LncRNA LOXL1-AS inhibits proliferation of PDLSCs and downregulates IL-1β in periodontitis patients

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Research

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Abstract

Background: Oncogenic role of lncRNA LOXL1-AS has been characterized in several types of cancer. Our preliminary deep sequencing data revealed the downregulation of LOXL1-AS in periodontitis and its inverse correlation with IL-1β, a critical inflammatory mediator in periodontitis. This study was therefore performed to investigate the potential interaction between LOXL1-AS and IL-1β in periodontitis.

Methods: Research subjects of this study included 30 periodontitis patients and 30 healthy controls. Cell transfection, RT-qPCR, luciferase activity assay and western blots were performed in this study.

Results: LOXL1-AS was downregulated in periodontitis-affected periodontal ligament stem cells (PDLSCs) than that in healthy PDLSCs. While IL-1β was upregulated in periodontitis-affected PDLSCs and was inversely correlated with LOXL1-AS. Overexpression of LOXL1-AS mediated the downregulation of IL-1β in PDLSCs. IL-1β treatment did not affect the expression of LOXL1-AS. Moreover, overexpression of LOXL1-AS led to inhibited proliferation of periodontitis-affected PDLSCs.

Conclusion: This study is the first to report the downregulation of LOXL1-AS in periodontitis. Moreover, we also propose that LOXL1-AS may inhibit the proliferation of periodontitis-affected PDLSCs and downregulate IL-1β to improve periodontitis.

Background

Periodontitis, also known as gum disease, is a common type of gum infection that is caused by bacterial infection and can damage the bone structure that supports the teeth [1]. Periodontitis is a heavy burden on public health [2]. It is estimated that severe periodontitis threat more than 11% adults worldwide and the development of periodontitis can seriously affect self-esteem, speaking ability, quality of life and nutrition conditions [3]. Gingivitis management and dietary antioxidants are the primary preventative approaches for periodontitis [4]. However, the effective treatment of periodontitis requires an intensive intervention of patients' lifestyle, such as quit smoking and alcohol abstinence [5], which is practical in many cases. Therefore, novel therapeutic treatments are urgently needed.

Previous studies on the molecular pathogenesis of periodontitis have revealed a considerable number of genetic factors involved in the occurrence, development and progression of this disease [6-8]. The functional characterization of the key genetic players may lead to the development of novel targeted therapies [9]. MicroRNAs (miRNAs) regulate the expression of diverse target genes and play crucial roles in the development, as well as suppression of different kinds of cancer. MiR-203 was reported to suppress the growth and stemness of breast cancer via targeting cytokine signaling 3 (SOCS3) [10]. Besides mi-RNA, the progression of many diseases including periodontitis also requires the involvement of long (> 200 nt) non-coding RNAs (lncRNAs) [11, 12], which regulate gene expression to participate in diverse biological processes [13]. LncRNA LOXL1-AS is a newly identified critical player in thoracic aortic aneurysm [14]. And the role of LOXL1-AS in the pathogenesis of periodontitis is unknown. Our present data revealed the downregulation of LOXL1-AS in periodontitis and its inverse correlation with IL-1β,

which is a critical inflammatory mediator and unexpectedly elevated in chronic periodontitis [15]. In order to extend the existing investigation on the role of LOXL1-AS and link the inflammation with periodontitis, this study was then carried out to investigate the potential effects of LOXL1-AS and IL-1β on periodontitis.

Methods

Research subjects

This study was approved by the Ethics Committee of Shanghai Yangpu Shidong Hospital. All experiments on human subjects were conducted in accordance with the Declaration of Helsinki (http://www.wma.net). Research subjects of this study included 30 periodontitis patients (18 males and 12 females, age range of 34 to 44 years old, mean age of 39.3 ± 2.1 years old) and 30 healthy controls (18 males and 12 females, age range of 33 to 44 years, mean age of 39.2 ± 2.0 years old) who were admitted at aforementioned hospital between June 2017 and June 2019. Patients' inclusion criteria: 1) newly diagnosed cases; 2) no therapies for any diseases were initiated within 3 months before this study; 3) no other systemic diseases diagnosed; 4) the patients received tooth extraction at aforementioned hospital. Exclusion criteria: 1) recurrent cases; 2) multiple chronic diseases were diagnosed. The 30 patients underwent tooth extraction due to chronic periodontitis, while the 30 healthy controls received extraction during orthodontic treatment. All patients were informed of the experimental principle of this project and signed the informed consent.

