The miR-223-3p from Salivary Exosome Regulates Pyroptosis through NLRP3-Caspase 1-GSDMD signal axis in Periodontitis.

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Research Article

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Abstract

Salivary exosomes contain various components and play an important role in oral diseases. We found that the expression of miR-223-3p in salivary exosomes was down regulated in inflammatory gingival tissue, and NLRP3 was the target of miR-223-3p. It has been reported that NLRP3 was involved in the formation of inflammasome and induced a type of cell death by cleaving gsdemin D (GSDMD), which is called pyroptosis. The purpose of this study was to investigate the role of miR-223-3p in NLRP3 inflammasome activation and pyroptosis. We found that miR-223-3p down regulated the activation of NLRP3, IL-1 β and caspase-1, and then released the pyroptosis of THP-1-derived macrophages inducing by Porphyromonas gingivalis -LPS (P. gingivalis -LPS). In addition, NLRP3, and GSDMD was highly active in inflammatory gingival tissue compared with healthy controls. In summary, we hypothesized that miR-223-3p in salivary exosomes regulates GSDMD-mediated pyroptosis by targeting NLRP3. Detection of miR-223-3p expression in salivary exosomes could be used as an important non-invasive method to diagnose and evaluate the severity of periodontitis.

Introduction

Exosomes are nanovesicles with diameters of 30-100 nm secreted by multiple cells. Moreover, exosomes are widely present in various body fluids, including serum, saliva, urine, bile, breast milk, and sputum [1]. They are encapsulated in bilayer lipid membranes and can carry a variety of biomolecules, including mRNAs, miRNAs, proteins and other cellular components [2]. When exosomes are transferred to recipient cells, those cargoes remained functional and alter cellular behavior [3]. Therefore, exosomes are acknowledged to be important mediators of intercellular communication [4]. Salivary exosomes are mainly secreted by oral epithelial cells and granulocytes [5]. More and more studies have shown that the abnormal expression and potential mechanism of salivary exosomes may be considered as potential biomarkers for the diagnosis and treatment of oral diseases [6, 7]. The future research direction should be to further study the potential of exosomes in translational medicine, research and develop exosomes related diagnostic kits, and provide a new way for clinical non-invasive diagnosis and treatment [8]. Studies have shown that salivary exosomes not only play a preventive role in systemic inflammatory response [9], but also are reliable biomarkers for diagnosis and prognosis evaluation [10, 11]. However, there are fewer studies on the relationship between periodontitis and salivary exosomes.

Periodontitis is a complex infection driven inflammatory disease, which is caused by biofilm accumulation and inflammatory immune response [12]. Periodontitis is characterized by the activation of osteoclastogenesis and the loss of alveolar bone, which results in irreversible destruction of periodontal supporting tissue [13]. The global prevalence of severe periodontitis is stable at 11.2%, which has become a predictable and increasing burden [14]. Therefore, it is urgent to find non-invasive and effective periodontitis biomarkers to diagnose the severity and prognosis of periodontitis.
Our previous studies had shown that NLRP3 was upregulated in periodontitis patients compared with healthy control. Therefore, in this study, we aim to evaluate the concentration changes of miR-223-3p in salivary exosomes of periodontal disease patients (stage III / IV) and healthy controls, and to explore its role in the progress of pyroptosis by regulating the target gene NLRP3. The expression of miR-223-3p in salivary exosomes may be a potential biomarker for noninvasive clinical diagnosis of periodontitis.

**Materials And Methods**

1. **Subjects**

This protocol was approved by the Human Research Ethics Committee of Ninth People's Hospital. After reading information about the study and explaining the procedures, all participants signed a consent form. We used the spitting methods to collect saliva [15]. Briefly, we collected saliva in the morning (7:00 AM to 12:00 noon). The subjects sat upright in the dental chair and rinsed thoroughly with deionized water before collecting saliva according to instructions. Saliva is allowed to accumulate on the mouth floor. The subject spitted it out into the preweighed tube every 60 seconds. After collection of saliva, it was stored at 4°C for up to 6 h, after which we held it at -80°C until use.

