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Original Article

MiR-1246 involves in the pathogenesis of periodontitis by negative regulation of IGF2BP1 and NF-κB signaling pathway

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Abstract: The objective of this study was to investigate the expression of miR-1246 in periodontal tissues and its potential function in the pathobiology of periodontitis. The lymphocytes were isolated from gingival tissue of patients with periodontitis. The expression of miR-1246 in lymphocytes of periodontitis patients was detected by RT-PCR. The expressions of inflammatory factors (interleukin 1 (IL-1), IL-6, and tumor necrosis factor alpha (TNF-α)) in lymphocytes were also measured using RT-PCR. The effect of miR-1246 on apoptosis was determined by flow cytometry. The target gene of miR-1246 was predicted and validated using luciferase reporter assay. The expressions of nuclear factor-κB (NF-κB) signaling pathway associated proteins were detected by Western blot. MiR-1246 was down-regulated in lymphocytes of periodontitis patients. omiR-1246 overexpression significantly inhibited the expressions of inflammatory factors and promoted apoptosis of lymphocytes in periodontitis. The insulin-like growth factor 2 RNA binding protein1 (IGF2BP1) was a target gene of miR-1246, and was up-regulated in periodontitis. MiR-1246 could negatively regulate IGF2BP1 expression and inhibit the activation of NF-κB signaling pathway to regulate apoptosis of lymphocytes in periodontitis. The miR-1246 inhibits the inflammatory reaction and promoted apoptosis of lymphocytes in periodontitis by negatively regulating IGF2BP1 and NF-κB signaling pathway. The miR-1246 may be used as biomarkers in the diagnosis and treatment of periodontitis.

Keywords: Periodontitis, lymphocyte, miR-1246, apoptosis, inflammation, IGF2BP1, NF-κB

Introduction

Periodontitis is a chronic multifactorial inflammatory disease affecting the periodontium which is the tooth-supporting structures [1]. This disease is very prevalent, and is widely considered as the second most common dental disease around the world, affecting about 750 million people as of 2010 [2]. Periodontitis involves progressive destruction of the alveolar bone around the teeth, which can lead to the loosening and subsequent loss of teeth [3]. Importantly, severe periodontitis adversely affects systemic health, increasing the patients' risk for diabetes, atherosclerosis, and rheumatoid arthritis [4-6]. Treatments of periodontitis include mechanical removal of bacterial plaque, periodontal surgery and intensive oral hygiene instructions for the patients [7]. However, the treatment effect is not very ideal. Therefore, exploring the pathogenesis of periodontitis from the molecular level may provide beneficial theoretical basis for its treatment.

MicroRNAs (miRNAs) are small, non-coding RNA sequences that usually bind to the 3'-UTR of the mRNA and form complexes with mRNA species [8]. miRNAs play key roles in post-transcriptional regulation, serving to repress mRNA translation or induce mRNA degradation [9]. miRNA expression has been revealed to affect the pathobiology of several diseases in humans, such as inflammatory diseases and cancer [10, 11]. Additionally, miRNAs have also been demonstrated to involve in the regulation of inflammatory response, however, their potential function in periodontal inflammation as well as their expression in periodontal tissues are largely unexplored. Recently, Stoecklin-Wasmer et al. [12] predicted that miR-1246 was differentially expressed between healthy and periodontal gingival with bioinformatics methods.

Table 1. Primers used for targets amplification

Name	Forward primer (5'-3')	Reverse primer (5'-3')
IL-1	CCACTCCATGAAGGCTGCATG	GGTGCTGACCTAGGCTTGATG
IL-6	ACAACCACGGCCTTCCCTACTT	CACGATTTCCCAGAGAACATGTG
TNF-α	CAGGGGCCACCACGCTCTTC	CTTGGGGCAGGGGCTCTTGAC
Bcl-2	ACA ACA TCG CCC TGT GGA TGA	ATA GCT GATTCG ACG TTTTGC C
Bcl-XL	ATTCTTGCTAGCTAGTGTCTGGAAGCCACTGGG	ACCGCCAGATCTGCCTGTGTTTAGCGATTCTCTTC
Bax	GGAATTCTGACGGCAACTTCAACTGGG	GGAATTCTTCCAGATGGTGAGCGA GG
Caspase-3	TGTCATCTCGCTCTGGTACG	AAATGACCCCTTCATCACCA
IGF2BP1	CCTGCTGGCTCAGTATGGT	GACATTCACCACTGCCGTCTC
IKBα	GTGGGGTTTGTAGAAGGTTG	GGCAGATCCAGCTTCTATGC
IKKα	GCTCCCGCCCCATGGAGCG	TCGAGTCATTCTGCTAACCAAC
IKKβ	ACTTGGCGCCCAATGACCT	CTCTGTTCTCCTTGCTGCA
GAPDH	TGGACTCCACGACGTACTCAG	CGGGAAGCTTGTCATCAATGGAA

However, its potential pathogenic mechanism in periodontitis has not been fully investigated.