Cell culture

Periodontal ligament (PDL) tissues were extracted from one tooth of each patient and healthy controls during tooth extraction. PDL was used to make small pieces and were kept in minimal essential medium supplemented with 0.292 mg/mL glutamine, 100 μM/l ascorbic acid, 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin. Insolation of periodontal ligament stem cells (PDLSCs) was performed according to the methods described by Li et al [16].

Vectors and cell transfection

Two cases of periodontitis affected-PDLSCs with average expression of LOXL1-AS was selected to do transfection experiments. LOXL1-AS expression vector was constructed using pcDNA3.1 vector (Invitrogen) as backbone. Small interfering RNAs targeting the human IL-1β gene were designed by the Shanghai GeneChem Co., Ltd., China. The optimal sequence of small interfering RNAs against human IL-1β (5'-TCAAAGGAAAGAATCTATA-3') was then cloned into the plasmid pGCL-GFP. IL-1β lentivirus shRNA was purchased from Shanghai GeneChem Co., Ltd., China. The negative control lentivirus shRNA was constructed by a similar process (5'-TTCTCCGAACGTGTCACGT-3'). PDLSCs were harvested at 75 - 85% confluence and were counted, followed by the transfection of 10 nM vector into 10^6 cells using lipofectamine 3000 (Thermo Fisher Scientific). Control (C) cells were untransfected cells. Negative control (NC) cells were transfected with empty pcDNA3.1 vector. The following experiments were performed using cells harvested at 48 h post-transfection.

RNA preparations and RT-qPCR

RNeasy Mini Kit (QIAGEN) was used to extract total RNAs from PDLSCs. LookOut® DNA Erase (Sigma-Aldrich) was used to digest all RNA samples to remove genomic DNAs. In cases of IL-1β treatment, PDLSCs were treated with 0, 10 and 50 ng/ml IL-1β for 48 h before the following RNA extractions. NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific) was applied to measure the concentration of RNA samples. With 1000 ng total RNAs as template, RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific) was used to perform reverse transcriptions to synthesize cDNA. With cDNA samples as template, QuantiTect SYBR Green PCR Kit (Qiagen) was used to prepare all qPCR reaction systems. The expression levels of LOXL1-AS and IL-1β mRNA were measured with GAPDH as endogenous control. All PCR reactions were repeated 3 times. Fold changes of gene expression levels of LOXL1-AS and IL-1β mRNA were calculated using $2^{-\Delta\Delta CT}$ method. The primers sequences were as follows: forward: 5'-TTCCCATTTACCTGCCCGAAG-3' (forward) and 5'-GTCAGCAAACACATGGCAAC-3' (reverse) for LOXL1-AS; 5'-ATAAGCCCACTCTACACCT-3' (forward) and 5'-ATTGGCCCTGAAAGGAGAGA-3' (reverse) for IL-1β; 5'- ATGGGTGTGAACCATGAGAA-3' (forward) and 5'- GTGCTAAGCAGTTGGTGGTG-3' (reverse) for GAPDH.**Cell proliferation assay**

PDLSCs were harvested at 48 h post-transfection and CCK-8 assay was performed to analyze the effects of transfection or infection on the proliferation of PDLSCs. Each well of one 96-well plate was filled with 10^4 PDLSCs in 0.1 ml cell suspension, followed by cell culture at 37 °C in a 5 % CO_2 incubator. Three replicate wells were set for each experiment. CCK-8 solution (Sigma-Aldrich) was added into each well to reach the final concentration of 10% at 4 h before the end of cell culture. Cells were harvested every 24 h until 96 h. OD values of cell culture medium were measured at 450 nm.

Western blots analysis

PDLSCs were harvested at 48 h post-transfection and RIPA solution (Sigma-Aldrich) was used to resuspend cell pellets containing 10^5 PDLSCs to extract total proteins. BCA assay (Sigma-Aldrich) was performed to measure protein concentrations. RNA samples were incubated in boiling water for 10 min to reach protein denaturation. Electrophoresis was then conducted using 10 % SDS-PAGE gel to separate protein molecules, followed by gel transfer to PVDF membranes. After blocking in TBST containing 5% skim milk, the membranes were incubated with rabbit primary antibodies of GAPDH (ab9845, Abcam) and IL-1β (ab9722, Abcam) at 4 °C for 18 h, followed by incubation with secondary antibody of HRP Goat Anti-Rabbit (IgG) (ab6721, Abcam) at 24 °C for 2 h. Signals were produced using ECL (Sigma-Aldrich, USA) and data were processed using Quantity One software.

Statistical analysis

Three biological replicates were included in each experiment. Mean values of 3 replicates were calculated and were used for all data analysis. Unpaired t-test was used to explore differences between two groups. Differences among multiple groups were explored by ANOVA (one-way), followed by Tukey's post-hoc

test. Correlations were analyzed by Pearson's correlation coefficient. $P < 0.05$ was considered as statistically significant.

