Long noncoding RNA KCNQ1OT1 targets miRNA let-7a-5p to regulate Osteoarthritis development and progression

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Abstract

Background Osteoarthritis (OA) is a common disease of the joints among old populace until today. The treatment possibilities and roles of miRNA and long non-coding RNA (lncRNA) in therapy of OA has previously been explored. However, the functional roles of Long noncoding RNA KCNQ1OT1 and miRNA let-7a-5p on Osteoarthritis development and progression remains unclear. This study aimed at investigating the influence of KCNQ1OT1 on let-7a-5p in moderation of OA development and advancement.

Materials and Methods RT-qPCR examined expression of KCNQ1OT1 and let-7a-5p in cultured human primary chondrocyte cell lines. Cell transfection overexpressed or knocked down the genes and CCK-8 assay measured cell viability in the proliferation biomarkers Ki87 and PCNA. While caspase-8 and caspase-3 activity determined rate of apoptosis. Furthermore, luciferase assay analyzed the luciferase activity and western blotting analysis determined the protein expression of KCNQ1OT1 and let-7a-5p in proliferation and apoptosis biomarkers.

Results The results demonstrated that KCNQ1OT1 is upregulated in OA-mimic cells and promotes the cell viability. KCNQ1OT1 knockdown suppresses cell viability of OA cells. Furthermore KCNQ1OT1 directly binds the 3'-UTR of let-7a-5p to negatively regulate let-7a-5p expression and OA progression. While upregulated let-7a-5p abolishes the proliferation effect of KCNQ1OT1 in OA cells.

Conclusion In summary, our study provides further insights into the underlying molecular mechanisms of KCNQ1OT1 and let-7a-5p suggesting a novel therapeutic approach to OA.

Keywords: KCNQ1OT1; let-7a-5p; Osteoarthritis; cell viability

Introduction

Osteoarthritis (OA) continues to be the prevalent disease of the human joints until today. It commonly affects about 10 to 18 percent of the population aged at least 60 years and a lot of times the hip and knee joints are the most commonly distressed joints [1]. Functional disability
as a consequence of major structural changes of the joint (i.e. progressive loss of articular cartilage) have been attributed to Osteoarthritis and it poses a huge burden on the quality of life [1-4]. Although it cannot be cured, advances in understanding and treatment have improved the outlook for people with this condition to reduce symptoms [5-7]. For example, diagnosis and treatment of osteoarthritis has been reported and treatment options classified as pharmacologic, non-pharmacologic, surgical, and complementary and/or alternative, typically used in combination to achieve optimal results [8]. The use of physical therapies, technical aids (walking sticks, etc.) and simple analgesics, opium alkaloids, and anti-inflammatory drugs have demonstrated effectiveness in controlling pain, improving physical function and quality of life and their use is clearly indicated in the treatment of osteoarthritis [9]. The existing evidence on the features related to the disease and its pathogenesis have been exhaustively reviewed based on clinical and surgical therapy with much stress on the recuperation development [10].

The therapeutic potential and role of miRNA; long non-coding RNA (IncRNA) and circular RNA (circRNA) in gene therapy of OA has been discussed including the effects on gene expression, inflammatory reaction, apoptosis and extracellular matrix in OA [11]. However the functional roles of let-7a-5p on Osteoarthritis development and progression remains unclear. Since let-7a-5p is lowly expressed and KCNQ1OT1 is highly expressed in disease cells our study investigates their interaction in OA. This study aimed at investigating the influence of KCNQ1OT1 on let-7a-5p in moderation of OA development and advancement. The outcome from this study can be used as a therapeutic approach to lessen the burden of OA.

**Materials and Methods**

This analytical study was conducted in Medicine, Unit-3, Lahore General Hospital / PGMI / AMC and IMBB, Lahore.