In this study, we aimed to investigate the expression of miR-1246 in periodontal tissues and its potential function in the pathobiology of periodontitis. In addition, we explored the underlying molecular mechanism of miR-1246 affecting the development of periodontitis in order to further understand the etiology of periodontitis as well as to provide beneficial theoretical basis for the treatment of this disease.

Materials and methods

Cell culture and transfection

The lymphocytes were isolated from gingival tissue of patients with periodontitis as previously described [13]. Briefly, the separated gingival tissue samples were placed in AIMV medium containing 125 ng/mLamphotericin B, proteaseinhibitor (1 μg/mL α_2-macroglobulin, 0.1 μg/mL α_1-antitrypsin, 200 μg/mL EDTA-2 Na, 0.5 μg/mLaprotinin, and 0.5 μg/mLE-64) and 10 U/mL heparin (Sigma, USA). The tissue samples were cutintoslice with thickness of 1 mm using surgical scalpels. Then mononucleararcells were isolated using Ficoll-Paque centrifugation, and were analyzed using flow cytometry analyzer. The majority of the isolated cells (more than 99%) showed forward and side scatter identical to those of peripheral blood lymphocytes, which were considered as lymphocyte-rich fractions (periodontal lymphocytes).

The isolated lymphocytes (5×10^7/L) were cultured into 6-wells plates for 24 h. Cell transfection was performed using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. In brief, 4 μg plasmids coated with 10 μL Lipofectamine 2000 were transfected into lymphocytes. The culture medium was changed after 6 to 8 h. The cells that were not transfected and were transfected with scrambled miRNA were used as positive and negative controls, respectively.

Cell apoptosis assay

Cell apoptosis was detected with a flow cytometry using the Annexin V-FITC/PI cell apoptosis kit (Invitrogen, USA) according to manufacturer's protocol. Briefly, cells were seeded into 24-wells plates (1×10^5 cells/well) and cultured with fresh medium until logarithmic phase. Then cells were harvested with trypsinand centrifugation at 12,000 g for 5 min. The cell concentration was adjusted into 1×10^9 and mixed with 5 μL annexin-V-FITC and 5 μL propidium iodide (PI). After 1 h, the mixtures were analyzed using the FACS can flow cytometry.

PT-PCR

Total RNA was isolated from cells with TRIZOL reagent (Takara Bio, Dalian, China) according to the manufacturer's protocol, and then was treated with RNase-free DNase I. The purity and concentration of total RNA was detected using ultraviolet spectrophotometer. The cDNA was synthesized using PrimerScript 1st Strand cDNA Synthesis Kit. The amplification of tar-

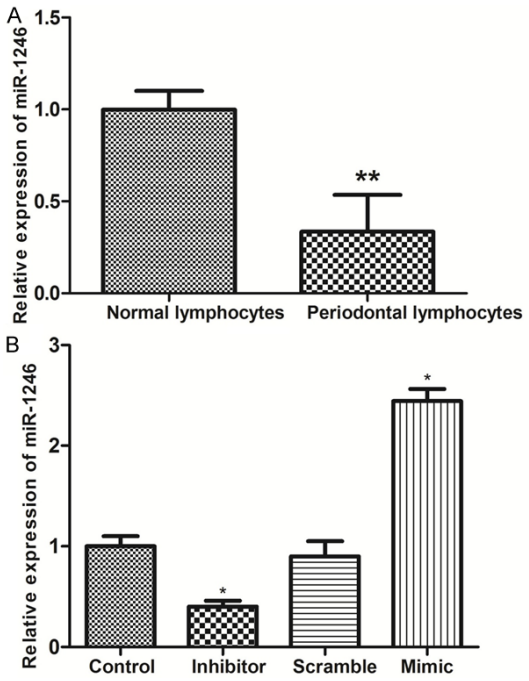


Figure 1. A: Relative expression level of miR-1246 in normal and periodontal lymphocytes detected by RT-PCR. B: Relative expression level of miR-1246 after lymphocytes were transfected with miR-1246 inhibitor and mimic detected by RT-PCR. **P < 0.01, *P < 0.05 compared to the control.

gets wasperformed with the SYBR ExScript RT-qPCR Kit (Takara Bio, Dalian, China). GAPDH and U6 were used as the internal controls. The set of primers used for each amplification was shown in **Table 1**.

Western blotting

Lymphocytes were lysed with RIPA assay lysate and centrifugated at 12,000 g for 5 min at 4°C. The supernatant was collected for the measurement of protein concentrations using BCA Protein Assay Kit. Then 50 μg protein samples were separated on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes. After blocking in TBST containing 5% bovine serum albumin for 1 h, the membranes were probed with rabbit anti human primary antibodies (1:100 for Bcl-2, Bcl-XL, Bax, Caspase-3, insulin-like growth factor 2 RNA binding protein1 (IGF2BP1), IκB kinase (IKK), IKBα and IKBβ) at 4°C overnight. Thenthe membranes were incubated with secondary antibodies (1:1000) at room temperature for 1 h. Finally, the immunoreactive protein bands were developed by enhanced chemiluminescence and analyzed using densitometer. GAPDH was used as the internal control. All reagents and antibodies used in western blot were purchased from Takara Bio, Dalian, China.