2. **Salivary Exosomes Isolation and Detection**

According to the manufacturer's instructions, we used Umibio® exosome isolation kits (Umibio, Cat. No: UR52121, China). In short, each sample was centrifuged at 3000 g 4°C for 10 minutes and then at 10000 g 4°C for 20 minutes to remove cells and debris. According to the manufacturer's instructions, we added the corresponding amount of reagent proportional to the volume of the starting sample. The mixture was well mixed, incubated at 4°C for 2 hours, then centrifuged at 10000 g at 4°C for 60 minutes to precipitate exosomes. The precipitate was resuspended with 1 x PBS and purified by exosome purification filter at 3000 g 4°C for 10 minutes. The initial volume of exosome particles was 5 ml and the resuspension volume was 200 μL. All exosomes were stored at -80°C immediately after extraction until further analysis. Zetaview PMX 110 (particle metrix, meerbusch, Germany) and transmission electron microscopy (TEM, jeol, jem-1230, TEM, Peabody, MA) were used to measure the exon size by nanoparticle tracking analysis (NTA).

3. **The miR-223 expression in Salivary Exosomes**

We used the microRNA Reverse Transcription Kit (EZBioscience, Cat. NO.: EZB-miRT2USA) and TB Green™ Premix Ex Taq™ (TaKaRa, Cat. NO.: RR420A, China) for the real time-PCR analyses. U6 was choosed as an internal reference for detecting miR-223-3p expression by the $2^{-\Delta\Delta Ct}$ method.
4. Target gene prediction and dual-luciferase reporter assay

Target gene prediction software, including miR-Base (http://www.mirbase.org/), TargetScan 4.2 (http://www.targetscan.org/), and PicTar (http://pictar.mdcberlin.de/) were used to predicted the miR-223-3p's potential molecular targets. Among the potential targets, we focused on NLRP3 because it is highly expressed in inflammatory gingival tissue, as our previous studies have shown [16].

5. Plasmid vectors construction and Dual-Luciferase Reporter Assays

The plasmids of wild type (wt)-NLRP3 clone ID: BK295, pmirGLO-NLRP3-3UTR-WT and mutant (mut)-NLRP3-fused luciferase genes (clone ID: BK296, pmirGLO-NLRP3-3UTR-MU) were constructed using conventional methods. One day before transfection, HEK293 cells in logarithmic growth phase were collected. After centrifugation and suspension, the cell density was adjusted, and the density of 1 x 10^5 cells at per hole was inoculated in 48 well plates. According to the manufacturer’s instructions, all transfections were performed with Lipofectamine 3000 (Invitrogen). The cells were transfected with 1 μg pmirGLO luciferase expression vector containing 3'UTR of human NLRP3 (Promega) and 60 nM hsa-miR-223-3p mimics or blank control (Ribobio, China). After 48 hours of transfection, the Dual-Luciferase reporter analysis system (Promega) was used to measure the luciferase activities normalizing to Renilla luciferase activity. All the experiments were conducted three times independently, and the data came from three independent experiments.

6. Integrated Bioinformatics Methods

The NCBI Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) database were used in Oct. 2019 using a single keyword "chronic periodontitis", and we found two relative datasets GSE10334 and GSE16134. Both microarray datasets were used GPL570 Platforms (HG-U133_Plus_2 Affymetrix Human Genome U133 Plus 2.0 Array). Both studies had obtained informed consent from all patients and been performed with the ethics committee's acquired approval in their institutions. The two gene expression datasets were further employed to identify the disease-associated pathways using the Gene Set Enrichment Analysis (GSEA; v4.0.2; http://software.broadinstitute.org/gsea/index.jsp). |NES| ≥ 1, NOM p-value ≤ 0.05, and FDR q value ≤ 0.25 were considered statistically significant.

