

EXPERIMENTAL STUDY

The function of microRNA-34a in osteoarthritis

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ABSTRACT

AIM: To discuss the effects and mechanism of microRNA-34a in cell apoptosis induced by osteoarthritis.

METHODS: Collection of the normal and osteoarthritis synovial tissues and measurements of the miRNA-34a and TGIF2 gene expression. In the cell experiment, the cells were divided into Control, Blank and miRNA inhibitor group. The cell proliferation and apoptosis of the different groups were measured by MTT and flow cytometry and the TGIF2 protein expression in the different groups was evaluated by WB assay. The correlation between TGIF2 and miRNA-34a was analyzed by Double luciferase experiment.

RESULTS: Compared with normal synovial tissues, the miRNA-34a gene expression was significantly up-regulated and TGIF2 gene expression was significantly suppressed in osteoarthritis synovial tissues ($p < 0.001$, respectively). The cell proliferation was significantly depressed and the cell apoptosis rate was significantly increased in miRNA inhibitor group compared with the Control group ($p < 0.001$, respectively). Using the WB assay it was shown that the TGIF2 protein expression of miRNA inhibitor group was significantly suppressed compared with that of Control group ($p < 0.01$). By Double luciferase assay, TGIF2 gene was one target gene of miRNA-34a. CONCLUSION: miRNA-34a could induce osteoarthritis synovial cell apoptosis via regulation of TGIF2 in vitro (Fig. 6, Ref. 29). Text in PDF www.elis.sk.

KEY WORDS: miRNA-34a, osteoarthritis, TGIF2.

Introduction

Osteoarthritis (OA) is a common degenerative joint disease characterized by a degenerative change in articular cartilage. OA not only causes articular cartilage damage, but also involves the entire joint, resulting in articular cartilage degeneration, fibrosis, and joint surface damage (1, 2). However, the treatment of OA is limited to symptomatic treatment. The clinical application of non-steroidal anti-inflammatory drugs and hormones only alleviates pain symptoms and cannot reverse the pathological process of OA (3, 4). MicroRNA (miRNA) is an endogenous non coded RNA with a length of 18–25 nucleotide fragments, which regulates gene expression by binding to the 3' UTR region of the target mRNA (5). The current clinical trials based on miRNA and therapy have been conducted in a variety of diseases, with great clinical diagnosis and treatment. However, similar treatments for arthritis are relatively scarce. In OA cartilage, it has been found that miRNA may be involved in the pathological process of OA (6–10). Some studies reported that miRNA-34a can suppress cell proliferation by stimulating cell apoptosis (11, 12). TGIFs belong to the TALE domain protein family, which includes TGIF, TGIF2/TGIF2LX/Y in the human genome. TGIFs can participate in the proliferation of cells in a variety of ways, and can interact with Smads proteins

and TGF signal regulates the occurrence of tumor (13–18). However, the effect and mechanism of TGIF2 in the development of OA has been unclear by now.

Materials and methods*Materials*

The synovial tissues were taken from osteoarthritis and joint replacement patients who were treated in the Second Hospital of Tangshan City. The synovial tissues were divided into 2 parts: one part was stored at -80°C until measuring miRNA-34a and TGIF2 gene expression and separation of the synovial cells; another part was fixed in 10 % polyoxymethylene and embedded in paraffin. The paraffin block was cut to 4 μm sections, the section was used for H&E staining.

RT-PCR assay

The total RNA was extracted from synovial tissues from osteoarthritis and joint replacement patients by Trizol total RNA extraction kit (Invitrogen, USA), taking 1.5 μg RNA following by Taq RNA PCR Kit (AMV) Ver 3.0 (Bao biological engineering Co., Ltd., China) operation method, configuration reaction system and placed in the PCR instrument reaction system as follows: 30°C for 10 min, 42°C for 30 min, 99°C for 5 min, 5°C for 5 min, the cDNA was synthesized. The relative genes' primers were added as follows: miRNA-34a: F: 5'-TCTATTTGCCATCGTCTA-3'; R: 5'-CAGGCAGCTCATTGGAC-3'. TGIF2: F: 5'-AGAGT-GCACTGCATGATAGCGTTCT-3'; R: 5'-CCAATGATAAAA-CAACCATTGG-3'. β -actin: F: 5'-GGACTTCGAGCAAGAGA-

