ORIGINAL RESEARCH

Dysregulation of IncRNA GATA3-AS1 is Involved in the Pathogenesis of Pulpitis by the Regulation of miR-17-3p

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Purpose: When the pulp is inflamed or injured, cell morphology, gene expression, and synaptic connections change occur in the medullary dorsal horn, causing inflammation pain and formatting the pulpitis pain. To examine the impact of lncRNA GATA3-AS1 regulation of miR-17-3p on bioactivity and inflammation of lipopolysaccharides (LPS)-stimulated human dental pulp stem cells (hDPSCs).

Patients and Methods: The GATA3-AS1 expression in serum samples from patients with pulpitis, dental caries, and healthy control was examined using RT-qPCR. The GATA3-AS1 expression was verified using the GSE198359 dataset. hDPSCs were exposed to LPS to mimic in vitro pulpitis model. The viability and apoptotic rates of hDPSCs were determined by CCK-8 method and Flow cytometric analysis. The inflammatory cytokines levels were quantified using ELISA-based approach. A SOD assay kit was utilized to measure the activity of SOD. Bioinformatic analysis and dual-luciferase reporter assay were performed to explore the interaction between GATA3-AS1 and miR-17-3p, along with the potential mechanism.

Results: Serum and tissue GATA3-AS1 levels were elevated in patients with pulpitis. Silencing GATA3-AS1 overturned the LPS stimulation inhibited viability and promoted apoptosis, inflammation, and oxidative stress in hDPSCs. GATA3-AS1 could target miR-17-3p, and miR-17-3p downregulation reversed silencing GATA3-AS1-mediated effects in LPS-induced hDPSCs. The GATA3-AS1-miR-17-3p axis might mediate the progression of pulpitis by many potential pathways, such as the PI3K-Akt signaling pathway and MAPK signaling pathway.

Conclusion: GATA3-AS1 knockdown might have a protective effect on bioactivity, LPS-triggered inflammation, and damage in hDPSCs by regulating miR-17-3p, which might be a promising target for the treatment of pulpitis.

Keywords: GATA3-AS1, pulpitis, inflammation, proliferation, miR-17-3p, oxidative stress

Introduction

The dental pulp tissue is a loose connective tissue that provides nourishment and nerves to the dentin.¹ It is composed of a variety of cell types, including odontoblasts, fibroblasts, stem cells, and various immune cells.² When diseases of the hard dental tissues such as caries, as well as bacteria and their metabolic by-products from the periodontal tissue, invade the pulp tissue, the pulp becomes infected, leading to pulpitis, which refers to an inflammatory response of the dental pulp tissue caused by bacteria and their by-products.^{3,4} Pulpitis usually processes from a reversible to an irreversible stage, and even in the early stages of microbial infection, bacterial components can spread through dentinal tubules and cause a local pulpitis response.⁵ Lipopolysaccharide (LPS) can promote the expression of inflammatory factors within the infected root canal of the dental pulp, thereby participating in the process of pulpitis.⁶ Root canal therapy is one of the effective treatments for pulpitis, but this method has many limitations.⁷ The pathogenesis of pulpitis has not been fully

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elucidated, and the treatment of pulpitis has always been a research hotspot. Hence, it is essential to investigate the molecular mechanisms of the onset and progression of pulpitis and find therapeutic targets, which may be helpful to reduce the incidence and improve the treatment effect.

Serum indicators can reflect the status of the disease, which can be used to monitor the early condition of irreversible pulpitis and help clinical diagnosis and treatment.^{8,9} Long non-coding RNA (lncRNA) and microRNAs (miRNAs) are members of non-coding RNAs (ncRNAs) that are devoid of protein-coding function. LncRNAs were demonstrated to participate in various pathological and biological processes by interacting with miRNAs or directly binding to DNAs.¹⁰ The aberrant expression of lncRNAs and their molecular function were implicated in numerous diseases, including pulpitis.^{11,12} For instance, TFAP2A-AS1 was elevated in patients with pulpitis and could repress odontogenic differentiation while facilitating inflammation in hDPSCs.¹³ The molecular function of GATA3-AS1 was reported in many diseases, such as tumors, multiple sclerosis, rheumatoid arthritis, and preeclampsia.^{14–17} However, the molecular function of GATA3-AS1 in pulpitis remains elusive.

