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# *ABCA1* IS DIRECT TARGET GENE OF MIR-144-3P IN CHONDROCYTE

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SUMMARY

Osteoarthritis (OA) is the most frequent disease of the musculoskeletal system, affecting millions of individuals around the globe. As a chronic disease, OA develops steadily over decades, eventually leading to joint degeneration. MicroRNAs are 20–25 nucleotide noncoding RNAs that modulate gene expression at the post-transcriptional level by binding to the 3'-UTR of target mRNAs, thereby inhibiting translation or inducing mRNA degradation. MicroRNA-144-3p was reported to be associated with osteoarthritis (OA) since it was upregulated in this disease. *ABCA1* was also found to be involved in OA and a potential target of miR-144-3p by bioinformatics algorithms. This study aims to experimentally demonstrate that ABCA1 is the direct target of miR-144-3p. Initially, a mimic or inhibitor of miR-144-3p was transfected into the chondrocyte. Subsequently, the expression of *ABCA1* was determined by Real Time RT-PCR. The 3'UTR containing several binding sites of miR-144-3p and its mutated version were subcloned. A luciferase assay was performed to check the ability of miR-144-3p to bind to *ABCA1*. Real-time RT-PCR revealed that miR-144-3p overexpression inhibited *ABCA1* expression. In contrast, when endogenous miR-144-3p decreased, *ABCA1* level increased. This result indicated that *ABCA1* was the target of miR-144-3p. Particularly, luciferase assay results showed that miR-144-3p interacted directly with *ABCA1* via its binding sites on the 3'UTR. These findings confirmed that miR-144-3p is the direct target of *ABCA1*.

**Key word:** *ABCA1*, miR-144-3p, osteoarthritis, target gene

## INTRODUCTION

OA is the most common form of arthritis and causes damages to the cartilage of joints (Lane *et al.*, 2017). This is a chronic disease and may spontaneously develop over the life course. Moreover, OA is also reported to involve changes in the cartilage remodeling of joints (Loeser *et al.*, 2012; Sharma *et al.*, 2013). A report in 2016 published by the

International Association for Orthopedic Research (OARSI) described OA as a serious disease due to its global burden (Hawker, 2019). An estimated 10-15% of adults have symptoms of OA. According to WHO, Vietnam is ranked in the group of countries with the highest rate of population suffering from osteoarthritis. OA is usually diagnosed by clinical imaging facilities such as X-rays, MRI and endoscopy. However, there are limitations in clinical diagnosis due to its less accuracy in early stages, as well as its low sensitivity and specificity. For the treatment, OA patients can only use medication to relieve pains, maintain a balanced diet and healthy lifestyle, and wear assistive devices to reduce pressure on the joints. Therefore, it is necessary to develop an early, accurate diagnosis as well as effective treatments at early stages to benefit people with OA.

MiRNAs are small non-coding RNAs, 20-25 nucleotides in size, which regulate gene expression at the post-transcriptional level by binding to the 3'-UTR of target mRNAs, suppressing translation or inducing the degradation of the mRNAs (Ambros, 2004; Bartel, 2004). A study examining the role of miR-144-3p in OA development in mice found that miR-144-3p was increased in the early stages of OA (Zhang *et al.*, 2017). Our previous study also demonstrated that miR-144-3p is involved in the development of OA (Le *et al.*, 2018). However, the role and function of the miRNA in OA pathogenesis remains unclear.

ABCA1 is a transmembrane protein involved in cholesterol metabolism, specifically mediating lipid transport from cells to APO (Apolipoprotein). *ABCA1* is located on chromosome 9, position 9q31.1. ABCA1 is predicted by multiple software as a potential target of miR-144-3p (Ngân, 2022). Bioinformatics analysis showed that the 3'UTR of *ABCA1* mRNA contains five recognition sites for miR-144-3p (Ngân, 2022), suggesting a possibility that *ABCA1* is a direct target for miR-144-3p.

This study aims to confirm that *ABCA1* is a direct target gene of miR-144-3p. The obtained results may help to develop a fundamental understanding for further research on the role and function of miR-144-3p in OA.

## MATERIALS AND METHODS

### Cell culture

The human primary chondrocyte and chondrosarcoma cell line SW1353 were maintained in DMEM (Dulbecco's modified Eagle's, Thermo Fisher Scientific, UK) containing 10% (v/v) FBS (Fetal Bovine Serum, Thermo Fisher Scientific, UK) and 1% Pen/Strep antibiotics (Penicillin and Streptomycin, Thermo Fisher Scientific, USA) at 37$^{o}$C and 5% (v/v) $CO_2$. Cells were passed to a new flask or seeded in a 6-well plate for further experiments when reaching 90% confluency.

