18].

Analysis of bioinformatics data from TargetScan, which predicts the target of miRNAs in human disease, the WISP1 gene was a target gene of miR-515-5p as previously described [22]. The target of miR-515-5p expression promoted cell growth and migration, and the microtubule affinity regulatory kinase 4 (MARK4) was a target gene of miR-515-5p associated with reduced patient prognosis [19–21].

Therefore, this study aimed to investigate the effects of miR-515-5p on the expression of WISP1 in rheumatoid arthritis fibroblast-like synovial (RAFLS) cells following treatment with RANKL.

Material and Methods

Cell transfection

Rheumatoid arthritis (RA) fibroblast-like synovial (RAFLS) cells (BNCC340230) were purchased from BnBio (Hangzhou, China) and 293T normal human embryonic kidneys cells (GNHu17) were purchased from the Chinese Academy of Sciences (Shanghai, China). When the cells reached 70% confluence, they were used in the cell transfection studies. The normal culture medium was replaced with Opti-MEM reduced serum medium (Thermo Fisher Scientific, Waltham, MA, USA).

Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA), and 12.5 μl of RNA powder dissolved in diethylpyrocarbonate (DEPC) (125 μl/1OD) was added at 4°C. The mixture was added into the wells of the six-well plate, and the cells were cultured for 4 h. Then, complete medium containing 20% serum was added to the six-well plate. After 48 h, the transfections were verified by the polymerase chain reaction (PCR).

Mimics and an inhibitor of microRNA-515-5p (miR-515-5p) were synthesized by Anhui General Bioengineering Co., Ltd. The sequences of miR-515-5p mimics were UUCUCCAAA AGAAAGACUUUCUG (5'-3', F) and CAGAAAGGCUUUUCU UUUGAGAA (5'-3', R). The sequence of miR-515-5p inhibitor was CAGAAAGGCUUUUCUUGGAGAA (5'-3').

Experimental groups

RAFLS cells were cultured in vitro and were divided into six study groups: a normal control group; a miR-515-5p mimic group; a miR-515-5p inhibitor group; a RANKL (50 ng/ml) treatment group; a miR-515-5p mimic+RANKL treatment group; and a miR-515-5p inhibitor+RANKL treatment group.

The Cell Counting Kit-8 (CCK8) assay

After treatment, cell proliferation was evaluated with the Cell Counting Kit-8 (CCK-8) assay (Gibco, Grand Island, NY, USA), as previously described [22]. The formazan crystals were dissolved in dimethyl sulfoxide (DMSO), and the absorbance was measured with a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 450 nm.

Detection of the double luciferase reporter gene

The supernatant was centrifuged at 12,000×g for 2 min at 4°C. The luciferase substrate was added to the enzyme in the detection tube at room temperature. The supernatant was carefully absorbed into the detection tube or enzyme label plate. After rapid mixing, the reporter gene activity of Firefly luciferase was detected in the fluorescence or enzyme label instrument.
The Renilla luciferase reporter gene activity was detected by fluorescence detection or enzyme labeling immediately after mixing with the newly configured Renilla substrate. Renilla luciferase was used as the internal reference, and the relative luminometer unit (RLU) value was determined by firefly luciferase. The degree of activation of the target reporter gene between different samples was compared, according to the ratios obtained.

**Flow cytometry**

The phases of the cell cycle were determined using flow cytometry with propidium iodide (PI) staining using a NovoCyte® 2060R flow cytometer (ACEA Biosciences, Inc., Hangzhou, China). The cells were washed in phosphate-buffered saline (PBS) and digested in trypsin and Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS. Then, 1–5×10⁵ cells were fixed overnight in 70% cold ethanol. After staining with PI for 5 min in the dark, the cell cycle was determined by flow cytometry. After treatment, the cells were digested with trypsin solution containing EDTA at 37°C, and the cell suspension was collected. The cells were stained with 5 μL of Annexin V-fluorescein isothiocyanate (FITC) and 5μL of PI in the dark for 10 min. Cell apoptosis was detected by flow cytometry.

