Long non-coding RNA HOTAIRM1-1 silencing in cartilage tissue induces osteoarthritis through microRNA-125b

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Abstract. Aberrations in long noncoding RNA (lncRNA) expression have been recognized in numerous human diseases. In the present study, the role of the long noncoding RNA HOX antisense intergenic RNA myeloid 1 variant (HOTAIRM1-1) in regulating the pathological progression of osteoarthritis (OA) was investigated. The aberrant expression of HOTAIRM1-1 in OA was demonstrated, but the molecular mechanisms require further analysis. The aim of the present study was to explore the function of miR-125b in modulating chondrocyte viability and apoptosis, and to address the functional association between HOTAIRM1-1 and miR-125b as potential targets. A miR-125b inhibitor was used, which laid the foundation for the following investigation. The study confirmed that HOTAIRM1-1 and miR-125b are inversely expressed in chondrocytes. The expression of HOTAIRM1-1 was downregulated and the expression of miR-125b was upregulated in tissues from patients with OA. HOTAIRM1-1 directly interacted with miR-125b in chondrocytes. The expression of HOTAIRM1-1 was downregulated and the expression of miR-125b was upregulated in tissues from patients with OA. HOTAIRM1-1 directly interacted with miR-125b in chondrocytes. HOTAIRM1-1 knockdown was associated with chondrocyte proliferation and extracellular matrix degradation. Furthermore, miR-125b reversed the effect of HOTAIRM1-1 on cell proliferation and apoptosis. In conclusion, the present study indicates that the loss of HOTAIRM1-1 function leads to aberrant increases in the proliferation and apoptosis of chondrocytes. miR-125b may be a potential downstream mechanism that regulates the function of HOTAIRM1-1, and this finding provides a therapeutic strategy for OA.

Introduction

Osteoarthritis (OA) is the most common type of arthritis, and it affects >10% of the adult population (1). Physicians consider the disease to be a degenerative disease that involves all the tissues of the joint. In OA, cartilage within a joint begins to break down, and the underlying bone begins to change (2). OA can induce substantial limb pain, stiffness, disability, and even loss of whole-body mobility. OA occurs most frequently in the hands, hips and knees, leading to difficulties in patients’ daily activities (3). Therefore, numerous management strategies to prevent cartilage degeneration, such as microfracture surgery to stimulate a healing response, stem cell therapy to repair damaged articular cartilage, cartilage transplantation, and complementary treatments, have been developed (4-6).

Long noncoding RNAs (lncRNAs) are transcripts longer than 200 nucleotides and are noncoding RNAs. Aberrant expression of lncRNAs participate in numerous biological processes and various diseases by posttranscriptional or posttranslational regulation (7,8). Jiang et al (9) and Marques-Rocha et al (10) reported that patients with OA have aberrant expression of certain lncRNAs, which indicates that lncRNAs may play a key role in articular cartilage degeneration. Recently, the novel lncRNA HOX antisense intergenic RNA myeloid 1 (HOTAIRM1) was found to be expressed in cells of the myeloid lineage (11,12). Additional evidence has confirmed that HOTAIRM1 plays a crucial role in the pathological progression of colorectal cancer and several other diseases, such as acute myeloid leukemia (13-15). Nonetheless, the potential function of HOTAIRM1 in the development of osteoarthritis remains unknown.

HOTAIRM1 is located between the HOXA1 and HOXA2 loci and has been identified as a myeloid-specific regulator in the HOXA gene family. HOTAIRM1 targets gene transcription during the chromosome remodeling that occurs when myeloid cells are induced to undergo chondrogenic differentiation (16). Furthermore, previous studies in mesenchymal stem cells showed that miR-125b is a miRNA target of HOTAIRM1 during osteoblastic differentiation (16,17). In the present study, the hypothesis that HOTAIRM1-1 and miR-125b have a functional association and that miR-125b is a potential therapeutic target of articular cartilage denegation was tested.
Materials and methods

Tissue collection. The present study was approved by the human ethics committee of Tianjin Hospital. All the patients provided written informed consent and participated in the study according to their own will. A total of 15 articular cartilage samples were obtained from patients with OA undergoing total knee arthroplasty (n=15; mean age, 65.8 ± 6.2 years; 5 males and 10 females). Healthy control articular cartilage samples were collected from trauma patients who underwent amputation and did not have a history of rheumatoid arthritis or OA (n=8; mean age, 41.2 ± 9.1 years; 5 male and 3 female). There was no significant difference between the OA group and the control group in terms of sex or age.