7. THP-1 culture and Differentiation
Human monocyte macrophage (THP-1) cell lines were obtained from ATCC and cultured in RPMI 1640 medium (Gibico, USA) containing 10% fetal bovine serum (Gibico, USA) and 2 mmol / L glutamine (Gibico, USA). THP-1 in logarithmic growth phase was collected for differentiation stimulation. Cells were collected and centrifugated. Cells were resuspended in a complete medium containing 200 nm phorbol-12-myristate-13-acetate (PMA, sigma Aldrich). The cell density was adjusted to $1 \times 10^4$ cells / ml, and the cells were inoculated for differentiation stimulation. Three days after inoculation, PMA treated THP-1 cells were gently washed with phosphate buffer (PBS) for three times, and replaced with fresh RPMI 1640 (10% FCS, 1% L-glutamine) containing Lipofectamine 3000 (Invitrogen life technologies, USA), and transiently transfected with miR-223-3p mimics, mir-223-3p inhibitor or control mimics (ribobio, China) for 48 hours. After transfection, THP-1-derived macrophages were treated with lipopolysaccharide (LPS) of $P. gingivalis$ ($1 \, \mu g / ml$) for 6 h, and real-time PCR was performed.

8. Real time-PCR

THP-1-derived macrophages treated with $P. gingivalis$-LPS were collected and extracted RNA by Trizol reagent (Invitrogen). The PrimeScript™ RT Kit (Shanghai Roche Pharmaceutical Co., Ltd., Shanghai, China) was used to prepare the cDNA. The expression levels of Caspase-1, IL-6, IL-1 β and NLRP3 mRNA were detected by real-time PCR. Three parallel replication wells were prepared for each sample, and the reaction was performed on the PCR instrument. TaqMan microRNA Analysis Kit (Applied Biosystems, Foster City, CA, USA) was used to measure gene expression level by $2^{-\Delta \Delta Ct}$ method. The primer series are shown in Table 1. GAPDH was used as an internal reference for detection.

9. Tissue Sampling and Immunochemical staining

We obtained the gingival tissues from periodontitis patients (n=4, stage III/IV) and healthy control (n=3). All of the participants signed a consent form after confirming information about the study. All procedures are in accordance with the rules and requirements of the Human Research Ethics Committee of Ninth People’s Hospital. All inclusion and exclusion criteria were listed in Table 2. The detailed procedures of gingival tissue collection and immunohistochemical staining were described in the previous study [16].

10. Statistical methods and data analysis

The results in the study were expressed as mean ± standard deviation (M ± SD). First of all, we test the homogeneity of variance using Levene’s test by SPSS 25.0.0. The results showed that the variation within each population was equal ($P > 0.05$). So we used one-way ANOVA and Bonferroni post hoc test to evaluate the statistical significance between groups. All the experiments were repeated three times independently and then analyzed statistically ($P < 0.05$).
Result

Expression of miR-223-3p in periodontitis-derived salivary exosomes was lower than in healthy control.

We isolated exosomes from saliva of periodontitis patients and healthy subjects according to the instructions (Fig. 1A). Nanoparticle tracking analysis (NTA) of salivary exosomes showed that the diameter of most particles was in the expected size range of 50-150 nm, and the maximum peak value of salivary exosomes detected by NTA was about 110 nm (Fig. 1B). Transmission electron microscopic images of the salivary exosomes showed typical round vesicles surrounded by double lipid membranes, with a diameter of 50-150 nm (Fig. 1C). Subsequently, we detected the expression of mir-223 in exosomes by real-time fluorescent quantitative PCR. We found that the expression of mir-223 in saliva of CP patients decreased compared with healthy controls (Fig. 1D).

miR-223-3p mediated NLRP3 down-regulation is critical for inflammation in periodontitis.

It was predicted that NLRP3 might be the potential molecular target of miR-223-3p (Fig. 2A). To prove the speculation, wild type (wt)-NLRP3 and mutant (mut)-NLRP3 were fused with luciferase and co-expressed with hsa-miR-223-3p mimic. We found that hsa-miR-223-3p could down-regulate wt-NLRP3 expression but not mut-NLRP3, which indicated that NLRP3 was a direct target of miR-223-3p (Fig. 2B). To mimic the inflammation regulatory function of miR-223-3p in periodontitis, we detected IL-6, IL-1β, and NLRP3 expression in THP-1-derived macrophages after P. gingivalis lipopolysaccharide (Pg LPS) treatment when miR-223-3p was overexpressed with miR-223-3p mimics or knockdown with miR-223-3p inhibitor. The data showed that miR-223-3p might regulate inflammation in macrophages through NLRP3 expression (Fig. 2C).