Results

The expression of LOXL1-AS and IL-1β mRNA were altered in periodontitis-affected PDLSCs

The differential expression of LOXL1-AS and IL-1β mRNA in 30 cases of periodontitis-affected PDLSCs and 30 cases of control PDLSCs was detected by qPCR. It was shown that both LOXL1-AS and IL-1β were dysregulated in periodontitis patients. Specifically, LOXL1-AS was significantly downregulated in periodontitis group relative to healthy controls. (Fig. 1A, control: mean: 4.175, 95% CI: [3.220, 4.990]; periodontitis: mean: 2.205, 95% CI: [1.960, 2.280], $p < 0.001$). In contrast, the expression of IL-1β was remarkably upregulated in periodontitis group than that in the control group (Fig. 1B, control: mean: 2.227, 95% CI: [1.720, 2.450]; periodontitis: mean: 4.379, 95% CI: [3.720, 4.820], $p < 0.001$), which suggested that dysregulation of LOXL1-AS and IL-1β might be two pathological factors in the formation or development of periodontitis.

The expression of LOXL1-AS and IL-1β were significantly and inversely correlated across periodontitis-affected PDLSCs

Linear regression was used to analyze the correlation between the expression levels of LOXL1-AS and IL-1β mRNA across 30 cases of periodontitis-affected PDLSCs and 30 cases of control PDLSCs. It was observed that the expression levels of LOXL1-AS and IL-1β mRNA were significantly and inversely correlated in periodontitis group (Fig. 2A). By contrast, there was no significant correlation between the expression of LOXL1-AS and IL-1β in healthy group (Fig. 2B), which suggested that the relationship between the expressions of LOXL1-AS and IL-1β was more indicatable in periodontitis condition but not in normal condition.

Overexpression of LOXL1-AS mediated the downregulation of IL-1β

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Overexpression of LOXL1-AS or suppression of IL-1β led to inhibited proliferation of periodontitis-affected PDLSCs

The promoted proliferation of PDLSCs in periodontitis has been demonstrated in previous literature[17]. Also, as we found the downregulated expression of LOXL1-AS in periodontitis patient, whether LOXL1-AS had any effects on the proliferation of PDLSCs was investigated. CCK-8 assay was performed to analyze

the effects of overexpression of LOXL1-AS on the proliferation of 2 cases of periodontitis-affected PDLSCs. Compared to C and NC groups, overexpression of LOXL1-AS led to significantly inhibited cell proliferation (Fig. 4, $p < 0.05$). In order to investigate the effects of IL-1β on the proliferation of periodontitis-affected PDLSCs, endogenous IL-1β was knocked down (Fig. 5A, $p < 0.05$). Indeed, suppression of IL-1β could also inhibit the proliferation of periodontitis-affected PDLSCs (Fig. 5B, $p < 0.05$), suggesting that inflammation might play negative roles in the pathogenesis of periodontitis. Overall, the inhibitory effects of LOXL1-AS on the proliferation of PDLSCs with periodontitis indicates upregulation of LOXL1-AS might be an effective approach on the therapy of periodontitis.

Discussion

In this study, we investigated the function of LOXL1-AS in periodontitis. We found that LOXL1-AS was downregulated in periodontitis and might regulate the expression of IL-1β and the proliferation of PDLSCs to participate in periodontitis.

The expression pattern and function of LOXL1-AS have only been investigated in thoracic aortic aneurysm [14]. The expression of LOXL1-AS is significantly upregulated in thoracic aortic aneurysm and can induce aortic smooth muscle cell apoptosis and promote proliferation to participate in the pathogenesis of this disease [14]. A previous study proposed that LOXL1-AS might play different roles in regulating proliferation of different cell types [17], in which it was also shown that the abnormally increased proliferation rate of PDLSCs is involved in the development of periodontitis [17]. Our study also focused on the regulatory functions of LOXL1-AS in periodontitis condition. In this study, we firstly found that the expression levels of LOXL1-AS was decreased in periodontitis patients, which led us to infer that LOXL1-AS might be a positive and even a protective factor for periodontitis. Therefore, we overexpressed LOXL1-AS in periodontitis affected-PDLSCs to evaluate cell proliferation. Interestingly, LOXL1-AS could significantly inhibit periodontitis affected-PDLSCs proliferation in both two cases, indicating that LOXL1-AS is a potential positive regulator in periodontitis at least in the aspect of inhibiting cell proliferation. Therefore, upregulating the expression of LOXL1-AS might serve as a therapeutic approach for periodontitis. And inhibiting proliferation of PDLSCs by overexpressing LOXL1-AS might benefit the recovery from periodontitis.