**Inflammation model in vitro for cell culture and transfection**

Human chondrocytes primary cultures preserved with IL-1β (10ng/mL) (a crucial pro-inflammatory cytokine associated with OA pathogenesis) were utilized in order to create an in vitro inflammation model. The model was adapted to our experiment following a description of the procedure. Specifically, $2.5 \times 10^4$ cells per cm$^2$ were planted in 24 well tissue dishes having biotechnological chondroitin (1%w/v) and/or chondroitin sulfate (1%w/v) with no any
supplement in control wells. Then, after 2 hours, IL-1β was supplemented at 10ng/mL in every well, excluding negative control wells (at best three per trial) and several wells were nurtured for 24 hours. Supernatants were gathered for cytokines multiplex analyses so as to assess cell reaction in various conditions of this in vitro inflammation assay. Likewise oxidative stress of cells was also assessed via gene expression measurements by RT-qPCR of the enzyme superoxide dismutase 2 (SOD-2) from cell extracts.

The KCNQ1OT1 and miRNA let-7a-5p mimics and corresponding negative control mimics were purchased from Guangzhou Fulengen Co. Ltd. Cell transfection of treated human chondrocytes cells was performed using Lipofectamine 3000 (Beyotime, Shanghai, China) to transfect KCNQ1OT1 and miRNA let-7a-5p mimics or inhibitors including negative control mimics following the manufacturer’s guidelines and transfection efficiency was analyzed after 48 hrs.

**Quantitative Reverse Transcription PCR assay**

Quantitative Real Time-PCR (qRT-PCR) assays were conducted using Beyofast™ SYBR Green QPCR Mix (Beyotime, China). The associated mRNA qRT-PCR detection kit (Beyotime, China) was used to measure quantities of KCNQ1OT1 and miRNA let-7a-5p gene expression for IL-1β treatment, respectively on an Applied Biosystems Vii7 RT-qPCR instrument (ABI, Vernon, CA, USA). Table 1 presents the primer sequences. U6 and GAPDH were normalized controls and the $2^{-\Delta\Delta CT}$ method was followed. All experiments were performed thrice.

**Table 1 Primer sequences**

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primers</th>
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<tr>
<td>KCNQ1OT1</td>
<td>CTGGCCCTGGAGATTATTTCA</td>
</tr>
<tr>
<td>KCNQ1OT1</td>
<td>AGCCCTAAAATGGAGGAATAGG</td>
</tr>
<tr>
<td>miRNA let-7a-5p</td>
<td>GGGGTGAGGTAGTAGGTTG</td>
</tr>
<tr>
<td>miRNA let-7a-5p</td>
<td>TCGTGTCGTGGAGTC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ACCCAGAAGACTGTGGATGG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TCAGCTCAGGGATGACCTTG</td>
</tr>
<tr>
<td>U6</td>
<td>ACAGAGAAGATTAGCATGGC</td>
</tr>
<tr>
<td>U6</td>
<td>TGGACCATTTCCTCATGTTG</td>
</tr>
</tbody>
</table>
**Western blotting assay**

Western blot was performed to evaluate the expression level of the proteins of cell cycle and apoptosis-related biomarkers, including Ki67, PCNA, caspase-3 and caspase-8. Human chondrocytes cells were exposed to IL-1β of for varying periods of time and then the proteins were extracted and washed twice with cold PBS and lysed in sample loading buffer that. The expression of the loading buffer was 1.5% SDS, 10% glycerol, 5 mM β-mercaptoethanol, bromophenol blue and 75 mM Tris (pH 7.0). Whole cell lysates were separated by SDS-PAGE of 12% gel and the proteins were moved onto a polyvinylidene fluoride membrane. Additionally, the membranes were nurtured and probed with the following antibodies: Ki67, PCNA, caspase-3 and caspase-8 enlisted in the Table 2 under the temperature of 4°C overnight. The immunoblots were established and seen by ECL Western blot medium (Thermo Fisher Scientific). U6 and GAPDH were used as internal control. The analysis of each group was repeated in three fold. The image J detection system was employed to determine the concentration of the bands.