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TGG-3'; R:5'-AGCACTGTGTTGGCGTACAG-3'. PCR reaction conditions were as follows: 95 °C for 3 min, 94 °C for 40 s, 54 °C for 40 s, 72 °C for 1 min, 72 °C for 3 min, the total cycles were 29 cycle in ABI step one plus real-time system (Applied Biosystems, USA). The mRNA-level of the genes of interest were expressed as the ratio of a gene of interest to β -actin mRNA for each sample. The comparative Ct ($\Delta\Delta$ Ct) method was used to determine the expression fold change.

OA synovial cell isolation and culture

Synovial tissue of the knee joint was aseptically removed, washed by PBS, cut, 2 ml DMEM culture fluid and 2 ml I type collagenase (2.5 mg/ml) were added, the cells were cultured in incubator (37 °C, 5% CO₂) for 4–6 h, filtered with 200 mesh gauze, Centrifuged and the supernatant was abandoned. After adding DMEM culture fluid to re-suspend cells, cells were inoculated into a culture bottle as 1×10⁶/ml and cultured in the incubator (37 °C, 5 % CO₂). The cells were divided into 3 groups: Control group treated with normal treatment; Blank group transfected with blank vector and miRNA inhibitor group transfected with miRNA-34a inhibitor.

Morphological observation and identification of synovial cells

The cell suspension was added in the 96 hole cell culture plate (Prefabricated 10mm slide), was cultured for 24h, the slide was remove, rinsed with PBS, dehydrated in graded ethanol, fixed,

stained with conventional HE, xylene transparent after mounting and cell morphology was observed under microscope.

Synovial cells transfection

T cells were inoculated on a 6-hole plate. There were 1×10⁶ cells/ml in every hole, Before transfection, the cell density was 80 – 90 %. Depending on Lipofectamine 2000 (Sigma, USA) instruction manual, the synovial cells of Blank and miRNA inhibitor groups, respectively, were transfected with empty vector and miRNA-34a inhibitor (Guangzhou Ruibo company. China).

MTT assay

After transfection for 24 h, the cells of different groups were collected, washed by PBS, digested by 0.25 % trypsin, centrifuged resulting in a single cell suspension, then the cell suspension was adjusted to concentration of 1×10⁶ cells/ml, inoculated in 96-well plate, cultured overnight, then 20 μ l MTT solution (5 mg/ml) was added and continued to culture for 4 h at 37 °C, after that the supernatant was removed, 100 μ l DMSO were added to every hole, after shaking for 10 min, absorbance by Enzyme-linked immunosorbent at 570 nm was evaluated. The cell proliferation of different groups was evaluated afterwards.

The cell apoptosis test

Annexin V-fluorescein isothiocyanate/propidium iodide (Annexin V-FITC/PI) Double staining method was used to measure