Luciferase reporter assay

The 3'-UTR of IGF2BP1, with wild-type (WT) or mutant (Mut) binding sites for miR-1246, was amplified and cloned into the pGL3 vector (Promega, USA), generating the plasmid pGL3-WT/Mut-IGF2BP1-3'-UTR. For the luciferase reporter assay, lymphocytes were cotransfected with luciferase reporter vectors and miR-1246 mimic or corresponding negative control (GenePharma, Shanghai, China). The pRL-TK plasmid (Promega, USA) was used as a normalizing control. The luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega, USA).

Statistical analysis

All experiments were conducted three times independently. The data were expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using Prism 6.0. Differences for data among groups were calculated using a one-way analysis of variance (ANOVA). P < 0.05 was considered as statistically significant.

Results

Expression of miR-1246 in periodontal lymphocytes

The expression of miR-1246 in lymphocytes from patients with periodontitis was detected using RT-PCR. As shown in **Figure 1A**, the relative expression level of miR-1246 in periodontal lymphocytes was significantly decreased compared with that in lymphocytes from gingival tissues f normal persons (P < 0.01), which suggested that the abnormal expression of miR-1246 may be associated with the occurrence of periodontitis. Subsequently, we transfected the periodontal lymphocytes with miR-1246 inhibitor or mimic and found that the miR-1246 expression decreased or increased significantly (P < 0.05) (**Figure 1B**).

Expressions of inflammatory factors in periodontal lymphocytes

The expressions of inflammatory factors (interleukin 1 (IL-1), IL-6, and tumor necrosis factor

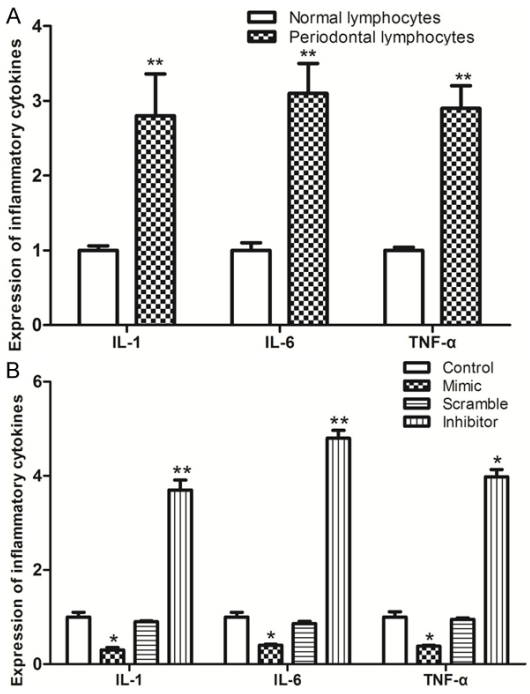


Figure 2. The expressions of inflammatory factors (interleukin 1 (IL-1), IL-6 and tumor necrosis factor-α (TNF-α)) in normal and periodontal lymphocytes detected by RT-PCR. **P < 0.01, *P < 0.05 compared to the control.

alpha (TNF-α)) in periodontal lymphocytes were detected using RT-PCR. The results showed that the expressions of three inflammatory factors in periodontal lymphocytes were significantly higher than that in normal lymphocytes (P < 0.01) (**Figure 2A**), indicating that periodontitis was associated with inflammatory reaction. However, when periodontal lymphocytes were transfected with miR-1246 mimic, the expressions of the three inflammatory factors decreased significantly compared with control (P < 0.05) (**Figure 2B**), which suggested that overexpressed miR-1246 could inhibit the inflammatory reaction in periodontitis.

Effect of miR-1246 on apoptosis

The effect of miR-1246 on apoptosis was detected by flow cytometry. As shown in **Figure 3A** and **3B**, when miR-1246 overexpressed, the percentage of apoptotic periodontal lymphocytes increased significantly compared with that in control (P < 0.01). On the contrary, when periodontal lymphocytes were transfected with miR-1246 inhibitor, the percentage of apoptotic cells decreased significantly (P < 0.05). The results suggested that low expression of miR-1246 was positively correlated with the apoptosis of periodontal lymphocytes.

To further explore the underlying mechanisms of miR-1246 suppression inhibiting apoptosis, the expressions of apoptosis-related proteins (Bcl-2, Bcl-XL, Bax and caspase-3) were detected. As shown in **Figure 3C** and **3D**, miR-1246 inhibitor significantly increased the mRNA and protein expressions of Bcl-2 and Bcl-XL (P < 0.01), and decreased the mRNA and protein expressions of Bax and caspase-3 (P < 0.05) in periodontal lymphocytes in comparison with the other groups. MiR-1246 mimic transfection showed an opposite result.