**Real-time polymerase chain reaction (RT-PCR)**

Total RNA was extracted using an UltraPure mRNA extraction kit (CoWin Biosciences Co., Ltd., Jiangsu, China). The purity of RNA was assessed by measuring the optical density (OD) at 280/260 nm. The RNA (1 μg) was reverse transcribed into cDNA using an Avian Myeloblastosis Virus Reverse-Transcriptase kit (cat. no. KL041; Shanghai Kang Lang Biological Technology Co., Ltd., Shanghai, China). The reaction system included 9.5 μl of RNase-Free distilled H₂O, 1 μl cDNA/DNA, 2 μl of primer, and 12.5 μl of UltraSYBR Mixture (cat. no. 00081405; CWBIO, Taizhou, China) and PCR was performed using the following thermocycling conditions: 40 cycles of denaturation at 95°C for 30 sec; annealing at 58°C for 30 sec; and extension at 72°C for 10 sec; annealing at 58°C for 30 sec; and extension at 72°C for 30 sec. The expression of TLR4, WISP1, and JNK were calculated using statistical significance for the difference.

**Statistical analysis**

Data were expressed as the mean ± standard deviation (SD). Multivariate analysis was performed by one-way analysis of variance (ANOVA) followed by Bonferroni’s post hoc test. Statistical analysis was performed using SPSS version 19.0 statistical software (IBM Corp., Armonk, NY, USA). P<0.05 indicated statistical significance for the difference.

**Results**

In rheumatoid arthritis fibroblast-like synovial (RAFLS) cells, microRNA-515-5p (miR-515-5p) acted on 3’-UTR of WISP1 to inhibit gene expression

Western blot

After treatment, protein was extracted from cell lines using the TriplePrep extraction kit (cat. no. 28-9425-44; GE Healthcare Life Sciences, Logan, UT, USA). The protein levels were quantified with a BCA protein assay kit. A total of 25 μg/lane protein was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as previously described [24]. The proteins were then transferred onto a nitrocellulose membrane. The membranes were blocked with 5% dried skimmed milk powder at room temperature for 2 h. The following primary antibodies were incubated with the membrane overnight at 4°C: rabbit monoclonal anti-WISP1 (1: 1,000) (DF12503; Affinity Biosciences, Cincinnati, OH, USA); rabbit monoclonal anti-TLR4 (1: 1,000) (bs-20594R; BioSS, Edinburgh, UK); rabbit monoclonal anti-JNK (1: 2,500) (ab199380; Abcam, Cambridge, MA, USA); and rabbit monoclonal anti-p-JNK (1: 5,000) (ab124956; Abcam, Cambridge, MA, USA).

**Statistical analysis**

Data were expressed as the mean ± standard deviation (SD). Multivariate analysis was performed by one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. Statistical analysis was performed using SPSS version 19.0 statistical software (IBM Corp., Armonk, NY, USA). P<0.05 indicated statistical significance for the difference.

**Results**

In rheumatoid arthritis fibroblast-like synovial (RAFLS) cells, microRNA-515-5p (miR-515-5p) acted on 3’-UTR of WISP1 to inhibit gene expression

TargetScanHuman bioinformatics software was used to analyze the 3’-UTR and miR-515-5p targeting binding sites of WISP1. The binding sites are shown in Figure 1A. The miR-515-5p mimics were effective in promoting miR-515-5p expression (about 5.2-fold (Figure 1B). The inhibitor reduced miR-515-5p expression by 20% of the control level. As shown in Figure 1C, the 3’-UTR of WISP1 was constructed and linked to the 3’-end luciferase vector of the fluorescence protein. Then, the fluorescence protein was expressed in the RAFLS cells. The relative fluorescence intensity of the cells transfected with the miR-515-5p mimics was lower than that of normal and blank control groups, indicating that miR-515-5p acted on the 3’-UTR of WISP1 to inhibit gene expression.
The miR-515-5p mimics promoted cell proliferation, reduced apoptosis and promoted the cell cycle.