In this study, GATA3-AS1 expression was evaluated in serum samples from patients with pulpitis. Serum GATA3-AS1 abundance was enhanced in patients with pulpitis. A pulpitis cell model in vitro was established by stimulating hDPSCs with LPS. The functional interaction between GATA3-AS1 and miR-17-3p in the progression and inflammation was evaluated. The potential mechanisms of GATA3-AS1 were briefly explored by bioinformatics analysis.

Materials and Methods

Study Subject's Enrollment and Specimens' Collection

Ninety patients who suffer from pulpitis admitted to Dongying District People's Hospital from January 2021 to July 2023 were selected as the pulpitis groups, including 53 cases of reversible and 37 cases of irreversible. The age of the patients is from 21 years to 50 years old and there are 41 males and 49 females. Another 80 patients with dental caries were included in the study. Furthermore, 80 healthy individuals who underwent routine physical examinations were collected as the control group. Both the patients with dental caries and healthy control groups were age and sex-matched with the pulpitis group. The enrollment criteria for pulpitis patients include: 1) All of them met the diagnostic criteria of pulpitis. 2) The root tip has fully developed. 3) Individuals who drink, smoke, with other periodontal diseases, and have previous dental treatment history were excluded.

Informed written consent was obtained from all the participants and their families. This study was granted ethical clearance by the Dongying District People's Hospital Ethics Committee (No.20210032) and adhered to the tenets of the Declaration of Helsinki.

The peripheral blood from pulpitis patients and those with dental caries before treatment, as well as the control group on the day of physical examination, were drawn under fasting conditions. The samples were subjected to centrifugation at 1000 g for 10 min and the upper layer of serum was collected and stored in a low-temperature freezer for study.

Expression Validation and Bioinformatics Analysis

The keywords "ncRNA" and "Pulpitis" were used to search GEO datasets and the GSE198359 dataset was obtained. The GSE198359 dataset includes 4 normal tissues, 4 carious tissues, and 4 pulpitis human dental pulp tissues and the platform was GPL23227 BGISEQ-500. The data was analyzed with GEO2R and the data about the expression of GATA3-AS1 among three groups was obtained.

The downstream miRNAs of lncRNA GATA3-AS1 were predicted and the binding sites of GATA3-AS1 and miR-17-3p were displayed by lncRNASNP2 database. Besides, the downstream targets of miR-17-3p were predicted by TargetScan and miRDB (binding score \geq 80), as well as these targets were overlapped using the Venn diagram. Following, the overlapped targets were included in GO enrichment and KEGG enrichment analyses using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) and graphed using online SRplot.¹⁸

Cell Culture and LPS Treatment

The human dental pulp stem cells (hDPSCs, PT-5025) cell line was purchased from Lonza and incubated with a human dental pulp stem cell basal medium (Lonza) plus 10% PBS (Gibco). The hDPSCs were stimulated with different concentrations of LPS (Solarbio). Besides, to investigate the changes of GATA3-AS1 over time, hDPSCs were stimulated with 5 μ g/mL LPS for 0 h, 12 h, 24 h, and 48 h. Finally, hDPSCs were treated with 5 μ g/mL LPS for 24 h for further cellular experiments.

Cell Transfection

siRNAs targeting GATA3-AS1 (si-GATA3-AS1), siRNA negative control (si-NC), mimic NC, miR-17-3p mimic, inhibitor NC (anti-miR-NC), and miR-17-3p inhibitor (anti-miR-17-3p) were designed and synthesized by Shanghai Sango Biotech (China). Before transfection, hDPSCs were incubated in 6-well plates with a density of 1×10^{5} /well and cultured to the logarithmic phase. Then, transfection was performed utilizing Lipofectamine 2000.

Total RNA Isolation and RT-qPCR

The total RNA was obtained from serum specimens and hDPSCs with the help of Trizol reagent (Invitrogen). The complementary DNA (cDNA) synthesis was reverse transcribed utilizing SuperScript II reverse transcriptase (Invitrogen) or miScript reverse transcription Kit (Qiagen). The RT-qPCR was implemented with SYBR Green One-Step qPCR Kit (Invitrogen) and primers. The relative quantification data were normalized using GAPDH for lncRNA, and U6 for miRNA, with $2^{-\Delta\Delta Ct}$ method.