Periodontitis is an inflammatory disease. The production of some pro-inflammatory factors, such as IL-1β, is increased in periodontitis patients to promote the inflammatory responses [18]. Consistently, our study also showed the upregulation of IL-1β in periodontitis-affected PDLSCs compared to control PDLSCs. Besides, our study reveals that upregulation of LOXL1-AS can inhibit the expression of IL-1β, which further confirm our inference that LOXL1-AS is a positive regulator for periodontitis. However, the mechanism behind this modulation is still unclear and need to be explored. In our study, it was observed that the expression levels of LOXL1-AS and IL-1β mRNA were significantly and negatively correlated only across periodontitis-affected PDLSCs but not control PDLSCs, which suggests that a certain pathological mediator might participate in the regulation of IL-1β by LOXL1-AS in periodontitis. Therefore, further studies are required to verify our inference. As mentioned above, the expression levels of IL-1β are

increased in periodontitis patients to promote the inflammatory responses. Therefore, other two cases of periodontitis-affected PDLSCs with average expression levels of IL-1β were selected to conduct the knockdown of IL-1β and evaluate the effect of IL-1β on periodontitis-affected PDLSCs proliferation. As expected, downregulation of IL-1β could significantly reduce the cell proliferation rate, which suggests that inflammation might be a pessimistic factor for periodontitis, and alleviating inflammatory response would be an effective approach for periodontitis therapy. The limitations of our study are that only two cases of periodontitis-affected PDLSCs with average expression levels of LOXL1-AS were used for transfection to assess the effects of LOXL1-AS on the expression of IL-1β and cell proliferation, and more cases are needed in further research. Moreover, why IL-1β can be regulated by LOXL1-AS is still unknown, and more efforts are needed to explore the behind mechanism. Lastly, the evidence for LOXL1-AS to participate in other pathological processes of periodontitis is still required.

Conclusions

This study is the first to report the downregulation of LOXL1-AS in periodontitis. As a positive regulator, LOXL1-AS inhibits the expression of inflammation factor IL-1β and suppresses the proliferation of PDLSCs, Accordingly, the mechanism of this research was described as the following scheme (Fig. 6).

Abbreviations

PDLSCs: periodontitis-affected periodontal ligament stem cells

FBS: fetal bovine serum

NC: Negative control

Declarations

Acknowledgements

Not applicable.

Authors' contributions

TZ: experiment studies, clinical studies, Data collection and Manuscript writing; JTH, LLY, and XXL: clinical studies, Data collection and Manuscript writing; LKX and JHW: experiment studies and clinical studies; BB: Project development, data analysis, manuscript editing, and literature research. All contributing authors have read and agreed to the final version of the manuscript.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by Shanghai Yangpu Shidong Hospital Ethics Committee and all the patients or parents/ guardians of patients provided written informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