**Table 2 Antibodies**

<table>
<thead>
<tr>
<th>Protein Examined</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCNA</td>
<td>Rabbit Polyclonal, (PA5-27214) (ThermoFisher,</td>
<td>Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed</td>
</tr>
<tr>
<td></td>
<td>Scientific, NY, US) diluted at 1:1000.</td>
<td>Secondary Antibody, Alexa Fluor Plus 488, (ThermoFisher,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Scientific, NY, US) diluted at 1:1000.</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Rabbit polyclonal to Ki67 (ab15580), (Abcam,</td>
<td>Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed</td>
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<tr>
<td></td>
<td>Cambridge, US) diluted at 1:900.</td>
<td>Secondary Antibody, Alexa Fluor Plus 647, (ThermoFisher,</td>
</tr>
<tr>
<td>caspas-3</td>
<td>Rabbit polyclonal to Caspase-3 (ab13847) (Abcam,</td>
<td>Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed</td>
</tr>
<tr>
<td></td>
<td>Cambridge, UK) diluted at 1:1000.</td>
<td>Secondary Antibody, Alexa Fluor Plus 488, (ThermoFisher,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Scientific, NY, US) diluted at 1:1000.</td>
</tr>
<tr>
<td>caspas-8</td>
<td>Rabbit polyclonal to Caspase-8 (ab25901),</td>
<td>Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Secondary Antibody,</td>
</tr>
<tr>
<td>Antibody/Reagent</td>
<td>Dilution Rate</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>--------------</td>
<td></td>
</tr>
<tr>
<td>GAPDH Mouse monoclonal (6C5) to GAPDH - Loading Control, (ab8245, Abcam, Cambridge, UK)</td>
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<td></td>
</tr>
<tr>
<td>Rabbit monoclonal (R18-2) Anti-Rat IgG Fc (ab125900, Abcam, Cambridge, UK)</td>
<td>1:1000.</td>
<td></td>
</tr>
</tbody>
</table>

**CCK-8 assay**

The transfected cells at a density of 4x10^3 per well were seeded into 96-well multiplying plates, and cultured between 0 to 48 hours. Cell proliferation was determined using Cell Counting Kit (#C0037, Beyotime), following standard protocol specified by manufacturer. The microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) was employed to quantify the wavelength absorbance at 450 nm.

**Bioinformatics analysis**

The target putative binding sites for KCNQ1OT1 and miRNA let-7a-5p were predicted using bioinformatics analyzing tool StarBase.

**Luciferase assay**

The target sequence let-7a-5p with the wild type (WT) or mutant type (MT) and KCNQ1OT1 binding sites were synthesized and replicated onto a pGL3 Dual luciferase Target Vector (Promega, Madison, WI, USA), to create Wild Type and Mutant Type let-7a-5p plasmids. These Wild Type or Mutant Type let-7a-5p plasmids were co-transfected into treated human chondrocytes cells along with NC mimics or KCNQ1OT1 mimic (SigmaAldrich; Merck KGaA) using Lipofectamine 6000 following manufacturer’s instructions. After 48 hours, luciferase assay was conducted with DualLuciferase Reporter Assay method (Promega), following protocol specified by the manufacturer.

**Statistical analysis**

The experiments were performed in triplicates and independently. The experimental data has been shown as mean and standard error (SE). The statistical evaluation was performed by GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS 18.0 version (SPSS...
Inc., Chicago, IL, USA). Student's t-test and ANOVA analysis were applied. The level of significance was P<0.05 to show a statistical significant difference.