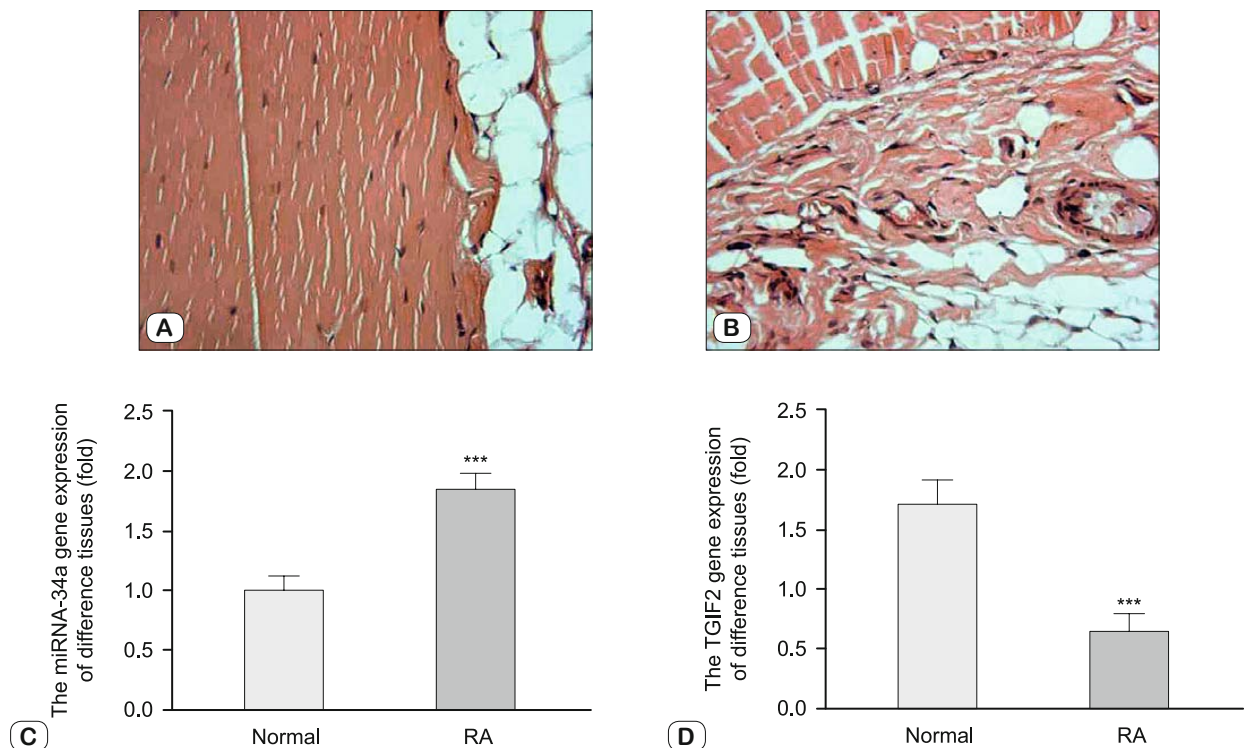


Fig. 1. The clinical data and analysis. A) Normal synovial tissue by H&E staining (×200), B) OA synovial tissues by H&E staining (×200), C) the miRNA-34a gene expression of different tissues, *** $p < 0.001$, vs normal tissue, D) the TGIF2 gene expression of different tissues, *** $p < 0.001$, vs normal tissue.

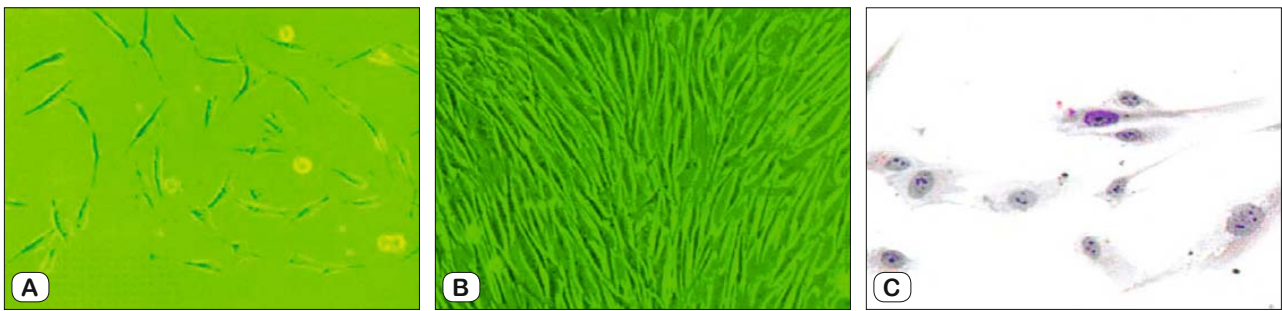


Fig. 2. Synovial cell morphology. A) Undyed ($\times 200$); B) undyed ($\times 100$); C) H&E staining ($\times 400$).