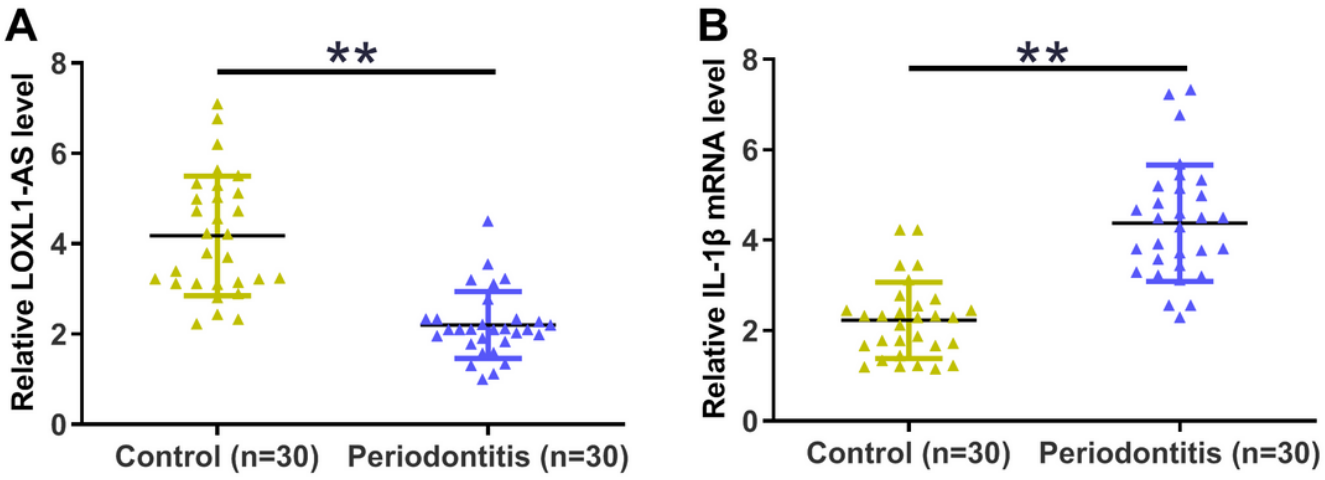

Figure 1

Expressions of LOXL1-AS and IL-1β mRNA were both altered in periodontitis-affected PDLSCs. The differential expressions of LOXL1-AS (A) and IL-1β mRNA (B) in 30 cases periodontitis-affected PDLSCs and 30 cases of control PDLSCs was analyzed by performing qPCR. PCR reactions were repeated 3 times and mean values were presented. *, $p < 0.05$.

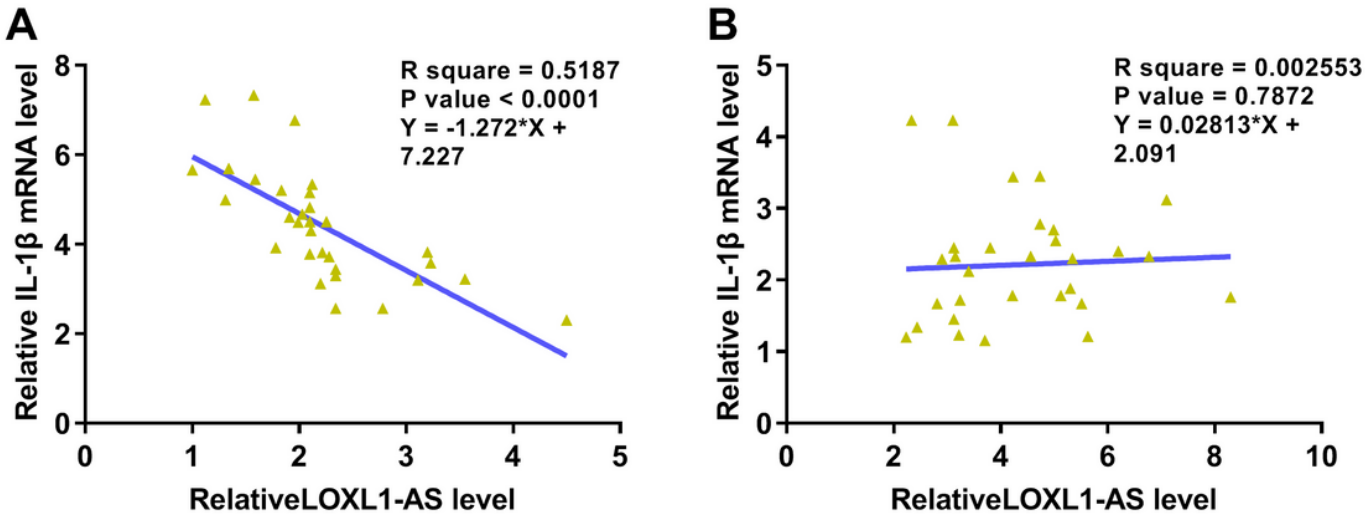

Figure 2

Expression levels of LOXL1-AS and IL-1β mRNA were significantly and inversely correlated across periodontitis-affected PDLSCs. Linear regression was used to analyze the correlation between LOXL1-AS mRNA level and IL-1β mRNA level across 30 cases of periodontitis-affected PDLSCs (A) and 30 cases of control PDLSCs (B). The expressions of LOXL1-AS and IL-1β were negatively correlated in periodontitis patients but not healthy controls.