Cell Viability Detection

The cell viability in treated hDPSCs was assessed by cell counting kit-8 (CCK-8; Beyotime) assay. The hDPSCs in 96well plates were added CCK-8 kit at specific points in time. After 1 hour of incubation, cell absorbance was measured by a microplate reader.

Apoptosis Assay

Flow cytometry was conducted to analyze the apoptosis phenomenon utilizing Annexin V-FITC Apoptosis Detection Kit (Beyotime). The cells of each group were collected and digested with 0. 25% trypsin, and the cells (1×10^5 cells) were collected. The cells were suspended in a 500 µL binding buffer, and the apoptosis rate was detected according to the instructions of the apoptosis detection kit.

ELISA Assay

HDPCS in good growth condition were plated into 6-well plates (1×10^5 cells/well), and the cell culture supernatant was collected when the cells adhered to the wall and grew. The levels of tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) were detected by ELISA.

SOD Assay

Superoxide dismutase (SOD) activity was measured to assess oxidative injury using SOD Assay Kit (Beyotime) in culture supernatant. Cell absorbance was detected at 450 nm.

Dual-Luciferase Reporter Assay

Based on the binding sites of GATA3-AS1 and miR-17-3p, the wild-type GATA3-AS1 (WT-GATA3-AS1) and mutant GATA3-AS1 (MUT-GATA3-AS1) luciferase reporter vectors were constructed. Then the vectors were co-transfected with miR-17-3p mimic or mimic NC in hDPSCs, respectively. The hDPSCs were cultured in an incubator for 48 h, and the dual luciferase activity for each group was detected.

Statistical Analysis

All experimental data were expressed as mean \pm standard deviation, and each experiment was conducted a minimum of three times. GraphPad Prism version 9.0 was utilized for statistical analysis. Two groups of data were compared by *t*-test, and multiple groups of data were compared by one-way or two-way analysis of variance. A *P* value below 0.05 was deemed to indicate statistical significance.

Results

GATA3-AS1 Expression and Validation

RT-qPCR analysis revealed that GATA3-AS1 expression was elevated in patients with pulpitis in contrast to healthy control and patients with caries (Figure 1A). Moreover, the levels of serum GATA3-AS1 were higher in irreversible pulpitis patients than in reversible pulpitis patients (Figure 1B). Furthermore, the expression of GATA3-AS1 was validated using the GSE198359 dataset, which included 4 pulpitis tissues, 4 deep caries tissues, and 4 normal control tissues. As displayed in Figure 1C, GATA3-AS1 expression values were higher in pulpitis tissues compared to either deep caries tissues or normal tissues.

GATA3-AS1 Was Increased in LPS-Induced hDPSCs and Regulated Cellular Activities

In this study, different concentrations of LPS were used to stimulate hDPSCs with RT-qPCR employed for the quantification of GATA3-AS1 expression. With the increase of LPS concentration, the levels of GATA3-AS1 increased continuously in a concentration-dependent manner, compared with the control group (Figure 2A). hDPSCs were treated with 5 μ g/mL LPS for 0, 12, 24, and 48 h, and the results showed that the level of GATA3-AS1 was upregulated, especially at 24 and 48 h, and the difference was statistically significant (Figure 2B).

To explore the influence of GATA3-AS1 on cellular activities, si-GATA3-AS1 was transfected into hDPSCs. As shown in Figure 2C, LPS treatment increased the GATA3-AS1 expression, while si-GATA3-AS1 repressed GATA3-AS1 levels. Then, hDPSCs were exposed to LPS (5 μ g/mL) for 24 h to stimulate the pulpitis model. CCK-8 assay results showed that LPS exposure repressed the viability of hDPSCs, while downregulation of GATA3-AS1 reversed the LPS-decreased cell viability (Figure 2D). Furthermore, flow cytometry assay indicated that LPS stimulation increased hDPSCs apoptosis while silencing GATA3-AS1 decreased the increased apoptosis caused by LPS (Figure 2E).