### Gain- and loss- of function assay

Primary chondrocyte cells were cultured in 6-well plates at 2 x $10^5$ cells per well. Once the culture reached 90% confluency, the cells were transfected with 50 nM miR-144-3p-mimics (Qiagen) or 50 nM miR-144-3p-inhibitor (Qiagen, Denmark) and control (Qiagen, Denmark) using Lipofectamine 2000 (Thermo Fisher Scientific, USA). After 24 hours of transfection, total RNAs were extracted using TRIzol Reagent (Invitrogen, USA) and then converted into cDNA using the SuperScript II RT kit (Thermo Fisher

Scientific, USA). The expression levels of *ABCA1* were observed by Realtime RT-PCR with F-primer 5'-GCCCCTACAGTATA GATGATGTA-3' and R-primer 5'-GTGCAGGGTCCGAGGT-3' on LightCycler 480 System (Roche, UK). The Real-time RT-PCR components in a total volume of 10 µL were: 5 µL 2X Sensi Mix (Bioline, Singapore); 0.4 µL forward primer; 0.4 µL reverse primer; 4.2 µL of cDNA (0.5 ng/µL) with thermal cycles as follows: 95°C for 5 min, 40 cycles of denaturation at 95°C for 15 s and annealing/stretching at 56°C for 30 s, 72°C for 1 min, and then cooled at 4°C for 10 seconds. Three different primary chondrocyte cell lines were used to conduct the assay.

**Luciferase assay**

The wildtype 3'UTR and the mutant 3'UTR of *ABCA1* harboring the miRNA recognition sites were co-transfected with miR-144-3p mimic or control into SW1353 cells to validate a direct target of miRNAs using Luciferase assay.

The wildtype *ABCA1* 3'UTR sequence region containing the miR-144-3p recognition sites or the mutant 3'UTR of *ABCA1* was cloned into the pmirGLO vector (Promega, USA) using the In-Fusion HD Cloning plus kit (Takara, Japan) with forward and reverse primer sequences of 5'-GCTCGCTAGCCTCGATTGGCTTTGCA GATATTGG-3′ and 5'-CGACTCTAG ACTCGATGAGGGCCAATGATGAAC-3′, respectively. The fused vectors were transformed into *E. coli*, after which recombinant colonies were identified by colony PCR using primers: 5'-AAAGAAGCCATGAGGTCTTCAATACT G-3' and 5'- CGACTCTAGACC GACCAGTAGAGAACAAATAAGC-3'.

Transfection was carried out in a 96-well plate (5 x $10^4$ cells per well). After reaching 80% confluency (approximately 24 h), SW1353 cells were transfected with 100 ng pmirGLO- *ABCA1* or 100 ng pmirGLO-*ABCA1* mutant along with 50 µM miR-144-3p-mimic or 50 µM control using Lipofectamine 2000 (Thermo Fisher Scientific, USA). After 24 hours of transfection, luciferase activities were estimated using the Dual-luciferase Reporter Assay kit (Promega, USA) on EnVision 2103 Multilabel equipment (Perkin Elmer) according to the manufacturer's instructions. The experiments were performed in triplicate.

**Statistics analysis**

All data was evaluated by unpaired two tailed Student's t-test using GraphPad Prism version 5.

## RESULTS

**The expression of *ABCA1* suppressed by miR-144-3p**

MicroRNAs are expected to suppress the expression of its direct targets. Thus, miRNA target gene expression may be inversely associated with miRNA levels. To investigate the correlation of miR-144-3p and *ABCA1* expression, in this study the elevation or inhibition of miR-144-3p in primary chondrocyte and the *ABCA1* expression level were investigated. Human primary chondrocytes were transfected with miR-144-3p mimics or the inhibitor of the endogenous miR-144-3p along with the non-targeting controls for 24 hours. The expression of *ABCA1* was then quantified by Real Time RT- PCR.

The results (Figure 1) showed that ectopic expression of miR-144-3p repressed

the expression of *ABCA1*, and conversely, the introduction of the inhibitor against the endogenous miR-144-3p was able to increase *ABCA1* expression. Taken together, these data suggested that *ABCA1* is suppressed by miR-144-3p.