Cell culture. Chondrocytes from the cartilage of patients with OA were cultured following a previously published protocol (18). In brief, small sections of OA cartilage tissues were digested with trypsin (0.25%). Then, type II collagenase (0.2%; Gibco; Thermo Fisher Scientific, Inc.) was incubated with the samples. The chondrocytes were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS and maintained in a cell incubator containing 5% CO2 at 37°C.

Cell transfection. The plasmids containing small interfering (si)RNA targeting HOTAIRM1-1 (si-HOTAIRM1-1, 5’‑CAC CGGAGACTGTGAGCTTATATTCAAGAGATAAAG CTACCAGTCTCTTTTTTG‑3’) or a si-HOTAIRM1-1 negative control sequence (si-NC, 5’‑CACCTCTCCGGAC GTACGTCAAGATTACGTGACACGTTCCGGAGAT TTTTT‑3’) were purchased from Gene-Pharma (Shanghai GenePharma Co., Ltd.). Briefly, siRNA was transfected into cultured chondrocytes by using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) on ice for 30 min. The protein concentration of each cell lysate was determined by a BCA Assay kit (Thermo Fisher Scientific, Inc.) to measure the luciferase activity. The results are shown as the firefly/Renilla ratio normalized to the Renilla luciferase activity.

Western blotting. Proteins were obtained from cultured cells with radioimmunoprecipitation assay (RIPA) buffer (Pierce; Thermo Fisher Scientific, Inc.) on ice for 30 min. The protein concentration of each cell lysate was determined by a BCA Assay kit (Thermo Fisher Scientific, Inc.). The protein samples (50 µg) were separated on 8% polyacrylamide gels by electrophoresis for 5 min at 50 V, and then the voltage was increased to 100 V for 1 h. The protein samples were then transferred to the membranes. The membranes were incubated with anti-collagen II (cat. no. ab185430; 1:500 dilution; Abcam) and anti-aggrekan (cat. no. ab36861; 1:10,000 dilution; Abcam) primary antibodies for 4 h at room temperature. The membranes were washed twice and then incubated with Goat Anti-Rabbit IgG H&L secondary antibodies (cat. no. ab205718; 1:1,000; Abcam) for 60 min at room temperature. A chemiluminescent substrate was applied to the blots with an ECL detection kit (Thermo Fisher Scientific, Inc.). The chemiluminescent signals were captured using a Chemi-Doc XRS camera-based imager (Bio-Rad Laboratories, Inc.). Image analysis software was used to analyze the bands of the targeting proteins with Empiria Studio® Software (9141-500E; LI-COR Biosciences), and β-actin was used as the internal control to normalize the band intensity.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Cells were washed twice with cold PBS, after which samples were fixed with 4% paraformaldehyde for 2 min at 23°C and washed twice with PBS. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed on cells with an In-Situ Cell Death Detection kit (Roche Diagnostics). A total of 200 cells were analyzed for each sample.
Table I. Primer sequences.

| Gene   | F, 5'-3'                           | R, 5'-3'                      |
|--------|------------------------------------|------------------------------|
| HOTAIRM1-1 | AAACGAGGGATGGAAGGGAGCG           | CCAGGCATTTCGGCATGGTG       |
| miR-125b | GCCTTGCTGCCTACTTCCA               | GTCCACACGGGTTCAGA           |
| MMP-13  | GGCTCGACACCCGTGTA                 | CGTCAAACCTCTGTGCACTCCA     |
| IL-10   | GTAGAGGACACGGGCAAGAT              | TTCACGAACTGTCACTGCAC       |
| U6      | CTCGGCTCGGGACGCAAA                | AACCCTCCAGAATGGCAGT       |
| GAPDH   | AGAAGGCTGGGGCATTTTG              | AGGGCCACTCCACAGTCTTC      |

HOTAIRM1-1, HOX antisense intergenic RNA myeloid 1 variant; miR, microRNA; MMP-13, matrix metalloprotease-13; IL-10, interleukin-10; F, forward; R, reverse.