Figure I IncRNA GATA3-AS1 expression in patients with pulpitis. (A) Serum GATA3-AS1 expression was increased in patients with pulpitis rather than in patients with dental caries and healthy control. (B) Serum GATA3-AS1 levels were higher in irreversible pulpitis than in reversible pulpitis patients. (C) The increased GATA3-AS1 expression was verified in pulpitis tissues from the GSE198359 dataset. ***P < 0.001.



Figure 2 The effect of GATA3-AS1 knockdown on LPS-stimulated hDPSC cells. (A) Different doses of LPS stimulated hDPSCs and GATA3-AS1 levels increased in a concentration-dependent manner. (B) hDPSCs were exposed to 5 μ g/mL LPS for 12, 24, and 48 h, and the level of GATA3-AS1 was upregulated, especially at 24 and 48 h. (C) GATA3-AS1 levels were detected by RT-qPCR after transfection. (D) Silencing GATA3-AS1 reversed the decreased proliferative ability caused by LPS. (E) GATA3-AS1 downregulation decreased the LPS-increased apoptosis in hDPSC cells. *P < 0.05, **P < 0.01, ***P < 0.001.

Silencing GATA3-AS1 Engaged the LPS-Induced Inflammation and Oxidative Stress of hDPSCs

ELISA assay was utilized to detect the IL-6 and TNF- α expression, and the LPS exposure induced the release of IL-6 and TNF- α inflammatory cytokines, while the increased IL-6 and TNF- α were inhibited by knockdown GATA3-AS1 (Figures 3A and B). The SOD assay showed that LPS exposure reduced the activity of SOD, while si-GATA-AS1 diminished the inhibitory role of LPS on SOD activity (Figure 3C).

miR-17-3p Was a Downstream miRNA of GATA3-AS1

IncRNASNP2 database displayed the interaction sites between GATA3-AS1 and miR-17-3p (Figure 4A). Dual-luciferase reporter assay revealed that miR-17-3p decreased the luciferase activity of GATA3-AS1-WT reporter construct, but had no effect on the GATA3-AS1-MUT reporter construct (Figure 4B). Besides, LPS treatment reduced miR-17-3p expression, while downregulation of GATA3-AS1 reversed the decreased miR-17-3p expression in hDPSCs (Figure 4C). Furthermore, the levels of serum miR-17-3p were decreased in patients with pulpitis (Figure 4D) and had a negative correlation with GATA3-AS1 expression in pulpitis patients (Figure 4E).



Figure 3 The influence of GATA3-AS1 on inflammation and oxidative stress in LPS-stimulated hDPSCs. (A) Downregulation of GATA3-AS1 repressed the LPS-enhanced IL-6 levels. (B) LPS-caused upregulation of TNF- α levels was decreased by si-GATA3-AS1. (C) The activity of SOD was detected by a SOD assay kit. **P < 0.01.



Figure 4 miR-17-3p was a downstream miRNA of GATA3-AS1. (**A**) The binding sites between GATA3-AS1 and miR-17-3p. (**B**) Dual-luciferase reporter assay was conducted to verify the target relationship between GATA3-AS1 and miR-17-3p. (**C**) miR-17-3p expression was downregulated in LPS-exposed hDPSCs, while was increased in LPS-exposed hDPSCs transfected with si-GATA3-AS1. (**D**) Serum miR-17-3p expression was decreased in patients with pulpitis. (**E**) A negative correlation between GATA3-AS1 and miR-17-3p was observed in patients with pulpitis. *P < 0.01, ***P < 0.01.

Interfering miR-17-3p Partially Reversed the Effect of Si-GATA3-AS1 on LPS-Stimulated hDPSCs Cell Activity, Inflammation, and Oxidative Stress

As shown in Figure 5A, LPS stimulation reduced miR-17-3p expression, and knockdown GATA3-AS1 reversed the decreased miR-17-3p levels, while silencing miR-17-3p repressed the miR-17-3p expression. Cellular experiments



Figure 5 Silencing GATA3-AS1-mediated protective effects in LPS-stimulated hDPSCs were partially diminished by the downregulation of miR-17-3p. (**A**). The expression of miR-17-3p was detected after cotransfection in LPS-induced hDPSCs. (**B**) Cell viability was assessed by CCK-8 assay. (**C**) The apoptosis of hDPSCs was analyzed by flow cytometry. (**D** and **E**) The release of IL-6 and TNF- α inflammatory cytokines was analyzed utilizing ELISA in the culture supernatant of hDPSCs. (**F**) A SOD assay kit was utilized to analyze the activity of SOD. *P < 0.05, **P < 0.01, ***P < 0.001.

indicated that knockdown miR-17-3p partially diminished the influence of si-GATA3-AS1 on LPS-stimulated hDPSCs cell viability (Figure 5B) and apoptosis (Figure 5C).