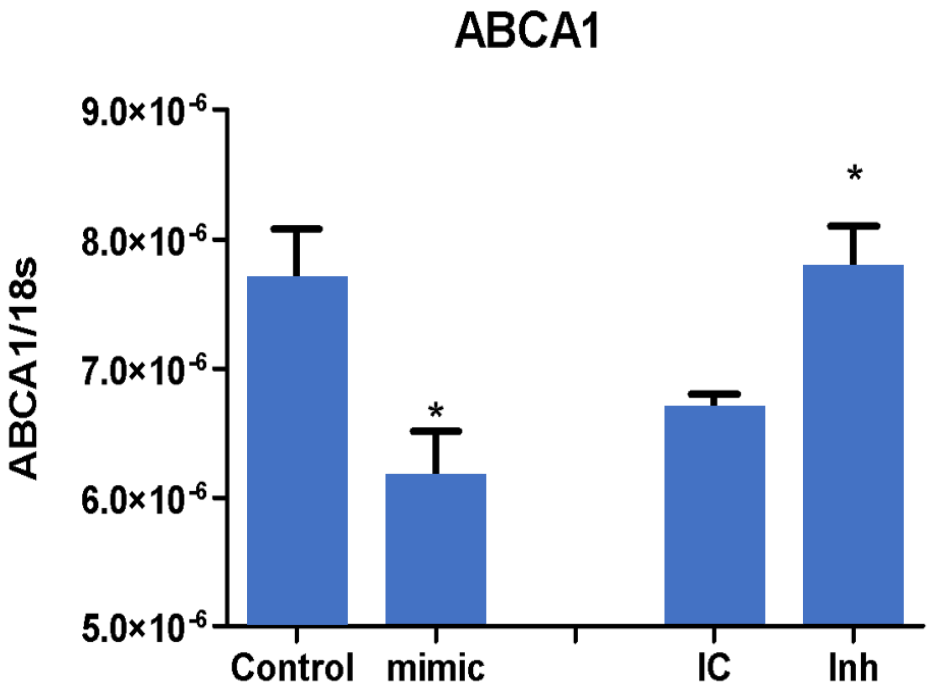


**Figure 1.** The expression of *ABCA1* in the cells transfected with *miR-144-3p* mimic and inhibitor of the endogenous *miR-144-3p.* Relative expression of ABCA1 in human primary chondrocyte cells transfected with miR-144-3p mimic or non-targeting control. Data were analyzed using unpaired Student's t-test, n=3. *p < 0.05. mimic: miRNA 144-3p mimic; IC: miR-144-3p inhibitor; Inh: Inhibitor control.

**Interaction of miR-144-3p with its binding sites on the 3'UTR of *ABCA1***

In order to verify *ABCA1* as a cognate target of miR-144-3p, the 3'UTR of ABCA1 containing binding sites of miR-144-3p was subcloned into the downstream of the luciferase encoding gene in the pmirGLO vector. This vector was then co-transfected with either the miRNA mimic or non targeting control for 24 hours before measuring luciferase activity. Result (Figure 2A) showed the relative luciferase activity was significantly decreased with miRNA mimic, suggesting that miR-144-3p directly binds to its binding sites in the *ABCA1* 3'UTR.

In addition, we hypothesized that if miR-144-3p truly bound to its binding sites in the 3'UTR of *ABCA1*, the relative luciferase activity would be rescued when the binding sites were mutated. The data (Figure 2B) showed that miR-144-3p no longer inhibited luciferase activity when all the binding sites of miR-144-3p in the 3'UTR of *ABCA1* were mutated. These suggested that *ABCA1* is a direct target of miR-144-3p since it could directly bind to its target sites in the 3' UTR of *ABCA1*.