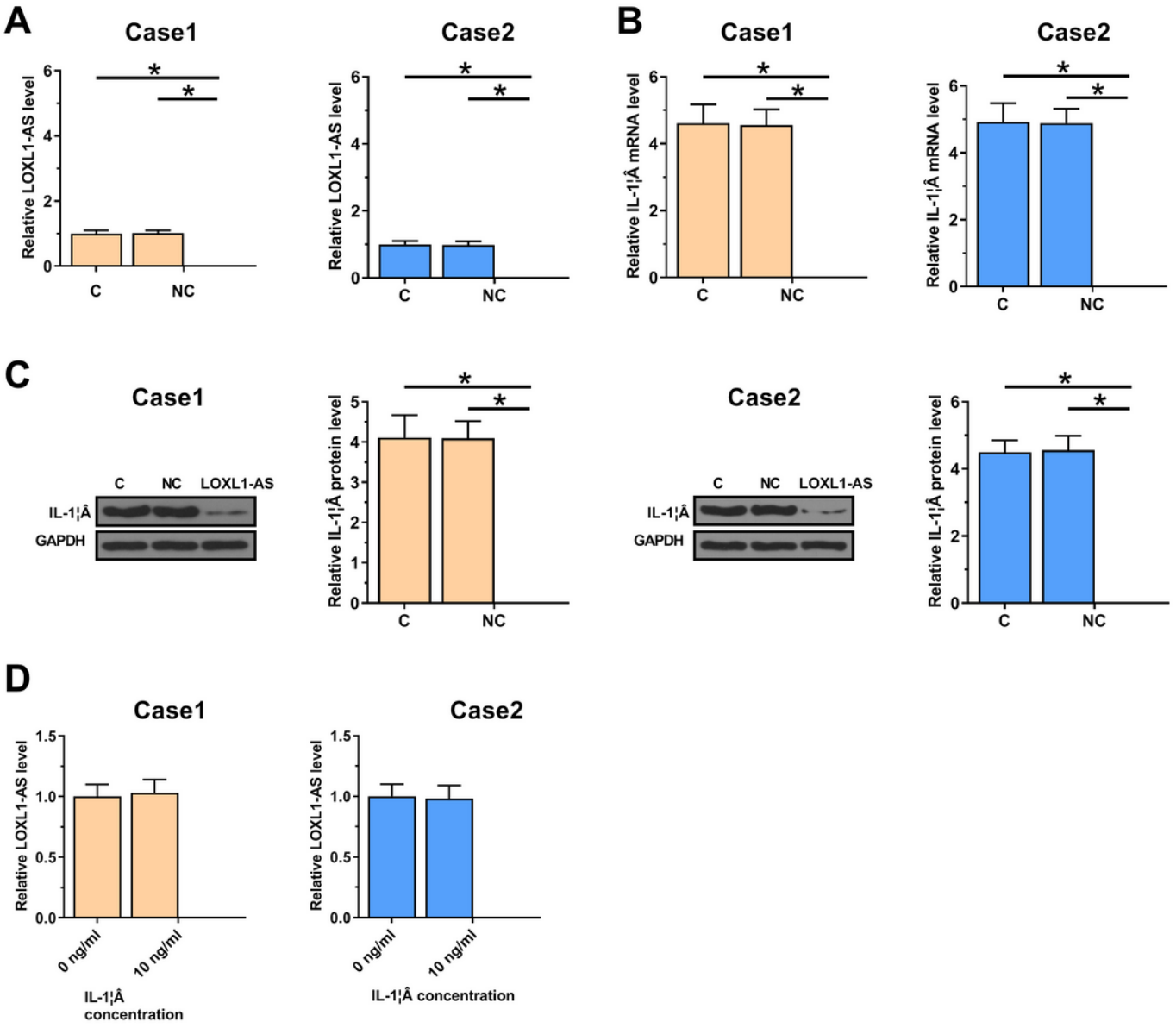

Figure 3

Overexpression of LOXL1-AS mediated the downregulation of IL-1β. Two cases of periodontitis-affected PDLSCs with average LOXL1-AS expression were transfected with LOXL1-AS overexpression vector and the mRNA level of LOXL1-AS was confirmed by qPCR at 48 h post-transfection (A). The effects of LOXL1-AS elevation the expression of IL-1β at both mRNA (B) and protein (C) levels were measured by qPCR and western blots. PDLSCs were treated with 0, 10 and 50 ng/ml IL-1β for 48 h, followed by the measurement of the expression levels of LOXL1-AS, and no alteration was observed in the effect of IL-1β on LOXL1-AS expression (D). Experiments were repeated 3 times and mean values were presented. *, $p < 0.05$.