50 µl reaction mixture (terminal deoxynucleotidyl transferase from calf thymus recombinant in E. coli. Buffer as enzyme solution and nucleotide; provided in the kit) was incubated for 1 h at 37°C. After staining, samples were mounted with 4,6-diamidino-2-phenylindole (DAPI) mounting medium (Vector Laboratories, Inc.). Nuclear staining was performed at the same time as mounting, at room temperature for 10 min. A microscope was used to visualize cell apoptosis under 10 fields of view per sample (magnification, x200).

Identification of miRNA-mRNA and miRNA-ncRNA interactions. To inspect genome-wide interactions between miRNAs and their target genes, the conserved miRNA target sites were predicted using algorithms from a public database (http://starbase.sysu.edu.cn/index.php). starBase was intersected with the aforementioned Ago CLIP clusters to gain CLIP-supported sites.

Statistical analyses. The sample size for each experiment was determined based on power analysis calculations. All the data are shown as the mean ± SD (standard deviation). Statistical analyses and graph generation were completed by Prism GraphPad (GraphPad Software, Inc.). Student's t test was used for the analysis of two groups. One-way ANOVA followed by the Tukey-Kramer test was used to compare multiple groups. Two-way ANOVA was used to determine the effect of time, the effect of treatment, and their interaction. The Pearson's correlation analysis test was used to evaluate the strength of the association between two quantitative variables. P<0.05 was considered to indicate a statistically significant in all the tests.

Results

Expression of HOTAIRM1-1 and miR-125b in chondrocytes is inversely affected by IL-6 and IL-1β treatment. To mimic OA in vitro, the effect of IL-6 or IL-1β on HOTAIRM1-1 and miR-125b expression was first tested in chondrocytes. Chondrocytes were treated with IL-6 or IL-1β for 96 h. The data confirmed that IL-6 could significantly decrease chondrocyte proliferation (Fig. 1A). Using the same method, IL-1β had a similar effect and decreased chondrocyte proliferation (Fig. 1B). It was observed that the expression of HOTAIRM1-1 was decreased in chondrocytes after IL-6 treatment, but the expression of miR-125b increased in chondrocytes (Fig. 1C and D). Additionally, the results demonstrated similar trends in the expression of HOTAIRM1-1 and miR-125b in the group of chondrocytes treated with IL-1β (Fig. 1E and F).

Expression of HOTAIRM1-1 is downregulated and the expression of miR-125b is upregulated in OA tissues. Moreover, the hypothesis that the expression of HOTAIRM1-1 is downregulated in OA tissue was tested. The data demonstrated that the expression of HOTAIRM1-1 was significantly lower in the OA tissue samples compared with the healthy control samples (Fig. 2A; P<0.05). In addition, miR-125b expression was higher in the OA tissue samples compared with the healthy control tissue samples (Fig. 2B; P<0.01). The correlation test showed that the expression level of miR-125b was negatively correlated with the expression level of HOTAIRM1-1 (Fig. 2C).

HOTAIRM1-1 directly interacts with miR-125b in chondrocytes. Studies have reported that miR-125b is a directly competitive endogenous RNA (ceRNA) that affects the function of HOTAIRM1. However, whether HOTAIRM1-1 has a potential role in OA progression remains unknown. To address this question, potential miRNA binding sites that correlated with HOTAIRM1-1 were searched for with the starBase online tool (http://starbase.sysu.edu.cn/index.php; Fig. 3A). Furthermore, the luciferase assay results indicated that HOTAIRM1-1 directly targeted miR-125b, which confirms a direct association between HOTAIRM1-1 and miR-125b (Fig. 3B and C).