MiR-1246 regulates apoptosis by negative regulation of IGF2BP1

Based on the public miRNA databases, we predicted that 3'-UTR of IGF2BP1 was a potential miR-1246 binding site, suggesting that IGF2BP1 may be a direct target of miR-1246. Then we detected the expression of IGF2BP1 in normal and periodontal lymphocytes, and found that IGF2BP1 overexpressed in periodontal lymphocytes (P < 0.01) (**Figure 4A**).

Subsequently, we carried out the dual-luciferase reporter assay to validate whether miR-1246 bound to the 3'-UTR of IGF2BP1 (**Figure 4B**). The results showed that miR-1246 overexpression (miR-1246 mimic) significantly decreased IGF2BP1-3'-UTR reporter luciferase activity (P < 0.05), while the mutant IGF2BP1-3'-UTR abrogated the suppressive effect of miR-1246 mimic (**Figure 4C**).

We then detected the IGF2BP1 expression in lymphocytes transfected with miR-1246 mimic/inhibitor. As shown in **Figure 4D**, there was a negative correlation between IGF2BP1 expression and miR-1246 expression (P < 0.05). After lymphocytes were transfected with si-IGF2BP1 (**Figure 4E**), the effects of miR-1246 mimic/inhibitor on apoptosis was reversed significantly (P < 0.01) (**Figure 4F** and **4G**). The results indicated that miR-1246 may regulate apoptosis of lymphocytes by negative regulation of IGF2BP1.

MiR-1246 involves in NF-κB signaling pathway to mediate apoptosis of lymphocytes in periodontitis

To further investigate the effects of miR-1246 on the biological process of lymphocytes in