In addition, the inhibitory effects of GATA3-AS1 knockdown on LPS-promoted IL-6 and TNF- α inflammatory cytokines were partially counteracted by interfering miR-17-3p expression (Figures 5D and E). As shown in Figure 5F, the opposite influence was observed in oxidative stress.

The Downstream Targets of miR-17-3p and Their GO and KEGG Enrichment Analysis

The online miRDB and TargetScan databases were used to predict the downstream mRNAs of miR-17-3p. From both databases, 135 targets were overlapped (Figure 6A). By utilizing DAVID functional annotation tools, the biological meaning behind the lists of 135 genes was understood. The GO enrichment indicated that these targets were enriched in multiple biological processes (BP), molecular function (MF), and cellular component (CC), such as axon extension, osteoblast differentiation, neuron migration biological process, microtubule organizing center and recycling endosome membrane CC, as well as GDP binding and microtubule binding MF (Figure 6B). KEGG enrichment results indicated



Figure 6 The downstream targets of miR-17-3p were predicted and enrolled in GO and KEGG enrichment analysis. (A) A total of 135 targets of miR-17-3p were overlapped. (B) The results of GO enrichment. (C) DAVID database was used to analyze KEGG enrichment.

that these targets were enriched in many pathways, including EGFR tyrosine kinase inhibitor resistance, FoxO signaling pathway, PI3K-Akt signaling pathway, and MAPK signaling pathway (Figure 6C).

Discussion

GATA3-AS1 is notably upregulated in various diseases. For instance, GATA3-AS1 was increased in human endometrial carcinoma and facilitated tumor cell invasion and migration via regulating miR-361/ARRB2 axis.¹⁶ In addition, GATA3-AS1 was overexpressed in preeclampsia and promoted trophoblast cell apoptosis and repressed cell proliferation and migration through miR-488-3p/ROCK1 axis.¹⁷ This research assessed the expression of serum GATA3-AS1 in patients with dental pulpitis and revealed that GATA3-AS1 was upregulated in patients with pulpitis rather than patients with dental caries and healthy control, especially in patients with irreversible pulpitis. Importantly, the data in GSE198359 verified its enhanced expression in pulpitis tissues. These data speculated that GATA3-AS1 might be involved in the pathogenesis of pulpitis. LPS is one of the strongest triggers of the human innate immune system and is often used to stimulate the inflammatory response of pulpitis.¹⁹ In addition, we treated hDPSC cells with LPS to mimic pulpitis in vitro. GATA3-AS1 levels were upregulated in hDPSCs with increasing LPS doses and increased after LPS stimulation for 12, 24, and 48 h, especially at 24 h and 48 h. Based on the above results, we speculate that GATA3-AS1 is aberrantly expressed in pulpitis and may be used as a novel biomarker for the diagnosis of pulpitis.

To probe the role of GATA3-AS1 in pulpitis progression and inflammation, si-GATA3-AS1 was transfected into LPSstimulated hDPSCs cells. Silencing of GATA3-AS1 enhanced LPS-induced proliferation decline, while inhibited the increase of LPS-stimulated apoptosis. As we all know, LPS induced a large number of cytokines, among which proinflammatory factors included TNF- α and IL-6.²⁰ The increased levels of TNF- α and IL-6 are closely related to the development of pulpitis and can be used as crucial auxiliary markers for the diagnosis of pulpitis.^{21,22} Besides, the expression of inflammatory factors (IL-6 and TNF- α) was decreased and the SOD value was increased after si-GATA3-AS1 transfection, which revealed that GATA3-AS1 positively regulates the pro-inflammatory cytokines and oxidative stress in LPS-stimulated hDPSCs and may have a promoting effect on inflammation and injury to promote pulp tissue to destruction.