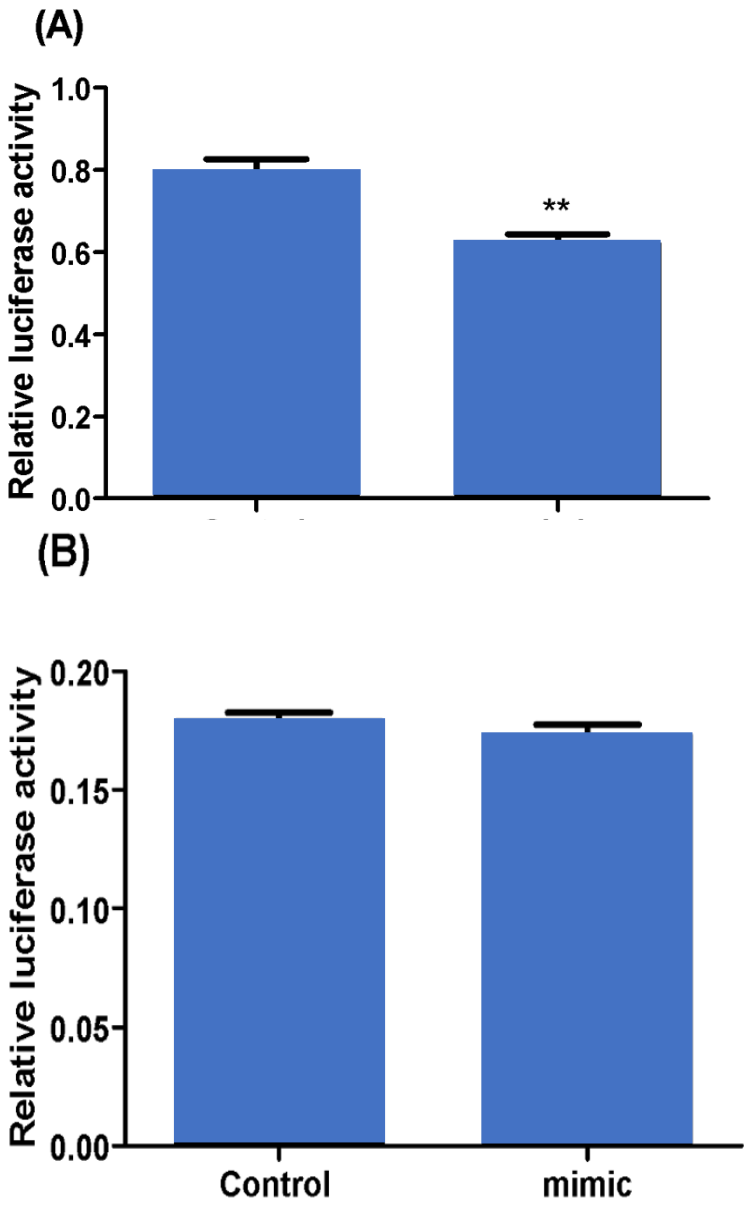


**Figure 2.** The expression of luciferase in case of direct binding of microRNA 144-3p to its binding sites in the 3'UTR of ABCA1. A. The pmiRGLo-ABCA1 3'UTR vector together with miR-144-3p mimics were transfected into SW1353 cells. In the cell, miR-144-3p mimics will complementarity bind to seed sites on the pmiRGLo-ADAMTSL3 vector, leading to mRNA degradation or translation inhibition of luciferase activity. B. The binding sites of miR-144-3p in the pmiRGLo-ABCA1 3'UTR vector were mutated. The mutant pmiRGLo-ABCA1 3'UTR vector was co-transfected with miR-144-3p mimics into SW1353 cells. In the cell, miR-144-3p will not be able to complementarity pair with mutated binding site, resulting in a rescue of luciferase expression. The luciferase activity was detected via luminescence. Renilla luciferase was used as an interval control. Data were analyzed using Student's unpaired t-test, n=3, **$p < 0.005$.

## DISCUSSION

MiR-144 has been shown to alter expression in Osteoarthritis (Lin *et al.*, 2021) . However, its function remains unclear. Understanding the function of miR-144-3p will help to develop a method to identify the disease at an early stage as well as to develop new therapeutics for efficient treatment. We previously found that there are five seed sites of miR-144-3p in the 3'UTR region of *ABCA1*. Therefore, it is likely that the miR-144/ *ABCA1* interaction is authentic 3p (Ngân, 2022). In this study the obtained results proved that miR-144-3p was directly targeting ABCA1. This was evidenced by the expression of *ABCA1* at mRNA level inversely correlated with miR-144-3p level in human primary chondrocyte due to miRNA-144-3p targeting its binding sites in the 3'UTR of the *ABCA1.* The expression of *ABCA1* is found to be regained when these binding sites are mutated. This is the first study to confirm that *ABCA1* is direct target of miR-144-3p in OA

There is evidence that abnormal *ABCA1* expression can lead to OA. ABCA1 is a transmembrane protein that facilitates the transfer of cholesterol from cells to apolipoproteins (APO). OA can develop when the amount of cholesterol entering the cells exceeds the amount of cholesterol absorbed. When compared to the normal control group, ABCA1 was found to have a significant reduction in OA articular cartilage group (Farnaghi *et al.*, 2017). MiR-144-3p, on the other hand, was found to increase in OA (Zhang, 2017). In the research, *ABCA1* is discovered to be a direct target of miR-144-3p. As a result, abnormal expression of the axis miR-144-3p/ABCA1 could explain a portion of the abnormal level of cholesterol accumulation in cells, eventually leading to OA. However, this hypothesis must be tested experimentally.

## CONCLUSION

MiR-144-3p directly modulated the expression of *ABCA1* via its binding sites on the 3'UTR of *ABCA1* mRNA. These data prove that *ABCA1* is a direct target of miR-144-3p. The study's findings contribute to a better understanding of miR-144-3p functions. This is critical for the future development of miRNA-based therapy for OA.

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