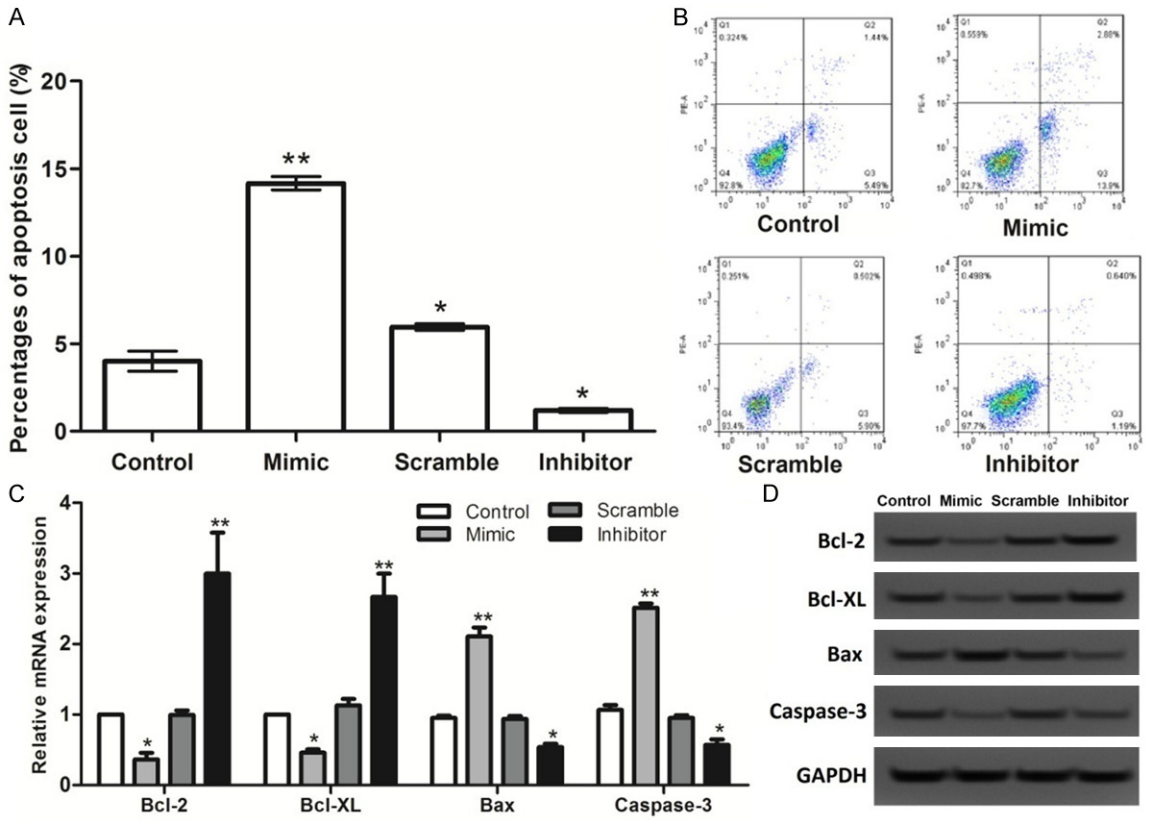


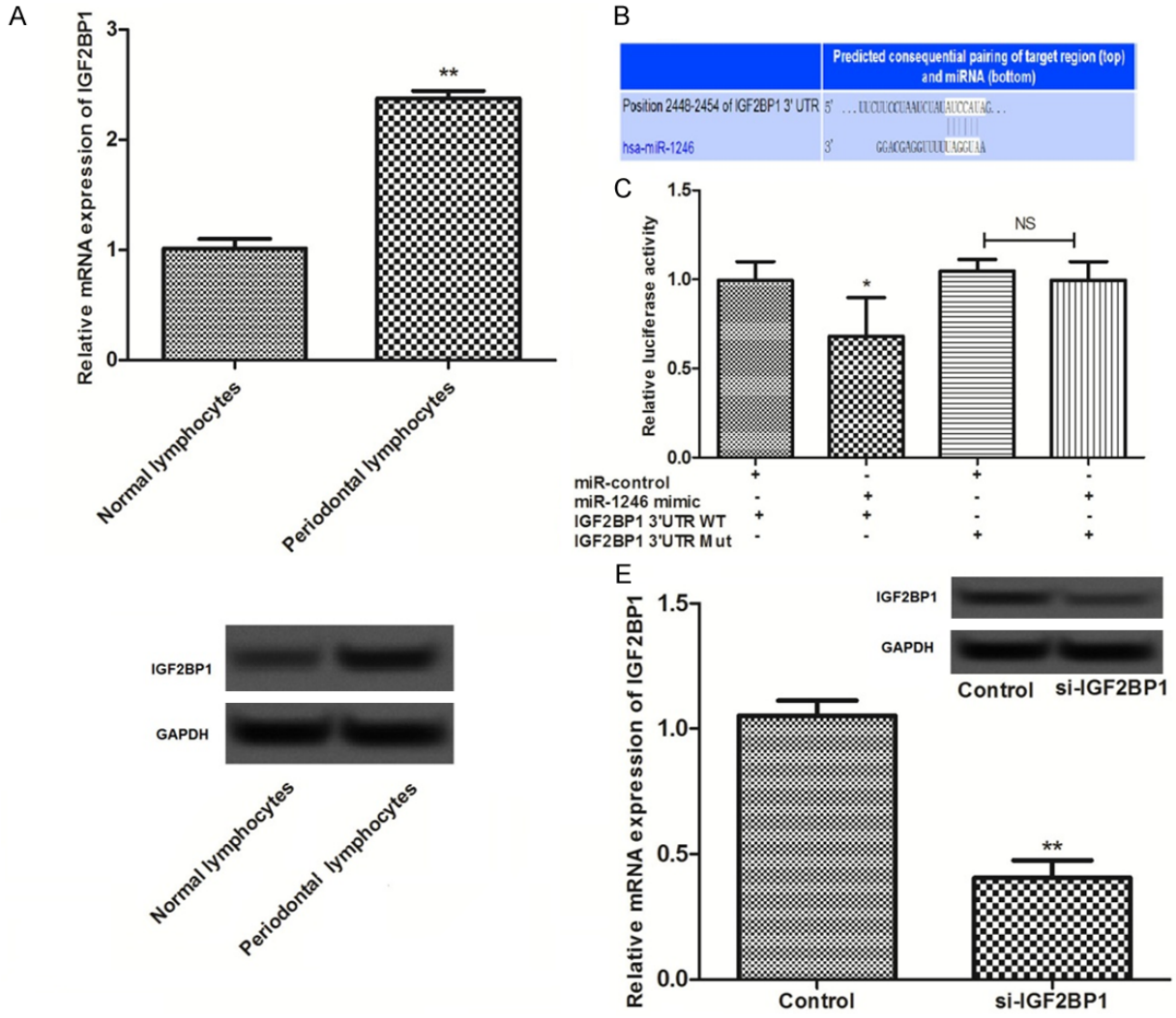
Figure 3. (A and B) Percentage of apoptosis cell in lymphocytes after cell transfection detected byflowcytometry. (C and D) The expressions of apoptosis-related proteins (Bcl-2, Bcl-XL, Bax and caspase-3) in lymphocytes detected by RT-PCR (C) and western blot (D). **P < 0.01, *P < 0.05 compared to the control.

periodontitis, we detected the expressions of nuclear factor-κB (NF-κB) signaling pathway associated proteins. As shown in **Figure 5A** and **5B**, the relative expressions of p-IKBα and p-IKKα/β decreased significantly when miR-1246 overexpressed (P < 0.05). On the contrary, when miR-1246 was inhibited, the relative expressions of p-IKBα and p-IKKα/β significantly increased (P < 0.01). Subsequently, we treated lymphocytes with 2 μmol/L of NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) to inhibit the activity of NF-κB (**Figure 5C** and **5D**). We found that PDTC could significantly reverse the effect of miR-1246 suppression on inflammatory reaction (**Figure 5E**) and apoptosis of lymphocytes (**Figure 5F** and **5G**) in periodontitis (P < 0.05). These results suggested that the dysregulated expressionof miR-1246 may mediate apoptosis of lymphocytes in periodontitis by regulating NF-κB signaling pathway.