HOTAIRM1-1 knockdown is associated with chondrocyte proliferation and extracellular matrix degradation. Next, the effect of HOTAIRM1-1 knockdown on chondrocytes treated with IL-6 for 48 h before transfection was determined. Chondrocytes were transfected with an siRNA targeting HOTAIRM1-1. The expression of HOTAIRM1-1 was significantly downregulated in the si-HOTAIRM1-1 group compared with the si-NC-HOTAIRM1-1 group (Fig. 4A). The proliferation results demonstrated that HOTAIRM1-1 knockdown significantly decreased cell proliferation (Fig. 4B) and decreased cell cycle progression in the chondrocytes (Fig. 4C). MMP-13 and IL-10 were identified as the major cartilage-degrading enzyme markers in chondrocytes. Collagen II and aggrecan are considered to be fundamental extracellular matrix (ECM) proteins in chondrocytes. The results revealed...
that MMP-13 and IL-10 expression was increased in the si-HOTAIRM1-1 group (Fig. 4D and E). Conversely, ECM degeneration marker expression was significantly lower in the si-HOTAIRM1-1 group compared with the control group (Fig. 4F). In conclusion, cartilage-degrading enzymes may be upregulated by HOTAIRM1-1 knockdown, which promotes ECM degradation.

miR-125b reverses the effects of HOTAIRM1-1 on proliferation and apoptosis in IL-6-treated chondrocytes. To further explore whether HOTAIRM1-1 regulates chondrocyte proliferation and apoptosis via miR-125b in OA a rescue experiment was performed. As shown in Fig. 5A, miR-125b mimics transfection effectively increased the expression of miR-125b compared with mimics control transfection. The miR-125b inhibitor effectively decreased the expression of miR-125b compared with inhibitor negative control group. A CCK-8 assay was conducted to determine the proliferation ability of the chondrocytes, as shown in Fig. 5B. Downregulation of miR-125b strikingly promoted chondrocyte proliferation. However, miR-125b inhibition dramatically blocked this effect. TUNEL staining is shown in Fig. 5C. The miR-125b inhibitor
significantly blocked chondrocyte apoptosis when the cells were treated with IL-6. Conversely, HOTAIRM1-1 knockdown increased cell apoptosis, while the miR-125b inhibitor obviously reversed this effect (Fig. 5d). It is postulated that miR-125b competitively binds to HOTAIRM1-1 to promote chondrocyte apoptosis.
Discussion

Understanding the association of key lncRNAs with disease pathogenesis would help with disease diagnosis and prognosis. Emerging reports have demonstrated that the lncRNA HOTAIRM1-1 is an important lncRNA in several tissues and diseases, such as colorectal cancer and acute myeloid leukemia (20,21). Additionally, HOTAIRM1-1 is involved in the biological processes associated with OA (22). Presently, many studies on OA focus on cartilaginous tissues, since cartilage is a key component of joints. Therefore, cartilage damage is thought to be a landmark event during OA pathology progression (23,24). Some studies have reported that the proinflammatory cytokines IL-6 and IL-1β may play a significant role in the pathology of OA (25). IL-6 is a compound that is characterized by omnidirectional interactions in the processes that occur in the human body. IL-6 is considered a cytokine that strongly activates the immune system and enhances the inflammatory response, although considering some of its effects, IL-6 may be classified as an anti-inflammatory cytokine (26). The production of IL-6 in tissues of joints affected by OA usually occurs in response to IL-1β and tumor necrosis factor (TNF)α and IL-6 and is mainly produced by chondrocytes (27). A study by Zanotti and Canalis (27) showed that IL-6 mediates the induction of MMP13 expression through Notch and contributes to the inhibitory effect of Notch on the mRNA levels in cells of the chondrocyte lineage. Simsa-Maziel and Monsonego-Ornan (28) suggest that interleukin-1β promotes the proliferation and inhibits the differentiation of chondrocytes through a mechanism involving the downregulation of fibroblast growth factor receptor-3 and p21.