Results

KCNQ1OT1 is upregulated in OA-mimic cells and promotes the cell viability

According to the RT-qPCR assay, the expression level of KCNQ1OT1 in OA IL-1β treated chondrocyte cell line was significantly increased with time (0h-72h) compared with the untreated cultured human primary chondrocyte cell line (Fig. 1A, *P< 0.05). However, the increase, improved with time and the highest level of expression was observed after 72 hours. Furthermore, following CCK-8 assay, results demonstrated that KCNQ1OT1 expression level in IL-1β treated chondrocyte cell line for OA was significantly increased with time (0h-72h) compared with the untreated cultured human primary chondrocyte cell line (Fig. 1B, *P< 0.05). However, the increase, improved with time and the highest level of expression was observed after 72 hours. To verify proliferation, RT-qPCR was performed on the proliferation biomarkers (Ki67 and PCNA). The results for Ki67 biomarker showed that the expression level of KCNQ1OT1 registered a significant increase with time (0h-72h) in the IL-1β treated chondrocyte cell line compared with the untreated cultured human primary chondrocyte cell line (Fig. 1C, *P< 0.05). However, the increase, improved with time and the highest level of expression was observed after 48 hours. For the PCNA biomarker, the expression level of KCNQ1OT1 registered a significant increase with time (0h-72h) in the IL-1β treated chondrocyte cell line compared with the untreated cultured human primary chondrocyte cell line (Fig. 1C, *P< 0.05). However, the increase, improved with time and the highest level of expression was observed after 72 hours. To verify apoptosis, the caspase-8 and caspase-3 activity assays were performed. The results for caspase-8, demonstrated that KCNQ1OT1 expression level in IL-1β treated chondrocyte cell line for OA was significantly decreased with time (0h-72h) compared with the untreated cultured human primary chondrocyte cell line (Fig. 1D, *P< 0.05). However, the decrease, improved with time and the lowest level of expression was observed after 72 hours. The results for caspase-3, demonstrated that KCNQ1OT1 expression level in IL-1β treated chondrocyte cell line for OA was significantly decreased with time (0h-72h) compared with the untreated cultured human primary chondrocyte cell line (Fig. 1D, *P< 0.05). However, the decrease, improved with time and the lowest level of expression was observed after 72 hours. The IL-1β treated chondrocyte
cell line was adopted for further experiments. In general these outcomes signified traits of upregulated KCNQ1OT1 expression in OA-mimic cells and enhanced cell viability and a notable pathological apoptosis.

Figure 1 KCNQ1OT1 is upregulated in OA-mimic cells and promotes the cell viability. A) RT-qPCR examined KCNQ1OT1 expression in human chondrocytes cells (untreated and IL-1β treated chondrocyte cell lines) at varying time periods (p<0.05). B) CCK8 examined cell viability based on KCNQ1OT1 expression in human chondrocytes cells (untreated and IL-1β treated chondrocyte cell lines) at varying time periods (p<0.05). C) RT-qPCR examined expression of proliferation biomarkers Ki67 and PCNA in untreated and IL-1β treated chondrocyte cell lines at varying time periods (p<0.05). D) RT-qPCR examined expression of apoptosis biomarkers caspase-8 and caspase-3 in untreated and IL-1β treated chondrocyte cell lines at varying time periods (p<0.05).

KCNQ1OT1 knockdown suppresses cell viability of OA cells

The influence of KCNQ1OT1 on IL-1β preserved chondrocyte cell viability was analyzed with CCK8 assays. At first, the IL-1β treated chondrocyte cells were transfected with control mimics.
or KCNQ1OT1-inhibitor at varying times (12h-48). Then, RT-qPCR was performed to evaluate the KCNQ1OT1 knockdown efficacy. The results showed that KCNQ1OT1 expression level was significantly down regulated for the KCNQ1OT1-inhibitor compared with control mimics in all the varied times (12h-48h) (Fig. 2A, P<0.05). Furthermore, the influence of KCNQ1OT1 on cell viability was determined by CCK-8 assay in IL-1β treated chondrocyte cell line in which observations were done at varying times from 12h-48. The results after 12h showed low cell viability in the KCNQ1OT1-inhibitor compared with control mimics (Fig. 2B, P<0.05). Thereafter, another observation was done after 24h has elapsed and results showed a significantly lower cell viability in the KCNQ1OT1-inhibitor compared with control mimics (Fig. 2C, *P<0.05). Next, after 48h the cell viability was significantly the lowest in the KCNQ1OT1-inhibitor compared with control mimics (Fig. 2D, **P<0.05). These results implied that down regulated KCNQ1OT1 expression inhibited cell viability of OA cells.