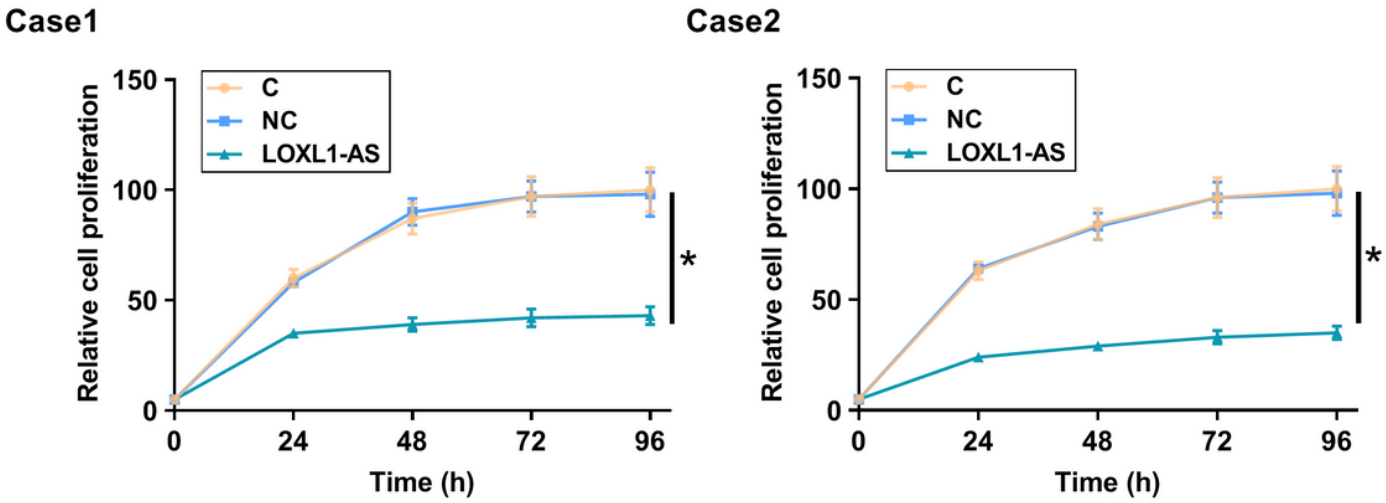

Figure 4

Overexpression of LOXL1-AS led to inhibited proliferation of periodontitis-affected PDLSCs. CCK-8 assay was performed to analyze the effects of LOXL1-AS overexpression on the cell proliferation of 2 cases of periodontitis-affected PDLSCs owing average LOXL1-AS expression. Increasing LOXL1-AS level could reduce the proliferation rate of periodontitis-affected PDLSCs. Experiments were repeated 3 times and mean values were presented. *, $p < 0.05$.