In the present study, an in vitro model was established to mimic OA pathogenesis in chondrocytes. The results demonstrated that the inflammatory mediators IL-6 and IL-1β suppressed HOTAIRM1-1 expression in chondrocytes.
In addition, the expression of miR-125b exhibited opposite changes, and its expression was increased after treatment with the inflammatory mediators (Fig. 1). Furthermore, the expression of HOTAIRM1-1 and miR-125b was investigated in cartilage samples from patients with OA and healthy subjects. It was found that the expression of HOTAIRM1-1 (downregulated) and miR-125b (upregulated) was inversely correlated in OA cartilage vs. healthy control cartilage (Fig. 2). The preliminary data suggest that miR-125b might stimulate the proliferation and apoptosis of chondrocytes through the development of osteoarthritis. In cells of the myeloid lineage, miR-125b represses interferon regulatory factor 4 and regulates inflammation by downregulating its direct target, TNFα (29). Nagpal et al (30) reported that miR-125b upregulation is critical for the TGF-β-induced fibroblast-to-myofibroblast transition, which implies a potential association between miR-125b and the TGF-β pathway. Zhen et al (31) demonstrated that inhibition of the TGF-β pathway in subchondral bone can attenuate OA progression. Moreover, in a bioinformatics study, the TNF signaling pathway was reported to be closely associated with HOX genes by interacting with HOX proteins (32). It was thus speculated that HOTAIRM1-1 and miR-125b physically interact with each other. The present study was performed to show an interaction between miR-125b and HOTAIRM1-1, and the results proved that HOTAIRM1-1 was a direct target of miR-125b (Fig. 3). Thus, HOTAIRM1-1 probably plays its role by directly targeting miR-125b, thus influencing chondrocytes in OA. As with many of the inflammatory cytokines whose levels increase with age, the precise consequences of dysregulated miR-125b expression on the functions of monocytes and naïve CD8 T cells, such as inflammatory states or altered migration, remain to be elucidated in OA.

Consistent with previous studies (25,31), HOTAIRM1-1 knockdown in chondrocytes promoted cell cycle-induced proliferation and cell apoptosis. ECM degradation is the main factor that changes during chondrocyte destruction in OA. MMP13 and IL-10 both play important roles in cartilage degradation (25,33,34). Aggrecan and type II collagen primarily contribute to the formation of cartilage tissue in the development of (35,36); the present study measured these markers when HOTAIRM1-1 was knocked down. MMP-13 and IL-10 expression was increased by downregulating HOTAIRM1-1 in chondrocytes. On the other hand, collagen II and aggrecan protein expression was decreased (Fig. 4). Thus, it was predicted that HOTAIRM1-1 may play a key role in the pathogenesis of OA. Recently, miR-125b was found to be stimulated as a possible consequence of the inhibitory role of the IL-6 receptor and activator of transcription 3 feedback loop (37,38). Rescue experiments demonstrated that the miR-125b inhibitor functionally reversed the impacts of HOTAIRM1-1 on cell proliferation and apoptosis in chondrocytes (Fig. 5). Furthermore, these results suggest that HOTAIRM1-1 downregulation may be required for cell proliferation and cell apoptosis in OA.

In conclusion, the present study indicates that the loss of HOTAIRM1-1 function leads to aberrant increase in the proliferation and apoptosis of chondrocytes and causes a comprehensive OA phenotype. miR-125b may be a potential downstream mechanism that regulates the function of HOTAIRM1-1, and this finding may provide a therapeutic strategy for OA.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
WBL contributed to the conception of the study and analyzed the data. GSL and PS performed the experiments and wrote the manuscript. YNL, FJZ and WBL analyzed the data and wrote the manuscript. YNL and WBL analyzed the data and provided constructive criticism. FJZ and PS confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The present study was approved by the human ethics committee of Tianjin Hospital (approval no. 2019-Yilunli-146). All the patients provided written informed consent and participated in the study according to their own will.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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