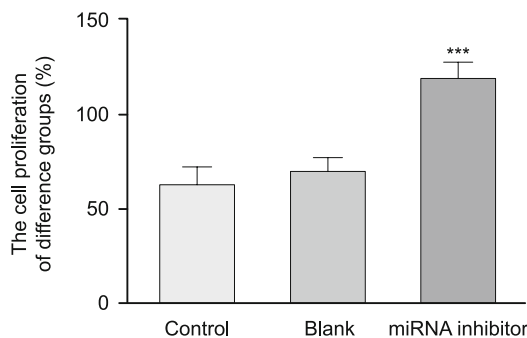


Fig. 3. The cell proliferation of different groups. *** $p < 0.001$; vs control group.

the cell apoptosis. The cells of different groups were collected and washed by PBS for 3 times, using 0.25 % pancreatin to digest, centrifuged at 1000r/min for 5 min, the cells were collected; adding the PBS in the tube to repeat again. 500 μ l combined buffer solution were added in the cells, and a single cell suspension was prepared with the concentration of 1×10^6 cell/ml. The single cell suspension was transferred to flow tube, 5 μ l Annexin V-FITC were added into the tube and it was stored for 30 min in the dark, then 2.5 μ l PI was added into the tube 5 min before measuring, the cell apoptosis of different groups was finally evaluated.

WB assay

The synovial cells of 3 groups were collected and washed by pre-cold PBS for 2 times, centrifuged at 1000 r/min for 5 min, the supernatant was removed 70 μ l pre-cold cell lysate were added to precipitate suspension cell, placed on ice for 30 min., centrifuged at 12000 r/min for 10 min at 4 $^{\circ}$ C, then the supernatant was collected. The total protein concentrations were measured by BCA method. The sample protein concentrations of different groups were adjusted to be similar, taking protein sample (25 μ g total protein/lane) and added the same volume buffer solution, SDS-PAGE electrophoresis, transferring film, using 5 % non-fat milk powder to close for 2 h, after that mice anti human TGIF2 and GAPDH antibodies (1:200) were added and cultured overnight at 4 $^{\circ}$ C, then washed and subsequently the corresponding alkaline phosphatase marked second antibodies were added to the culture for 2 h, alkaline phosphatase color rendering and imaging under automatic

gel imaging analysis system, using Scion Corporation analysis software to analyze the TGIF2 protein expression, GAPDH was considered as reference. The experiment was repeated for 3 times.

Statistical analysis

The relative data were analyzed by SPSS 22.0 software, the data are shown as mean \pm SD (standard deviation), and the Student–Newman–Keuls test and one-way ANOVA were used to compare the variables among different groups. $p < 0.05$ means there were significantly differences.

Results

Normal and RA synovial tissues and relative gene expressions

Using H&E staining, there were just 1–3 layers of cells in the normal synovial tissue (Fig. 1A); However, synovial layer was thickened in the RA synovial tissue (Fig. 1B). RT-PCR assay results showed that miRNA-34a gene expression of normal synovial tissues was significantly up-regulated and TGIF2 gene expression of normal synovial tissues was significantly down-regulated compared with those of RA synovial tissues ($p < 0.001$, respectively). Depending on this result, we inferred that miRNA-34a might be in negative correlation with IGIF2 in RA.

RA synovial cells Morphological observation and identification

The RA primary synovial cells were cultured in vitro, including macrophage like synovial cells (MCs/A type cell), fibroblast like synovial cells (FCs/B type cell) and dendritic cells (DCs/C cells) in inverted microscope. Under the microscope, the morphology of the cells was mainly spindle shaped, and the two poles were long and elongated. The end of the cells was connected to the adjacent cells and interwoven into a network (Figs 2A and 2B). H&E staining showed that most of the nuclei were round or oval, the nucleolus was clear and nucleolus number was 1–3 (Fig. 2C).