Pyroptosis and cytokine secretion participated in inflammation in diseased gingival tissues.

We analyzed two GEO databases (GSE10334 and GSE16134) by GSEA. We found that the infected gingival tissues had more related gene enriched in the "regulation of cytokines secretion" (Fig. 3A). The expression of GSDMD, IL1B, and NLRP3 was significantly upregulated in affected gingival tissues (Fig. 3B).

miR-223 was involved in inflammation through pyroptosis in Periodontitis patients

The activated form of GSDMD, N-terminal GSDMD (GSDMD-N), was increased significantly in gingival tissues from periodontitis patients compared with tissues from healthy controls (Fig. 4A and 4B) which indicated that the pyroptosis might participate in inflammation of periodontitis patients. Because GSDMD-mediated pyroptosis depends on caspase cleavage, we detected caspase 1 in THP-1-derived
macrophage after *P.gingivalis* LPS treatment when miR-223-3p was overexpressed with miR-223-3p mimics or knockdown with miR-223-3p inhibitor. We found that the expression of Caspase 1 was downregulated when miR-223 was overexpressed and upregulated when miR-223 was knockdown (Fig. 4C). Immunohistochemistry also showed more NLRP3 and GSDMD-N in gingival tissues from periodontitis patients (Fig. 4D).

**Discussion**

Previous studies showed that NLRP3 inflammasome complexes can induce pyroptosis by activating Caspase-1 [17, 18]. During Gram-negative bacteria infection, such as *P.gingivalis*, the cytosolic LPS was bonded and subsequently initiate the NLRP3 inflammasome activation and cleave GSDMD to drive pyroptosis [19]. In previous studies, we confirmed the high expression of NLRP3 inflammasome in inflammatory gingival tissue by immunochemical detection and real time-PCR. [20].

In our current manuscript, we found that miR-223 mediated NLRP3 down-regulation was critical for *P.gingivalis* LPS induced inflammation, which indicated the inflammatory regulation function of miR-223-3p in periodontitis. We detected the direct interaction between miR-223-3p and NLRP3 using the online database and verified by a dual-luciferase reporter assay (Fig. 2). The results showed that miR-223-3p could regulate NLRP3 by binding sites in the NLRP3 3'UTR (Fig. 2A). To further explore the regulatory mechanism of miR-223 and verify the prediction results of TargetScan database, Dual-Luciferase Reporter Assays was conducted. The results revealed that hsa-miR-223-3p could down-regulate wt-NLRP3 expression but not mut-NLRP3, which indicated the direct interaction between miR-223-3p and NLRP3 (Fig. 2B). To mimic the inflammation regulatory function of miR-223-3p in periodontitis, we found that inhibition of miR-223-3p might upregulate the inflammatory biomarkers IL-6 and IL-1β through NLRP3 expression (Fig. 2C). This suggested that miR-223 inhibitor could remarkably aggravate *P.gingivalis*-LPS induced macrophage inflammatory response. All these findings indicated that miR-223-3p could exert a therapeutic effect on periodontitis via downregulating NLRP3 expression.

Salivary exosomes having diverse components, including proteins, liquids, and nucleic acids, play essential roles in various biological mechanisms. Compared with other sources of exosomes, salivary exosomes have the characteristics of stable and non-invasive, which is a better and accessible tool in the diagnosis and treatment of diseases. [21]. Few studies are concentrating on the relationship between salivary exosomes and periodontitis. This study preliminarily revealed the difference of miR-223-3p expression from salivary exosomes between periodontitis patients and healthy control (Fig. 1D). The expression level of miR-223-3p in salivary exosomes of periodontitis patients might be related to the severity of periodontitis and might be an essential biomarker to evaluate the stage and grade of periodontitis. It is necessary to assess the correlation between miR-223-3p in salivary exosomes and the severity of periodontitis in a more detailed way. Further study needs to expand the sample size and
classify in various stage and grade to provide a noninvasive and efficient biomarker for the diagnosis and prognosis of periodontitis in the future.