As shown in Figure 2, the proliferation of the RAFLS cells was promoted by the miR-515-5p mimics when compared with the control. However, the miR-515-5p inhibitor and treatment with RANKL did not affect cell proliferation. The miR-515-5p mimics promoted the RAFLS cell proliferation in the cells treated with the receptor activator of nuclear factor-kappa-B ligand (RANKL). These results showed that the miR-515-5p mimics promoted cell proliferation of RAFLS cells.

The miR-515-5p mimics reduced cell apoptosis, while the miR-515-5p inhibitor promoted apoptosis of the cells when compared with the control. RANKL treatment did not affect apoptosis, but the miR-515-5p inhibitor promoted apoptosis in the...
cells treated with RANKL (Figure 3). These results indicated that the miR-515-5p mimics reduced cell apoptosis, while the inhibitor promoted apoptosis of RAFLS cells.

Flow cytometry analysis of the cell cycle showed that the miR-515-5p mimics promoted the entry of the RAFLS cells into mitosis, while the inhibitor reduced the entry of the cells into mitosis. RANKL also reduced the entry of RAFLS cells into mitosis (Figure 4). These results indicated that the miR-515-5p mimics promoted the cell cycle, while the inhibitor of miR-515-5p inhibited the cell cycle of RAFLS cells.

The miR-515-5p mimics reduced the expression of TLR4, WISP1, and JNK and the miR-515-5p inhibitor promoted the expression of TLR4, WISP1, and JNK at the mRNA level. As shown in Figure 5, TLR4 expression at the mRNA level was reduced by the miR-515-5p mimics compared with the control. RANKL did not affect TLR4, but the miR-515-5p inhibitor promoted TLR4 expression in the RAFLS cells. The miR-515-5p mimics reduced WISP1 expression, and the miR-515-5p inhibitor promoted WISP1 expression. The miR-515-5p mimics reduced cell expression of JNK, but the miR-515-5p inhibitor promoted JNK expression in the RAFLS cells. Also, the miR-515-5p mimics reduced JNK expression in the cells treated with RANKL. These results indicated that the miR-515-5p mimics reduced the expression of TLR4, WISP1, and JNK at the mRNA level in the RAFLS cells.

Figure 3. microRNA-515-5p (miR-515-5p) mimics reduced apoptosis, while the miR-515-5p inhibitor promoted apoptosis. * P<0.05; # P<0.05 (one-way analysis of variance).
The effects of the miR-515-5p mimics and the miR-515-5p inhibitor on the expression of AKT, JNK, TLR4, and WISP1

As shown in Figure 6, both the miR-515-5p inhibitor and the miR-515-5p mimics significantly promoted the phosphorylation of AKT in the RAFLS cells treated with or without RANKL when compared with the control. The miR-515-5p inhibitor promoted the phosphorylation of JNK in the RAFLS cells. The expression of TLR4 and WISP1 was significantly reduced by the miR-515-5p inhibitor when compared with the miR-515-5p mimics. The expression of TLR4 and WISP1 was inhibited by treatment with RANKL. These findings showed that the miR-515-5p mimics reduced the expression of TLR4, WISP1, and JNK, while the miR-515-5p inhibitor promoted the expression of TLR4, WISP1, and JNK at the protein level in the RAFLS cell in vitro.

Discussion

The findings from the present study showed a direct action of microRNA-515-5p (miR-515-5p) with the WISP1 gene in rheumatoid arthritis fibroblast-like synovial (RAFLS) cells in vitro. The use of the miR-515-5p mimics promoted cell proliferation, reduced apoptosis, and promoted cell the cell cycle in the RAFLS cells. The miR-515-5p mimics inhibited the expression of WISP1, and treatment with the receptor activator of nuclear factor-kappa-B ligand (RANKL) inhibited the TLR4/JNK signaling pathway.