The cell proliferation of difference groups by MTT assay

Compared with the Control group, the cell proliferation rate of miRNA inhibitor group in which the miRNA-34a was knocked down was significantly increased ($p < 0.001$); And there were no significant differences between Control and Blank group, this shows that the transfection had no effects on cell proliferation in this study. The relative data are shown in Figure 3.

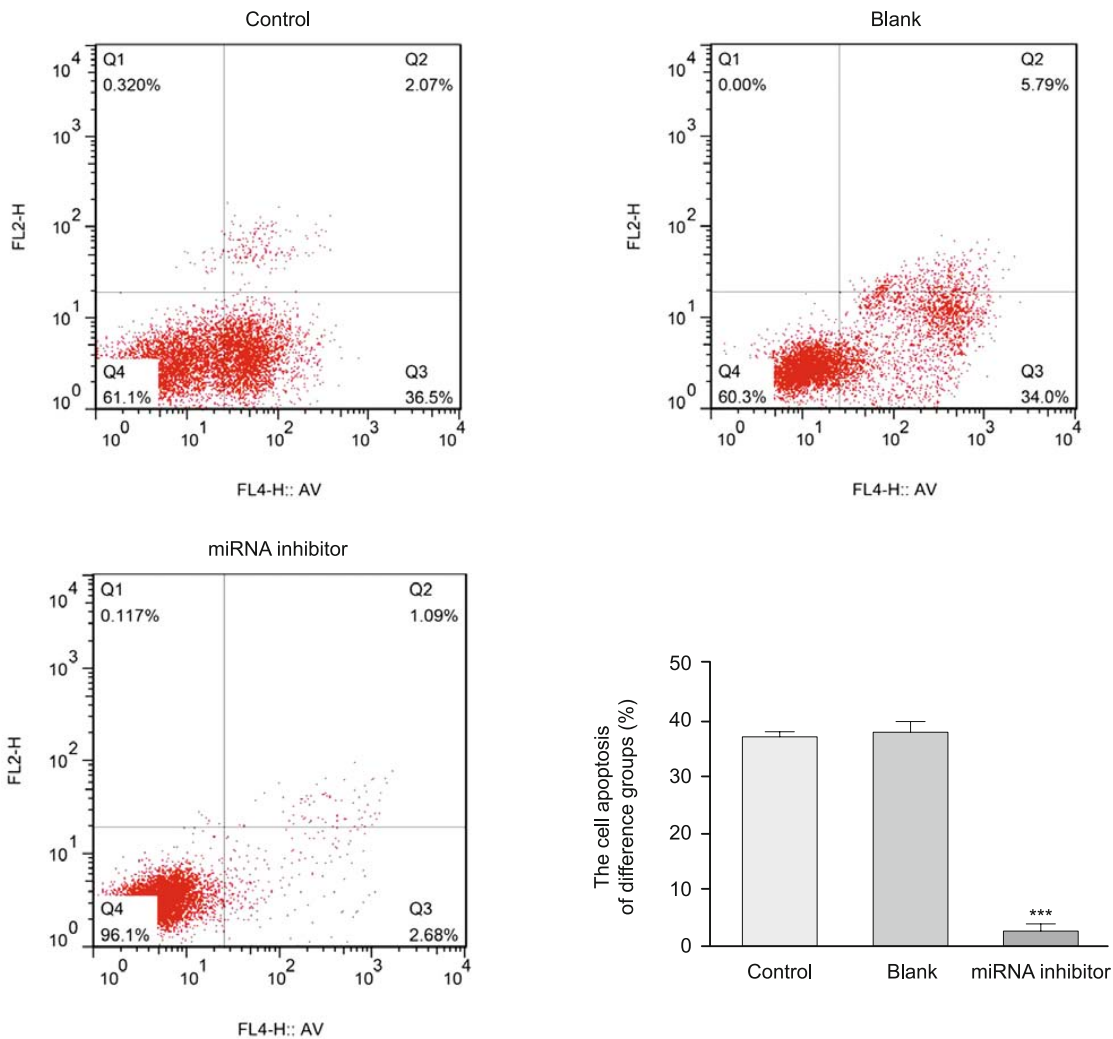


Fig. 4. The cell apoptosis rate of different groups. *** $p < 0.001$; vs control group.