Pyroptosis is one type of cell death pattern, which controls IL-1β secretion under inflammatory conditions [22, 23]. GSDMD, one of the gasdermin family members, reported function as the executor of inflammatory pyroptosis in various immune cells and non-immune cells [22]. Although enhanced IL-1β secretion and activated NF-kappa B signaling have been observed in many kinds of inflammatory diseases, such as periodontitis, there was insufficient pieces of evidence to prove pyroptosis's involvement in gingival inflammation. Therefore, we searched in the GEO database to obtain a large sample size of periodontitis and healthy control. The results showed that cytokine secretion regulation was highly stimulated in affected gingival tissues (Fig. 3A). After merging the two databases, we found the expression of GSDMD, IL-1B, and NLRP3 were upregulated in affected gingival tissues (Fig. 3B). It was consistent with the previous results that IL-1β has highly upregulated in P.gingivalis LPS-stimulated macrophage after miR-223-3p inhibitor treatment (Fig. 2C), which indicated the disruption of the inflammatory inhibitory function of miR-223-3p in infected gingival tissues. The data above suggested that pyroptosis-mediated inflammatory cytokines were critical for infected gingival tissues, and miR-223-3p was likely involved.

Because NLRP3 down-regulation by miR-223-3p was critical for P.gingivalis LPS induced inflammation, we hypothesized that miR-223-3p might be involved in the inflammation of periodontitis through pyroptosis. As showed in Fig. 1D, we found that expression of miR-223-3p reduced in salivary exosomes from periodontitis patients. It indicated that decreased expression of miR-223-3p in salivary exosomes from periodontitis patients might result in the upregulated NLRP3 and Caspase 1, which in turn enhanced the activation of GSDMD and inflammation. Recent studies have shown that pyroptosis accrued during the progress of periodontitis [24]. We found pyrototic phenomena reflected as high expression of NLRP3, caspase-1, GSDMD, and IL-1β in inflammatory gingival tissues as identified by immunohistochemistry (Fig. 4D) and real time-PCR (Fig. 2C and 4C).

In summary, our present work has suggested that the miR-223-3p from salivary exosomes could alleviate P.gingivalis-LPS induced inflammatory responses partly by inhibiting the NLRP3/Caspase-1/GSDMD pyroptosis pathway (see schematic diagram in Figure.S1). Moreover, our work indicated that the detection of miR-223-3p expression in salivary exosomes could be used as an important noninvasive method for diagnosis and assess the severity of periodontitis.

**Declarations**
**Funding:** This work was supported by National Natural Science Foundation of China (81991500, 81991503), Cross-disciplinary Research Fund of Shanghai Ninth People's Hospital, Shanghai JiaoTong University School of Medicine (JYJC201904), Science and Technology Commission of Shanghai Municipality (18ZR1422400) and Innovative Research Team of High-level Local Universities in Shanghai (SSMU-ZDCX20180900).

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

**Availability of data and material:** Not applicable.

**Code availability:** Not applicable.

**Authors' contributions:** Y.R Xia and K.C Zhou performed the experiments of molecular biology, including exosomes extraction, real time-PCR, western blotting and dual-luciferase reporter assays. R Shu performed the histochemistry examination of the inflammatory tissues. Q.J Lei and Y.F Xie contributed to the data statistics and write the manuscript. All authors read and approved the final manuscript.

**Ethics approval:** All procedures performed in studies involving human tissues were in accordance with the ethical standards of the Ethical Committee of Shanghai Ninth People's Hospital (the registration number is ChiCTR-OOR-1600992) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Consent to participate:** All the participates who donate the saliva and gingival tissues were confirmed with the information of this study and signed a consent form.