LncRNA could regulate the expression of downstream miRNAs to achieve their biological functions.¹⁰ The current study identified that miR-17-3p was a downstream miRNA of GATA3-AS1. MiR-17-3p was related to inflammatory reactions in many diseases.^{23–25} A recent miRNA expression profile study indicated that miR-17-3p was downregulated in individuals with aggressive periodontitis.²⁶ A previous study also indicated that mice lacking the miR-17-92 cluster manifest a dorsal shift in pMN/p2 boundary and impairment in the production of V2 interneurons.²⁷ Herein, serum miR-17-3p expression was decreased in patients with pulpitis, and it was negatively correlated to GATA3-AS1. These results suggest that GATA3-AS1 might achieve its function in pulpitis by regulating miR-17-3p expression. Functional experiments showed that interfering with GATA3-AS1 exerted a protective role in LPS-stimulated hDPSCs, which

was reversed by the downregulation of miR-17-3p, revealing that silencing GATA3-AS1 protected hDPSCs from LPSinduced inflammation and injury by upregulating miR-17-3p.

To investigate the underlying molecular mechanisms of GATA3-AS1/miR-17-3p in regulating the progression of pulpitis, the downstream mRNAs were predicted and overlapped. Bioinformatic enrichment analyses revealed that the identified targets were enriched in a variety of biological functions and signaling pathways. For instance, the axon extension, osteoblast differentiation, and neuron migration were significantly associated with hDPSCs differentiation and cellular mechanotransduction in health and diseases.^{28,29} A recent study by Nam, O. H et al indicated that Ginsenoside Rb1 alleviates LPS-induced inflammation through regulating PI3K-Akt, NF-KB, and MAPK signaling in human dental pulp cells.³⁰ Inflammatorv mediator-induced resistance to EGFR tyrosine kinase inhibitor was involved in various diseases and tumors.³¹ Many studies demonstrated that PI3K-Akt and MAPK signaling pathway plays a crucial role in the inflammatory response and pulpitis.^{32–35} The bioinformatics analysis results revealed that GATA3-AS1 knockdown might inhibit LPS-induced inflammation in hDPSC cells by targeting miR-17-3p to repress many important signaling pathways. Wnt1/β-catenin signaling was a signal transduction pathway that plays a key role in biological development and cancer occurrence. GATA3-AS1 facilitated tumorigenesis in pancreatic cancer and bladder uroepithelial cancer through modulating the Wnt/β-catenin signaling pathway.^{36,37} Although the top 10 GATA3-AS1 enriched pathways in pulpitis did not display the Wnt/β-catenin signaling pathway, the pathway was reported to impact the tooth density of pulpitis.³⁸ These pathways need to be confirmed in future studies. The present study provides a deeper theoretical basis for understanding the nature of dental pulp diseases. Detecting the expression level of GATA3-AS1 in dental pulp tissues or oral saliva of patients is helpful in realizing the early and accurate diagnosis of pulpitis, which may change the previous diagnostic model and improve the accuracy and sensitivity of diagnosis.

There are several limitations in this study. Firstly, we explored the role of GATA3-AS1 in pulpitis using a 2D in-vitro model. 2D cell cultures are a fundamental and widely used approach in cell-based research, which is an essential first step in exploring the mechanisms underlying our research questions. The 2D model allows for better control over experimental variables, such as cell-to-cell interactions and exposure to external stimuli, compared to more complex 3D in vivo models. However, the role of GATAS-AS1 in vivo remains to be clarified in the future. Secondly, the molecular mechanism of GATA3-AS1 regulating hDPSC cells was briefly discussed, which needs to be further confirmed through more experiments as well as in vivo experiments.

Conclusion

In conclusion, lncRNA GATA3-AS1 was upregulated in pulpitis and might have clinical diagnostic value in predicting patients with pulpitis. GATA3-AS1 knockdown relieved LPS-triggered inflammatory reaction and damage in hDPSCs by targeting miR-17-3p, which might be a promising target for the treatment of pulpitis.

Disclosure

The author(s) report no conflicts of interest in this work.

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