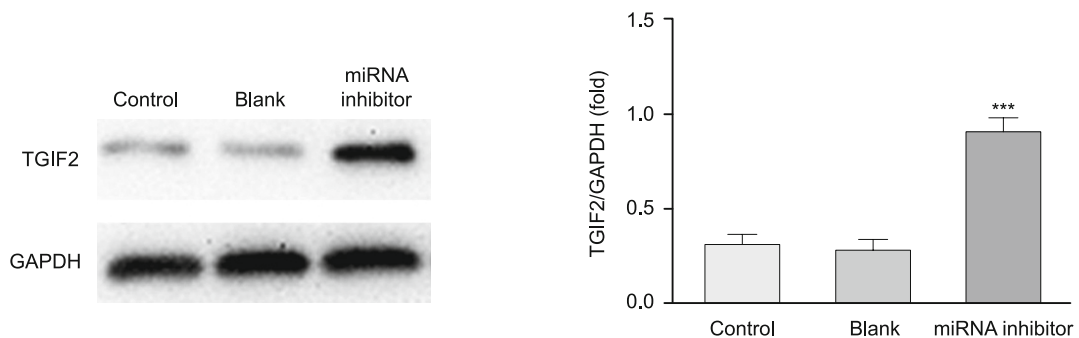


Fig. 5. The TGIF2 protein expression of different groups. *** $p < 0.001$; vs control group.

The cell apoptosis of different groups

Apoptosis is often accompanied by articular chondrocytes in OA. Flow cytometry was used to detect the apoptosis of OA synovial cells. The result was shown that the cell apoptosis rate of

miRNA inhibitor group was significantly suppressed compared with that of Control group ($p < 0.001$). However, there were no significant differences between Control and Blank groups ($p < 0.05$). The relative data are shown in Figure 4.

	predicted pairing of target region (top) and miRNA (bottom)	seed match
Position 580-614 of TGIF2 3' UTR	5' ... GUUCUGGUGAGCUGGGAAGGACCCACCACUGCCA...	8mer
hsa-miR-34a	3' UUGUUGGUCGAUUCU-----GUGACGGU	

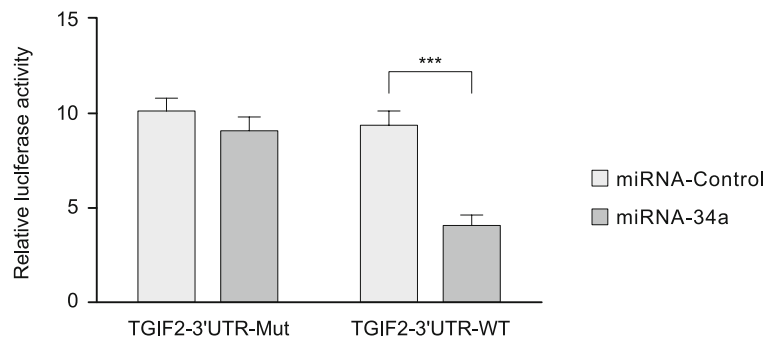


Fig. 6. Double luciferase target experiment. *** $p < 0.001$; vs miRNA-Control.

The TGIF2 protein expression in different groups

Compared with Control group, the TGIF2 protein expression of miRNA inhibitor group was significantly up-regulated ($p < 0.001$) and the TGIF2 protein expression of Blank group was not significantly different ($p > 0.05$) (Fig. 5).

Double luciferase assay

The luciferase activity of miRNA-34a was not significantly different compared with that of miRNA-Control group in TGIF2-3'UTR-Mut in which the TGIF2 gene mutation; compared with miRNA-Control, the luciferase activity of miRNA-34a was significantly suppressed compared with that of miRNA-Control in TGIF2-3'UTR-WT in which the TGIF2 gene was normal ($p < 0.001$). The results were shown that TGIF2 was the target gene of miRNA-34a (Fig. 6).