Discussion

The present study found that miR-1246 was down-regulated in lymphocytes of periodontitis patients compared with normal controls. Overexpression of miR-1246 significantly inhibited the expressions of inflammatory factors and promoted apoptosis of lymphocytes in periodontitis. Further study found that miR-1246 could negatively regulate IGF2BP1 expression and inhibit the activation of NF-κB signaling pathway to play a protective role in periodontitis.

Human genome has been suggested to encode more than 1000 miRNAs which are differentially expressed at a high dynamic range in diseases [14]. Recent studies have focused on the regulatory role of miRNAs in the inflammatory response [15, 16]. It is well known that periodontal tissue damages are associated with bacteria-induced inflammatory responses. The severity of periodontitis is somewhat rely on the dynamic equilibrium between the microbial challenge and the immuno-inflammatory responses of host [17]. The local balance is tipped toward periodontal breakdown by the excessive production of inflammatory factors, such as IL-1, IL-6, TNF-α, and matrix metalloproteinases


A
Relative mRNA expression of IGF2BP1
3
2
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0
**
Normal lymphocytes
Periodontal lymphocytes
IGF2BP1
GAPDH
Normal lymphocytes
Periodontal lymphocytes
B
Predicted consequential pairing of target region (top) and miRNA (bottom)
Position 2448-2454 of IGF2BP1 3' UTR
hsa-miR-1246
C
Relative luciferase activity
1.5
1.0
0.5
0.0
*
NS
miR-control
miR-1246 mimic
IGF2BP1 3'UTR WT
IGF2BP1 3'UTR Mut
E
Relative mRNA expression of IGF2BP1
1.5
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Control
si-IGF2BP1
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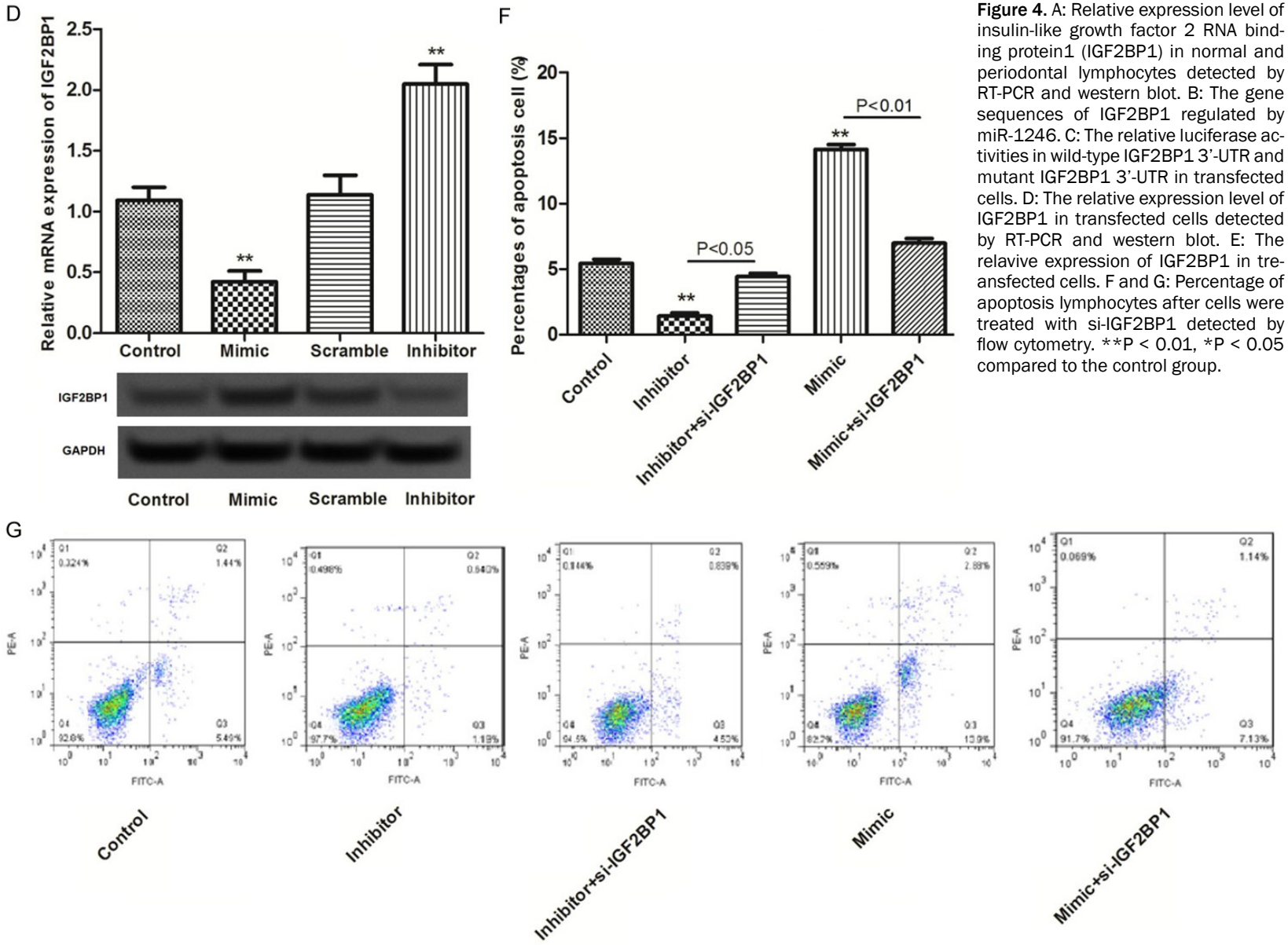