Figure 2 KCNQ1OT1 knockdown suppresses cell viability of OA cells. A) RT-qPCR examined detected KCNQ1OT1 knockdown efficiency at different time periods in IL-1β treated chondrocyte cell lines. B) CCK8 examined cell viability based on knocked down KCNQ1OT1 expression in IL-1β treated chondrocyte cell lines after 12h (p<0.05). C) CCK8 examined cell viability based on knocked down KCNQ1OT1 expression in IL-1β treated chondrocyte cell lines after 24h (p<0.05). D) CCK8 examined cell viability based on knocked down KCNQ1OT1 expression in IL-1β treated chondrocyte cell lines after 48h (p<0.05).
KCNQ1OT1 directly binds the 3'-UTR of let-7a-5p to negatively regulate its expression and OA progression

To confirm the assertion that KCNQ1OT1 might directly interact with let-7a-5p to regulate OA progression, bioinformatics analysis was performed using Starbase. It was found that there were matching pairing sequences between KCNQ1OT1 and 3’UTR of let-7a-5p (Fig. 3A). Thereafter, luciferase reporter vector having Wild Type or Mutant Type KCNQ1OT1 bonding locations in let-7a-5p were constructed in order to verify the connection. Subsequently, control mimics or KCNQ1OT1 mimics were transfected to Wild Type or Mutant Type to verify the luciferase activity. The luciferase activity results indicated remarkable increased luciferase activity of WT let-7a-5p 3’UTR for KCNQ1OT1 mimic transfected cells while decreased luciferase activity was observed in cells transfected with control mimics (Fig. 3B, P<0.05). However, no influence was noticed in the luciferase activity of MT- let-7a-5p (Fig. 3B, P<0.05). Then, RT-qPCR examined let-7a-5p expression. The results as determined by Real Time-qPCR showed decreased expression of let-7a-5p in IL-1β treated chondrocyte cell line compared with untreated cultured human primary chondrocyte cells (Fig. 3C, P<0.05). Additionally, the expression of let-7a-5p in chondrocyte cell lines was observed at varying times (12h-48h) by performing the RT-qPCR assay. The results indicated a significant decrease in let-7a-5p expression in IL-1β preserved chondrocyte cell line compared with untreated cultured human primary chondrocyte cells (Fig. 3C, P<0.05). However, the decrease, increased with time and the lowest expression was observed after 48h. These results suggested that let-7a-5p is directly targeted by KCNQ1OT1 to regulate OA progression.
Figure 3  KCNQ1OT1 directly binds the 3’UTR of let-7a-5p to negatively regulate its expression and OA progression. A) Bioinformatics analysis tools (StrarBase) produced target putative binding sites for KCNQ1OT1 and let-7a-5p. B) Dual luciferase reporter confirmed the actual binding sites in WT and MUT let-7a-5p luciferase activity after transfection with KCNQ1OT1 mimic. C) RT-qPCR examined let-7a-5p expression in human chondrocytes cells (untreated and IL-1β treated chondrocyte cell lines) (p<0.05). D) RT-qPCR examined let-7a-5p expression in human chondrocytes cells (untreated and IL-1β treated chondrocyte cell lines) at varying time periods (p<0.05).