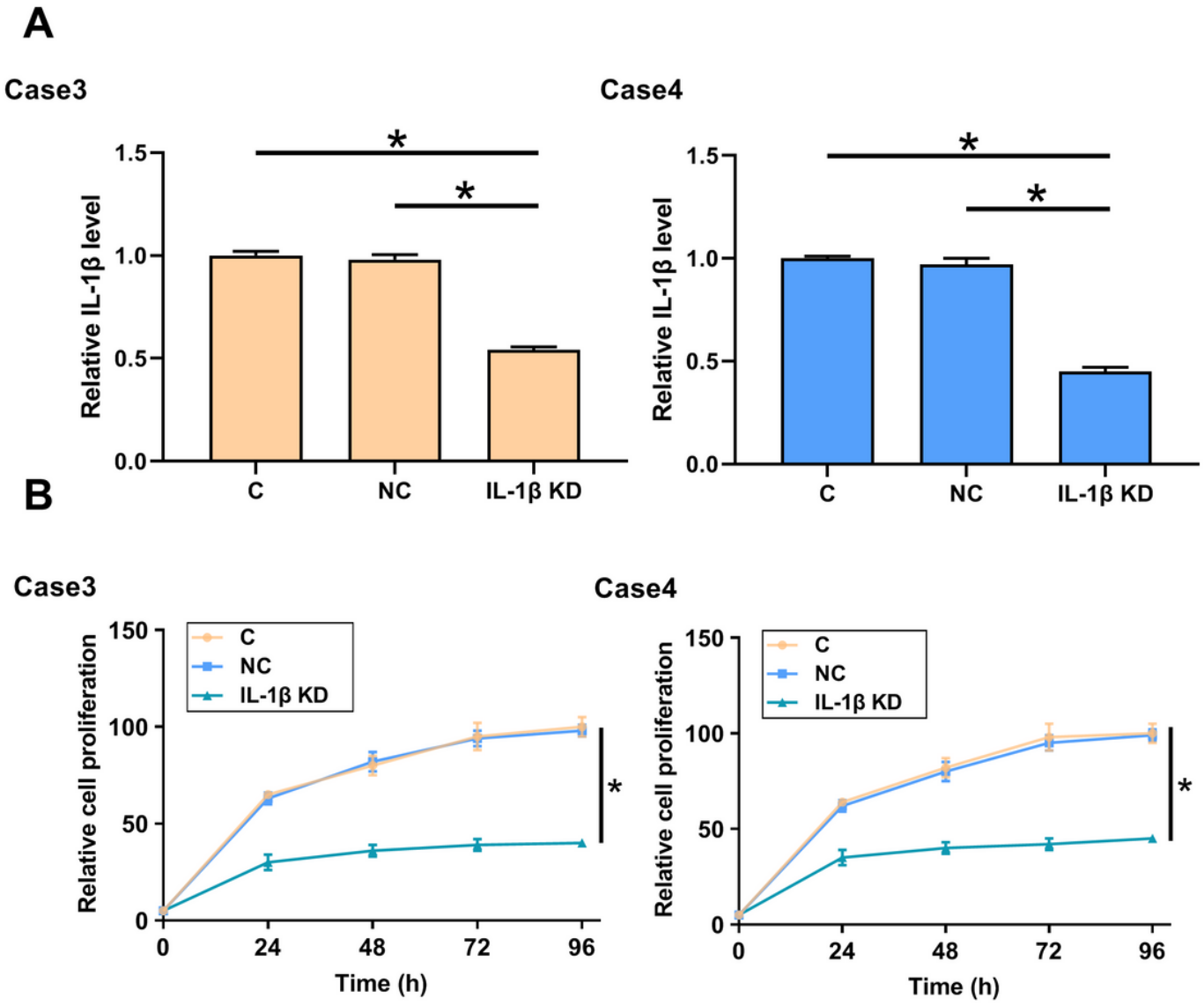

Figure 5

IL-1β knockdown resulted in the inhibited proliferation of periodontitis-affected PDLSCs. CCK-8 assay was performed to analyze the effects of IL-1β depression on the cell proliferation of periodontitis-affected PDLSCs with average IL-1β expression. Other 2 cases of PDLSCs with periodontitis was isolated and used to evaluate the effects of IL-1β knockdown on cell proliferation, and interfering endogenous IL-1β could also drive down the proliferation rate of periodontitis-affected PDLSCs. Experiments were repeated 3 times and mean values were presented. *, $p < 0.05$.

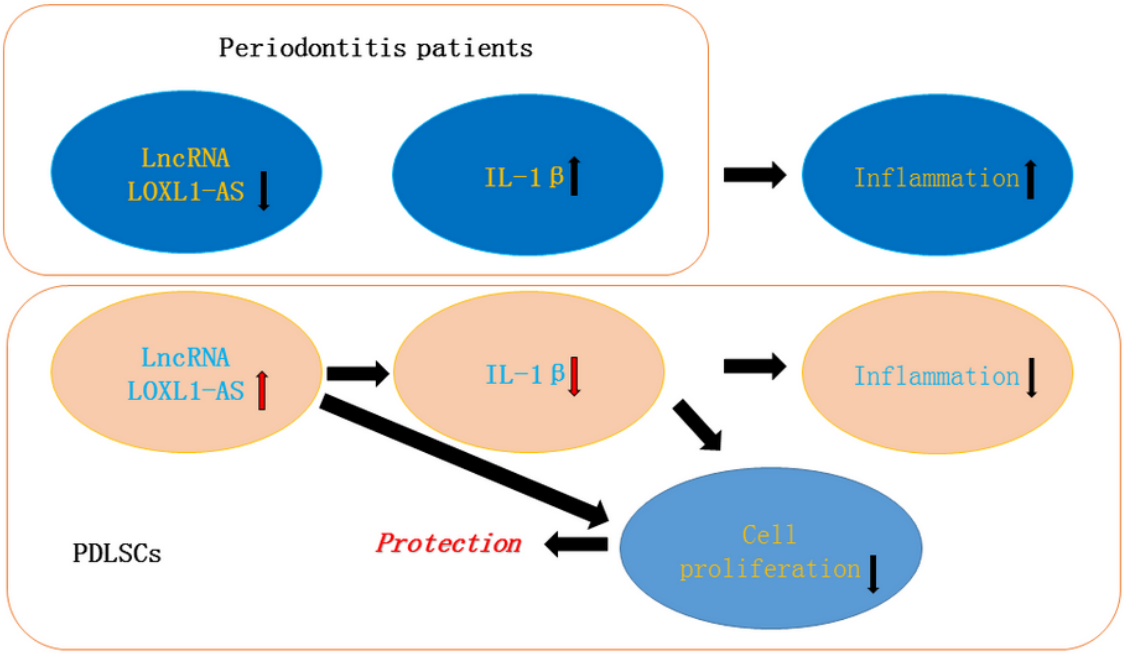

Figure 6

The scheme of the mechanism of the effect of LOXL1-AS and IL-1β on periodontitis. In periodontitis patients, LOXL1-AS displayed an elevated level. Also, a decrease in IL-1β was observed, which contributes to the high response of inflammation in these patients. On the other hand, overexpression of LOXL1-AS was able to downregulate IL-1β expression so as to suppress inflammatory effects in PDLSCs. In addition, LOXL1-AS elevation and IL-1β reduction prohibited PDLSCs proliferation to play protective roles in the progression of periodontitis.