Discussion

MiRNA can regulate gene expression through regulating protein translation process targeting mRNA or binding to specific mRNA. MiRNA is widely involved in human neural differentiation, lipid metabolism, cell proliferation and apoptosis, physiological and pathological processes, and is an important regulator.

As let-7 could regulate RAS gene expression in the lung cancer (19); miRNA-143 and miRNA-145 were down-regulated in the colon cancer tissues; miRNA plays an important role in human diseases (leukaemia, virus infection and others) (20, 21). The previous relative studies have reported that miRNA-34a overexpression could suppress cell proliferation by stimulating cell apoptosis enhancement (22–24). Studies have shown that miRNAs play an important role in the progress of OA (25, 26). Yamasaki et al reported that the expression of miRNA-146 is increased in the low grade OA articular cartilage, and the expression of miRNA-146

could be induced by IL-1 β stimulation (27). Miyaki et al confirmed that decreased OA in articular cartilage of miR-140 expression may lead to abnormal gene expression patterns characteristic of OA (28). Apoptosis of chondrocytes is a major factor in the progress of OA. Our present results have shown that miRNA-34a was highly expressed in the OA synovial tissues of OA patients and the synovial cell apoptosis was depressed with miRNA-34a knock-down in in vitro study.

Many studies have proved that TGIF2 might play key role in cell proliferation (16–18), TGIF2 was over-expressed in Non-small cell lung cancer (NSCLC), and was considered as a cancer gene to partake in cell proliferation, G1/S cell cycle process (29). In the present study, it was found that TGIF2 gene expression was down-regulation in the OA synovial tissues of OA patients that might be the results of OA synovial cell apoptosis enhancement and the cell apoptosis and TGIF2 protein were suppressed with miRNA-34a knock-down in in vitro study. By double luciferase target experiment, the TGIF2 gene was one target gene of miRNA-34a.

Conclusion

The miRNA-34a over-expression might induce OA synovial cell apoptosis during OA progression; meanwhile, the miRNA-34a inhibitor had effects to improve OA synovial cell apoptosis in in vitro study. Based on those results, we inferred that miRNA-34a knock-down might be a potential method to treat OA in clinical practice.

References

1. McAlindon TE, Bannuru RR. OARSI recommendations for the management of hip and knee osteoarthritis: the semantics of differences and changes. *Osteoarthritis Cartilage* 2010; 18 (4): 473–475.