The extracellular domain of RANKL has similar features to tumor necrosis factors (TNF), as the extracellular domain can be cleaved to form a soluble secretory protein [25]. The downstream signaling pathways regulated by WISP1 and RANKL are different from each other but also interact with each other,

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including through the TLR4/JNK signaling pathway [25]. WISP1 can activate Ras-related C3 botulinum toxin substrate 1 (Rac1) through the Wnt/planar cell polarity (PCP) signaling pathway to activate JNK [26]. It has previously been reported that WISP1 activates TIR-domain-containing adapter-inducing interferon-β (TRIF) downstream of TLR4 to activate NF-κB [27]. Similar to WISP1, RANKL also activates downstream JNK by activating the MAPK cascade through TNF/TRAF signaling pathway, and TRAF can, directly and indirectly, activate IkB kinase (IKK), resulting in the activation of NF-κB [28, 29].

WISP1 and RANKL may have similar effects on cell survival and the promotion of cell growth by activating JNK and NF-κB [30]. However, the results of the present study showed that the effects of WISP1 and RANKL on the RAFLS cells were different. In this study, the expression of WISP1 was regulated by the miR-515-5p inhibitor and mimics. The luciferase assay showed that 3’UTR inhibited the expression of WISP1. Subsequent experiments also found that the inhibitor of miR-515-5p could increase the expression of WISP1 in the RAFLS cells. These results supported the effectiveness of miR-515-5p in regulating the expression of WISP1 and showed that WISP1 expression, promoted by the inhibitor of miR-515-5p, could induce the RAFLS cells to enter mitosis, while RANKL significantly increased the phosphorylation levels of AKT and JNK in RAFLS cells, but inhibited the entry into mitosis. Following treatment with RANKL, the expression level of the WISP1 protein was relatively reduced, and the effect of the miR-515-5p inhibitor on the expression of WISP1 and JNK phosphorylation was also reduced.

WISP1 has previously been reported to promote cell growth and to inhibit apoptosis by activating the downstream PI3K/AKT signaling pathway [31]. These previous findings were supported by the results of the present study, which showed that the inhibitors of miR-515-5p increased the phosphorylation level of AKT, and RANKL treatment significantly inhibited the effect of the inhibitors of miR-515-5p. Also, inhibitors of miR-515-5p promoted apoptosis. The effects of inhibitors of miR-515-5p have previously been reported to be significantly inhibited following treatment with RANKL [32]. Also, it has previously been reported that there may be some competition between WISP1 and RANKL [33]. Following exogenous treatment with RANKL, the change in WISP1 expression in cells is relatively minor. These results suggest that both WISP1 and RANKL can activate the TLR4/JNK signaling pathway in RAFLS cells, but they have different, or even opposite, regulatory effects on cell growth. This finding may be due to the regulation of other downstream signaling pathways by WISP1 and RANKL [33]. Future studies should be performed to investigate the effects of regulation of components of the TLR4/JNK signaling pathway.

Figure 5. The microRNA-515-5p (miR-515-5p) mimics reduced the expression of TLR4, WISP1, and JNK, while the miR-515-5p inhibitor promoted the expression of TLR4, WISP1, and JNK at the mRNA level. (A) TLR4. (B) WISP1. (C) JNK. The relative expression levels of the target genes were obtained according to the objective internal reference. * P<0.05; # P<0.05 (one-way analysis of variance).
This study had several limitations. First, the findings from the expression of TLR4 protein and mRNA were different, which may indicate that the regulatory effects of WISP1 and RANKL on TLR4 involves processes other than transcription and translation [27,34]. In future studies, we aim to investigate whether processes such as ubiquitination are involved in TLR4 expression. Also, this in vitro study used a single cell line, and the roles of miR-515-5p in RA, using inhibitors and mimics, should be verified using more cell lines and in vivo models.

**Conclusions**

This in vitro study aimed to investigate the effects of microRNA-515-5p (miR-515-5p) on the expression of WNT1-inducible-signaling pathway protein 1 (WISP1) in RAFLS cells following treatment with the receptor activator of nuclear factor-kappa-B ligand (RANKL). In RAFLS cells, miR-515-5p inhibited the expression of the WISP1, and treatment with RANKL inhibited the TLR4/JNK signaling pathway. Further studies are required to determine whether the expression of miR-515-5p can reduce the inflammatory response in the synovium in rheumatoid arthritis in vivo.
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