Figure 4. A: Relative expression level of insulin-like growth factor 2 RNA binding protein1 (IGF2BP1) in normal and periodontal lymphocytes detected by RT-PCR and western blot. B: The gene sequences of IGF2BP1 regulated by miR-1246. C: The relative luciferase activities in wild-type IGF2BP1 3'-UTR and mutant IGF2BP1 3'-UTR in transfected cells. D: The relative expression level of IGF2BP1 in transfected cells detected by RT-PCR and western blot. E: The relavive expression of IGF2BP1 in treansfected cells. F and G: Percentage of apoptosis lymphocytes after cells were treated with si-IGF2BP1 detected by flow cytometry. **P < 0.01, *P < 0.05 compared to the control group.

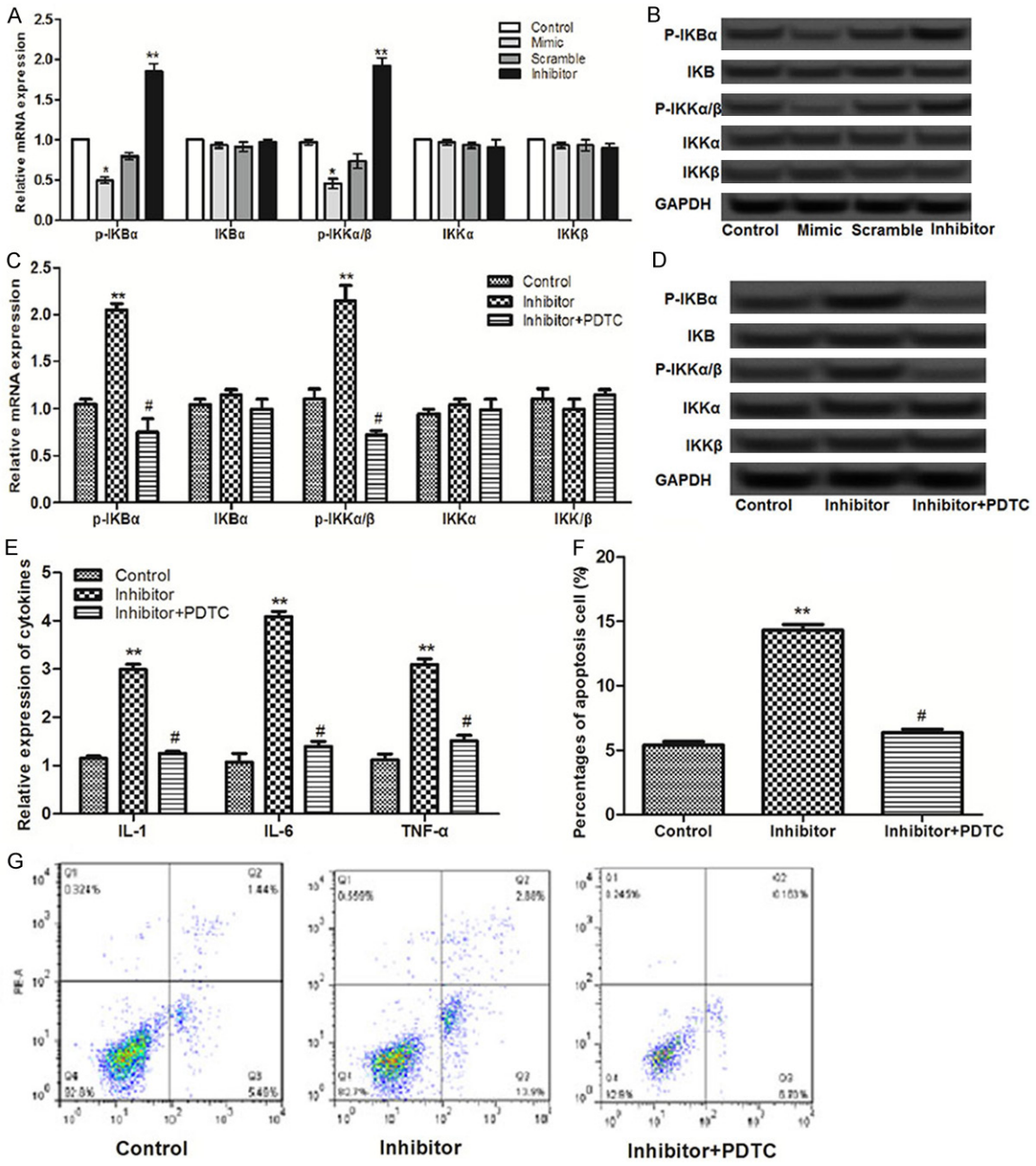

Figure 5. A and B: The relative expression levels of nuclear factor-κB (NF-κB) signaling pathway associated proteins after transfection detected by RT-PCR and western blot; C and D: The relative expression levels of NF-κB signaling pathway associated proteins after NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) was added. E: The expressions of inflammatory factors (IL-1, IL-6 and TNF-α) after PDTC was added. F and G: Percentage of apoptosis lymphocytes after PDTC was added. **P < 0.01, *P < 0.05 compared to the control group; #P < 0.05 compared to the miR-1246 inhibitor group.

[18]. In this study, the inflammatory factors including IL-1, IL-6 and TNF-α were up-regulated in lymphocytes of periodontitis patients, which suggested the inflammatory responses in periodontitis. MiR-1246 was found to be down-regulated in periodontitis, overexpression of which significantly decreased the expressions of inflammatory factors, suggesting the anti-inflammatory effect of miR-1246.

Apoptosis has a physiological role in lymphocyte development. Evidence has found that

apoptosis play an important pathogenic mechanism promoting inflammation [19]. In this study, we found that suppression of miR-1246 promoted the inflammatory responses in periodontitis and inhibited apoptosis of lymphocytesm, which was in accordance with a previous study. Sawa et al. [13] have reported that accumulated inflammatory cells may be resistant to apoptosis, leading the cells to a phenotype of longevity.

To further investigate the potential molecular mechanism, we detected the expressions of several apoptosis-associated proteins. The Bcl-2 protein is known to interact with Bax. The ratio of Bcl-2 to Bax plays an important role in regulating cell survival and death. Bcl-2 overexpression reduces the formation of Bax homodimers and inhibits apoptosis. On the contrary, Bax overexpression reduces Bcl-2 homodimers and accelerates apoptosis [20]. Bcl-XL is another member of the Bcl-2 family, which is reported to inhibit apoptosis in various cell types [21]. The caspase-3 is an executer of the programmed cell death and promote apoptosis [22]. Taken together, Bcl-2 and Bcl-XL are anti-apoptotic proteins while Bax and caspase-3 are pro-apoptotic proteins. In this study, the expressions of the four apoptosis-associated proteins in periodontitis were consistent with the result of flow cytometry.