2. **De Silva V, El-Metwally A, Ernst E et al.** Evidence for the efficacy of complementary and alternative medicines in the management of osteoarthritis: a systematic review. *Rheumatology (Oxford)* 2011; 50 (5): 911–920.
3. **Soifer HS, Rossi JJ, Saetrom P.** MicroRNAs in disease and potential therapeutic applications. *Mol Ther* 2007; 15 (12): 2070–2079.
4. **Erson AE, Petty EM.** MicroRNAs in development and disease. *Clin Genet* 2008; 4 (4): 296–306.
5. **Wienholds E, Plasterk RH.** MicroRNA function in animal development. *FEBS Lett* 2005; 579 (26): 5911–5922.
6. **Jones SW, Watkins G, Le Good N et al.** The identification of differentially expressed microRNA in osteoarthritic tissue that modulate the production of TNF-alpha and MMP13. *Osteoarthritis Cartilage* 2009; 17 (4): 464–472.
7. **Iliopoulos D, Malizos KN, Oikonomou P et al.** Integrative MicroRNA and proteomic approach identify novel osteoarthritis genes and their collaborative metabolic and inflammatory network. *PLoS One* 2008; 3 (11): e3740.
8. **Yamasaki K, Nakasa T, Miyaki S et al.** Expression of MicroRNA-146a in osteoarthritis cartilage. *Arthritis Rheum* 2009; 60 (4): 1035–1041.
9. **Miyaki S, Nakasa T, Otsuki S et al.** MicroRNA-140 is expressed in differentiated human articular chondrocytes and modulates interleukin-1 responses. *Arthritis Rheum* 2009; 60 (9): 2723–2730.
10. **Chang TC, Wentzel EA, Kent OA et al.** Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol Cell* 2007; 26 (5): 745–752.
11. **Kofman AV, Kim J, Park SY et al.** microRNA-34a promotes DNA damage and mitotic catastrophe. *Cell Cycle* 2013; 12 (22): 3500–3511.
12. **Kim HR, Roe JS, Lee JE et al.** A p53-inducible microRNA-34a down-regulates Ras signaling by targeting IMPDH. *Biochem Biophys Res Commun* 2012; 418 (4): 682–688.
13. **Seo SR, Ferrand N, Faresse N et al.** Nuclear retention of the tumor suppressor cPML by the homeodomain protein TGIF restricts TGF-beta signaling. *Mol Cell* 2006; 23 (4): 547–559.
14. **Kuribayashi H, Tshako A1, Kikuchi M et al.** Role of transcription factor Tgif2 in photoreceptor differentiation in the mouse retina. *Exp Eye Res* 2016; 152: 34–42.
15. **Koso H, Tshako A, Lyons E et al.** Identification of FoxR2 as an oncogene in medulloblastoma. *Cancer Res* 2014; 74 (8): 2351–2361.
16. **Lips EH, van Eijk R, de Graaf EJ et al.** Integrating chromosomal aberrations and gene expression profiles to dissect rectal tumorigenesis. *BMC Cancer* 2008; 8: 314.
17. **Wang Y, Wang L, Wang Z.** Transgenic analyses of TGIF family proteins in *Drosophila* imply their role in cell growth. *J Genet Genomics* 2008; 35 (8): 457–465.
18. **Hu Y, Pu Q, Cui B et al.** MicroRNA-34a inhibits tumor invasion and metastasis in gastric cancer by targeting Tgif2. *Int J Clin Exp Pathol* 2015; 8 (8): 8921–8928.
19. **Johnson SM, Grosshans H, Shingara J et al.** RAS is regulated by the let-7 MicroRNA family. *Cell* 2005; 120 (5): 635–647.
20. **MZ Michael, SM O' Connor, NG van et al.** Reduced accumulation of specific microRNAs in colorectal neoplasia. *Mol Cancer Res* 2003; 1 (12): 882–891.
21. **Calin GA, Dumitru CD, Shimizu M et al.** Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 2002; 99 (24): 15524–15529.
22. **Chang TC, Wentzel EA, Kent OA et al.** Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol Cell* 2007; 26 (5): 745–752.
23. **Raver-Shapira N, Marciano E, Meiri E et al.** Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Mol Cell* 2007; 26 (5): 731–743.
24. **He L, He X, Lim LP et al.** A microRNA component of the p53 tumour suppressor network. *Nature* 2007; 447 (7148): 1130–1134.
25. **Iliopoulos D, Malizos KN, Oikonomou P et al.** Integrative MicroRNA and proteomic approaches identify novel osteoarthritis genes and their collaborative metabolic and inflammatory networks. *PLoS One* 2008; 3 (11): e3740.
26. **Tazawa H, Tsuchiya N, Izumiya M et al.** Tumour-suppressive miR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells. *Proc Natl Acad Sci USA* 2007; 104 (39): 15472–15477.
27. **Yamasaki K, Nakasa T, Miyaki S et al.** Expression of MicroRNA-146a in osteoarthritis cartilage. *Arthritis Rheum* 2009; 60 (4): 1035–1041.
28. **Miyaki S, Nakasa T, Otsuki S et al.** MicroRNA-140 is expressed in differentiated human articular chondrocyte and modulates interleukin-1 responses. *Arthritis Rheum* 2009; 60 (9): 2723–2730.
29. **Lu YJ, Liu RY, Hu K et al.** MiR-541-3p reverses cancer progression by directly targeting TGIF2 in non-small cell lung cancer. *Tumour Biol* 2016; 37 (9): 12685–12695.

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