Upregulated let-7a-5p abolishes the proliferation effect of KCNQ1OT1 in OA cells

In the above sections the expression of KCNQ1OT1 was found to significantly upregulated after 48h in OA IL-1β treated chondrocyte cell line. It was also discovered that let-7a-5p expression is significantly knocked down after 48h in OA IL-1β treated chondrocyte cell line. Therefore in this section the IL-1β treated chondrocyte cell line (48h) exposed model was adopted. Moreover, the underlying molecular mechanisms between let-7a-5p and KCNQ1OT1 were explored by performing CCK-8 assay on cellular viability. Firstly, transfection was performed with KCNQ1OT1-NC, KCNQ1OT1 mimic +sh-control or KCNQ1OT1 mimic+oe let-7a-5p into the IL-1β treated chondrocyte cell line after 48h. This was followed by RT-qPCR to examine let-7a-
5p expression. The results demonstrated declined let-7a-5p expression in KCNQ1OT1 mimic +sh-control group compared to the KCNQ1OT1-NC and KCNQ1OT1 mimic+oe let-7a-5p groups (Fig. 4A, P<0.05). In addition, CCK-8 assay results demonstrated significant reduced cell viability in both KCNQ1OT1 silenced (KCNQ1OT1 mimic +sh-control) and let-7a-5p overexpressed (KCNQ1OT1 mimic+oe let-7a-5p) transfected groups compared to the control (KCNQ1OT1-NC) groups (Fig. 4B, P<0.05). The proliferation biomarkers confirmed the results for both Ki67 and PCNA after performing the RT-qPCR assay. The results showed let-7a-5p expression was significantly reduced in both KCNQ1OT1 silenced (KCNQ1OT1 mimic +sh-control) and let-7a-5p overexpressed (KCNQ1OT1 mimic+oe let-7a-5p) transfected groups compared to the control (KCNQ1OT1-NC) groups for both Ki67 and PCNA (Fig. 4C, P<0.05). The apoptosis biomarkers confirmed the results for both caspase-8 and caspase-3 after performing the RT-qPCR. The outcomes demonstrated significantly increased let-7a-5p expression in both KCNQ1OT1 silenced (KCNQ1OT1 mimic +sh-control) and let-7a-5p overexpressed (KCNQ1OT1 mimic+oe let-7a-5p) transfected groups compared to the control (KCNQ1OT1-NC) groups for both caspase-8 and caspase-3 (Fig. 4D, P<0.05).
**Figure 4** Upregulated let-7a-5p abolishes the proliferation effect of KCNQ1OT1 in OA cells. A) RT-qPCR examined relative expression of let-7a-5p in IL-1β treated chondrocyte cells after 48h transfected with either KCNQ1OT1-NC, KCNQ1OT1 mimic +sh-control or KCNQ1OT1 mimic+oe let-7a-5p (p<0.05). B) CCK8 examined cell viability in IL-1β treated chondrocyte cells after 48h transfected with either KCNQ1OT1-NC, KCNQ1OT1 mimic +sh-control or KCNQ1OT1 mimic+oe let-7a-5p (p<0.05). C) RT-qPCR examined relative expression of Ki67 and PCNA in IL-1β treated chondrocyte cells after 48h transfected with either KCNQ1OT1-NC.

**Discussion**

OA is one of the most prevalent joint diseases amid senior civilians, which is typified by degenerative adjustment of cartilage [12]. Recent studies established that irregular expression of miRNAs was essential in the development of OA, for instance, intra-articular dose of miRNA-140 alleviated osteoarthritis (OA) development via regulation of externa cell matrix (ECM) homeostasis in rats [13]. miR-20a regulated inflammatory in osteoarthritis by targeting the IkBβ and regulated NK-κB signaling pathway activation [14]. Additionally, long non coding RNAs have also been reported to be crucial in development OA, for example, LncRNA MALAT1 promoted osteoarthritis by modulating miR-150-5p/AKT3 axis. LncRNA PVT1 regulated chondrocyte apoptosis in Osteoarthritis by acting as a sponge for miR-488-3p [15]. LncRNA MIR4435-2HG was found to be downregulated in osteoarthritis and regulated chondrocyte cell proliferation and apoptosis. LncRNA SNHG1 alleviated IL-1β-induced Osteoarthritis by inhibiting miR-16-5p-mediated p38 MAPK and NF-κB signaling pathways.
However aberrant expressions of KCNQ1OT1 have been stated in several types of human cancers. For instance, silencing of long non-coding RNA KCNQ1OT1 suppressed chemo-resistance to paclitaxel in lung adenocarcinoma [16].

Let-7a-5p is a miRNA that has been reported to be involved in regulating the expression of tumor suppressors and oncogenes in various diseases.

**Conclusion**

In summary, our study provides further insights into the underlying molecular mechanisms of KCNQ1OT1 and let-7a-5p suggesting a novel therapeutic approach to OA

**Reference**