As we known, in mammalian cells, miRNAs usually regulate biological processes via inhibiting gene expression by translational repression [23]. In this study, we focused on a member of the RNA-binding proteins IGF2BP1 and found that miR-1246 overexpression significantly decreased the expression levels of IGF2BP1. To validate that IGF2BP1 is a direct target of miR-1246, we performed luciferase activity reporters. The results confirmed that miR-1246 binds to the 3'-UTR of IGF2BP1. To our best knowledge, this is the first time that IGF2BP1 is found to be a direct target of miR-1246. Interestingly, previous studies have suggested the role of IGF2BP1 in cell survival and apoptosis. For instance, Gutschner et al. [24] reported that silenced IGF2BP1 decreased the proliferation and promoted apoptosis in hepatocellular carcinoma cells. Additionally, Mongroo et al. [25] found that IGF2BP1 suppression involved in modulating cell survival by promoting caspase-3 cleavage in colon cancer cell line SW480. Our results were in accordant with the findings above. We found that IGF2BP1 depletion by siRNA increased the percentage of apoptosis cell in periodontitis.

NF-κB signaling pathway is an important cellular signaling pathway, of which protein phosphorylation is a major factor for the activation of downstream events [26]. The NF-κB transcription factor has attracted extensive attention of researchers due to its central role in immunological processes, and its involvement in human diseases [27]. It has been found to plays an important role inimmune and inflammatory responses and apoptosis [28-30]. A primary level of control for NF-κB is through interactions with IκB, an inhibitor protein [27]. IκB can be phosphorylated by the IKK which regulates many physiological processes, including inflammation and cell death [31]. In agreement with findings above, the present study found that NF-κB signaling pathway really played a role in inflammatory responses and apoptosis in periodontitis, which was regulated by miR-1246.

In conclusion, this study suggests thatmiR-1246 is down-regulated in periodontitis andmayplay an important roleinthe pathogenesis of periodontitis. The miR-1246 inhibits the inflammatory reaction and promoted apoptosis of lymphocytes in periodontitis by negatively regulating IGF2BP1 and NF-κB signaling pathway. The miR-1246 may be used as biomarkers in the diagnosis and treatment of periodontitis.

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Disclosure of conflict of interest

None.

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