**Consent for publication:** Not applicable

**References**


**Tables**

**Table 1. Primers for Real time-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-1</td>
<td>TTTCCGCAAGGTTCGATTTTCA</td>
<td>GCCATCTGGCGCTCTACCAC</td>
</tr>
<tr>
<td>NLRP3</td>
<td>GATCTTCGCTGCGATCAACAG</td>
<td>CGTGCATTATCTGAACCCAC</td>
</tr>
<tr>
<td>IL-6</td>
<td>CCTGAACCTTCCAAAGATGGCC</td>
<td>TTCACCAGGCAAAGTCTCCTCA</td>
</tr>
<tr>
<td>IL-1β</td>
<td>ATGATGGCTTATTACAGTGCGCAA</td>
<td>GTCGGAGATTCGTAGCTGGA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGAGCGAGATCCCTCCAAAT</td>
<td>GGCTGTTGTGATACACTTTCTCATGG</td>
</tr>
<tr>
<td>miR-223-3p</td>
<td>AACACGCTGTGCCTGAGA</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2. The inclusion and exclusion criteria for human subjects**

<table>
<thead>
<tr>
<th>Inclusion criteria for healthy specimens</th>
<th>Inclusion criteria for periodontitis (Stage III/IV)</th>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) with intact periodontium (without attachment loss)</td>
<td>(ii) Interdental CAL (clinical attachment loss) is detectable at ≥2 non-adjacent teeth, or buccal or oral CAL ≥3 mm with PD &gt;3 mm is detectable at ≥2 teeth</td>
<td>(i) systemic diseases such as diabetes mellitus or any metabolic syndrome affect periodontal tissue</td>
</tr>
<tr>
<td>(ii) Probing pocket depths (assuming no pseudo pockets) ≤3 mm and BOP (bleeding on probing) (+) % &lt;10%</td>
<td>(ii) Probing depth ≥ 5 mm</td>
<td>(ii) antimicrobial or medicinal treatments in the last 6 months</td>
</tr>
<tr>
<td>(iii) Without radiological bone loss</td>
<td>(iii) radiological bone loss &gt; 33%</td>
<td>(iii) history of smoking</td>
</tr>
<tr>
<td>(iv) crown lengthening surgery for aesthetic needs and BOP (-) at the surgical site</td>
<td></td>
<td>(iv) periodontal treatment in the last 3 months</td>
</tr>
</tbody>
</table>

**Figures**
Figure 1

The isolation of salivary exosomes and the expression of miR-223-3p in the salivary exosomes. A. Schematic diagram of exosomes isolation from human saliva. After centrifuging the saliva and removing the debris, the supernatant was mixed with reagent and then centrifuged to obtain pellets. B. Saliva exosome size distribution in purified pellets consistent with a size range of exosomes (average size 100 nm), measured by ZetaView® Particle Tracking Analyzer. C. Transmission electron microscopy images of salivary exosomes from healthy control and periodontitis patients. The scale bar is 200 nm, and the exosomes are 30-100 nm in size and spherical. D. The miR-223-3p expression in salivary exosomes. U6 was used as an internal reference. * P < 0.1.
miR-223-3p mediated NLRP3 down-regulation is critical for inflammation in periodontitis. A. We predicted the target of miR-223-3p by using target gene prediction software. It showed that miR-223 could regulate NLRP3 by binding sites in the NLRP3 3’UTR. B. The dual-luciferase reporter assays. The result showed that hsa-miR-223-3p could down-regulate wt-NLRP3 expression but not mut-NLRP3. C. The mRNA expression of NLRP3, IL-6, and IL-1β of THP-1 derived macrophages after transfected with miR-223 mimic or miR-223 inhibitor.

Figure 2
Figure 3

Pyroptosis and cytokine secretion participated in inflammation in diseased gingival tissues. A. The GESA analysis of GSE10334 and GSE16134. It showed that the affected gingival tissues were highly associated with cytokine secretion in both databases. B. We analyzed the mRNA expression of GSDMD, IL-1B, and NLRP3 from the affected gingival samples and unaffected samples in both databases.
miR-223 was involved in inflammation through pyroptosis in Periodontitis patients. A. The GSDMD-N activation from periodontitis gingival tissue and healthy control was detected by western blot. B. Quantitative analysis of GSDMD-N activation level in gingival tissues of periodontitis patients and healthy controls was performed. C. The mRNA expression of Caspase-1 of THP-1 derived macrophages after transfected with miR-223 mimic or miR-223 inhibitor. The immunohistochemistry results showed NLRP3 and GSDMD-N expression from gingival tissues of periodontitis patients and healthy controls.